Genetic and biological characterisation of a novel South African *Plutella xylostella* granulovirus (*Plxy*GV) isolate

A thesis submitted in fulfilment of the requirements for the degree of

Masters of Science in Microbiology

At

RHODES UNIVERSITY

By

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MARCH 2014

Abstract

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is an important pest of cruciferous crops worldwide. The prolonged use of synthetic chemical insecticides as a primary means of control has resulted in the development of resistance in pest populations. In addition, the pest has also evolved resistance to the bacterial insecticidal protein of *Bacillus thuringiensis* which is also widely used as a method of control. Baculoviruses are considered as effective alternatives to conventional methods of control when incorporated into integrated pest management (IPM) programmes. These viruses target the larval stages of insects, are generally host-specific and are safe for use in the environment. This study aimed to isolate a baculovirus from a laboratory-reared *P. xylostella* colony, characterise it genetically and then evaluate its virulence against neonate and fourth instar larvae.

A laboratory colony of *P. xylostella* was established using pupae and asymptomatic larvae collected from a cabbage plantation outside Grahamstown in the Eastern Cape province of South Africa. The colony flourished in the laboratory due to prime conditions and availability of food. The duration of development from egg to adult was determined by observation and imaging of the various life stages. The mean developmental time from egg to adult was observed to be 14.59 ± 0.21 days. The population of the insects increased rapidly in number leading to overcrowding of the insect colony, and hence appearance of larvae with viral symptoms. Occlusion bodies (OBs) were extracted from symptomatic larval cadavers and purified by glycerol gradient centrifugation. Analysis of the purified OBs by transmission electron microscopy revealed the presence of a granulovirus which was named PlxyGV-SA.

The virus isolate was genetically characterised by restriction endonuclease analysis of the genomic DNA, and PCR amplification and sequencing of selected viral genes. The complete genome sequence of a Japanese *P. xylostella* granulovirus isolate, PlxyGV-Japan, has been deposited on the GenBank database providing a reference strain for comparison with DNA profiles and selected gene sequences of PlxyGV-SA. BLAST analysis of the *granulin* gene confirmed the isolation of a novel South African PlxyGV isolate. Comparison of the restriction profiles of PlxyGV-SA with profiles of PlxyGV-Japan and other documented PlxyGV profiles obtained by agarose gel electrophoresis revealed that PlxyGV-SA is a genetically distinct isolate. The data obtained from the sequencing and alignment of *ecdysteroid UDP-glucosyltransferase* (*egt*), *late expression factor 8 (lef-8*) and *late*

expression factor 9 (*lef-9*) genes with those of PlxyGV-Japan also showed that PlxyGV-SA is a genetically different isolate.

In order to determine the biological activity of PlxyGV-SA against neonate and fourth instar *P. xylostella* larvae, surface dose bioassays were conducted. The median lethal concentration of the virus required to kill 50% (LC_{50}) and 90% (LC_{90}) of the larvae was estimated by feeding insects with a range of doses. In addition, the time to kill 50% of the larvae (LT_{50}) was determined by feeding insects with the LC_{90} concentration. Larval mortality was monitored daily until pupation. The data obtained from the dose response assays were subjected to probit analysis using Proban statistical software. The time response was determined using GraphPad Prism software (version 6.0). The LC_{50} and LC_{90} values for the neonate larvae were 3.56×10^5 and 1.14×10^7 OBs/ml respectively. The LT_{50} was determined to be 104 hours. The neonate larvae were found to be more susceptible to infection than the fourth instar larvae with the same virus concentration. The concentrations used for the neonate larvae assay did not have a significant effect on the fourth instar as no mortality was recorded.

This is the first study to describe a novel South African PlxyGV isolate and the results suggest that PlxyGV-SA has significant potential for development as an effective biopesticide for the control of *P. xylostella* in the field.

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List of abbreviations

General

BV – budded virus

DNA – deoxyribonucleic acid

GV – granulovirus

h - hours

IPM – integrated pest management programme

LC – lethal concentration

LC_{50} – median lethal concentration

LC_{90} – 90% lethal concentration

LD – lethal dosage

LT – lethal time

LT_{50} – median lethal time

Ltd – Limited

MNPV – multiple nucleopolyhedrovirus

NPV – nucleopolyhedrovirus

RNA - Ribonucleic acid

SNPV – single nucleopolyhedrovirus

ODV – occlusion derived viruses

OB – occlusion body

OV - Occluded virus

OD260nm - Optical density at 260nm

ORF - Open reading frame

PCR - Polymerase chain reaction

REN – restriction enzyme analysis

SDS – sodium dodecyl sulphate

SE – standard error

SNP – single nucleotide polymorphismViruses

Units and symbols

bp - base pairs

kb – kilobasepairs

% - percentage

°C - degrees Celcius

g - gram

x g - times gravity

L - litre

µl - microlitres

µM – micromolar

mM – millimolar

mg - milligrams

mm – millimetre

ml – millilitre

nm - nanometre

V – Volts

Viruses

AcMNPV - *Autographa californica* MNPV

AgMNPV - *Anticarsia gemmatalis* MNPV

BmNPV - *Bombyx mori* NPV

CpGV - *Cydia pomonella* GV

CrleGV - *Cryptophlebia leucotreta* GV

CuniNPV - *Culex nigripalpus* NPV

GmNPV - *Galleria mellonella* NPV

HearNPV - *Helicoverpa armigera* NPV

PhopGV - *Phthorimaea operculella* GV

PlxyGV - *Plutella xylostella* GV

PlxyMNPV - *Plutella xylostella* NPV

PrGV- *Pieris rapae* GV

SfMNPV - *Spodoptera frugiperda* MNPV

XcGV - *Xestia c-nigrum* GV

Research outputs

Conference Proceedings:

Abdulkadir F, Marsberg T, Knox CM, Hill MP and Moore SD. Genetic and biological characterisation of a novel baculovirus for the control of diamondback moth (*Plutella xylostella*). Oral presentation SASM 2013 conference, held at Forever Resorts Warm Baths, Bela-Bela, Limpopo, South Africa. 24-27 November 2013.

Research article:

Abdulkadir F, Marsberg T, Knox CM, Hill MP and Moore SD (2013) Morphological and genetic characterization of a South African *Plutella xylostella* granulovirus (PlxyGV) isolate. *African Entomology* **21**(1):168–171.

Acknowledgements

I would like to acknowledge the following people for their support towards the successful completion of this work.

- My supervisor Dr. Caroline Knox for professional advice, suggestions and comments. I cannot find words to express my gratitude for your kindness, patience and for bearing with my poor English skills
- My co- supervisors Prof. Martin Hill and Dr. Sean Moore for guidance, encouragement and criticism
- Tamryn Marsberg for putting me through on how to maintain the insect colony
- Dr. John Opoku-Debrah for assistance with molecular techniques and advice
- Tanya Fullard for assistance with the insect rearing and always willing to help
- Michael Jukes for giving me all the necessary help in the lab and for proof reading my chapters
- Brittany Jade Williams for giving me all the necessary help in the lab
- Boitumelo Moetlhoa and Dora Mkabili Mwangola for their assistance in the lab
- Tumi Motsoeneng, Lithabiso Motanyane and Caroline Ross for the wonderful conversations
- Marvin Randal and Shirley Pinchuck for assistance with electron microscopy work
- Candice Coombes for giving me all the necessary assistance in the entomology lab
- Claire Love and Kennedy Zimba for making the entomology lab lively
- River Bioscience for funding
- My husband Yusuf Hassan for motivation, words of encouragement, advice and for believing that I could to it
- My daughter Bilkisu Hassan for coping with my absence and for the sweet smiles that brighten my heart and keep me moving forward
- My parents for their incredible love, encouragement and prayers. I couldn't have made it without their support
- My siblings for their encouragement, concern, prayers and affection that gave sense of belonging

CHAPTER ONE

Literature review

1.1 *Plutella xylostella*

The diamondback moth, *Plutella xylostella* (L.), is the most destructive insect pest of cruciferous crops worldwide (Talekar and Shelton 1993). This insect occur wherever brassica crops are grown and is considered to be the most extensively distributed of all lepidopteran species (Shelton 2004). The annual cost for managing *P. xylostella* is estimated to be US $ 4-5 billion (Zalucki *et al.* 2012). Shelton (2004) reported that one of the most important factors contributing to the exceptionally high pest status of *P. xylostella* is the diversity and abundance of its host plants. The Brassicaceae (cruciferae) is an important family of flowering plants also known as the mustards, crucifers or the cabbage family. This family comprises of 350 genera and over 3500 species (Warwick *et al*. 2003). Species in this family include economically important crops such as canola, mustard, cabbage, cauliflower, radish and turnip (Sarfraz *et al*. 2005). They occur in temperate and tropical climates (Shelton 2004).

The production of brassica crops has increased by 39% from 1993-2009, and in 2009 about 3.4 million hectares were cultivated globally (FAO 2012). Over the same period, cabbage production increased by 27% and oil seed *Brassica rapa* by 59% (FAO 2012). Oil seed *Brassica rapa* provides 13% of the world vegetable oil and is rated second in importance after soybean (Raymer 2012). Factors that promote the pest status of *P. xylostella* are lack of effective natural enemies (Lim 1986), the high reproductive potential of the moth (over 20 generations per year in the tropics) and its genetic elasticity which facilitates rapid development of resistance to insecticides (Shelton 2004).

In many countries, *P. xylostella* has evolved resistance to most chemical insecticides and also to the bacterial insecticide *Bacillus thuringiensis* (Tabashnik *et al.* 1990). The lack of effective biological control agents in most regions could be as a result of the destruction of natural enemies by the use of broad spectrum insecticides (Talekar and Shelton 1993). For example, high parasitism levels of *P. xylostella* were observed in unsprayed cabbage fields from the Eastern Cape, Gauteng and North-West Provinces of South Africa (Kfir 1997;

Waladde *et al.* 2001; Smith and Villet 2002) and, in the same regions, high infestation of *P. xylostella* was recorded in cabbage fields that are regularly sprayed (Kfir 2005). The two key biotic factors that can regulate the population of *P. xylostella* in the field are availability of host plants and the action of the natural enemies (Harcourt 1986; Kfir 1997).

1.1.1 Origin of *Plutella xylostella*

Hardy (1938) suggested that *P. xylostella* originated in Europe, probably in the Mediterranean region and was accidentally spread around the world with cultivated brassicas. The European origin of *P. xylostella* has been questioned by research workers in South Africa and China (Kfir 1998; Liu *et al*. 2000). Kfir (1998) argues the European origin based on the rich and diverse fauna of *P. xylostella* parasitoids. Kfir (1997) recorded twenty-two species of parasitoids and hyperparasitoids reared from *P. xylostella* larvae and pupae in South Africa, some of which are specific to *P. xylostella* and known only from South Africa. Kfir states that these large numbers indicate a very long association between parasitoids and the pest in the region.

The first cultivated brassicas in South Africa were introduced approximately 300 years ago by the Dutch settlers (Kfir 1998). Kfir (1998) contends that the presence of rich and diverse parasitoids of *P. xylostella* in South Africa understates the possibility that *P. xylostella* spread to this region together or after the introduction of cultivated brassicas and suggested that this period of time is too short for such a diversity of parasitoids to evolve. He speculated that *P. xylostella* may have already existed in South Africa before the introduction of cultivated brassicas and proliferated on indigenous Brassicaceae.

The parasitiod *Diadromus collaris* (Gravenhorst) (Hymenoptera: Ichneumonidae) is arrhenotokous in South Africa which means that females produces both female and male progeny, and reproduction is sexual (Kfir 1996). *Diadromus collaris* has been used in the biological control of *P. xylostella* in Europe but only the thelytokous form exists, meaning that only female offspring are produced and reproduction is asexual (Kfir 1998). Kfir asserts that *D. collaris* may have originated in South Africa and dispersed to Europe because several incidences are known in which parasitoid species are sexual in their original geographic location and become asexual either when reared in the laboratory or when released for biocontrol in a different region. Moreover, according to Mayr (1965) all asexual organisms are derived from sexual forms as asexuality in organisms is a secondary phenomenon.

Kfir (1998) also questioned the European origin based on the presence of large number of indigenous brassicas in South Africa. Arnold and De Vet (1993) reported that South Africa is the smallest of the 6 floral kingdoms but the richest in terms of temperate flora containing approximately 10% of the flowering plants of the world. Out of the 175 plant species recorded in the Brassicaceae family in South Africa, only 32 are not considered as indigenous species (Jordaan 1993). By contrast, in Taiwan, where *P. xylostella* is an exotic and important pest, Liu and Ying (1976) recorded 19 brassicas, none of which are indigenous.

In summary, Kfir (1998) suggests that *P. xylostella* originated in South Africa. Using similar arguments, the presence of rich and diverse parasitoids of *P. xylostella* in China, the arrhenotokous form of *D. collaris* and the large number of native brassicas in the region, Liu *et al.* (2000) are of the view that *P. xylostella* originated in China.

1.1.2 Host range of *Plutella xylostella*

Plutella xylostella feeds only on the plants in the family Brassicaceae (Talekar and Shelton 1993). Plants from this family are extensively cultivated worldwide for various edible parts such as the root (turnip), stems (kohlrabi), leaves (cabbage), flowers (cauliflower), buds (brussel sprouts) and seeds (mustard and rape seeds). The *P. xylostella* host range is limited to wild and cultivated Brassicaceae that are characterised by having glucosinolates, which are sulphur-containing secondary plant compounds (Sarfraz *et al*. 2006) (Table 1.1).

Plutella xylostella feeds on brassica weeds in the absence of the cultivated host. These uncultivated host pants are important in maintaining *P. xylostella* populations in temperate regions during spring before planting of brassica crops (Talekar and Shelton 1993). The weeds that have been reported to support the feeding and reproduction of *P. xylostella* are listed in Table 1.2 (Talekar and shelton 1993).

Table 1.1 Plants reported as hosts of *P. xylostella* (Talekar and Shelton 1993)

Common name	Scientific name	Variety/cultivar
Brussels sprouts	*Brassica oleracea*	gemmifera
Brocolli	*Brassica oleracea*	italica
Cabbage	*Brassica oleracea*	capitata
Chinese cannabe	*Brassica rapa*	pekinensis
Cauliflower	*Brassica oleracea*	botrytis
Collard	*Brassica oleracea*	acephala
Kale	*Brassica oleracea*	alboglabra
Kohlrabi	*Brassica oleracea*	gongylodes
Mustard	*Brassica juncea*	-
Radish	*Raphanunus sativus*	-
Rapeseed	*Brassica napus*	-
Turnip	*Brassica rapapekinesis*	-
Watercress	*Nasturtium officinale*	-

Table 1.2 Brassica plants that are reported as uncultivated host of *P. xylostella* (Talekar and Shelton 1993)

Common name	Scientific name
Tower mustard	*Arabis glabra*
Horse radish	*Armoracia lapathifolia*
Winter-cress	*Barbarea vulgaris*
Malabar spinach	*Basella alba*
Sugar beet	*Beta vulgaris*
Charlock mustard	*Brassica kaber*
Rutabaga	*Brassica napobrassica*
Turkish rocket	*Bunias orientalis*
Shepherds purse	*Capsella bursa-pastoris*
Large bittercress	*Cardamine amara*
Heartleaf bittercress	*Cardamine cordifolia*
Cardamine pratensis	*Cardamine pratensis*
Wallflower	*Cheiranthus cheiri*
Tansy mustard	*Descurainia sophia*

1.1.3 Distribution of *Plutella xylostella*

Plutella xylostella has been reported from more than 80 countries (Harcourt 1956). Liu *et al*. (2005) developed a bioclimatic model for *P. xylostella* based on the countries where it has been reported as a pest. The model predicts the core distribution of *P. xylostella*, regions where it persists year-round and regions where it can be a seasonal pest (Figure 1.1). Areas shaded in red show regions of the world where the Ecoclimatic Index (EI) is positive meaning that *P. xylostella* can be present year-round, while regions where EI ≤ 15–20 are marginal for year-round *P. xylostella* survival. Areas shaded in blue show regions of the world where the EI is zero but the annual growth index (GI) is positive. In these regions *P. xylostella* cannot persist year-round but it can become a seasonal pest following migration of moths from elsewhere.

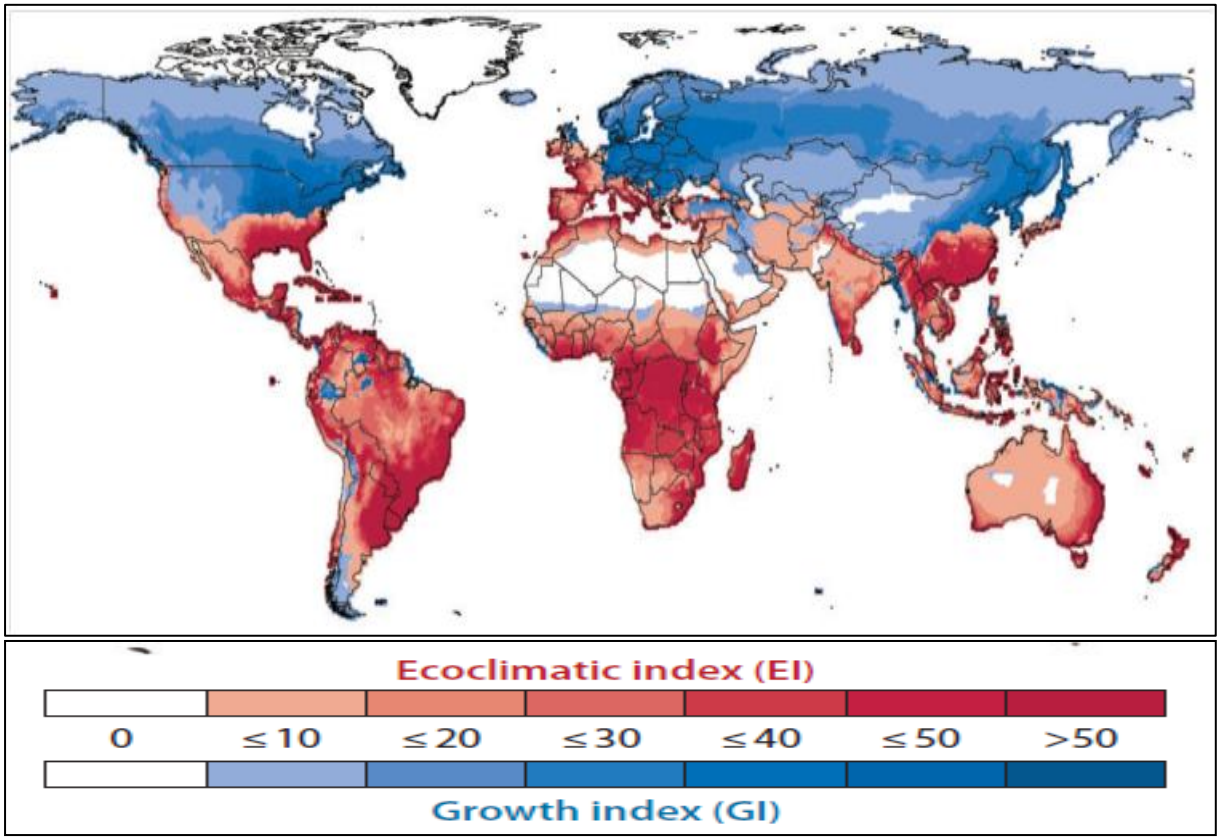


Figure 1.1 Predicted worldwide distribution of *P. xylostella* based on a bioclimatic model (Liu *et al*. 2005). Regions shaded in red indicate places where *P. xylostella* can persist year-round. Regions shaded in blue indicate areas where *P. xylostella* is a seasonal pest.

1.1.4 Nature and extent of injury of *Plutella xylostella* on host plants

Newly hatched *P. xylostella* larvae are leafminers feeding on spongy mesophyll tissues (Harcourt 1957). Older larvae feed on all plant parts (Talekar and Shelton 1993). The second, third and fourth instar larvae feed on the leaf tissue, making holes on the leaves that are window like in appearance (Figure 1.2). The entire leaf can be damaged in the case of a severe infestation. Larvae and pupae are found on damaged leaves and the feeding of larvae can deform the plant. *Plutella xylostella* larvae also feed on stems, growth tips, flowers and seed pods (Ramachandran *et al.* 1998). Different crops show varying degrees of susceptibility to damage at different stages in their growth (Mumford and Knight 1997).

Figure 1.2 *Plutella xylostella* infested cabbage with many holes caused by larval feeding. (Picture courtesy of Ryan Shaw, Department of Biochemistry and Microbiology, Rhodes University).

1.1.5 Life cycle of *Plutella xylostella*

Plutella xylostella adults emerge during the first eight hours of photophase and copulation occurs at night on the same day that the adult emerges (Pivnick *et al.* 1990). Female moths start laying eggs soon after copulation (Figure 1.3, panel A) and the oviposition period lasts for four days, during which the female lay about 188 eggs (Harcourt 1954). Most of the eggs are oviposited before midnight with peak oviposition occurring between 7 and 8 pm (Pivnick *et al.* 1990). Eggs are preferably oviposited in concavities of leaves rather than on smooth surfaces (Gupta and Thorsteinson 1960). Lack of light during normal daylight stimulates

oviposition, however light during the night does not entirely inhibit oviposition. Other factors that stimulate oviposition are plant volatiles, secondary chemicals, temperature and waxes on the leaf surface (Tabashnik 1985).

The incubation period of eggs lasts 5-6 days (Figure 1.3, panel B) and is mostly determined by temperature. Shortly after emergence, the first instar larvae (Figure 1.3, panel C) feed on spongy mesophyll tissue. The older larvae (second to fourth instar larvae) feed from the lower leaf surface and usually consume all tissue except the wax layer on the upper surface, creating a window in the leaf (Sarnthoy *et al.* 1989). Each larval instar survives for approximately four days and pupation occurs immediately after the fourth instar (Figure 1.3, panel D and E) (Safraz *et al.* 2005). The duration of pupation also depends on temperature and lasts for 4-15 days (Hoy 1988). After eclosion the adult moths (Figure 1.3, panel F) feed on water drops or dew and are usually short lived (Talekar & Shelton 1993).

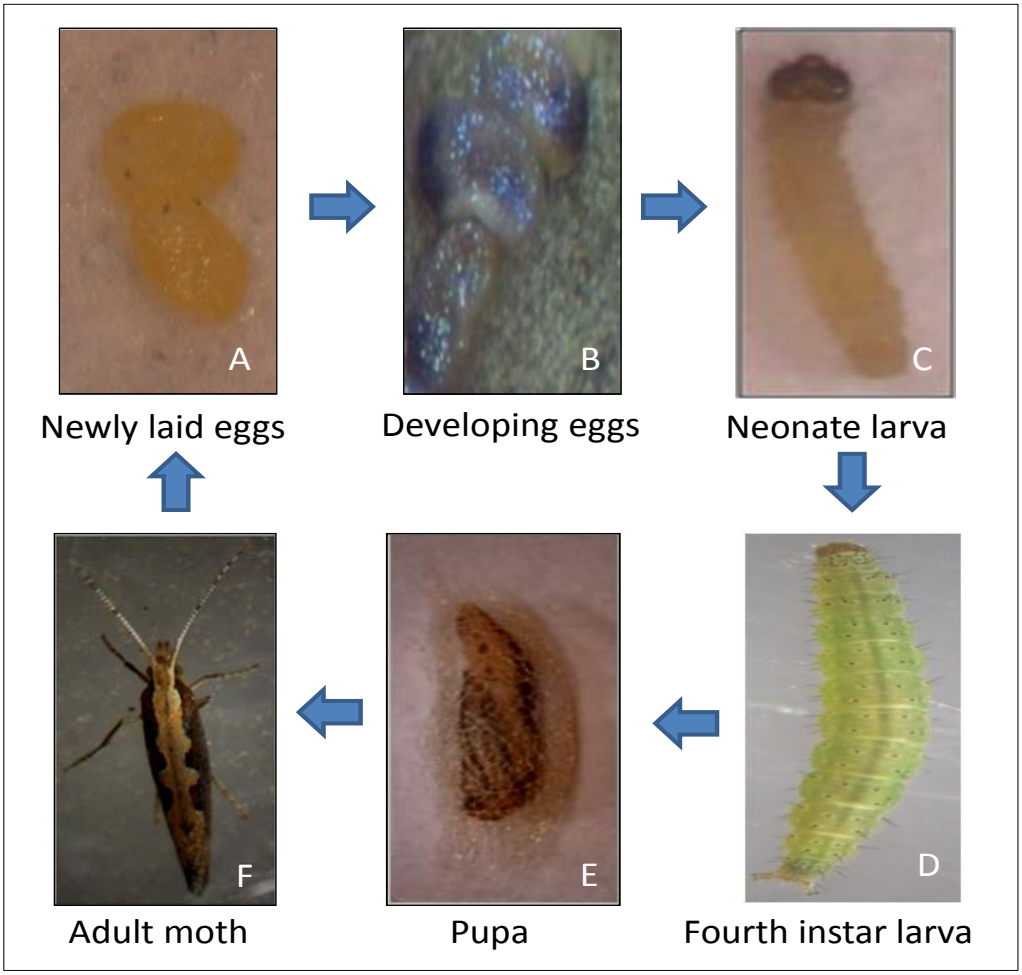


Figure 1.3 Life cycle of diamondback moth, *Plutella xylostella*. Pictures taken by Fatima Abdulkadir (2013)

1.1.6 Control of *Plutella xylostella*

The serious damage caused by *P. xylostella* on brassicas worldwide has led to farmers employing cultural, chemical and biological control as part of an integrated pest management (IPM) programme (Talekar and Shelton 1993). One single method cannot control pests completely; the best alternative is to employ an integrated approach. IPM is the combination of suitable techniques in a compatible manner to reduce pest status and maintain them below levels causing damage (Kogan 1998). IPM had success in suppressing *P. xylostella* populations on commercial farms in Asia (Talekar and Shelton 1993). The essential components of IPM include cultural methods such as crop rotation, sprinkler irrigation and intercropping, chemical methods, and biological control. These methods are described in the following sections.

1.1.6.1 Cultural methods

Cultural methods involve manipulation of environmental factors. These techniques include crop rotation, intercropping, sprinkler irrigation and the use of plants with repellent characteristics which inhibit the development of pests.

Crop rotation can play an important role in the management of *P. xylostella*, although it is not a standard practice in intensive crucifer-producing areas. This practice eliminates the continuous build-up of *P. xylostella* and resistance development due to the continual application of chemical insecticides (Lim 1986).

Heavy rainfall causes high *P. xylostella* mortality. As a result *P. xylostella* is only a serious pest during the dry season (Talekar & Shelton 1993). This factor is exploited by using overhead irrigation of the crop. Larvae on the leaf surface can be washed off the plant by water from an overhead sprinkler, thus reducing damage (Lim 1992). Mating and oviposition activities of the adult *P. xylostella* could also be disrupted when the sprinkler is left on from dusk through the early morning hours.

Intercropping is an ancient practice which can contribute significantly to reducing pest problems. The plants act as barriers to insect pest movement. Several field trials have been conducted using different intercrops such as tomato, garlic, coriander and carrot. In one study, garlic was grown in alternate rows with cabbage and the influence on *P. xylostella* was estimated. It was observed that intercropped plots had significantly lower numbers of *P.*

xylostella larvae and pupae and higher yields of good quality cabbage heads as compared to the control cabbage plots (Talekar & Shelton 1993). Trap crops can also be intercropped with commercial crops to lure pests away from the commercial product (Talekar & Shelton 1993). The trap crops are planted earlier than the commercial crop and must be available throughout the growing period of the commercial crop.

1.1.6.2 Chemical control

Insecticides have been developed due to significant yield losses of crops and high demand for income and consumer demand. In order to reduce the damage by *P. xylostella* and other insect pests, farmers use several different insecticides. The intense use of insecticides has led to the appearance of *P. xylostella* resistance to pyrethroids (Liu *et al.* 1982), organophosphates (OPs) (Miyata *et al.* 1982) and insect growth regulators (Lin *et al.* 1989).

