INACTIVATION OF *HELICOVERPA ARMIGERA* NUCLEOPOLYHEDROVIRUS ON CHICKPEA, *CICER ARIETINUM* L.

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A thesis submitted in partial fulfillment of the requirements of the University of Greenwich for the degree of Doctor of Philosophy

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Declaration

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy (PhD) being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

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Dedication

I dedicate this work to my wife and children (Bilal, Sabirah and Ismail)

Abstract

Insect specific viruses like nucleopolyhedrosisvirus (NPV) can provide an alternative and effective insect pest control compared to conventional chemicals; however, on certain crops like chickpea efficacy of the virus is limited. Previous studies have demonstrated that inactivation on chickpea was leaf surface based and rapid, however, the compound identified (isoflavonoid, sissotrin) could not account for the total inactivation that was recorded on chickpea leaves. The present study investigated further the legume-NPV insect interaction, with aim of identifying conclusively the compound(s) responsible for NPV inactivation on chickpea, and also to determine if the same plant chemistry also occurs on two other major African and Asian legumes, cowpea and pigeonpea, and to understand the mechanism involved. Laboratory results showed that chickpea leaf surface was more inactivating to NPV than cowpea or pigeonpea. Although both cowpea and pigeon also reduced the efficacy of NPV the effect was relatively small. Bioassays with the isoflavonoids (biochanin A and formononetin), identified to be present or induced at higher levels after spraying with *Hear*NPV, showed that although both compounds significantly reduced NPV efficacy, the effect was modest compared to that recorded on chickpea leaves. When the most abundant chickpea acids (malic and oxalic) were mixed with different concentrations of the isoflavonoids and tested on NPV, significant inactivation was observed which was comparable to that obtained on chickpea leaves. The inactivation of NPV by the combination of chickpea acids plus isoflavonoids was not dependent on high concentrations of isoflavonoid tested, suggesting that chickpea acids alone could be responsible. Therefore, further tests were carried out to determine the role of the acids alone on NPV. The most abundant chickpea acids were prepared at different concentrations and tested with the virus at high lethal concentration. The results showed that chickpea acids alone were responsible for the inactivation of NPV, and among the two acids tested, oxalic was found to be more active against the virus. Although the inactivation was shown to correlate negatively with pH of the acids, the results suggest that oxalic acid was exerting its effect independently of the pH. This is the first study to demonstrate that organic acids of chickpea were responsible for inactivation of NPV. The findings from this study will be helpful in identifying suitable formulation additives to improve the field persistence of NPV on chickpea and other crops.

Table of Contents

List of Figures

List of Tables

Abbreviations

B.t	*Bacillus thuringiensis*
BCAs	Biocontrol agents
BV	Baculovirus
CaOx	Calcium oxalate
DNA	Deoxyribonucleic acid
ds	Double stranded
EC_{50}	Effective lethal concentration of the virus required to kill 50% of the test insect
GV	Granulovirus
*Hear*NPV	*Helicoverpa armigera* nucleopolyhedrovirus
HPLC	High Performance Liquid Chromatography
HPR	Host plant resistance
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
ICTV	International Committee on Taxonomy of Viruses
IITA	International Institute for Tropical Agriculture
IPM	Integrated Pest Management
LC_{25}	Lethal concentration of the virus required to kill 25% of the test insects
LC_{75}	Lethal concentration of the virus required to kill 75% of the test insects
LC-MS	Liquid Chromatography linked to Mass Spectrometry
MNPV	Multiple envelope virus
NOV	Non-occluded virus
NPV	Nucleopolyhedrovirus
NRI	Natural Resources Institute
OB	Occlusion body
ODV	Occlussion derived virus
PDV	Polyhedral derived virus
pH	Power of Hydrogen (negative logarithmic value of the Hydrogen ion concentration)
PIB	Polyhedral inclusion body
pKa	Acid dissociation constant at logarithmic state
POD	Peroxidase
ppm	Part per Million (1 ppm = 1 μg/ml)

PPO	Polyphenol oxidase
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SEM	Scanning Electron Microscope
SNPV	Single envelope nucleopolyhedrovirus
UV	Ultraviolet
VAM	Vesicular arbuscular mycorrhizae

CHAPTER ONE

INTRODUCTION

1.1. Principal objectives

The principal objectives of the present study is to investigate further the tritrophic interactions between chickpea and its major field pest *Helicoverpa armigera* and nucleopolyhedrovirus (NPV) a pathogen of this pest used as biological insecticide, with the aim of improving the effectiveness of *Hear*NPV on chickpea and to enhance up-take of biopesticides for pest control.

1.2. Background of the study

Crop losses due to pests remain a serious feature of global agriculture (Fitt, 1989; Gowda, 2005). Food losses are expected to increase in the future unless sustainable farming practices are employed to meet the needs of the growing population. Crop production in many countries is held back due to the difficulty in controlling obstinate pests such as *Helicoverpa armigera* (Gowda, 2005; Sharma *et al*., 2005). This pod borer [*H. armigera* Hubner (Lepidoptera: Noctuidae)] has been described as one of the most important constraints to crop production globally (Fitt, 1989; Gowda, 2005), having developed high level of resistance to many of the commonly used insecticides (Kranthi *et al*., 2001; 2002; Srinivas *et al*., 2004). According to King (1994) the four major species of *Heliothis*/*Helicoverpa* were probably responsible for more indiscriminate use of chemical insecticides than any other insect genera.

Sustainable control of *H. armigera* through relying solely on the conventional chemical insecticides has become increasingly expensive and sometimes ineffective (King, 1994; Franzmann *et al*., 2008). At present, there is action by several governments to ban many toxic chemical pesticides due to environmental, human health and safety issues (Skovmand, 2007; Hillocks, 2012). Biopesticides based on entomopathogenic bacteria, fungi and insect viruses such as NPV are becoming

increasingly important in the management of legume pod borer (Grzywacz *et al*., 2005). One advantage of NPV is that they could reproduce and persist in the environment hence maintaining the pest population below the economic threshold (Payne, 1982; Huber, 1986). Viral insecticides are specific in action with no toxic residue which makes them safer compared to conventional chemical pesticides that are broad spectrum in action with negative effects on the environment (Hunter-Fujita *et al*., 1998; Copping and Menn, 2000). One important feature of microbial control agents such as baculovirus (BV) is that they can be produced using a relatively simple and cheap technology (Cunningham, 1995; Moscardi, 1999; Jenkins and Grzywacz 2000; Gelernter, 2007).

Interest is increasing in the use of NPVs for the control of several important pests like *Heliothis/Helicoverpa spp* (Grzywacz *et al*., 2005; Buerger *et al.,* 2007), due to the difficulty encountered during their control using conventional approaches. The use of viral insecticides is expected to increase in future because of the concern for the environment, problems of insecticide resistance and difficulty of developing new chemical pesticides (Hunter-Fujita *et al*., 1998, Glare *et al*., 2012). Field resistance to viral insecticides is not common (Abot *et al*. 1996; Buerger *et al.,* 2007 Cory and Franklin, 2012) and where the problem has been identified, resistance ratios are relatively low and unstable particularly where the selection pressure is absent (Fuxa and Richter, 1989; Fuxa and Richter, 1998). The inherent variability among organisms may substantially reduce the occurrence of resistance compared to chemical pesticides (Straus and Knight, 1997). Evidence has shown that viral insecticides do not create resistance problems in either target or non-target species nor show cross resistance with chemical compounds (Huber, 1986). According to Cory and Franklin (2012) the infection process in insect viruses unlike other entomopathogens, is a complex process involving many resistance mechanisms. Furthermore, they noted that viral insecticides are still not widely used for pest control hence risk for resistance developing is low. Even where viral insecticide has been used extensively, no resistance problem has developed (Huber, 1986; Abot *et al*., 1996; Buerger *et al*., 2007). This unique property along with their specificity and safety has contributed in promoting NPV as a desirable agent for use in Integrated Pest Management (IPM) (Franzman *et al*. 2008; Moscardi *et al*., 2011).

However, despite their potential for management of insect pests, the level of control achieved with NPVs is still relatively low compared to chemical insecticides (King and Coleman, 1989; Lacey *et al*., 2001, Moscardi *et al*., 2011). One major limitation which reduces their efficacy is their short-term persistence (Payne, 1982; Fuxa, 2004). Viral persistence is primarily affected by ultraviolent light (UV light) (Jacques, 1967; McLeod *et al*., 1977 ; Jacques, 1985; Young and Yearian, 1986) and by host plant chemistry (Duffey *et al*., 1995; Hoover *et al*. 1998abc; Ali *et al*., 2002; Raymond *et al*., 2002; Stevenson *et al*., 2010). In the tropics, UV portion of solar radiation can lead to a significant lost of viral activity in the field (Jones *et al*., 1993). In cotton, the mechanism has been attributed to oxidative processes influenced by foliar oxidative enzyme, particularly peroxidase (POD), leading to free radical generation and subsequent sloughing of infected midgut cells (Hoover *et al*., 1998bc; Hoover *et al*., 2000). In chickpea, it has been partially linked to action of leaf surface isoflavonoids (Stevenson *et al*., 2010). Promotion of alternatives to the conventional chemical pesticides will be difficult unless the solutions are effective and cheap to the primary users (Skovmand, 2007; Buerger, 2007). Increasing the use of viral insecticides will depend on further research on these key limiting issues. Future development is expected to occur in areas of recombinant Baculoviruses and *in vitro* commercial production, which will help to increase the speed of kill and widen the host range as well as decrease the cost of viral product (Moscardi, 1999; Bonning and Hammock, 1996; Szewczyk *et al*., 2006). However, biosafety issues of genetically engineered virus and technical difficulties for commercial development of *in vitro* viral insecticide production systems are still not yet resolved (Moscardi *et al*., 2011). In addition, wild-type and engineered viruses will still share a common problem of low persistence (Bonning and Hammock, 1996; Sun *et al*., 2004). Hence improving the stability of Baculoviruses in the field particularly on key crops through improved formulation chemistry could provide a better alternative (Moscardi, 1999; Cherry *et al*., 2000; Stevenson *et al*., 2010). If the crop factors that reduce the efficacy of viral insecticides are identified and the mechanism of action known, appropriate formulations could be developed to improve the effectiveness of viral insecticide (Grzywacz *et al*., 2005; Stevenson *et al*., 2010). Improved formulation could improve product stability during storage and at the same time enhance persistence and efficacy in the field (Jones, 1994; Jones *et al*., 1997; Szewczyk *et al*., 2006).

1.3 The cotton bollworm (*Helicoverpa armigera*) and other Heliothine species as crop pests

1.3.1 Biology

Adult moth

The life cycle of *H. armigera* is shown below (see Fig. 1.1). Adult moths have a stout body with male insects being smaller (Jayaraj, 1982). There is colour variability between the different *Heliothine* species (King, 1994). In *H. armigera*, female insects are differentiated from the males by the colour of their forewings, which is greenish in males and light to dark brown in females (Armes *et al*., 1992; King, 1994). The adult lifespan is largely determined by food availability. Where food is absent the moth dies after about 3-6 days due to rapid depletion of body fat, although female moths have a longer life span than males (Jayaraj, 1982). Provided the adult moths have fed, copulation occurs in about 1-4 days after emergence from pupae, although female moth usually lay infertile eggs before mating (Jayaraj, 1982). According to Abate and Ampofo (1996) fecundity is heavily influenced by environmental factors. They observed high number of eggs during the rainy season of 1226 per female against 198 in the dry season. Female moths continue to oviposit for 10-23 days in South Africa depending on the time of the year, each producing about 730 eggs on average and a maximum of 1600 (Jayaraj, 1982). According to Zalucki *et al*. (1986) maximum fecundity of 2899 was recorded at 24°C with a maximum number of egg produced in one day of 691 and an average of 112 eggs per female per day over 12.8 days and 83 eggs per female per day over 16.8 days at 24ºC and 19ºC respectively. Bergvinson (2005) observed that the life cycle of *H. zea* is completed in 28-30 days at 25°C, although he noted that development can be delayed or stopped by either drought or low temperatures and thus may increase the cycle under cold conditions.

Eggs

The colour of the egg changes as they develop from light yellow to dark brown and finally turns black as the embryo develops before hatching. The infertile egg usually remains yellow and shrivel within few days (Armes *et al*., 1992). Eggs are laid singly usually in the evening and mostly after 21:00 hour (Jayaraj, 1982), with majority laid

within first 12 days after mating and peaking on nights 3-4 (Armes *et al*., 1992). On many host plants, eggs are laid on leaf surfaces, stems, buds, flowers and fruits, while on chickpea they are usually found under surface of terminal leaflets (Reed *et al*., 1987). According to Bergvinson (2005) eggs are preferably laid on or near the fruiting structures or tender plant parts. This could be important when monitoring early larval instars for pest control. Jayaraj (1982) observed female moths to prefer deep green colour for oviposition. The duration of the egg incubation period is dependent on temperature (King, 1994). Therefore, hatching can be delayed by storing the eggs at low temperature (i.e. 4°C) so as to synchronize hatching of larvae and reduce variability during bioassay (Jones, 2000).

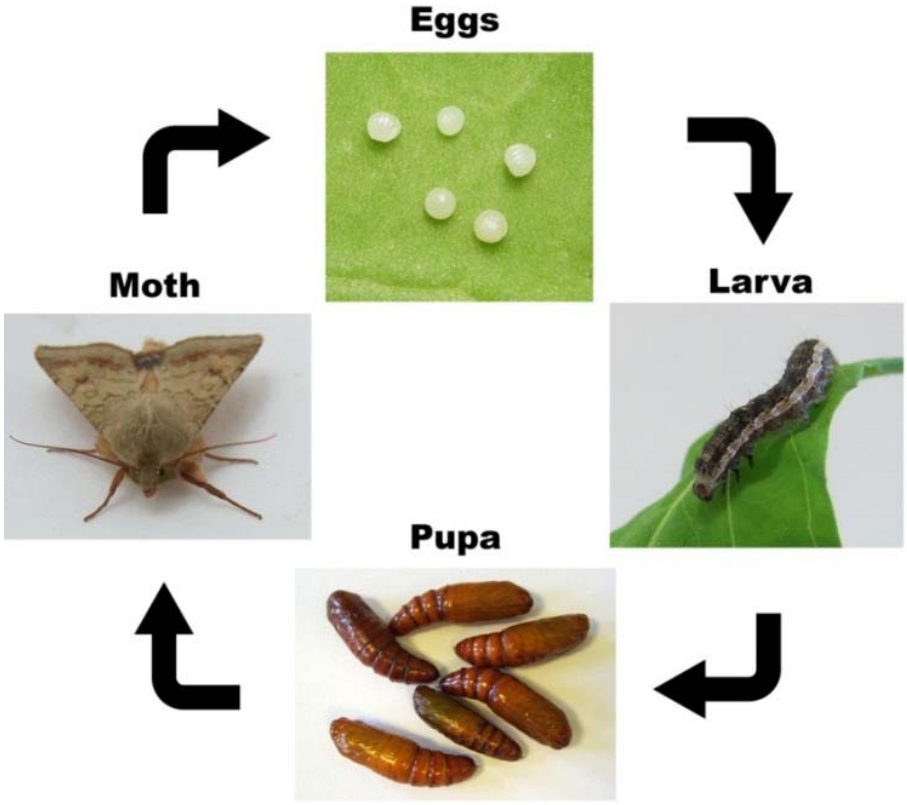


Figure 1.1 Life cycle of *Helicoverpa armigera.*

http://www.intechopen.com/source/html/37968/media/image1.jpeg

(Accessed: 04/02/2015)

Larvae

The newly hatched larva is semi-transparent and yellowish-white in colour, with faint darker longitudinal lines and a black head capsule. The general body colour of the full grown larva is pale green with one broken stripe along each side of the body and another line on the dorsal side, as well as presence of short white hairs found all over the body (Jayaraj, 1982). However, there is variability with regards to the colour of the last instar, ranging from shades of green to yellow, pinkish to reddish brown or black (Jayaraj, 1982; King, 1994; Reed *et al*. 1987; Bergvinson, 2005). This colour variability according to King (1994) is common to all the four *Heliothine* species (*H. amigera, H. zea, H. virescens and H. punctigera*). Larval colour variability has been attributed to be influenced by the colour and nutritional content of the host plant (King, 1994). After hatching, the larva feed on some or all of its empty shell before it settles down at a preferred site (Jayaraj, 1982; King, 1994). They noted that a fully grown larva usually feeds with only the front part of its body inside the hole it has made while the remaining part is outside, and this feeding habit can protect the larva against insecticide spray (see Fig. 1.2). Cannibalistic habit can increases as the larva develops which is an important consideration in culturing (see methods chapter 2). The larvae normally undergo six instars, but seven have also been recorded under cold conditions (Abate and Ampofo, 1996) but the target for most pesticides is second or third instar. The duration of larval development is dependent on the temperature as well as the nature and quality of the host plant (Jayaraj, 1982; King, 1994), and therefore maintaining insects in temperature controlled environment using artificial diet will help to reduce this variability. Prior to pupation, the fully-grown larva dropped to the ground and burrows into the soil to a depth of 2.5-17.5cm (Jayajaj, 1982; King, 1994).

Pupae

The pupae are reddish brown with a smooth-surface and round shape with two narrowing spines at the posterior end (Jayaraj, 1982; King, 1994). While larval size is determined by food quality, on average female pupae are reportedly heavier than the males (King, 1994). The pupae of all the four *Heliothine* species undergo facultative diapause, and the duration of the pupal stage depends upon whether diapause has been induced during the earlier stages and its intensity (King, 1994). Non-diapause pupal

period of *H. armigera* took 14-40 days in Sudan Gezeira, 14-57 days in Zimbabwe, 12-25 days in Tanzania, 14-37 days in Uganda and 5-8 days in India (King, 1994).

Figure 1.2 *Helicoverpa armigera* larva burrows into and feed on chickpea pods (Stevenson, 2006).

1.3.2 Distribution and host range

The noctuid genera *Heliothis* and *Helicoverpa* contain pests of world-wide importance, and their larvae are the most important bollworms globally (Fitt, 1989; King, 1994). *H. armigera* is distributed throughout the old world (i.e. Africa, India, China and Australia); while *H. zea* and *H. virescens* are confined to new world (i.e. North America) and *H. punctigera* is limited to Australia (King, 1994). Those four *Heliothine* species are the key pests of agricultural significance and therefore attracted most research work (Fitt, 1989). However, *H. armigera* is the most widely distributed of the group (see Fig. 1.3) occurring throughout Africa, India, central and south-eastern Asia, eastern and northern Australia, New Zealand, and many eastern Pacific Islands

(Zalucki, *et al*., 1986; Fitt, 1989; Armes *et al*., 1992). Other *Heliothis/Helicoverpa* species of lesser importance include *H. peltigera*, *H. viriplaca* (*dipsaceae*) and *H. assulta* (Fitt, 1989; King, 1994). The history of the nomenclature and taxonomy of the genus *Heliothis/Helicoverpa* is full of controversy (Nye, 1982). Until the middle of this century, *H. armigera* had been considered identical to the cotton bollworm or the corn earworm of the United States, which is now referred to as *H. zea* (Reed and Pawar, 1982; King, 1994). However it has been accepted generally that the two species are very similar in all aspects and are responsible for circling the earth, with *H. zea* distributed across the Americas and *H. armigera* stretching across all over the tropical and sub tropical countries (Reed and Pawar, 1982). Recently, mitochondrial DNA sequence data supports the single species status of *H. armigera* across Africa, Asia and Australia (Behere et al., 2006, Behere *et al.,* 2007), although both *H. armigera* and *H. zea* were known have high genetic similarities between them (Behere *et al.,* 2007).

The four major *Heliothis/Helicoverpa* species are highly polyphagous and collectively they attack a wide range of food, fibre, oil and fodder crops and many other horticultural and ornamental crops (Fitt, 1989; King, 1994; Sharma *et al*., 2005). Zalucki *et al.* (1986) recorded a total of 159 plant species from 49 families, as host plants for both *H. armigra* and *H. punctigera* in Australia. The most complete list of host plants based on published reports shows *Heliothis/Helicoverpa* species to feed on 235 plant species in 36 families (Kogan *et al*., 1989). In relation to areas of cultivation, cotton, soybean, tobacco and pulses, account for crops that incur most of the economic damage caused by *Heliothis* species (Fitt, 1989). According to King (1994) the prominent host families include Leguminosae, Solanaceae, Malvaceae, Asteraceae and Gramineae. The families with the most species reported to be hosts include Asteraceae (24 species), Convolvulaceae (10 species), Cucurbitaceae (7 species), Leguminaceae (39 species), Malvaceae (25 species), Rosaceae (41 species), Scrophulariaceae (7 species) and Verbenaceae (4 species) (Bergvinson, 2005). According to Sharma *et al*. (2005) the host range of *H. armigera* includes such economically important crops as cotton, chick pea, pigeon pea, maize, peas, cowpea, sunflower, sorghum, groundnut, field beans, tobacco and range of vegetables, fruits and tree crops. However, in this study interest is on chickpea, pigeon pea and cowpea. Kogan *et al*. (1989) also listed crops of research importance based on the number of papers published which include; maize, cotton, soybean, tomato, tobacco, common bean, alfalfa, peanut and sugarcane.

Despite the ability to breed on a wide range of host plants, species linked preference exist (Zalucki *et al*., 1986), although this is modified by seasonal availability of hosts in the preferred developmental stage (King, 1994). The feeding preference of *H. armigera* has been recorded in descending order to include; pigeon pea, field bean, cotton, sunflower, sorghum, chickpea, mungbean, urdbean (blackgram/black lentils) and tomato (Jayaraj, 1982). Cotton often supports the highest population but only after other alternative hosts have been harvested (Fitt, 1989).

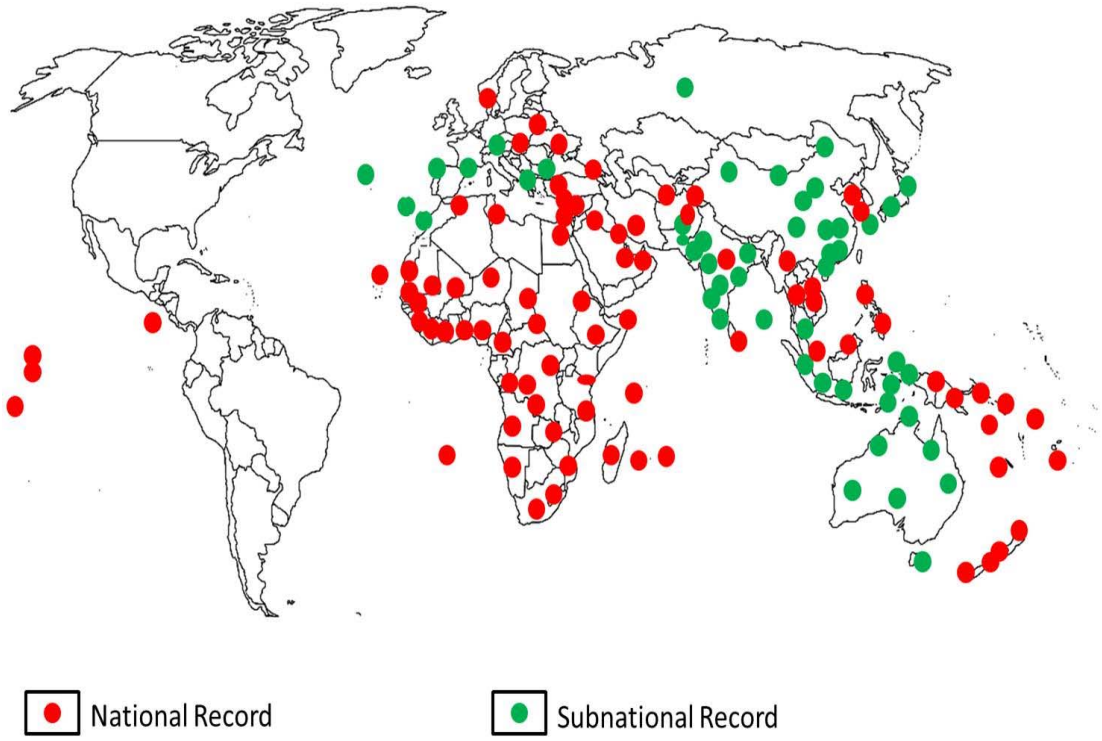


Figure 1.3 Global distribution of *Helicoverpa armigera* across different continents including Africa, Australia, Asia and parts of Europe

https://secure.fera.defra.gov.uk/phiw/riskRegister/plant-health/documents/helicoverpa.pdf

(Accessed: 15/03/2015)

1.3.3 Economic impact and existing control

The pest status of the four major *Heliothine* species is derived from the nature of their biology (King, 1994). They have high mobility and fecundity, as well as being

polyphagous and can undergo facultative diapause (Fitt, 1989, King and Colemam, 1989, King, 1994). Such unique physiological, behavioural and ecological characteristics enable them to survive in unstable habitats and to successfully colonise and exploit agricultural systems (Fitt, 1989). The feeding preference of *Heliothine* larva for the fruiting and reproductive structures of their host plants has a direct effect on the yield (Fitt, 1989, King and Coleman, 1989, King, 1994). Zalucki *et al*., (1986) noted that the tendency of the larvae to move from one fruit to another can result in high economic damage even where the number of final instars decreases (see Fig. 1.3). Damage caused to important subsistence crops like maize, sorghum and pulses as well as small farmers' cash crop can be severe in socio-economic terms (King, 1994). Economic losses due to *Heliothine* pests can result from a direct reduction in crop yield and also from the cost of pest monitoring and control, (Fitt, 1989, King, 1994). King and Coleman (1989) noted that structures damaged by *Heliothine* species are greatly reduced in quality and this can lead to a reduction in their market value and often rendered them susceptible to diseases. Ujagir (2005) observed that 2-3 larvae on a cotton plant can cause damage to all the bolls within 15 days, while a single larva can damage 2-34 grains on maize plant. Ujagir (2005) also reported about 30% pod damage in northern India by *H. armigera* and a total of 33-50% pod damage across India. In India, losses due to chickpea and pigeon pea may exceed $ US 300 million per annum (Reed and Pawar, 1982). Recently, an estimated loss of $ US 5 billion annually was attributed to *Heliothis/Helicoverpa*, despite the use of more than $ US 1 billion worth of pesticides for its control (Sharma *et al*., 2005). In addition, there are other indirect losses as a result of the negative effect of chemical pesticides on the environment, human and animals' health which are not accounted for (Gowda, 2005).

Effective management of *Heliothine* pests, especially on high-value crops still relies heavily on chemical pesticides (Gowda, 2005, Sharma *et al*., 2005). However, indiscriminate and repeated spray with chemicals has resulted in the development of insecticide resistance and resurgence of pest populations (Kranthi *et al*., 2001; Kranthi *et al*., 2002). They noted that use of poor application equipment and unreliable products in the market coupled with poor choice of chemicals have exacerbated the problems. Where insecticide resistance has developed, efficacy of pesticides application declines thus, farmers resort to the use of different potentially more toxic chemicals and more frequent applications to achieve control (Fitt, 1989; Kranthi *et al*.,

2005). *Heliothis/Helicoverpa* infestations are difficult to control even with insecticides as the larvae burrow into fruiting structures making it difficult to reach with insecticide (Sharma *et al*., 2005). King and Coleman (1998) also observed that insecticide applications in field corn against *H. armigera* and *H. zea* are usually not economically feasible because of the reason mentioned above.

1.3.4 Biopesticides and *Helicoverpa/Heliothis* control

Bacteria, viruses and fungal organisms have many features which make them ideal for use in pest management (Copping and Menn, 2000). They are specific and highly virulent on the target the host, safe to non-target organisms and compatible with other pest management options (Groner, 1990; Lacey *et al*., 2001). Although it can be said that certain degree of success has been achieved with the use of biopesticides for insect pest control, still their potential has not matched with use compared to conventional chemical pesticides (Copping and Menn, 2000; Grzywacz *et al.,* 2005).

Bacillus thuringiensis

Biological insecticides based on entomopathogenic bacteria represent 95% of the microbial insecticide market and are mainly based on one species, *Bacillus thuringiensis* (*B.t*) (Bravo *et al*., 2011). *B.t* is closely related but differs from other members of *B. cereus* group (common soil bacterium) due to its enthomopathogenic properties and production of insecticidal δ- endotoxin protein during sporulation (Lacey *et al*., 2001; Grzywacz *et al*., 2005). These δ- endotoxins are highly specific to their target insect but safe to human, other organisms and the environment (Bravo *et al*., 2011). There are several *B.t* isolates or subspecies, the well-known of which include *B.t kurstaki* and *B.t azaiwai* with activity against lepidopteran larvae, *B.t israelensis* active against dipteran larvae and *B.t tenebrionis* that shows activity towards coleopteran larvae (Smits, 1997). Despite many advantages of *B.t*, it has some limitations, the major of which is the risk of resistance developing as it is sprayed regularly like the conventional chemical pesticides (Smits, 1999; Navon, 2000; Koul and Dhaliwal, 2002). They also observed that unlike virus and fungi, *B.t* lacks the desirable biological property of reproducing and maintaining itself in the environment. Hence, industry has focused more on the strength of its insect killing toxin by

encouraging its repeated use like a chemical product (Koul and Dhaliwal, 2002). Field persistence of *B.t* is generally poor, due to action of solar UV light, rainfall and foliar metabolites (Smits, 1997; Navon, 2000). However, one important factor which leads to the success of *B.t* as a microbial insecticide is the ability to produce it in large quantities at low cost using a variety of simple media in large scale fermenters (Smits, 1997; Sanchis, 2011). Lately, the isolation of new strain of *B.t* with virulent toxins and development of recombinant technology has provided the opportunity to manipulate *B.t* genes that encode toxin production as well as incorporating these genes into crop plants has contributed to overcome some of the inherent limitations of *B.t* as a biopesticide and widen its market (Georgis, 1997; Navon, 2000). For example, one limitation of *B.t* microbial preparation such as low field persistence and lack of ability to control cryptic feeding pests has been overcome through expression of *B.t* in transgenic plants (Sanchis, 2011).

Enthomopathogenic fungi

From over 700 species of entomopathogenic fungi reported (Copping and Menn, 2000; Lacey *et al*., 2001; Rosell *et al*., 2008), only a few have been considered for development commercially as biological control agents (Smits, 1997; Copping and Menn, 2000). The most important group of insect pathogenic fungi with high potential for commercial production as biological control agents are the *imperfecti* fungi (Deuteromycotina: Hyphomycetes) (Bell, 1982; Smits, 1997; Lacey *et al*., 2001). Among these groups, several have been tested for *Heliothis* management but the common ones observed causing natural epizootics include; *Beauvaria bassiana* (Balsamo*)* Vuillemin*, Metarhizium anisopliae* (Metschnikoff) Sorokin and *Nomuraea rileyi* (Farlow) Samson (Bell, 1982; Goettel *et al.,* 2005). Like *B.t* most fungi can be mass-produced using fermentation techniques (Bell, 1982; Copping and Menn, 2000). However, their most important feature which differentiates them from entomopathogenic bacteria or virus is that they can penetrate the insect cuticle hence ingestion is not required for infection (Inglis *et al*., 2001; Cory and Hoover, 2006). Therefore, fungi could be active against other stages of insects such as adults and pupae (Goettel *et al.,* 2004). The main limitation of fungi as a biocontrol agent is their dependence on environmental conditions such as high humidity and moderate temperature (Smits, 1997). Generally, fungi have their greatest potential where

microclimate of the host area provides optimum conditions such as high humidity and moderate temperature (Butt and Copping, 2000; Goettel *et al.,* 2005).

Nucleopolyhedrovirus

Some of the major entomopathogens (i.e. virus, bacteria and fungi) have been reported to have potential for use in *Heliothis* management (Bell, 1982; Carner and Yearian, 1989; Grzywacz *et al*., 2005). Recently however, interest has shifted more on nucleopolyhedrovirus (NPV) for the control of *H. armigera* (Ratanasatein *et al*., 2005; Grzywacz *et al.,* 2005; Buerger *et al*., 2007; Kessler *et al*., 2008). NPV appears to have the most potential of all the microbial agents available due to its virulence against early larval instars and its property of causing epizootics (Carner and Yearian, 1989, Rowley *et al*., 2011). Those viruses are natural pathogens of *H. armigera* and are commonly found in Asia, Africa and Australia (Grzywacz *et al*., 2005; Buerger *et al.,* 2007; Kessler *et al*., 2008). NPV has been found to be highly specific to *Heliothis/Helicoverpa* spp, and has been shown to be effective against those pests on sorghum, cotton, chickpea and maize (Cherry *et al.,* 2000, Buerger *et al*., 2007), as well as on mungbean, tomato, okra, asparagus, grape vine, tangerine, rose and marigold (Ratanasatein *et al*. 2005; Kessler *et al.,* 2008). *Heliothis* NPV was the first viral insecticide to be registered in the U.S.A, and has been shown to be effective against *Heliothis* spp on sorghum, cotton, corn, soybean, tobacco and tomato (Ignoffo and Couch, 1981). Presently, strains of these viruses have been commercially developed for pest control in Australia, America, China, India and Thailand (Grzywacz *et al.,* 2005; Buerger *et al*., 2007; Sun and Peng, 2007). The number of registered baculovirus products has been increasing globally, with about more than fifty different products, even though many are of the same virus sold under a different brand name (Szewczyk *et al*., 2009; Moscardi *et al*., 2011) However, apart from Australia and Brazil their use has been restricted to specific markets and are yet to be established on major field crops (Grzywacz *et al*., 2005; Buerger *et al.,* 2007; Moscardi *et al*., 2011). Some of the constraints identified to be responsible for the limited uptake of NPV for pest control include; certain technical, perceptual, regulatory and commercial obstacles (see Section 1.4) (Cherry *et al*., 2005; Cherry and Gwynn 2007; Chandler *et al*., 2008), as well as limitations on the efficacy, cost and availability of viral insecticide (Cherry *et al*., 2000; Grzywacz *et al*., 2005). Expansion of viral insecticides

use will depend on exerting more research effort to overcome some of those limiting issues (Moscardi, 1999; Grzywacz *et al.,* 2005; Buerger *et al.,* 2007). Although technological advancement as well as socio-political attitudes has brought changes in favour of environmental friendly pest control options like baculovirus (Glare *et al*., 2012), more is still required to bring the transformational change that will enhance their uptake into the mainstream agriculture. Already NPV selectivity and cost of production has deterred its commercial development by industries (Lisansky 1997; Lacey *et al*., 2001). This could be the reason for its high cost and limited use in many countries, especially in the developed world. Where as in developing countries where cost of production could be relatively low, use of biocontrol agents (BCAs) has been hindered primarily by lack of awareness due to the absence or breakdown of extension services which promote the transfer of technology to farmers (Cherry *et al*., 2005; Grzywacz *et al.,* 2014). While specificity of NPV has been a limiting factor to their development, this has not been the case for *Hear*NPV because about 40% of the world`s insecticide is used for *Heliothis/Helicoverpa* spp control on different crops globally (Mettenmeyer, 2002).

Enthomopathogens have many features which makes them suitable for use in pest management programmes. Such as their human safety, environmental friendliness and relative cheapness to develop and registered compared with chemical pesticides (Georgis, 1997; Ignoffo, 1999). Recently microbial insecticides are facing competition from new technologies, prominent of which are new biorational pesticide chemicals and transgenic plants (Georgis, 1997; Grzywacz *et al*., 2005). However they noted that there is a high probability of insect pests developing resistance to such products if used extensively and exclusively. Furthermore, new chemicals could be more expensive hence improving the market potential of biopesticides (Grzywacz *et al*., 2005). Also, the need for *refugia* (areas of non-treated/non-transgenic crops set aside to ensure survival of susceptible insects) as a means of resistance management strategy could provide new opportunity for biopesticides as they are incorporated in to a sustainable transgenic plant management (Gelernter, 1997; Narayan, 2004; Grzywacz *et al*., 2005). By and large, transgenic crops are not a choice for certain agricultural practices such as organic production and also not all countries have subscribe to this technology (Bravo *et al*., 2011) in these cases, microbial pesticide will still continue to play a key role in insect pest control.

1.4 Advantages and limitations of baculovirus insecticides

In most instances, baculovirus (BV) cost-effectiveness and performance are always compared directly to chemical pesticides. However, in reality some of the attributes that are considered to be the strength of the chemical compounds have now turned out to be their weakness due to the negative effects they have on non-target organisms and the environment (Lacey *et al.,* 2001). Some of the advantages and limitations of BV include the following:

Specificity and safety

The family Baculoviridae only infects insects and mainly of the same order, therefore their host range is limited making them safe to non-target organisms (i.e. natural enemies and beneficial insects) and other vertebrates (Groner, 1990; Thiem and Cheng, 2009). Viral insecticides are therefore compatible with other pest control measures and also ideal for use in IPM programmes (Jones, 1990, 1994). Because they produce no toxic residue and have relatively short environmental persistence, BVs are considered as safe to human health and the environment (Ignoffo 1999; Moscardi, 1999). On the other hand, although specificity is seen as one of their greatest advantage, this has also discouraged some product development due to small market size and limited profit potential (Huber, 1990; Gelernter, 2007).

Production

As an obligate pathogen, BV can only be produced in living cells (*in vitro* or *in vivo*), but for now it is only produced in insect host-larvae. Although this production system is relatively easy using simple technology (Jones, 1994; Cunningham, 1995; Grzywacz *et al.,* 2014), it is more feasible economically in cottage industry of developing countries due to high labour cost (Huber, 1990, Moscardi, 1999; Grzywacz *et al.,* 2005). Although it is possible to produce viral insecticide using insect cell lines (*in vitro*) on a small scale, presently this technique is not commercially viable due to difficulties in terms of both technical and economic aspects and this has discouraged some potential producers (Inceoglu *et al.,* 2006; Reid 2014). One major limitation of baculovirus using *in vitro* technology is the lack of cost-effective production technology (Reid, 2014). The scale in which *in vitro* production is currently feasible is relatively small and costly due to high cost of specialized reactors, media and patent

license fees (Ravensberg, 2011). Similarly, studies carried recently have shown that due to instability of the virus after anumber of passages and the fact that cell lines do not produce high enough titres, commercial production is still not yet commercially feasible (Ravensberg, 2011).

Sustainable control

When applied for pest control, BV can reproduce in host insects from where it spreads horizontally and become established in pest populations creating a natural epizootic that helps to keep the target pest below economic threshold level (Jones, 1990; Fuxa, 2004), where it can ensure durable and sustainable control. Viral insecticides are not known to cause major resistance problem in the absence of strong selection pressure (Fuxa *et al.,* 1989; Fuxa and Richter, 1998), since BVs infection is considered to be complex process involving many diverse and polygenic resistance mechanisms (Cory and Franklin, 2012), therefore field resistance to viral insecticides is uncommon. Resistance did appear to Codling moth GV (granulovirus) in Europe after 20 years of sustained use but this was overcome by switching to a different strain (Eberle *et al.,* 2006; Eberle *et al.,* 2008). Therefore, viral products have often been used to provide effective control of insect pests where chemical products failed due to insecticide resistance (Buerger *et al.,* 2007). Conversely, viral insecticides are perceived by many as slow acting insecticides (Straus and Knight, 1997; Moscardi, 2007), because they needs to be ingested to be effective hence timing of application is important and need to be targeted at early instar larvae which are more susceptible. Although a number of virus products can give pest control equal to that of chemical pesticides (Cherry *et al.,* 2000; Lacey *et al.,* 2001; Grzywacz *et al.,* 2008), overall viral insecticides are seen by many primary users as products with unpredictable performance (Straus and Knight, 1997, Grzywacz et al. 2005). This is because their effectiveness is usually influenced by solar radiation and host-plant factors (Young and Yearian, 1974; Entwistle and Evans, 1985a,b; Cory and Hoover, 2006). However applied to the right targets and as part of a validated IPM system they can be used effectively in pest control (Lacey *et al.,* 2008; Moscardi *et al.,* 2011; Glare *et al.,* 2012).

Adaptable to genetic manipulation

Genetic enhancement of BVs through recombinant DNA technology could overcome some of the inherent limitations of viral insecticides such as increasing the host range and shortening the time required to kill the host insect (Bonning and Hammock, 1996; Szewczyk *et al.,* 2006), as well as ensuring quality and cost effective viral product (Inceoglu *et al.,* 2006). However, this technology could only be economically feasible when the *in vitro* production of BV in insect cell line is resolved (Inceoglu et al. 2006). One important consideration of genetically modified (GM) viral insecticide is biosafety issues (Szewczyk *et al.,* 2006). Therefore, while research in this area has made a lot progress, no any GM viral insecticide product has yet been commercialised due to strict regulations (Glare *et al.,* 2012)**.**

Registration

Generally, BV products are faster and cheaper to develop and register compared to conventional chemical products or even other microbial products such as *B.t* and fungal products (Ignoffo, 1999; Ravensberg, 2011). Developing a registration dossier for baculovirus active substances is relatively easy (compare to *B.t* or entomopathogenic fungi) since viral insecticide is not known to produce metabolites, toxin or reproduce outside the insect host (Ignoffo *et al.,* 1999; Ravensberg, 2011). The request for data waivers (i.e. justification for non-submission of data) could be a means of building registration dossier (Ravensberg, 2011). In this case, data waivers are relatively easier for baculoviruses compare to either *B.t* or entomopathogenic fungi (Ravensberg, 2011). However, registration is still one of the major difficulties encountered in the development and commercialization of microbial pest control products including BV, particularly in developed countries (Buerger *et al.,* 2007; Glare *et al.,* 2012). Primarily because still in many countries entomopathogens are still registered using the inappropriate chemical pesticide model and many regulatory staffs often lack the expertise of microbial pest control agents so that registration in these cases can be more costly due to the requirement for too much inappropriate data (Chandler *et al.,* 2008; Glare, 2012).

1.5 Baculoviridae

1.5.1 General features

A unique feature of many insect viruses which distinguish them from other viruses associated with plants or vertebrates is the occlusion of virus particles (virions) within a proteinaceous coat called the occlusion body (OB) (Entwistle and Evans, 1985a; Hunter-Fujita *et al*., 1998; Crook, 1999). Among this group the family Baculoviridae is the largest and most widely studied by virtue of its biological potential for the control of arthropod pests particularly the holometabolous insects (Entwistle and Evans, 1985; Federici, 1986; Smits, 1997). The Baculovirus family is unique because it shares no overt structural or biochemical similarities linked to vertebrates or plants (Payne, 1982; Crook, 1999; Slack and Arif, 2007). This family has a large circular double-stranded (ds) DNA genome and a structurally complex rod-shaped enveloped virus particle (Federici, 1986; Hunter-Fujita *et al*., 1998). Previously, Baculoviridae was classified into two genera (see Fig. 1.4); nucleopolyhedrovirus (NPV) and granulovirus (GV) (Murphy *et al*., 1995). The two genera are distinguished by the size and morphology of their OB (Rohrmann, 1999). The OB of GV is smaller (0.3-0.5µm in length) to that of NPV (0.15-15µm in diameter) and usually contain single envelope nucleocapsid (virus particle), while that of NPV contain several hundred virus particles each containing single (SNPV) or multiple (MNPV) nucleocapsids within a viral envelope (Blissard and Rohrmann, 1990; Rohrmann, 1999). OBs of NPV are polyhedral in shape and are referred to as polyhedral inclusion body (PIB) and those of GVs are ovicylindrical and are known as capsule or granule (Tanada and Kaya, 1993; Jones, 1994; Crook, 1999). Recently, the classification of the family Baculoviridae has been revised and the NPV genus is now divided into three different genera consisting of lepidopteran, dipteran and hymenopteran NPVs (Jehle *et al.,* 2006). They noted that based on morphological, biological and phylogenetic features, Baculoviridae will now have four genera which include: Alphabaculovirus (lepidopteran-specific NPVs), Betabaculovirus (lepidopteran-specific GVs), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dipteran-specific NPV). This classification has now been approved by the International Committee on Taxonomy of Viruses (ICTV) (Harrison and Hoover, 2012; Herniou *et al.,* 2012).

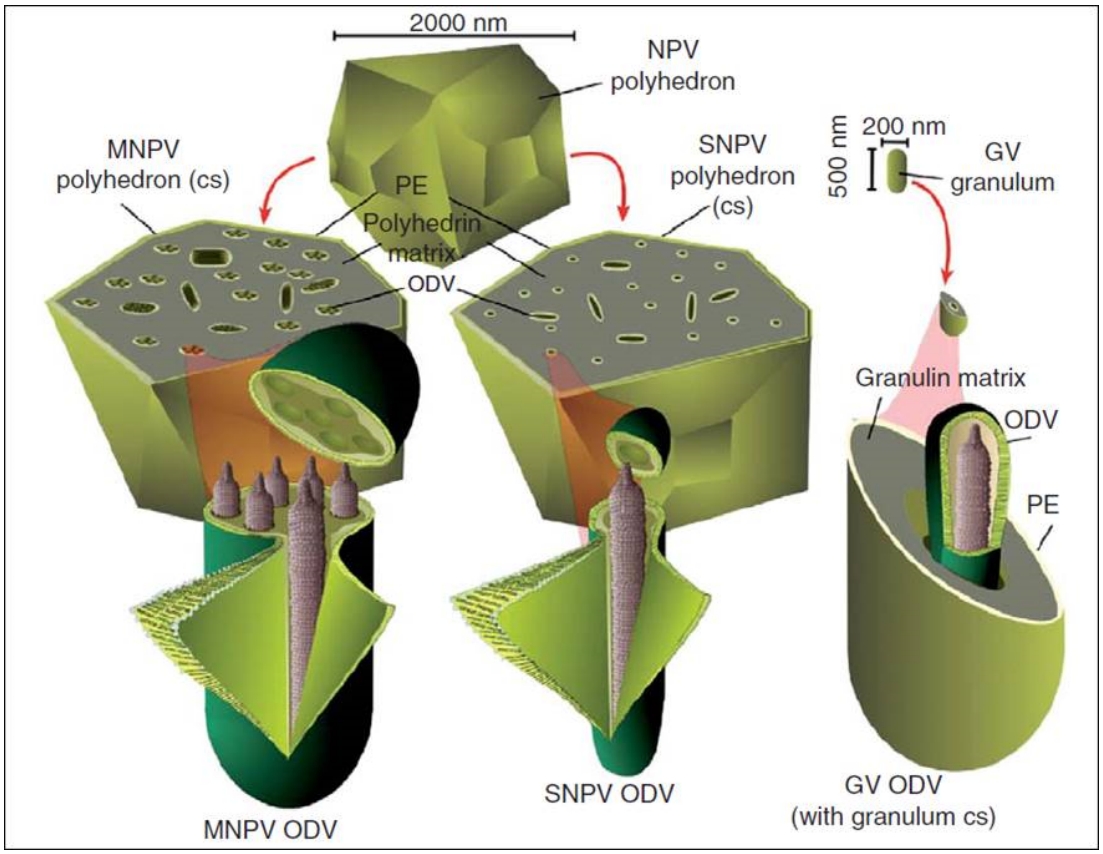


Figure 1.4 Three major occlusion-derived virion (ODV) forms are illustrated in the background. The nucleopolyhedrovirus (NPV) occlusion bodies (OBs) are larger than the Granulovirus (GV) OBs due to the fact that they contain multiple numbers of ODVs. The OBs of GVs are capsule shaped and contain single virions. The OBs of NPVs are multisided or polyhedral. The NPVs are further divided into the multiple nucleopolyhedroviruses (MNPVs) and single nucleopolyhedroviruses (SNPVs). The multiple (M) and single (S) designations are in reference to the number of nucleocapsids that are found in each virion. (Slack and Arif, 2007)

1.5.2 Mode of Action

Understanding the mode of action of baculovirus (see Fig. 1.5) is a key step towards optimizing their use as insecticides so as to achieve better results and avoid some of the inherent limitations that are linked to their biological features. In the most extensively studied Baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), the life cycle involves the production of two morphologically distinct but genetically identical viral forms (Blissard and Rohrmann, 1990; Jehle *et al*., 2006; Rohrmann, 2008). The virions found within the polyhedral (OB) are known as occlusion or polyhedral derived virus (ODV or PDV) particles and

are responsible for initiating infection in the midgut epithelial cells, while the non-occluded virus (NOV) particles are responsible for systemic spread of infection within the host insect (Hunter-Fujita *et al*., 1998; Rohrmann, 1999). The replication process can be said to be biphasic in nature, with tissue-to-tissue spread carried out by NOV and host-to-host transmission carried out by ODV (Cory and Myers, 2003; Jehle *et al*., 2006).

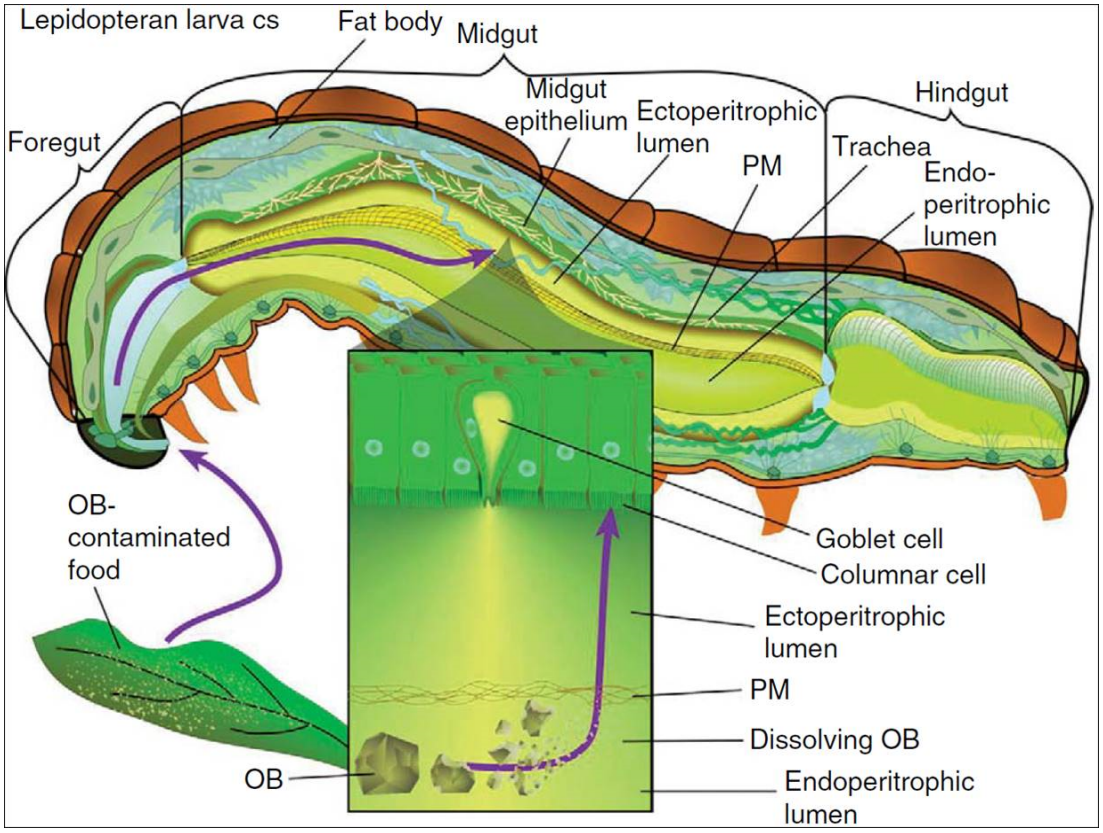


Figure 1.5 Process of oral infection by baculoviruses. A cross-sectional representation of the anatomy of an insect larva is depicted. A baculovirus occlusion body (OB) enters by the per os route in contaminated food. OBs pass through the foregut and enter the midgut where they dissolve in the alkaline midgut lumen and release occlusion-derived virions (ODVs). The insect figure depicts the translocation of released ODVs past the peritrophic membrane (PM) to midgut columnar epithelial cells. The midgut region surrounded by the PM has been referred to as the endoperitrophic lumen and the region outside the PM has been referred to as the ectoperitrophic lumen (Slack and Arif, 2007).

Although the two virions are similar in their nucleocapsid structure, they differ in the origin and composition of their envelopes as well as their role in virus life cycle (Blissard and Rohrmann, 1990; Rohrmann, 1999; Rohrmann, 2008). When OBs are ingested by the host insect, they are dissolve by the action of the alkaline gut pH and protease (Tanada and Kaya, 1993; Cory and Myers, 2003; Rohrmann, 2008). The virions released from the OB fuse with the specific receptors of midgut columnar cells and the DNA containing nucleocapsids moves to the nucleus to initiate infection (Blissard and Rohrmann, 1990; Rohrmann, 1999; Cory and Myers, 2003). The ODV uncoat either after (NPV) or before (GV) passing through the nuclear pores (Hunter-Fujita *et al.,* 1998). Once assembled in the nucleus, nucleocapsids destined to become NOV are transported through the cytoplasm by budding through the nucleus obtaining an envelope from the nuclear membrane, which is later shed in the cytoplasm and another envelope obtained from the cytoplasmic membrane and a virus-coded glycoprotein spike is acquired by budding through the midgut plasma membrane (Rohrmann, 1999; Hunter-Fujita *et al*., 1998). This NOV is released into the haemolymph and further spreads the infection to other susceptible tissues (i.e. fat body, muscles, tracheal matrix, hemocyctes and epithelial), via the cell-mediated endocytosis (Hunter-Fujita *et al*., 1998; Slack and Arif, 2007). Late in the infection cycle, PDV is formed and subsequently become occluded and later released in to the environment as the host insect dies and disintegrates (Hunter-Fujita *et al*., 1998; Rohrmann, 1999). In contrast to Lepidopteran infection, Hymenopteran infection is confined to gut cells (Smits, 1997; Rohrmann, 1999). A common symptom in the late stage of the infection process is the movement of the host larvae to the top of its host plant and finally many tissues liquefy extensively and many OBs are released in to the environment (see Fig. 1.6) as the cuticle become fragile and ruptures due to destruction of the epidermal cells (Fuxa, 2004). Death of larvae occurs 5-14 days after infection and a single Lepidopteran larva can produce about 10^9 OB in NPV or 10^{11} OB for GV (Smits, 1997), depending on the insect species, larval instar, viral dose and ambient temperature (Rohrmann, 1999)

Figure 1.6 *Helicoverpa armigera* larva killed by NPV on pigeonpea (*Cajanus cajan*) (Grzywacz *et al.*, 2004)

1.5.3 *H. armigera* nucleopolyhedrovirus (HearNPV)

The complete nucleotide sequence and organisation of *Hear*SNPV (single enveloped nucleocapsid isolated from *H. armigera*) genome (see Fig. 1.7) has been studied (Chen *et al*., 2001). Size of the genome was in agreement with the previous estimates (Chen *et al*., 2000) and also similar to that of a genotypic variant single enveloped NPV isolated from *H.* zea *(Hz*SNPV) (Chen *et al*., 2002). They noted that the two viruses are similar in both their nucleotide and amino acid identity. Going by their high overall homology, it can be said that the two are variants of the same virus species (Chen *et al*., 2002). Analysis of the genome revealed 326 methionine-initiated open reading frames (ORFs) out of which 135 ORFs with no overlap with other ORFs accounting for about 87% of the genome identified (Chen *et al*., 2001). Five homologous repeat (hr) regions were also discovered (Chen *et al*., 2000) and have been found to be distributed along the genome, usually at AT-rich intergenic regions (Chen *et al*., 2001). They also highlighted the presence of two types of repeats (type A and type B), which are unique to this NPV and do not share any homology with other baculoviruses. The

genome shares similarities with other baculoviruses in the presence or absence of putative ORFs (Chen *et al*., 2001). However, *Hear*SNPV does not contain duplicate genes of virion structural proteins or the homologue of the baculovirus envelope surface glycoprotein gp64 (Chen *et al*., 2001). They also noted that although the NPV contains homologues of 16 out of the known 19 *Ac*MNPV *lef* (late expression factor) genes, it lacks *ie* (immediate early gene)-*2*, P35 and *lef-12* genes which are also absent in *Spodoptera exigua* (*Se*) MNPV and *Xestia c-nigrum* (*Xc*) GV, indicating that those genes only occur in group I NPV members. Auxiliary genes encoding chitinase, cathepsin and egt found in *Se*MNPV are also present in *Hear*SNPV (Ijkel *et al*., 1999). Three bro-related genes were identified in *Hear*SNPV genome, and the presence of this group of related genes is one of the common features of baculovirus genomes (Chen *et al*., 2001). Twenty ORFs were found to be unique to *Hear*SNPV and do not have significant homology to any other sequence in the GenBank (Chen *et al*., 2001). They observed that those ORFs are small or contain no common baculovirus transcription initiation sites for early or late gene expression. Comparison of relative gene order of *Hear*SNPV to those of other baculoviruses revealed the presence of certain clusters in all the genomes (Chen *et al*., 2001). Hence they observed that when the gene cluster is taken to represent the baculovirus genome organisation the common structure of group II NPV members become apparent. By and large, from the gene and genomic phylogeny result it can be concluded that there is no separate ancestral lineage between SNPVs and MNPVs, suggesting that both forms (SNPV and MNPV) occurred many times independently (Chen *et al*., 2000). Therefore, they concluded that in baculoviruses morphotype (different individuals of same species) is only useful for naming purpose and not for phylogeny.

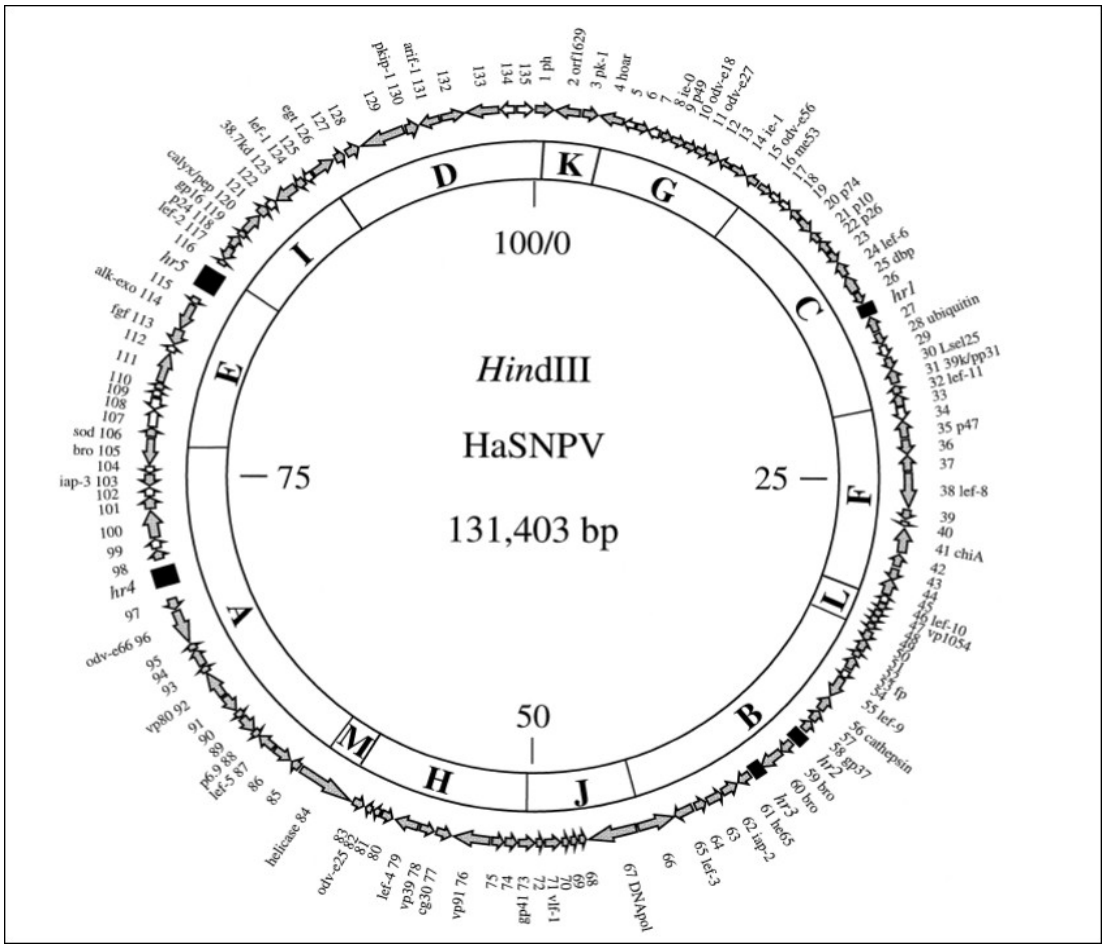


Figure 1.7 Circular map and genomic organisation of the *Ha*SNPV (*Helicoverpa armigera* single enveloped nucleocapsid isolated from *Helicoverpa armigera*) DNA genome. The positions of the 135 identified ORFs are indicated with arrows that also represent the direction of transcription. Shaded arrows indicate that the ORF has a homologue in other baculoviruses in the protein sequence databases. Open arrows represent ORFs unique to *Ha*SNPV. The corresponding number along the ORF represents the *Ha*SNPV ORF number. The positions of the *hr* sequences are indicated by black boxes. The scale on the inner circle is in map units (Chen et *al.*, 2001).

1.6 Factors affecting the efficacy and persistence of baculovirus insecticides

1.6.1 Abiotic factors

The efficacy of pest control by entomopathogens depends largely on their stability and persistence (Jaques, 1985; Tanada and Kaya, 1993). This condition is even more

relevant to baculoviruses, since as an obligate pathogen they cannot multiply outside a living tissue (Jaques, 1985; Jones, 2000). Persistence of baculovirus in the habitat depends to a great extent on the substrate on which it is located, the environmental factors and whether it is occluded or not (Jaques, 1985; Entwistle and Evans, 1985). Some of the major abiotic (environmental factors) affecting the persistence of baculovirus insecticides include the following:

UV Sunlight radiation

Exposure to UV portion of sunlight appears to be a major factor affecting the persistence of viral insecticide for pest control (Jaques, 1967; Young and Yearian, 1974; McLeod *et al*., 1977). Radiant energy in the UV wave length has been identified to be the most detrimental to viral insecticides (Jaques, 1985; Entwistle and Evans, 1985a). The absorption by atmospheric ozone layer prevents the light of less than 290nm (i.e. in the near UV range) from reaching the earth's surface (Jacques, 1985). Therefore, inactivation of viral insecticide by sunlight has been linked to action of wave lengths above 290nm (Ignoffo and Couch, 1981). Ignoffo (1992) observed that the half-life of most microbial pest control inoculums such as virions exposed to natural sunlight to be about one hour for the most sensitive entomopathogen to about 96 hour for the most resistant type. The effects of sunlight could be direct such as deletions, cross-linking, strand breakage and or formation of labile sites on DNA to indirect effects which could be linked to generation of reactive radical species (Ignoffo, 1992). McLeod *et al*. (1977) observed a decrease in viral activity with time of exposure to UV irradiation. They recorded loss in viral activity of 78.9, 86.9 and 84.1% after exposure to between 0 and 12, 12 and 24 and 24 and 48 hour respectively. About half of the viral activity was found to be lost after exposure to sunlight for about 25 hour (Tanada, 1971). This is in agreement to Ignoffo and Couch (1981) who noted that the half-life of *Heliothis* NPV to be less than 24 hour under natural or simulated sunlight. Griego *et al*. (1985) also recorded an increase in loss of viral activity after exposure of virus to the same fluence but at different wavelengths, with the highest activity remaining at 320nm (71.8%) and the lowest at 290nm (15%). They attributed this increase to differences in the wavelengths, since larvae of the same batch were used in the experiment.

Temperature

Baculoviruses appear to be stable at temperatures prevailing in most agricultural ecosystems (Tanada, 1971; McLeod *et al*., 1977; Jacques, 1985; Ignoffo, 1992), except high temperatures prevalent in desert areas (McLeod *et al*., 1977). Since viruses are mostly crystalline in structure and usually having no life processes or enzyme activities they are partially affected by low temperatures compared to other entomopathogens (Jacques, 1985). However, Jacques (1985) noted that being made up of nucleoprotein, viruses are liable to denaturation at high temperatures. Exposure of baculovirus to high temperature (i.e. above 60ºC) for few minutes can lead to inactivation (Entwistle and Evans, 1985a). McLeod *et al*. (1977) observed a significant interaction between UV irradiation and temperature on virus activity. They noted that exposure of virus to UV irradiation at either 15°C or 30°C did not cause a significant loss of activity. Ignoffo (1992) also confirmed that temperatures above 30°C can be detrimental to entomopathogens, particularly if they are negatively influence by action of water, sunlight, chemicals and other abiotic or biotic factors.

Rainfall/Humidity

Water, apart from its role as a dispersal and diluting agent or in combination with other environmental factors, has low influence on entomopathogens like baculovirus. Ignoffo (1992) noted that *Heliothis* NPV was found to be stable in water at 30 ºC over one year but however, significantly decreased in activity by about 3-fold was observed when exposed to sunlight. He further noted that viral inclusion bodies persist longer under dry conditions. Usually the lost of viral activity encounter in some instances by action of rainfall has been attributed particularly to the physical abrasion caused by wind and sand, which could lead to loss of about more than 90% of virus preparation within one week (Jones, 1994). Rainfall does not appear to be an important factor affecting persistence of virus on foliage, since virus deposits seem to adhere well on leaf surfaces (Tanada, 1971). *Trichoplusia ni* NPV deposit on plant leaves exposed to sunlight and kept wet with a sprinkler did not retain activity longer than dry deposits of the virus or deposits on leaves exposed to constant flowing water kept under controlled light in a controlled environment (Jacques, 1967), indicating that surface moisture resulting from dew or rainfall may contribute to inactivation of viral insecticides exposed to sunlight (Jaques, 1985).

1.6.2 Phytochemical factors

pH/Inorganic ions exudates from leaf surface

Microhabitats that are strongly acidic or alkali can affect the stability of BV (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968). Analysis of cotton dew on leaf surface indicated a high pH as well as high concentration of cations (McLeod *et al*., 1977; Young et al., 1977; Entwistle and Evans, 1985a,b). The high pH observed was linked to excretory activities of numerous salt glands on cotton leaf surface (Entwistle and Evans, 1985b). They also noted that there are differences in surface pH between species and age of leaves, with younger leaves being less acidic. The most frequent inorganic ions secreted were found to be cations particularly $Mg^{2+,}$ $Ca^{2+,}$ Na and K, usually as carbonates and hydrogen carbonates (McLeod *et al*., 1977; Entwistle and Evans, 1982). Almost no viral activity remains in bioassay carried out immediately after cotton dew was dried compared to virus not exposed to the cotton dew (McLeod *et al*., 1977; Young *et al*., 1977). Most of the loss in activity was observed after the virus suspension in cotton dew was dried and resuspended in water (McLeod *et al*., 1977; Entwistle and Evans, 1985). According to Elleman and Entwistle (1985) the increase in ionic concentration that occurs during the drying process contributed to the increase in inactivation of the PIBs. They further stated that the significant inactivation of the virus activity observed without a corresponding significant decrease in the number of PIBs after treatment with the cotton leaf dust is an indication that high pH was not the only agent responsible for the inactivation. Addition of chelating agent (EDTA), acid or pH buffer to the cotton dust mixed with the virus suspension helped to prevent inactivation of virus, indicating that both pH and the inorganic ions on the cotton leaf surface might be contributing to the inactivation (Elleman and Entwistle, 1985).

Chickpea leaf surfaces emit large quantities of organic compounds that are extremely acidic in nature ($pH \leq 1.0$) (Rembold and Winter, 1982; Launter and Munns, 1986; Rembold *et al*., 1990a,b). Qualitative analysis of those compounds showed that they are primarily made up of malic and oxalic acids (Rembold *et al*., 1990a,b; Rembold and Weigner, 1990). Despite the mentioned above high concentration of organic acid secreted by the chickpea leaf surface exudates, and existing evidence linking low pH and NPV inactivation (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968), it

is surprising that others reported that *Hear*NPV was not inactivated after it was exposed to most abundant chickpea acids (malic and oxalic acids) and used against *H. armigera* larvae (Stevenson *et al*., 2010).

Plant secondary chemicals

*Ortho*dihydroxyphenolic compounds like chlorogenic acid, quercetin, rutin and caffeic acid in combination with plant oxidative enzymes (i.e. polyphenoloxidases and peroxidases) and physicochemical properties of the insect gut environment were reported to be detrimental to lepidopteran larvae through generation of free radicals and other reactive products (Duffey and Stout, 1996; Hoover *et al*., 1998ab; Hoover *et al*., 2000). However, many mechanisms of action of plant chemicals upon host insect are more relevant when considered in the context of other chemical components (i.e. reducing agents, prooxidants, unsaturated lipids and amino acids), catalytic role of foliar oxidative enzymes and physicochemical environment of the insect gut (Duffey *et al*., 1995; Duffey and Stout, 1996; Hoover *et al*., 1998ab).

1.6.3 Host plant-insect pathogen interactions

Proper understanding of the mechanisms of interactions between plants chemicals with NPV and their insect host is essential for their effective use for pest management (Duffey *et al*., 1995; Hoover *et al.,* 1998a,b,c). Understanding the mechanisms involved will help to reduce the instability and unpredictability associated with the use of viral products for pest control (Duffey *et al*., 1995; Cory and Myers, 2003). Different studies have shown that insects are intimately associated with their host plant and likewise their pathogens (Cory and Myers, 2003; Cory and Hoover, 2006). Evidence has also shown that disease infection caused by entomopathogens such as virus is 3-way (tritrophic) interaction between the plant, insect and enthomopathogenic agent (i.e. virus). Using this idea, Duffey *et al*. (1995) proposed that the three major components mentioned above are interacting with each other under three random but orderly stages of disease infection namely; pre-infectional, infectional and post-infectional stages.

In the pre-infectional stage, the plant contains both nutrients and many other constitutive chemicals in preparation for defence against attack by either insects or pathogens (Duffey *et al*., 1995). They also reported that plant respond to insect`s attack by triggering a corresponding wound respond in form of defence chemicals. Host plant can mediate interactions between insects and their pathogens in various ways (Cory and Hoover, 2006). According to them, this depends on the ability of the plant to influence either directly or indirectly such key processes like infections and environmental persistence of the pathogens. However, previous studies have shown that susceptibility of lepidopteran larvae to baculoviral infection can be significantly affected by type of host plant consumed along with the virus (Keating et al., 1988; Keating *et al*., 1990; Forschler *et al.,* 1992).

The herbivory process of insect on the plant sets in series of chemical reactions in plant tissues, these reactions subsequently lead to the generation of free radicals and other reactive products in the insect midgut which serve as a means of plant defence against insects (Duffey *et al*., 1995; Duffey and Stout 1996; Bi *et al*., 1997). Interactions between the plant secondary chemicals and the insect gut environment could influence the infection of the insect via redox cycling (Hoover *et al*., 1998ab; Cory and Hoover, 2006). However, once infection has started the ability of the insect to resist disease depends on its physiological condition (Duffey, *et al*., 1995). Hence, they noted that the chemical composition of the plant may be a major factor affecting the course and extent of disease, either by direct interaction with the virus or indirectly through the insect physiology. According to Mehdy (1994) plants adopt two major defence responses against insect attack; either through rapid but prolonged oxidative burst (release of reactive oxygen species) leading to production of hydrogen peroxide (H_2O_2) at the point of attack, or via immediate responses that involve phenolic and lipids oxidation by hydrolytic enzymes which produce barriers against microbial invasion. Once the ingested virus enters the insect's midgut, the alkaline condition of the gut (pH 9.5-11.5) helps to solubilise the PIBs thus releasing the virion particles (Entwistle and Evans, 1985; Cory and Myers, 2003). Movement of virion particles through the peritrophic membrane as well as their binding to receptors on epithelium gut wall before passing into epithelial cell could be affected by chemicals derived from plant or insect origin (Duffey *et al*., 1995; Cory and Hoover, 2006). Virions could be inactivated by phenolic binding or redox cycling via the generation of free radicals

probably with other chemical processes such that they are unable to bind to midgut receptors (Felton and Duffey, 1990; Cory and Hoover, 2006).

Plant-derived chemicals that disrupt the physico-chemical integrity of the insect gut cell wall may permit the speedy entry of virus particles into cells (Duffey *et al*., 1995). They also noted that such processes could also encourage secondary infection. Furthermore, oxidation of phenolics by foliar oxidative enzymes, particularly peroxidase (POD) could lead to generation of reactive chemicals (i.e. free radicals and reactive oxygen species etc) causing complete sloughing of midgut cells hence inhibiting infection process (Hoover *et al*., 1998bc; Hoover *et al*., 2000; Cory and Hoover, 2006). In addition feeding behaviour (Duffey *et al*., 1995), and food ingested by the insect may also have influence on the infection process (Lee *et al*., 2006).

Although the post-infectional stage of viral disease has not been fully investigated (Duffey *et al*., 1995). They proposed some mechanisms at this stage to include factors that relate to virus persistence on the plant leaves and soil which may include; pH, redox potential, chelation of ions, generation of reactive quinones, free radicals, reactive oxygen species, (particularly in the presence of UV ionizing radiation), phenolics, tannins, coumarins and unsaturated lipids to be important.