Mechanisms of resistance to chemical insecticides include changes in sensitivity of the target or binding site (Noppun *et al.* 1983) and enhanced metabolic detoxification (Chiang *et al.* 1993; Huang *et al.* 1998). Nappun *et al.* (1983) reported the insensitivity of the *P. xylostella* acetylcholinesterase (AChE) as a resistance mechanism to organophosphorous and carbamate insecticides which target this enzyme. AChE mediates the hydrolysis of the neurotransmitter acetylcholine at the postsynaptic membrane to terminate nerve impulses (Baek *et al.* 2005). Weill *et al.* (2002) reported that structural alteration of AChE by mutation can result in an insensitive enzyme. Two types of AChEs have been identified in *P.* xylostella, namely AChE1 and AChE2, and these enzymes differ in their substrate and inhibitor specificity (Baek *et al.* 2005). A study by Ni *et al.* (2003) found two amino acid changes in the AChE1 protein sequence of *P. xylostella* and speculated that the mutations are involved in target-insensitive resistance in *P. xylostella* to prothiofos which is an organophosphate insecticide. Lee *et al.* (2007) identified three mutations in the AChE1 protein sequence of a prothiofos-resistant *P. xylostella* strain, and three-dimensional modelling and functional expression of the protein in *Spodoptera frugiperda* (armyworm) cell line (Sf9 cells) suggested that two of the mutations were involved in prothiofos-resistance.

Decreased in target site sensitivity associated with the nervous system, known as knock down resistance (kdr), has been reported as one of the major mechanisms of pyrethroid resistance of *P. xylostella* (Sun 1992). Knock down resistance occurs due to a point mutation in the gene encoding the voltage sensitive sodium channel protein of the nerve membrane in the central

nervous system, the target site of pyrethroids and dichlorodiphenyltrichloroethane (DDT) (Bloomquist 1996). Schuler *et al*. (1998) observed two mutations in the sodium channel gene associated with the pyrethroid resistance after comparison of sodium channel gene sequences from susceptible and kdr resistant *P. xylostella*.

Resistance in *P. xylostella* can also be by increased activity of metabolic detoxification systems such as glutathione S-transferases (GSTs). GSTs are a family of isoenzymes that are responsible for detoxification of endogenous and xenobiotic compounds in all animals (Rushmore and Pickett 1993). GSTs have high binding affinity to various hydrophobic compounds and can conjugate a variety of compounds that bear an electrophilic site with reduced glutathione (Mannervik *et al*. 1990; Rushmore and Pickett 1993). GSTs have been associated with the degradation and resistance of some organophosphates in *P. xylostella* (Koa and Sun 1991; Chiang and Sun 1993). Many pesticides have now been abandoned because of resistance development by various pests (Kfir 1997).

1.1.6.3 Biological control

Biological control is defined as the manipulation of natural enemies for pest management, and is one of the most effective and oldest methods of insect control. Biological agents such as parasites, plants, fungi, bacteria, viruses and even molecules like pheromones and other hormones have been used to control insect pests (Sarfraz *et al.* 2005).

1.1.6.3.1 Parasitoids

Parasitiods are used for the control of *P. xylostella* in many countries including Indonesia (Vos 1953), Australia (Wilson 1960), Taiwan (Talekar *et al*. 1992), Philippines (Ventura 1997), India (Chandramohan 1994), Malaysia (Ooi 1992), Laos, Vietnam, China (Talekar 2004), Kenya (Lohr *et al*. 2008) Australia (Furlong and Zalucki 2007), Japan (Talekar 2004), South Africa (Kfir 1997) and North Korea (Furlong *et al.* 2008). The potential of parasitoids as biological control agents varies depending on the interrelationships between them and the environment. For example, thermal tolerance studies by Chua and Ooi (1986) revealed that parasitism of *P. xylostella* and survival of the parasitoid *Diadegma semiclausum* (Hellen) is low at temperatures greater than 25°C. Over 135 parasitoid species have been documented worldwide to attack various life stages of *P. xylostella* (Delvare 2004). The major parasitoids of *P. xylostella* are listed in Table 1.3. Egg parasitiods of *P. xylostella* contribute little to

control because they are not host specific (Goulet and Huber 1993), and require periodic mass releases (Talekar and Shelton 1993). Species are restricted to the genera *Trichogramma* and *Trachogrammatoidea* (Sarfraz *et al.* 2005). The larval parasitiods of *P. xylostella* have the greatest control potential, and the most effective belong to the genera *Diadegma, Cotesia* and *Microplitis* (Lim 1986). The pupal parasitiods produce moderate levels of parasitism. They include *Diadromus plutellae* (Ashmead), *Diadromus subtilicornis* Gray, *T. sokolowskii* and *T. collaris* (Sarfraz *et al.* 2005).

Kfir (1997) recorded 21 species of parasitoids and hyperparasitoids that were reared from *P. xylostella* larvae and pupae in South Africa, and speculated that they have a positive impact in repressing the population of *P. xylostella* in the country. A previous study by Kfir (1996) revealed that the pest status of *P. xylostella* in South Africa is lower than in other countries with a similar climate.

Table 1.3 Major parasitoids of *P. xylostella* and their worldwide distribution (Grzywacz *et al*. 2010; Furlong *et al.* 2013)

SPECIES	Distribution in the world
Larval parasitoids	
Diadegma semiclausum (Hellen)	United Kingdom, France, Austria, Serbia, Netherlands, Romania, Bulgaria, Turkey, Finland, Russia; Uzbekistan, Nepal, Iran, Taiwan, Indonesia, Malaysia, Thailand, China, *India*, Egypt, Kenya; Australia, Australia, New Zealand, Papua and New Guinea
Diadegma fenestrale (Holmgren)	United Kingdom, France, Germany, Romania, Greece, Poland, Finland, Russia Japan and India
Diadegma mollipla (Holmgren)	Eritrea, Kenya, Malawi, Tanzania, South Africa, Reunion Island and Mauritius
Diadegma insulare (Cresson)	Canada, United States, Mexico, Honduras, Venezuela, Puerto Rico, Cuba and Jamaica
Diadegma leontiniae (Brethes)	South Brazil, Uruguay, Argentina and Chile
Diadegma novaezealandiae (Azidah)	Australia and New Zealand
Diadegma rapi (Cameron)	Australia and Australia
Cotesia vestalis Haliday (Kurdjumov)	France, Austria, Finland, Serbia, Bulgaria, Russia, Ukraine, Turkey, Japan, Sri Lanka, China, Taiwan, Vietnam, Indonesia, Malaysia, Philippines, Thailand, Myanmar, India, Pakistan, Cape Verde Islands, Benin, Senegal, South Africa, Reunion Island, Venezuela, Brazil, Martinique, Barbados, Leeward Islands, St. Kitts, St. Lucia, Trinidad, Guadeloupe and Australia
Microplitis plutellae (Muesbeck)	Canada, United States and Laos
Apanteles halfordi (Ullyett)	South Africa
Apanteles piceotrichosus (Blanchrad)	Argentina, Brazil and Venezuela
Oomyzus sokolowskii (Kurdjumov)	France, Switzerland, Italy, Hungary, Romania, Pakistan, India, Sri Lanka, Japan, China, North Korea, Egypt, Cape Verde Islands, Senegal, Benin, Kenya, South Africa, Canada, Brazil, Chile, Jamaica, Dominican Republic, Barbados, Trinidad,Martinique and Australia
Pupal parasitoids	
Diadromus collaris (Gravenhorst)	United Kingdom, Netherlands, Germany, France, Austria, Serbia, Romania, Bulgaria, Turkey, India, Japan, China, Malaysia, North Korea, South Africa, Australia, New Zealand, Fijic and Cook Islands
Diadromus subtilicornis (Gravenhorst)	Germany, Poland, Serbia, Romania, Finland, Russia, Japan, Canada and Dominican Republic

1.1.6.3.2 Bacteria

The gram-positive soil bacterium *B. thuringiensis* (*Bt*) produces parasporal crystalline inclusions during sporulation. These inclusions contain one or more glycoproteins known as insecticidal crystal proteins (ICPs) that are important in *Bt* pathogenicity to insects (Bauer 1995). The *Bt* formulations used against *P. xylostella* are based upon *Bt* kurstaki (*Bt*k) and *Bt* aizawai (*Bt*a) (Grzywacz *et al*. 2010). Most *Bt* formulations are mixtures of toxins, for example, *Bt*k is a cocktail of Cry 1 Aa, Cry 1Ab, Cry 1Ac, Cry 2a2A and Cry 2B toxins while *Bt*a is a mixture of Cry 1Aa, Cry 1Ab, CryIC, Cry ID and Cry 2B toxins (Heckel *et al*. 2004). The *Bt* toxin must be ingested by a susceptible host to be effective. Mechanism of action involves dissolving of the toxin upon ingestion, and cleavage of the toxin with midgut proteases before binding to the midgut receptors. The toxin is inserted into the membrane of cells creating pores which lead to death of the larvae (Schnepf *et al*. 1998). *Bt* toxins have a narrow host range, they can be genetically manipulated and are environmentally safe because they have little or no effect on humans and beneficial insects (Tabashnik 1994; Bauer 1995).

The prolonged and frequent use of *Bt* products has led to the appearance of resistance in field populations of *P. xylostella* in the Philippines (Kirsch and Schmuttere 1998), Malaysia (Iqbal *et al*. 1996), Thailand and China (Liu *et al*. 1996; Wright *et al*. 1997; Perez and Shelton 1997). Resistance to *Bt* has not yet been reported from Africa (Grzywacz *et al*. 2010). Tabashnik (1994) speculated that any interference in the process of solubilisation, proteolytic activation, passage through the peritrophic membrane, receptor binding, membrane insertion, pore formation and osmotic lysis of the midgut cells can help the insect to develop resistance. A study by Van Rie *et al.* (1990) showed that resistance in a laboratory-resistant strain of *Plodia interpunctella* is associated with a change in a membrane receptor. Other possible mechanisms of resistance include inactivation of the toxin (Oppert 1999), degradation of toxin by increased protease activity (Shao *et al*. 1998) and removal of toxin by precipitation (Milne *et al*. 1998) or coagulation (Ma *et al*. 2005). In other cases, it has been shown that resistance can be due to the inability of the toxin to bind to the receptor or to form pores in the cell membrane (Ferre and Van Rie 2002; Griffitts and Aroian 2005), shedding off of the binding site from the midgut epithelium (Valaitis 1995) and rapid replacement of dead midgut cells by increased activity of stem cells (Loeb *et al*. 2001).

1.1.6.3.3 Virus

There are twelve virus families that infect insects (Erlandson 2008). Only six of the virus families are frequently observed in the insect population namely, ascovirus, iridovirus, polyonavirus, baculovirus, cypovirus and entomopoxvirus (Ibarra and Del Rincon-Castro 2008). However, baculoviruses are the most common viruses used for biocontrol of insect pests (Szewczyk *et al*. 2006). These viruses are usually highly host specific and pose little risk to beneficial insects and the environment (Groner 1990). A number of baculoviruses have been reported to infect *P. xylostella*. Wilding (1986) reported that granuloviruses (GV), a member of the baculovirus family showed promising levels of pathogenicity to *P. xylostella* and the *P. xylostella* granulovirus (PlxyGV) has been shown to be effective in controlling *P. xylostella* in small-scale field trials in Japan (Su *et al*. 1991) and in India (Rajagopalbabu *et al*. 2003). PlxyGV fed larvae were shown to consume less foliage compared with their healthy counterparts (Lu *et al.* 2004).

1.2 Baculoviruses

The discovery of the "wilting disease" of silkworms in the 16th century raised concern about insect disease (Benz 1986). In the early 20th century this disease was attributed to a virus infection, and in 1947, rod-shaped virions were visualised which are now known to be characteristic of *Baculoviridae* (Miller 1996). The family *Baculoviridae* comprises of a diverse group of arthropod-specific viruses which have been reported worldwide from over 600 host species (Martignoni 1986). Baculoviruses have been isolated from the insect orders of Lepidoptera, Diptera, Hymenoptera and the crustacea (Decapoda) (Blissard *et al.* 2000). The name *baculum*, meaning rod or rod shaped is used to describe the rod-shaped nucleocapsid that is characteristic of the family (Winstanley and O'Reilly 1999).

Baculoviruses have circular, double-stranded DNA genomes ranging in size from approximately 80 to 180 kbp, depending on the species, they are predicted to encode for up to 180 genes. The viral genome associates with protein, forming a nucleocapsid which is surrounded by a membrane envelope to form a rod-shaped virion. The enveloped virions are occluded in a granulin or polyhedrin protein coat which provides protection against ultraviolet light and mechanical stress (Van Regenmortel *et al*. 2000).

1.2.1 Taxonomy of baculoviruses

Baculoviruses used to be divided into two genera based on the morphology of the different occlusion bodies: nucleopolyhedrovirus (NPVs) and granulovirus (GVs). Recently, the International Committee on Taxonomy of Viruses (ICTV) accepted a new classification of *Baculoviridae* based on DNA sequence data. It preserves correlation with both occlusion body morphology and host taxonomic classification. In this new classification the family *Baculoviridae* is subdivided into four genera: alphabaculovirus (nucleopolyhedroviruses isolated from lepidoptera), betabaculovirus (granulovirueses isolated from lepidoptera), gammabaculovirus (nucleopolyhedroviruses isolated from hymenoptera) and deltabaculoviruses (nucleopolyhedroviruses isolated from diptera) (Jehle *et al.* 2006). Baculovirus isolates are named based on the host species from which they are isolated and the type of occlusion body they were first associated with either NPV or GV (Hunter-Fujita *et al.* 1998).

The major protein found in the nucleopolyhedrovirus is polyhedrin, which is expressed very late in infection. Multiple occluded virus (OV) particles are each embedded within a polyhedron and may contain one or more nucleocapsid. This observation led to a grouping of the NPVs as single nucleopolyhedrovirus (SNPVs) containing one enveloped nucleocapsid per virion and the multiple nucleopolyhedrovirus (MNPVs) with multiple nucleocapsids per virion (Figure 1.4a). The other historic baculovirus genus, GV, has characteristic occlusion bodies that appear as ovoidal granules, with granulin as the major protein component. Usually GVs contain a single virion per occlusion body with only one nucleocapsid (Figure 1.4b) (Funk *et al.* 1997).

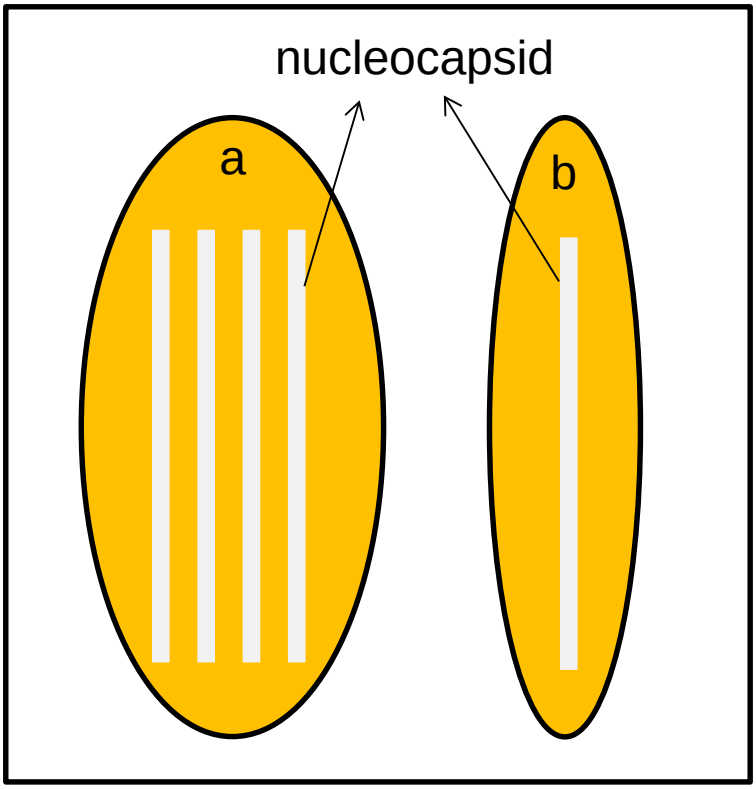


Figure 1.4 Baculovirus occlusion bodies (a) nucleopolyhedrovirus with multiple nucleocapsid (b) granulovirus with single nucleocapsid.

1.2.2 Baculovirus genome

A total of 58 baculoviral genomes have been fully sequenced and are available on the GenBank database. Forty one belong to the Alphabaculovirus, thirteen to the Betabaculovirus, three to Gammabaculovirus and one to the Deltabaculovirus (Ferelli *et al*. 2012). Baculoviruses encode 89 to 183 predicted open reading frames in both strands. Open reading frames (ORFs) are numbered consecutively in a clockwise direction.

Baculovirus genomes encode for 31 genes, known as the core genes, which represent a distinctive characteristic of the virus family and may play a role in some important biological functions (Miele *et al.* 2011). The core genes can be classified according to their functions: replication, transcription, packaging, cell cycle arrest and oral infectivity. There are some genes that are only present in the GVs, and not found in NPVs, which may be associated in the differential pathogenesis of these viruses. The most extensively studied baculovirus genome is that of *Autographa californica* MNPV (AcMNPV), which infects the alfalfa

loopers species and is often used as a model to describe baculovirus genome structure (Possee and Rohrmann 1997; Friesen 2007).

1.2.2.1 Replication genes

Baculovirus DNA replicates in the nucleus of the host cell using its own complement of genes encoding DNA replication proteins. There are four DNA replication genes found in all sequenced baculovirus genomes, namely *DNA polymerase*, *DNA helicase* (*p143*), *lef-1* and *lef-2* (Herniou *et al*. 2003). The LEF-1 protein is associated with primase activity while the LEF-2 protein is a primase accessory factor (Mikhailov and Rohrmann 2002). The LEF-1 and LEF-2 proteins are believed to form hetero-oligomeric complexes during baculovirus DNA replication (Evans *et al*. 1997). The DNA *polymerase* is responsible for polymerisation activity and DNA *helicase* for the unwinding of the DNA during replication (McDougal and Guarino 1999).

1.2.2.2 Transcription genes

Baculovirus transcription is divided into three stages namely, early, late and very late. The early genes are transcribed by host RNA polymerase II (Friesen 1997), while the late and vary late genes are transcribed by a viral RNA polymerase (Fuchs *et al.* 1983; Guarino *et al*. 1998). The late RNA polymerase consists of four subunits, coded by four core genes: *lef-4*, *lef-8*, *lef-9*, and *p47* (Guarino *et al*. 1998). The LEF-8 and LEF-9 proteins have motifs common to the subunits of the DNA-dependent RNA polymerases of prokaryotes and eukaryotes, with LEF-8 containing the essential C-terminal region conserved in RNA polymerases (Passarelli *et al*. 1994). The Mg^{2+} binding site of the catalytic centre of LEF-9 is similar to those found in other RNA polymerases (Ferrelli *et al.* 2012). The LEF-4 protein is an RNA capping enzyme and P47 protein does not show similarity with other RNA polymerase subunits and its function is not known (Van Oers and Vlak 2007).

Two other core genes are involved in late transcription, *lef-5* and *vlf-1*. The LEF-5 protein is proposed to be an initiation factor in AcMNPV (Guarino *et al.* 2002). VLF-1 protein is required for very late transcription and the expression of the very late genes *polyhedrin/granulin* and *p10* which is implicated in the morphogenesis of the occlusion body (McLachlin and Miller 1994). VLF-1 acts by interacting with promoter sequences which leads to the burst of expression observed from very late promoter driven genes (Yang and

Miller 1999). Transient expression assays revealed that other proteins such as LEF-6, LEF-10, LEF-12 and PP31 are also required for late transcription (Rohrmann 2011a).

1.2.2.3 Structural genes

Budded virus (BV) and occluded derived virion (ODV) differ in the protein composition of their envelope and origin, but their nucleocapsids are the same (Ferrelli *et al*. 2012). Structural proteins are not needed for the initiation of transcription of the viral DNA in the nucleus of the cell, hence the structural proteins present in virions are implicated in overcoming the barriers for cell entry (Ferelli *et al*. 2012). Many genes are encoded in the baculovirus genome whose products are included in the virion and occlusion body structure, as well as genes whose proteins are not present in the final structure but are essential for its assembly (Funk *et al*. 1997).

1.2.2.3.1 Occlusion body (OB)

Polyhedrin is the major OB protein of NPV, while granulin is the major OB protein of GV. Several other proteins apart from the major occlusion protein play a role in OB morphogenesis. A protein layer surrounds the polyhedrin/granulin which enhances the ability of the OB to dissolve in the midgut (Gross *et al.* 1994). The protein layer that encloses the major occlusion protein is calyx/PE which is associated with fibrillar structures formed by P10 during OB formation. P10 is not part of the OB but plays a role in its correct morphogenesis (Williams *et al.* 1989).

Other proteins associated with the polyhedrin/granulin are virus enhancing factor (VEF) and proteinases, these proteins depend on the species (Ferelli *et al*. 2012). For example, *Xestia c-nigrum* GV (XcGV) contains four copies of enhancins (Hayakawa *et al.* 1999). Enhancins help in degrading mucin and aiding ODVs to pass through the PM (Wang and Granados 1997). OBs were found to be associated with alkaline proteinases which aid in the dissolution of OBs and therefore the release of ODVs (Rohrmann 2011b). However there is no gene identified in baculovirus genome coding for these enzymes and it is thought that they may be of bacterial or insect cell origin (Rohrmann 2011b).

1.2.2.3.2 BV and ODV

Baculovirus genomes are enclosed within viral protein coats to form nucleocapids. The nucleocapsids acquire a virus modified plasma membrane envelope as they bud from infected cells to form budded virus (BV) (Ferelli *et al*. 2012). Occluded viruses (OVs) are produced during late stage of infection and their nucleocapsid obtains an envelope from the nuclear membrane prior to being occluded (Herniou *et al*. 2003).

Genomic DNA interacts with proteins to form the nucleocapsid. Four genes are required for the formation of nucleocapsid structure namely, *p6.9, vp39, vp1054* and *vp91* (Herniou *et al*. 2003). Condensation of DNA within the nucleocapsid is facilitated by the P6.9 protein (Kelly *et al.* 1983). VP39 is a major nucleocapsid protein (Thiem and Miller 1989; Faulkner *et al*. 1997). VP1054 and VP91 are associated with BV and OV (Olszewski and Miller 1997; Russell and Rohrmann 1997). Other core gene products associated with the nucleocapsid and found in both BVs and ODVs are GP41 (tegument protein), 38K, P49 and ODV-EC27 which form part of the virion structure (Ferelli *et al*. 2012).

Studies on the proteins included in ODV structure of AcMNPV (Braunagel *et al*. 2003), *Culex nigripalpus* NPV (CuniNPV) (Perera *et al*. 2007), *Helicoverpa armigera* NPV (HearNPV) (Deng *et al*. 2007) and *Pieris rapae* GV (PrGV) (Wang *et al*. 2011), and the BVs of AcMNPV (Wang *et al*. 2010) by mass spectrometry revealed that baculovirus virions are complex and may contain some host proteins in addition to virally encoded proteins.

1.2.2.3.3 Genes essential for oral infectivity

Six core genes are involved in the oral infection of insects namely *p74*, *pif-1*, *pif-2*, *pif-3*, *pif-4* and *pif-5* (*odv-e56*) (Faulker *et al*. 1997; Miele *et al*. 2011). PIF-1, PIF-2 and PIF-3 proteins associate with P74 protein to form a stable complex on the surface of AcMNPV ODV (Ferelli *et al*. 2012). These four proteins were proposed to form an evolutionarily conserved complex on the ODV surface that may play an important role in the initial stage of infection (Pang *et al*. 2001). Fang *et al.* (2009) observed that PIF-4 was essential for oral infection of AcMNPV in *Trichoplusia ni* larvae. Studies by Sparks *et al.* (2011) and Xiang *et al*. (2011) revealed that ODV-E56 was necessary for oral infection in AcMNPV and *Bombyx mori* NPV (BmNPV).

1.2.2.4 Auxiliary genes

Auxiliary genes are genes whose products are not directly required in viral gene expression, genome replication or formation of the progeny virus (Herniou *et al*. 2003). Analysis of 57 baculovirus genomes revealed the presence of 895 different genes and 31 core genes that are present in all the 57 genomes (Miele *et al*. 2011). Due to the high number of different genes Ferelli *et al*. (2012) suggested that some genes may not be essential but can modulate the infection. In addition, some genes might have developed a particular function and play a role only in specific virus-host interactions.

1.2.2.5 Genes affecting cellular metabolism

Apoptosis is one of the defence mechanisms of host cells against the invasion and spread of viral pathogens. All baculoviruses genomes encode for anti-apoptotic genes in order to complete their replication cycle. Anti-apoptotic genes are divided in to *P35/P49* homologues and *IAPs* (Ferelli *et al*. 2012). The action of caspases is directly inhibited by P35/P49 and this protein has been found in some NPVs and one GV (Escasa *et al*. 2006). IAPs act at a later stage in the apoptotic pathway (Van Oers and Vlak 2007).

The removal of free radicals in infected haemocytes is aided by a gene coding for a superoxide dismutase (*sod*) and this gene is widely distributed among baculoviruses (Rohrmann 2011c). Lepidopteran baculoviruses encode a viral ubiquitin which inhibits steps in the host degradative pathways in order to stabilise viral proteins that could have been degraded by the host enzymes (Haas *et al.* 1996).

1.2.2.6 Genes affecting the insect host

Lepidopteran baculoviruses cause systemic infection of the host by spreading of BVs through the insect tracheal system (Miller and Lu 1997). Some baculoviruses such as alpha and betabaculoviruses carry genes that encode for viral *fibroblast growth factor* homologues (*v-fgf*) in their genomes. This gene is absent in Gamma and Delta baculoviruses which cause midgut-restricted infections (Ferrelli *et al*. 2012). Passarelli (2011) observed that the presence of *v-fgf* accelerates larval death as deletion of these genes in AcMNPV and BmNPV caused retardation in host death compared to infection with wildtype viruses.

Another gene product that affects the insect host is ecdysteroid-UDP-glycosytransferase (egt) which mediates the inactivation of the moulting hormone ecdysone in insects in order to produce large amounts of progeny virus (O'Reilly and Miller 1989), this gene is described in detail in Chapter 4 Section 4.4. The liquefaction of larvae after death is mediated by the two viral encoded enzymes namely cathepsin and chitinase (Hawtin *et al.*1997).

1.2.3 Baculovirus life cycle

Baculoviruses produce two different virion phenotypes during their life cycle (Figure 1.5). The budded virus (BV) is responsible for the spreading of the disease inside the insect body and appears early in infected cells. The second virion, occluded virus (OV), is produced very late in the infection and becomes enclosed in a protein matrix forming a structure known as an occlusion body (OB) which is responsible for the horizontal transmission of the virus from one insect to the other (Figure 5). OBs protect the virions from damage in the environment during the non-infectious stage of the viral life cycle (Ferelli *et al*. 2012).

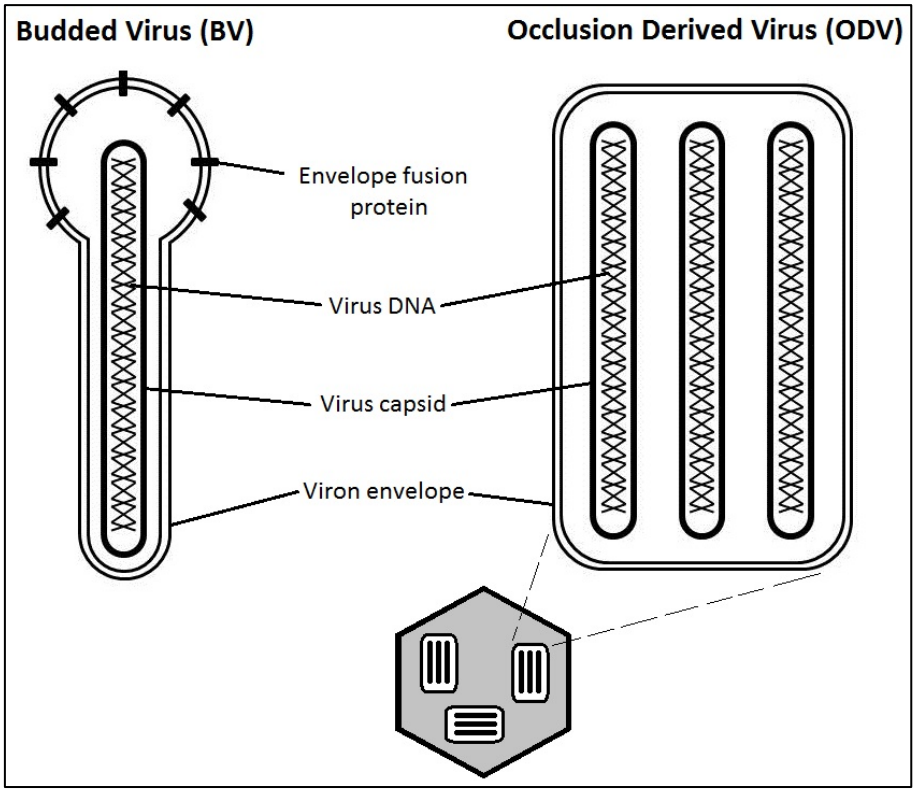


Figure 1.5 Budded virus and occlusion derived virus structure of a typical NPV (Funk *et al.* 1997).

Baculoviruses infect insects when the OBs are ingested by susceptible species along with the food (Figure 1.6, panel A). The highly alkaline conditions of the insect midgut dissolve the protective protein of the OB (Figure 1.6, panel B) and release the infectious virion referred to as an occlusion derived virion (ODV) (Pritchett *et al*. 1984; Rohrmann 2008a). The released ODV passes through the peritrophic membrane of the gut (Figure 1.6, panel C) and fuses with the microvilli on the columnar epithelial cells (Faulkner *et al*. 1997; Haas-Stapleton *et al*. 2004). This releases the nucleocapsids, which enter the cytoplasm and migrate to the nucleus. This initiates the first replication cycle of the virus that is restricted to cells of the midgut. The nucleocapsid then unwinds, the DNA is exposed and virus replication begins (Friesen 1997; O' Reilly *et al*. 1992). The nucleus of the infected cell becomes swollen and enlarged at 8 hours post infection as progeny virus starts to be produced. These progeny virus particles are budded virus and consist of naked nucleocapsids. These migrate to the outside of the cell where they acquire an envelope and protein structures called peplomers containing a particular 64 k protein at one end of the virion before leaving the cell (Washburn *et al*. 2003). At this point the first cycle of viral replication is complete and the second phase of infection in the other body tissues follows.

Just as the OB is the form of the virus designed to carry the infection from one insect larva to the other, the BV is the form in which the virus spreads from the initial site of infection in the midgut to the other tissues of the body. After the midgut cycle, the BV particles spread throughout the body in the haemolymph and infect other tissues of the host (Figure 1.6, panel D) (Harrap 1970; Washburn *et al*. 2003). It is in these tissues that a second cycle of infection occurs and the OBs are produced. The nucleocapsids acquire their envelope from the nuclear membrane, polyhedrin/granulin is synthesised at a later stage of infection and condenses to form crystals in which the nucleocapsid become embedded to form new OBs (Figure 1.6, panel E) and so complete the cycle. Cells containing large amounts of OBs burst and disintegrate and the massive destruction of the body that accompanies the production of OBs eventually kills the insect. The OBs are spread on the host's food material and the viral life cycle begins again. The virus can however also be present in the host without showing any viral symptoms.

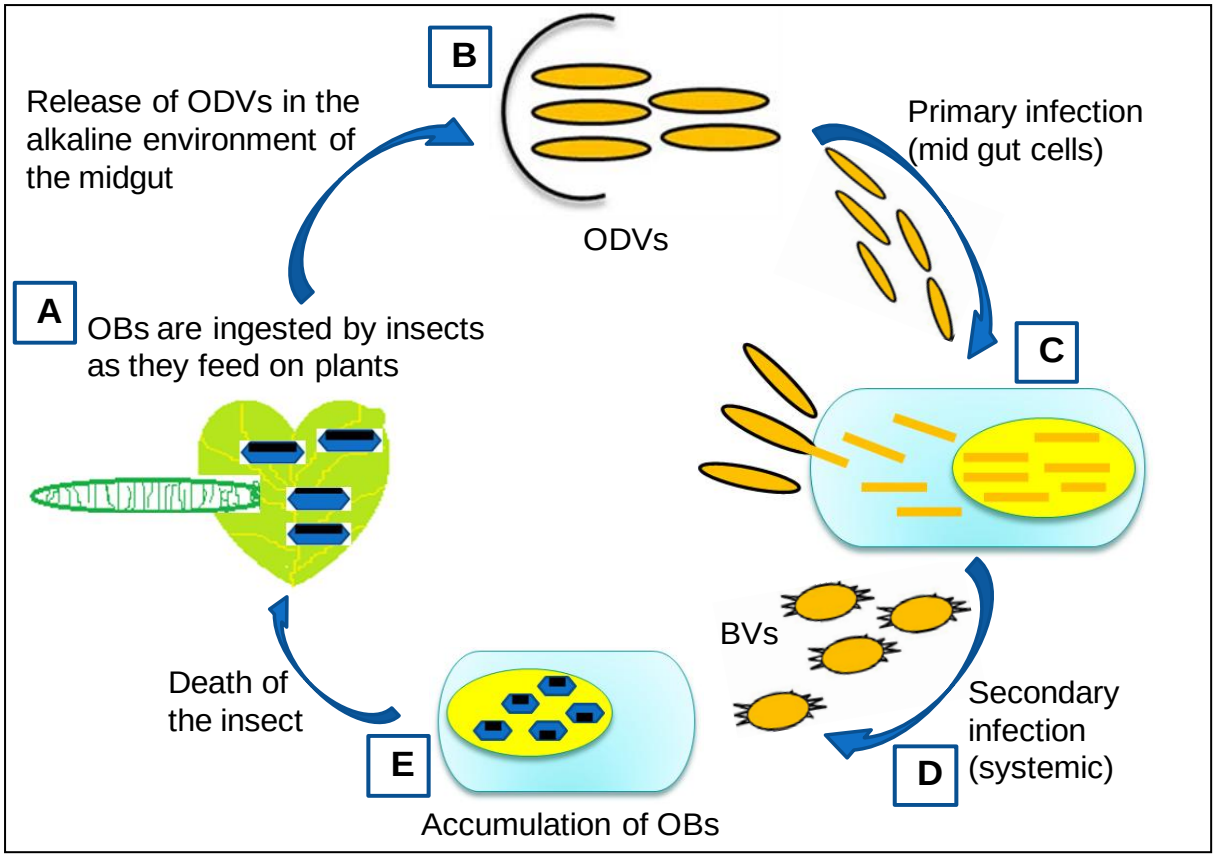


Figure 1.6 Summarised lifecycle of baculoviruses; (A) occlusion bodies are ingested orally by larvae along with food material, (B) alkaline conditions of the insect midgut dissolve the OBs, (C) ODVs infect the epithelial cells of the midgut, (D) BV is produced which infects other cells of the body, (E) OBs are produced and finally the larva disintegrates and the OBs are spread in the environment.

1.2.4 Covert Baculovirus infection

Virus infection does not always result in the death of the host insect and some insects are observed to complete their developmental cycle and reproduce. This type of infection is referred to as covert infection. Covert infection is a persistent sub-lethal infection that is asymptomatic and can be vertically transmitted. The mechanism of baculovirus covert infection in an insect population is not well understood (Il'inykh 2007). However, covert infection is speculated to result from overcoming the stage of acute virus infection by immune response or metamorphosis. It is also thought that a convert infection results from a compromise between the apoptotic cell responses of the virus infected insect and the ability of baculoviruses to block apoptosis (Il'inykh and Ul'yanova 2005). For instance Lee *et al.* (1998) reported that deletion of the apoptotic suppressor gene p35 can result in covert virus infection. During covert infection viral genes are expressed at low levels and the insect host

do not show any obvious symptom of the virus (Berretta *et al*. 2013). Covert infection can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR) as was confirmed in a study conducted in England by Burden *et al.* (2003) where gene expression of *Mamestra brassicae* NPV was detected by RT-PCR amplification of the MbNPV polyhedrin gene in 10 geographically distinct field populations of cabbage moth (*Mamestra brassicae*).

Unfavourable conditions such as stress can result in the activation of covert virus infection to lethal overt state which can cause death of host carriers (Il'inykh and Ul'yanova 2005). Low temperature was observed to induce nuclear polyhedral disease in gypsy moth (*Lymantia dispar dispar*) (Il'inykh 2000). Co-infection with another virus was found to induce overt virus infection in gypsy moth (Wood 1988) and forest tent caterpillar (*Malacosoma disstria*) (Cooper *et al.* 2003). Alteration of diet was also found to be effective in virus induction. Lindroth *et al.* (1991) observed that rearing of gypsy moth on a vitamin-deficient artificial diet can activate virus infection. Similarly, treatment of food with 0.6% copper sulphate was also found to be effective in inducing baculovirus infection in gypsy moth (Il'inykh 2000). Recently Opoku-Debrah *et al.* (2013) used overcrowding as a means of inducing baculovirus infection in false codling moth (*Cryptophlebia leucotreta* (Meyrick)) and recovered five new isolates of CrleGV-SA.

1.2.5 Resistance and reduced susceptibility to baculovirus infection

Variation in susceptibility to baculovirus infection has been reported among geographically distinct field populations of insects (Briese 1986). Recently, resistance has been reported from some field populations of codling moth, (*Cydia pomonella* (Linn)) to a Mexican isolate of *Cydia pomonella* granulovirus (CpGV-M) (Fritsch *et al*. 2005; Eberle and Jehle 2006; Berling *et al.* 2009). Bioassays in the laboratory showed that the median lethal concentrations (LC_{50}) of field populations increased up to 1000-fold suggesting a high resistance to CpGV (Eberle and Jehle 2006). However, there are some reported cases of host resistance under laboratory conditions. Some laboratory strains of potato tuber moth, *Phthorimaea operculella* (Zeller) were found to show low susceptibility to *Phthorimaea operculella* granulovirus (PhopGV) by having an LC_{50} value 30 times greater than that of the field populations (Briese and Mende 1981). In some cases, it was observed that resistance to virus infection decreased in subsequent generations when exposure to the virus was discontinued (Briese 1986; Fuxa and Richter 1989).

Some of the mechanisms that insects use to overcome virus infection include physical barriers and systemic response (Rohrmann 2008b). Shedding off of the epithelial cells of the midgut during moulting can lead to the destruction of virus infected cells (Federici 1997; Sun 2005). Reduced susceptibility of velvet bean larvae (*Anticarsia gemmatalis* (Hubner)) to AgMNPV was observed to be correlated with increasing thickness of the peritrophic membrane (Levy *et al.* 2007). Late instar larvae possess a thicker peritrophic membrane (protective lining of the midgut) which makes it difficult for the virus to penetrate (Federici 1997). A study by Haas-Stapleton *et al*. (2005) showed that the ODVs of AcMNPV were observed to have a reduced affinity to the midgut cells of fall armyworm (*Spodoptera frugiperda* (J.E Smith)) compared to ODV of fall armyworm (SfMNPV) indicating that SfMNPV might bind to a different receptor.

1.2.6 *Plutella xylostella* granulovirus

A granulovirus infecting *P. xylostella* was first isolated in Japan in 1970 (Asayama and Osaki 1970). The virus was later isolated from *P. xylostella* populations in India (Rabindra *et al*. 1997), China (Kadir *et al.* 1999a) and Kenya (Parnell *et al*. 2002; Muthamia *et al*. 2011). To date, PlxyGV-Japan is the only full genome sequence available in the National Centre for Biotechnology Information (NCBI) database. The genome of PlxyGV-Japan is 100999 nucleotides in size and has a G + C content of 41% (Hashimoto *et al.* 2000b). The PlxyGV-Japan genome contains 120 ORFs; 63 of the ORFs are oriented to the right while the remaining 57 are oriented to the left (Hashimoto *et al.* 2000b). Comparison of the genomes of the Taiwanese and Chinese isolates (Kadir *et al.* 1999a), the Kenyan and Taiwanese isolates (Parnell *et al.* 2002), and the Kenyan and Indian isolates (Subramanian *et al*. 2008) by REN analysis revealed that all the isolates contain major band similarities along with some unique sub-molar bands. The symptoms of infection for larvae infected with PlxyGV include swelling of the larval integument, colour change from bright green to pale yellow and finally death of the larvae (Asayama and Osaki 1970; Parnell *et al*. 2002; Dezianian *et al*. 2010).

1.2.7 Morphological and genetic characterisation of baculovirus isolates

Transmission electron microscopy can be used to classify baculoviruses as NPVs or GVs based on the morphology of the OB. Restriction endonuclease (REN) analysis can be used to determine genetic variation among different baculovirus isolates (Goto *et al*. 1992). This

technique also allows the estimation of the genome size. REN analysis is a process whereby total genomic DNA is extracted from the virus and digested with a range of restriction enzymes. The resulting DNA fragments can be separated according to their sizes by agarose gel electrophoresis to form specific restriction patterns known as DNA fingerprints.

Another method of identifying and distinguishing between different baculovirus isolates is by full genome sequencing, it provides an enormous amount of information about the organism. The nucleotide sequence obtained is subjected to BLAST (Basic local alignment search tool), for comparison and alignment with other related organisms. Single nucleotide polymorphisms (SNPs) can be used to track polymorphisms between organisms. Full genome sequencing produces accurate and reliable data but is expensive and the data analysis is time consuming. Alternatively, specific genes of interest can be amplified and sequenced using the polymerase chain reaction (PCR). PCR is the process of amplifying a segment of DNA, using sequence specific oligonucleotides, and the amplified segment can then be sequenced to determine the exact order of nucleotide bases within the DNA molecule.

1.2.8 Determination of biological activity of baculoviruses

Biological assays are methods of estimation of dose-response or time-response relationships (Finney 1971). Bioassays are necessary in order to determine the virulence and speed of kill of virus isolates. Dose response assays are performed in order to determine the median lethal concentration (LC_{50}) which is the concentration required to kill 50% of the test insect population. This assay can also be used to estimate the median lethal dose (LD_{50}) which is the dose required to kill 50% of a given population of test insect. Where the determination of the precise dosage ingested by the larva is not possible, the LC_{50} is calculated. Moreover the LC_{50} value is considered more important than the LD_{50} when determining the amount of virus to be formulated because, in the field, it is difficult to determine the dosage of virus ingested by each larva (Moore 2002).

Time response assays estimate the median lethal time (LT_{50}) which is the time taken to kill 50% of the test insect population. A typical bioassay involves the application of the stimulus at a specified concentration to a subject, in this case insects and monitoring for response either death or growth (Finney 1971). The methods used for the determination of time-response relationship are the same as those applied for estimation of the dose-response;

techniques include surface dose, droplet feeding, diet incorporation, diet plug and egg dipping bioassays (Hunter-Fujita *et al*. 1998; Jones 2000).

For surface dose bioassay, a known concentration of virus is evenly spread on the surface of the leaf or on artificial diet. The inoculum is allowed to air dry before feeding the larva for a certain period of time. The larva is then transferred to fresh food material and monitored for response until pupation. With this method only the LC_{50} value can be determined. This method portrays the natural feeding habit of insects that are internal feeders (Moore 2002).

The diet incorporation bioassay, a known concentration of virus is directly mixed with the artificial diet. Larvae are fed with the virus treated diet until response or pupation, and LC_{50} is determined. This method is similar to the surface dose method except that the virus is incorporated in the diet rather than on the surface (Hunter-Fujita *et al*. 1998; Jones 2000).

A known concentration of virus suspension is evenly spread on a small portion of diet that can be consumed by the test larva within a specified period of time in the diet plug bioassay. After consumption of the diet the larva is transferred onto fresh diet and monitored for response until pupation. With this method both, lethal concentration and lethal time can be calculated (Hunter-Fujita *et al*. 1998; Jones 2000).

In an egg dipping bioassay, eggs are dipped in a known concentration of virus for a specific period of time and allowed to air dry. After hatching, the larvae are transferred onto fresh food material and are monitored for response until pupation. Lethal concentration can be calculated using this method (Jones 2000).

While in the droplet feeding bioassays neonate larvae are fed with a known concentration of virus suspension administered as droplets. The virus suspension is mixed with food dye in order to identify the larvae that ingest the inoculum by the colouration of the gut (Hunter-Fujita *et al*. 1998). The larvae are transferred to virus free food material and monitored for response until pupation. Lethal dose can be calculated using this method. The LD value is more suitable for comparison of different experiments because the amount of virus ingested by each larva can be calculated.

1.3 Baculoviruses in biocontrol

Baculoviruses are a beneficial family of viruses in terms of their potential for pest control (Funk *et al.* 1997). Several baculovirus products are commercially available for the control of

different insect pests (Szewczyk *et al.* 2011) (Table 1.4). Both NPVs and GVs are used as pesticides but the number of registered NPV formulations is larger than that for GV (Szewczyk *et al.* 2011). Some formulations such as Cryptogran® and Cryptex® contain the same baculovirus species but different isolates, and are distributed under different trade names.

The major baculovirus biopesticide products used in South Africa are Cryptogran® (River Bioscience, South Africa), Cryptex® (Andermatt, Switzerland), Helicovir™ (River Bioscience), Bolldex®, Madex® (both Andermatt) and Carpovirusine ® (Arysta LifeScience, France). Cryptogran® and Cryptex® are formulated with South African isolates of *Cryptophlebia leucotreta* granulovirus (CrleGV) as the active ingredient. They are used for the control of *Thaumatotibia leucotreta*, an important pest of citrus in South Africa. Moore *et al*. (2004a) reported a 70% reduction in *Thaumatotibia leucotreta* infestation within a period of 7 weeks post treatment with Cryptogran®. Helicovir™ and Bolldex ® are registered biopesticides in South Africa that are used for the control of *Helicoverpa armigera* which is a pest of economically important crops such as cotton, citrus fruits, wheat, tomatoes, maize and sorghum. Helicovir™ was formulated with a South African isolate of *Helicoverpa armigera* NPV. Field trials with HearNPV in the Eastern Cape showed an 84% reduction in crop damage and a 96% reduction in export rejection (Moore *et al*. 2004b). Two field trials with the formulated product Helicovir™ revealed a 67% and 99% increase in yield compared to untreated trees (Moore and Kirkman 2010). Madex® and Carpovirusine ® are used for the control of *Cydia pomonella* which is an important pest of apple, walnut, pear, apricot and peach.

Table 1.4 Examples of some commercial baculovirus biopesticides (Moscadi *et al*. 2011)

Host insect	Baculovirus	Product name	References
Adoxophyes orana	GV	Capex® 2	Cunningham (1995); Erlandson (2008)
Anticarsia gemmatalis	NPV	Coopervirus®	Moscardi (1999, 2007)
Cydia pomonella	GV	Madex®, Virosoft®	Vincent *et al.* (2007)
Cryptophlebia leucotreta	GV	Cryptex®, Cryptogran®	Kessler and Zingg (2008); Moore *et al*. (2004a)
Helicoverpa zea	NPV	Elcar™, GemStar®	Moscardi (1999); Erlandson (2008)
Helocoverpa armigera	NPV	Helicovir™	Moore and Kirkman 2010
Lymantria dispar	NPV	Gypcheck®	Erlandson (2008)
Neodiprion abietis	NPV	Abietiv®	Lucarotti *et al.* (2007)
Phthorimaea operculella	GV	Matapol®	Moscardi (1999)
Spodoptera exigua	NPV	Spod-X®, Ness-A®	Erlandson (2008)

1.3.1 Advantages and disadvantages of baculovirus biopesticides

The host specificity of baculoviruses makes them safer for use and good candidates for IPM programmes because non-target organisms are not threatened. Baculoviruses are recognised by the European Union as being amongst the safest biocontrol agents (Black *et al.* 1997). Several baculoviruses have been tested against non-target organisms in order to assess their safety and no adverse effect has been reported (McWilliam 2007). Baculoviruses are useful tools for pest management since they can control specific insects and leave beneficial insects unharmed.

Secondly, baculoviruses are safe for use in the environment because they do not leave toxic residues in the environment (Szewczyk *et al.* 2011). This is a major advantage over chemical pesticides which leave active chemical residues in the field and on the crops that may be harmful to humans and other beneficial organisms.

Another advantage of baculoviruses is that they can easily be applied along with other pesticides, providing that those pesticides do not degrade the virus particles. Research has shown that the viral activity of baculoviruses on the host is not affected when mixed with most other pesticides (Moscadi and Sosa-Gomez 1992). This approach is important in

resistance management and IPM programmes. Mixing of baculoviruses with chemical pesticides will also reduce the harmful effect of those chemicals in the environment because the concentration of the chemicals used will be reduced.

On the other hand, the disadvantages of baculoviruses biopesticides includes their narrow host range (Szewczyk *et al.* 2011), which limits their acceptability to farmers who want a broad spectrum control agent. This factor also makes it difficult for private companies to invest in the production and sales of some viral pesticides (Cunningham 1995). Also, the cost of production of biopesticides is expensive in developed countries because of the high cost of labour that is required in maintaining a large insect colony, thereby making the end product expensive. This factor does not allow biopesticides to be cost competitive with chemicals (Black *et al*. 1997; Moscardi *et al.* 1997). Another limitation of baculoviruses is that they infect larval stages only and should be targeted towards the first instar because older larvae require higher concentrations of virus to be effective (Washburn *et al*. 2003). The short persistence of the virus particles in the environment can also be regarded as a disadvantage because farmers may want a control agent that can persist for a long time in the field. The virus particles are rapidly degraded in the field mostly by solar radiation (Moscadi 1999). The half-life of virus particles is between 2-5 days in the environment (Jaques 1985). A study by Shapiro (1986) showed that baculoviruses are inactivated by ultraviolet radiation at wavelengths 280-310 nm. Generation of highly active radicals such as peroxide, hydroxyls and single oxygen by the sunlight have also been shown to contribute in the degradation of virus particles in the environment (Ignoffo and Gracia 1994). Finally, slow speed of kill is regarded as one of the major shortcoming of baculoviruses (Ignoffo and Gracia 1992). Infected larvae continue feeding on the plant throughout the period of infection, therefore continuing to cause damage to the plant.

1.3.1.1 Methods of overcoming baculoviruses limitations

Baculovirus activity can be enhanced by certain substances such as boric acid, extracts of neem tree (*Azidarachta indica* (A. Juss)), optical brighteners of the stilbene group and chitinase (Shapiro 1995; Cook *et al*. 1996; Morales *et al*. 1997). A study by Shapiro (1995) showed that formulation of baculovirus particles with optical brighteners of the stilbene group at the concentration of 0.01% protects the virus against solar radiation and increases its speed of kill. Viral enhancing substances have been shown to improve the virulence and

speed of kill of baculoviruses such as NPVs of *Helicoverpa zea*, *Heliothis virescens*, *Autographa californica*, *Spodoptera exigua*, *Trichoplusia ni*, and *Pseudoplusia includes* (Shapiro 1995; Zou and Young 1996; Fuxa and Richter 1998). Morales *et al.* (2001) reported that a mixture of optical brighteners with AgMNPV can also be used to manage resistance of *Anticarsia gemmatalis*, enhancing of virus activity and speed of kill.

Genetic engineering of baculoviruses also makes it possible to increase the speed of kill and reduce feeding of the insect host (Bonning and Hammock 1996). Genetic engineering involves the production of viruses that can express specific toxins such as *Bacillus thuringiensis* and arthropod venom toxins (Inceoglu *et al.* 2001), insect hormones such as prothoracic, diuretic and eclosion hormones (Elvira *et al.* 2010) and deletion of the *egt* gene (O´Reilly and Miller 1991). The feeding damage caused by larvae infected with a recombinant virus containing an arthropod toxin from scorpion *Androctonus australis* was reduced by 60 % and the speed of kill was 40% faster compared to the wild type baculovirus (Cory *et al*. 1994; Inceoglu *et al*. 2001). A study by Maeda (1989) showed that a recombinant *Bombyx mori* MNPV (BmNPV) containing a gene that encodes a diuretic hormone killed larvae 20% faster than the wild type baculovirus. Deletion of the *egt* gene can result in 30% faster killing of the insect host than the wild type virus (Szewczyk *et al.* 2011), and because the *egt* gene is not essential for virus replication, it can be replaced by a virulent gene that can enhance the insecticidal activity of the virus (Sun *et al.* 2004).

1.3.2 Potential of PlxyGV for the control of *P. xylostella*

A number of studies have been conducted in which the virulence of different PlxyGV isolates against laboratory populations of *P. xylostella* is described. A study by Parnell (1999) showed that Kenyan isolates of PlxyGV are pathogenic to the second instar larvae of *P. xylostella* using the surface dose method. Field trials with one of the Kenyan isolates (Nya-01) applied weekly at the rate of 3.0×10^{13} OBs/hectare revealed that PlxyGV is more effective than chemical insecticides in controlling *P. xylostella* populations. Muthamia *et al*. (2011) determined the biological activity of two Kenyan PlxyGV isolates, one from the field and the other from the laboratory against the second instar larvae using the surface contamination method. Both isolates were found to be pathogenic to *P. xylostella* with the potential to be developed as biocontrol agents. In another study, the median lethal concentration and speed of kill of the Kenyan and Indian PlxyGV isolates were compared by the surface dosing

method using early and late third instar larvae (Subramanian *et al*. 2008). It was also shown that the isolates did not differ in virulence but that the Kenyan isolates had a significantly faster speed of kill compared to the Indian isolate. It was concluded that these PlxyGV isolates have potential to be developed as biopesticides for the control of *P. xylostella*. In another study, a surface dose leaf disc method was used to evaluate the effect of different doses of PlxyGV-Taiwan on first, second and third instar larvae (Dezianian *et al*. 2010). It was found that younger larvae were more susceptible to infection than older larvae using the same virus concentration. In addition, the LC_{50} and LT_{50} values showed the virus to be highly pathogenic to its larval host indicating that it has potential for use as a biopesticide. A study by Kadir *et al*. (1999b) determined the susceptibility of *P. xylostella* neonate larvae to infection with PlxyGV-Taiwan, GmNPV and AcMNPV using the droplet feeding method. Neonate *P. xylostella* larvae were found to be susceptible to all the three viruses with GmNPV killing faster than the other two viruses. The result indicates that PlxyGV can be used as a selective biopesticide while GmNPV can be used where *P. xylostella* is a pest with other lepidopteran pests. Although results from the field trials of PlxyGV are promising, the only country that has registered PlxyGV as a commercial product is China (Sun and Peng 2007; Yang *et al*. 2012).

1.4 Statement of research problem

Plutella xylostella is a serious world-wide pest of Brassica crops. The global estimated cost of controlling this insect is estimated to be US $ 4-5 billion annually. The dependence on chemical pesticides for control has resulted in the pest developing resistance to most chemical insecticides. In addition, these chemicals have negative environmental implications and may affect non target species. Baculoviruses can be used as an alternative to chemical insecticides. Several baculovirus based products are used as effective pest control agents of a wide range of insects. These viruses are usually host specific and pose little or no risk to beneficial insects and the environment in general. Although research has been conducted in Taiwan, China, India and Kenya on PlxyGV, to date the virus has not been identified in South Africa where *P. xylostella* is a significant problem. This study will attempt to isolate a virus specific for *P. xylostella* and characterise it in terms of morphology, genotype and virulence.

1.4.1 Overall aim

The aim of this study was to isolate and genetically characterise a South African *P. xylostella* granulovirus and to test its biological activity against laboratory-reared insect hosts

1.4.2 Specific objectives were

1. To establish and maintain a stable laboratory population of *P. xylostella* for virus isolation and biological assays
2. To stress the insects and monitor them for viral symptoms
3. To purify virus occlusion bodies (OBs) from larval cadavers showing viral symptoms and to determine the size and morphology of the viral OBs using transmission electron microscopy
4. To genetically characterise the virus isolate using restriction endonuclease analysis of genomic DNA and PCR amplification and sequencing of selected viral genes
5. To determine the biological activity of the virus against the host population

The results of this research are described in the following chapters:

Chapter Two describes the establishment and maintenance of a laboratory colony of *P. xylostella*. The population of the insects increased rapidly due to prime growing conditions in the laboratory which in turn led to overcrowding and the appearance of larvae with viral symptoms.

Purified OBs obtained from larval cadavers showing viral symptoms were analysed in **Chapter Three** by transmission electron microscopy. The morphology and size of the viral OBs revealed the presence of a granulovirus.