1.7 Improving performance of biopesticides on crops that produce chemical inhibitors

Effective formulation of baculovirus is required to achieve reliable and efficient control over different environmental conditions (Jones, 1994), although, results from the field have shown that this is not essential for their success (Jones *et al*., 1997; Cherry *et al*., 2000). However, it has become necessary for large-scale commercial application as well as to build farmers' confidence and to increase up take (Jones *et al*., 1997). Since the goal of formulation has not been clearly defined, many different types of additives have been tried (Burges and Jones, 1998) usually based on individual`s preference and availability of materials (Jones, 1994). In most cases, formulation additives were selected based on the conventional chemicals model with little consideration of their compatibility with living organisms in the biopesticide

product (Hynes and Boyetchko, 2006). Jones and Burges (1998) has identified four main functions of a formulation which include; to stabilise the microbial agent during production, distribution and storage, to aid handling and application, to improve persistence under adverse environmental conditions in the field and to increase the activity of the agent in the field, through increasing its reproduction, contact and interaction with the target pest. According to Jones (1994), formulation additives that have proven to be effective in the field contain several additives hence are usually not feasible to use and in most cases they are costly. Jones (1994) identified some of those additives used in a formulation to include; wetting and thickening agents as well as antievaporants (aid dispersion and suspension as well as improve contact with target), stickers and UV protectants (improve persistence in the field), gustatory stimulants and attractants (encourage ingestion of the virus), chemical insecticides (improve potency) and lastly antifungal and antibacterial agents (prevent the growth of microbial contaminants). However, of the various formulations tested in the field, none was consistently effective compared to unpurified aqueous suspensions (Cherry et al. 2000; Silver and Moscardi, 2002) and this has been linked to the protective effect of the insect-derived proteins and debris retained in the formulation (Cherry *et al*., 2000). Young and Yerian (1986) were of the opinion that provided this type of formulation if properly stored, it could be equal or even superior to other standard formulations. However, because of the reason cited above, there is a need for standard formulations. Over the years, there have been calls for the need to develop simple formulations that could match the stability of chemical pesticides (Ignoffo and Couch, 1981; Jenkins and Grzywacz, 2000). Persistence of viral insecticides has been found to be impaired by two important factors; UV solar radiation (Jacques, 1985, Entwistle and Evans, 1985a, Young and Yerian, 1986) and by host plant chemistry (Cory and Hoover, 2006; Stevenson *et al*. 2010). Therefore, expanding the use of biopesticides like baculovirus will depend on developing suitable and cheap formulation to improve their persistence on some key crops like cotton and chickpea (Grzywacz *et al*., 2005; Stevenson *et al*., 2010).

1.8 Research objectives

Research carried out at Natural Resources Institute (NRI) has shown that the efficacy of *Hear*NPV is limited on some key crops (i.e. cotton and chickpea). The mechanism for this effect has been described in detail for cotton, but not for legumes. The inactivation process in chickpea differs from that described previously in cotton. For example in chickpea, inactivation is directed at the virus particle and not mediated through the host midgut cells and also that the inactivation in chickpea is permanent. These facts strongly demonstrate that a different mechanism is at work in chickpea to those previously reported in cotton (Young *et al.,* 1977; Elleman and Entwistle, 1985; Hoover *et al*., 1998b,c; Hoover, *et al.,* 2000). Preliminary data has identified a possible role of a specific isoflavonoid compound, sissotrin. Although *in vitro* studies suggested that this accounted for at most 10% of the observed effect, leading to the conclusion that other unknown plant factors might be involved (Stevenson *et al.,* 2010). Therefore, the specific objectives of this study include:

- To compare the inactivation of *Hear*NPV on chickpea with that on two other major African and Asian legumes, cowpea and pigeonpea, for which NPV is currently developed for pest control.
- To identify potential inactivating chemicals in chickpea leaves.
- To evaluate the effectiveness of potential inactivating chemicals on inactivation of *Hear*NPV both individually and in combination
- To discuss the importance of these results in relation to future use of *Hear*NPV in pest management.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Introduction

To study the tritrophic interactions between host plants- insects and NPV, an adequate supply of insects, host plants and NPV were required. Details of insect rearing, plant culture and stock virus preparation are described in this chapter. Similarly, bioassay method that was common throughout the experiments is also described. However, more detail on bioassay procedures that are specific to each chapter was explained in the relevant chapter.

2.2 *Helicoverpa armigera* insect culture

The method described by Armes *et al.* (1992) and that had been used successfully for earlier work at NRI by Stevenson *et al*. (2010) was used for rearing the insects, with slight modification.

2.2.1 Source of insects

H. armigera insects for laboratory culture were obtained as eggs from Australia (Ag. Biotech. Australia Pty Ltd.). Eggs arrived as neonate larvae because they hatched on transit. On arrival, the first instars were placed on modified semi-synthetic wheat germ diet (Hoffman *et al*., 1966) in 250 ml transparent plastic containers (Polarcup Gmbh, Germany), and reared in a group at about 50 larvae per container for 5-7 days, before they were transferred individually to a fresh cube of diet in 29 ml translucent plastic containers (Pactiv Corporation, Illinois, USA). Two attempts were made to establish the insect culture obtained from India, first in 2008 when the programme initially started and two years after in 2010 when the programme restarted. However, difficulty in sourcing disease free insects made the task of rearing more difficult.

2.2.2 Experimental conditions

Insects were maintained in the insectary at Natural Resources Institute (NRI), University of Greenwhich, UK. The environmental conditions were maintained on 26±2°C (constant dark and light temperature), 14h:10 h light: dark photoperiod.

2.2.3 Larvae

Second instar larvae (5-7 days old) were individually transferred to fresh cube of diet in a 29 ml polypot (Plastic container) using blunt steel forceps. Ventilation holes were made on the pot lids to prevent larvae from suffocating. A piece of tissue paper was also placed in between the lid and the pot to absorb any moisture in the polypot. Any larva that was found to be developing poorly was removed (frozen). Forceps used for handling the larvae were sterilized in 5% sodium hypocholorite to reduce risk of infection. Transferring larvae into individual rearing pots at the second stadium helped to reduce cannibalistic behaviour associated with *Helicoverpa* spp. Individual pots containing larvae were kept in trays which were labelled with hatching date until pupation.

2.2.4 Pupae

Only properly-formed and healthy pupae (hardened and red-brown) were removed from the individual rearing pots, using a blunt nosed forceps, sterilizing and changing the forceps occasionally to reduce the risk of infection. Pupae were removed carefully from within the diet where they burrow to pupate and sterilized using 0.5% sodium hypochlorite solution for about 5 min, followed by rinsing twice in distilled water. Rinsed pupae were dried on a filter paper before they were sexed by observing the tip of the ventral side of their abdomen, as described by Armes *et al.* (1992). About 25 pairs of pupae (i.e. equal males and females), were placed on moist vermiculite in a 250 ml polypot at the rate of 50 pupae per pot (see Fig. 2.1). Pots containing the pupae

were transferred to a 36 cm high × 20 cm diameter Perspex cylinder with ventilated lids (adult moth rearing cage).

Figure 2.1 *Helicoverpa armigera* pupae on moist vermiculite in 250 ml plastic container.

2.2.5 Adult moths

Three nappy liners (Boots Nottingham, UK) were hung vertically downward within the cylinder to serve as a site for oviposition and also for the adult moths to dry their wings after emergence (Fig. 2.2). Few days (2-3 days) before adult emergence, adult diet (10% sugar solution containing vitamin and preservative) was provided in a 29 ml polypot with absorbent cotton wool placed within the pot and extended through the lid to provide easy access and prevent adult drowning in to the pot while feeding. Once oviposition commenced, eggs were collected every other day, by removing the nappy liners and cutting them in to medium sizes and storing them in 250 ml polypots and labelled with date of collection. New nappy liners were then replaced. Eggs were incubated at 26±2°C where they usually hatched within 3-5 days.

Figure 2.2 *Helicoverpa armigera* adult moths in rearing cylinder with nappy liners for oviposition

2.3 Colony collapse

As mentioned in sectioned 2.2.1 twice the insect culture obtained from India collapsed. The insects were obtained as pupae and disease symptoms were not observed until later when most of the adult insects failed to emerge and those that emerged were showing symptoms such as reduced vigour, low fecundity, decreased feeding and retarded growth. It was later learned that these insects did not come from an established colony but were obtained from the field, and it is usually difficult to determine their health status, particulary that of microsporidian infections which not manifest visible symptoms (Maddox *et al.*, 1998; Gouli *et al.*, 2011).

Most of the pathological symptoms that were observed were similar to those described as characteristics of microsporidian infection (Lacey *et al.*, 1997; Franzen, 2008). Examination of wet smears of diseased larvae showed the presence of distinct microsporidian spores (Fig. 2.3). The spores were oval in shape and uniform in size, which could be that of either *Nosema* spp or *Variamorpha* spp.

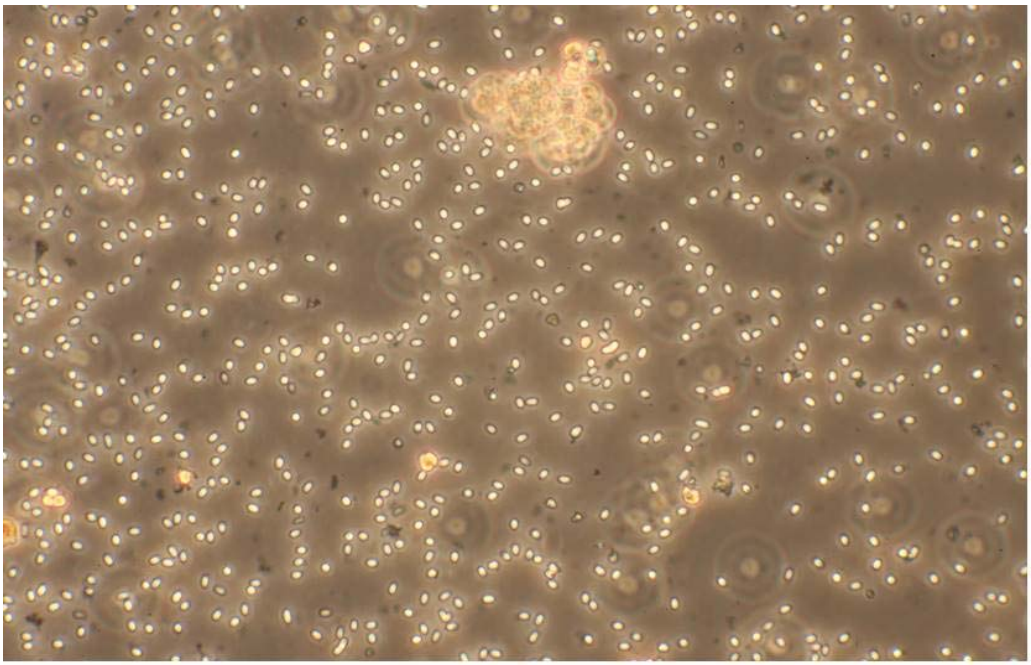


Figure 2.3. Light micrograph of body tissue from *Helicoverpa armigera* larva showing microporidian spores (× 100).

Immediately the disease infection was confirmed, all the insects were autoclaved and all rearing equipments and materials were sterilized using 1% Virkon® solution, and new insects were sourced from a reliable and disease free insect culture (Ag. Biotech Autralia pty Ltd.). This insect culture was successfully maintained and used throughout the research period (> 3 years) with no sign of disease infection; however, strict measures were taken against diseases.

2.4 Plant culture

Chickpea (*Cicer arietinum* L.) cultivar 'WR-315' and pigeonpea (*Cajanus cajan*) cultivar 'ICPL-87' were provided by International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India, and cowpea (*Vigna unguiculata* (L.) Walp) variety IT84E-124 was provided by IITA, Cotonou, Benin, and tomato (*Lycopersicum esculentum*) 'Money maker', were used for the experiments. The seeds of the three plants (chickpea, cowpea and tomato) were sown directly in plastic pots on John Innes No. 2 potting compost at the rate of two seedlings per pot and maintained at 28±2°C in a glasshouse under 14 h photoperiod and a relative humidity of 60% (Fig.

2.4.). While for pigeonpea, seeds were first sown in between a moist folded tissue paper and covered with two sand wich box lids. This process helped to achieve nearly 100% germination that was difficult to obtain using the direct seeding method. After germination, seedlings were transplanted into the pots containing the compost at one seedling per pot. All the plants were used for the experiments when they were about 4-5 week old.

Figure 2.4 Chickpea plants in plastic pots

2.5 *Heliothis armigera* nucleopolyhedrovirus

2.5.1 Source and preparation of stock virus

The initial *Hear*NPV strain (NRI#210) was obtained from Tamil Nadu Agricultural University, India. However, this isolate becomes very difficult to count due to excessive clumping of the occlusion bodies, which has been attributed to certain additives used in the formulation. This aggregation could not be dispersed even after several attempts of shaking the virus suspension using vortex mixer (Stuart SA7,

Bibby Scientific Ltd., Staffordshire, UK) for about 30 s, followed by sonication on a water bath and use of Darvan® 2 (10 mg ml^{-1}; R.T. Vanderbilt Co., Los Angeles, California) or by forcing the virus suspension three to four times using a hypodermic syringe as suggested by Jones (2000). This led to the use of a commercial *Hear*NPV isolate (Helicovex® from Andermatt, Switzerland). The virus was used after the ingredients in the formulation were removed by spinning the virus formulation in centrifuge (Mistra 3000i, bench top centrifuge, Fisons, England) at 2500 g at 5°C for 30 minutes (Hunter-Fujita *et al*., 1998) for at least three times while removing the supernatant and re-suspending the virus in sterile distilled water (SDW).

2.5.2 Determination of virus concentration

The concentration of the *Hear*NPV was determined by using the improved Neubauer haemocytometer (Weber Scientific International Ltd. England). This method has been described in detail (Hunter-Fujita *et al*., 1998; Jones, 2000). Sample of the NPV suspension was first sonicated in a sonicator (Decon Laboratories Ltd., East Sussex, UK) to disperse the OBs for about one minute, followed by mixing of the virus suspension by vortex mixer until the virus particles were evenly distributed. Ten-fold dilutions of the NPV suspension were prepared in separate 1.5 ml Eppendorf tubes. The haemocytometer and the cover slip were cleaned with 70% ethanol using non fibrous tissue. Breath was gently blown on to the cover slip which was used to cover the grids lines drawn on both sides of the haemocytometer using even pressure. The diluted sample of the NPV suspension in the Eppendorf tube was mixed thoroughly using a vortex mixer and 10 µl of the virus was dispensed at the edge of the cover slip and the surface of the slide using a micropipette, allowing the NPV suspension to move via surface tension covering the grid surface. The haemocytometer was then placed on a microscope stage (DMR Leica Mikroskopie and System, GmbH, Wetzlar, Germany.) and left to there for about 10 minute to allow the NPV occlusion bodies (OB) to settle on the slide. Numbers of OB were then counted under phase-contrast optics (×400) (see Fig. 2.5).

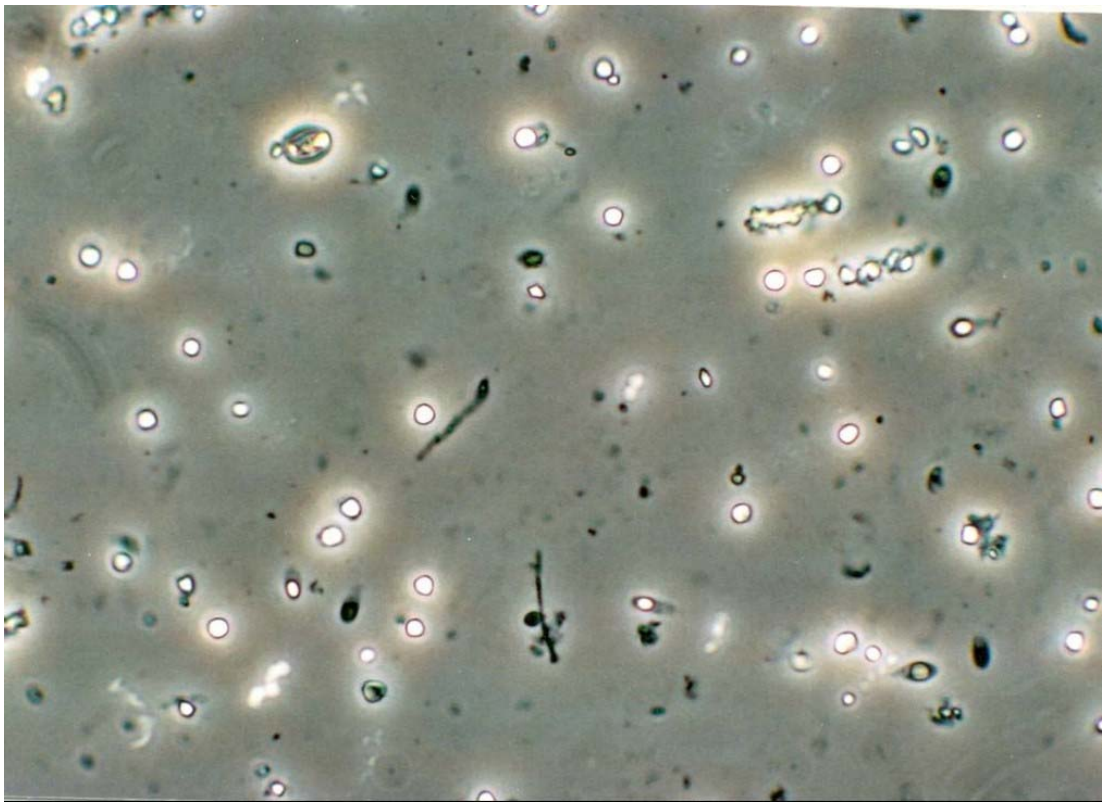


Figure 2.5 Infective occlusion body (OB) of NPV as seen under phase contrast microscopy (× 400) (Grzywacz *et al.,* 2014)

The OBs were identified as bright round objects, often with a black dot in the centre. Counts were made of 20 large squares (320 small squares, 160 from each of the grids drawn on the haemocytometer), following a predefined pattern. OBs touching the top and right (central) line were counted, while those touching the bottom and left lines were not. A total of 3 separate counts were made using different sub-samples of the same virus preparation and the average was taken. A minimum of 300 OB were counted.

The concentration of the NPV suspension was calculated using the following formula (Jones, 2000).

$$\text{OB ml}^{-1} = \frac{D \times X}{N \times K}$$

Where: D = dilution factor (dilution of NPV suspension dispensed into the haemocytometer); X = number of OB counted; N = number of small squares counted; K = volume above a small square in cm^3.

Since area of each small square is $\frac{1}{400}$ mm^2 = 0.0025 mm^2, depth of chamber is 0.1 mm then volume of liquid above a single small square is 0.0025 mm^2 × 0.1 mm = 0.00025 mm^3. To convert to cm^3 multiply by $\frac{1}{1000}$ to get a volume of 2.5×10^{-7} cm^3

2.6 Bioassay

As an obligate pathogen, the infectivity and virulence of viral insecticide can only be measured using a living host or insect cell (*in vitro*). Thus bioassay using live insects is the key means of measuring the activity of an infectious agent like NPV. To be consistent, bioassay needs to be standardized in terms of the insect, rearing conditions and method used, so as to reduce the variations and reflect the natural conditions as well as to quantify the effects one intends to measure (Jones, 2000). To obtain this standardization, all bioassays were conducted using laboratory-reared, neonate larvae (18 h old).

2.6.1 Bioassay to determine the median lethal concentration (LC_{50}) of *H. armigera* larvae.

Mass dosing using the surface dosing bioassay was used, this was the method used in a similar study (Stevenson *et al*., 2010). Fivefold serial dilutions of the stock virus (*Hear*NPV) was prepared in sterile distilled water using five Eppendorf tubes (1.5 ml), each tube was labelled with its corresponding treatment as follows:

T1= 3.2×10^4 OB ml^{-1}

T2= 6.4×10^3 OB ml^{-1}

T3= 1.28×10^3 OB ml^{-1}

T4= 2.56×10^2 OB ml^{-1}

T5= 5.10×10 OB ml^{-1}

For each treatment dilution three polypots (29 ml) containing artificial diet for polypots (McKinley *et al*., 1984) was set out, including three controls. Each pot was labelled according to its treatment. Starting with the lowest concentration (T5) the virus suspension was mixed using a vortex mixer for at least 30 seconds, and 100 µl aliquot was dispensed using a micropipette onto the surface of the diet in the polypots, until all the three pots set for the treatment were dosed. The pots were tilted and rotated making sure the whole diet surfaces were covered by the virus suspension. The process was repeated until all the treatments were dosed with their corresponding virus concentrations, moving from lower (T5) to top concentration (T1). Control treatment was dosed with 100 µl sterile distilled water only. All the dosed pots were kept in a tray to dry under room temperature. Using a paint brush (No. 2) 30 unfed neonate larvae (18 hour old) were transferred to the control treatment first at the rate of 10 larvae per pot. Followed by the lowest virus concentration to the top (T5-T1) the process continued until each treatment contained 30 neonates at 10 larvae per pot. To avoid the build-up of condensation in each pot, and reduce the risk of neonate drowning, a small hole was made on the top of the polypot lid and a piece of tissue paper was inserted between the lid and the pot. Moving from lowest concentration treatment to the top (T5-T1) the process was repeated until each treatment had 30 neonate larvae with each pot containing 10 larvae. For each treatment, a separate brush was used to prevent the risk of contamination and used brushes were sterilised in 1% Virkon solution and rinsed in distilled water. Dosed pots containing the neonates were maintained at 25 ± 2°C and 14 h photoperiod. After 24 h numbers of larvae death were counted by gently tapping the pots on bench surface and larvae that did not move were considered dead and hence removed from the mortality estimation. The number deaths were also checked and recorded fifth day after dosing. Bioassays were repeated at least five times under same conditions.

CHAPTER THREE

EFFECT OF HOST PLANT ON EFFICACY OF NUCLEOPOLYHEDROVIRUS

3.1 Introduction

A number of studies have shown that variability existing between host plants can be an important mediating factor determining the susceptibility of insects to baculovirus infection (Forschler *et al.,* 1992; Hoover *et al.,* 1998b,c; Ali *et al.,* 2002). Since BVs are relatively stable in their protective covering (polyhedra), influence of host plant on viral disease depends on whether plant factors can affect the environmental persistence and subsequent infection of the occlusion bodies (i.e. occlusion derived virions) in the insect midgut (Cory and Hoover, 2006). Maintaining viral OBs stability on host plant substrate could enhance its persistence before infection in another susceptible insect.

Field trials have demonstrated that control of *H. armigera* using *Hear*NPV to be higher on susceptible compared to resistant genotypes of chickpea (Rabindra *et al.,* 1992; Cowgill and Bhagwat, 1994, Cherry *et al.,* 2000). Similarly, levels of control provided by insect viruses has been shown to be higher on tomato, intermediate on legumes like common bean (*Phaseolus vulgaris*) and least on cotton (Forschler *et al.,* 1992; Farrar and Ridgway, 2000). Previous laboratory studies have also shown that both cotton and chickpea leaf surfaces are detrimental to the stability of viral OBs (Young and Yearian, 1977; Ellerman and Entwistle, 1985a,b; Rabindra *et al.,* 1994, Stevenson *et al.,* 2010). While both crops have been found to reduce the efficacy of viral insecticides used against *H. armigera* larvae, the degree of inactivation was much greater on chickpea compared to cotton (Stevenson *et al.,* 2010). The mechanism of action in chickpea is also quite different from that reported in cotton. In chickpea, inactivation is leaf surface related and permanent and is directed at the viral particles (Stevenson *et al.,* 2010), while in cotton the inactivation is caused by internal leaf factors and is mediated through the insect's midgut cells (Hoover *et al.,* 1998b,c; Hoover *et al.,* 2000).

Understanding how viral insecticide persistence is affected by the host plant is essential to its successful utilisation and development for management of insect pests,

particularly on crops like chickpea on which NPV has short chemically mediated persistence. Improving the stability of baculovirus on such crops could be a step towards obtaining a better and more persistent formulation and improve control efficacy. Improved or lower cost control could increase uptake of this environmentally benign pest management option by users. Additionally such knowledge may also help to reduce the optimum rate of *Hear*NPV application on chickpea in the field so as to save cost and manage *H. armigera* more effectively.

Therefore this study was carried out to extend earlier findings that showed that chickpea leaf surface affect *Hear*NPV efficacy (Stevenson *et al.,* 2010), and also to determine if the same inactivation effect observed in chickpea is the same as in other major legumes (i.e. pigeonpea and cowpea). Thus this work will also test the hypothesis that the inactivation previously reported for chickpea also occurred on other major legume crops grown in Africa and Asia specifically cowpea and pigeonpea.

Additionally NPV OBs were also examined after being exposed to chickpea leaf surfaces at different time intervals under electron microscope, to test the hypothesis that exposing viral OBs to chickpea leaf surfaces leads to a major change in the morphological structure of the viral particles. To determine this viral OBs exposed on chickpea leaves where compared to those of tomato which has no significant inactivating effects on the virus (Forschler *et al.,* 1992; Stevenson *et al.,* 2010) and unexposed *Hear*NPV.

3.2 Materials and Methods

3.2.1 Exposure of HearNPV to leaf surfaces of chickpea, cowpea, pigeonpea and tomato

Chickpea, cowpea, pigeonpea and tomato leaf surfaces were sprayed with *Hear*NPV suspended in sterile distilled water at a concentration of 3×10^8 OB ml^{-1} in 0.02% Triton using a 10 ml chromatographic reagent sprayer (Fig. 3.1) a variant of a technique previously used with chickpea (Stevenson *et al.,* 2010). The plants were sprayed at the rate of 2 ml per plant for chickpea, 2 ml per branch of plant for tomato and 2 ml per trifoliate leaf for pigeon pea (only the largest trifoliate leaves were selected), which

coincided with equal spray on each plant. Spraying was carried out such that the plants or selected parts were evenly wet. All treated plants were maintained at 25˚C and 14 h photoperiod, and the *Hear*NPV was left on the leaves of the treated plants for periods of 2, 8, 24 or 48 h.

Figure 3.1 Chromatographic sprayer used for spraying *Hear*NPV suspension on plant leaf surfaces

3.2.2 Recovery of *Hear*NPV from chickpea, cowpea, pigeonpea and tomato leaf surfaces

*Hear*NPV OBs were recovered from the plants after the specific exposure period (48 h for cowpea and pigeonpea and 2-48 h for chickpea) by cutting the leaves of each treated plant and putting them in 50 ml conical centrifuge tube (Falcon^{TM}, Fisher Scientific, UK). Thirty ml of 0.1% sodium dodecycl sulphate (SDS) in sterile distilled water (SDW) was added to each tube; suspended leaves were then sonicated for 3 min and put on a rotator for 60 min at 30 rpm (Fig. 3.2). After one hour of washing, the leaves were removed and the virus suspended in SDS was concentrated by

centrifugation at 2500 g at 5˚C for 25 min (Hunter-Fujita *et al.*, 1998). The supernatant was discarded and the virus re-suspended in SDW in 1.5 ml Eppendorf tube and concentrated again in micro centrifuge at 2550 g for 25 min at room temperature. The process was repeated for at least 3 times and each time the supernatant was discarded and the virus pellet re-suspended in SDW. Finally the recovered virus pellet was re-suspended in 1 ml of SDW in 1.5 ml micro centrifuge tube and stored at -20˚C until needed for bioassay or examination by scanning electron microscopy (SEM).

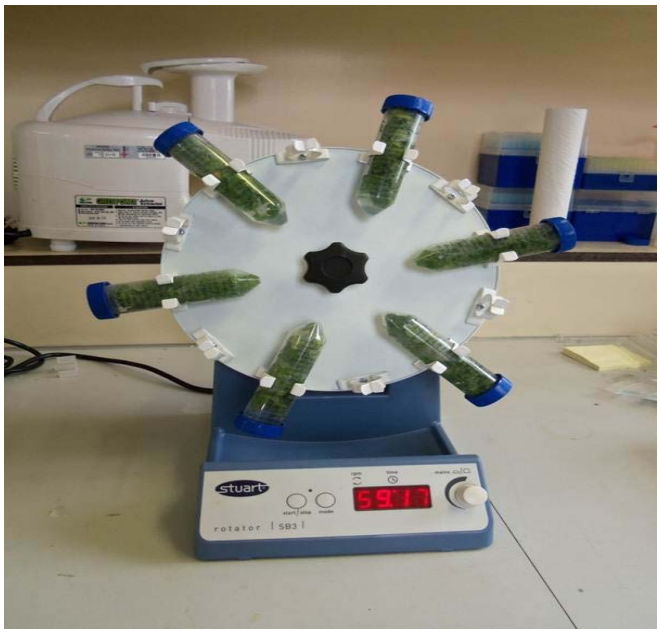


Figure 3.2 Washing of *Hear*NPV suspension from chickpea leaves using a rotator

3.2.3 Bioassay to determine the effect of chickpea, cowpea, pigeonpea and tomato leaf surfaces on *HearNPV* efficacy against *H. armigera* larvae

Surface dosing bioassay using the mass dosing method was used. This method has been described in details in section 2.6.1. The 5-fold dilution series was prepared from the stored virus recovered from exposed plant leaves (see section 3.2.2). The same method as mentioned previously in section 2.6.1 was also adopted for cowpea, pigeonpea and tomato. Procedure was same as in the previous bioassay (see section 2.6.1 in chapter 2), except for chickpea where the following 5-fold dilution series was

prepared from the *Hear*NPV recovered from chickpea leaves after different exposure period;

T1 = 2.0×10^9 OB ml^{-1}

T2 = 4.0×10^8 OB ml^{-1}

T3 = 8.0×10^7 OB ml^{-1}

T4 = 1.6×10^7 OB ml^{-1}

T5 = 3.2×10^6 OB ml^{-}

3.2.4 Preparation of *Hear*NPV for Scanning Electron Microscopy after being exposed to chickpea and tomato leaves

*Hear*NPV exposed to leaf surfaces of chickpea and tomato for 2 h or 48 h and unexposed *Hear*NPV were used as sample treatments. The procedure was as follows:

1. The exposed virus were centrifuged three times, to remove plant debris, using a micro centrifuge at 2550 g for 25 min, each time the supernatant was discarded and the virus pellet was re-suspended in distilled water in 1.5 ml Eppendorf tube.
2. The virus was then sonicated for 1 min and mixed using a vortex mixer for at least 30 seconds to disperse any NPV OB clumps.
3. Three aluminium stubs were used as the primary substrate for each of the sample treatments (i.e. chickpea, tomato and NPV). Silicon wafer (5 mm squares) was used as a secondary support for each sample, and attached to the aluminium stub by means of a carbon adhesive (Leit-C Agar Scientific). For each sample treatment (exposed virus on chickpea for 2 and 48 h and tomato for 48 h), 1 µl was pipetted on the silicon wafer which was attached to the aluminium stub and each time the tip of the pipette was replaced to avoid contaminating the samples. The unexposed NPV was mounted on a separate stub via the silicon wafer. All the samples were labelled accordingly.

4. The samples were all dried using gentle heat from the reading lamp for about 45 min.
5. Samples were examined using a Hitachi model SU8030 cold-cathode field emission gun scanning electron microscope, using the following modes; accelerating voltage of 1.5kV, decelerating voltage of 1.0 kV and landing voltage of 0.5 kV. The following nominal magnifications and working distances were also used; specimen A (×15,000; 3.1mm); specimen B (×10,000; WD=3mm); specimen C (×10,000; WD=4.1) and for specimen D (×15,000; WD=3mm).

3.2.5 Statistical analysis

Data from bioassays were analysed by calculating the mean LC_{50} using the Poloplus insect bioassay software (Robertson *et al.,* 2007) a method previously used for such bioassays (Stevenson *et al.,* 2010). For comparison of experimental treatments LC_{50} were obtained from a minimum of five separate replicate assays carried out on different days. Following accepted protocols for bioassays the results of all assays that did not include three partial kills needed for accurate estimation of LC_{50} were discarded (Roberston *et al.,* 2007).

The differences between the treatment means were detected using analysis of variance and differences were separated using multiple comparison tests. In experiments where, LC_{50} varied by orders of magnitude and the data were not of equal variance, the results were log transformed to equalise the variances and analysed using ANOVA procedures in Sigmastat software (11.0). In cases where variances were not equal even after transformation, appropriate non-parametric tests including Kruskal-Wallis or Holm-Sidak were used to test for significant differences as suggested by the software package.

3.3 Results

3.3.1 Bioassay to determine the effects of different plant leaf surfaces on *Hear*NPV efficacy against *H. armigera* larvae

The *Hear*NPV recovered after exposure to the leaves of cowpea and pigeonpea was enough to obtain at least three partial kill necessary for bioassay analysis (after 48 h). On chickpea even after spraying more than 300 plants the virus recovered was not active enough even with concentrations up to 1×10^9 ml^{-1} to produce the three partial kills necessary to get an LC_{50} estimate by computing for individual treatments. Instead, all *Hear*NPV sprayed on chickpea leaf surfaces and recovered after 2-48 h were pooled together and used for bioassay. The mean LC_{50}s from two different bioassays were used for comparison between chickpea and the other treatments. However, to increase the sample size for LC_{50} mean estimate, an additional estimate of three more LC_{50}s were obtained using the graphical extrapolation as the partial kills on these did not straddle the estimated LC_{50} as required by the LC_{50} estimation package (Poloplus software).

The average LC_{50} of *Hear*NPV exposed to chickpea, cowpea, pigeonpea and tomato leaf surfaces were log transformed to allow comparison before analysed using Kruskal-Wallis one way Analysis of Variance on Ranks (Fig. 3.3), the results showed that there were significant differences between the treatment groups ($H= 21.619$, $df= 4$, $P<0.001$). The results for the LC_{50} of the chickpea exposed samples are significantly higher than those for the other treatments as shown by Dunn's multiple comparison test ($P <0.001$). For each treatment group, the experiment was replicated at least five times. This validates the earlier finding (Stevenson *et al.*, 2010), that exposure of NPV to chickpea leaf surface inactivates BV OB even with exposures as short as two hours giving >99% inactivation.

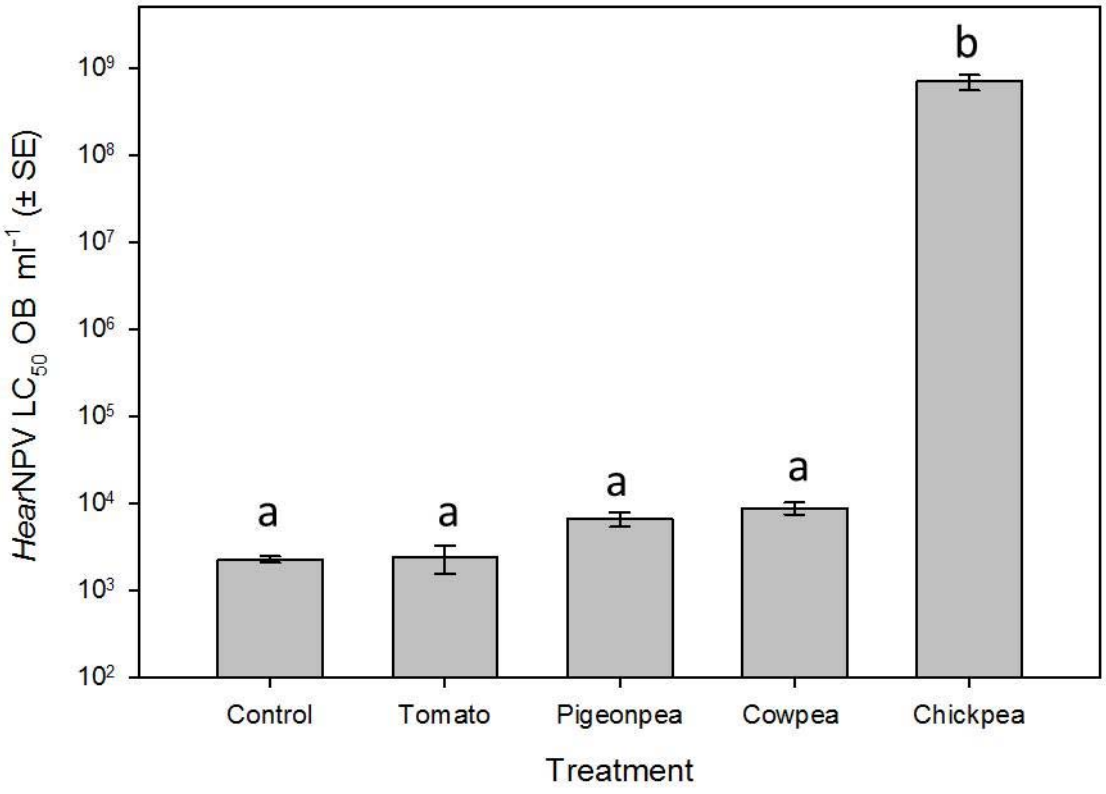


Figure 3.3 Average median lethal concentrations (LC_{50}±SEM) of *H. armigera* neonates on artificial diets containing either untreated *Hear*NPV OBs or with *Hear*NPV OBs that had been exposed to leaf surfaces of chickpea (> 2 h), cowpea, pigeonpea and tomato (48 h). Bars with different letters are statistically significant (P < 0.001).