In **Chapter Four** the virus isolate was genetically characterised by restriction endonuclease analysis of the genomic DNA and PCR amplification and sequencing of selected viral genes. Comparison of the REN profiles obtained with other profiles of PlxyGV isolates and BLAST analysis of amplified gene sequences confirms the isolation of a novel PlxyGV isolate in South Africa that is genetically distinct from other PlxyGV isolates.

The biological activity of PlxyGV-SA against the host neonate and fourth instar larvae was determined in **Chapter Five**.

Chapter Six is a general discussion of the major findings of the previous chapters and their relevance to *P. xylostella* management in South Africa.

CHAPTER TWO

Establishment and maintenance of a laboratory colony of *Plutella xylostella*

2.1 Introduction

This chapter describes the establishment of a laboratory colony of *P. xylostella* which is a prerequisite for a continuous supply of clean and healthy insects for downstream applications such as virus production, monitoring of insect life-cycle and laboratory bioassays.

Plutella xylostella can be reared either on artificial diet or cruciferous seedlings. A study by Shelton *et al*. (1991) compared percentage survival rates of a single population of *P. xylostella* reared on artificial diet and rape seedlings. The percentage survival of *P. xylostella* reared on artificial diet and on rape seedlings ranged between 4.40–54.20% and 24.00–56.50% respectively. Due to difficulties in establishment of *P. xylostella* colonies on artificial diet, several researchers prefer to maintain *P. xylostella* on cruciferous seedlings (Carpenter and Bloem 2002). For instance, Shelton *et al*. (1991) reported that *P. xylostella* colony reared on artificial diet requires a long time to flourish due to lower larval survivorship in the first generation.

Rearing of *P. xylostella* has been found to be effective using different cruciferous seedlings. For example rapeseed seedlings were used to rear *P. xylostella* (Koshihara and Yamada 1976), cauliflower and cabbage (Golizadeh *et al*. 2009), broccolis leaves (Marchioro and Foerster 2011), canola (Niu *et al*. 2013), rapeseed (Ebrahimi *et al*. 2008) and cabbage (Parnell 1999; Sow *et al*. 2013). In this rearing method, the various plants serve as both the oviposition substrate and food source for the *P. xylostella*. Some of the important factors to consider when setting up a laboratory colony include optimal growing temperatures and oviposition preference of the females. Temperature has been shown to be the most important factor influencing growth, development rate and survival of *P. xylostella* (Liu *et al.* 2002). *Plutella xylostella* was found to develop between temperatures 10-32.5°C (Liu *et al.* 2002), with shorter developmental time between 25 - 32.5 °C (Golizadeh *et al.* 2007). Female moths prefer to lay eggs on rough rather than on smooth surfaces (Gupta and Thorsteinson 1960) and lack of light during the day can stimulate oviposition (Talekar and Shelton 1993).

The aim of this chapter was to establish and maintain a healthy laboratory colony of *P. xylostella* from insects collected from a cabbage plantation outside Grahamstown. Specific objectives were first to monitor the life cycle of *P. xylostella* in order to estimate the developmental time from egg to adult and secondly, to measure the width of the head capsule in order to monitor development of the different instar stages.

2.2 Materials and methods

2.2.1 Maintenance of a laboratory colony of *Plutella xylostella*

Insects were collected from a cabbage plantation at a single site on Varnam Farm in the Belmont Valley outside Grahamstown (33°18'S 26°32'E) in the Eastern Cape, South Africa. Insects (pupae and larvae) were maintained in rearing chambers in a controlled environment (CE) room at 26 ± 1°C, 12:12 light and dark cycle and 50 ± 10% relative humidity. Adults were maintained in an adult emergence chamber (Figure 2.1, panel A and C). Canola seedlings (Klein Karoo Seed Marketing, South Africa) were placed in the chamber for oviposition, and cotton wool soaked in sugar solution was supplied as a source of nutrition. The adult nutrition and oviposition substrates were replaced every two days. Eggs were moved to a larval rearing chamber (Figure 2.1, panel B) and allowed to hatch on the canola seedlings before transfer to cabbage leaves (Figure 2.1, panel D) or new canola plants depending on what food source was available. Larvae were transferred to fresh cabbage leaves or canola plants at two day intervals with the aid of a paint brush and left there until pupation. Pupae were collected and returned to the adult rearing chamber for emergence and the cycle was repeated for later generations. Death larvae were collected and stored at -20°C for subsequent analysis.

Figure 2.1 *Plutella xylostella* rearing chambers (A) adult rearing chamber, (B) larval rearing chamber, (C) front view of the rearing chamber, (D) Larvae fed with cabbage leaves in 2L ice cream tub.

2.2.2 Life-cycle and identification of larval instar using head capsule size

A new canola plant was placed in the adult rearing chamber in order to obtain eggs laid on the same day. Sixty eggs were transferred into a Petri dish lined with moist filter paper, and the eggs were monitored until they hatched. The first 48 larvae were transferred into 24-cell well plates individually with the aid of a paint brush. Paper towelling was placed in between the wells and the lid of the plates to restrict larval movement, and masking tape was used to secure the lid firmly to the plate (Figure 2.2, panel A and B). Larvae were fed with cabbage

leaves only and leaves were changed at two day intervals. Images of all the life stages were captured. In order to determine the duration of each instar, images of the larvae were taken daily using a dissecting microscope (Leica® EZ4D). The width of the head capsules were measured using AnalySIS software (Olympus SZX16).

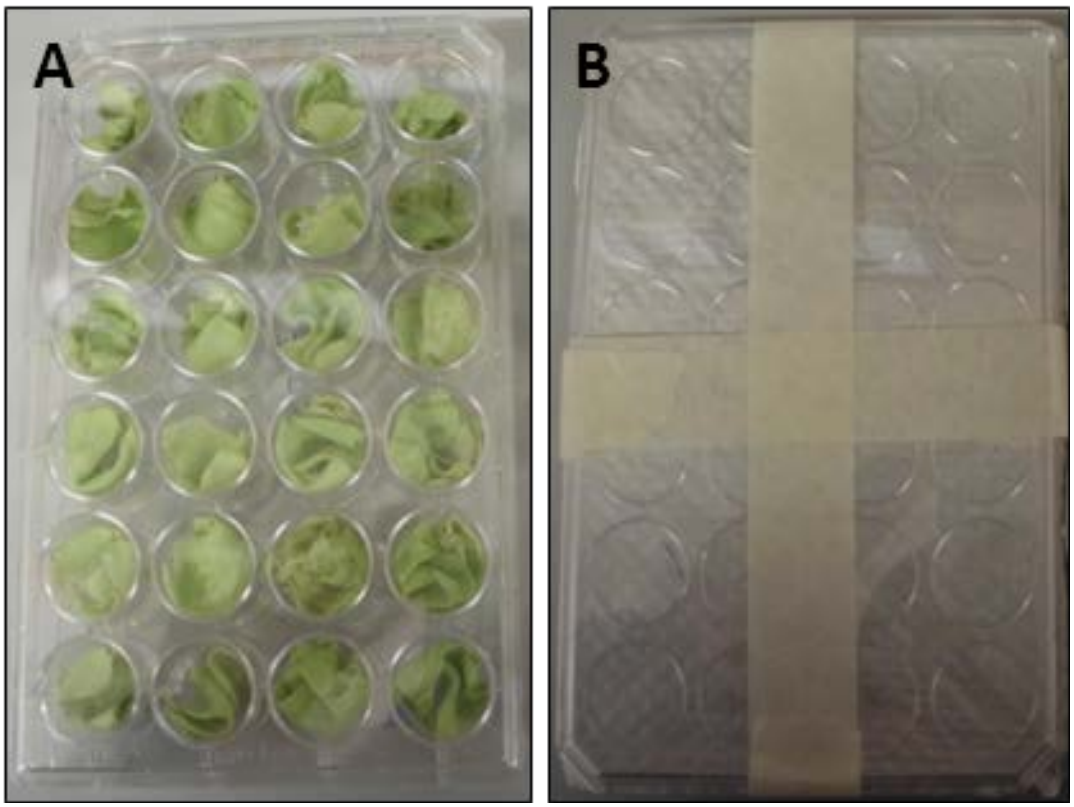


Figure 2.2 Controlled life monitoring of *P. xylostella* larvae on cabbage leaves (A) larvae placed individually in 24-cell well plates, (B) the 24-cell well plate sealed with paper towel and masking tape to prevent larval movement.

2.3 Results

2.3.1 Establishment and maintenance of a laboratory colony of *Plutella xylostella*

A laboratory population of *P. xylostella* was established. The population size increased rapidly due to females laying many eggs, availability of food and optimal growing conditions in the CE room. As the population increased, dead larvae were observed and collected daily. The larval cadavers were stored at -20°C for subsequent analysis. The number of dead larvae increased as the population of the colony increased in number. Sudden change of colour accompanied by swelling of larval segment was observed in larvae prior to death (Figure 2.3). First to third instar larvae changed from whitish yellow (Figure 2.3, panel A) to

yellowish brown with enlarged larval segments (Figure 2.3, panel B) while the fourth instar larvae changed from green (Figure 2.3, panel C) to pale yellow with swollen body segments (Figure 2.3, panel D). Overcrowding of the population eventually led to death of the colony which was subsequently re-established using pupae and asymptomatic larvae collected from the same field.

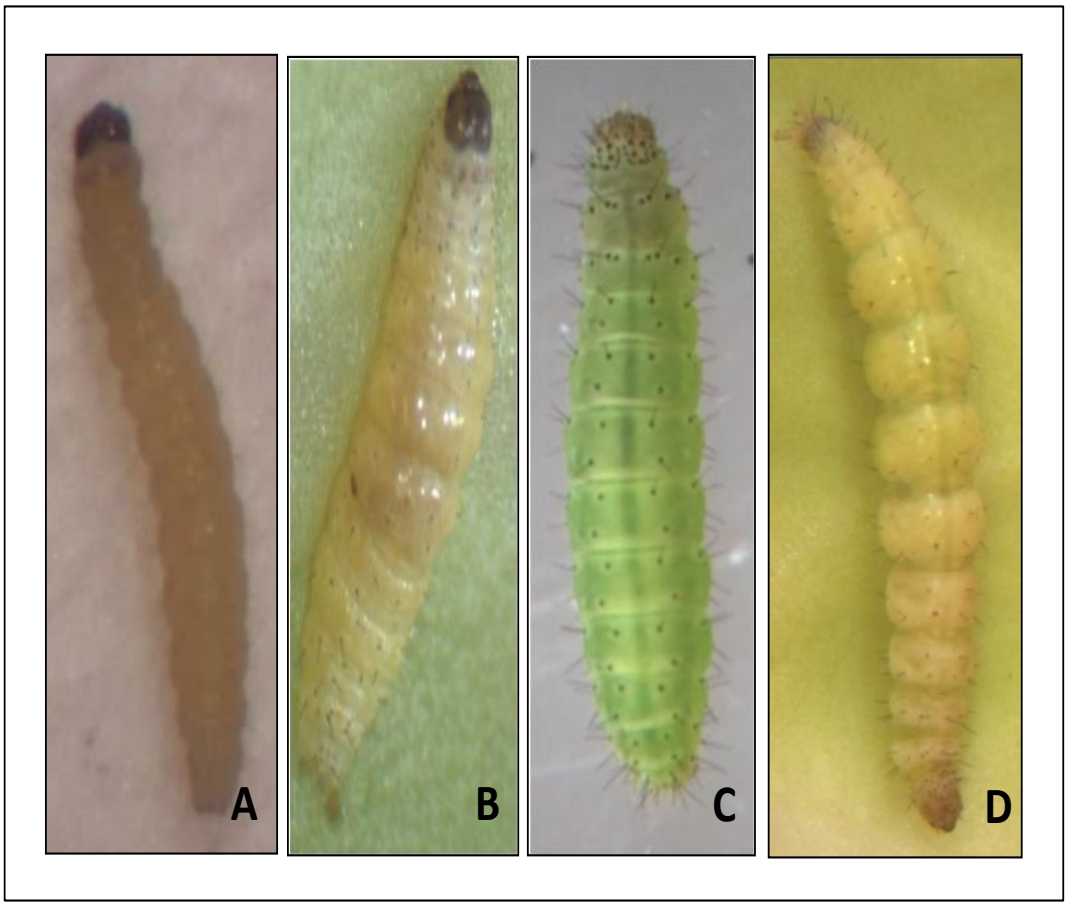


Figure 2.3 Comparison of healthy and diseased *P. xylostella* larvae (A) first or second instar larva before colour change, (B) first or second instar larva after colour change, (C) fourth instar larva before colour change, (D) fourth instar larva after colour change. Pictures taken by Fatima Abdulkadir (2013)

2.3.2 Controlled life cycle monitoring

Controlled life-cycle monitoring was performed in order to determine the duration of each stage from egg to adult. Eggs were observed to be oval in shape and yellow in colour, which changed to dark brown or black as they developed (Figure 2.4, panel A and B). First, second and third instar larvae were whitish yellow (Figure 2.4, panels C, D and E) while the fourth instar larvae were green in colour (Figure 2.4, panel F). Pupae were green or pale yellow

(Figure 2.4, panel G and H), changing to a brown colour as they developed (Figure 2.4, panels I). The adult moths were greyish brown with diamond patterns along the back (Figure 2.4, panel J and K). The diamond patterns were more distinct in the males (Figure 2.4, panel K) than in the females (Figure 2.4, panel J).

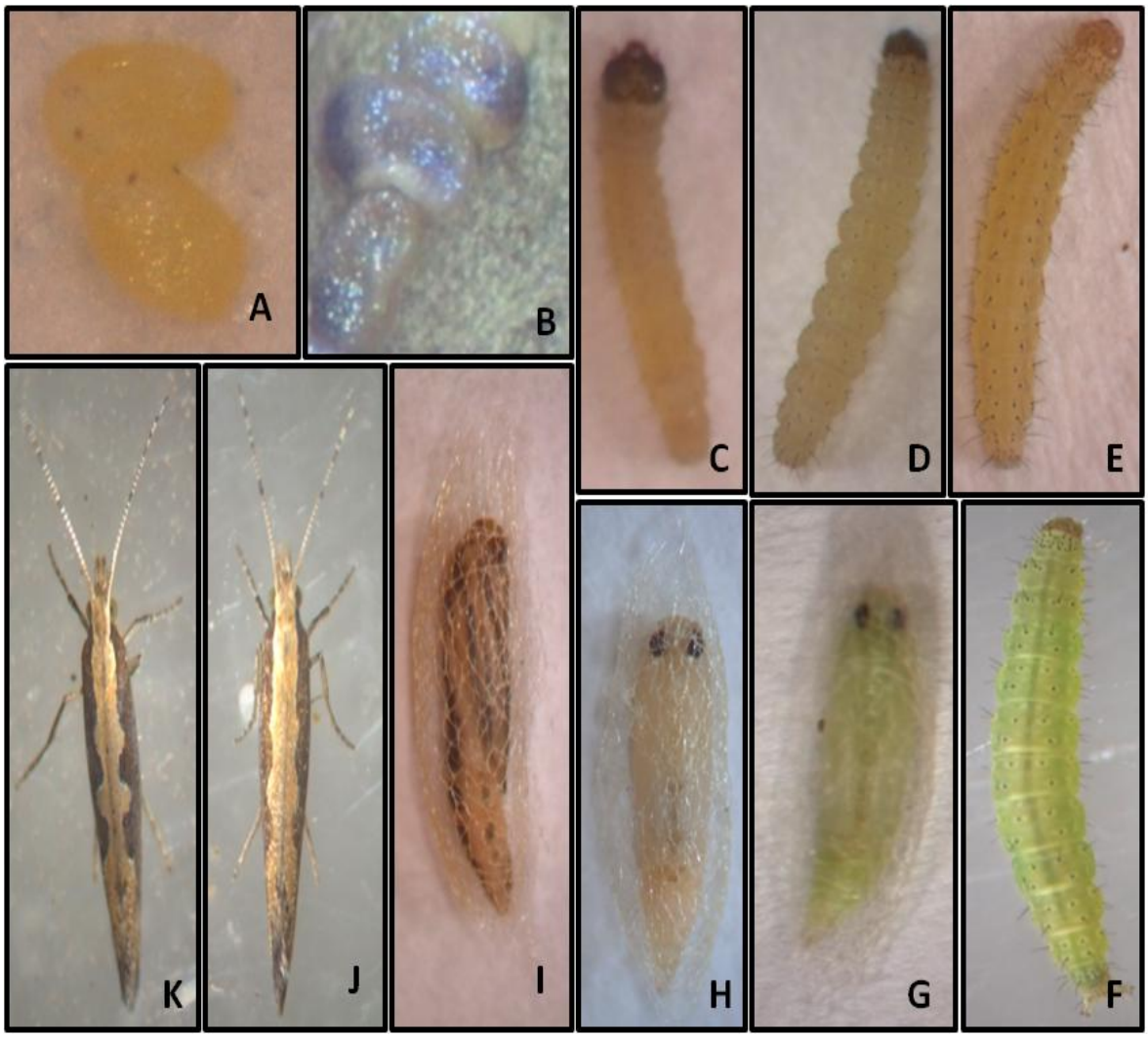


Figure 2.4 Various stages in the life cycle of *P. xylostella* (A) newly laid eggs, (B) developing egg, (C) fisrt instar larva, (D) second instar larva, (E) third instar larva, (F) fourth instar larva, (G) green pupa, (H) brown pupa, (I) developed pupa, (J) female adult moth, (K) male adult moth. Pictures taken by Fatima Abdulkadir (2013)

2.3.2.1 Duration of development of *Plutella xylostella*

The developmental time of the egg and the larval stages were determined at a temperature of 26 ± 1°C. It was observed that 51 out of the 60 eggs hatched successfully. The mean incubation period of the 51 eggs was 2.65 ± 0.12 days. Forty-eight larvae were used to

determine developmental time, but due to handling only 32 survived to the end of the experiment. The average developmental time of the 32 larvae for each of the four larval instars were 2.43 ± 0.08, 2.15 ± 0.09, 2.37 ± 0.08 and 1.81 ± 0.11 days respectively. The average total developmental time from the first instar to the fourth instar was 8.78 ± 0.12 days (Table 2.1). The average developmental time of the pupae was 3.25 ± 0.07 days and the total developmental time from egg to adult was 14.59 ± 0.21 days.

Table 2.1 Mean developmental time of *P. xylostella* on cabbage leaves

Stage	**Duration (day ± SE)**
Egg - first instar	2.65 ± 0.12
First - second instar	2.43 ± 0.08
Second - third instar	2.15 ± 0.09
Third - fourth instar	2.37 ± 0.08
Fourth - pupae instar	1.81 ± 0.11
Total larval stage	8.78 ± 0.12
Pupae – adult	3.25 ± 0.07
Total from egg to adult	14.59 ± 0.21

2.3.2.2 Measurement head capsule width

The four larval instars were identified based on measurement of the head capsule of 32 larvae. The head capsule widths for the first, second, third and fourth instar were 0.15 ± 0.01 mm, 0.25 ± 0.02 mm, 0.36 ± 0.01 mm and 0.61 ± 0.02 mm respectively (Figure 2.5, panel A, C, E and G). Towards the completion of each instar the outgrowth of a new head capsule resulted in pushing of the old one forward which gradually detached from the body as the instar changed. It was found that the width of the new head capsule that outgrew during the first instar was 0.21 ± 0.02 mm (Figure 25, panel B). Removal of the old head capsule resulted in the expansion of the new capsule to 0.25 ± 0.02 mm (Figure 2.5, panel C). The width of the head capsule that outgrew during the second instar was 0.33 ± 0.01 mm (Figure 2.5, panel D). Completion of the second instar resulted in increment of the head capsule width to 0.36 ± 0.01 mm (Figure 2.5, panel E). The diameter of the head capsule that outgrew during the third instar was 0.55 ± 0.03 mm (Figure 2.5, panel F). Removal of the old head

capsule resulted in enlargement of the outgrowing head capsule to 0.61 ± 0.02 mm (Figure 2.5, panel G).

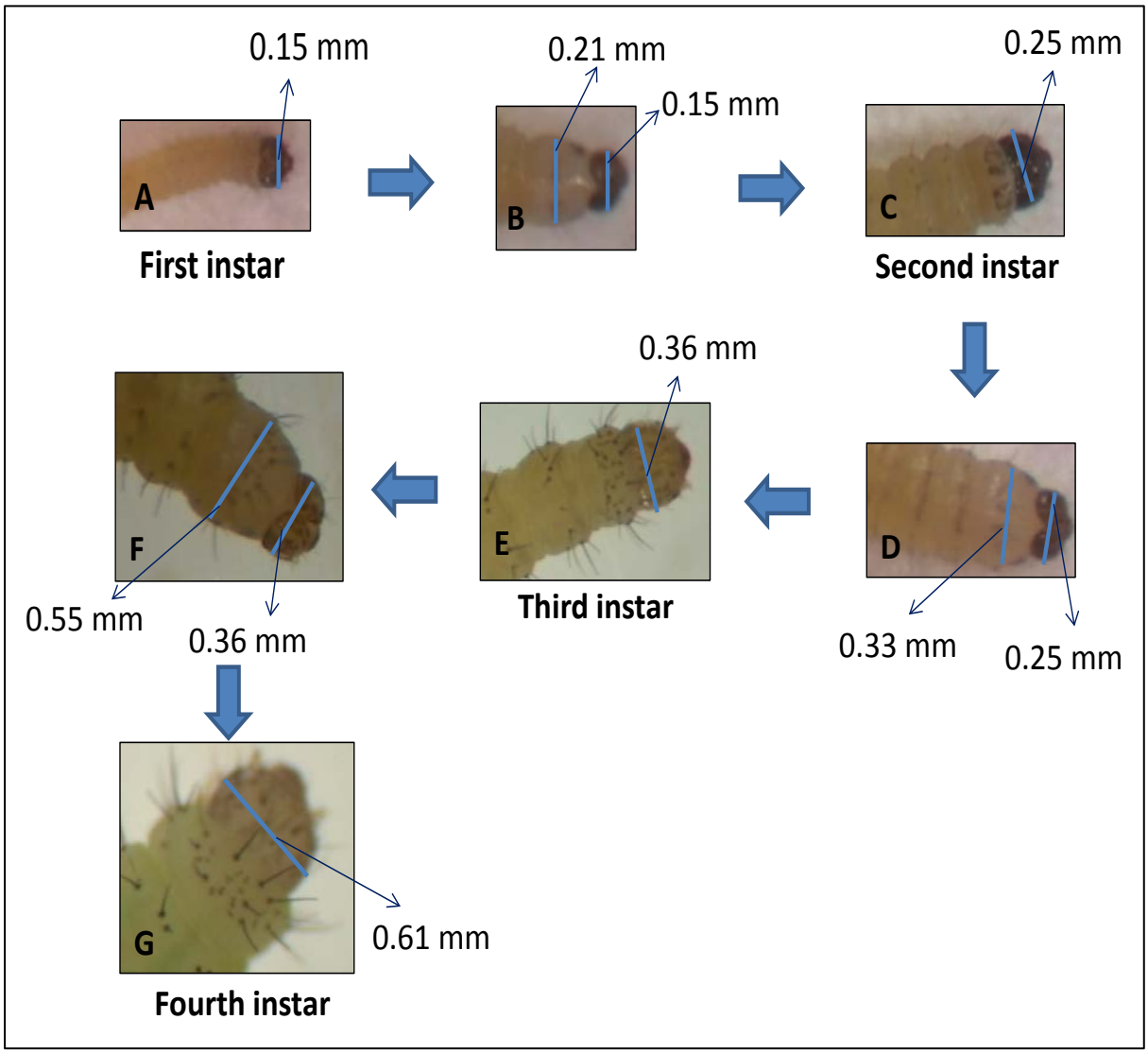


Figure 2.5 Head capsule width of *P. xylostella* larvae (A) early first instar larva (B) late first instar larva (C) early second instar larva (D) late second instar larva (E) early third instar larva (F) late third instar larva (G) fourth instar larva

2.3.2.3 Moulting of *Plutella xylostella* larvae

Plutella xylostella larvae moult three times during the larval stage (Talekar and Shelton 1993). The neonate larvae shed the exoskeleton and head capsule on completion of the first instar stage (Figure 2.6). The head capsule is replaced on completion of the second and third instar without shedding of the exoskeleton. The old head capsule of the first instar larva is pushed forward as a result of the outgrowth of a new one (Figure 2.6, panel A) on completion

of the first instar, the larva moults by removing the old head capsule (Figure 2.6, panel B). Immediately after the removal of the head capsule the exoskeleton is removed by quick movements of the larva (Figure 2.6, panels C, D and E). The larva appeared to be creamy white in colour after moulting (Figure 2.6, panel G) and after some few hours changes to the normal whitish yellow colour.

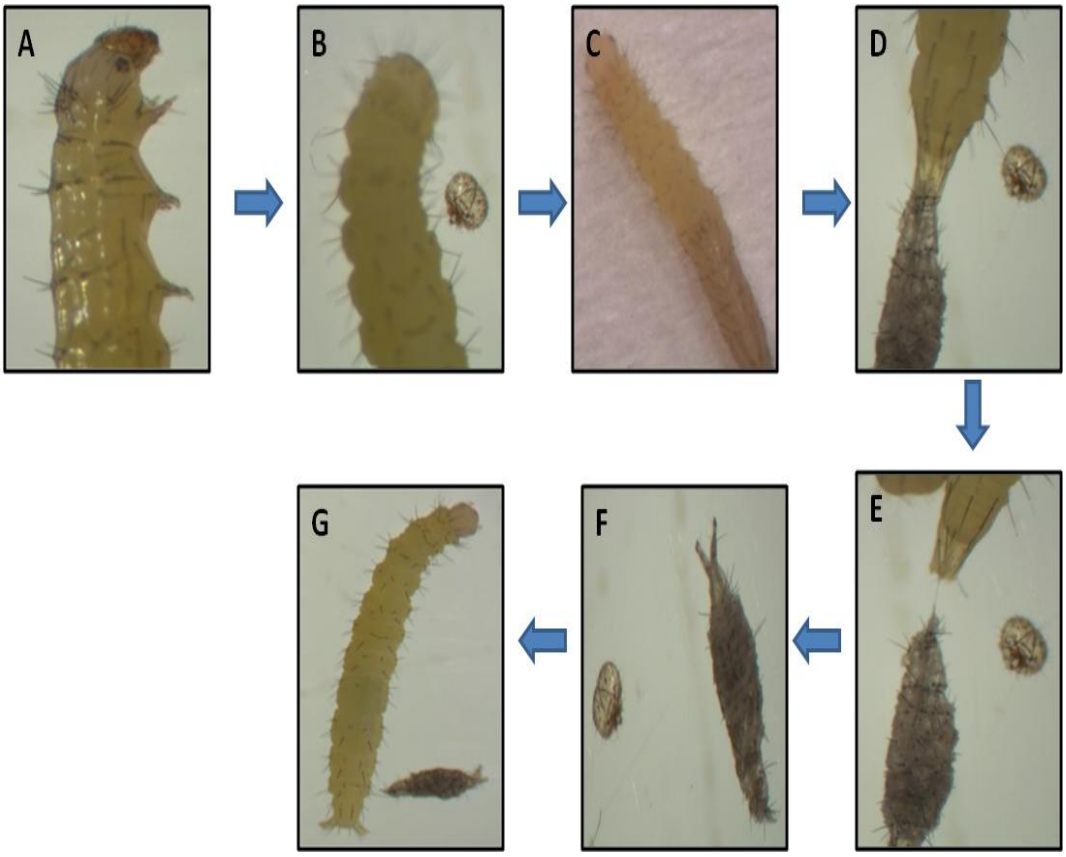


Figure 2.6 Moulting of a *P. xylostella* neonate larva (A) larva before moulting (B) larva sheds head capsule (C and D) larva shedding off exoskeleton (E) old exoskeleton is completely removed (F) shed exoskeleton and head capsule (G) larva after moulting

2.4 Discussion

A laboratory colony of *P. xylostella* was successfully established and the life-cycle monitored for a period of three weeks. The rapid increase of the population size in this study is mainly attributed to the optimal temperature (26 ± 1°C) at which the insects were reared. A previous study on the biology of *P. xylostella* by Talekar and Shelton (1993) reported that the developmental time and survival depends on temperature. Golizabeh *et al.* (2007) studied the development of *P. xylostella* under eight constant temperatures and found shorter

developmental time for temperatures between 25-32.5°C, longer developmental time for temperatures below 20°C and no growth at 35°C and above. A similar study by Marchioro and Foerster (2011) reported a high survival rate of *P. xylostella* larvae between temperatures 15-25°C and low survival rate at temperature 10°C and between 30-35°C. Liu *et al*. (2002) also reported a similar result and concluded that temperature can influence the population density of *P. xylostella.*

The second factor that may have favoured the rapid growth of this insect in the laboratory is the diet on which they were reared. Awmack and Leather (2002) stipulated that host plant quality is an important factor that determines the fertility and fecundity of herbivore species. The reproduction and development of *P. xylostella* has been reported to depend on the type of host plant. For example, Niu *et al*. (2013) studied the effect of different cruciferous crops on the survival and development of *P. xylostella*. They found that developmental time from egg to adult was shortest on canola and cabbage. The survival rate was higher on pak-choi and canola while the lowest survival was recorded on cabbage. In a similar study conducted by Golizabeh *et al.* (2009), it was reported that the longest developmental time and lowest survivorship of *P. xylostella* larvae was on canola. The variation between the two studies could be due to differences in the nutritional content of the host plant cultivar. A study by Fathi *et al.* (2011) investigated the performance of *P. xylostella* on nine cultivars of canola and found differences in the survival and developmental rate of *P. xylostella* larvae on the cultivars. Similar results were reported for *P. xylostella* on rapeseed cultivars (Sarfraz *et al.* 2007) and on cabbage cultivars (Hamilton *et al.* 2005). This suggests that the host plant can influence the rate of population growth of the insect.