3.3.2 Bioassay to determine the effects of cowpea, pigeonpea and tomato leaf surfaces on *Hear*NPV efficacy against *H. armigera* larvae

Because time of *Hear*NPV exposure on chickpea and other plants (cowpea and chickpea) differed, data from both experiments were analysed separately. Average LC_{50}s of *Hear*NPV exposed to cowpea, pigeonpea and tomato leaf surfaces and unexposed *Hear*NPV (Fig. 3.4) showed that there were significant differences between the treatment groups (ANOVA; F= 12.089, df= 3,20, P < 0.001) with the LC_{50} for cowpea and pigeonpea being significantly greater than those for unexposed *Hear*NPV and tomato. Using Turkey HSD multiple comparison test, it was shown that there was a significant difference (P < 0.001) between unexposed *Hear*NPV and *Hear*NPV exposed to cowpea leaf surfaces. Similarly, there was significant difference (P < 0.016)

between unexposed *Hear*NPV and *Hear*NPV exposed to pigeonpea leaf surfaces. However, there was no significant difference ($P > 0.05$) between unexposed *Hear*NPV and *Hear*NPV exposed to tomato leaf surfaces. Similarly, there was no significant difference ($P > 0.430$) between *Hear*NPV exposed to cowpea and pigeonpea leaf surfaces. Thus this result is consistent with the hypothesis that some inactivation of *Hear*NPV occurs when OB are exposed to cowpea and pigeonpea leaf surfaces although this effect is much lower for virus exposed pigeonpea and cowpea than that seen in chickpea. This is the first demonstration and quantification of direct inactivation of OB or any microbial pesticide on pigeonpea and cowpea.

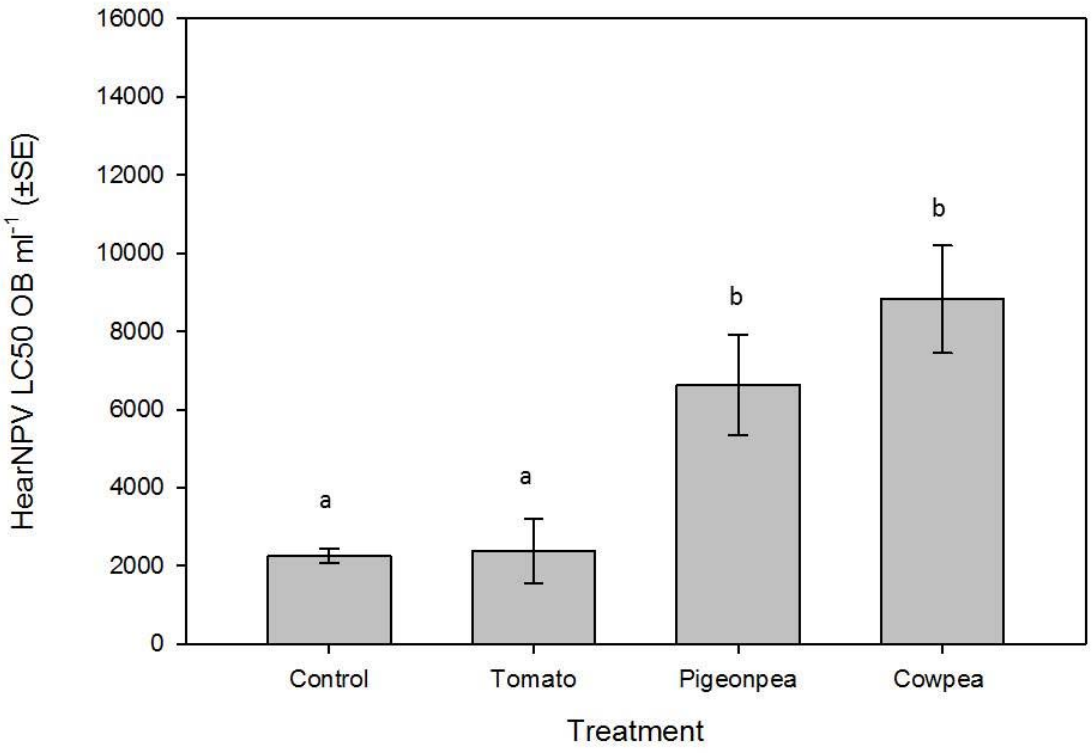


Figure 3.4 Average median lethal concentrations (LC_{50}±SEM) of *H. armigera* neonates on artificial diets containing untreated *Hear*NPV or with *Hear*NPV OBs exposed to leaf surfaces of cowpea, pigeonpea and tomato for 48 h. Bars with different letters differ significantly ($P < 0.001$).

3.3.3 Scanning electron microscopy of *Hear*NPV after being exposed to chickpea and tomato leaf surfaces

From the scanning electron micrograph (Fig. 3.5) both the exposed and unexposed *Hear*NPV OBs shows some degree of aggregation or clumping. However, the exposed viral OBs on chickpea appeared to be more tightly held together compared to those exposed to tomato leaf surfaces or the unexposed *Hear*NPV. Similarly, OBs on chickpea treatments (A and B) are more compacted compared to OBs exposed to either tomato (C) or unexposed virus (D). Most of the OBs in either the exposed and unexposed virus were spherical in shape, with few having irregular shape. Some of the NPV OBs in the unexposed virus (D) have hollows or empty spaces evenly distributed on their surfaces; While NPV OBs exposed to host plant leaves have few of those empty spaces which are also very few and small in size. From both the size and shape of all the OBs in both the exposed and unexposed virus, no differences of changes were detected in the physical structure of the *Hear*NPV OBs. Thus the hypothesis that the inactivation of OB after exposure to chickpea surfaces is correlated with major physical changes in the OB structure is not supported by these findings.

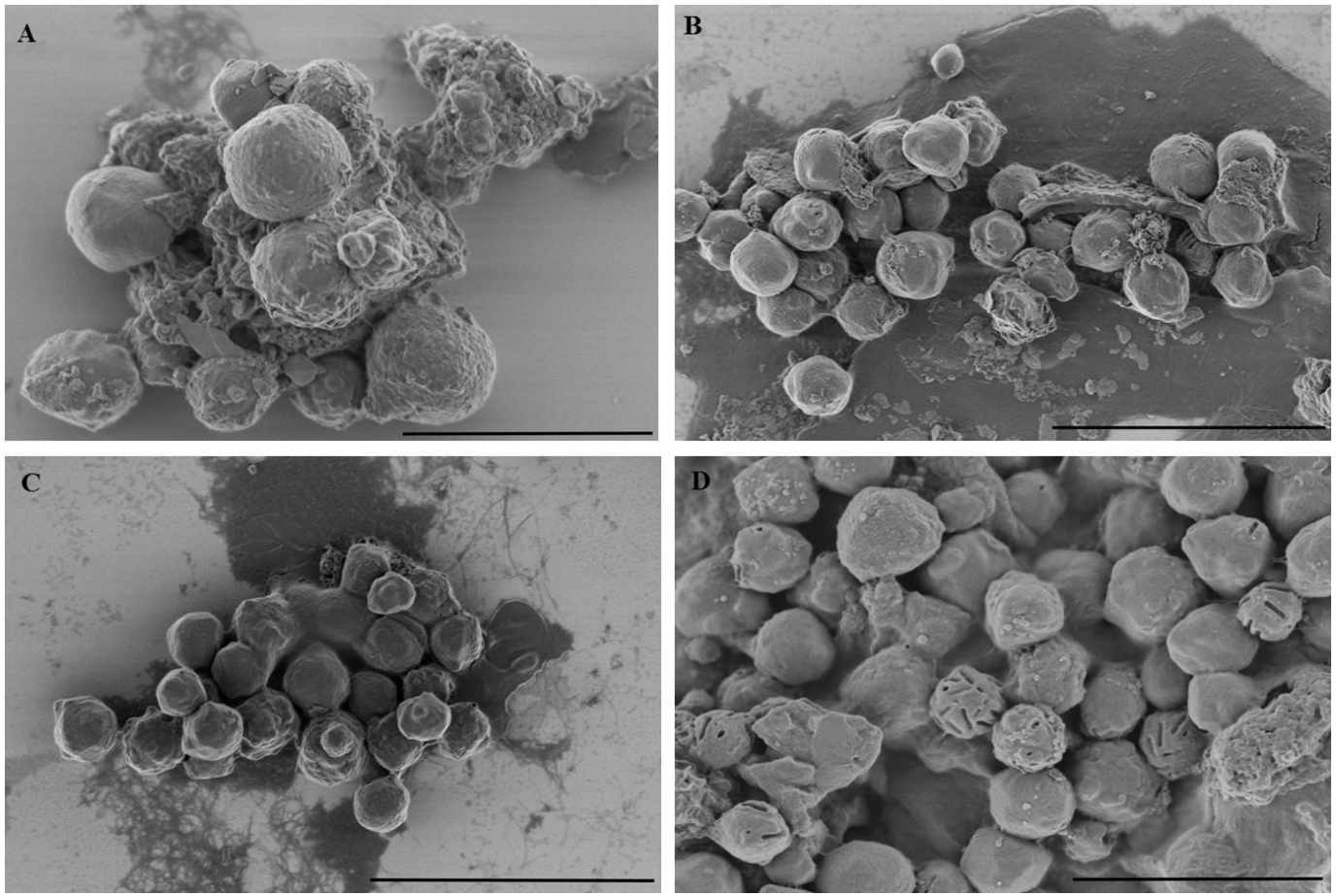


Figure 3.5 SEM images of unexposed *Hear*NPV OBs and *Hear*NPV OBs exposed to different host plant leave surfaces at different time intervals: (A) *Hear*NPV OBs exposed to chickpea leaf surfaces for 2 hour (Bar= 3µm); (B) *Hear*NPV exposed to chickpea leaf surfaces for 48 hour (Bar= 5µm); (C) *Hear*NPV OBs exposed to tomato leaf surfaces for 48 hour (Bar= 5µm);(D) Unexposed *Hear*NPV (Bar= 3µm).

3.4 Discussion

The present results showed that leaf surfaces of chickpea, cowpea and pigeonpea are detrimental to *Hear*NPV infectivity against *H. armigera* larvae in comparison to tomato leaf surfaces or unexposed *Hear*NPV. The leaf surfaces of chickpea showed a significantly greater level of inactivation compared to the leaf surface of either cowpea or pigeonpea. Previous results have also shown that chickpea leaf surfaces reduced the efficacy of *Hear*NPV against *H. armigera* larvae (Rabindra *et al.,* 1994; Stevenson *et al.,* 2010). Inactivation by chickpea occurred when the virus was exposed to chickpea leaf surfaces for more than 2 h, while for pigeonpea and cowpea lower but significant inactivation of the NPV was recorded when the viral OBs were exposed on their leaves but only after 48 h, demonstrating that on chickpea the inactivation effect was considerably higher and takes place within a short period. Rapid inactivation of *Hear*NPV on chickpea leaf surface, was also reported earlier with the effect peaking within 1 h with no significant differences in LC_{50s} between OBs exposed on chickpea leaf surfaces for 1 h and those exposed for 24 h, when the exposed virus was recovered and inoculated in diet and bioassayed against *H. armigera* larvae (Stevenson *et al.,* 2010). In the present study, an increase in LC_{50} of 8.48×10^4-fold was recorded between the virus exposed to chickpea leaves and those exposed to pigeonpea and 6.37×10^4-fold difference in LC_{50} between chickpea and cowpea leaf surfaces. Additionally, a 2.36×10^5-fold difference in LC_{50}s was recorded between chickpea and tomato and 2.49×10^5-fold difference between chickpea and unexposed *Hear*NPV. Conversely, only a 3-fold increase in LC_{50} was recorded between pigeonpea and tomato leaf surfaces and between pigeonpea and unexposed *Hear*NPV. Also, about 4-fold increase in LC_{50} was recorded between cowpea and tomato leaf surfaces and beween cowpea and unexposed *Hear*NPV. The result showed that there was no significant difference in LC_{50}s between cowpea and pigeonpea leaf surface or between *Hear*NPV exposed to tomato leaf surfaces and unexposed *Hear*NPV. While this study has shown a small but significant effect of pigeonpea leaf surfaces on *Hear*NPV efficacy, however Rabindra *et al.* (1994) observed that although exposing viral OBs to pigeonpea leaf surfaces could lead to an increase in LC_{50} when used in bioassay against *H. armigera* larvae, the difference was not significant when compared to unexposed *Hear*NPV in this study. However, differences in methodology between the two studies could account for those results. For example in previous work second instar larvae were used

while in present study first instar larvae (neonates) were used. Similarly, in Rabindra *et al.* (1994) study, NPV was not sprayed but rather smeared on the leaves surfaces.

A previous study has attributed the reduced efficacy of *Hear*NPV on chickpea to the low rate of food consumption by the larvae, particularly on the resistant genotype (Rabindra *et al.,* 1992). However, no antifeedant deterrent was observed when the two most abundant chickpea acids (malic and oxalic) were used in a paper feeding test on *H.armigera* larvae (Yoshida *et al.,* 1995) suggesting these components were not causing the reduced food consumption effect. The secretion of highly acidic exudate (pH approximately 1.3) on all the green parts of chickpea plants with its corresponding high malic acid content has been attributed to be the resistant mechanism of the plant against *Helicoverpa* larvae (Rembold, 1981; Rembold and Winter, 1982; Lateef, 1985). Suprisingly, even with this high acid content, *H. armigera* still remains the most damaging pest of chickpea (Lateef, 1985). It has been reported that chickpea plant character (organic acid exudates and isoflavonoids) could be an important resistant mechanism of the plant against *H. armigera* larvae (Stevenson *et al.,* 2005). However, the relative concentration between the two acids varies depending on varieties, diurnal cycles and growth stage of the plant (Rembold *et al.,* 1990b).

Stevenson *et al.* (2010) showed that chickpea leaf extracts could affect the efficacy of *Hear*NPV used against *H. armigera* larvae. They attributed the inhibitory effect on the viral OBs to be caused at least partly by their direct interactions with chickpea leaf surface isoflavonoids (sissotrin and biochanin A), since the two minor constituents of chickpea leaf extracts significantly reduced the activity of the virus when incubated together with viral OBs on artificial diet used against *H. armigera* larvae. Those isoflavonoids were reported to be induced on chickpea leaf surfaces at a higher level after spraying the virus suspension (Stevenson *et al.,* 2010). The study proved that the reduced efficacy of *Hear*NPV was leaf surface related and permanent because OBs were still less active after being removed from the leaf and bioassay against *H. armigera* larvae, and this is in agreement with the present work. Although the previous result have shown that NPV inactivation takes place within one hour (Stevenson *et al.,* 2010), the present work could not confirm the one hour data, due to difficulty encountered in obtaining enough infective virus OBs required to obtained the LC_{50} after exposing *Hear*NPV to chickpea leaves as mentioned earlier. The reason why it is

difficult to obtain enough virus in the present study could not be explained. However, other possibile suggestions could be that in the previous study, *Hear*NPV strain (NPV #0210) was used while in the present work a different strain obtained from a commercial isolate (Helicovex®, Andermatt Biocontrol, Switzerland) was used. Similarly, while in the other study chickpea plants were sprayed using a hydraulic hand sprayer, in the present study 10 ml glass chromatographic reagent sprayer was used (see Fig. 3.1). Also in the previous study, mortality was recorded after the seventh day but in the present work mortality was taken after the fifth day to eliminate the cannibalism behaviour observed during longer bioassays (i.e 7 days), which obscured treatment results. Pooling the concentration of the exposed virus on chickpea leaf surface is unlikely to have affected the present result, because previously Stevenson *et al.* (2010) showed that there was no significant difference between the virus exposed to chickpea leaf after 1 h and 24 h. However, although the two isoflavonoids were shown to reduce the activity of *Hear*NPV, both compounds could not account entirely for the observed inactivation seen on the chickpea leaf surface, indicating that other leaf surface compounds might be involved (Stevenson *et al.,* 2010). This problem could relate to some of the inconsistency mentioned about the differences in results obtained between experiments carried out with host plant tissue and and those with artificial diets (Ali *et al.,* 1999).

An important feature of leguminous plants is their widespread ability to produce isoflavonoid phytoalexins as a defensive mechanism against infection by microorganisms (Ingham, 1982; Williams and Harbone, 1989) and these could be produced in respond to exposure to NPV OBs. In relation to this, cowpea tissues have been reported to respond to infection by tobacco necrosis virus (TNV), inducing different isoflavonoid compounds like phaseollidine, kievitone and phaseollin (Bailey, 1973). The same type of phytoalexin were also observed to be the major compounds in eight different species of *Vigna*, from which phaseollin was detected in leaf tissue of *Vigna unguiculata* (Seneviratne and Harbone, 1992). Similarly, three phytoalexin compounds were isolated via bioassay-guided fractionation of chloroform extracts of pigeonpea leaves (Kong *et al.,* 2010). The study reported that of the three phytoalexins isolated, coumarin and stilbene compounds (cajanuslactone and cajaninstilbene) were found to be active antimicrobial constituents of pigeonpea leaf extracts. In addition, four isoflavonoid phytoalexins (hydroxygenistein, genistin, cajanin and cajanol) were

isolated from pigeonpea cultivars inoculated with fusarium wilt pathogen, *Fusarium udum*, from which cajanol was consistently identified to be present at higher concentration in the wilt resistant cultivar (Marley and Hillocks, 1993). It is suggested that there is a link between the ability of phenolic compounds to bind with protein coat of viruses and the ability of viral OBs to initiate infection in insect midgut cells (Pierpoint *et al.,* 1977; Felton and Duffey, 1990). Since it is known that viral OB is mainly made of protein (Vlak and Rohrmann, 1985; Whitt and Manning, 1988), occlusion body is susceptible to being permananetly bound by phenolic compounds such as isoflavonoids (Felton and Duffey, 1990). It was also proposed that virions released from the OBs bind to each other and to semiquinones thereby preventing them from attaching to the midgut epithelial cells to initiate an infection (Hoover *et al.,* 1998c).

Therefore one hypothesis for the reduced efficacy of *Hear*NPV on the legume crops observed in the present study could be due to the ability of the leaf surface chemical compounds (phenolics) induced in the presence of the virus to bind with virus OBs which then prevents the release of ODV and subsequently inhibits primary infection of the insect midgut. Interestingly, while the inhibition of the virus seen on the leaf surfaces of the two legumes (cowpea and pigeonpea) may be caused by the leaf surface compounds such as the phytoalexin isoflavonoids mentioned above, the fact that inhibition was observed within short period (i.e. within 1 h) on chickpea, suggest that other unknown and more potent leaf surface compounds might be involved. Furthermore, the two isoflavonoids previously identified in bioassays do not account for the whole inactivation effect observed on the chickpea leaf surface (Stevenson *et al.,* 2010).

It was observed that viral OBs were inactivated when consumed on both chickpea and cotton leaf, but OBs from cotton leaf surfaces showed no sign of inactivation when bioassayed on diet (Stevenson *et al.,* 2010). This is in agreement with the previous studies (Hoover *et al.,* 1998b; Forschler *et al.,* 1992), that shows that ingestion of viral inoculum along with host plants determined whether an insect will be susceptible to virus infection. This is consistent with the hypothesis that inhibition of viral disease in cotton is mediated by plant foliar enzymes (i.e. peroxidase), which lead to the

generation of free radicals and susequent sloughing of midgut cells before infection commences (Hoover *et al.,* 1998c; Hoover *et al.,* 2000).

Inhibition of viral disease has been shown to be a 3-way interaction involving; the host plant foliar constituents, larval midgut conditions and the viral OBs hence, treating BV in isolation from the insect did not result in viral disease inhibition (Keating *et al.,* 1990; Forschler *et al.,* 1992). However, the reduced activity of the viral OBs recorded on chickpea leaf surfaces observed in this study have demonstrated that the efficacy of the viral OBs were significantly reduced by their contact with the plants surface chemicals. Therefore, a different mechanism of inactivation which is not mediated via the insect midgut physiology must be involved in chickpea, which is in agreement with the previous study (Stevenson *et al.,* 2010). None of the results shows that tomato leaf surface reduce the efficacy of *Hear*NPV used against *H. armigera* larvae. This is in agreement with earlier studies that have also demonstrated that both leaf surfaces of cotton or tomato does not significantly affect the efficacy of the viral OBs before ingestion by the insect larvae (Forschler *et al.,* 1992; Stevenson *et al.,* 2010).

The results from the electron microscope images also shows that the mechanism of inactivation observed when the viral OBs were exposed to chickpea leaf surfaces was not linked to any physical change in the morphological structure of the viral polyhedral structure. Similarly, Forschler *et al.* (1992) also noted through electron microscopy that the decreased mortality seen when *H. zea* larvae were fed and dosed on cotton foliage was not correlated with the dissolution of particle inclusion bodies (PIBs) structure. Although earlier laboratory results have linked NPV inactivation when exposed to cotton dew with dissolution of viral OBs and liberation of virions within 48 h (Young *et al.,* 77) however, electron microscopical examination of the polyhedra on the upper leaf surface of cotton showed little degradation even after seven days (Young *et al.,* 1977).

CHAPTER FOUR

ANALYSIS OF CHICKPEA LEAVES TO IDENTIFY COMPOUNDS THAT INACTIVATE *HEAR*NPV

4.1 Introduction

Chemical screening of chickpea leaf was carried out using chromatographic and spectroscopic techniques, with the aim of investigating further the phytochemical compounds with possible antiviral activity. Previous studies have shown that chickpea leaf surface reduces the efficacy of *Hear*NPV used against *H. armigera* larvae but the observed effect could not be fully accounted for by either of two isoflavonoids (sissotrin and biochanin A) that had been found to be present at high levels on chickpea leaf after treatment with *Hear*NPV suspension (Stevenson *et al*., 2010). There was some indication that both sissotrin and biochanin A were induced in greater quantities on the chickpea leaf surface in response to spraying with *Hear*NPV and or Triton X-100, the spray surfactant.

This study was carried out with the aim of identifying whether other chickpea plant chemical compounds might be involved in the inactivation of *Hear*NPV. This could possibly explain the greater inactivation recorded on the chickpea leaf surface. At the same time the study will help to find out conclusively which compounds were induced at higher level after *Hear*NPV spraying and also to determine the elicitor that is responsible for their induction. In addition, the study will determine whether spraying or wetting with either Triton surfactant or water could be responsible for the induction of phytochemical compounds with antiviral properties as suggested in the previous study (Stevenson *et al*., 2010).

Therefore, this study was carried out to test the following hypotheses;

1. That spraying chickpea plants with water alone is responsible for elevated levels of isoflavonoids,
2. That spraying chickpea plants with Triton surfactant is responsible for elevated levels of isoflavonoids,

3. That spraying chickpea plants with *Hear*NPV is responsible for elevated levels of isoflavonoids.

To test these hypotheses, chickpea plants were sprayed either with water, Triton surfactant or *Hear*NPV, and the sprayed leaves were excised and extracted in methanol after different time interval. Extracted leaves were analysed using LC-MS.

4.2 Materials and Methods

4.2.1 Chickpea leaf surface and whole leaf extraction

Chickpea plants were sprayed with *Hear*NPV suspended in distilled water at a concentration of 3×10^8 OB ml^{-1} in 0.02% Triton and with water or 0.02% Triton only using a 10 ml glass chromatographic reagent sprayer (Sigma- Aldrich, UK) at the rate of 2 ml per plant, while the control treatment was left unsprayed. Spraying was carried out such that the leaves were evenly wet. Chickpea plants were first sprayed with water followed by Triton surfactant and finally with *Hear*NPV suspension to prevent the risk of contamination between the treatments. Plants were maintained in the green house conditions as mentioned in section 2.4 (Chapter 2). For each treatment 20 compound leaves of chickpea were randomly excised within 1 h (hour) and after 48 h for the unsprayed control while the sprayed plants were excised after 2, 8, 24 and 48 h of spraying and the leaf surfaces were extracted in 5 ml HPLC grade methanol by rinsing the leaves individually in a 7 ml glass vial for 1 min each. The whole leaf extracts were obtained by soaking the 20 rinsed leaves from the extracted leaf surface in 10 ml HPLC grade methanol using 21.5 ml glass vial for 18 h at room temperature. Each treatment was replicated 5 times and for each replicate two different plants were used to avoid putting too much stress on the plants. Both chickpea leaf surface and whole leaf extracts were evaporated to dryness by heating the samples at 40°C in 7 ml glass vials using sample concentrator (DB-3 series Techne Dri-Block heater) and passing a stream of nitrogen on to the vials via stainless steel 76 mm needles (Techne, Bibby Scientific Ltd., Staffordshire, UK). Dried filtrates of chickpea leaf surface extracts were re-suspended into 500 µl HPLC grade methanol, making them 10 times their previous concentration. When no compound was detected, the concentrated leaf surface extracts

were again evaporated under room temperature, by opening the lid of the 1 ml auto sampler vial (low volume) for 24 h and the dried sample re-suspended for the final time in 100 µl of HPLC grade methanol (making it 50 times its initial concentration). While the dried samples from chickpea whole leaf extracts were re-suspended into 500 µl (HPLC grade methanol) making them ten times their original concentration. The concentrated samples were sonicated before they were transferred into 1.5 ml Eppendorf tubes and spun on a rotator for 5 min. The supernatant was transferred in to 1 ml glass vials using a glass Pasteur pipette and stored at 4°C.

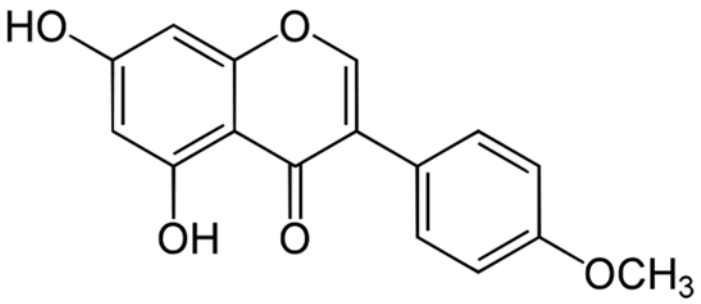
4.2.2 HPLC analysis of chickpea leaf extracts after spraying with water, Triton surfactant and *Hear*NPV

High-performance liquid chromatography is one of the several chromatographic techniques used for the separation and analysis of chemical mixtures of compounds, with the aim of identifying, quantifying and purifying the individual components of the mixture. When combined with mass spectrometry (MS), liquid chromatography (LC) becomes a powerful tool for compound identification and this was used for analysis of chickpea extracts.

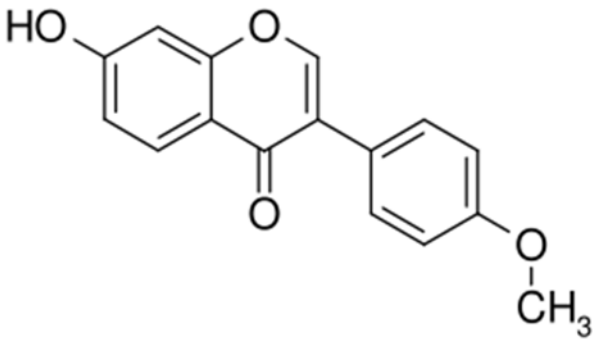
Filtered extracts from both chickpea leaf surface and whole leaf extracts in methanol were co-chromatographed with authentic samples of daidzein, biochanin A and formononetin (Fig. 4.1.; Sigma-Aldrich, UK), at 100 ppm, 33 ppm, 10 ppm, 3.3 ppm and 1 ppm. Aliquots (10 µl) of the filtered samples (as explained in 3.2.1 above) were injected on to a reverse phase column (Zorbax Eclipse Plus, analytical column; C18, 4.6mm i.d. × 150 mm; 5 µl particle size) and flow at rate of 0.4 ml min^{-1} using the gradient 90% A: 10% C at t= 0 min to 90% D: 10% C at t = 30 min to 90% A: 10% C at t = 31 min (A = 100% water, C = 1% formic acid in acetonitrile and D = 100% acetonitrile). Using LC-MS analysis, unknown compounds in the chickpea leaf extracts were identified by comparing their retention times, UV spectra and mass spectra to those of the authentic standards. Graphs of peak area versus treatments were plotted.

4.2.3 Calibration of LC-MS with authentic standards of biochanin A and formononetin

Peak area of the authentic standards of biochanin A and formononetin prepared at five different concentrations (100 ppm, 33 ppm, 10 ppm, 3.3 ppm and 1 pmm) as mentioned in section 4.2.2 above were plotted against concentrations (mg ml^{-1}) to construct the calibration curve. Using regression line equation obtained from the calibration graph, the natural amounts of both compounds were then calculated based on the equivalent concentration of the compounds on chickpea leaf area (1,250 mm^2) of artificial diet used for bioassay, this was the same method used by Stevenson *et al.* (2010). This forms the bases for subsequent bioassays.



Biochanin A



Formononetin

Figure 4.1 Isoflavonoids identified to be induced at high levels (formononetin) or present at high levels constitutively (biochanin A) after treatment with a biotic elicitor (*Hear*NPV).

4.2.3 Statistical analyses

Data from LC-MS were analysed using analysis of variance (ANOVA), having first tested the data was suitable with equal variance and normal distribution. Where significant differences were detected among the treatment means, differences were separated using Holm-Sidak multiple comparison tests. Where data were not of equal variances, results were log transformed before being analysed using ANOVA or by using appropriate non-parametric tests like Krukal-Wallis. All analysis was carried out using Sigmaplot software (11.0).

4.3 Results

This section of results is concerned with whether the presence of isoflavonoids can be induced by spraying with water, surfactant (Triton) or *Hear*NPV. Previous work has reported elevated levels of two isoflavonoids linked to NPV inactivation occurred after spraying with a mixture of water, surfactant and *Hear*NPV but which component induced these elevated levels has not been determined, nor for how long did these increased levels occur (Stevenson *et al*., 2010).

Hypothesis 1: spraying chickpea plants with water alone induces increased levels of bioachanin A and formononetinon on leaf surfaces

Analysis of the formononetin levels using Kruskal-Wallis one way analysis of variance on ranks shows that there was no significant difference ($H = 8.041$, $df = 3$, $P = 0.045$) between unsprayed leaves and those sprayed with water (Fig. 4.2). Although on leaves 24 h after spraying with water there was an increase in formononetin over that of untreated leaf surfaces (1.7-fold), the increase was not significant. Samples taken 2 h and 48 h after water spraying showed formononetin levels to be lower but were not different to controls (Fig. 4.2). Thus spraying with water caused a non significant transient rise in formononetin at 24 h but this is temporary and modest.

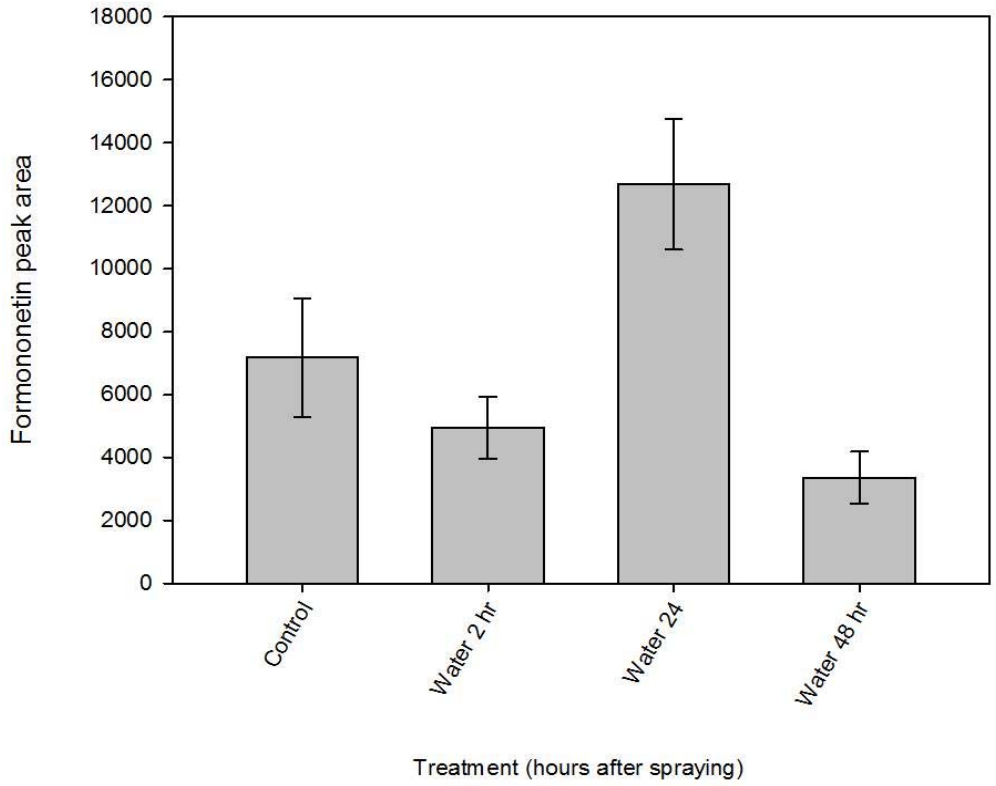


Figure 4.2 Comparison of formononetin level induced on chickpea leaf surfaces between untreated control leaves and chickpea leaves treated with water after different time intervals as determined by LC-MS. There is no significant differences between the treatment groups ($P > 0.05$).

To test the hypothesis that there was no significant differences in the levels of biochanin A in chickpea leaf surfaces, the data was analysed using Kruskal-Wallis analysis of variance on ranks. The result shows that there were no significant differences ($H = 16.202$, $df = 12$, $P = 0.182$) between the median value among the treatment groups. Thus for biochanin A there is no evidence of elevated production in response to spraying with water, even though a high level of biochanin A was recorded 24 h after spraying, and samples 2 h after water treatment shows lower biochanin A (Fig. 4.3) than in other samples.

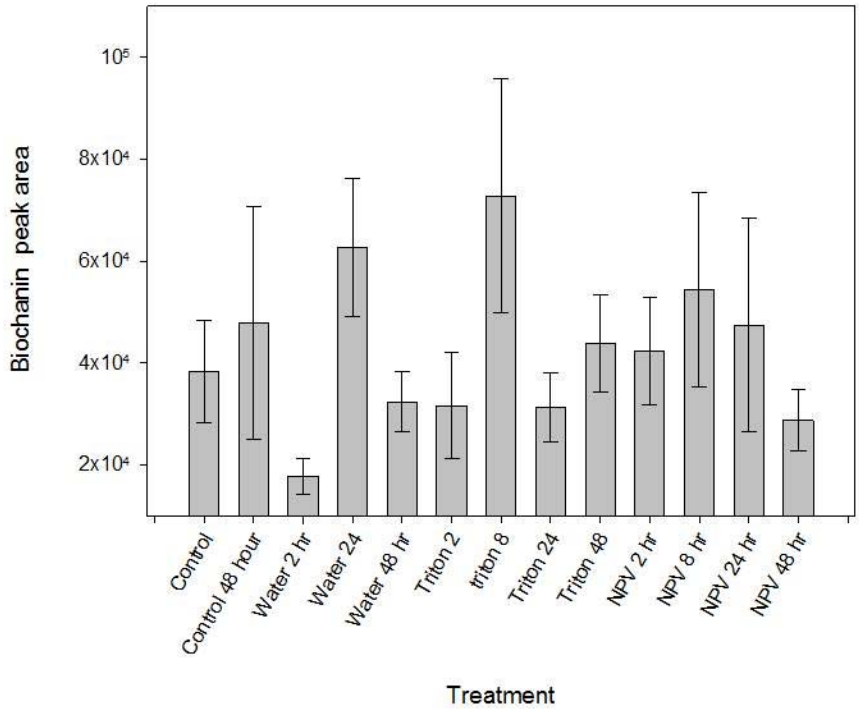


Figure 4.3 Comparison of biochanin A levels induced on chickpea leaf surfaces between untreated control leaves and chickpea leaves treated with water, Triton and *Hear*NPV OBs at different time intervals as determined by LC-MS. There is no significant difference between the treatment groups (P> 0.05).

Hypothesis 2: Spraying with the surfactant Triton is associated with elevated levels of formonomentin and biochanin A.

Results from ANOVA shows a significant increase in formononetin between untreated chickpea leaf surface and leaf surfaces treated with Triton surfactants ($F = 9.040$, df = 4, 19, $P < 0.001$). Furthermore, Holm-Sidak multiple comparison test shows a significant increase in formononetin level on chickpea leaf surfaces, between those sprayed at 8 h and all the other treatment groups ($P < 0.001$) and also between 48 h and untreated control leaves ($P = 0.012$). However, no significant increased in formononnetin levels was recorded between untreated control chickpea leaf surfaces and chickpea leaf surfaces sprayed with Triton for either 2 h or 24 h (Fig. 4.4).