Another factor that may have influenced the population density of the *P. xylostella* in this study is the high reproductive potential of the adult moth. It has been reported that the each female moth can lay up to 188 eggs within a period of four days (Harcourt 1954; Talekar *et al*. 1994; Justus *et al*. 2000). *Plutella xylostella* has been reported to complete four to twenty generations per year in the temperate and tropical regions (Harcourt 1986; Vickers *et al*. 2004).

The width of the head capsule changes only when the instar changes. This is in accordance with the findings of Dyar and Rhinebeck (1890) who studied 28 species of lepidopteran larvae and found that the size of the larval head followed an orderly progression in growth. The head capsule is a rigid sclerotised structure, and continuous growth of larvae results in

moulting of the structure and replacement of the old head capsule by a new one. The widths of the head capsule for the first, second, third and fourth instar larvae recorded in this study were in accordance with the values obtained in a similar study by Capinera (2000) for the American *P. xylostella* larvae.

In conclusion, a laboratory population of *P. xylostella* was successfully established and maintained. The colony flourished due to prime conditions in the laboratory, females laying many eggs and availability of food. The rapid increase in number resulted in overcrowding and stress due to the high population density, led to the death of the laboratory colony. The next chapter describes an investigation into the cause of larval death by purification of viral occlusion bodies (OBs) and transmission electron microscopy.

CHAPTER THREE

Isolation and morphological characterisation of *Plutella xylostella* granulovirus (PlxyGV-SA)

3.1 Introduction

In chapter two the successful establishment and maintenance of a laboratory colony of *P. xylostella* was described. The population increased rapidly due to optimal growing conditions in the laboratory leading to overcrowding and death of the colony. Numerous larvae showing symptoms similar to that of a viral infection were observed and collected for analysis. This chapter describes the purification of virus occlusion bodies (OBs) by glycerol gradient centrifugation and their morphological examination by transmission electron microscopy.

Overcrowding of insect colonies has been shown to result in the development of covert virus infection to overt lethal state. For example, a recent study by Opoku-Debrah *et al.* (2013) used overcrowding to recover baculovirus infection in false codling moth (*Cryptophlebia leucotreta* (Meyrick)). Larval cadavers in this study showed symptoms similar to those reported in other studies where larvae were collected in the field (Asayama and Osaki 1970; Parnell *et al*. 2002) and where colonies of *P. xylostella* were reared in the laboratory (Rabindra *et al*. 1997; Dezianian *et al*. 2010). A study by Parnell *et al*. (2002) surveyed 27 brassica farms in Kenya and collected 127 larvae showing symptoms such as change in colour from bright green to pale yellow accompanied by swelling of larval integument. Microscopic examination of the larval cadavers revealed that 95 larvae succumbed to a granulovirus infection. Baculovirus epizootics are speculated to be due to a covert baculovirus in the insect population which becomes apparent when the insects are subjected to stress conditions (Fuxa 1989).

In view of the above, the aim of this chapter was to investigate the cause of larval death by purifying baculovirus OBs from larval cadavers. The occlusion bodies were analysed by transmission electron microscopy to determine size and morphology. The concentration of OBs was determined by light microscopy.

3.2 Materials and methods

3.2.1 Purification of occlusion bodies (OBs)

Occlusion bodies were purified using a protocol described by Opoku-Debrah *et al.* (2013). Approximately 1.8 g of insect larvae was homogenised in 10 ml of 0.1 % sodium dodecyl sulphate (SDS), and the homogenate filtered through cheese cloth. The resulting insect debris was homogenised again in 10 ml of 0.1% SDS and filtered. The filtrate was transferred into two JA-20 (Beckman®) centrifuge tubes, the tubes were then filled up with double distilled water (ddH_2O) and centrifuged at 7840 ×*g* for 30 minutes at 4ºC using a Beckman® Coulter centrifuge (J-E Avanti). The supernatant was discarded, the pellet re-suspended in ddH_2O and re-centrifuged to remove any remaining insect debris. The supernatant was again discarded and pellet re-suspended in 3 ml of ddH_2O. A 30-80% (v/v) glycerol gradient was prepared in 0.1% SDS in a Beckman ultra-clear centrifuge tube (14 x 89 mm) by placing the gradient solutions on top of each other from the densest (80% v/v glycerol) to the least dense solution (30% v/v glycerol). The gradient was stored overnight at 4°C. Ultracentrifugation of the pellet was done on a 30-80% (v/v) glycerol gradient at 29774 ×*g* for 15 minutes at 4ºC using a Beckman® Coulter Optima (L-90 K) ultracentrifuge. Occlusion bodies visible in a band in the middle of the tube were collected using a pipette and transferred into Beckman JA-20 tubes. The tubes were filled with ddH_2O and centrifuged at 7840 ×*g* for 30 minutes at 4ºC. Centrifugation was repeated three times to remove all traces of glycerol. The resulting pellet was re-suspended in 750 µl of ddH_2O and stored at -20°C.

3.2.2 Transmission electron microscopy of occlusion bodies

Transmission electron microscopy by negative staining was done using the protocol described by Dezianian *et al.* (2010) with some modification. Approximately 5 µl of purified OBs was placed onto a carbon coated grid for 60 seconds, and the grid dried using filter paper. A drop of approximately 5 µl of 1% aqueous uranyl acetate was used to stain the OBs for 60 seconds, and the grid was dried again using filter paper. The grids were viewed using a Zeiss Libra 120 transmission electron microscope at 80000 kV and images captured using Mega view (G2) Olympus analysis software. The size of OBs was measured using Olympus analysis software (Olympus SZX16) and the mean size and standard error was calculated using Microsoft Office Excel® 2007.

3.2.3 Enumeration of occlusion bodies

The concentration of OBs was determined using the light microscopy method described by Hunter-Fujita *et al*. (1998) and Jones (2000). The virus stock was vortexed to mix virus particles before making a 1:5 dilution of one part virus and four parts double ddH_2O. The suspension obtained was used to make another 1:5 dilution with 1% SDS. A further dilution of 1:60 was made with the resulting virus-SDS suspension and ddH_2O. The Helber counting chamber (0.02 mm depth, Hawksley®) and the cover slip was cleaned with 70% ethanol before placing 5 µl of the diluted OBs onto the centre of the counting chamber. The cover slip was placed over the drop and the slide was allowed to stand for 5 minutes to sediment the virus particles. The slide was viewed at 400 x magnification under dark field microscopy. Moving virus particles from five blocks were counted: four blocks from the corners of the counting chamber (top right, bottom right, top left and bottom left) and one block from the middle which was randomly selected. The procedure was repeated using the same sample and the mean number of particles was used to calculate the concentration of the virus using the following formula.

$$OBs/ml = \frac{D \times X}{N \times V}$$

Where D: dilution of suspension, X: number of occlusion bodies counted
N: number of small squares counted, V: volume in ml

3.3 Results

3.3.1 Purification of viral occlusion bodies

Viral occlusion bodies were successfully purified on a 30-80% glycerol gradient. Cell debris was observed to sediment at the bottom of the tube while a white milky virus band appeared in the middle of the tube as indicated by the arrow in Figure 3.1.

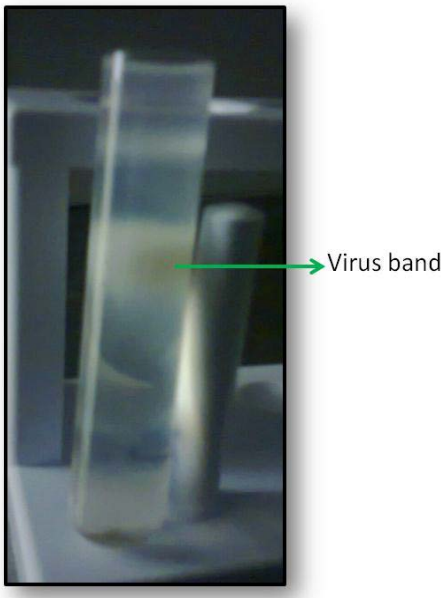


Figure 3.1 Purified PlxyGV occlusion bodies on 30-80% glycerol gradients; the green arrow is pointing to the virus band

3.3.2 Transmission electron microscopy

Transmission electron microscopy of purified OBs revealed the presence of numerous virus particles that are ovicylindrical in shape (Figure 3.2.). Single nucleocapsids surrounded by a granulin protein matrix with a prominent granulin envelope were observed in some particles (Figure 3.2.). The mean length of 60 virus OBs was determined to be 302 ± 3.18 nm.

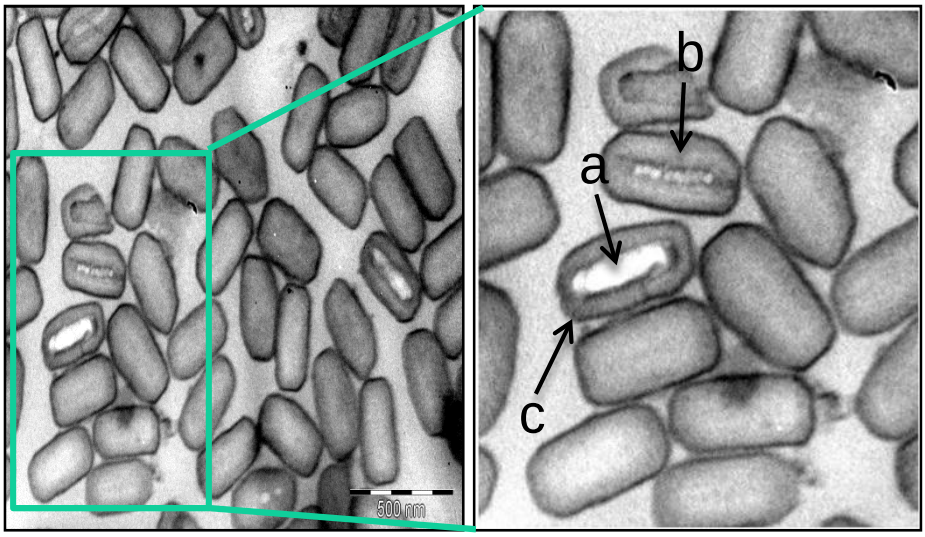


Figure 3.2 Transmission electron micrograph of PlxyGV occlusion bodies (a) nucleocapsid (b) granulin protein matrix (c) granulin envelope

3.3.3 Concentration of occlusion bodies

In order to calculate the concentration of the OBs, the number of OBs in five blocks of the counting chamber was counted twice. The mean number of OBs from two counts was 95.0, 105.0, 100.5, 92.0 and 93.0 respectively. Using the formula in section 3.2.3 the concentration of purified virus particles was determined to be 1.82×10^{11} OBs/ml. This was the yield from 1.8 g of larval cadavers which was approximately 70 – 80 larvae.

3.4 Discussion

The dead larvae collected in this study were observed to show viral symptoms such as change of colour from dull green to pale yellow and swelling of larval segment. The observed symptoms were similar to those of a granulovirus infection described by Asayama and Osaki (1970), Federici (1997), Parnell *et al*. (2002) and Dezianian *et al.* (2010).

Viral occlusion bodies were successfully purified and analysed by transmission electron microscopy. The morphology of the OBs in this study was similar to that of a typical granulovirus. The virus particles were ovicylindrical shape and observed to contain one nucleocapsid per occlusion body indicating that it is a granulovirus. The mean length of the viral OBs in this study was determined to be 302 ± 3.18 nm which is larger than 272.8 ± 12 nm recorded for the Taiwanese isolate (Dezianian *et al.* 2010). The length of the virus particle in this study is smaller than the 350 nm recorded for the Kenyan isolate (Muthamia *et al*. 2011) and the 411 ± 17 nm for the Japanese isolate (Asayama & Osaki 1970). Kioaukia *et al.* (1995) speculated that differences in size of virus particles might be due to the physiological state of the host cell at the time of infection.

Purified virus particles were successfully enumerated and the concentration was determined to be 1.82×10^{11} OBs/ml. In this study, it was possible to obtain 1 ml of virus stock at this concentration from approximately 1.8 g of larval cadavers. This concentration is lower than the 1.18×10^{12} OBs/ml obtained for PlxyGV-USA reported by Farrar *et al*. (2007). The difference in number of virus particles recovered from diseased larvae could be due to the differences in methods used for virus extraction and enumeration.

The cause of death of the laboratory colony in this study was confirmed to be as a result of granulovirus infection. Rabindra *et al*. (1997) also reported a sudden appearance of

granulovirus in two laboratory populations of *P. xylostella* which resulted in the death of both insect colonies. It is possible that stress due to overcrowding resulted in the developmentof covert virus infection to overt disease in the larvae as the population increased in number. This was also reported by Opoku-Debrah *et al.* (2013) where overcrowding was used as a method to recover baculovirus in false codling moth larvae (*Thaumatotibia leucotreta*). The death of the laboratory colony may be attributed to horizontal transmission of the virus from one larva to another via ingestion of contaminated food material. This is probably because during late infection of the virus, cells containing large amounts of virus particles lyse. The destruction of cells accompanied by the production of virus particles gradually kills the insect and the virus is spread onto the host food material (Szewczyk *et al.* 2006). There are also possibilities of vertical transmission of the virus from one generation to the other. The vertical transmission could be either transovum, which is transfer of virus to progeny on the surface of the egg or transovarial which involves transfer of virus to progeny by the parent ovary (Fuxa *et al*. 2002). Vertical transmission could result in either covert or overt infection depending on the growing conditions of the larvae.

In conclusion OBs were extracted from larval cadavers collected before the death of the colony. Identification by transmission electron microscopy confirmed the isolation of *a* granulovirus. The next chapter describes genetic characterisation of the granulovirus by restriction endonuclease analysis of genomic DNA, and amplification and sequencing of selected viral genes.

CHAPTER FOUR

Genetic characterisation of *Plutella xylostella* granulovirus (PlxyGV-SA)

4.1 Introduction

In chapter three, occlusion bodies (OBs) were purified from larval cadavers showing viral symptoms, and transmission electron microscopy confirmed the isolation of a granulovirus. This chapter describes the genetic characterisation of the virus isolate using restriction endonuclease analysis of genomic DNA and PCR amplification and sequencing of selected viral genes.

It is essential to characterise novel virus isolates in order to determine the genotype and to differentiate between isolates of the same species. It has been reported that the *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) differs in genotype between geographically distinct host populations (Opoku-Debrah *et al*. 2013). Virulence against host insect populations can differ between genetically different virus isolates with important implications for insect resistance management. For example, some field populations of codling moth (*Cydia pomonella* (L.)) were found to be resistant to the Mexican isolate of *Cydia pomonella* granulovirus (CpGV-M). Bioassays in the laboratory revealed that resistance can be managed by challenging resistant insects with different virus isolates of the same species (Eberle *et al*. 2008; Berling *et al*. 2009)

Several methods can be used to genetically characterise viruses including single restriction endonuclease (REN) analysis, amplification and sequencing of selected viral genes and whole genome sequencing. REN analysis of genomic DNA is a convenient method used to establish genetic differences between virus isolates by observation and comparison of DNA profiles produced. The DNA fragments are separated according to their sizes which form specific restriction patterns also known as DNA fingerprints. The band sizes generated by single restriction digestion can be compared if at least one complete genome sequence is available as this can then be digested *in silico*. REN analysis has been used by various researchers to characterise baculovirus isolates. For example Kadir *et al*. (1999a) used REN analysis to establish genetic differences between Taiwanese and Chinese PlxyGV isolates. Comparative

analysis of the restriction profiles of the two isolates following digestion with *Eco*RI, *Pst*I and *Hin*dIII showed that they differ by the presence or absence of one to three major bands. Another study by Subramanian *et al*. (2008) compared the DNA profiles of the Kenyan and Taiwanese isolates and observed considerable differences between these isolates. Fourteen Kenyan isolates of PlxyGV were found to be genetically different on the basis of sub-molecular bands in the REN profiles generated with *Pst*I and *Eco*RI (Parnell *et al.* 2002).

The most accurate method of characterising virus isolates is by complete genome sequencing and pairwise comparison of nucleotide and amino acid residues. However, this method is expensive and requires significant analytical expertise. Alternatively, selected genes of interest can be amplified and sequenced. Many studies have shown that specific viral genes are prime targets for sequencing and comparison in order to distinguish between virus isolates. For example Eberle *et al*. (2009) amplified and sequenced the *granulin*, *late expression factor 8 (lef-8*) and *late expression factor 9* (*lef-9*) genes of eight isolates of *Cydia pomonella* granulovirus (CpGV) and classified these isolates into four genome types, A to D, based on the single nucleotide polymorphisms (SNPs) present in the gene sequences. In another study, different CrleGV-SA isolates were placed into two genome groups based on SNPs observed in the nucleotide sequences of their *granulin* and *ecdysteroid UDP-glucosyltransferase* (*egt*) genes (Opoku-Debrah *et al*. 2013).

The aim of this chapter is to establish the genetic identity of PlxyGV-SA by restriction endonuclease analysis of genomic DNA, and sequencing of selected viral genes. To date, the only sequence data available for PlxyGV is the full genome sequence of the Japanese isolate (GenBank Accession number AF 270937.1; Hashimoto *et al*. 2000b), and this was used as a reference strain for comparison with PlxyGV-SA.

The first objective of this chapter was to extract genomic DNA from purified PlxyGV-SA OBs and generate DNA profiles by single restriction endonuclease digestion of genomic DNA for comparison with those of PlxyGV-Japan generated in silico. The second objective was to amplify the *granulin, egt, lef-8* and *lef-9* genes and compare their sequences with those of PlxyGV-Japan.

4.2 Materials and methods

4.2.1 Genomic DNA extraction using CTAB

A CTAB DNA extraction protocol described by Aspinall *et al.* (2002) with some modification was used to extract genomic DNA from purified OBs. A 200 µl sample was transferred into a 1.5 ml tube and 90 µl of sodium carbonate (1M) was added. The mixture was incubated at 37ºC for 30 minutes. Afterwards, the samples were equilibrated with 120 µl of Tris-HCl (1 M, pH 6.8), 90 µl of SDS (10% w/v), 50 µl of Proteinase K (25 mg/ml) and incubated at 37ºC for 30 minutes. Thereafter, 10 µl of RNaseA (10 mg/ml) was added and the sample incubated again for another 30 minutes at 37ºC. The sample was then micro-centrifuged at 11800 × g for 3 minutes and the supernatant transferred into new tubes. Pre-warmed CTAB buffer (400 µl) [2% w/v, CTAB, 10 mM Tris (pH 8.0), 20 mM Na_2EDTA, 1.4 M NaCl] was added and samples incubated at 37ºC for 60 minutes with mixing every 10 minutes. Chloroform (400 µl) (stored at 4ºC) was added and tubes inverted briefly before micro-centrifugation at 6700 x g for 10 minutes. The upper phase was transferred into a new tube and DNA precipitated overnight at -25ºC by the addition of 400 µl of ice-cold isopropanol. Samples were micro-centrifuged at 11800 x g for 20 minutes, the pellet washed with 70% ice-cold ethanol and re-centrifuged at 11800 x g for 5 minutes. The DNA pellet was air-dried to evaporate traces of ethanol and the pellet re-suspended in 25 µl of 10 mM Tris- HCl (pH 8.0) and stored at 4ºC.

4.2.2 Determination of DNA concentration

A 1:100 dilution of viral DNA was made using double distilled water (ddH_20). The concentration of DNA was determined using a spectrophotometer (Genequant, Pharmacia Biotech®) and the absorbance of three independent samples was read at 260 nm. Mean absorbance of the three samples was used to calculate DNA concentration using the equation: OD_{260} nm × dilution factor × 50 µg / ml.

4.2.3 Restriction endonuclease analysis and agarose gel electrophoresis

Single restriction enzyme digestions were carried out in a total volume of 30 µl containing 3 µl 10x restriction enzyme (RE) buffer and 3 µl of either *Pst*1, *Eco*R1, *Bam*H1 or *Hin*dIII, 10 µl (± 4.8 µg) genomic DNA and 14 µl of ddH_20. Reactions were micro-centrifuged briefly

and incubated for 4 hours at 37°C before analysis by 0.6% agarose gel electrophoresis in 1x TAE buffer (40 mM Tris-acetate, 20 mM acetic acid, 1 mM EDTA) at 30 V for 16 hours. DNA marker II and 1 Kb DNA marker (Fermentas®) were used to estimate the size of the DNA fragments. Gels were stained with ethidium bromide, visualised under a UV trans-illuminator (Uvitec®) and images captured using UVI Prochemi software. The sizes of the fragments obtained were measured using scanning and imaging analysis software UVIband version 11.9 (UVItec 2004). The complete genome of PlxyGV-Japan was analysed *in silico* using A-plasmid Editor Version 2.046 (Davis, MW 2013) and the fragment sizes were compared to each other.

4.2.4 Amplification of the PlxyGV-SA *granulin, egt, lef-8* and *lef-9* gene sequences

The *granulin, egt, lef-8* and *lef-9* genes were amplified by polymerase chain reaction. The function and sizes of the genes selected for amplification are described in Table 4.1.

Table 4.1 Function and sizes of viral genes selected for amplification

Gene	Function	Gene size (nt)
Granulin	structural gene, forms part of the protein matrix	747
Ecdysteroid UDP-glucosyltransferase (*egt*)	auxiliary gene, delays larval moulting	1289
Late expression factor 8 (lef-8)	transcriptional gene, takes part in late transcription of the viral DNA	2517
Late expression factor 9 (*lef-9*)	transcriptional gene, takes part in late transcription of the viral DNA	1485

Virus-specific oligonucleotides were designed for amplification of the *granulin, egt, lef-8 and lef-9* gene sequences according to the complete genome sequence of PlxyGV-Japan. The oligonucleotides were designed to flank the genes, in order to obtain the full gene sequence (Table 4.2).

Table 4.2 Oligonucleotide primers used for the amplification and sequencing of *granulin, ecdysteroid UDP-glucosyltransferase (egt), late expression factor 8 (lef-8)* and *late expression factor 9 (lef 9)* genes of PlxyGV-SA

Primer name	Sequence (5' to 3')	Binding site on the genome	Location on the genome	Amplicon size (nt)
granulinF	5' TGGTGAACTCCGTACCCAACATAC 3'	821-844	945-1691	1000
granulinR	5' GCTTTGACAGTTCCGTCAGCCTCG 3'	1824-1801		
egtF	5' CACCGTGCTTATCATTAGTCTCG 3'	98261-98283	98354-	1500
egtR	5' CGAGTCTTCATCGTGAATGATGC 3'	99761-99739	99643	
lef-8F	5' GTATCACGTTTTTATCGCCGTGC 3'	91078-91100	91271-	2900
lef-8R	5' CGTGATAGTTGAGAAGCTGTTCG 3'	93924-93902	93787	
lef-9F	5' ACGCCCGATTACTACAAACTACCG 3'	81317-81340	81501-	1900
lef-9R	5' TTTGTACGTCGCCTTCACAACGTC 3'	83115-83092	82985	

Amplification of genes was carried out using PCR on an MJ Mini Personal Thermal Cycler (BIO-RAD®). Reactions were carried out in a total volume of 25 µl containing 7.5 µl ddH_20, 12.5 µl 2× KAPATaq (Kapa Biosystems) and 2 µM of each oligonucleotide. The same cycling parameters were used for amplification of the *granulin*, *egt*, *lef-8* and *lef-9* genes with the only difference being the annealing temperature, the elongation time and number of cycles for the *lef-8* gene (Table 4.3). Different annealing temperatures were used for each gene because of different melting temperatures of the oligonucleotides. The number of cycles was increased to 45 for the *lef-8* gene because it is a much larger gene. Amplified products were analysed by 0.7% AGE and sequenced by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa), using the forward and reverse primers of each gene. An additional internal primer was designed by Inqaba Biotechnical for the sequencing of the *lef-8* gene because of its size.

Table 4.3 Cycling parameters used for amplification of *granulin, egt, lef-8* and *lef-9* genes from PlxyGV-SA

Stage		Temperature °C	Time (min)	Cycle
stage 1	step 1	95	5 : 00	1X
stage 2	step 1	95	0 : 30	
	step 2	45/59/58/59*	0 : 30	30/45 X#
	step 3	72	1 : 30/ 2 : 30$	
stage 3	step 1	72	5 : 00	1X

* Annealing temperature of *granulin* gene (45 °C), *egt* gene (59 °C), *lef-8* gene (58 °C) and *lef-9* gene (59 °C). $ 2:30 min elongation time for *lef-8* gene and 1:30 min for *granulin, egt* and *lef-9* genes. # 45 cycles for *lef-8* gene and 30 cycles for *granulin, egt,* and *lef-9* genes

4.2.5 Sequence alignment of *granulin, egt, lef-8* and *lef-9* gene sequences of PlxyGV-SA

Two overlapping sequence products were obtained for *granulin, egt* and *lef-9* genes, one in the forward direction and the other in the reverse direction. Three sequences were obtained for the *lef-8* gene including the internal sequence. The sequences were viewed using Chromas Lite (Version 2.1). Forward and reserve sequences were assembled and aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 5.05. Sequences obtained were subjected to nucleotide BLAST on the National Centre for Biotechnology Information Database (NCBI) to detect single nucleotide polymorphisms (SNPs). Nucleotide sequences of PlxyGV-SA were then translated using MEGA and subjected to protein BLAST to detect changes in amino acid sequence.

4.3 Results

4.3.1 Genomic DNA extraction and DNA concentration

Genomic DNA was successfully extracted from PlxyGV-SA purified occlusion bodies and analysed by 0.7% AGE (Figure 4.1). The extracted DNA was observed to be of high molecular weight, as it is above the 10000 nt band (Figure 4.1. lane 2). The concentration of DNA was determined according to the method described in section 4.2.2. The absorbance of PlxyGV-SA genomic DNA read at 260 nm of three independent samples was 0.099, 0.096

and 0.098 respectively, giving a mean absorbance of 0.097. The concentration of DNA was determined to be 485 µg/ml.