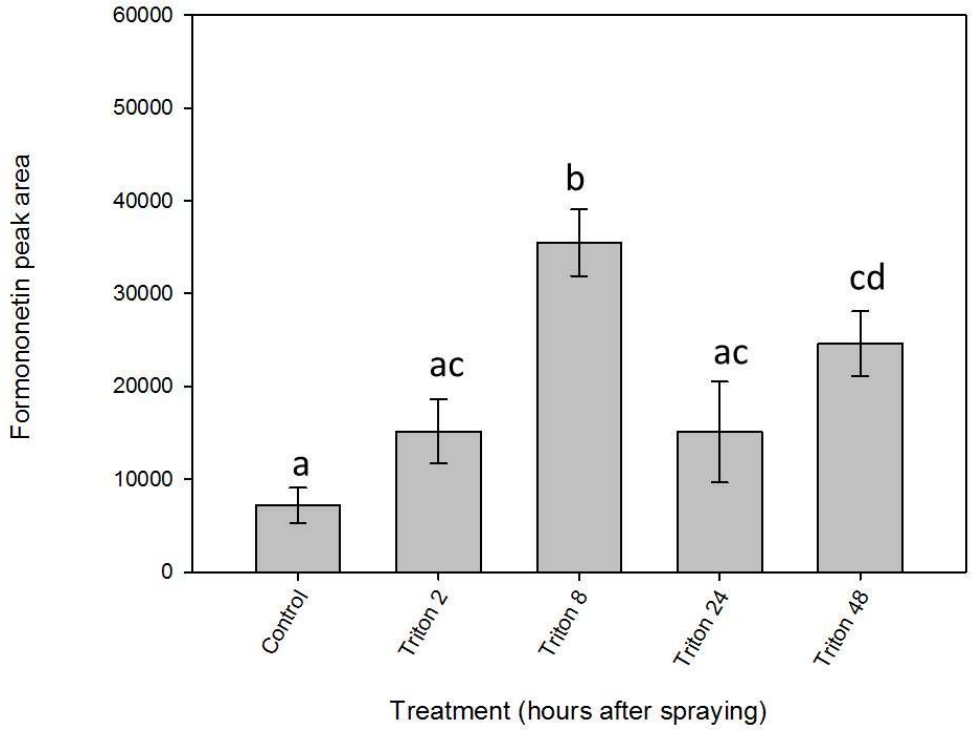


Figure 4.4 Comparison of formononetin level induced on chickpea leaf surfaces between untreated control leaves and chickpea leaves treated with Triton after different time intervals as determined by LC-MS. Bars with different letters differed significantly ($P < 0.05$).

The concentration of biochanin A was higher 8 h after spraying with Triton on chickpea leaf surfaces than 2 h or 24 h after spraying although this was not significantly different compared to each other treatments and the untreated control, as shown by Kruskal-Wallis one way analysis of variance on ranks ($H = 16.202$, $df = 12$, $P = 0.182$). Thus for biochanin A there was no statistical significant evidence to support the hypothesis that spraying with Triton surfactant is associated with a subsequent increase in biochanin A level on leaf surfaces (Fig. 4.3).

Hypothesis 3: Spraying with HearNPV is associated with elevated levels of formononentin and biochanin A.

Treatment with *Hear*NPV was associated with significantly higher levels of formononetin on chickpea leaf surfaces compared to those of untreated chickpea leaf

surfaces (Fig. 4.5). Results from Kruskal-Wallis one way analysis of variance on ranks shows that there are significant differeces between the median values among the treatment groups (H = 13.697, df = 4, P = 0.008). Similarly, Dunn's multiple comparison tests shows that there is significant difference (P < 0.05) between untreated control chickpea leaf surfaces and those sprayed with *Hear*NPV at 8 h and 24 h. But there was no significant difference (P > 0.05) between untreated control chickpea leaves and leaves sprayed with *Hear*NPV at 2 h and 48 h.

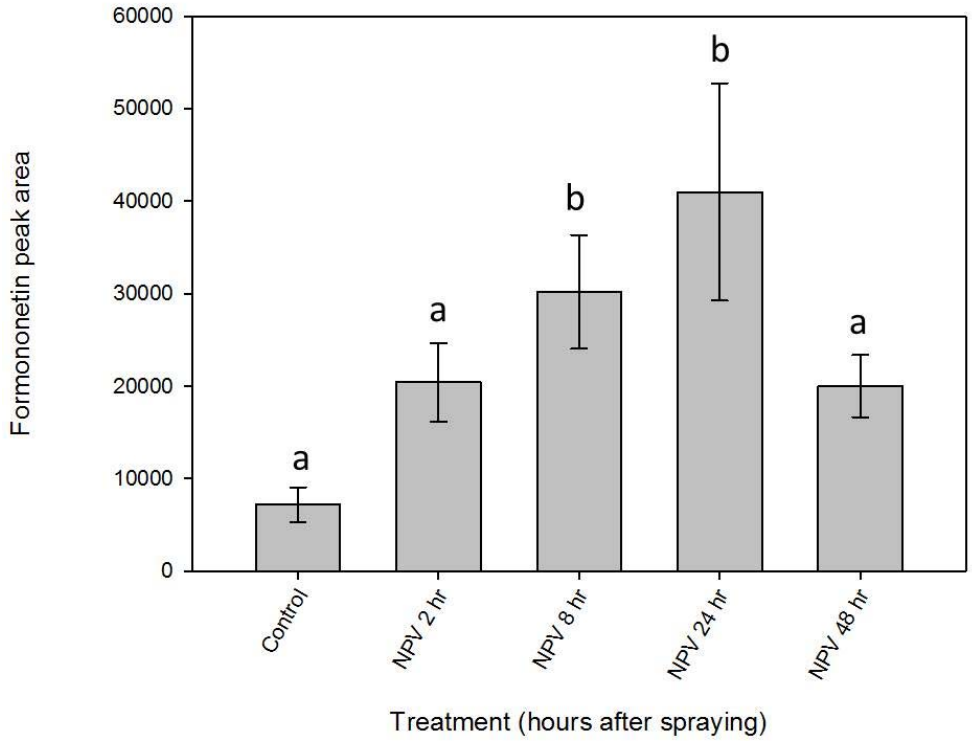


Figure 4.5 Comparison of formononetin level induced on chickpea leaf surfaces between untreated control leaves and chickpea leaves treated with *Hear*NPV OBs at different time intervals as determined by LC-MS. Bars with different letters differed significantly (P< 0.05).

In contrast spraying with *Hear*NPV produced no significantly elevated levels of biochanin A over controls after treatment and there was no significant differences in the chickpea leaf surfaces (H = 16.202, df = 12, P = 0.182) between the median values of all the treatment groups (Fig. 4.3).

To determine which among the three treatments (water, Triton, *Hear*NPV) induced higher levels of formononetin on chickpea leaf surfaces, means from the three treatment groups were log transformed and subjected to ANOVA. The results showed that there were significant differences ($F = 27.790$, $df = 2, 51$, $P < 0.001$) in the levels of formononetin induced between the three treatment groups. Holm-Sidak multiple comparison between water treatment and other treatments groups shows that treatment with either *Hear*NPV or Triton surfactant induced significantly higher ($P < 0.001$) levels of formononetin compared to treatment with water. Thus in conclusion these experiments showed that only Triton and *Hear*NPV were associated with increased levels of formononetin on chickpea leaf surfaces. Three way ANOVA also shows that there was an association between time and spray ($P = 0.016$), and in this respect *Hear*NPV spraying induced significantly ($P < 0.05$) higher levels of formononetin compared to Triton on chickpea leaf surfaces at 24 h.

The amounts of biochanin A recorded on the chickpea leaf surface after spraying with each of the three treatments (water, Triton or *Hear*NPV) as well as the untreated control were found to be higher compared to that of formononetin on the chickpea leaf surfaces treated similarly. The differences between the amounts of biochanin A and formononetin were more pronounced in the untreated control and the water treated for both leaf surface and whole leaf extracts, with the differences between the leaf surface extracts of the two compounds increasing by up to 12 and 9-fold respectively for the water treatment and untreated control after 48 h. However, the levels of the two compounds were similar in three out of four *Hear*NPV treatments, in both the leaf surface and whole leaf extracts.

Hypothesis 4: spraying water, Triton and HearNPV changes level of isoflavonoids in the whole leaf of chickpea

Result of formononetin analysis from chickpea whole leaf extracts (Fig. 4.6) was analysed using ANOVA, after the data were log transformed to normalise the variance. The results from the analysis shows there were significant differences ($F = 8.072$, $df = 12.50$, $P < 0.001$) among the treatment groups.

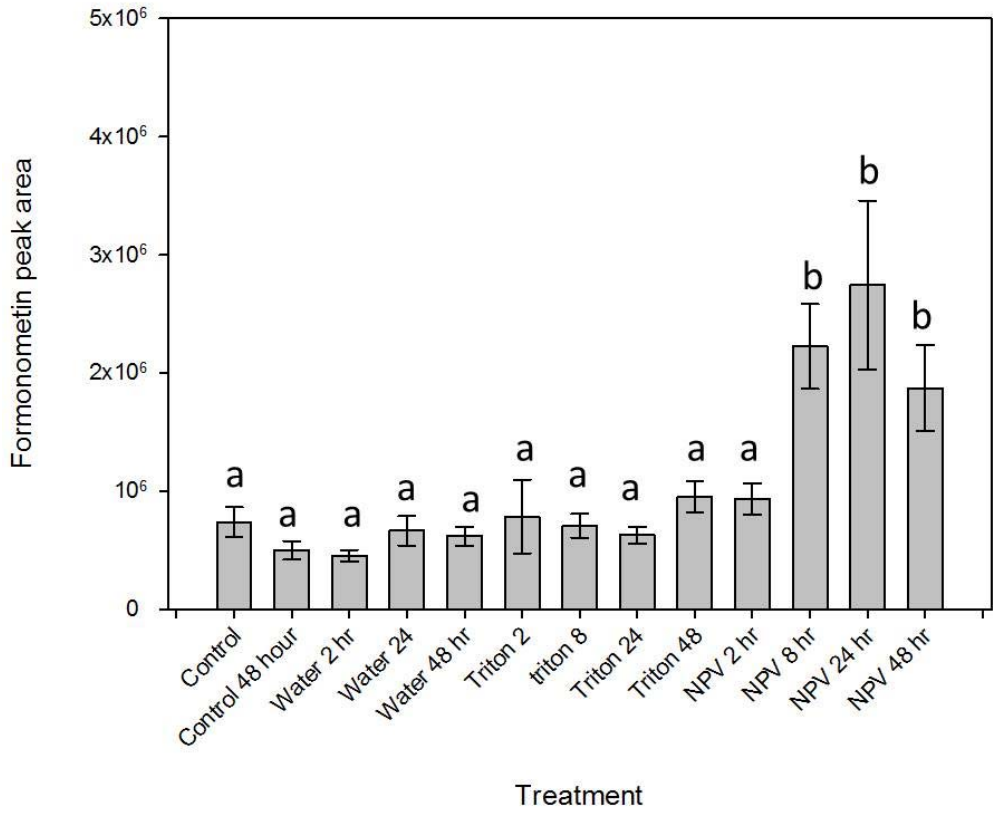


Figure 4.6 Comparison of formononetin level induced in untreated chickpea whole leaf extracts (control) and extracts from whole leaves of chickpea treated with either water, Triton or *Hear*NPV OBs at different time intervals as determined by LC-MS. Bars with different letters differed significantly ($P< 0.05$).

Using the Holm-Sidak multiple comparison test the difference between each treatment and the control were separated. The result shows that formonometin levels were significantly higher in all NPV treatments at 8 h, 24 h ($P < 0.001$) and 48 h ($P = 0.018$) after spraying compared to the untreated control. The levels of formononetin in water

or Triton sprayed leaves were not significantly different to the untreated control leaves ($P < 0.05$).

The concentrations of formononetin induced in chickpea whole leaf extracts were calculated to be 83 ppm, 102 ppm and 69 ppm after treatment with *Hear*NPV suspension at 8, 24 and 48 h respectively.

ANOVA result of the biochanin A levels in chickpea whole leaves (Fig. 4.7) shows that there were no significant differences among the means of all the treatment groups ($F = 1.576$ df = 13,54, $P = 0.121$).

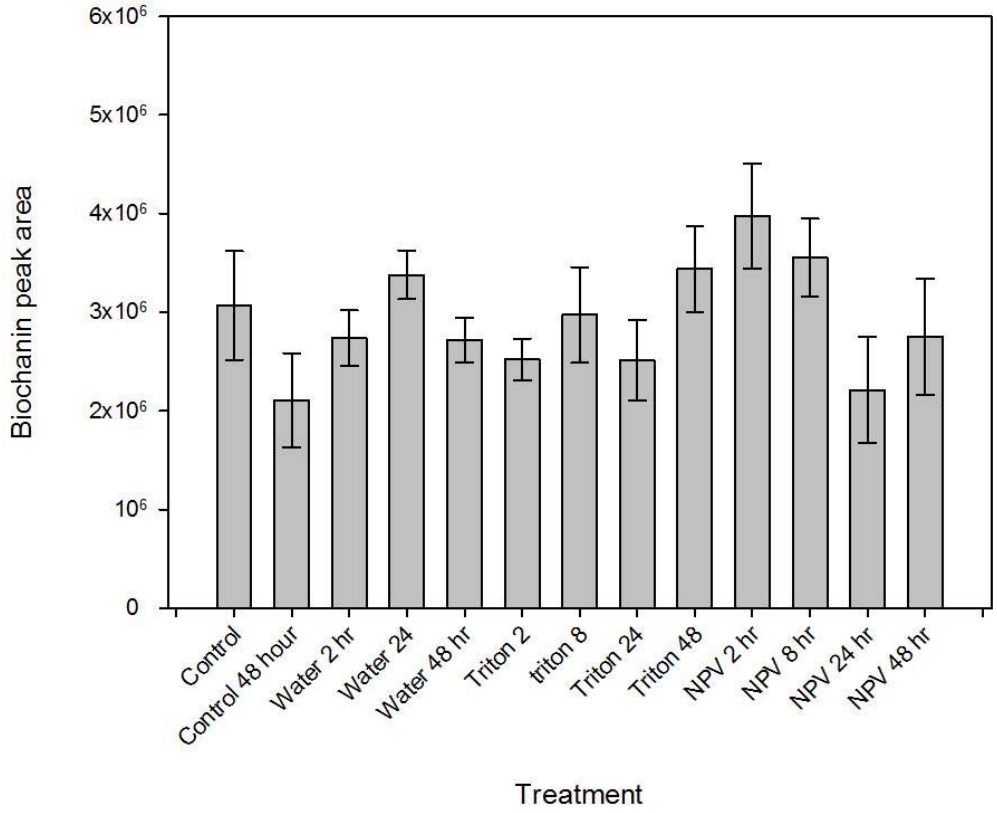


Figure 4.7 Comparison of biochanin A level in untreated whole leaves of chickpea extracts (control) and extracts from whole leaves treated with either water, Triton or *Hear*NPV OBs after different time intervals as determined by LC-MS. There is no significant difference between the treatment groups ($P > 0.05$).

The concentrations of biochanin A present were calculated to be 225 ppm, 202 ppm, 127 ppm and 157 ppm in chickpea whole leaf extracts after treatment with *Hear*NPV at 2, 8, 24 and 48 h respectively.

4.4 Discussion

The results from the chemical analysis showed that the concentration of formononetin increased on the leaf surface of chickpea after treatment with *Hear*NPV suspension in 0.02% Triton or even when spraying the surfactant alone. Both Triton and *Hear*NPV induced higher levels of formononetin compared to the water treatment or untreated control.

Therefore, the study demonstrated that spraying water alone was not responsible for induction of high level of the formonometin hence the plant is not responding simply to wetting as previously suggested (Stevenson *et al*., 2010), but that chickpea plant is reacting to the presence of Triton surfactant (Hargreaves, 1981) and *Hear*NPV. Furthermore, the study showed that there was an association between spray and time. In this regard, spraying of *Hear*NPV induced higher levels of formononetin compared to Triton 24 h after treatment. However, formononetin accumulated for only a short period (8 h) after treatment with Triton on chickpea leaf surfaces. Comparing both the *Hear*NPV and the Triton induction of the compound, it can be said that *Hear*NPV has a stronger and longer effect, with the maximum induction expressed at 24 h compared to the Triton induction which was transient in nature (i.e. 8 h) after which it started to decline.

Previous study using chickpea cell suspension culture has shown that maximum accumulation of pterocarpan phytoalexins, medicarpin and maackiain were observed consistently to be 24 h after treatment with elicitor (Kessman and Barz, 1987). Although the increases in formononetin level on chickpea leaf surface after Triton and virus treatment were found to be significant compared to the untreated control leaves, this was not the case for biochanin A. However, both compounds were induced at very low concentrations on the leaf surface and could only be detected after the leaf extracts were concentrated to about 50 times the initial concentration.

In the case of chickpea whole leaf, the result shows clearly that substantially elevated formononetin levels only occur in response to spraying with *Hear*NPV. Therefore only treatment with the virus produced significant level of formononetin induction compared to spraying with either water or Triton surfactant. Thus, increase in the

concentration of formononetin on both the leaf surface and in whole leaf of chickpea could be the plant defence response to external stimuli.

Stress metabolites (phytoalexin) have been reported to be produced by plants not only as a result of interactions with biotic agents such as plant pathogens but also in response to other forms of stress like chemicals or UV radiation (Bailey, 1982; Soylu *et al*., 2002). Short-wave UV radiation has been shown to induced high level of pisatin in excised pea pods within few hours after treatment (Hadwiger and Schwochau, 1971), as well as increase in coumestrol in the leaves of common bean (Beggs *et al*., 1985). Furthermore, silver nitrate ($AgNO_3$) was found to be the most effective elicitor of phytoalexin, methoxybrassinin and cyclobrassinin in Brassica plant compared to infection by biotic agents like *Leptosphaeria maculatus*, causal organism of stem canker disease (Dahiya and Rimmer, 1989). Similarly, copper salt ($CuCl_2$) has been shown to be the most effective inducer of phytoalexin (i.e. coumarin compounds) in the leaves of *Corchorus olitorius* compared to treatment with fungal pathogen (*Helminthosporium turcicum*) or mercury choride (Hussein and Abou Zeid, 2002). Similarly, concentrations of pterocarpan, maakiain and medicarpin as well as cicerfuran and judaicin were shown to increase significantly in the roots of resistant Cicer species in the presence of fungal pathogens (Stevenson *et al*., 1997; Stevenson and Veitch., 1998a; Stevenson and Haware, 1999). Thus a range of chemical and biotic agents have been shown to be capable of eliciting phytoalexins.

Previous study has shown that both cowpea and pea produced different antifungal compounds following treatment with either tobacco necrosis virus (TNV) or pea early browning virus (PEBV) (Bailey, 1973). The study shows that while TNV triggered many stress metabolites like phaseollidin, kievitone and phaseollin, in pea only pisatin was induced with PEBV. Ataga *et al*. (1999) also reported that treatment of celery plants with celery mosaic virus reduce the plants response to blight disease by *Septoria apiicola*.

This study also showed that biochanin A was present constitutively on both the chickpea leaf surface and whole leaf, and its concentration was consistently found to be higher than that of formononetin, after spraying with any of the three treatments (water, Triton or *Hear*NPV). However, the difference between the levels of biochanin A among the three treatments was not significant. While, the levels of formononetin

in the whole leaf are likely related to the concentrations recorded on the leaf surface, only the virus treatment group recorded significantly higher whole leaf concentrations compared to the untreated control. Both formononetin and biochanin A together with their glycosides were identified to be present at high concentrations in both the roots and cell suspension cultures of *Cicer* species (Kessman and Barz, 1987; Stevenson and Veitch, 1998b). However, significant difference was only observed between resistant and susceptible *Cicer* cultivars with regards to phytoalexin accumulation (Kessman and Barz, 1987; Stevenson and Veitch, 1998b). Based on these findings, biochanin A may occur as a pre-infectional defence mechanism or a preformed component, while formononetin could be responding like a post-infectional defence (phytoalexin) against the virus. Weideman *et al*. (1991) showed that phytoalexins are subject to increased concentration from either the aglycone or the glycosides as a result of elicitor effects. Furthermore, analysis of chickpea cell suspension cultures at preinfectional state also indicates the presence of polar conjugates of biochanin A and formononetin at higher concentration compared to the aglycone which were detected in small quantities (Weideman *et al*., 1991). Since facile cleavage of the pterocarpan glycosides has been proposed to be a direct source of the antifungal aglycone maackiain (Stevenson and Veitch, 1998b), this mechanism could also assumed to be the source of formononetin aglycone from the formononetin 7-*O*-glucoside, which has been observed to be one of the major components of *Cicer* spp (Stevenson and Veitch, 1998b).

In a related study, Tebayashi *et al*. (2001) have shown that treatment of red clover roots with biotic and abiotic elicitors triggered a rapid consumption of formononetin and maakiain conjugates followed by increase in their aglycones. They suggested that the free formononetin and maakiain were produced from the conjugate pools via hydrolysis during the process of infection. Availability of stored conjugates of formononetin and daidzein could facilitate the rapid biosynthesis of more complex isoflavonoids in response to various biological elicitors (Graham *et al*., 1990; Phillip and Kapulnik, 1995). They noted that free formononetin and daidzein released from such conjugates may play a role as precursors of more complex compounds such as medicarpin and glyceollin which are known to act as postinfectional inhibitors against many plant pathogens.

CHAPTER FIVE

EFFECTS OF ISOFLAVONOIDS ON *HEAR*NPV EFFICACY USED AGAINST *H. ARMIGERA* LARVAE

5.1 Introduction

Biological screening was carried out using laboratory reared *H. armigera* larvae to determine the antiviral activities of the two isoflavonoids, formononetin and biochanin A that were found to be either present or induced at high concentrations on chickpea leaf after spraying with *Hear*NPV suspension.

Previous research has shown that the efficacy of baculovirus for pest control can be reduced by host-plant chemistry (Duffey *et al*., 1995). They noted that both the course and severity of disease can be affected by host-plant. In addition to reducing the efficacy, host-plant also affects the virulence and yield of OB production (Raymond *et al*., 2002), which has been attributed to the intra-specific variations existing between plants, (Ali *et al*., 2002). Although a number of studies have demonstrated that interactions between plants and enthomopathogens can reduce the efficacy of pathogens (Duffey *et al*., 1995; Hoover *et al*., 1998a,b, 2000; Cory and Hoover, 2006), these authors noted that mechanisms involved still remains complicated due to the number of chemicals involved. However, two specific mechanisms have been identified in cotton such as cation inactivation and action of plant foliar enzyme, peroxidase (Young *et al.,* 1977; Entwistle and Evans, 19985b; Hoover *et al*., 1998b,c)

This study was carried out to determine the effect of formononetin and biochanin A identified to be induced or present at high levels after treatment with *Hear*NPV elicitor and quantify their effect as a basis for comparison to that of sissotrin identified previously. To quantify the effect of formonometin and biochanin A surface contamination bioassay was carried out using *H. armigera* neonate larvae. The formonometin and biochanin A were diluted in acetone at different concentrations and used to dose the diet surface. After the solvent had evaporated, known concentration of the *Hear*NPV was incubated with the isoflavonoid and later the neonates were

released into the diet surface. Mortality was counted and the LC_{50} was calculated and the result compared to that of untreated *Hear*NPV.

5.2 Materials and Methods

5.2.1 Bioassay to determine the effect of formononetin and biochanin A on *Hear*NPV efficacy against *H. armigera* larvae

A fivefold serial dilution was prepared from the stock suspension of *Hear*NPV in SDW into five treatments (T1-T5) as mention in section 2.6.1. Standards of formononetin and biochanin A (Sigma Aldrich, USA) were diluted into 500 ppm, 50 ppm and 5 ppm concentrations in acetone (analytical grade) using separate glass vials (7 ml) which were labelled accordingly.

Three polypots (29 ml) containing artificial diet for polypot were set out for each treatment plus six other for control treatments, three each for water and acetone control. Starting with the top concentration of formononetin or biochanin A to the least, 100 µl aliquot of the standard compound was dispensed using micropipette onto the centre of the diet in each of the three pots set for each treatment.

Dosed pots were tilted and rotated gently until the applied compound solution spread evenly over the entire surface of each pot. The process was continued until all the diet polypots set for each treatment (T1-T5) were dosed with the three different concentrations (i.e. 500 ppm, 50 ppm and 5 ppm) of either formononetin or biochanin A.

Control treatments were dosed with 100 µl of SDW or acetone only to indicate whether the solvent used to solubilise compounds had any independent effect on the insects. A separate micropipette tip was used to dispense different compounds and concentration dilution in order to prevent the risk of contamination between the compounds and within their concentration dilutions. All the dosed pots were arranged according to their treatments in a tray on a flat surface allowing the acetone solvent to evaporate from the diet surface. Starting with the top formononetin or biochanin A concentration, 100 µl aliquot of the least virus concentration (T5) was dispensed using a micropipette

into the centre of the diet dosed with the top formononetin or biochanin A concentration dilution (i.e. 500 ppm).

The process continued until each virus concentration (T1-T5) was dosed with three different concentrations of formononetin or biochanin A (i.e. at 500 ppm, 50 ppm and 5 ppm). After the virus suspension had dried, 30 newly hatched neonate (18 h old) were transferred into the dosed pots at the rate of 10 larvae per pot using a paint brush (No. 2). All other procedures were the same as mentioned in section 2.6.1.

5.2.2 Statistical analysis

Data from bioassays were analysed by calculating the mean LC_{50}s obtained from atleast five different experiments carried out on different days using the Poloplus software (Robertson *et al.,* 2007). The difference between the treatments means were detected through analysis of variance, difference in the means were separated by comparing the untreated *Hear*NPV control with the other treatments using Holm-Sidak multiple comparison test (Sigmaplot 11.0). However, difference between water and acetone treated diet was analysed using t-test.

5.3 Results

5.3.1 Bioassay to determine the effect of acetone solvent against *H. armigera* larvae

To determine whether acetone used in preparing different concentrations of formononetin or biochanin A had a significant effect against *H. armigera* larvae used in the experiment, bioassay was carried out comparing the effect of SDW and acetone on *H. armigera* neonate. The data from the bioassay was analysed using t-test, the result shows that there was no significant difference (P = 0.587) between the mean values of the two groups (Fig. 5.1).

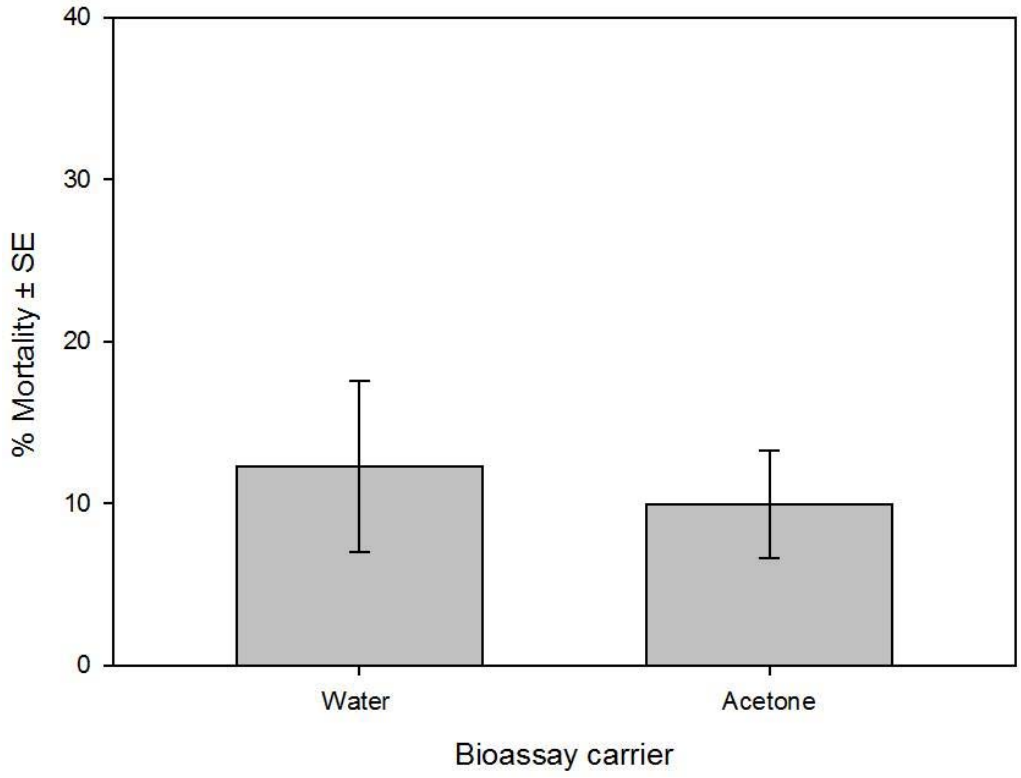


Figure 5.1 Mean percentage mortality (± SEM) of *H. armigera* neonates on artificial diets containing either water or acetone carrier. There is no significant difference between the two groups (P> 0.578).

5.3.2 Bioassay to determine the effect of formononetin on *Hear*NPV efficacy against *H. armigera* larvae

The mean LC_{50}s of *Hear*NPV after exposure to different concentrations of formononetin (Fig. 5.2), shows that there was significant difference between the treatment groups (ANOVA; F = 3.145, df = 3, 22, P = 0.046). Using Holm-Sidak multiple comparison test, it was shown that there was significant difference (P= 0.017) between the untreated *Hear*NPV and the *Hear*NPV treated with formononetin at 500 ppm, and between untreated *Hear*NPV and *Hear*NPV treated with formononetin 50 ppm (P= 0.050). However, there was no significant difference (P = 0.056) between the untreated *Hear*NPV and the *Hear*NPV treated with formononetin at 5 ppm.

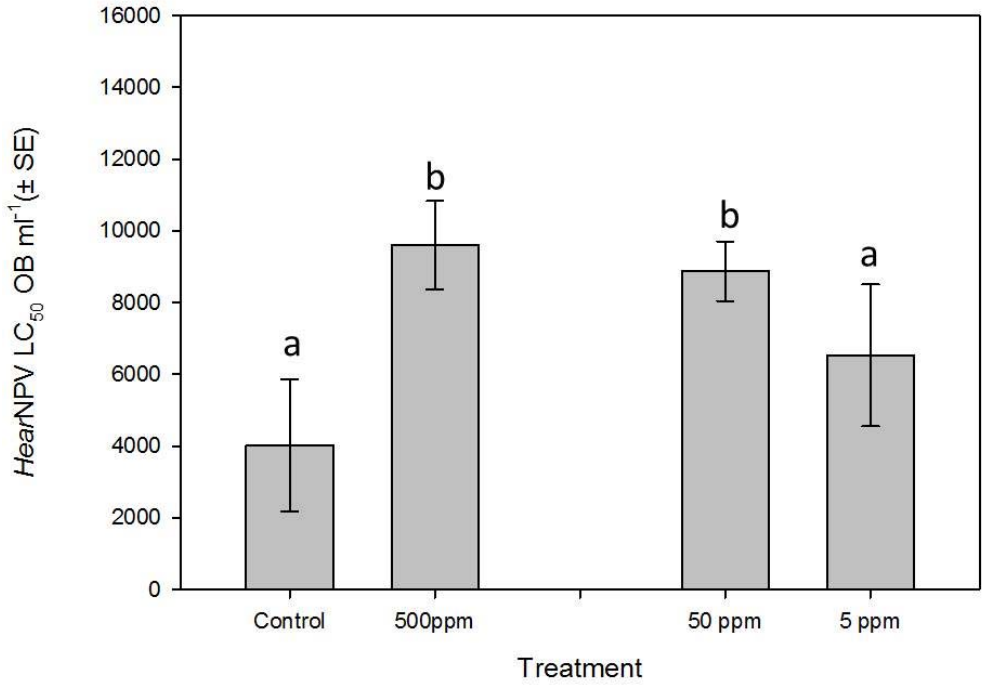


Figure 5.2 Average median lethal concentrations (LC_{50} ± SEM) of *Helicoverpa armigera* neonates on artificial diets containing either untreated *Hear*NPV OBs or *Hear*NPV OBs exposed to different concentrations of formononetin. Bars with different letters are statistically different ($P < 0.046$).

5.3.3 Bioassay to determine the effect of biochanin A on *Hear*NPV efficacy against *H. armigera*

The mean LC_{50} of *Hear*NPV after exposure to different concentrations of biochanin A (Fig. 5.3) was log transformed before being analysed using ANOVA, the result shows that there was significant difference ($F = 13.637$, $df = 2,9$, $P = 0.002$) between the treatment groups, and using Holm-Sidak multiple comparison test the result suggest that there was significant difference ($P= 0.001$) between the untreated *Hear*NPV and the *Hear*NPV treated with biochanin A at 500 ppm and between untreated *Hear*NPV and *Hear*NPV treated with *Hear*NPV at 50 ppm ($P= 0.013$).

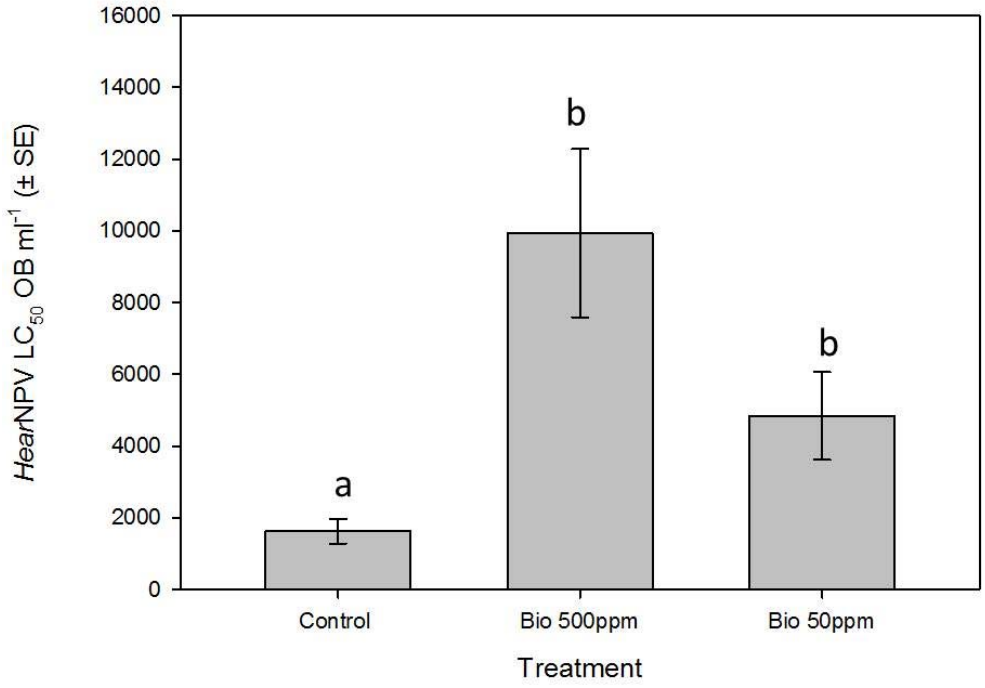


Figure 5.3 Average median lethal concentrations (LC_{50} ± SEM) of *Helicoverpa armigera* neonates on artificial diets containing either *Hear*NPV OBs or with *Hear*NPV OBs exposed to different concentrations of biochanin A. Bars with different letters are statistically different ($P < 0.002$).

5.4 Discussion

The study showed that both biochanin A and formononetin reduced the efficacy of *Hear*NPV used against *H. armigera* neonate at a concentration of 50 ppm or more. However this is less than the concentrations of biochanin A identified to be present (225 ppm, 202 ppm, 127 ppm and 157 ppm) in chickpea whole leaf extracts after treatment with *Hear*NPV at 2, 8, 24 and 48 h respectively. Similarly, the amount was also less than the concentrtions of formononetin induced in chickpea whole leaf extracts (i.e. 83 ppm, 102 ppm and 69 ppm) after treatment with *Hear*NPV suspension at 8, 24 and 48 h respectively. The concentration of both compounds were based on their natural concentrations obtained by calculating the equivalent amounts of the compounds in chickpea leaf surface area (whole leaf) of artificial diet, so that quantities used in bioassays could match the amounts in chickpea leaf (see section 4.2.3). Because the amounts of both compounds in chickpea leaf surface were very small (see section 4.2.1), only the concentrations in chickpea whole leaf extracts were

used as bases for the bioassay. Furthermore, in the bioassay, three concentrations of the authentic standards (5 ppm, 50 ppm, and 500 ppm) were used, by adding both low (5 ppm) and high (500 ppm) concentrations so as to determine whether increasing the quantities will have more inactivating effect on the NPV.