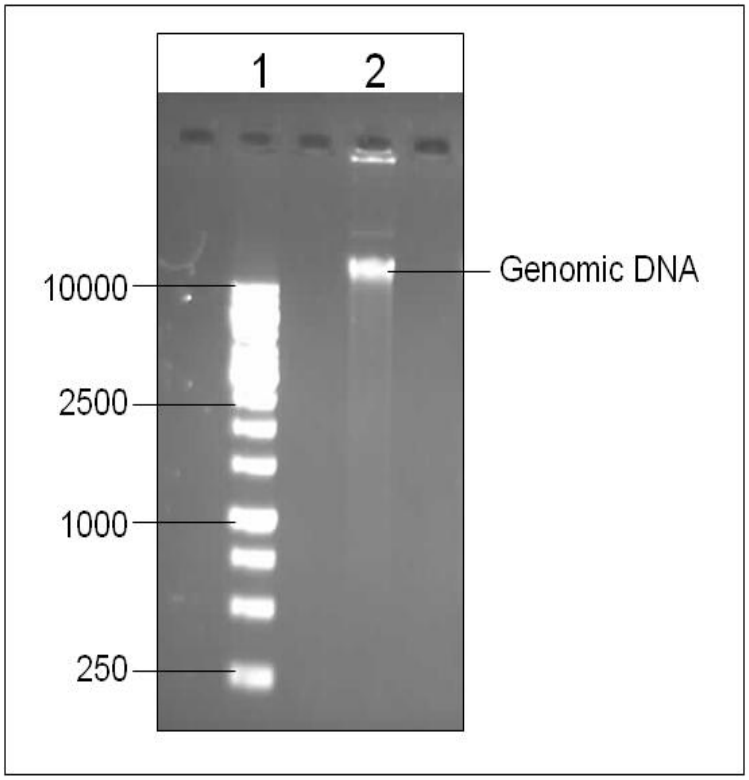


Figure 4.1 Agarose gel electrophoresis of PlxyGV-SA genomic DNA. Lane 1- 1 kb DNA marker, lane 2- PlxyGV-SA genomic DNA. Electrophoresis was carried out by 0.7% agarose gel electrophoresis at 90V for 30 minutes.

3.3.2 Restriction endonuclease (REN) analysis of genomic DNA

REN analysis was done in order to obtain profiles of PlxyGV-SA genomic DNA for comparison with other PlxyGV isolates. Single REN digestion of PlxyGV-SA genomic DNA with *Bam*H1, *Hin*dIII, *Pst*1 and *Eco*R1 generated several prominent and fainter fragments (Figure 4.2). DNA marker II and 1kb DNA marker were run alongside the restriction digest in order to estimate the sizes of the DNA fragments. Panel A of Figure 4.2 shows the DNA profiles obtained by digestion with *Bam*H1 and *Hin*dIII. Restriction digestion with *Bam*H1 revealed the presence of seven prominent fragments and three faint fragments (Figure 4.2, panel A, lane 3). The *Hin*dIII profile generated ten prominent fragments and two faint fragments (Figure 4.2, panel A, lane 4). Panel B of Figure 4.2 shows the DNA profiles obtained by digestion with *Pst*1 and *Eco*R1. REN analysis with *Pst*1 produced ten prominent fragments and four faint fragments (Figure 4.2, panel B, lane 3). The *Eco*R1 profile generated

seven prominent fragments and five faint fragments (Figure 4.2, panel B, lane 4). The next section describes a comparison of each PlxyGV-SA profile with that of the reference strain PlxyGV-Japan which was generated *in silico*.

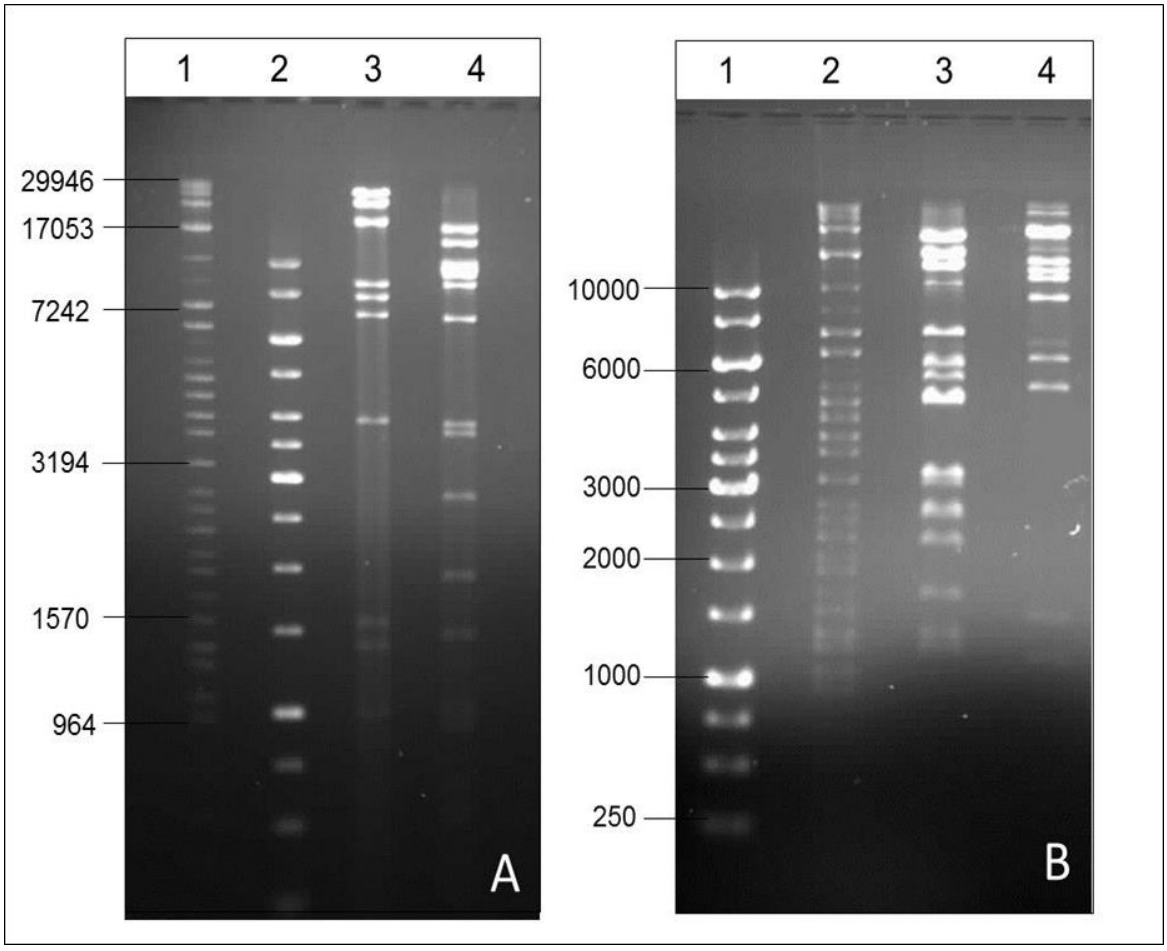


Figure 4.2 Restriction endonuclease profile of PlxyGV-SA genomic DNA. Electrophoresis was carried out on 0.6% agarose gels for 16 h at 30 V. [A] lane 1- DNA marker II, lane 2- 1 Kb DNA marker, lane 3- *Bam*HI and lane 4- *Hin*dIII. [B] lane 1- 1 Kb DNA marker, lane 2- DNA marker II, lane 3- *Pst*I, lane 4- *Eco*RI.

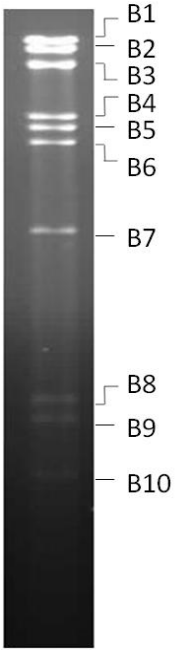
4.3.3 Comparison of PlxyGV-SA and PlxyGV-Japan *Bam*HI profiles

Restriction digestion of PlxyGV-SA genome DNA with *Bam*HI generated 10 fragments. The estimated fragment sizes ranged between 24145-1000 nt and the total genome size was approximately 93800 nt. *In silico* digestion of PlxyGV-Japan produced 12 fragments ranging from 30401- 98 nt with a total genome size of 100999 nt (Table 4.4). Analysis of the two profiles showed that all the fragments present in PlxyGV-Japan profile were also present in

the PlxyGV-SA profile with the exception of fragment A11 (140 nt) and fragment A12 (98 nt), which were not present possibly due to their low molecular weight. It was observed that the size of fragment A1 (30401 nt) and fragment A2 (22833 nt) of PlxyGV-Japan were larger than B1 (24145 nt) and B2 (20421 nt) of PlxyGV-SA, making the total genome size of PlxyGV-Japan larger than that of PlxyGV-SA.

Table 4.4 *Bam*HI DNA restriction profiles of PlxyGV-Japan and PlxyGV-SA. DNA fragments are arranged in order of decreasing size.

PlxyGV-Japan		**PlxyGV-SA**	
Fragment	**Size (nt)**	**Size (nt)**	**Fragment**
A1	30401	± 24145	B1
A2	22833	± 20421	B2
A3	16092	± 17592	B3
A4	8646	± 8687	B4
A5	7979	± 7952	B5
A6	6809	± 7104	B6
A7	3978	± 3909	B7
A8	1574	± 1567	B8
A9	1424	± 1423	B9
A10	1025	± 1000	B10
A11	140	-	-
A12	98	-	-
Number of fragments	12	10	
Total	**100999**	**± 93800**	



4.3.4 Comparison of PlxyGV-SA and PlxyGV-Japan *Hin*dIII profiles

The *Hin*dIII profile of PlxyGV-SA generated 12 fragments with estimated fragment sizes ranging between 15706-951 nt, and the total genome size was approximately 97746 nt. *In silico* digestion of PlxyGV-Japan with *Hind*III revealed the presence of 19 fragments ranging in size between 15600-414 nt (Table 4.5). It is possible that fragment B3 (9457 nt) of

PlxyGV-SA contains three bands because the fragment is brighter and thicker than the fragments above it. Analysis of the two profiles suggests that fragments B5 (7007 nt) and B9 (1931 nt) of PlxyGV-SA may be doublet bands containing fragments of similar size that cannot be resolved. This assumption was made from the observation of similar sized fragments in the PlxyGV-Japan profile for A7 (7039 nt) and A8 (6927 nt), A12 (1903 nt) and A13 (1698 nt). It was observed that fragment B12 (951 nt) was not present in the *Hind*III profile of PlxyGV-Japan. The last four fragments of PlxyGV-Japan profile were not visible on the PlxyGV-SA profile, possibly due to their low molecular weight but are suspected to be present. The possibility of triple and double bands in the PlxyGV-SA profile for B3, B5 and B9 increased the number of bands to sixteen.

4.3.5 Comparison of PlxyGV-SA and PlxyGV-Japan *Pst*I profiles

The *Pst*1 profile of PlxyGV-SA generated 14 fragments ranging in size from approximately 19892-1344 nt and the total genome size was approximately 101732 nt. The *in silico* digestion of PlxyGV-Japan produced 22 fragments ranging in size from 18489-123 nt (Table 4.6). It was observed that fragment B2 (17609 nt) of *Plxy*GV-SA was not present in the PlxyGV-Japan profile. The *Pst*1 profile also revealed that fragments A8, A10, A12, A13, A14, A18, A20, A21 and A22 of PlxyGV-Japan were absent in the PlxyGV-SA profile.

4.3.6 Comparison of PlxyGV-SA and PlxyGV-Japan *Eco*RI profiles

The *Eco*RI profile of PlxyGV-SA revealed the presence of 12 fragments. The size of estimated fragments ranged from approximately 29946-1299 nt and the total genome size was approximately 144653 nt which is much larger than the genome size of PlxyGV-Japan with approximately 43654 nt. The *Eco*RI profile of PlxyGV-Japan generated 10 fragments, ranging from 20654-84 nt. (Table 4.7). It was observed that fragments B1 (29946 nt), B8 (7329 nt) and B10 (5447 nt) of PlxyGV-SA were not present in the PlxyGV-Japan profile. All other fragments present in the PlxyGV-Japan profile were also present in PlxyGV-SA profile except for the smallest fragment A10 (84 kb).

Table 4.5 *Hind*III DNA restriction profiles of PlxyGV-Japan and PlxyGV-SA. DNA fragments are arranged in order of decreasing size.

PlxyGV-Japan		**PlxyGV-SA**	
Fragment	**Size (nt)**	**Size (nt)**	**Fragment**
A1	15600	± 15706	B1
A2	15527	± 13346	B2
A3	10422	± 9457	$B3^1$
A4	9956	± 9457	$B3^2$
A5	9490	± 9457	$B3^3$
A6	7985	± 8541	B4
A7	7039	± 7007	$B5^1$
A8	6927	± 7007	$B5^2$
A9	3954	± 3904	B6
A10	3754	± 3695	B7
A11	1989	± 2816	B8
A12	1903	± 1931	$B9^1$
A13	1698	± 1931	$B9^2$
A14	1517	± 1494	B10
A15	1044	± 1046	B11
-	-	± 951	B12
A16	678	-	-
A17	569	-	-
A18	533	-	-
A19	414	-	-
Number of fragments	19	17	
Total	**100999**	**± 97746**	

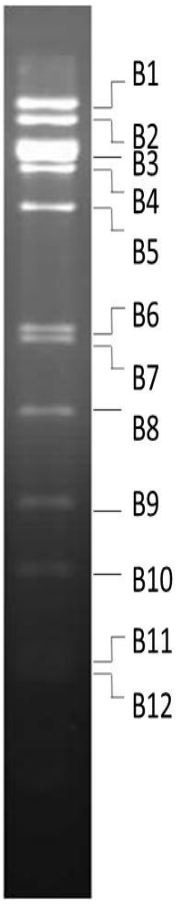


Table 4.6 *Pst*I DNA restriction profiles of PlxyGV-Japan and PlxyGV-SA. DNA fragments are arranged in order of decreasing size.

PlxyGV-Japan		**PlxyGV-SA**	
Fragment	**Size (nt)**	**Size (nt)**	**Fragment**
A1	18489	± 19892	B1
-	-	± 17609	B2
A2	14341	± 14866	B3
A3	12696	± 11613	B4
A4	7268	± 7685	B5
A5	5977	± 6256	B6
A6	5339	± 5748	B7
A7	4979	± 4973	B8
A8	4826	-	-
A9	3312	± 3316	B9
A10	3147	-	-
A11	2769	± 2757	B10
A12	2671	-	-
A13	2424	-	-
A14	2404	-	-
A15	2352	± 2379	B11
A16	1761	± 1825	B12
A17	1499	± 1469	B13
A18	1420	-	-
A19	1311	± 1344	B14
A20	948	-	-
A21	943	-	-
A22	123	-	-
Number of fragments	22	14	
Total	**100999**	**± 101732**	

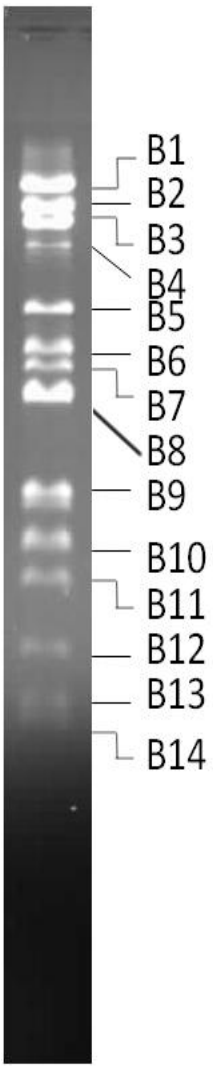
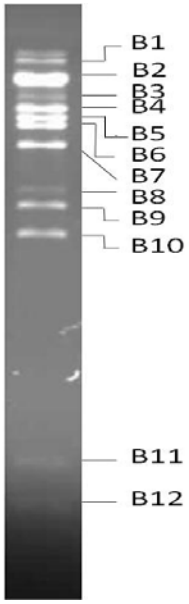


Table 4.7 *Eco*RI DNA restriction profiles of PlxyGV-Japan and PlxyGV-SA. DNA fragments are arranged in order of decreasing size.

PlxyGV-Japan		**PlxyGV-SA**	
Fragment	**Size (nt)**	**Size (nt)**	**Fragment**
-	-	± 29946	B1
A1	20654	± 20164	B2
A2	19702	± 18645	B3
A3	17411	± 16543	B4
A4	13140	± 14300	B5
A5	11279	± 13036	B6
A6	9437	± 9842	B7
-	-	± 7329	B8
A7	6518	± 6507	B9
-	-	± 5447	B10
A8	1563	± 1595	B11
A9	1211	± 1299	B12
A10	84	-	-
Number of fragments	10	12	
Total	**100999**	**± 144653**	



4.3.7 Amplification of the PlxyGV-SA *granulin, egt, lef-8* and *lef-9* gene sequences

The *granulin, egt, lef-8* and *lef-9* genes were successfully amplified by PCR (Figure 4.3). The amplified products were analysed by 0.7% AGE. The *granulin* gene amplicon produced a bright band which was estimated to be 1000 nt (Figure 4.3, panel A) and the amplified product of the *egt* gene formed a thick bright band of approximately 1500 nt (Figure 4.3, panel B). The amplicons of the *lef-8* and *lef-9* gene produced very bright and thick bands (Figure 4.3, panel C and D) and the sizes of the amplicons were estimated to be 2900 and 1900 nt respectively. The sizes of the *granulin, egt, lef-8* and *lef-9* genes are 747, 1289, 2517 and 1485 nt respectively (Table 4.1). It was observed that each amplicon was larger than the

actual gene size. This outcome was expected as oligonucleotides were designed to flank the gene of interest.

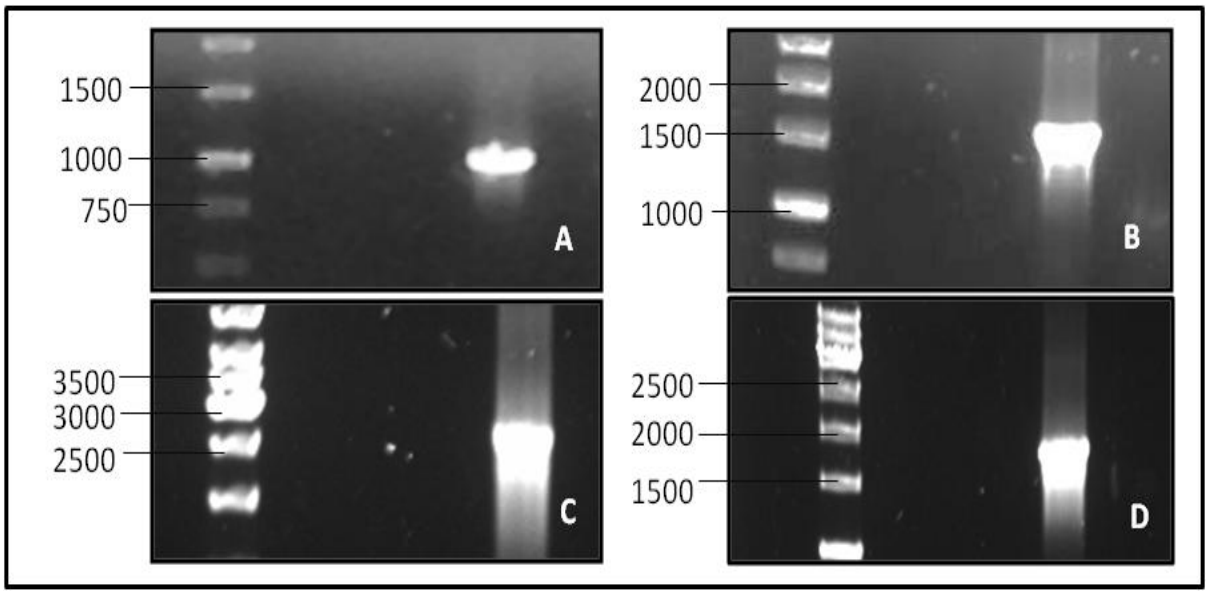


Figure 4.3 Agarose gel electrophoresis of the amplified products of *granulin*, *egt*, *lef-8* and *lef-9* genes from PlxyGV-SA (A) *granulin* gene amplicon, (B) *egt* gene amplicon (C) *lef-8* gene amplicon and (D) *lef-9* gene amplicon.

4.3.8 Analysis of nucleotide and amino acid sequences of the PlxyGV-SA *granulin* gene

The forward and reverse sequences of each gene were assembled and edited using MEGA 5 analysis software. Several transitions (A↔G or C↔T) and transversions were observed. BLAST analysis of the PlxyGV-SA *granulin* gene sequence revealed a 99% nucleotide identity with PlxyGV-Japan. Alignment of the *granulin* gene sequence with PlxyGV-Japan showed four transitions from guanine to adenine (G to A) at nucleotide positions 6, 68, 187 and 221, three transitions from cytosine to thymine (C to T) at nucleotide positions 146, 148 and 246 and one transversion from G to T was detected at nucleotide position 197. It was observed that the single nucleotide polymorphisms in this gene did not alter the predicted amino acid sequence of the protein (Table 4.8).

Table 4.8 Single nucleotide polymorphisms (SNPs) found in PlxyGV-SA *granulin* gene after alignment with PlxyGV-Japan

Nucleotide position/codon	Isolate		Amino acid
	Japan	South Africa	
6	TC**G**	TC**A**	Serine
68	AA**G**	AA**A**	lysine
146	GC**C**	GC**T**	Alanine
148	GCC	GC**T**	Alanine
187	CA**G**	CA**A**	Glutamine
197	TT**G**	TT**T**	Phenylalanine
221	GA**G**	GA**A**	Glutamic acid
246	CC**C**	CC**T**	Threonine

Letters in red bold colour indicate single nucleotide polymorphisms

4.3.9 Analysis of nucleotide and amino acid sequences of the PlxyGV-SA *egt* gene

BLAST comparison of the PlxyGV-SA *egt* gene sequence showed a 99% nucleotide identity to the PlxyGV-Japan sequence. Analysis of the nucleotide sequence revealed the presence of three transitions from A to G at nucleotide positions 21, 56 and 321, two transitions from G to A at nucleotide positions 209 and 240, one transition from T to C at nucleotide position 356 and two from C to T at nucleotide positions 261 and 373. The analysis also showed the presence of two transversions at nucleotide positions 95 (A to T) and 129 (G to T). It was observed that the SNPs at nucleotide positions 56, 129 and 321 resulted in amino acid changes from asparagine to aspartic acid, methionine to isoleucine and glutamic acid to glycine respectively. The remaining SNPs did not change the amino acid sequence of the protein (Table 4.9).

Table 4.9 Single nucleotide polymorphisms (SNPs) found in PlxyGV-SA *ecdysteroid UDP-glucosyltransferase* (*egt*) gene after alignment with PlxyGV-Japan

Nucleotide position/codon	Isolate		Amino acid
	Japan	South Africa	
21	GT**A**	GT**G**	Valine
56	**A**AT	**G**AT	Asparagine » Aspartic acid
95	AT**A**	AT**T**	Isoleucine
129	AT**G**	AT**T**	Methionine » Isoleucine
209	GA**G**	GA**A**	Glutamic acid
240	TT**G**	TT**A**	Leucine
261	TT**C**	TT**T**	Phenylalanine
321	G**A**A	G**G**A	Glutamic acid » Glycine
356	TT**T**	TT**C**	Phenylalanine
373	TT**C**	TT**T**	Phenylalanine

Letters in red bold colour indicate single nucleotide polymorphisms. (») indicates amino acid changes.

4.3.10 Analysis of nucleotide and amino acid sequences of the PlxyGV-SA *lef-8* gene

Alignment of PlxyGV-SA *lef-8* and PlxyGV-Japan *lef-8* gene sequences showed a 99% nucleotide identity. The alignment also indicated the presence of seven transitions from C to T at nucleotide positions 80, 133, 189, 460, 493, 521 and 641, one transition from T to C at nucleotide position 366, two transitions from A to G at nucleotide positions 81 and 461 and seven transitions from G to A at nucleotide positions 119, 289, 313, 337, 352, 360 and 658. Five transversions were also detected in the alignment at nucleotide positions 289 (G to C), 376 (A to T), 576 (T to G), 596 (T to A) and 655 (C to A). Sixteen out of the 21 SNPs were observed to be silent while the SNPs at nucleotide positions 81, 289, 461, 596 and 655 resulted in amino acid changes from threonine to alanine, Valine to Isoleucine, lysine to glutamic acid, isoleucine to asparagine and phenylalanine to leucine respectively (Table 4.10).

Table 4.10 Nucleotide polymorphisms (NPs) found in PlxyGV-SA *late expression factor 8* (*lef-8*) gene after alignment with PlxyGV-Japan

Nucleotide position/codon	Isolate		Amino acid
	Japan	South Africa	
80	TT**C**	TT**T**	Phenylalanine
81	**A**CG	**G**CG	Threonine » Alanine
113	GC**C**	GC**T**	Alanine
119	AG**G**	AG**A**	Arginine
189	CG**C**	CG**T**	Arginine
289	**G**T**G**	**A**T**C**	Valine » Isoleucine
313	CT**G**	CT**A**	Leucine
337	AA**G**	AA**A**	lysine
352	AC**G**	AC**A**	Threonine
360	AA**G**	AA**A**	lysine
366	**T**TA	**C**TA	Leucine
376	GT**A**	GT**T**	Valine
460	TT**C**	TT**T**	Phenylalanine
461	**A**AA	**G**A**A**	lysine » Glutamic acid
493	AC**C**	AC**T**	Threonine
521	TA**C**	TA**T**	Tyrosine
576	CT**T**	CT**G**	Leucine
596	A**T**T	A**A**T	Isoleucine » Asparagine
641	GG**C**	GG**T**	Glycine
655	TT**C**	TT**A**	Phenylalanine » Leucine
658	CT**G**	CT**A**	Leucine

Letters in bold red colour indicate single nucleotide polymorphisms. (») indicates amino acid changes

4.3.11 Analysis of nucleotide and amino acid sequences of the PlxyGV-SA *lef-9* gene

Comparison of PlxyGV-SA *lef-9* gene sequence with that of PlxyGV-Japan revealed a 99% nucleotide identity. The comparison also showed the presence of three transitions from T to C

at nucleotide positions 38, 232 and 301, three transitions from C to T at nucleotide positions 316, 409 and 463 and two transitions from G to A at nucleotide positions 160 and 395. Three more transitions from A to G were observed at nucleotide positions 320, 333 and 377. The analysis also showed the presence of two transversions at nucleotide positions 129 (C to A) and 145 (T to G). It was observed that the SNPs at nucleotide positions 320 and 333 led to amino acid changes from asparagine to serine and glutamine to arginine respectively. The remaining eleven SNPs were silent and did not alter the amino acid sequence of the protein (Table 4.11).

Table 4.11 Single nucleotide polymorphisms (SNPs) found in PlxyGV-SA *late expression factor 9* (*lef-9*) gene after alignment with PlxyGV-Japan

Nucleotide position/codon	Isolate		Amino acid
	Japan	South Africa	
38	GC**T**	GC**C**	Alanine
129	CT**C**	CT**A**	Leucine
145	GT**T**	GT**G**	Valine
160	GT**G**	GT**A**	Valine
232	AC**T**	AC**C**	Threonine
301	**T**TG	**C**TG	Leucine
316	AA**C**	AA**T**	Asparagine
320	A**A**T	A**G**T	Asparagine » Serine
333	C**A**A	C**G**A	Glutamine » Arginine
377	GT**A**	GT**G**	Valine
395	CA**G**	CA**A**	Glutamine
409	AC**C**	AC**T**	Threonine
463	GA**C**	GA**T**	Aspartic acid

Letters in red bold colour indicate single nucleotide polymorphisms. (») indicates amino acid changes

4.4 Discussion

Genomic DNA was successfully extracted using the CTAB method. A total of 25 µl of DNA at a concentration of 485 µg / ml was obtained from 200 µl of purified virus particles. This is a typical yield from 0.36g of larval cadavers which is approximately 35-40 larvae. This

experiment was repeated three times in order to provide enough genomic DNA for restriction endonuclease analysis and PCR amplification of gene sequences. Similar concentrations of DNA were obtained for each batch of DNA extracted, indicating that the method is reproducible and reliable. This result correlates with the reports of Aspinall *et al.* (2002), Parnell *et al*. (2002) and Opoku-Debrah *et al*. (2013) who found the CTAB method effective for the extraction of genomic DNA from baculoviruses.

In this study, restriction endonuclease analysis was used to characterise PlxyGV-SA in order to compare the banding patterns obtained by agarose gel electrophoresis with other PlxyGV isolates available. Comparative analysis of the *Bam*H1, *Hin*dIII, *Pst*1 and *Eco*R1 banding profiles of PlxyGV-SA with profiles obtained for the Japanese, Taiwanese, Chinese, Indian and Kenyan isolates using agarose gel electrophoresis (Kadir *et al*. 1999a; Hashimoto *et al*. 2000a; Parnell *et al.* 2002; Subramanian *et al*. 2008) revealed several differences in banding patterns. The *Bam*HI profiles of PlxyGV-SA and PlxyGV-Japan were similar, with the only difference being the presence of one extra fragment in the profile of PlxyGV-Japan (Hashimoto *et al*. 2000a). The *Hin*dIII profile of PlxyGV-SA resembled that of the Taiwanese isolate (Kadir *et al*. 1999a) with the exception of three extra fragments in the profile of PlxyGV-SA. The *Pst*1 profile was similar to the Indian isolate (Subramanian *et al*. 2008) with the difference of two additional fragments in the PlxyGV-SA profile and the *Eco*R1 profile was also similar to the Indian isolate with the only difference being the presence of four extra fragments in the PlxyGV-SA profile. The profiles of PlxyGV-SA were different from the Kenyan profiles (Parnell *et al.* 2002; Subramanian *et al*. 2008) which is also an African isolate.