Previous study also demonstrated that both biochanin A and its polar conjugate, sissotrin were induced on chickpea leaf surface after treatment with *Hear*NPV and that both isoflavonoids reduced the efficacy of the virus against *H. armigera* neonate (Stevenson *et al*., 2010). The study reported that sissotrin caused a significant inactivation of the *Hear*NPV, with a 5-fold difference compared to the untreated *Hear*NPV. Similarly, the study also showed that biochanin A was also active against *Hear*NPV even at a lower concentration (10 ppm). However, this effect was not concentration dependent since no significant difference was observed between the highest and lowest dose (500 ppm and 10 ppm) of the compound used against the virus (Stevenson *et al*., 2010). Probably this is a clue that biochanin A could be a more potent compound that is active against the virus compared to sissotrin. In the present study, biochanin A at 5 ppm did not produce a significant increased in LC_{50}. Furthermore, this study suggests that biochanin A could be more potent compared to formononetin, because in the bioassays carried out both at high and low concentration (500 ppm and 50 ppm), biochanin A produced 6- and about 3-fold increase in LC_{50} respectively compared to the untreated *Hear*NPV, while the formononetin produced only 2-fold increase in LC_{50} at the same concentrations. Similarly, 5-fold increase in LC_{50} was observed when the virus was pre-treated with sissotrin compared to the untreated *Hear*NPV (Stevenson *et al*., 2010). This observed increase in LC_{50} caused by sissotrin and biochanin A did not explain fully the effect observed when the virus was exposed to chickpea leaf surface for one hour and then removed and used for bioassay against the *H. armigera* neonate, from which it was hypothesised that other unknown compounds might be involved (Stevenson *et al*., 2010). The difference in the LC_{50s} in the two studies could be due to the fact that in the previous study, mortality of the *H. armigera* larvae was counted after the seventh day while in this study it was counted earlier, after the fifth day so as to reduce the effect of the cannibalistic behaviour observed among the dosed larvae. Furthermore, in the previous study methanol was used as the solvent to dilute the isoflavonoids while in the present study acetone was

used due to its volatile nature hence, it will allow maximum contact between the virus OB and the plant compounds (formononetin and biochanin A).

In a related study, resistance to the wilt pathogen, *Fusarium oxysporum f. sp. ciceri* in resistant chickpea cultivars was demonstrated to be as a result of induced production of the pterocarpans, medicarpin and maackiain (Stevenson *et al*., 1997). The authors showed that both compounds inhibited the spore germination and growth of fungal hyphae at their natural induced concentrations they occurred in the plant. Furthermore, cicerfuran and judaicin were found to be effective against the wilt-fungal pathogen, at their natural induced concentration in the presence of the pathogen (Stevenson and Veitch 1998a). They demonstrated that those two compounds were significantly induced at higher levels in roots of wild *Cicer* species grown in presence of the fungal pathogen hence they suggested that both compounds could be responsible for the natural resistance of wild *Cicer* species against the soil borne fungi. Although judaicin was found to be less active compared to maackiain or cicerfuran against the fungal spores (Stevenson and Veitch, 1998a). Similarly, Elmer (2002) reported that formononetin was partially responsible for a reduced fungal infection and improved plant growth in replanted asparagus field hence consider it to be useful in re-establishing asparagus in abandoned asparagus field. The study observed that formononetin helped to stimulate colonisation of vesicular arbuscular mycorrhizae (VAM) fungi in the root area of the plant thereby reduced the root infection caused by *Fusarium* pathogen compared to the untreated control plants. Another study also demonstrated that formononetin released from stressed alfalfa (*Medicago sativa* L.) roots significantly inhibited spore germination of two VAM fungi (Tsai and Phillips, 1991).

CHAPTER SIX

EFFECTS OF ISOFLAVONOIDS IN COMBINATION WITH CHICKPEA ORGANIC ACIDS ON EFFICACY OF NUCLEOPOLYHEDROVIRUS USED AGAINST *H. ARMIGERA* LARVAE

6.1 Introduction

A significant body of research has reported that host plants can influence the interactions between insect herbivores and their pathogens (Ramoska and Todd, 1985; Tanada and Kaya, 1993; Duffey *et al.,* 1995; Krischick *et al.,* 1988). It has been proposed that plant defence chemicals can reduce the infectivity of enthomopathogens against insects, so that the use of microbial pest control agents might be incompatible with plants that have these plant defence chemicals (Felton *et al.,* 1987). Therefore, before proposing any strategy that will combine host plant resistance with microbial control agents against herbivore insects, it is important to have a proper understanding of the tritrophic level of interactions between the three components involved (Hoover *et al.,* 1998; Cory and Hoover, 2006; Bauce *et al.,* 2006).

Host plant chemistry has been identified as one of the potential limitations in the use of viral insecticides for pest control (Duffey *et al.,* 1995; Hoover *et al.,* 1998a,b). Earlier researchers have demonstrated that interactions between host plant chemistry such as plant oxidative enzymes (PPO and POD) and phenolic substrates supports the idea that plant chemical factors influence viral infection in a manner that is multiplex, interactive and multifunctional each depending upon chemical context (Hoover *et al.,* 1998a,b). They noted that, a given phytochemical may enhance or attenuate viral infection depending upon chemical context.

*Hear*NPV has been reported to be inactivated when larvae consumed NPV OBs on chickpea leaves; however, the degree of inactivation recorded when *Hear*NPV OBs were treated on the surface of artificial diet after being exposed to chickpea leaves was much greater (Stevenson *et al.,* 2010). The authors demonstrated that the mechanism

of inactivation on chickpea was different from that reported previously in cotton (Hoover *et al.,* 1998b,c; Hoover *at al.,* 2000). Although the study showed that inactivation of the *Hear*NPV could occur after exposure to chickpea leaf surface isoflavonoids (biochanin A and sissotrin), the degree of inactivation by both compounds *in vitro* was much less than the observed effects on the leaf surface. From which they concluded that other unknown plant factors must be involved (Stevenson *et al.,* 2010). Similarly, the level of inactivation observed after exposure to biochanin A and another isoflavonoid (formononetin) on their own as shown earlier did not explain the full inactivation effect seen on actual chickpea leaf surfaces (see chapter 3 and 5).

Since both isoflavonoids have been identified to be present on chickpea leaves either as constitutive or induced defence compounds (see Chapter 4; Stevenson *et al.,* 2010), the possibility that those isoflavonoids may interact to produce a greater combined effect may be important. For example, if small concentrations of each of those compounds interact together in an additive or synergistic manner, this would enhance their activity on the NPV OBs. Evidence of this combined inhibitory activity has been demonstrated for isoflavonoids from other chickpea species that were shown to have antifeedant activity against noctuid larvae, particularly *H. armigera* (Simmonds and Stevenson, 2001). Therefore, understanding how plant chemicals reduce the activity microbial pathogen could be a first step to selecting appropriate adjuvants that could be included in NPV spray formulations that will assist in suppressing such inhibitory activities and thus obtain the full effect of NPV application on crops such as chickpea.

To summarise, earlier work had shown that.

1. The isoflavonoids known to be present either as preformed or induced compounds on the chickpea leaf surface could cause some degree of inactivation of NPV OBs, but not to the same extent seen after exposure to the leaf surfaces (chapter 3 and 5; Stevenson *et al.,* 2010).
2. The work reported by Stevenson *et al.* (2010) had shown no evidence that inactivation involved the low pH on the chickpea leaf surface.

The present study was carried out to resolve the mechanism of inactivation of *Hear*NPV observed on chickpea leaf surface, and to understand the role of chickpea

leaf isoflavonoids and identify what other factors might be involved in the inactivation of NPV OB.

Therefore, this work tested the following hypotheses;

1. That NPV inactivation recorded on chickpea leaf is due to the action of leaf isoflavonoids (biochanin A and or formononetin) plus some other as yet unidentified factor(s) or chemical(s)

2. Given hypothesis (1) it will test further whether chickpea organic acids have a role in the inactivation process

3. That isoflavonoids inactivate NPV in a concentration-dependent manner.

To test the above hypotheses, three different bioassays were designed to investigate the effect of varying the concentrations of chickpea leaf isoflavonoids (either singly or their combination) along with natural concentration of chickpea most abundant acids (malic and oxalic) at low and high virus concentrations (LC_{75} and LC_{25}) used against *H. armigera* larvae. To test the effect of isoflavonoids in combination with chickpea organic acids on high NPV lethal concentration (LC_{75}), two different bioassays were used.

6.2 Materials and Methods

6.2.1 Bioassay to determine the effects of isoflavonoids in combination with most abundant chickpea organic acids on *Hear*NPV efficacy (LC_{25}).

A solution of the most abundant chickpea acids, malic and oxalic (Sigma Aldrich, USA) present on chickpea leaf surface were mixed together in sterile distilled water in 50 ml reusable glass media bottle (Fisher Scientific, USA), at the concentration present on the leaf surface (Rembold and Weigner, 1990). The pH was measured using a microprocessor pH meter (Hanna Instrument, Bedfordshire, UK) and was found to be pH 1.40. A solution of the two acids were then used in combination with isoflavonoids, biochanin A and formononetin (Sigma Aldrich, USA) that had been previously found (See Chapter 5) to significantly reduce the efficacy of *Hear*NPV against *H. armigera*

at ≥ 50 ppm, either individually at 50 ppm or their combination at 25 ppm each, to determined their combined effects on *Hear*NPV efficacy bioassayed against *H. armigera* neonate. Mass dosing bioassay was used; using diet surface contamination method of the test materials on artificial diet for polypot (Mckinley *et al.,* 1984). Each isoflavonoid (biochanin A or formononetin) was solubilised according to the concentration mentioned above in acetone (analytical grade) in separate glass vial (7 ml) and labelled accordingly. The *Hear*NPV concentration (LC_{25}) was determined by analysing data from seven different LC_{50} bioassays using *H. armigera* neonates, and results was analysed using Poloplus software package. (See section 5.2.1).

The treatments used were as follows:

1. Control, organic acids (malic + oxalic acids combination) = 119.60 mg ml^{-1} (oxalic acid) and 3.80 mg ml^{-1} (malic acid) with no *Hear*NPV
2. *Hear*NPV (LC_{25}) = 1.1×10^3 OB ml^{-1}
3. Organic acids + *Hear*NPV (LC_{25})
4. Formononetin 25 ppm and biochanin A 25 ppm + organic acids + *Hear*NPV (LC_{25})
5. Formononetin 50 ppm + organic acids + *Hear*NPV (LC_{25})
6. Biochanin A 50 ppm + organic acids + *Hear*NPV (LC_{25})

Five polypots (29 ml) containing artificial diet were set out for each treatment and labelled accordingly as shown above. Starting with the organic acids combination, 100 µl aliquot of the organic acids mixture was dispensed using a micropipette on to the centre of each of the five polypots set for the treatment. Dosed pots were tilted and rotated gently until the applied organic acid solution spread evenly over the entire surface of each of the five treatment pots.

Moving to the next treatment, *Hear*NPV suspension at LC_{25} (1.1×10^3 OB ml^{-1}) was sonicated for 1 min and mixed using a vortex mixer for about 30 s, using a separate micropipette tip, 100 µl aliquot was dispensed on to the centre of each of the five treatment pots using a micropipette, until all the five pots were dosed making sure the NPV suspension has covered the entire diet surface of each pot as mentioned in the first treatment.

For the third treatment, a separate micropipette tip was used to dispensed 100 µl aliquot of the organic acids mixture on to the centre of each of the five polypots diet set for the treatment, until the entire surface of each pot was covered with the organic acids combination as described in the first treatment.

Immediately after all the five treatment pots were dosed with the acid mixture, 100 µl aliquot of the *Hear*NPV suspension was dispensed on to the surface of the diet using a separate micropipette tip, after it was mixed properly for 30 s using a vortex mixer. The aim was to obtain a maximum interaction between the organic acids mixture and the NPV OBs suspension, before the acids dries out. To optimise the interaction between the two compounds, each of the five dosed pot was tilted and rotated gently until both compounds mixture spread evenly over the entire surface of each pot.

For the fourth treatment, isoflavonoids (formononetin 25 ppm plus biochanin A 25 ppm) mixture solubilised in acetone were first dispensed on to the surface of each of the five polypots set for the treatment using a separate micropipette tip at 100 µl aliquot, making sure the entire surface of the diet was covered as described in the first treatment above.

After the acetone solvent used to solubilise the compounds has evaporated from the diet surface, using a separate micropipette tip 100 µl aliquot of the organic mixture was then dispensed on to the surface of each of the diet pots.

After all the five pots were dosed with both the isoflavonoids combination (at 25 ppm each) and organic acids mixture, 100 µl of the NPV OBs suspension was dispensed immediately on the surface of the diet to mix with the organic acids combination after being mixed properly as described in treatment three above. Each of the five dosed pots was then tilted and rotated to make sure the surface of the each pot was properly covered with the combination of the organic acids and virus suspension as mentioned in treatment three above.

For the fifth treatment, 100 µl of formononetin (50 ppm) diluted in acetone was dispensed using a separate micropipette tip on to the centre of each of the five polypots set out for the treatment, ensuring that the solution of the compound covered the surface of each diet pot, by gently tilting and rotating the pot as mentioned in the first treatment above.

After the acetone solvent has evaporated from the diet surface, 100 µl aliquot of the organic acids mixture was dispensed on the surface of each diet pot dosed with the formononetin using a separate micropipette tip. This was followed immediately by dispensing 100 µl of the NPV suspension after it was properly mixed, and the process was the same as in fourth treatment above.

Finally, in the sixth treatment, all the procedures were the same as in the fifth treatment, except that instead of formononetin, here biochanin A (50 ppm) was used. All the dosed polypots for each treatment were kept on a tray to dry at room temperature. After the compounds in each of the treatments have dried (about 1 h), starting with the first treatment to the last (1-6), 50 newly hatched *H. armigera* neonates (18 h old) were transferred into the dosed pots at the rate of 10 larvae per pot using a paint brush (No. 2), giving a total of 50 larvae per treatment. All other procedures were the same as mentioned in section 5.2.1.

6.2.2 Second bioassay to determine the effects of isoflavonoids in combination with most abundant chickpea organic acids on *Hear*NPV efficacy (LC_{75})

In this experiment, the effects of isoflavonoids (formononetin and biochanin A) in combination with organic acids mixture on *Hear*NPV efficacy was determined using diet surface contamination bioassay. In this assay, the concentration of the isoflavonoids used either separately or in combination was increased from 50 ppm as in 6.2.1 above to 100 ppm and also at a higher concentration (500 ppm) to find out if there was concentration effect, and also from 25 ppm each when the isoflavonoids were combined together to 100 ppm. In addition, the concentration of the NPV was increased from LC_{25} (1.1×10^3 OB ml^{-1}) to LC_{75} (5.8×10^3 OB ml^{-1}) to determine if there would be a greater effect at higher concentration of the compounds either singly or when combined together with the organic acid on the efficacy of the NPV OBs. This experiment will also test whether the NPV at higher concentration can overcome the effects of the isoflavonoids and the organic acids combination. To optimise the interactions between the three compounds, all the test materials were mixed together in Eppendorf tube (1.5 ml), before being dispensed on the diet surface. To obtain the LC_{75}, seven separate bioassays were carried out at different dates, within short period

intervals (10 days). From these bioassays the LC_{50} was determined (see section 2.6.1), and from this result the average LC_{75} was estimated from the graph (Poloplus software).

The treatments used were as follows:

1. Sterile distilled water (SDW) control
2. *Hear*NPV $LC_{75} = 5.8 \times 10^3$ OB ml^{-1}
3. Formononetin 100 ppm and biochanin A 100 ppm + organic acids + *Hear*NPV $LC_{75} \times 2$ (1.1×10^4 OB ml^{-1})
4. Formononetin 100 ppm + organic acids + *Hear*NPV $LC_{75} \times 2$ (1.1×10^4 OB ml^{-1})
5. Biochanin A 100 ppm + organic acids + *Hear*NPV $LC_{75} \times 2$ (1.1×10^4 OB ml^{-1})
6. Formononetin 500 ppm + organic acids + *Hear*NPV $LC_{75} \times 2$ (1.1×10^4 OB ml^{-1})
7. Biochanin A 500 ppm + organic acids + *Hear*NPV $LC_{75} \times 2$ (1.1×10^4 OB ml^{-1})

Five polypots (29 ml) containing artificial diet were set out for each of the treatments and labelled as shown above. Starting with the SDW control, 100 µl of the SDW was dispensed using a micropipette on to the centre of each of the five treatment pots. Dosed pots were tilted and rotated gently until the water spread evenly over the surface of each of the pots.

Moving to the *Hear*NPV treatment, the virus OBs (5.8×10^3 OB ml^{-1}) was sonicated in a water bath for 1 min then mixed with a vortex mixer for about 30 s, using a separate micropipette tip, 100 µl aliquot was dispensed on to the centre of each of the five pots and the remaining procedure were the same as mentioned in 6.2.1 above.

For the third treatment, 500 µl of organic acids mixture was dispensed in Eppendorf tube (1.5 ml) using a separate micropipette tip, and in the same tube another 500 µl of *Hear*NPV suspension at a concentration of 1.16×10^4 OB ml^{-1} (i.e. $LC_{75} \times 2$) was added to the organic acids mixture using a separate micropipette tip after the NPV OBs was mixed properly for 30 seconds using a vortex mixer. The concentration of the

NPV suspension was doubled to allow for dilution in the final mixture. Isoflavonoids (formononetin and biochanin A) were prepared at higher concentrations separately (1000 ppm each), from this high concentration, 100 µl of each compound (formononetin and biochanin A) were mixed together, to obtain the isoflavonoid combination (formononetin 100 ppm plus biochanin A 100 ppm). From this combination of isoflavonoids, 100 µl was taken using a separate micropipette tip and added into the Eppendorf tube containing the organic acids and the NPV suspension, giving a total volume of 1.1ml of the three compounds together. However, since the isoflavonoids were diluted in acetone and knowing that the acetone is volatile, it is expected that the acetone would evaporate quickly leaving only the isoflavonoids (solute) to interact with the other compounds. The idea was to add the isoflavonoids either individually or their combination into the organic acids mixture together with the NPV suspension at 10 part aliquot of the total mixture.

All the three compounds were mixed thoroughly using vortex mixer for about 30 s to optimise their interaction. From this mixture of the three compounds, 100 µl was taken using separate micropipette tip and dispensed on to the centre of each of the five diet polypots. All the other procedure was the same as in 6.2.1 above.

For the fourth treatment, organic acids mixture and *Hear*NPV suspension at a concentration of 1.16×10^4 OB ml^{-1} ($LC_{75} \times 2$), were mixed together in 1.5 ml Eppendorf tube at a 500 µl aliquot of each compound using separate micropipette tip for each as described in treatment three above. However in this treatment, 100 µl aliquot of formononetin was taken from the higher concentration of the compound (1000 ppm formononetin diluted in acetone). All the remaining procedure was the same as described in treatment three above as well as in section 6.2.1.

Treatment five was prepared exactly as mentioned in treatment four above, except that instead of formononetin, biochanin A was used at 100 µl, which was also taken from the higher concentration of the compound (1000 ppm biochanin A diluted in acetone).

Treatments six and seven were prepared exactly in the same procedure as in treatments four and five, except that in treatments six and seven higher isoflavonoid concentrations were used (5000 ppm each for formononetin and biochanin A). To get this concentration, each of the isoflavonoids (formononetin or biochanin A) was

diluted separately in acetone at 1000 ppm, the acetone was allowed to evaporate under room temperature, and then each of the compounds was then resuspended in 200 µl acetone. From this concentration, 100 µl aliquot was suspended into each Eppendorf tube for each treatment (6 or 7) using a separate micropipette tip after the organic acids and *Hear*NPV suspension were added as mentioned above in treatments four and five. All other procedures were the same as in sections 6.2.1 and 2.6.1.

6.2.3 Third bioassay to determine the effects of isoflavonoids in combination with most abundant chickpea organic acids on *Hear*NPV efficacy (LC_{75}).

Because the isoflavonoids were not easy to solubilise particularly at higher concentration (1000 ppm) when suspended in aqueous mixture of organic acids and *Hear*NPV suspension, diluting the isoflavonoid first in acetone before adding on the diet surface could minimize the problem. This experiment was carried out to minimize the effect of poor solubility of the isoflavonoids when mixed together with organic acids and *Hear*NPV suspension.

The effect of isoflavonoids (formononetin and biochanin A) in combination with organic acids mixture on *Hear*NPV efficacy was determined as in 6.2.2 above. However in this bioassay, only two of the test materials (organic acids and *Hear*NPV) were mixed together in 1.5 ml Eppendorf tube, while the isoflavonoid either individually or their combination were added separately to the diet surface. All the treatments were the same as in 6.2.2.

For the water control and the *Hear*NPV treatments, all the procedures were same as detailed in 6.2.2. For treatment three, the isoflavonoids combination (formononetin and biochanin A) were diluted together at 100 ppm each, from this concentration 100 µl aliquot was dispensed using a micropipette on the surface centre of each of the five polypots set out for the treatment, each pot was gently tilted and rotated until the entire diet surface was covered with the compound mixture.

All the dosed pots were then arranged on a tray to allow the acetone to evaporate under room temperature for about 45 minutes. After the acetone evaporated, organic acids mixture and *Hear*NPV suspension were mixed together as mentioned in section 6.2.2.

After the acetone used to dilute the isoflavonoids combination had evaporated, the organic acids and *Hear*NPV combination were mixed using vortex mixer for about 30 s and 100 µl aliquot was dispensed using a separate micropipette tip on the centre of each of the five pots dosed with isoflavonoids.

All the remaining procedure was same as in 6.2.2. For treatments four and five, same procedure as explained in treatment three above was used except that each of the isoflavonoid (formonononetin and biochanin A) was solubilised separately at 100 ppm, from which 100 µl aliquot was dispensed into the treatment polypots. Similarly for treatments six and seven, same procedure was used as mentioned in three above, except that the isoflavonoids were also solubilised separately in acetone at 500 ppm and from this concentration, 100 µl aliquot was dispensed for each of the isoflavonoids to its corresponding treatments as mentioned treatments four and five above. All the other procedures are same as described in sections 6.2.1 and 2.6.1.

6.2.4 Statistical analysis

Treatment effects were measured by comparison of mean percentage mortality between at least five different experiments for each treatment group. Data were analysed by ANOVA, having previously tested that key ANOVA assumptions of equal variance (Shapiro-Wilko test) and normality were passed. Differences arising from treatment means were separated using Holm-Sidak multiple comparison tests. All analysis were carried out using Sigmastat (11.0). For the effects of isoflavonoids plus organic acids on low lethal concentration of *Hear*NPV (LC_{25}), data were corrected for untreated control mortality using Abbott's formula before analysis.

6.3 Results

6.3.1 Determine the effects of isoflavonoids in combination with most abundant chickpea acids on *Hear*NPV efficacy (LC_{25}).

Results from the bioassay (Fig. 6.1) showed that the mortality of *Hear*NPV exposed to chickpea organic acids alone or with biochanin A and/or formononetin were significantly lower than unexposed *Hear*NPV (ANOVA; F= 5.326, df= 5, 36, P< 0.001). The untreated *Hear*NPV gave the highest larval mean mortality (31.9%), which was close to the intend LC_{25}, compared to all the other treatment groups.

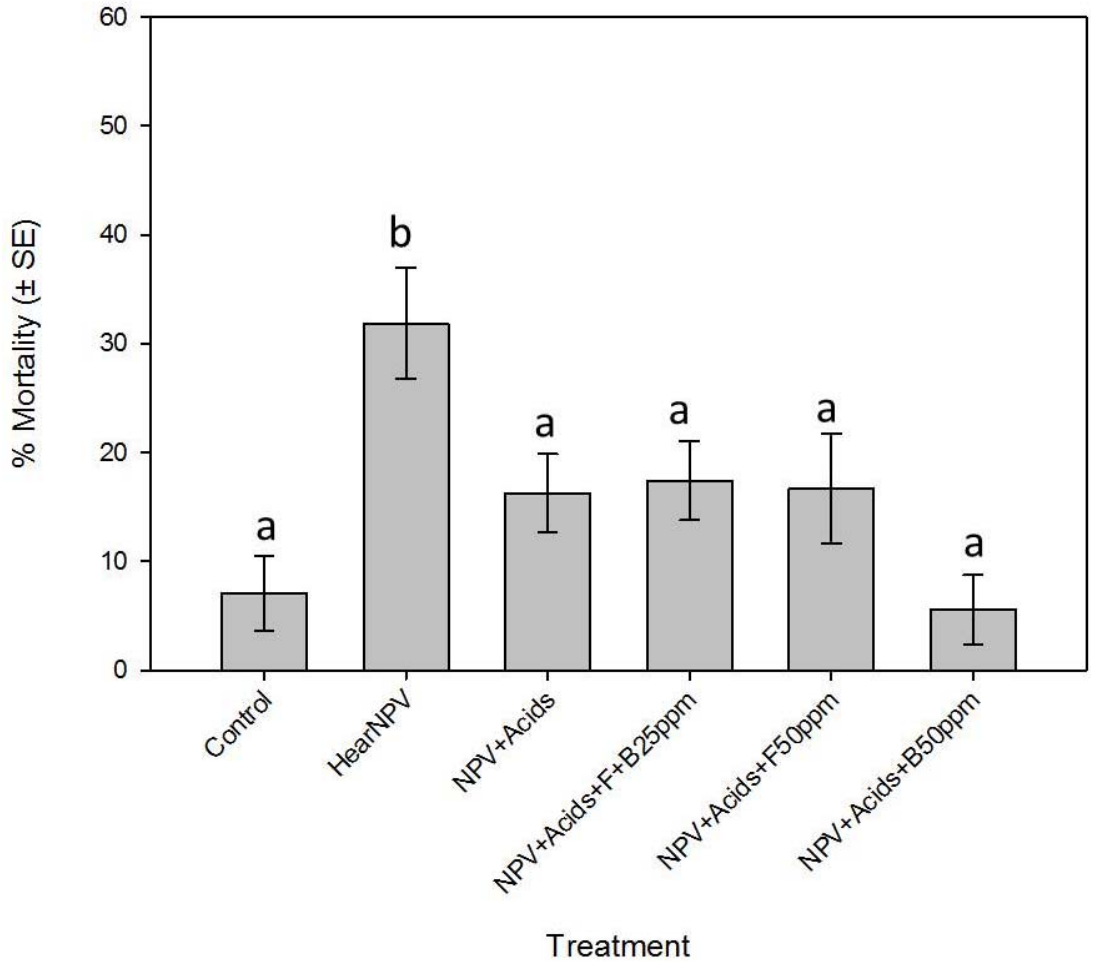


Figure 6.1 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets containing organic acids (control), untreated *Hear*NPV OBs or with *Hear*NPV OBs exposed to biochanin A (B), formononetin (F) or their mixture (F+B) in combination with most abundant chickpea acids (Acids). Bars with different letters differed significantly (P< 0.001).

The novel finding from this experiment is that chickpea organic acids can produce NPV inactivation when OBs were exposed to natural concentrations of the acids. These results show that inactivation did not increase if isoflavonoids were also present at concentration of 50 ppm, except for biochanin A.

Average mortality of 17.4% was obtained when the isoflavonoids were mixed together (biochanin A at 25 ppm plus formononetin at 25 ppm each) in combination with organic acids and added together with the NPV and bioassayed against *H. armigera* larvae. This was similar to mean mortality of 16.7% recorded when formononetin at 50 ppm and organic acids were added together with the NPV, and also similar to the mean larval mortality obtained (16.2 %) when only the organic acids was exposed to the NPV. Holm-Sidak multiple comparisons differences shows that there was significant differences ($P < 0.05$) between mean larval percentage mortalities of all the three treatments mentioned above and the unexposed *Hear*NPV (LC_{25}).

The lowest mean percentage larval mortality was obtained when biochanin A at 50 ppm in combination with organic acids were exposed to the NPV OBs (5.6% mortality), which was very similar to the mortality obtained when *Hear*NPV was exposed only to organic acids (7.0%). The differences between mortalities in these two last treatments and the other acid plus isoflavonoid treatments were not significant ($P < 0.001$) in this experiment. Thus there is no statistically significant evidence of an additive effect of combining organic acids and isoflavonoids. However this experiment used a sample size ($N = 5$) which combined with the high variability meant that the limit of statistical discrimination was low. It could be therefore that if this experiment was repeated with larger samples size ($N > 10$) a significant statistical difference might become apparent. Also the use of the LC_{25} as a treatment rate might limit the degree of inactivation that could be detected. Thus increasing the NPV rate used to say LC_{75} and repeating with more replicates might increase the chances of detecting significant treatment effects. The implication of the finding that mortality in the NPV + biochanin + organic acid treatment equalled that in an acid only control suggests that exposing OB *in vitro* to a mixture of organic acid and isoflavonoids can produce complete inactivation previously not seen *in vitro* with isoflavonoids (Stevenson *et al.,* 2010) but only seen with *in vivo* NPV exposure.

6.3.2 Second bioassays to determine the effects of isoflavonoids in combination with most abundant chickpea acids on *Hear*NPV efficacy at LC_{75}.

The results from this second bioassay using higher levels of isofavonoids and a higher NPV concentration confirmed that both biochanin A and formononetin (either used singly or their combination) when added together with organic acids significantly reduced the infectivity of *Hear*NPV.

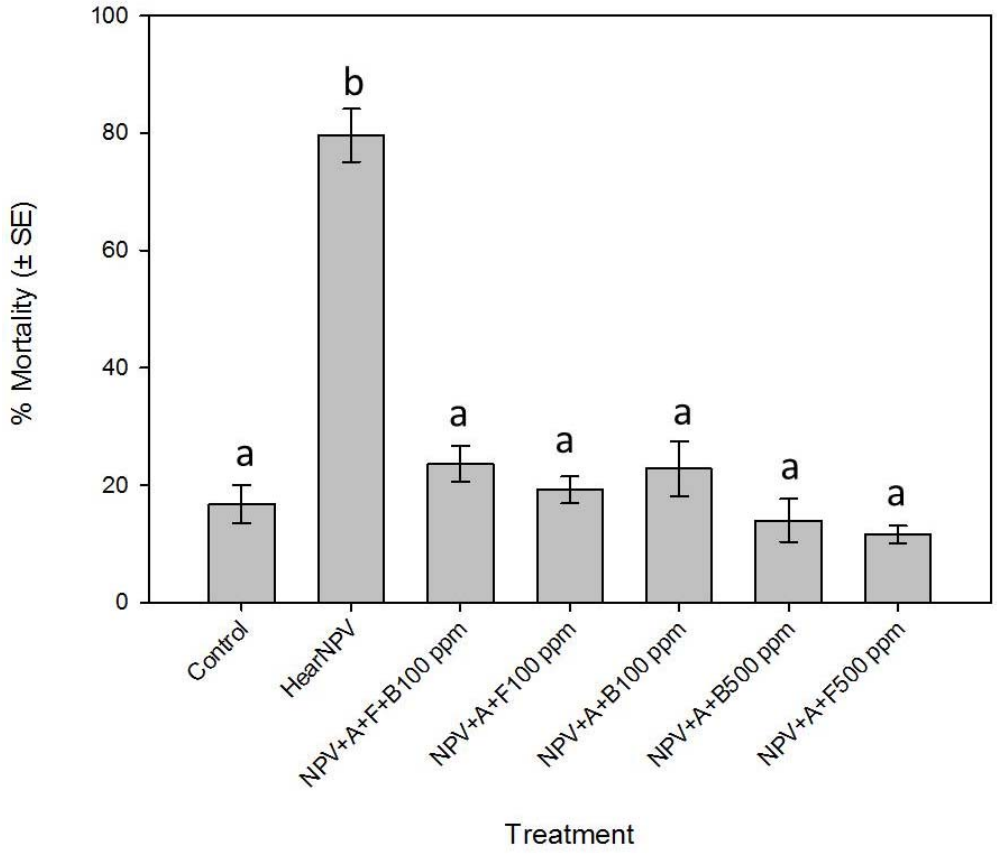


Figure 6.2 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets containing untreated *Hear*NPV OBs or with *Hear*NPV OBs (NPV) exposed to biochanin A (B), formononetin (F) or their mixture (F+B) in combination with most abundant chickpea acids (A). Bars with different letters differed significantly ($P< 0.05$).

The mean percentage mortalities (Fig. 6.2) for the different treatment groups were lower and significantly different to that of the untreated control *Hear*NPV (ANOVA; $F= 48.570$, $df= 6, 27$, $P< 0.001$). The untreated control mortality at 79.6% was close to the planned LC_{75} concentration. Holm-Sidak multiple comparison differences show

that there were highly significant differences (P< 0.001) between the mean percentage mortality obtained with untreated *Hear*NPV and *Hear*NPV exposed to biochanin A and formononetin (at 100 ppm and 500 ppm) or their combination (at 100 ppm each) mixed together with organic acids.

Although there was no statistically significant concentration effect (P> 0.05) when the concentration of the isoflavonoids were increased (100 ppm to 500 ppm) however, both biochanin A and formononetin showed a lower larval mortality when their concentrations was increased (from 100 ppm to 500 ppm). For example, biochanin A at high concentration (500 ppm) in combination with organic acids showed a lower mortality by 2-fold (11.6%) in comparison to the lower concentration of the compound (22.8%). Similarly, formononetin at higher concentration (500 ppm) in combination with organic acids gave a slightly lower larval mortality (14%) compared to mortality (19.2%) obtained with lower concentration (100 ppm). Although this data did not give a statistically significant proof of a concentration effect with biochanin A between 100 ppm and 500 ppm it could be consistent with such a hypothesis.

Thus the data again failed to show any significant concentration effect over the range 100-500 ppm for biochanin A or formonometin. One possibility considered was that the technique of mixing the isoflavonoids and acids plus NPV in the Eppendorf was not efficiently done, due to the difficulty in solubilising the isoflavonoids in the organic acids and NPV suspension. Thus a third bioassay series of experiments was carried out where the acids and NPV suspension were mixed in an Eppendorf while the isoflavonoids were applied directly to the diet surface.

6.3.3 Third bioassay to determine the effects of isoflavonoids in combination with most abundant chickpea acids on *Hear*NPV efficacy at LC_{75}.

Results from this third bioassay also confirmed that the major organic acids of chickpea in combination with isoflavonoids (biochanin A and formononetin) significantly reduced the efficacy of *Hear*NPV used against *H. armigera* larvae. Results from ANOVA shows that the mean percentage mortality (Fig. 6.3) for the treatment groups were different (F= 36.122, df= 6, 28, P< 0.001). The highest percentage mean larvae mortality of 87% was obtained when *Hear*NPV OBs not

exposed to acids or isoflavonoids was used against *H. armigera* larvae. Holm-Sidak multiple comparison tests also shows that there were highly significant differences (P< 0.001) between the percentage mean mortalities obtained with the unexposed *Hear*NPV OBs and that obtained when *Hear*NPV was exposed to the isoflavonoids at both low or high concentrations (100 ppm and 500 ppm) or their mixture (formononetin at 100 ppm plus biochanin A at 100 ppm) in combination with the organic acids.

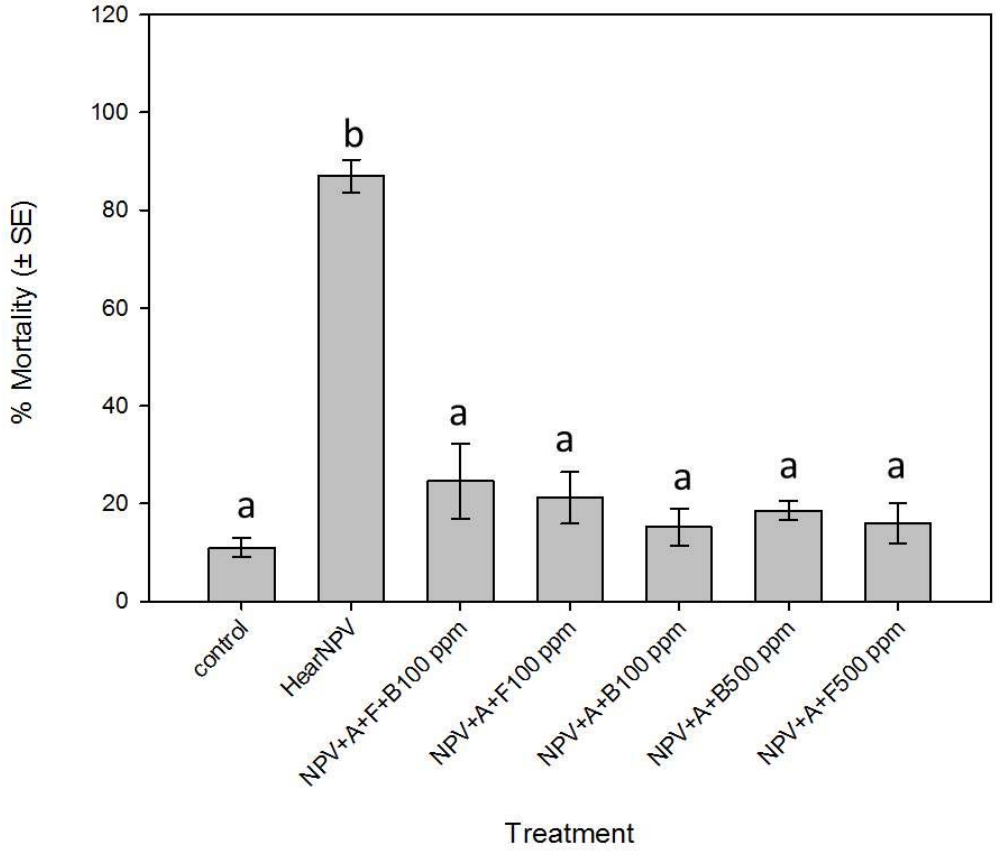


Figure 6.3 Mean of percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets containing untreated *Hear*NPV OBs or with *Hear*NPV OBs exposed to biochanin A (B), formononetin (F) or their mixture (F+B) in combination with most abundant chickpea acids (A). Bars with different letters differed significantly (P< 0.05).