Although the above comparison showed that the profiles of PlxyGV-SA are different from those of the Japanese, Taiwanese, Chinese, Indian and Kenyan isolates, comparing DNA profiles by this method is complicated because of the varying conditions between experiments, the amount of DNA used for the analysis and different DNA markers used for band size estimation. A more accurate method for comparison of DNA profiles is *in silico* digestion of complete genome sequences. This method is effective and reliable, but expensive and time consuming. Due to time constraints, full genome sequencing of PlxyGV-SA was not performed in this study. The only complete genome sequence of PlxyGV available to date is that of the Japanese isolate (Hashimoto *et al.* 2000b) and this was used to generate *in silico* profiles for comparison with those of PlxyGV-SA.

Comparison of the *Bam*H1, *Hin*dIII, *Pst*1 and *Eco*R1 fragments sizes of PlxyGV-SA with the *in silico* profile of PlxyGV-Japan revealed similarities and differences in banding patterns between the two isolates. A total genome size of 100999 nt was obtained for PlxyGV-Japan following digestion with *Bam*H1, *Hin*dIII, *Pst*1 and *Eco*R1. The total genome size of PlxyGV-SA was 93800 nt after REN analysis with *Bam*HI which is less than the total genome size of PlxyGV-Japan. A possible explanation for this may be that the sizes of the top two bands of the PlxyGV-SA profile (24145 nt and 20421 nt) were under-estimated due to the poor resolution of the DNA marker in that region. A total genome size of 97746 nt was obtained for PlxyGV-SA following digestion with *Hin*dIII which is also less than the total genome size of PlxyGV-Japan. This difference may be due to the four missing bands in the PlxyGV-SA profile which add an extra 2194 nt to the genome. REN analysis of PlxyGV-SA genomic DNA with *Pst*1 revealed an estimated total genome size of 101732 which is approximately the same as the genome size of PlxyGV-Japan. A total genome size of 144653 nt was obtained for PlxyGV-SA following restriction digestion with *Eco*R1 which is larger than the total genome size of PlxyGV-Japan. The PlxyGV-SA profile contained a large band of approximately 29946 nt in size that was not present in the PlxyGV-Japan profile. This result may be due to partial digestion of PlxyGV-SA genomic DNA possibly due to insufficient enzyme in the reaction mixture. The difference in genome size observed in this study may be because measurement of band size by REN analysis is an estimation, and the calculated genome size can be less than or more than the actual genome size. For example, Hashimoto *et al*. (2000a) reported a total genome size of 102200 nt for PlxyGV-Japan using REN analysis which is larger than 100999 nt that was obtained after full genome sequencing (Hashimoto *et al*. 2000b).

This study showed that the banding patterns obtained for PlxyGV-SA following restriction analysis with *Pst*1, *Eco*R1, *Bam*HI and *Hin*dIII by agarose gel electrophoresis were different from the patterns obtained for other PlxyGV isolates. These results indicate that PlxyGV-SA is a genetically different isolate. Kadir *et al*. (1999a) compared the *Eco*R1, *Bam*HI and *Hin*dIII profiles of the Taiwanese and Chinese PlxyGV isolates and also found differences in banding patterns. Similar studies on Kenyan, Indian and Taiwanese PlxyGV isolates (Parnell *et al*, 2002; Subramanian *et al*. 2008) also established major band similarities and differences between isolates and concluded that these results indicate the existence of genetically different isolates.

In order to gain more information about the PlxyGV-SA genome, the *granulin, egt, lef-8* and *lef 9* genes were amplified and sequenced. The *granulin* gene of PlxyGV is located in open reading frame four (ORF 4) and encodes for 249 amino acids (Hashimoto *et al*. 2000b). It is expressed in the late stages of infection and forms a crystalline lattice around the virion which protects the viral nucleocapsid in the environment and is dissolved upon ingestion of the virus by a susceptible host (Rohrmann 2008a). Sequence alignment of the *granulin* gene of PlxyGV-SA with that of PlxyGV-Japan revealed the presence of eight SNPs which did not change the predicted amino acid sequence and showed a 99% nucleotide identity with PlxyGV-Japan. This result is expected because the *granulin* gene is known to be highly conserved (Federici 1997). Any change in the amino acid sequence of the *granulin* gene can compromise the function of the gene. A study by Opoku-Debrah *et al.* (2013) where seven *granulin* gene sequences for different South African *Cryptophlebia leucotreta* granulovirus (CrleGV) isolates were analysed also found that, although there were several single nucleotide polymorphisms, these did not alter the amino acid sequence of the protein.

The *egt* gene is an auxiliary gene that encodes an enzyme which mediates the inactivation of the moulting hormone in insects. The expression of the *egt* gene allows the virus to delay the development of the insects thereby increasing the feeding time which is beneficial to the virus as it allows the propagation of progeny virus (Ferrelli *et al.* 2012). In PlxyGV, the gene is encoded in ORF 118 and it encodes for 429 amino acids (Hashimoto *et al*. 2000b). Several SNPs were observed after comparison of the *egt* gene sequence of PlxyGV-SA with PlxyGV-Japan which resulted in three amino acid changes. This result is not surprising because the *egt* gene sequence has been found to vary between virus isolates in other baculoviruses. Carpio *et al.* (2013) analysed 20 *egt* gene sequences of *Phthorimaea operculella* granulovirus (PhopGV) and identified three distinct groups of *egt* genes that differ in size and structure. In another study, amino acid differences in egt were reported between geographically different CrleGV isolates (Opoku-Debrah *et al*. 2013). The change in amino acid sequence of the egt protein further indicates that PlxyGV-SA is genetically different from PlxyGV-Japan.

Lef-8 and *lef 9* are essential genes that are present in all baculovirus genomes (Miele *et al.* 2011). In PlxyGV, the *lef-8* and *lef-9* genes are encoded by ORFs 109 and 99 respectively (Hashimoto *et al*. 2000b). These genes encode subunits of the viral RNA polymerase, which is highly conserved and responsible for late transcription of the viral genome (Guarino *et al.* 1998). The *lef-8* gene encodes a protein of 880 amino acids and *lef-9* gene a protein of 516

amino acids (Hashimoto *et al*. 2000b). Several SNPs were observed after comparison of the *lef-8* and *lef-9* gene sequences of PlxyGV-SA with PlxyGV-Japan which altered the amino acid sequence of both proteins. The observation of amino acid change in the lef-8 protein of PlxyGV-SA is in agreement with a study by Eberle *et al.* (2009) where the partial *lef*-8 gene sequences of nine *Cydia pomonella* granulovirus (CpGV) isolates were compared and one amino acid change from threonine to methionine was recorded. Another study by Jukes *et al*. (2014) found six SNPs in the *lef*-8 gene sequence of PhopGV-SA which resulted in two amino acid changes when compared to sequences of *Phop*GV-1346 isolate (unpublished). The alteration of the amino acid sequence of PlxyGV-SA lef-9 protein is in contrast to the result obtained by Eberle *et al.* (2009) who found that the *lef*-9 gene sequence was identical in nine *Cp*GV isolates. The comparison of genes in this study was done with the only full genome sequence of PlxyGV available to date and the PlxyGV-SA genes were only subjected to sequencing once, therefore it is not possible to confirm whether the SNPs observed in the PlxyGV-SA genome are valid, or result from sequencing errors

In conclusion, comparison of REN analysis profiles obtained by agarose gel electrophoresis and *in silico* digestion confirmed that PlxyGV-SA is genetically different from PlxyGV isolates identified in other countries. The data obtained from the sequencing of selected viral genes also supports that PlxyGV-SA is a genetically different isolate. In order to formulate the virus into a biopesticide for application in the field, it is important to estimate the biological activity of the virus isolate against the host population in terms of median lethal concentration and time. The next chapter describes laboratory bioassays used to determine biological activity of PlxyGV-SA against *P. xylostella* neonate and fourth instar larvae.

CHAPTER FIVE

Determination of biological activity

5.1 Introduction

In chapter four, PlxyGV-SA was genetically characterised by restriction endonuclease analysis of genomic DNA and sequencing of selected viral genes. The data obtained confirmed the isolation of a novel PlxyGV isolate in South Africa. This chapter describes the determination of the biological activity of PlxyGV-SA against *P. xylostella* neonate and fourth instar larvae.

Biological assay is a technique used in measuring the virulence of a substance, in this case a virus, on a living organism. This is necessary firstly to determine the dose-response relationship and lethal concentration of the virus that will kill 50% and 90% of the population (LC_{50} and LC_{90} respectively). Secondly, it is important to determine the time-response relationship where median lethal time (LT_{50}) is estimated, proving an indication of the speed of kill of the virus. Bioassays performed in a laboratory setting are valuable because they can be used to compare virulence between different isolates and can also give an indication of concentration for application in the field. Neonate and fourth instar larvae were chosen for the bioassays in this study in order to assess the effect of the virus on the youngest and oldest larval stages, as developmental stages of the insect in the field may be overlapping.

Several studies have shown that PlxyGV is pathogenic to all the larval stages of *P. xylostella*. For example studies by Parnell (1999) and Muthamania *et al*. (2011) have shown that the Kenyan isolate of PlxyGV was infective to the second instar *P. xylostella* larvae. Kadir *et al*. (1999b) determines the biological activity of PlxyGV-Taiwan against the first instar larvae and Dezianian *et al*. (2010) reported that the Taiwanese isolate was pathogenic to all larval instars of Malaysian population of *P. xylostella*.

This aim of this chapter was to determine the biological activity of PlxyGV-SA against *P. xylostella* neonate and fourth instar larvae. The specific objectives included performing a dose response bioassay to determine the LC_{50} and LC_{90} of PlxyGV-SA and secondly, to perform a time response bioassay on neonate larvae to determine the LT_{50} of PlxyGV-SA

5.2 Materials and methods

5.2.1 Dose preparation for the neonate bioassay

A 1000 fold dilution of the virus stock was made using ddH_20 in order to lower the concentration of the virus stock as described by Parnell (1999). The concentration obtained after the dilution was 1.82×10^8 OBs/ml. The tube was labelled A and six five-fold serial dilutions were made from the virus suspension, each in a total of 5000 µl (Figure 5.1) so as to obtain different concentrations of the virus for the dose response bioassays. The dilutions were made using 0.02% Triton- X100 in ddH_20 for the surface dose bioassay but for the droplet feeding bioassay the dilutions was made in ddH_20 only. Triton is used as a wetting agent which aids the spread of virus on the leaf surface. Six sterile culture bottles were labelled B, C, D, E, F and G. Thereafter 400 µl of 0.02% Triton- X100 was placed into each tube. Tube A was vortexed prior to pipetting 1000 µl of the suspension into tube B. The mixture was vortexed to achieve homogeneity and 1000 µl was transferred into tube C, this procedure was repeated for D, E, F and G.

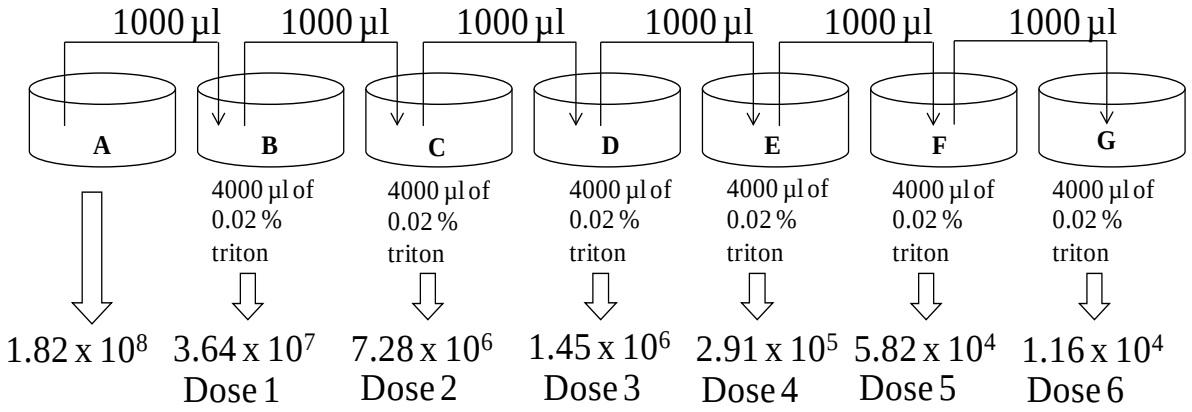


Figure 5.1 A fivefold serial dilution of the virus stock with 0.02% Triton-X100, virus suspension from tube A, C, D, E, F and G were used for neonate dose response bioassays

5.2.2 Dose preparation for fourth instar larvae bioassay

The doses for the fourth instar bioassays were made directly from the virus stock in order to obtain higher doses. Four ten-fold dilutions containing one part virus and nine parts 0.02% Triton-X100 in ddH_20 were made (Figure 5.2). Four sterile culture bottles were labelled A, B, C and D. Ttriton-X100 0.02% (4500 µl) was placed in each tube. The virus stock was vortexed to mix content prior to transferring 500 µl of the virus stock into tube A. The

mixture was vortexed before transferring 500 µl into tube B, the process was repeated for tube C and D.

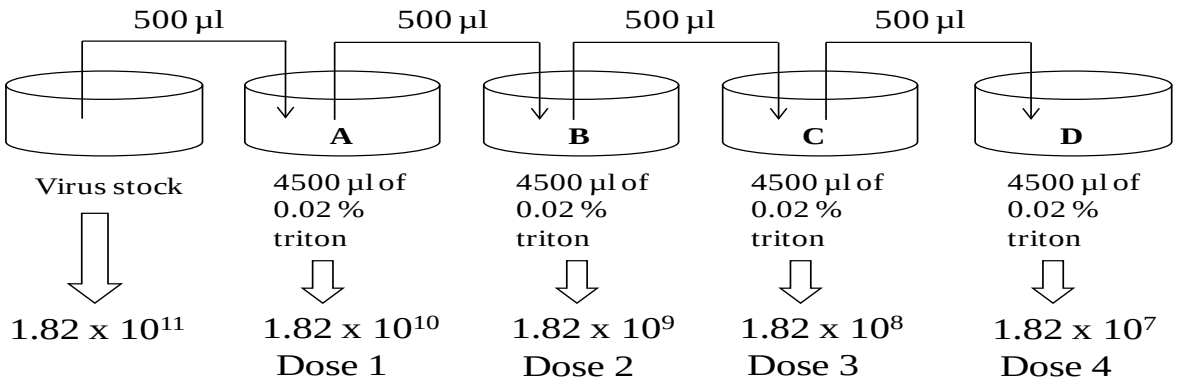


Figure 5.2 A tenfold dilution used for fourth instar dose response bioassay (A) dose 1, (B) dose 2, (C) dose 3 and (D) dose 4

5.2.3 Droplet dose-response bioassay with neonate larvae

The droplet dose-response bioassay method described by Pereira-da-Conceicoa *et al*. (2012) was initially used for the bioassay. The oviposition substrate was replaced every day in order to obtain eggs of the same day. The eggs were allowed to hatch and the neonate larvae were starved for 24 hours (h) before feeding them with 2 µl virus-dye droplets that were randomly placed on parafilm. The virus-dye suspension was made by mixing 50 µl of brilliant blue dye with 600 µl of virus from each dilution which resulted in 650 µl virus-dye solution. Control larvae were fed with double distilled water-dye solution. Larvae that ingest either the virus-dye solution or ddH_20-dye solution were identified by blue colouration of the gut. Thereafter larvae were transferred with aid of a paint brush individually in to a 24-cell well plates (sterilin) containing fresh cabbage leaves. They were then monitored for larval mortality until all the surviving larvae pupated.

5.2.4 Surface dose bioassay with neonate larvae

Surface dose bioassays were conducted as described by Parnell (1999) with some modifications. Fresh canola seedlings were placed in the adult rearing chamber in order to obtain eggs that were laid of the same day. Every day plants were replaced and the plants containing the eggs were placed in a separate larval rearing chamber and eggs allowed to hatch. The virus concentrations used for neonate bioassays were 3.64×10^7, 7.28×10^6, 1.45

$\times 10^6$, 2.91×10^5, 5.82×10^4 and 1.16×10^4 OBs/ml. Cabbage leaves with the dimensions of 50 × 70 mm were cut from the cabbage seedling, washed and cleaned with a paper towel before placing in each in individual Petri dishes. Thereafter the leaf was inoculated with 50 µl from one of the different concentrations on both sides and air dried. Twenty four neonate larvae were fed per virus- treated leaf for 24 h. Control larvae were fed with ddH_20 treated leaves. All larvae were then transferred individually into 24 well plates containing fresh cabbage leaves (Figure 5.3). Larvae were monitored for larval mortality until the surviving larvae pupated. The experiment was replicated three times with 24 larvae per dose.

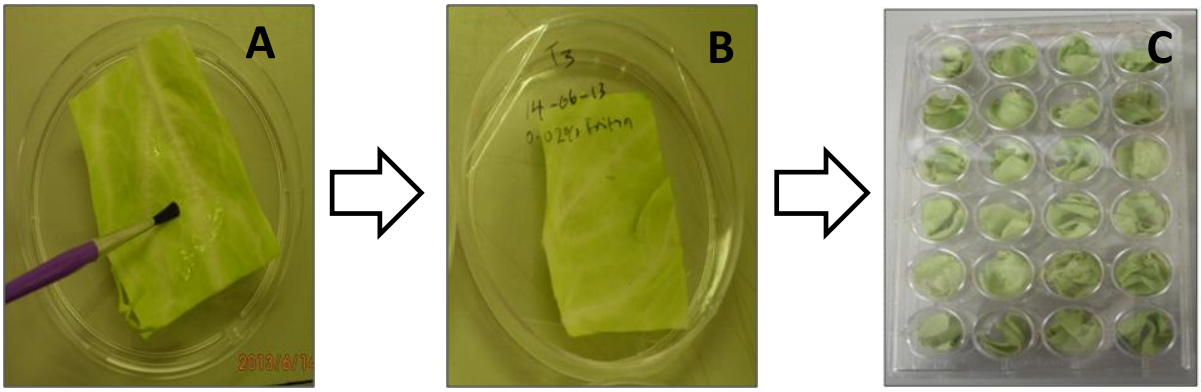


Figure 5.3 Surface dose bioassay procedure: (A) known concentration of virus spread on leaf disc; (B) larvae are fed with virus for 24 h; (C) larvae are transferred to 24-cell plate containing fresh leaves.

5.2.5 Surface dose bioassay with fourth instar larvae

The surface dose method was performed in the same way as for the neonate with the only difference being the size of the leaves, volume of virus inoculated on the leaves and the concentrations of virus. For the fourth instar bioassays, the size of the leaves was increased to dimensions of 125 × 175 mm and placed in large tuper-ware. To each 250 µl of virus was inoculated onto both sides using the following concentrations of virus: 1.82×10^{10}, 1.82×10^9, 1.82×10^8 and 1.82×10^7 OBs/ml.

5.2.6 Time response bioassay with neonate larvae

Time response bioassays were conducted using the surface dosing method as described in section 5.2.4. Neonate larvae were fed with two concentration of virus (3.64×10^7 and 1.14×10^7 OBs/ml) for 24 h before transferring them onto fresh cabbage leaves. Control larvae were fed with ddH_20 and both control and treatment larvae were observed after 12 h for larval

mortality. Subsequently they were inspected three times a day at 8 hour intervals. The virus dose of 3.64×10^7 OBs/ml was found to elicit 100% mortality in the dose response assay. The second dose of 1.14×10^7 OBs/ml was the LC_{90} concentration estimated from the dose response bioassay described in section 5.2.4. The experiment was replicated three times with 24 larvae per treatment.

5.2.7 Statistical analysis

The data obtained from the dose response assays were subjected to probit analysis using Proban statistical software (Van Ark 1995). Concentrations were transformed to $\log_{10}$ and percentage mortality to empirical probits. Percentage mortality was also adjusted according to control mortality using Abbott's formula (Abbott 1925).

$$Corrected\ mortality\ (\%) = \frac{Treatment\ mortality\ (\%)\ -\ Control\ mortality\ (\%)}{100 - Control\ mortality\ (\%)} \times 100$$

The regression lines from the three replicate experiments were compared by the Bartlett's and Chi squared tests (x^2), in order to determine whether they are comparable before pooling the replicate values. The mean value was used to calculate the LC_{50} and LC_{90} values. The time response relationship was determined using GraphPad Prism software (version 6.0). The data obtained from the three replicates on a total of 72 larvae were used to determine the median lethal time (LT_{50}) taking into account the control mortality.

5.3 Results

5.3.1 Droplet dose-response bioassay

The droplet dose-response bioassay was performed as described in section 5.2.3 in order to determine the virulence of the virus isolate interms of LD_{50} and LD_{90}. Larvae that ingested the virus-dye solution or ddH_20-dye solution were identified by blue colouration of the gut when viewed under the microscope. Larvae were observed to be crawling away from the drops. Very few larvae ingested the solution and some were found to ingest very little. Therefore this method was considered to be ineffective and the surface dosing method was used instead.

5.3.2 Surface dose bioassay with neonate larvae

The surface dosing bioassay as described in section 5.2.4 was used to estimate the LC_{50} and LC_{90} values of the neonate larvae. It was observed that a virus concentration of 3.64×10^7 OBs/ml resulted in 100% mortality of neonate larvae. This concentration was therefore not used for subsequent bioassays because mortality for dose response bioassays must range from 1 – 99% in order to enable estimation of the dose-response relationship. The mortality of the larvae was observed to increase with increasing virus concentration for all the three replicate experiments (Figure 5.4).

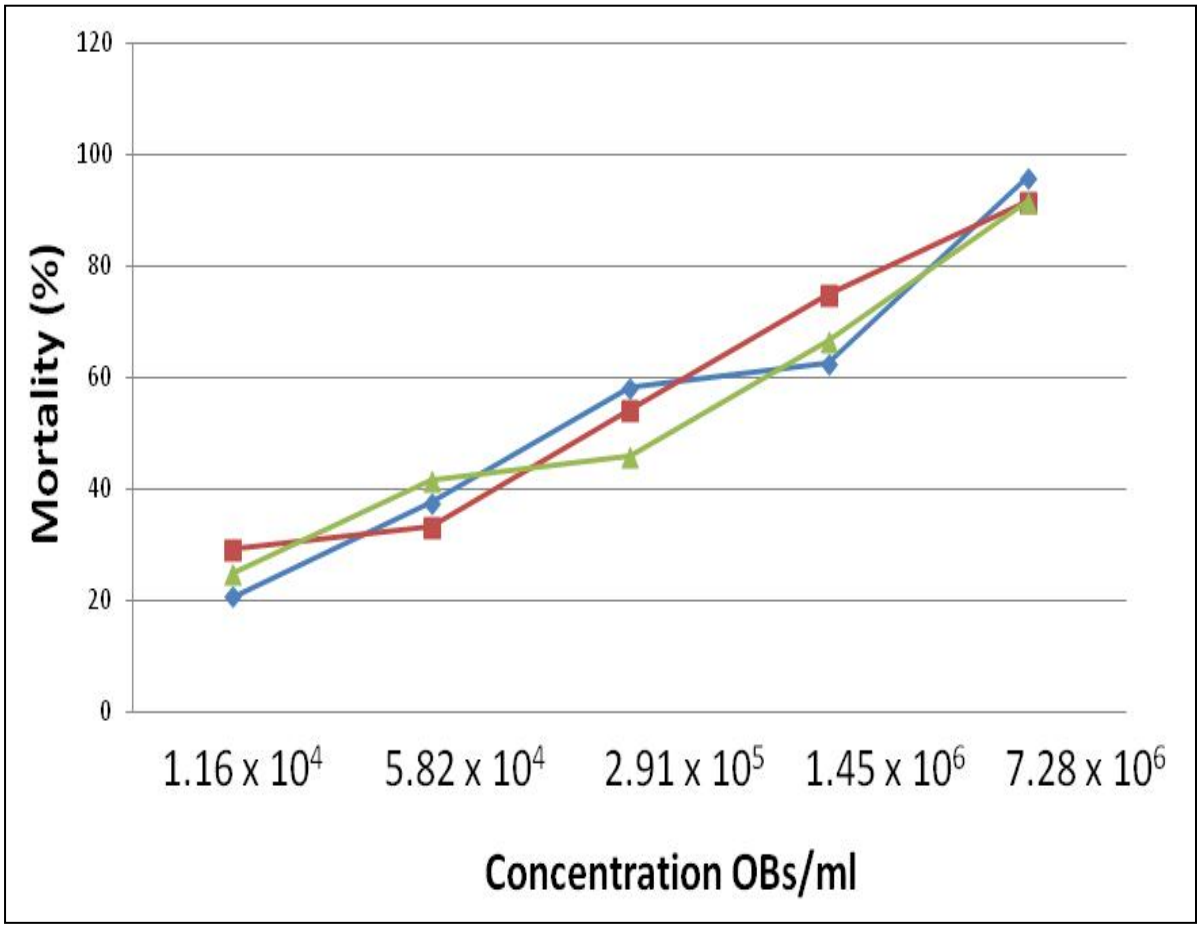


Figure 5.4 Mortality from the three replicates of neonate dose response bioassays (▬) replicate 1, (▬) replicate 2, (▬) replicate 3

The control mortality for the first replicate was 12.50% and treatment mortality ranged from 20.80 – 95.83% (Table 5.1). The G for fiducial limit was 0.1690. According to Van Ark (1995) the experimental procedure or the value of probit line is questionable if G value exceeds 0.25 while a G value greater than 0.025 indicates a large difference in mortality. The

deviations from the regression line were homogeneous. The equation for regression line was y = 0.1691 + 0.8915x (SE of slope = 0.1876).

Table 5.1 Mortality of neonate larvae from the first replicate of dose response bioassays

Dose	Number exposed	Larval mortality	Mortality (%)	Corrected mortality
control	24	3	12.50	-
7.28×10^6	24	23	95.83	95.21
1.45×10^6	24	15	62.50	56.90
2.91×10^5	24	14	58.33	52.11
5.82×10^4	24	9	37.50	28.16
1.16×10^4	24	5	20.80	9.20

Treatment mortality for the second replicate ranged from 29.19-91.67% respectively for the five concentrations. The control mortality was again 12.50% (Table 5.2). The G for fiducial limit was 0.1712 and the equation for the regression line was y = 0.4098 + 0.8405x (SE of slope = 0.1774). The deviations from the regression line were homogeneous.

Table 5.2 Mortality of neonate larvae from the second replicate of dose response bioassays

Dose	Number exposed	Larval mortality	Mortality (%)	Corrected mortality
control	24	3	12.50	-
7.28×10^6	24	22	91.67	90.42
1.45×10^6	24	18	75.00	71.26
2.91×10^5	24	13	54.17	47.32
5.82×10^4	24	8	33.33	23.37
1.16×10^4	24	7	29.19	18.58

The control mortality for the third replicate was 16.66% and the treatment mortality for the lowest and highest concentrations were 25.0% and 91.67% respectively (Table 5.3). The

deviations from the regression line were also homogenous. The G for fiducial limit was 0.2088 and the equation for regression line was y = 0.1513 + 0.8535x (SE of slope = 0.1990).

Table 5.3 Mortality of neonate larvae from the third replicate of dose response bioassays

Dose	Number exposed	Larval mortality	Mortality (%)	Corrected mortality
control	24	4	16.66	-
7.28×10^6	24	22	91.67	89.96
1.45×10^6	24	16	66.67	59.84
2.91×10^5	24	11	45.83	34.74
5.82×10^4	24	10	41.67	29.72
1.16×10^4	24	6	25.00	9.64

The regression lines from the three replicates were compared and the residual variance of the lines was determined to be homogenous. Chi square test confirmed that the lines were parallel and their elevations were comparable ($x^2 = 7.82$, df = 3 and p = 0.08). The test also revealed that there was no significant difference between elevations and therefore slopes were comparable. The average results from the three replicate experiments was then used to calculate the LC_{50} and LC_{90} values of the virus isolate.The LC_{50} and LC_{90} values were determined from the mean of the three replicates to be 3.56×10^5 and 1.14×10^7 OBs/ml respectively (Table 5.4).