When both isoflavonoids were added at high concentration (500 ppm) to the NPV together with organic acids as well as when biochanin A at low concentration (100 ppm) was used in combination with the organic acids, similar mean larval mortalities

of 16%, 18.6% and 15.2% were obtained respectively. However, the combination of the isoflavonoids (formononetin at 100 ppm and biochanin A at 100 ppm) in combination with organic acids gave slightly higher larval mortality (24.6%), similar to that obtained with formononetin alone exposed to the virus at a lower concentration (100 ppm) in combination with the acids (21.2%). The lowest larval mortality of 11% was obtained with water (SDW) control.

Thus overall, while there is clear evidence that combinations of chickpea organic acids with formonmetin and biochanin can completely inactivate *Hear*NPV at LC_{75} concentrations, but there is no evidence that increasing the concentrations beyond 100 ppm increases the effect.

6.4 Discussion

This study showed that the efficacy of baculovirus insecticide (*Hear*NPV) was significantly reduced by most abundant chickpea acids in combination with leaf isoflavonoids (biochanin A and formononetin). This is the first study to report on the inactivation of *Hear*NPV by the combination of those two compounds.

The rate of *Hear*NPV inactivation observed in this study when it was exposed to isoflavonoids in combination with most abundant chickpea acids showed that both compounds could account for the inactivation reported previously on chickpea leaf surfaces (chapter 3; Stevenson *et al.,* 2010). This is because the inactivation observed when the isoflavonoids in combination with chickpea acids were exposed to *Hear*NPV at high lethal concentrations resulted in larval mortality not significantly different to that of control treatment.

The effect was also observed when NPV was treated with those chickpea natural products (isoflavonoids plus organic acids) at either low or high NPV concentrations (LC_{25} and LC_{75}). Both isoflavonoids alone have been reported previously to significantly reduce *Hear*NPV efficacy used against *H. armigera* larvae (see chapter 5; Stevenson *et al.,* 2010).

The data from Fig. 6.1 shows that organic acid plus biochanin A at 50 ppm when exposed to *Hear*NPV at low NPV concentration (LC_{25}) produces a lower mean mortality (5.6%) than the formonmetin at 50 ppm (16.7%), although given the small sample size this was not statistically significant. This indicates the possibility that at these concentrations which are lower than those found on the leaves, that biochanin A was more potent inactivator. It might also be an indication that inactivation may be more important when NPV is used at low concentrations. This was in line with an earlier study which shows that two major orthodihydroxyphenolics of tomato, rutin and chlorogenic acids significantly reduced the activity of *Hz*SNPV used against *H. zea*, and the greatest effect on the viral OBs was observed at low virus doses (Felton *et al.,* 1987). Furthermore, this study reported that while both phytochemicals shows inhibitory effect at low virus doses (< LD_{50}), the effect of the two compounds were not significantly different to the control at high virus dose (LD_{90}). Similarly, Hoover *et al.,* (1998a) also observed differential influence of host plants on baculovirus infection with greatest impact occurring at lower NPV doses. The study observed that except at high NPV inoculum, larval mortality was greater on lettuce compared to cotton. They opined that the lack of significant host plant effect on larval mortality observed when NPV was applied at higher dose (LD_{99}), could suggest that when epizootics builds up in natural environment producing high concentrations of OB on the plant surface, it is unlikely that host plant can significantly inactivate the virus OBs to reduce infection rates. Phenolic substrate and two foliar oxidative enzymes (POD and PPO) were identified as factors that could significantly modify baculovirus infection in noctuid larvae fed either cotton or lettuce (Hoover *et al.,* 1998a). Additionally, the study proved that of the two phenolases tested, POD was more influential in predicting infection. However, the influence of POD on viral disease was found to be greater at lower NPV concentration (Hoover *et al.,* 1998a).

The influence of host plant factors on baculovirus efficacy at low NPV concentration reported in this study as well as in other related work as mentioned above is not limited to NPV alone. Gallardo *et al*. (1990) also shows that at LC_{50}, alpha-tomatine (a plant natural product present in large quantities in solanaceae plant family, particularly tomato and potato) significantly reduced *H. zea* larval mortality against *N. rileyi* fungus compared to the larvae fed on control diet without the chemical. The study further demonstrated that while at low levels of fungal inoculum the allelochemical

has the ability to protect the insect larvae against the fungus, at high concentration of the fungus (LC_{90}) alpha-tomatine was able to decrease the development of *N. rileyi* by reducing the production of fungal conidia. Similarly, high concentration of another plant allelochemical, nicotine was reported to reduce larval mortality of *Manduca sexta* fed diet modified with enthomopathogenic bacteria, *B.t* var. *kurstaki* (Krischik *et al.,* 1998).

In addition the present study shows that at low *Hear*NPV concentration (LC_{25}), NPV OBs were sensitive to the most abundant chickpea acids (malic and oxalic acids). When those acids were treated together with the *Hear*NPV OBs on diet surface and fed to *H. armigera* neonate, there was significant reduction in larval mortality compared to the unexposed *Hear*NPV OBs. The inhibitory effect observed on the virus when the organic acids were treated alone with the viral OBs were similar (16.2%) to that observed with formononetin at 50 ppm (16.7%) or when the isoflavonoids were mixed together (at 25 ppm each) in combination with the organic acids (17.4%), except when biochanin A (50 ppm) was combined with organic acids along with the virus (5.5%). This could suggest that the lower larval mortality seen when the viral OBs were treated with the organic acids in combination with isoflavonoids may partly be due to the influence of the chickpea major organic acids rather than the effect of the isoflavonoids on the virus. However, because the lowest larval mortality was recorded with the combination of biochanin A (50 ppm) in combination with organic acids compared to the percentage mortality observed when the organic acids was used alone (7.0%) suggest that the organic acids combination might be enhancing the effect of the biochanin A. Similarly, it has been shown previously that both biochanin A and formononetin when used individually significantly reduced the efficacy of *Hear*NPV OBs used against *H. armigera* larvae (see chapter 5 and Stevenson *et al.,* 2010). However, the degree of inactivation seen in the present study (when organic acids plus isoflavonoids were incubated with NPV) was much greater than that reported previously (chapter 5; Stevenson *et al.,* 2010).

Both biochanin A and formononetin were found to significantly reduce the efficacy of *Hear*NPV OBs used against *H. armigera* at a concentration of $\geq$ 50 ppm (see chapter 5), and this was the basis for the concentration that was chosen for each isoflavonoid in the present study in the first bioassay. This was less than the minimum natural

concentration of both compounds that was identified to be induced or present in chickpea whole leaves after spraying the virus suspension, for formononetin at induced state (about 100 ppm) and for biochanin A at preformed state (about 200 ppm). Both preinfectional and postinfectional inhibitors have been reported to act as plant's defence mechanism against pathogens (Mansfield, 1982; Harborne, 1993). Furthermore the results also shows that when biochanin A and formononetin were exposed to the NPV singly at 50 ppm, 3-fold and 2-fold increase in LC_{50} were recorded for biochanin A and formononetin respectively compared to the untreated NPV control. Previously Stevenson *et al.* (2010) reported that biochanin A alone was able to reduce the infectivity of the NPV OBs even at a very low concentration (10 ppm), all these are indications that biochanin A could be the more potent inactivator to the NPV OBs compared to formononetin. Although the reason why biochanin A was more inhibitory than formononetin is not clear, other studies have related the antimicrobial and deterrent activity of natural plant products against some insects, human or plant pathogens to the presence of hydroxyl group in their structure. For example, Wang *et al.* (1998) identified 10 isoflavones with deterrent activity from the methanol extract of trifoliates of subclover, *Trifolium subterraneum* resistant variety (SEO14). The compounds include biochanin A, genistein, formononetin and their 7-*O*-glucosides and 7-*O*-glucoside-6-malonates derivatives. The study found out that among those compounds, genistein and biochanin A were more active against the red legged earth mite, *Halotydeus destructor*. The activity of the two compounds was linked to the presence of a hydroxyl group at the C-5 position of their chemical structure (Wang *et al.,* 1998). Similarly, Aslam *et al.* (2009) determined the antimicrobial activity of Cicerfuran and other five related 2-arylbenzofuran analogues as well as nine stilbenes against bacterial and fungal pathogens. The result from the study shows that three of the nine stilbenes were active against the fungal and bacterial pathogens and the activity was associated to the presence of a free hydroxyl group in their structure. Cicerfuran was the only compound among the six 2-arylbenzofuran tested with antimicrobial activity and was also the only one amongst the six with free hydroxyl group (Aslam *et al.,* 2009). The importance of 2-hydroxyl group for the antiprotozoan activity of stilbenes against human parasite (*Leishmania promastigotes*) has been reported (Getti *et al.,* 2005). Previously, Carter *et al.* (1978) also attributed the antifungal property of another analogues of the related 2-arylbenzofuran, Vignafuran to the presence of hydroxyl group.

The present study shows that there was no significant concentration-dependent effect of the isoflavonoids in combination with organic acids on NPV efficacy. In one of the experiments where all the test materials were mixed together in Eppendorf tube; both isoflavonoids at 500 ppm in combination with organic acids recorded a lower larval mortality at high NPV concentrations (LC_{75}). When biochanin A was included at a high concentration (500 ppm) larval mortality was lower by about 2-fold (11.0%) compared to the lower concentration (100 ppm), which recorded a higher larval mortality (22.8%). However, the difference in mortality was relatively small when formononetin at 500 ppm was administered (14.0%) in combination with organic acids along with the NPV compared to the mortality of 19.2% recorded at a lower concentration of the compound (100 ppm). When the isoflavonoids were added directly on the diet formononetin in combination with organic acids at high concentration (500 ppm), this was associated with a lower larval mortality (16.0%) compared to when the compound was combined with organic acids at lower concentration of 100 ppm (21.6%).

A possible reason for a lack of concentration effect is that since both isoflavonoids are not soluble in water, to add them to the aqueous mixture of organic acids and *Hear*NPV the two compounds were first solubilised in acetone. However at high concentration (500 ppm), the two compounds did not dissolve completely in the aqueous combination. Both isoflavonoids formed colloidal suspension in the Eppendorf tube. The poor solubility was more pronounced with formononetin, and this might be the reason for the small decrease in mortality seen when formononetin concentration was increased to 500 ppm compared to the biochanin A. Stevenson *et al.* (2010) also noted that there was no dose effect when biochanin A was bioassayed alone with *Hear*NPV against *H. armigera* larvae between four different concentrations of the compounds used (10, 100, 250 and 500 ppm).

CHAPTER SEVEN

ROLE OF CHICKPEA ORGANIC ACIDS ON NUCLEOPOLYHEDROVIRUS EFFICACY USED AGAINST *H. ARMIGERA* LARVAE

7.1 Introduction

Chickpea is the only plant known to have trichomes whose main function is to secrete acidic exudate (Lazzaro and Thompson, 1989). The acid in chickpea exudate is reported to be made of about 66% malic and 33% oxalic acids (Rembold *et al.,* 1990a; Rembold and Weigner, 1990). The acidic compounds have also been implicated in the reduced podborer (*H. armigera*) damage on chickpea (Rembold, 1981; Rembold and Winter, 1982; Rembold *et al.,* 1990 a,b). In addition, the acidic exudates of chickpea surfaces have also been reported to produce an unsuitable habitat for searching by the egg parasitoid, *Trichogramma chilonis* (Romeis *et al.,* 1999). They observed that the parasitoid spent longer time on washed chickpea leaves compared to unwashed leaves. Furthermore they noted that when placed on unwashed leaves, 6.8% of the parasitoids were trapped and killed by the chickpea exudates. Additionally, filter paper bioassay showed that searching behaviour of parasitoids were deterred by high concentrations of the major components of the acid exudates (malic and oxalic acids) (Romeis *et al.,* 1999).

Previous studies have noted that for effective insect pest control, NPV suspension must be maintained at near neutral pH (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968). They observed that low pH (≤ 2) conditions could lead to a significant reduction in baculovirus efficacy against insect pests. Field work has shown that the efficacy of *Anticarsia gemmatalis* nucleopolyhedrovirus (*Ag*MNPV) was significantly affected by the pH of the spray suspension (Silva and Moscardi, 2002). They noted that NPV-induced larval mortality was significantly higher in plots treated with NPV at near neutral conditions compared to plots treated at high or low pH (pH 2 and 10). Since differences in diet pH may cause differences in larval midgut pH (Schultz and Lechowics, 1986) variation in midgut pH could lead to either enhanced or reduced

mortality caused by NPV. It has been suggested that this could act in various ways; such as influencing the rate of virion release, virion survival in the midgut environment (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968), virion binding to the midgut epithelial cells and it could also influence the activity of gut proteases, which help in the dissolution of NPV OBs (Pritchett *et al.,* 1984).

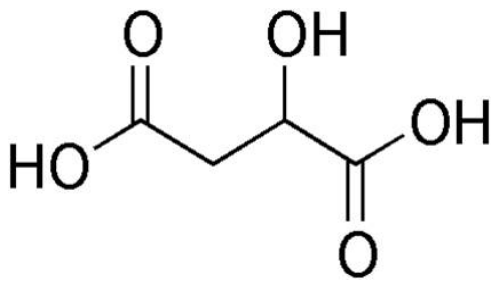
Chickpea leaf surfaces have been shown previously to reduce the efficacy of *Hear*NPV used against *H. armigera* (Stevenson *et al.,* 2010; Chapter 3). However, the reduction in efficacy was reported to be specifically due to the action of leaf surface isoflavonoids (sissotrin and biochanin A) and not due to the chickpea organic acids (Stevenson *et al.,* 2010). This was surprising since earlier studies had demonstrated that extreme acidity could significantly reduce the efficacy of NPV (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968). Also the inactivation produced by the chickpea leaf surface isoflavonoids *in vitro* could not account for the total inactivation observed on the chickpea leaf surface *in vivo* (Stevenson *et al.,* 2010; Chapter 3-5). Thus it was possible that other factors in addition to the presence of isoflavonoids were responsible for the inactivation recorded on chickpea leaf surfaces. The present study (Chapter 6) has demonstrated that chickpea organic acids in combination with isoflavonoids (biochanin A and/or formononetin) can significantly reduce the efficacy of *Hear*NPV. It may be that the results at low NPV lethal concentration (LC_{25}) were due to the action of the major organic acids and not that of the isoflavonoids, because similar larval mortality was recorded when the organic acids were mixed with NPV as well as when all the three components (NPV + organic acids + isoflavonoids) were mixed together and bioassayed against *H. armigera* neonate on diet surface. Similarly, when the organic acids were mixed with isoflavonoids and NPV at high lethal concentration (LC_{75}) results also showed that there was no significant difference between the exposed NPV (NPV + organic acids + isoflavonoids) and the water control treatment. From which it may be inferred that both the organic acids and the isoflavonoids were acting together to cause the total inactivation observed. The results also showed that the inactivation effect was not isoflavonoid concentration dependent, because increasing the concentration of the isoflavonoids (from 100 ppm to 500 ppm) did not lead to a significant increase in larval mortality. Nor was a significant additive effect obtained when the two isoflavonoids (biochanin A and formononetin) were

combined together. Hence, it was decided to determine whether the acids alone could be responsible for the inactivation of *Hear*NPV.

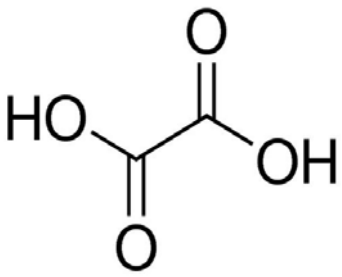
Therefore, this study was carried out to test the following hypotheses;

1. That most abundant chickpea organic acids are responsible for the inactivation of *Hear*NPV
2. That malic acid concentration is associated with *Hear*NPV inactivation
3. That oxalic acid concentration is associated with *Hear*NPV inactivation

To test these hypotheses, the most abundant chickpea acids (malic and oxalic) were combined according to their natural concentration on the leaf surface (Rembold and Weigner, 1990) and at three other concentrations and mixed together with high lethal concentration of *Hear*NPV (LC_{75}) (See section 7.2) before being fed to *H. armigera* on diet surface.



A. Malic acid (pKa 3.40)



B. Oxalic acid (pKa 1.25)

Figure 7.1 Most abundant organic acids found on chickpea leaf surface exudate (A. Malic and B. Oxalic).

7.2 Materials and methods

This experiment was carried out to determine the effect of most abundant chickpea organic acids (malic and oxalic) in the exudate of chickpea leaf on *Hear*NPV efficacy, and also to find out if either of the two most abundant acids is involved in the inactivation process.

Previously, Rembold and Weigner (1990) determine the chemical composition of chickpea exudate from the resistant chickpea genotype (ICC 506) using HPLC. The exudate was wiped from the plant surface using cotton plugs which were squeezed into glass vials, the vials were sealed with paraffin and stored at 4°C. Eight different organic acids were identified from the exudate by comparison with authentic materials. Malic (119.60 mg ml^{-1}) and oxalic (3.80 mg ml^{-1}) acids were identified as the most abundant acids both of which represent 61.2% and 28.6% of chickpea acid respectively. This was the concentration of chickpea acids used in this study as well as in the work of Stevenson *et al.* (2010).

Organic acids were prepared (See section 6.2.1) according to the concentrations reported on leaf surface (Rembold and Weigner, 1990), and also at three different concentrations; (1) by increasing the natural concentration by three, (2) reducing it by half and (3) exchanging the relative concentration between the two acids as shown below. The pH of each organic acids combination was also recorded (See Table 7.1). The *Hear*NPV was prepared at 5.8×10^3 OB ml^{-1} (LC_{75}). For each organic acid treatment, aliquot (500 µl) of each of the organic acids concentrations (treatments 3-6 below) were mixed together with 500 µl of *Hear*NPV suspension ($LC_{75} \times 2 = 1.16 \times 10^4$ OB ml^{-1}) in Eppendorf tube (1.5ml). All other procedures were same as mentioned in section 6.2.1

The treatments used were as follows:

1. Sterile distilled Water (SDW) control
2. Virus only (*Hear*NPV) $LC_{75} = 5.8 \times 10^3$ OB ml^{-1}
3. Organic acids combination = malic (120 mg ml^{-1}) + oxalic (4.0 mg ml^{-1}) + *Hear*NPV ($LC_{75} \times 2 = 1.16 \times 10^4$ OB ml^{-1})
4. Organic acids combination = malic ($120 \times 3 = 360$ mg ml^{-1}) + oxalic ($4.0 \times 3 = 12$ mg ml^{-1}) + *Hear*NPV ($LC_{75} \times 2 = 1.16 \times 10^4$ OB ml^{-1})

5. Organic acids combination = malic ($\frac{120}{2}$ = 60 mg ml^{-1}) + oxalic ($\frac{4.0}{2}$ = 2.0 mg ml^{-1}) + *Hear*NPV ($LC_{75} \times 2 = 1.16 \times 10^4$ OB ml^{-1})
6. Organic acids combination = malic (4.0 mg ml^{-1}) + oxalic (120 mg ml^{-1}) + *Hear*NPV ($LC_{75} \times 2 = 1.16 \times 10^4$ OB ml^{-1})

Table 7.1 Acidity test of the major organic acids of chickpea leaf exudate with or without *Hear*NPV as measured by pH meter or universal indicator paper

Treatment	Oxalic acid (mg ml^{-1})	Malic acid (mg ml^{-1})	Total acids conc.(mg ml^{-1})	pH (OAs)*	pH (final) ** OAs+NPV+ Diet
3	4.0	120	124	1.4	2.0
4	12	360	372	0.8	1.5
5	2.0	60	62	1.6	2.0
6	120	4.0	124	0.4	1.0

OAs = Organic acids (Malic + Oxalic), NPV = *Hear*NPV, * measured by pH meter, ** measured by pH indicator paper

7.2.1 Statistical Analysis

Treatments effects were measured by comparing the mean bioassay percentage mortality using four different replicate experiments for each treatment group. Data were analysed using ANOVA. Differences arising from treatment means were separated using Tukey multiple comparisons tests. Relationship between pH and *H. armigera* larvae survival were analysed using logit regression analysis, data were corrected for untreated control mortality using Abbott's formula before subjected to Analysis of deviance. Data were analysed using R Statistics (2.10.0) R Development core Team (2012).

7.3 Results

Hypothesis 1: Chickpea major organic acids are responsible for the inactivation of HearNPV *used against* H. armigera

Results from the bioassay (Fig. 7.2) showed that larval mortality of *Hear*NPV exposed to different treatment concentrations of organic acids were significantly lower compared to mortality recorded with unexposed *Hear*NPV (ANOVA; F= 25.57, df= 5, 18, P< 0.001). Larvae reared on the untreated NPV recorded the highest larval mortality (83%), which was close to the intended LC_{75}. The result shows that as the natural concentration of the acids decreased, mean percentage mortality also increased (Fig. 7.2). The highest larval mortality (44.5%) among the organic acid plus *Hear*NPV treatments was obtained at treatment (a). The mean percentage larval mortality among all the acids plus NPV treatments were not significantly different (P < 0.05), except when the natural concentration of the acids was half in treatment (a). When the natural concentration of chickpea acids was reduced by half in treatment (a) and tested with the NPV, larval mortality (44.5%) was significantly (P= 0.02) higher compared to mortality obtained with water control (16%). However, larval mortality (25.5%) recorded when the natural concentration of the acids was exposed to *Hear*NPV in treatment (b) was not significantly different (P= 0.20) to the larval mortality obtained (44.5%) when the concentration of organic acids was reduced by half and treated with NPV in treatment (a). The least larval mortality (9%) was recorded in treatment (d) when the natural concentration of the acids was exchanged between the two acids before being exposed to NPV. At this treatment (d) the mean larval mortality (9%) was not significantly different (P= 0.99) with the larval mortality (12.5%) recorded when the natural concentration of the acids was increased by 3-fold in treatment (c). Similarly, the larval mortality (16%) obtained with water control treatment (w) was not significantly (P> 0.05) different with either the natural concentration of the acids in treatment (b), or when the natural acids was increased by 3-fold in treatment (c) or when the natural concentrations of the acids was exchanged between the two acids in treatment (d).

The novel finding from this experiment is that major organic acids in chickpea can inactivate *Hear*NPV and do so in the absence of isoflavonoids.

Hypothesis 2: Malic acid concentration is associated with the inactivation of HearNPV *used against* H.armigera

The results from the bioassay showed that malic acid concentration was not strongly associated with larval mortality (Fig. 7.2). For example, the least larval mortality (9.0%) was recorded with the least concentration of malic acid (4 mg ml^{-1}) in the organic acids plus virus treatment (d). Similarly, the larval mortality (12.5%) recorded with highest concentration of malic acid 360 mg ml^{-1} (c) in organic acids combination was lower than the mortality obtained with the highest oxalic acid combination 120 mg ml^{-1} in treatment (d). Also, when the natural concentration of the acids was exposed to NPV in treatment (b), larval mortality was 25.5% and this corresponds with malic acid concentration of 120 mg ml^{-1}. However, at same concentration of oxalic acid 120 mg ml^{-1} in treatment (d), larval mortality was lower (9.0%). Similarly, results from logit regression analysis (Fig. 7.3) also showed that there is a highly significant negative correlation ($r = - 0.78$, $P < 0.001$) between pH and larval survival, meaning that higher proportion of larvae survived at lower pH.

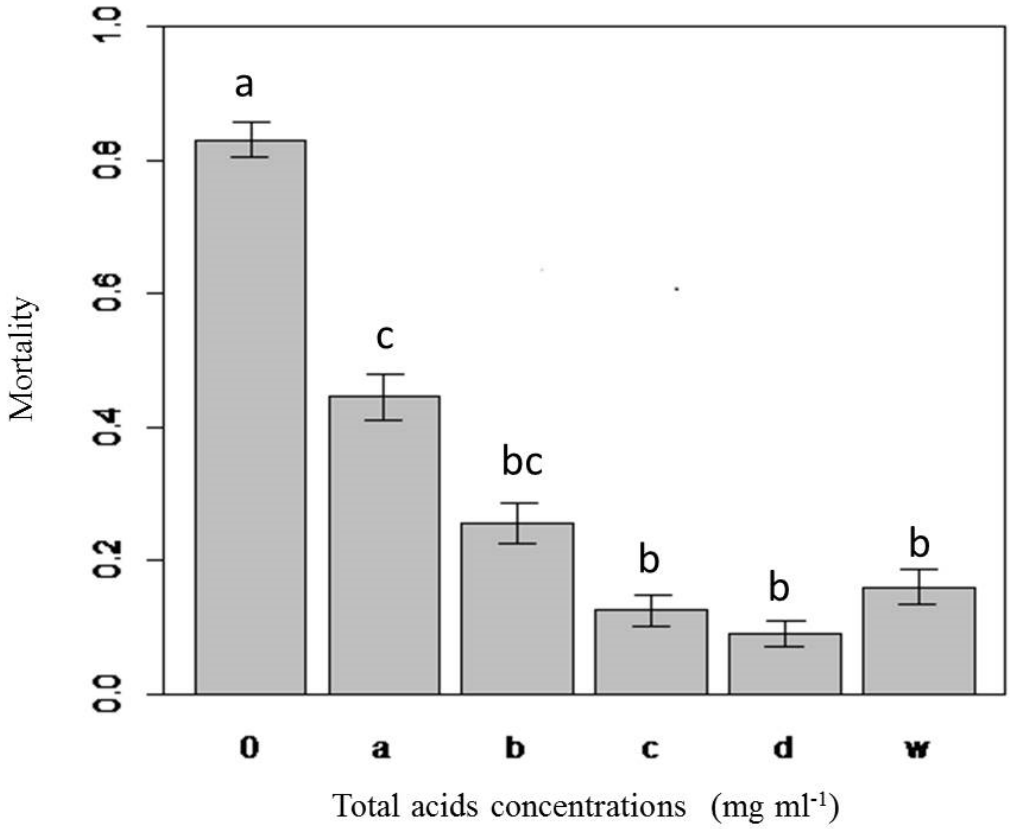


Figure 7.2 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets containing either untreated *Hear*NPV OBs or with *Hear*NPV OBs that had been exposed to the major chickpea organic acids, malic (ML) and oxalic (OX) at different concentrations [a= (ML) 60 + 2 (OX), b= (ML)120 + 4 (OX), c= (ML)360 + 12 (OX), d= (ML) 4 + 120 (OX)]. Bars with different letters are statistically significant ($P< 0.05$).

At same concentration of organic acids tested (b and d), oxalic acid was found to be more acidic (pH 0.4) than malic acid (pH 1.4). Results from pH mortality graph (Fig. 7.4) also shows that larval mortality was reduced at pH 0.4, compared to mortality that was recorded at pH 1.4.

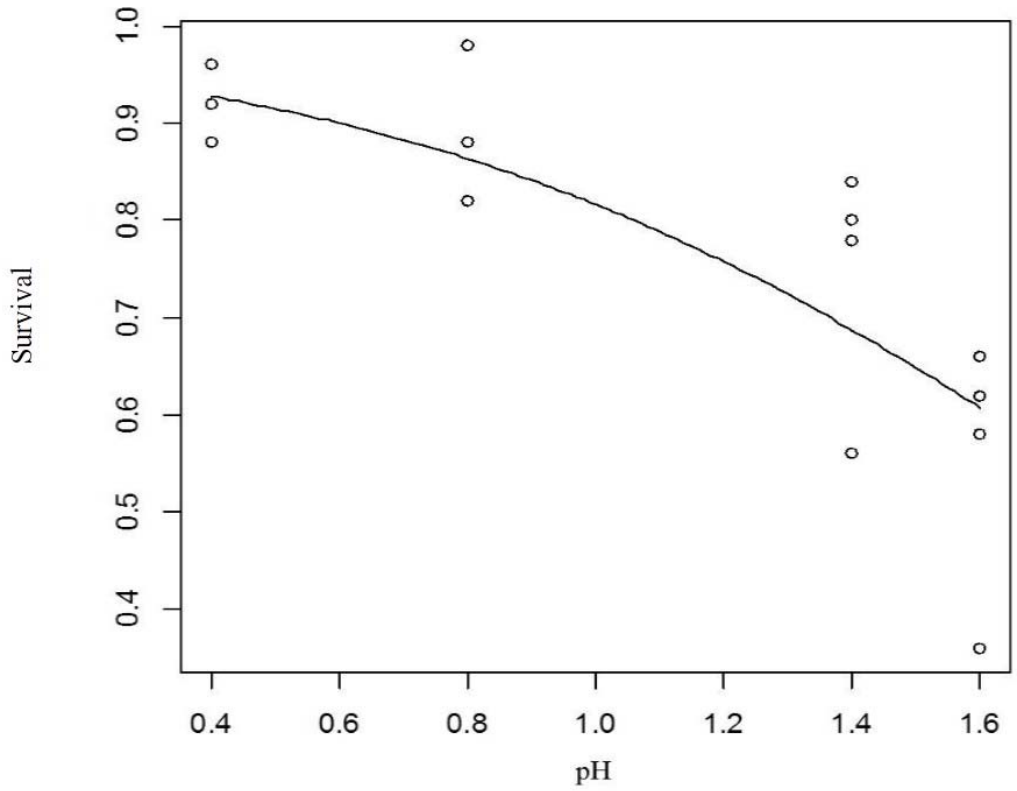


Figure 7.3. Relationship between mean proportion survival of *Helicoverpa armigera* neonates and pH as obtained on artificial diets containing *Hear*NPV OBs exposed to different combinations of most abundant chickpea acids (malic and oxalic). The trend is statistically significant ($P < 0.001$).

Hypothesis 3: Oxalic acid concentration is associated with the inactivation of HearNPV used against H.armigera

Larval mortality was significantly associated with concentrations of oxalic acids in the organic acids combinations (Fig. 7.2). Larval mortality was consistently reduced on diets containing *Hear*NPV that had been treated with different organic acid concentrations, and this was oxalic acid-concentration dependent. The lowest mean larval mortality (9.0%) was obtained when *Hear*NPV was exposed to the highest concentration of oxalic acid (120 mg ml^{-1}) in the organic acids combination (d).

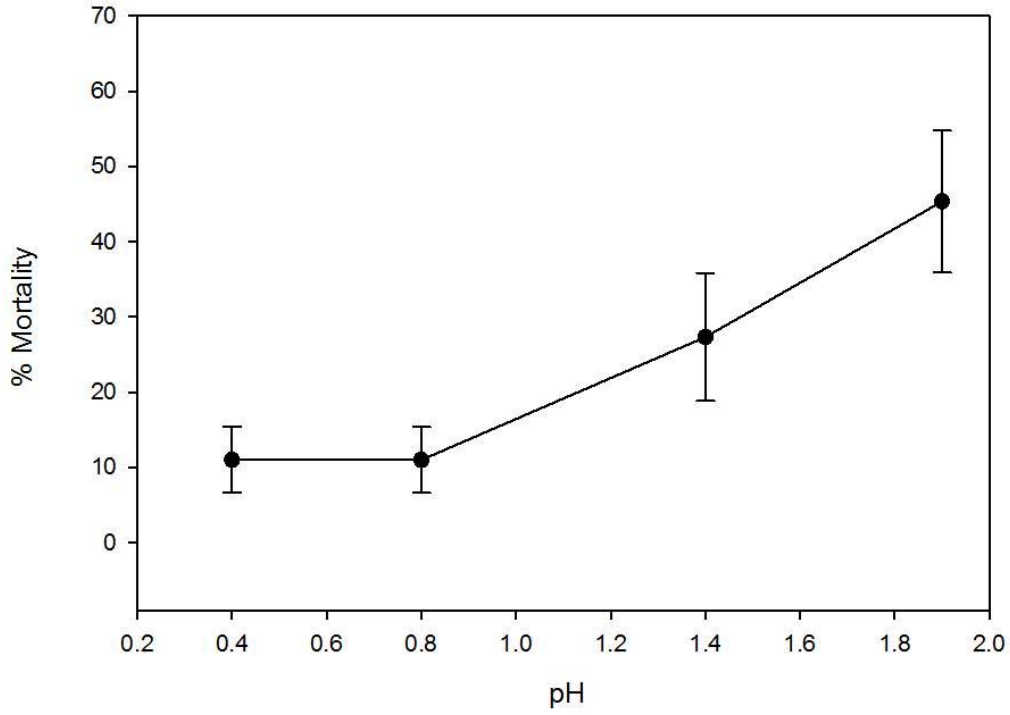


Figure 7.4. Graph of mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on diets containing *Hear*NPV OBs exposed to different pH conditions obtained from different combinations of chickpea organic acids (malic and oxalic).

Similarly, when the oxalic acid concentration was reduced in treatment (a) to 2 mg ml^{-1} from 120 mg ml^{-1} in treatment (d), mortality increased from 9% to 44.5%. In addition, when the natural concentration of the acids was reduced by half in treatment (a) and treated with NPV, larval mortality (44.5%) was significantly lower (P= 0.003) compared to mortality (9.0%) recorded with highest concentration of oxalic acid in treatment (d). The result from the logit regression analysis (Fig. 7.3) also shows a highly significant nagative relationship ($r = - 0.78$, $P < 0.001$) between larval survival and pH of the organic acids combinations. Which means that the lower the pH (higher acidity) the higher the proportion of larvae that survived. Oxalic acid concentration correlates strongly with lower pH. For example, the highest concentration of oxalic acid tested (120 mg ml^{-1}) gave the highest acidity (pH 0.4), while the least oxalic acid gave the lowest acidity (pH 1.6) among all the organic acids treatments. This trend was also similar in the results obtained with pH mortality graph (Fig. 7.4), which showed

lowest larval mortality at lowest pH 0.4 compared to mortality (44.5%) recorded at highest pH 1.6.

7.4 Discussion

This study has shown that *Hear*NPV was inactivated by the highly acidic conditions present in chickpea leaf surface exudate. This is the first time organic acids exudates as part of a plants normal function have been shown to inactivate insect viruses and this effect occurred in the absence of other compounds on the leaf surfaces such as isoflavonoids (i.e. biochanin A or formononetin), that had been reported earlier (chapter 6) and in previous work (Stevenson *et al.,* 2010) as being involved in *Hear*NPV inactivation. Of the two most abundant acids present at high concentration in the chickpea leaf exudate, oxalic acid had the most significant inactivating effect compared to malic acid in the *Hear*NPV inactivation process.

Previous studies showed that extreme pH conditions reduced the efficacy of NPV (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968). Chauthani *et al.,* (1968) also showed that NPV OBs incubated in human gastric juice (pH 2.1) were inactivated rapidly by 50% within 40 min, and became completely inactivated by 120 min. They noted that since neutralized gastric juice (pH 7) has no significant effect on *Hz*NPV efficacy is an indication that extreme acidity of the human gastric juice was responsible for the inactivation. The present results demonstrated that larval mortality was significantly lower when *Hear*NPV was exposed to different concentrations of chickpea most abundant organic acids compared to mortality obtained with unexposed *Hear*NPV, but since the two acids (malic and oxalic) differed in their inactivating effects it may not simply be an issue of pH in this specific case but could be related to other chemical properties. However, oxalic acid was shown to be more acidic (pH 0.4) compared to malic acid (pH 1.4) when both acids were at the same concentrations (see Table 1).

Previous results had showed no evidence that chickpea organic acids have a significant effect on *Hear*NPV efficacy used against *H. armigera* larvae (Stevenson *et al.,* 2010). In seeking an explanation for the different findings obtained here one factor may be the difference in the protocol of bioassay used. In the previous study, larval mortality

was counted after seventh days while in the present work mortality was recorded after the fifth day. This change was adopted to reduce the effect of larval cannibalism which was observed to occur after five days, as this larval cannibalism can mask the treatment effects and reduce the ability to detect significant results in trials counted at seventh day post treatment. In addition, previous work did not mentioned the pH of the organic acid combination that was used. Another previous study has shown that more than 90% mortality of neonate larvae (*Heliothine* species) was obtained within the first five days after exposure to *Hz*NPV (Ignoffo, 1966). Therefore, they suggested that five days period should be adopted as the ideal cut-off date for recoding mortality when first-instar bollworms are used for bioassay with NPV.