Table 5.4 LC_{50} and LC_{90} of neonate larvae

Lethal concentration	OBs/ml	Fudicial limits	
		Upper	Lower
LC_{50}	3.56×10^5	8.38×10^5	1.33×10^5
LC_{90}	1.14×10^7	2.15×10^8	3.53×10^6

5.3.3 Surface dose bioassay with fourth instar larvae

The fourth instar larvae were initially assayed with the same virus doses used for the neonate larvae assay. It was observed that these concentrations did not affect the fourth instar larvae

as no larval mortality was recorded for both control or treatment larvae and all of the larvae were able to pupate successfully. Therefore the concentration of the virus was increased by making a one in ten dilution of the virus stock as described in section 5.2.2. It was found that mortality of the fourth instar larvae increased with increasing virus concentration. No mortality was recorded for control larvae in any of the three replicates and all were able to pupate (Table 5.5). Treatment mortality ranged from 4.1 – 25.8%. It was observed that some virus infected larvae were able to pupate successfully.

Table 5.5 Dose response bioassay with fourth instar larvae

Dose	**Number exposed**	**Replicate 1**		**Replicate 2**		**Replicate 3**	
		Number of response	**Mortality (%)**	**Number of response**	**Mortality (%)**	**Number of response**	**Mortality (%)**
Control	24	0.0	0.0	0.0	0.0	0.0	0.0
1.82×10^{10}	24	5	20.8	5	20.8	6	25.0
1.82×10^{9}	24	5	20.8	5	20.8	5	20.8
1.82×10^{8}	24	3	12.5	2	8.3	3	12.5
1.82×10^{7}	24	1	4.1	1	4.1	2	8.3

5.3.4. Time response bioassay for neonate larvae

The time response bioassay was performed as described in section 5.2.6. Figure 5.5 and 5.6 represent the percentage survival versus time. The time zero on the survivorship graph is 36 h post infection. The LT_{50} using 3.64×10^{7} OBs/ml was calculated to be 92.00 h with a 95% confidence interval ratio of 89 – 100 h (Figure 5.5). This concentration of virus was able to elicit 100% mortality of the neonate larvae. Comparison of the survival curve of the control and treated larvae showed that the curves were significantly different ($P < 0.0001$). The Log-rank and Gehan Breslow-Wilcoxon tests revealed Chi square values of 43.1 and 25.55 ($p = 0.0001$ and $df = 1$).

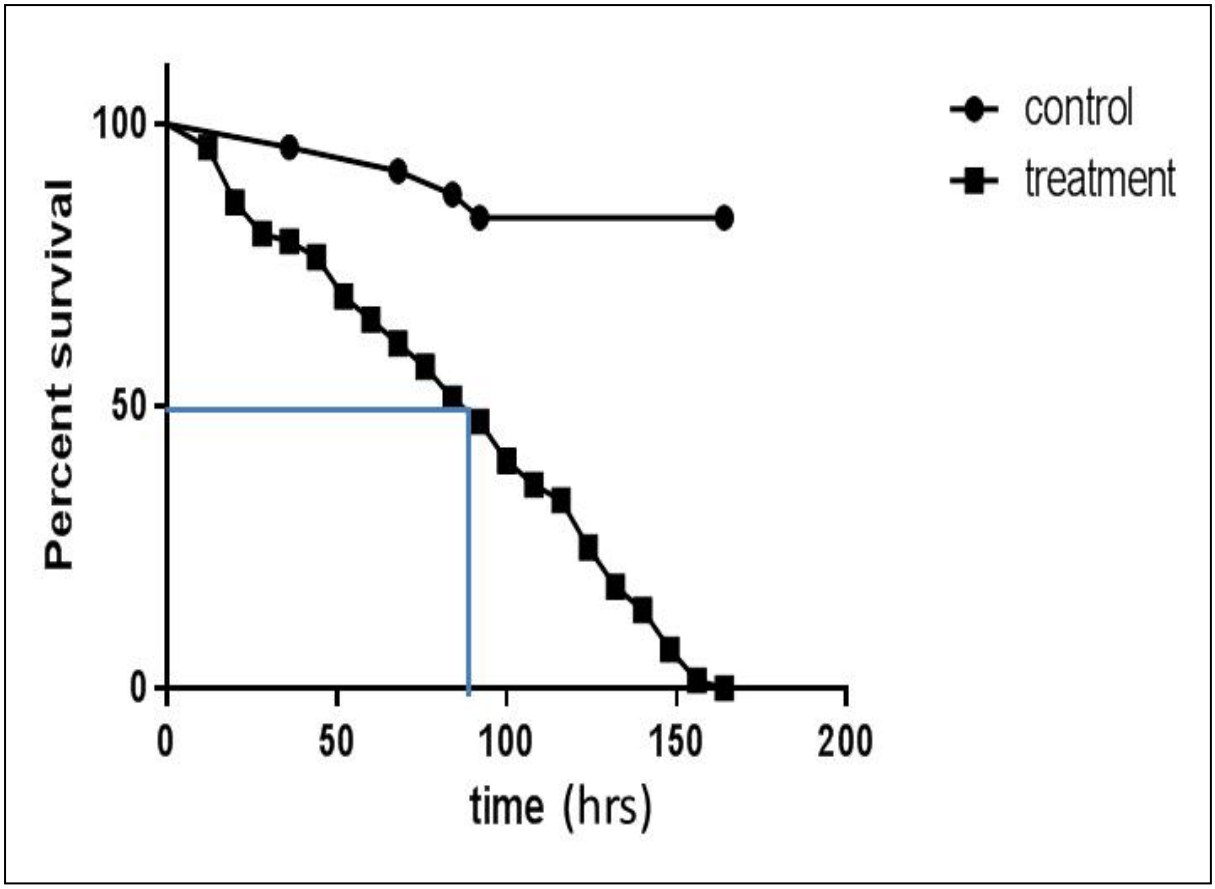


Figure 5.5 Survival curve of neonate larvae fed with virus dose of 3.64×10^7 OBs/ml

The time to kill 50% of the population using a dose of 1.14×10^7 OBs/ml (the LC_{90} concentration obtained from the dose response bioassay) was determined to be 104 h which is 4 days 8 h (Figure 5.6). The lower and upper 95% confidence intervals were 101 – 112 h. It was observed that 10 larvae out of the 72 treated larvae were able to survive and pupate successfully. The Log-rank test and Gehan Breslow-Wilcoxon test confirmed the Chi square value of 36.06 and 24.11 ($p = 0.0001$ and $df = 1$). The test also revealed that the control and treatment curves were significantly different ($P < 0.0001$).

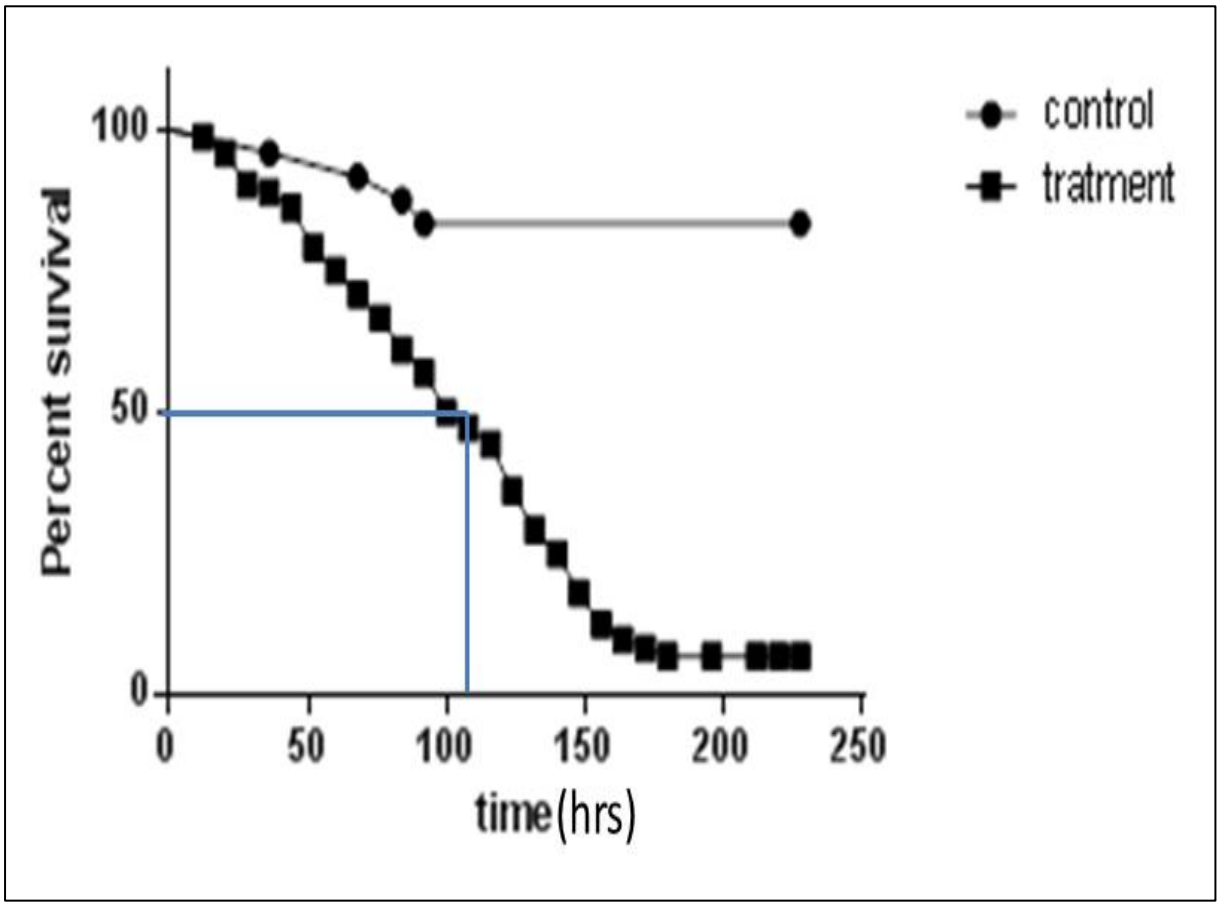


Figure 5.6 Survival curve of neonate larvae fed with virus dose of 1.14×10^7 OBs/ml

5.4 Discussion

In order to determine the pathogenicity of PlxyGV-SA against neonate and fourth instar larvae, the virus stock was further diluted to different concentrations for dose response bioassays. Bioassays can be done by droplet feeding (Kadir *et al*. 1999b; Pereira-da-Conceicoa *et al.* 2012), surface dosing (Dezianian *et al*. 2010; Muthamia *et al*. 2011) or an egg dipping method (Mark *et al.* 2005). In this study, the droplet feeding method was observed to be less effective on the *P. xylostella* neonate larvae as very few larvae were observed to ingest the virus. This is in contrast to the reports of Kadir *et al*. (1999b) who used the droplet feeding method to assay *P. xylostella* neonate larvae. The reason for this is not clear, but it could be attributed to the difference in technique used for the assay or variation in experimental conditions.

The surface dosing method was therefore used for bioassays, as this technique mimics the feeding behaviour of the larvae. In this method, different concentrations of virus were

inoculated on the host food material. The surface dose method was successful as larvae ingested the virus particles with the food material. This observation is in agreement with Muthamia *et al*. (2011) who used surface dosing to determine the biological activity of two Kenyan isolates of PlxyGV against *P. xylostella* second instar larvae. The major limitation of the surface dosing method is that the amount of virus ingested by the larvae cannot be quantified and so only lethal concentration (LC) as opposed to lethal dose (LD) can be estimated from the data obtained. The droplet feeding method, allows the quantification of virus ingested by each larva, therefore the lethal dose per larva can be calculated. On the other hand, determining the lethal concentration is more relevant for determining rates for application in the field.

Dose response bioassays revealed that susceptibility to infection of neonate larvae increased with increasing virus concentration. This is in accordance with the findings of Dezianian *et al*. (2010) who found that mortality increased with increasing virus concentration for first, second and third instar larvae of *P. xylostella*. These results are also similar to those reported by Parnell (1999) and Muthamia *et al*. (2011) where mortality increased with increasing virus dose for PlxyGV-Kenya against the second instar larvae. The most probable explanation for this observation may be that, as the virus dose is increased, more larval cells are infected and more progeny virus are produced to initiate a systemic infection that kills the larvae (Rohrmann 2008a).

The concentration that can kill 50% of the neonate population in this study was determined to be 3.56×10^5 OBs/ml with confidence intervals of 8.38×10^5 - 1.33×10^5 OBs/ml. This result cannot be compared to Parnell's (1999) because neonates as opposed to second instars were used in this study. This value is lower than the LC_{50} of $3.82 \times 10^5 – 3.42 \times 10^6$ OBs/ml obtained for PlxyGV-Taiwan on neonate *P. xylostella* larvae by Kadir *et al*. (1999b) and may therefore suggest that PlxyGV-SA is slightly more virulent to *P. xylostella* neonate larvae than PlxyGV-Taiwan. However, Kadir *et al*. (1999b) used the droplet feeding method, which might explain this difference.

The LC_{50} of 3.56×10^5 OBs/ml recorded in this study for neonate larvae is lower than the LC_{50} of 1.39×10^6 and 1.90×10^7 OBs/ml for PlxyGV-Taiwan against second and third instar *P. xylostella* larvae reported by Dezianian *et al.* (2010). The LC_{50} value recorded in this study is also lower than the LC_{50} of $2.36 \times 10^6 – 3.95 \times 10^7$ OBs/ml of the Kenyan PlxyGV isolate against second instar *P. xylostella* larvae reported by Parnell (1999). Comparison of the LC_{50}

values of different larval instars indicates that neonate larvae are more susceptible to the virus infection than the second and third instar larvae, as lower doses can kill the neonate larvae whereas the older larvae require higher concentrations of virus to elicit mortality.

The LC_{50} value of 3.56×10^5 OBs/ml recorded in this study is similar to the LC_{50} value of 5.86×10^5 and 2.31×10^5 OBs/ml recorded by Opoku-Debrah (2011) for two commercial biopesticide products Cryptogran® (River Bioscience, South Africa) and Cryptex® (Andermatt, Switzerland) against *Thaumatotibia leucotreta* neonate using the droplet feeding method. Cryptogran® and Cryptex® are formulated using South African isolates of the *Cryptophlebia leucotreta* granulovirus (*Crle*GV) and are considered effective for the control of their host (Moore *et al.* 2004a; Kessler and Zingg 2008). This comparison suggests that PlxyGV-SA can be used as a biological control agent against *P. xylostella* in South Africa.

When dose response assays were conducted against fourth instar larvae, it was observed that these larvae were less susceptible to virus infection. It is interesting to note that control mortality was zero in this experiment possibly because larvae at this stage were able to withstand handling and experimental conditions. This result correlated with the findings of Dezianian *et al.* (2010) who also observed very low mortality of fourth instar larvae fed with virus concentrations of 3.11×10^9 and 3.11×10^7 OBs/ml. The resistance of fourth instar larvae to infection may be due to the fact that they pupate within 2-3 days which may be a very short period for a productive infection cycle of the virus. This is also similar to the findings of Engelhard and Volkman (1995) who reported that the fourth instar cabbage looper larvae *Trichoplusia ni* were resistant to oral infection but sensitive to budded virus (BV) infection via the intrahaemocoelic route. The resistance may also be due to the presence of the peritrophic membrane, the protective lining of the midgut. This membrane is much thicker in older larvae making it difficult for the virus to penetrate and initiate infection (Federici 1997).

The time response bioassays showed that the time to kill 50% of the neonate population with the dose of virus that can kill 100% of the population was 92 h or 3 days 20 h. This concentration was determined from bioassay trials and so was considered to be a high dose. In order to get a more accurate or precise result, the LT_{50} was determined using the LC_{90} concentration from the dose response bioassay and the time to kill 50% of the population was 104 h or 4 days 8 h, which is longer than that of the higher dose. This result is in agreement with the findings of Dezianian *et al.* (2010), Farrar *et al.* (2007) and Kadir *et al.* (1999b) who

reported that LT_{50} values decline with increasing virus dose. The lethal time recorded in this study is similar to the 4.9 days for PlxyGV-Taiwan against neonate *P. xylostella* larvae at virus dose of 3.11 × 107 OBs/ml (Dezianian *et al.* 2010). The survivorship curve in this study appears to be different compared to that of Dezianian *et al.* (2010), where survival remains high for some period of time before the declination of the population. This is because the time zero on the survivorship graph in this study is 36 h post infection.

In conclusion PlxyGV-SA was determined to be highly pathogenic against the host *P. xylostella*. The LC_{50} was 3.56×10^5 OBs/ml, which is a very low concentration compared to the virus stock of 1.82×10^{11} OBs/ml indicating that sufficient amount of virus for field use can easily be produce. Comparison of the LC_{50} value obtained in this study with the LC_{50} values of two commercially available biopesticides indicates that PlxyGV-SA can potentially be developed into a biopesticide. The results also show that application of the virus in the field should be targeted towards the neonates as the LT_{50} value of the virus was 4 days 8 h. From chapter two, the mean developmental time of the first and second instar larvae was found to be 4.23 ± 0.07 days, indicating that the virus can kill 50% of the neonate population before the move to the third instar. The next chapter discuss the significance of the major findings in this study and their relevance to the *P. xylostella* management in South Africa.

CHAPTER SIX

General discussion

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is one of the most serious pests of brassica crops worldwide (Talekar and Shelton 1993). It is among the few insect species that has evolved resistance to all major classes of synthetic insecticides and it has been reported as second in the Arthropod Pesticide Resistance Database for resistance to multiple insecticides in one population (APRD 2012; Furlong *et al*. 2013). *Plutella xylostella* is one of three insect species that has developed field resistance to Bt-based products, and the first to evolve field resistance to the Cry toxins of *Bacillus thuringiensis* (*Bt*) (Furlong *et al*. 2013; Talekar and Shelton 1993). Resistance development, together with public demand for a safer environment and strict regulatory policies by government agencies worldwide for pesticide usage has resulted in growing interest towards alternative control technologies (Lapointe *et al*. 2012).

The ultimate aim of this research was to establish a laboratory colony of *P. xylostella* and isolate a baculovirus that could be used as a component of IPM programmes for the control of *P. xylostella* in the field. According to Moore (2002), the objectives that must be achieved in order to commercialise a virus product include genetic characterisation of the virus isolate, determination of the biological activity of the virus, establishment of an efficient and cost-effective method for mass rearing, field trials of the virus isolate and evaluation of the product's feasibility in integrated management programmes. In this study, the first two objectives have been achieved.

The establishment and maintenance of a laboratory colony of insects is a vital prerequisite for virus isolation and production, as well as virulence assessment using biological assays. A laboratory population of *P. xylostella* was successfully established using pupae and asymptomatic larvae collected from the field. However, the population increased rapidly in number due to optimal growing conditions in the laboratory, availability of food and females laying many eggs. This led to overcrowding, microbial contamination and death of the colony in a short period of time.

A new colony was established and maintained on a small scale using pupae and asymptomatic larvae from the same field. Several precautions were taken in order to avoid overcrowding and microbial contamination in the colony. Firstly, in order to avoid frequent contact between individual insects that can lead to stress, the number of larvae placed on each plant was reduced to 9-12 larvae per plant as opposed to 18-20 larvae per plant that was used before the death of the colony. Secondly, excess neonate larvae were discarded in order to minimise the population and maintain the colony on a small scale. Lastly, microbial contamination was prevented by use of chemically treated canola seeds and the paint brushes used for transferring larvae were disinfected in bleach before and after use. These precautions enabled the maintenance of a health colony and resulted in a decrease in the number of dead larvae collected daily.

The second method of rearing *P. xylostella* in this study was effective for a continuous supply of insects for conducting biological assays. However, it is questionable whether this method will be effective for large scale production of insects in order to produce sufficient virus for field application. Firstly, rearing of insects on canola seedlings is time consuming because the seedlings take a number of days to grow. Therefore cabbage leaves had to be provided when the seedlings were not available. Secondly, this method is labour intensive since the rearing requires the transfer of larvae onto fresh food material at two days intervals. Thirdly, the cost of virus production using this method will be expensive because of the number of rearing chambers required and the time taken to transfer larvae onto to fresh feeding material. An artificial diet for the rearing of *P. xylostella* has been described in the literature (Carpenter and Bloem 2002; Dunhawoor and Abeeluck 2003) and newly hatched larvae (neonates) can be reared until pupation. However, it was not used in this study because of the prolonged time required to establish a colony of the size suitable for downstream experiments on artificial diet. One advantage of the artificial diet is that, it is a consistent source of nutrition, readily available and easy to prepare, and it is possible to raise many individuals in a small space. For this reason, it is recommended that mass rearing of *P. xylostella* should be on artificial diet rather than on canola plants, as has been described for the rearing of false codling moth (FCM) and many other Lepidoptera. FCM is currently mass reared on artificial diet for the commercial production of *Cryptophlebia leucotreta* granulovirus (*Crle*GV) with considerable success; up to 5.38 million moths are produced per month for virus production (Moore *et al.* 2014).

During the rearing of the laboratory colony, dead larvae were observed and collected daily. Because the symptoms resembled that of a baculovirus infection, occlusion bodies were extracted and examined by transmission electron microscopy. This analysis confirmed the first isolation of a South African *P. xylostella* granulovirus. Before formulating a virus into a biopesticide for field application, it is important to characterise it genetically in order to establish the genetic identity of the virus isolate. PlxyGV-SA was genetically characterised by restriction endonuclease analysis (REN) and amplification and sequencing of selected viral genes. REN analysis of genomic DNA is widely used to differentiate between baculovirus isolates of the same species by generating profiles that can be compared by the band sizes. It is easy to perform and inexpensive, and it does not require the use of advanced instrumentation. The disadvantages of this method include requiring large amounts of viral DNA which means large samples of occlusion bodies are required in order to extract enough genomic DNA for the analysis. In addition, it is difficult to obtain the exact enzyme: DNA ratio in the reaction mixture which can result in incomplete digestion and poor resolution of the restriction digest on the gel. Moreover, electrophoretic separation of DNA restriction digests is slow and the DNA sample may be subject to degradation during electrophoresis or storage. Another disadvantage of this technique is that comparison of REN profiles between isolates is not accurate unless all digested samples are analysed on the gel in the same experiment.

It was not possible to obtain DNA from other PlxyGV isolates from other countries (Taiwan, India, Kenya and China) for comparison with PlxyGV-SA because of import restrictions. Although the comparison between profiles of PlxyGV-SA and those of published isolates indicated that they were different, it is more accurate to compare REN profiles with a profile generated *in silico,* if a full genome sequence is available. For the purpose of this study, the complete genome sequence of PlxyGV-Japan was used, and comparison of the two profiles confirmed that PlxyGV-SA is a genetically novel isolate.

PlxyGV was further characterised by amplification and sequencing of selected viral genes followed by comparison of nucleotide and amino sequences with those of PlxyGV-Japan. This analysis based on *granulin*, *ecdysteroid UDP-glucosyltransferase* (*egt*), *late expression factor 8 (lef-8*) and *late expression factor 9* (*lef-9*) genes also confirmed that PlxyGV was genetically different from the PlxyGV-Japan. This method has several advantages including that much less viral DNA is required because the PCR is capable of amplifying sequences from minute amounts of DNA. In addition, the method is relatively easy to perform and is not

expensive. The drawback of this method is that errors can occur in the sequencing reaction creating false single nucleotide polymorphisms (SNPs) which may not actually be there. Therefore, this method should involve multiple sequencing of the same gene segment. A second disadvantage is that only a small section of the genome can be analysed. The most accurate method of genetic comparison is sequencing and assembly of the complete viral genome. However, this was not done in this study due to time constraints but is currently being performed by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The full genome sequencing will provide more information on the PlxyGV-SA genome and will be used to validate the SNPs observed previously in the *granulin*, *egt*, *lef-8* and *lef-9* genes.

In order to formulate a biopesticide for field application, it is also important to evaluate the biological activity of the virus isolate against the host population under laboratory conditions. This is necessary in order to determine the virulence and speed of kill of the virus isolate. The surface dosing as opposed to the droplet feeding method proved to be effective in determination of the dose and time response in this study because the larvae readily ingested the virus along with food material whereas they did not drink the virus presented in droplets. The surface dosing bioassay method has been used in several studies to determine the biological activity of PlxyGV (Parnell 1999; Dezianian *et al*. 2010; Muthamia *et al*. 2011). The main limitation of the surface dosing method is that it only allows an estimation of the lethal concentration (LC). Because the amount of virus ingested by each larva cannot be quantified, it was not possible to determine LD_{50} values. Bioassays in this study showed that PlxyGV-SA was pathogenic to neonate PlxyGV-SA larvae with the LC_{50} value of 3.56×10^5 OBs/ml. This value is comparable with other LC_{50} values obtained in other studies indicating that the virus has potential as a biocontrol agent (Kadir *et al*. 1999b; Parnell 1999; Dezianian *et al*. 2010; Muthamia *et al*. 2011). The time to kill 50% of the neonates was observed to be 4 days and 8 hours. The mean developmental time of the first and second instar stage was 4.23 ± 0.07 days, indicating that when the virus is targeted against the neonate population it has the potential to kill 50% of the population before the transition to the third instar.

In this study, neonate larvae were observed to be more susceptible to infection than the fourth instar larvae, as indicated by the fact that the fourth instar larvae were not susceptible to the virus concentration that could elicit 95.83% mortality of the neonate larvae. This indicates that control should be aimed at the early instar larvae. Dezianian *et al*. (2010) also suggested that application of PlxyGV in the field should be targeted at the early stages of larval development in order to obtain the full potential of the virus. Timing of application is

regarded as one of the most important factors influencing the successful use of baculoviruses because susceptibility to infection is greatly reduced as larvae mature (Moscardi and Sosa-Gomez 1992). Monitoring of adult populations and egg counts usually helps in determining biopesticide application times in the field (Jaques 1990).

Conclusions and future work

In this study, a novel *P. xylostella* granulovirus, PlxyGV-SA, was isolated and genetically characterised, and its biological activity was determined under laboratory conditions. Although several objectives were successfully achieved, there is a large body of future work to be completed before commercialisation of the virus as a biopesticide. Firstly, it is necessary to establish a colony of *P. xylostella* maintained on artificial diet to facilitate the large scale production of virus. Secondly, a more thorough analysis of the biological activity of PlxyGV-SA must be conducted, namely on the second and third larval stages of the insect in order to test the pathogenicity of the virus during the entire developmental time of the insect. In addition, it will be necessary to establish a greater mortality in fourth instar larvae by feeding them with a higher dose of virus because the mortality observed in this study was low even with the highest virus concentration. It will also be necessary to conduct field trials in order to determine the pathogenicity of the virus under field conditions. Several factors such as stability of the virus in the environment, concentration required to elicit mortality in the field, and timing of application must be investigated in different geographical regions, under various conditions of insect infestation and in different plant stages. Once the field trials have been completed, the compatibility of the virus formulation must be tested with synthetic pesticides and other control techniques, in order to incorporate the virus product into an integrated management programme for the control of *P. xylostella*.

Finally, it will be vital to bioprospect for different PlxyGV isolates if the virus is to be used on a commercial scale. In this study, *P. xylostella* was collected from a single site and only one South African isolate was recovered and characterised. This can be a disadvantage in insect resistance management. For example, some field populations of codling moth, *Cydia pomonella* (L.) were reported to developed resistance to a Mexican isolate *Cydia pomonella* granulovirus (CpGV-M) in Europe, after repeated field applications (Fritsch *et al*. 2005; Eberle and Jehle 2006; Berling *et al*. 2009). Subsequent, studies have shown that resistance can be managed by exposing the virus resistant insects to a genetically different virus isolate

from the same virus species (Eberle *et al*. 2008; Berling *et al*. 2009). Therefore, a future aim of this research is to bioprospect for more novel PlxyGV isolates and to test their pathogenicity against a range of *P. xylostella* populations in the field.

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