The fact that NPV induced mortality reduced as the concentration of the organic acids increases is an indication that the high concentration of the acids in the chickpea leaf exudate could be responsible for the *Hear*NPV inactivation on the chickpea leaf surfaces (see chapter 3; Stevenson *et al.,* 2010). As the concentration of the organic acids increased, the acidity also increased (see table 7.1). However, lowest pH was obtained with the highest concentration of the oxalic acids in the organic acid combination (120 mg ml^{-1}, pH 0.4), and not with the highest concentration malic acids tested (360 mg ml^{-1}, pH 0.8). When all the acids combinations were mixed with *Hear*NPV suspension prior to placement on the diet surface, although the pH slightly increased, the extreme acidity ($pH \leq 2$) was still maintained (see table 7.1). High acidity of chickpea exudate has been reported to be within the ranges of pH 0.4 to 1.3 (Launter and Munns, 1986; Rembold and Weigner, 1990), and this is within the pH range used in the present study. Additionally, the effect of chickpea leaf exudates may not be relevant to NPV alone. Armstrong and Gossen (2005) demonstrated that the germination of *Ascochyta rabiei* fungal conidia was completely inhibited at high concentration of the exudate (1.5 mg ml^{-1}, pH 2.8) and partially inhibited at 0.3 mg ml^{-1} (pH 3.1) and might be a function of these acids in defence. They noted that germination of the fungal pathogen decreased as the concentration of the exudate increased from 0.012 mg ml^{-1} (pH 4.4) to 0.06 mg ml^{-1} (pH 3.6).

In addition, present results showed that the lowest NPV-induced larval mortality (9.0%) was obtained when the natural concentration of the most abundant organic acids was exchanged, and this corresponds with the highest concentration of oxalic

acid in the organic acids combination (120 mg ml^{-1}). However, at same concentration of malic acid (120 mg ml^{-1}), larval mortality was higher (25.5%), although the two mortalities obtained were not significantly different to the mortality (16%) obtained with water control. Similarly, increasing the concentration of the natural concentration of the organic acids by 3-fold (372 mg ml^{-1}) did not lead to a significant decreased in larval mortality (12.5%) compared to mortality obtained with the highest concentration of oxalic acid (9.0%) or that which was obtained with the natural concentration of the most abundant chickpea acids (25.5%). However, reducing the natural concentration of the acids by half (62 mg ml^{-1}), led to higher larval mortality (44.5%) that was significantly different with that of all the other acids plus NPV treatments or the water control. Surprisingly, this mortality was not significantly different from the mortality obtained with the natural concentration of the two most abundant chickpea acids (124 mg ml^{-1}). However, at this reduced concentration of the organic acids, there was only slight difference in acidity (pH 1.4) which was similar to that recorded with the natural concentration of the acids (pH 1.6), and this could account for the non-significant difference recorded between the two treatments. Therefore, present results demonstrated that all the organic acid treated NPV led to complete inactivation of *Hear*NPV with mortality equivalent to that obtained with water control except when the natural concentration of the acids was reduced by half.

Interestingly, previous study correlated high malic acid concentration (> 290 mg ml^{-1}) in chickpea leaf exudate to be an important factor responsible for low podborer (*H. armigera*) larvae damage in some chickpea varieties (Rembold *et al.,* 1990 a,b) . While susceptible chickpea varieties were reported to have low malic acid concentrations (60 - 120 mg ml^{-1}) in their exudates (Rembold *et al.,* 1990 a,b). However they noted that some chickpea genotypes with malic acid concentrations of between 120 - 290 mg ml^{-1} of the exudate were inconsistent with podborer damage, and some have low malic acid content and low acidity in their exudates. Therefore, they suggested that other factors might be involved. This is consistent with the results obtained in the present study, which shows that malic acid in the organic acids combination does not consistently correlates with larval mortality compared to oxalic acid, when different concentrations of both acids were treated with NPV prior to being fed to larvae on diet surface. Since previous studies (Rembold *et al.,* 1990a,b) that correlated high malic acid in chickpea leaf exudates with resistance to podborer did not mentioned the

concentration of another important acid (oxalic acid) in the chickpea exudates, it is therefore possible to suggest that oxalic acid might be the other factors that were not identified in previous studies that could account for low pod damage on some chickpea genotypes. Already others have demonstrated through both laboratory and field studies that there was no significant correlation between podborer damage and malic acid levels (Yoshida *et al.,* 1995, 1997). However, they reported that high concentration of oxalic acid in the chickpea leaf exudate to be an important resistant factor of chickpea against *H. armigera* larvae. Feeding test with chickpea genotypes resistant to *H. armigera* showed significant inhibition of larval growth and extension of larval period compared to those larvae fed on susceptible genotypes (Yoshida *et al.,* 1995). They noted that oxalic acid showed significant inhibition of larval growth when included in to artificial diet, while malic acid has no significant effect compared to larvae fed on control diet. Chemical analysis shows that resistant chickpea genotypes contained higher levels of oxalic acids on the leaf surface compared to the susceptible genotypes (Yoshida *et al.,* 1995; 1997). Therefore, they proposed that oxalic acid on the leaves is responsible for the reduced larval growth observed on the resistant chickpea genotype. Since no antifeedant effect was observed among the two acids (malic and oxalic) in the filter paper feeding test, it was suggested that oxalic acid in the leaves of the resistant genotype could be acting as antibiotic compound (Yoshida *et al.,* 1995). Another study provided evidence of this proposed effect, that the leaf of resistant chickpea genotype was responsible for antibiotic effect on *H. armigera* larvae (Srivastava and Srivastava, 1990). They demonstrated variable larval survival, weight, pupal weight, egg viability, adult longevity and larval period among different chickpea genotypes that differs in susceptibility to podborer, however in that study the factor responsible for the antibiotic effect in the resistant chickpea genotype was not identified. Although malic acid concentration was reported to have no significant correlation with podborer damage on chickpea (Yoshida *et al.,* 1995; 1997), malic acid was shown to contribute in chickpea resistance against *H. armigera* larvae by acting as oviposition deterrent at high concentration (Yoshida *et al.,* 1997).

The mechanism by which oxalic acid inactivates *Hear*NPV is presently not known and was not determined in this study, however, other studies have demonstrated that the antiviral effect of tannic acid might be acting on the viral particles directly, because larval mortality was reduced when tannic acid and viral OBs (*Hz*NPV) were mixed

together in aqueous suspension (Young *et al.,* 1995). They observed that for tannin to have any effect in NPV efficacy in the insect midgut, it must either be present at or before exposure of larvae to the virus. This is similar to the *Hear*NPV inactivation that was observed in the present study with the acidic exudates of chickpea leaf surface. Therefore the effect of the most abundant chickpea acids (malic and oxalic) on NPV might be due to their contact with the virus directly and not primarily due to the low pH of the acids per se, although the highly acidic effect will not be excluded. This also in line with the previous findings which noted that NPV inactivation on chickpea was leaf surface based and permanent, since viral OBs were still inactive after their being exposed and removed from chickpea leaves (Stevenson *et al.,* 2010). However, present study also suggests that of the two most abundant chickpea acids tested with the virus, oxalic acid was shown to be more active on *Hear*NPV compared to malic acid.

CHAPTER EIGHT

GENERAL SUMMARY OF RESULTS AND DISCUSSIONS

8.0 Introduction

Insect pests have been identified as the main cause of global food losses (Fitt, 1989; Gowda, 2005), and *H. armigera* is one of the most important insect pests attacking several key crops such as chickpea, tomato, maize and cotton, particularly in Asia, Africa and Australia (King, 1994; Gowda, 2005). This pest has become increasingly important over the years due to its ability to become resistant to many chemicals products used for its control (Kranthi *et al.,* 2001; 2002) so that sustainable control using conventional chemicals has recently become costly and sometimes ineffective (Hunta-fujita *et al.,* 1998; Grzywacz *et al.,* 2005). Furthermore, concerned for the environment, human health and safety issue has contributed in making use of broad spectrum chemicals unacceptable in many countries (Grzywacz *et al.,* 2005; Hillocks, 2012)

A result of such limitations with the use of conventional chemicals in controlling *H. armigera,* has been the search for alternatives such as insect specific viruses like *Hear*NPV (Jones, 1994; Moscardi, 1999; Grzywacz *et al.,* 2005), that have been found to be effective in controlling *H. armigera* larvae. However, despite their potential for insect pest control, the use of insect viruses has been largely restricted to niche-markets and high-value crops (Grzywacz *et al.,* 2005; Buerger *et al.,* 2007). Two factors identified for the limited use of viral insecticides is their low-field persistence (Cherry *et al.,* 2000), attributed largely to inactivation by solar UV light (Young and Yearian, 1974; McLeod *et al.,* 1977), and host-plant chemical factors (Hoover *et al.,* 1998a,b,c; Cory and Hoover, 2006). Host-plant chemistry has been identified to be one of the factors affecting the efficacy of baculovirus, however, determining the agents responsible or mechanism involved has remain unresolved, primarily due to experimental difficulty in establishing cause and mode of action (Duffey *et al.,* 1995; Hoover *et al.,* 1998b,c).

On cotton the mechanism of NPV inactivation has been linked to sloughing of damaged infected insect midgut cells, due to the action of reactive end products formed by phenolic oxidation catalyzed by foliar enzymes (i.e. peroxidase) (Hoover *et al.,* 1998b,c; 2000). In contrast, on chickpea the inactivation has been linked to leaf surfaces isoflavonoids, sissotrin and biochanin A (Stevenson *et al.,* 2010). However, it was noted that the degree of inactivation by both compounds could not account for all the inactivation observed on chickpea leaves when *Hear*NPV was sprayed on the leaves and washed off and used against *H. armigera* larvae on diet surface. It was also suggested that wetting the plants by spraying with water and wetting agent (i.e. Triton surfactant) also leads to induction of higher levels of isoflavonoids.

Therefore, this study investigated further the inactivation of *Hear*NPV on chickpea to identify the compounds involved in the inactivation of *Hear*NPV on chickpea and to determine if similar phytoinactivation also occurs in other major African and Asia legumes (i.e. cowpea and pigeonpea). Finally this work set out to determine how the plant chemicals act to inactivate *Hear*NPV. The research described in this thesis evaluated the following;

1: Effect of host-plant on *Hear*NPV efficacy (Chapter 3),

2: Analysis of chickpea leaves to identify the compounds that inactivate *Hear*NPV (Chapter 4),

3: Effect of isoflavonoids on *Hear*NPV efficacy (Chapter 5),

4: Effects of isoflavonoids in combination with chickpea organic acids on *Hear*NPV efficacy (Chapter 6) and

5: Role of chickpea organic acids on *Hear*NPV efficacy (Chapter 7).

This chapter summarises the results, interprets the findings and discusses their broader practical implications with the aim of facilitating future development and improvement of *Hear*NPV formulations for insect pest control, particularly on chickpea. Finally, areas of further studies were also identified.

8.1 Summary of results

*Hear*NPV exposed to chickpea leaf surfaces was almost completely inactivated compared to the *Hear*NPV exposed to other host-plants (i.e. cowpea, pigeonpea or tomato). This demonstrated that chickpea leaf surface was more detrimental to *Hear*NPV compared to leaf surfaces of the other three host-plants. The study did show that other legumes tested (i.e. cowpea and pigeonpea) also reduce the efficacy of *Hear*NPV however; the effect was modest compared to that of chickpea leaf surface. No inactivation was seen on tomato leaf. To determine whether exposing *Hear*NPV OBs to chickpea leaf surface could result in any major change to the morphological structure of the viral particles. *Hear*NPV exposed to chickpea were compared to those of tomato and unexposed *Hear*NPV. The result shows that *Hear*NPV inactivation does not lead to any major change in the OBs physical structure.

Triton surfactant and *Hear*NPV induced higher levels of formononetin compared to either water or non-sprayed control leaves. The results from whole leaves conclusively demonstrated that only spraying with *Hear*NPV elicited increased levels of formononetin in chickpea leaves. In contrast, levels of another compound (biochanin A) on chickpea was not changed significantly after spraying with any of the treatments compared to unsprayed control leaves. Furthermore, result from chemical analysis also shows that biochanin A was present at higher levels than that of formononetin in both the leaf surface and whole leaf.

Bioassay results showed that both formononetin and biochanin A reduced the efficacy of *Hear*NPV at $\geq$ 50 ppm. This is less than the concentration of both isoflavonoids present on chickpea leaves either before or after treatment with *Hear*NPV. Although the result shows that both formononetin and biochanin A were inhibitory to *Hear*NPV, however neither of the two compounds could account for the whole inactivation effect that was recorded on chickpea leaves. This led to the conclusion that other phytochemical compound(s) might be involved.

To test this hypotheses, biochanin A and formononetin were tested in combination with chickpea organic acids either individually (50 ppm) or in combination (at 25 ppm each) together with *Hear*NPV at low lethal concentration (LC_{25}) against *H. armigera* neonates on the diet surface. Chickpea organic acids were also tested in combination with *Hear*NPV (LC_{25}) against *H. armigera* neonate larvae on diet surface. The results showed that although both isoflavonoids in combination with chickpea organic acids also reduced the efficacy of *Hear*NPV, except for the combination biochanin A with organic acids, all the other isoflavonoids and organic acids combinations produced almost equal effect on *Hear*NPV efficacy equivalent to that obtained with organic acids and *Hear*NPV combination.

Furthermore, when high concentrations of isoflavonoids (100 ppm and 500 ppm) plus chickpea acids were mixed with aqueous suspension of *Hear*NPV (LC_{75}), before used against *H. armigera* neonate on diet surface, all the treatments significantly reduced the efficacy of *Hear*NPV. The rate of inactivation obtained by the mixture of these three components together could account for the total inactivation that was previously reported on chickpea leaf surfaces. The inactivation effect was not isoflavonoid concentration-dependent.

Earlier results in this study have shown that organic acid alone could reduce the efficacy of low lethal concentration of *Hear*NPV (LC_{25}) comparable to that seen with that of the isoflavonoids and organic acids combinations when added to the diet with *Hear*NPV. Increasing the concentration of isoflavonoids in the organic acids mixture did not led to a significant reduction in NPV-induced larval mortality.

This required further investigation to determine if the inactivation effect caused by the combination of the isoflavonoids plus organic acids on *Hear*NPV was due primarily to the action of the organic acids rather than that of the isoflavonoids. Thus another experiment was carried out to determine whether chickpea organic acids alone could account for the inactivation of *Hear*NPV, and also determine which of the two most abundant acids (malic and oxalic) is more important in the inactivation effect. The subsequent results showed that organic acids of chickpea alone could produce total inactivation of *Hear*NPV. Furthermore, the study demonstrated that the inactivation was significantly and positively correlated with pH (high acidity) of the treatment

mixtures and that oxalic acid is more important than malic acid in the inactivation process.

8.2 General discussion

8.2.1 Effects of host-plants on Nucleopolyhedrovirus efficacy

The results of the present study confirm previous findings which showed that the leaf surface of chickpea reduced *Hear*NPV efficacy (Rabindra *et al.,* 1994; Stevenson *et al.,* 2010). However, the present study went further and demonstrated that cowpea and pigeonpea leaf surfaces also significantly reduced the efficacy of NPV compared to leaf surface of tomato. This is the first study to demonstrate that *Hear*NPV efficacy is reduced by these two important and widely cultivated crop species. Although Rabindra *et al.* (1994) reported that on pigeonpea there was an increase in the lethal time of larval mortality it did not report significant inhibition of *Hear*NPV on pigeonpea leaves. Identifying how viral insecticides are affected by phytochemical factors is important to their successful utilisation and future development for pest control, particularly on chickpea the most detrimental crop compared to either of the two legumes (cowpea or pigeonpea). Previous studies have also reported that tomato was not inhibitory to *Hear*NPV efficacy (Forschler *et al.,* 1992; Farrar and Ridgway, 2000; Moore *et al.,* 2004).

Although this and a previous study have shown that chickpea leaf surface rapidly reduces the efficacy of NPV within an hour (Chapter 3; Stevenson *et al.,* 2010), others surprisingly reported that *Hear*NPV was effective on chickpea when used against *H. armigera* larvae in the field (Cherry *et al.,* 2000). However, one of the reasons could be that they used *Helicoverpa*-susceptible variety (Shoba), and this variety has been reported to be compatible with *Hear*NPV (Rabindra *et al.,* 1992; Cowgill and Bhagwat, 1994). In addition, they also noted that use of *Hear*NPV on resistant chickpea genotypes was found to lead to variable and sometimes ineffective control. This could be attributed to the differences in the chemical compounds in both varieties, since production of defence compounds such as isoflavonoids and organic acids were known to differ among different chickpea varieties (Stevenson *et al.,* 1997; Yoshida *et al.,* 1995; 1997; Simmonds and Stevenson, 2001). The present work also supports

the previous study (Stevenson *et al.,* 2010), which shows that the mechanism of NPV inactivation on chickpea is leaf surface related and permanent, because viral OBs were still inactive after being exposed and removed from chickpea leaves and used against *H. armigera* larvae on diet surface. This is different from the midgut based inactivation mechanism that was reported earlier on cotton (Hoover *et al.,* 1998b,c; Hoover *et al.,* 2000).

Inhibition of NPV viral disease on plants has been hypothesised to be caused by the ability of reactive oxygen species formed through phenolic oxidation catalysed by foliar oxidized (peroxidase) to bind with the viral OBs directly, thereby preventing the viral particles to initiate primary infection in midgut cells (Hoover *et al.,* 1998b,c; Cory and Hoover, 2006). Since it is possible for phenolics such as isoflavonoids to be induced after spraying with NPV on chickpea (Chapter 4), it is also likely that those defence compounds could reduce the efficacy of viral insecticides on cowpea and pigeonpea leaves after spraying with *Hear*NPV (Chapter 3), through binding with viral OBs hence preventing infection taking place in insect midgut cells (Hoover *et al.,* 1998b,c; Cory and Hoover, 2006). Hoover *et al.* (1998c) have also demonstrated using *in vitro* studies that treatment of viral OBs with peroxidase (POD) and phenolic substrate together in mixture diminished viral-induced mortality. They suggested that viral OBs stability could be affected directly by free radicals during phenolic oxidation by POD. However on tomato, which has high amounts of antioxidants such as catalase in its foliage (Duffey and Stout, 1996), viral disease could be protected from the oxidative action of POD by catalase there by preventing the generation of free radicals that could be detrimental to the NPV OBs (Hoover *et al.,* 1998b,c; Cory and Hoover, 2006).

Present study has also demonstrated that plant type and plant chemistry could influence the extent of baculovirus disease and this could be responsible for the different *Hear*NPV inactivation that was recorded in this study. Most leguminous crops like chickpea, cowpea and pigeonpea are known for their widespread production of isoflavonoids as defence mechanisms against microbial infection (Ingham, 1982; Dakora and Phillips, 1990). These isoflavonoids could account for the inactivation observed in this study, at least for cowpea and pigeonpea, however on chickpea, the inactivation effect is far greater than what could be attributed to either of the

isoflavonoids reported in the present work (see chapter 5) or previous study (Stevenson *et al.,* 2010). However, control of the velvet bean caterpillar, *Articarsia gemmatalis* using *Ag*MNPV on another legume (soybean) was reported to be the most successful in the use of viral insecticide for insect pest control, covering about 2 million hectares of land (Moscardi, 1999; Moscardi, 2007). In this plant higher NPV-induced mortality was reported when *A. gemmatalis* larvae were fed on diet containing *Ag*MNPV with extracts from resistant soybean genotypes (PI 274454 and PI 227687) as compared to larvae fed on control diet (Piubelli *et al.,* 2009). It was reported that the resistant genotypes enhanced the efficacy of the *Ag*MNPV against *A. gemmatalis* larvae. Similarly, Ali *et al.* (2004) investigated the effects of host-plant mediated variation in larval susceptibility to *Bacillus thuringiensis* between three different insect pests (*H. zea, S. exigua* and *Pseudoplusia includes*), and reported that LC_{50} did not differ significantly between soybean and tomato for any of the larval species. However on cotton the LC_{50} was greater compared to the other two host-plants (soybean and tomato) for all the three larval pests (Ali *et al.,* 2004). Other studies have reported that soybean leaves exudates has a neutral pH as compared to cotton leaves, and they attributed this to be the reason why viral OBs were not inactivated on soybean leaves compared to cotton which has high alkaline pH (Young *et al.,* 1977; McLeod *et al.,* 1977).

The above results have shown that chickpea can influence the efficacy of *Hear*NPV used for *H. armigera* control, and among all the crops tested in this study, chickpea was found to be the most detrimental to the viral OBs, while cowpea and pigeonpea were modest in their inactivation effect on NPV. This should be considered when planning to use NPV on chickpea in IPM programme, since chickpea has been reported to reduce the efficiency of other IPM components like egg parasitoid, *Trichogramma chilonis* (Romeis *et al.,* 1999), chemical insecticides (endosulfan) (Cherry *et al.,* 2000) as well as other arthropod predators (Lateef, 1985; Reed *et al.,* 1987), and could affect the persistence of NPV when used to control *H. armigera* on chickpea in the field. Understanding how chickpea host plant factors affect NPV efficacy will facilitate choice of formulation adjuvants to enhance control efficacy. This will also help to set the optimum application rate of NPV to apply for effective management of *H. armigera* on chickpea.

8.2.2 Effects of chickpea isoflavonoids on nucleopolyhedrovirus efficacy

Chemical analysis of chickpea leaf extracts (chapter 4) shows that spraying with both Triton surfactant and *Hear*NPV suspension induced higher levels of formononetin on chickpea leaf surfaces. However, results from whole leaves proved that formononetin was only induced at significantly higher levels after treatment with *Hear*NPV. In contrast, the concentration of biochanin A was not significantly altered after spraying with any of the three treatments, and was also present at higher concentrations than formononetin in both the leaf surfaces and whole leaf extracts of chickpea. The result also shows that chickpea was not reacting to wetting as suggested earlier (Stevenson *et al.,* 2010), because there was no significant differences between water treated leaves and unsprayed control leaves for formononetin or between unsprayed chickpea leaves and all the other three treatments for biochanin A.

Previous studies have identified both compounds (formononetin and biochanin A) along with their conjugates (7-*O*-glucoside and 7-*O*-glucoside-6-malonate esters) to be the main constitutive phenolics of chickpea (Kessmann and Barz, 1986). Another study noted that the level of biochanin A was twice that of formononetin in the roots of chickpea seedlings (Armeo *et al.,* 2001). Weigand *et al.* (1986) studied the accumulation of biochanin A and formononetin together with their conjugates between resistant and susceptible chickpea cultivars after infection with the fungal pathogen (*Ascochyta rabiei*). The result shows that there was no significant difference in the level of either the isoflavone aglycones (biochanin A and formononetin) or their conjugates. However, the levels of both aglycones showed a marked decrease in the susceptible cultivars compared to the resistant cultivars after infection with the fungal pathogen (Weigand *et al.,* 1986). Based on their concentrations in both cultivars, the study suggested that the two isoflavonoids are not important resistant factors of chickpea against the fungal pathogen. While no significant difference in the levels of formononetin and biochanin A was observed after treatment with virulent strain of *A. rabiei* (Weigand *et al.,* 1986), in contrast, another study demonstrated that treatment of sliced cotyledons of chickpea with crude extracts *A. rabiei* leads to a marked increase in both formononetin and biochanin A (Kessman and Barz, 1986). However they noted that none of the isoflavone conjugates levels changed after treatment with the fungal elicitor. This demonstrated that isoflavonoids are also subject to induction

to higher levels after treatment with pathogen; hence, since both could be induced after treatment with a biotic elicitor (i.e., fungal pathogen) it is possible that both compounds could respond to the presence of another pathogen, albeit an insect pathogen (*Hear*NPV) in a similar way. Because none of the isoflavone conjugate levels shows any sign of decrease after treatment with the fungus (Kessman and Barz, 1986), this excludes their possible released from their respective conjugates as result of enzyme activities (Hosel and Barz, 1985), which suggest that both compounds (biochanin A and formononetin) could also be induced after treatment with biotic elicitor.

Both formononetin and biochanin A were shown to significantly reduced *Hear*NPV infectivity when used against *H. armigera* on diet surface at ≥ 50 ppm, which was lower than the natural concentrations of both compounds in chickpea leaves after treatment with NPV suspension (formononetin) or that was present constitutively (biochanin A). Although exposure to both formononetin and biochanin A showed significant reduction in *Hear*NPV infectivity, none of the two isoflavonoids could account for the degree of inactivation that was recorded when NPV was exposed to chickpea leaf surface (Chapter 3; Stevenson *et al.,* 2010), indicating that other chickpea leaf compounds could be involved. Because formononetin was demonstrated to reduce *Hear*NPV infectivity at a concentration lower than its natural induced state after treatment with NPV, this suggest that formononetin could be acting as post-infectional (phytoalexin) defence mechanism of chickpea against biotic stress. On the other hand, based on its ability to reduced NPV infectivity at a concentration which is lower than what was found to be present on chickpea leaves at preformed state, this suggest that biochanin A could be the plant's pre-infectional (phytoanticipin) defence mechanism against biotic stress. Although none of the two isoflavonoids could account for the whole *Hear*NPV inactivation on chickpea leaves, identification of another compound (formononetin) as a defence compound of chickpea against *Hear*NPV has added to the number already existing in the literature.

Isoflavonoids such as formononetin and biochanin A which occur commonly in legumes such as chickpea are usually regarded as precursors of phytoalexins (Dakora and Phillips, 1996). They proposed that since such compounds have potential activity against the growth of many microorganisms, therefore they could also be referred to

as phytoalexin. Similarly, based on a phytopathological perspective, any part of plant can accumulate high concentrations of isoflavonoids if it is likely to be attacked by a pathogen (Dakora and Phillips, 1996), and this could be the reason for the high concentrations of biochanin A which was identified to be present constitutively on leaves of chickpea that is susceptible to attack by many fungal pathogens (Akem, 1999; Pande *et al.,* 2006). Furthermore, the concepts of phytoalexins and phytoanticipin have now been complicated due the discovery that isoflavonoids could have other biological roles in addition to being active against microorganisms. For example, it was shown that daidzein, another isoflavonoid, could stimulate the germination of *Glomus* spores (Kape *et al.,* 1992), however, equivalent concentration of formononetin significantly inhibited germination of two other *Glomus* species (Tsai and Phillips, 1991). Another complication in differentiating between the two concepts (phytoalexin and phytoanticipin) is that two identical molecules could have different functions by virtue of being released at different times (before or after infection) or in different concentrations (Dakora and Phillips, 1996; Strack, 1997). A good example of this has been demonstrated with soybean seedlings, which synthesized two isoflavones (daidzein and genistein) constitutively as preformed compounds and stored both in conjugated forms, using one as phytoalexin and the other as phytoanticipin (Graham *et al.,* 1990).

In addition, the presence of both formononetin and biochanin A were identified on both the control and infected leaf tissues of red clover (*Trifolium pratense*) (Debnam and Smith, 1976). However, they observed that only biochanin A shows any antifungal property based on its concentration on the chromatograms of the healthy leaves. They further demonstrated that although both formononetin and biochanin A were released from their conjugates in healthy tissue after fungal infection, the two compounds have little activity against virulent fungal pathogen of red clover (*Sclerotinia trifoliorum*). The activity of both compounds was investigated using *in vitro* test, the results shows that neither of the two compounds (biochanin A or formononetin) or another well-known phytoalexin (maackiain) was active against *S. trifoliorum* (Debnam and Smith, 1976). However, they noted that medicarpin was active against the pathogen at effective lethal concentration (ED_{50}) of 50-60 μg ml^{-1}, and was completely inhibitory to the pathogen at 100 μg ml^{-1}. Similarly, none of the isoflavonoids or maackiain have significant effect on the germination of another pathogen (*Botrytis cinerea*), although

the three compounds significantly reduced growth of the pathogen at relatively low concentrations (40 μg ml^{-1}), with biochanin A being the most active compound, reducing the growth by 70% and the other two by 30% compared to the control (Debnam and Smith, 1976). They observed that medicarpin was again more active against both germination and growth of *B. cinerea* at ED_{50} of between 20 - 30 μg ml^{-1}. The results shows that both biochanin A and formononetin have almost same antifungal effect with a well-known phytoalexin (maackiain), with biochanin A being the most active of the three compounds against *Botrytis* growth (Debnam and Smith, 1976). Similarly, another study determined the effect of stem nematode (*Ditylenchus dipsaci*) on white clover (*Trifolium repens*) seedlings, with reference to isoflavonoid metabolism (Cook *et al.,* 1995). The study observed that the plant mainly accumulate glycosidic conjugates (formononetin 7-*O*- glucoside-6-malonate (FGM) and medicarpin-3-*O*-glucoside-6-*O*-malonate (MGM), with no differences between the resistant or susceptible cultivars in accumulating the two compounds. However, infection with either the virulent (clover race) or avirulent (oat race) pathogen led to induction of higher levels of medicarpin and both glycosidic conjugates to a similar degree in the meristem where majority of the pathogens were found (Cook *et al.,* 1995). They noted that infection with the virulent pathogen led to higher levels of formononetin within short interval (3 to 7 days), and that of medicarpin and the respective conjugates 10 days later. Although no difference in concentration of formononetin was observed in the meristem of the susceptible cultivar after infection with either race of the pathogen, however the level of formononetin was increased on the resistant clover after infection with virulent pathogen (Cook *et al.,* 1995). They suggested that in clover, accumulation of isoflavonoids such as formononetin and medicarpin with their respective conjugates is associated with race specific resistance. This shows that formononetin could act as antimicrobial defence mechanism in certain host plants against pathogens and could be induced to higher level after infection as demonstrated to occur on chickpea leaves after treatment with *Hear*NPV. The above study also demonstrated just like in chickpea, both pre-infectional and post-infectional chemcals could be used by plants as defence mechanism against microorganisms (Strack, 1997).

When the two natural leaf compounds of chickpea (isoflavonoids and organic acids) were either added to the diet surface together with the virus directly or mixed together with aqueous suspension of *Hear*NPV prior to feeding the *H. armigera* neonates on the diet surface, the result shows that the combination of both plant compounds significantly reduced mortality caused by the virus. Furthermore, *Hear*NPV infectivity was significantly reduced when all the three components (Isoflavonoids + organic acids + NPV) were mixed together in an aqueous suspension prior to being used against the insects on diet surface. In this instance, *Hear*NPV was completely inactivated as previously reported on chickpea leaf surface (Chapter 3; Stevenson *et al.,* 2010), because NPV-induced mortality was not significantly different to that obtained with water control. This demonstrated that the combination of the three compounds (isoflavonois, organic acids plus NPV) *in vitro* was able to reciprocate the *in vivo* effect on chickpea leaves, suggesting that these compounds may account for the complete inactivation effect that was observed in the present and previous studies (Chapter 3; Stevenson *et al.,* 2010). Although both isoflavonoids (biochanin A and formononetin) were reported to reduce the efficacy of *Hear*NPV significantly (Chapter 5) when treated on the diet surface with the virus OBs, however, the inhibition effect was lower compared to when all the three components (isoflavonoids, organic acids and NPV) were mixed together in aqueous suspension together with NPV (Chapter 6). The significance of the findings is that both isoflavonoids plus organic acids reduced larval mortality. However, the effect of isoflavonoids was not concentration-dependent, and organic acids alone in combination with *Hear*NPV could account for the low larval mortality similar to that obtained with isoflavonoid plus organic acid combination or the mixture of both isoflavonoids plus organic acids. This strongly suggested that the inactivation could be attributed to organic acids alone and not that of the isoflavonoids *per se.*

Another similar *in vitro* study have shown that viral-induced *H. zea* larval mortality was significantly reduced when tannic acid was added to *Hz*NPV suspension that larvae ingested prior to being placed on the diet (Young *et al.,* 1995). They noted also that larval mortality did not differ significantly with the concentration of tannic acids in the viral suspension. However they observed that, larval mortality was not altered significantly when larvae were exposed to virus suspension alone prior to being placed on diet containing tannic acid (Young *et al.,* 1995). They suggested that much of the

antiviral effect of tannic acid might have been directed against the polyhedra itself as mortality was reduced when tannic acid was added to the viral suspension before being used against the larvae on the diet. Similarly, others demonstrated that *in vitro* treatment of NPV OBs with peroxidase and phenolic substrates in mixture only prevented viral disease, indicating that NPV stability could be affected directly during redox cycling such as phenolic oxidation leading to the generation of free radicals (Hoover *et al.,* 1998c). Because phenolics such as formononetin and biochanin A could bind to viral OBs preventing them from initiating primary infection in the insect midgut cells (Pierpoint *et al.,* 1977; Felton and Duffey, 1990), this could possibly explain the reduction of *Hear*NPV induced mortality that was observed when NPV OBs were mixed together with isoflavonoids and organic acids combination prior to placement on diet surface. Hoover *et al.* (1998) also proposed that virus particles released from the OBs could bind to each other and to semiquinones thereby preventing them from initiating infection in midgut cells. However, the degree of *Hear*NPV inactivation observed when isoflavonoids alone were added with the viral OBs on the diet surface or when isoflavonoids plus organic acids were added together with NPV and fed to the larvae on diet surface was lower compared to that recorded when both isoflavonoids and organic acids were mixed with aqueous suspension of the virus prior to placing on the diet surface, this demonstrates some of the experimental difficulties mentioned in establishing course and effects (Duffey *et al.,* 1995), particularly in tritrophic interactions where *in vitro* and *in vivo* results are sometimes inconsistent (Hoover *et al.,* 1998b,c; Ali *et al.,* 1999). One possible reason given for the limited impact in using in *vitro* experiments to demonstrate effects of host plant on viral disease, is that it is difficult to simulate practically all the physical and chemical interactions that occurs simultaneously *in vivo* (Hoover *et al.,* 1998b,c). This is particularly true for phenolics which could sometimes act as antioxidants (Ahmed, 1992) or prooxidants (Ahmed, 1992; Summers and Felton, 1994), depending on physiochemical context of host plant tissue and insect's digestive system (Johnson and Felton, 1996; Duffey and Stout, 1996).

8.2.3 Role of chickpea organic acids on nucleopolyhedrovirus efficacy

When different concentrations of the most abundant chickpea organic acids (malic and oxalic) were mixed with aqueous *Hear*NPV suspension at high lethal concentration (LC_{75}) prior to feeding the insect larvae on diet surface, NPV infectivity was significantly reduced compared to unexposed NPV. All the organic acids treated *Hear*NPV significantly reduced larval mortality, and the effect was concentration-dependent for oxalic acids. The study also demonstrated that of the two organic acids, oxalic acid had a more potent inactivating effect. Larval mortality was found to be strongly correlated with the pH of the organic acids, which meant that the lower the pH (high acidity) the lower the efficacy of the virus and higher the larval survival, and at same concentration, oxalic acid had a lower pH than malic (see table 7).

The most striking findings from this study is that the most abundant organic acid of chickpea completely inactivate *Hear*NPV activity at high lethal concentration (LC_{75}), bringing the larval mortality equivalent to that obtained with water control treatment. The high degree of inactivation observed when the organic acids were mixed together with the virus, could account for the level of *Hear*NPV inactivation previously reported on chickpea leaf surfaces (Chapter 3; Stevenson *et al.,* 2010). The absence of organic acids on cowpea and pigeonpea could be the reason for the significant but lower reduction in *Hear*NPV efficacy that was recorded on those two crops. The fact that inactivation on chickpea was reported to occur rapidly (within 1 h) (Stevenson *et al.,* 2010), suggests that the presence of the organic acids constitutively on the leaf surface of chickpea is responsible for the rapid and marked effect observed. The result also shows that reducing the natural concentration of the organic acids by half significantly reduced efficacy of *Hear*NPV to about 45%, possibly because at this concentration the pH was the highest among all the organic acid treatments tested (pH 1.6). The present study has shown that the natural concentration of the chickpeas most abundant organic acids (Rembold and Weigner, 1990) could totally inactivate NPV, however, the concentration of those acids was reported to vary depending on location, temperature, or growth stage of the plant (Rembold, 1981; Kaudal and Sinha, 1981; Rembold *et al.,* 1990a,b). This could account for the differences in NPV efficacy that have been reported in different chickpea varieties (Rabindra *et al.,* 1992; Cowgill and Bhagwat, 1994; Cherry *et al.,* 2000). This is in line with previous findings that highly

acidic conditions (p H ≤ 2) could significantly reduce NPV efficacy substantially (Ignoffo and Garcia, 1966; Gudauskas and Canerday, 1968). Similarly, human gastric juice (pH 1.2) was also reported to significantly inactivate viral OBs (Chauthani *et al.,* 1968) and that of fungal spores of *Nomuraea rileyi* (Ignoffo and Garcia, 1977). Others have also shown that low pH of the viral suspension in the tank mixes used in the field also significantly reduced *Ag*MNPV efficacy (Silva and Moscardi, 2002). Similarly, others have also demonstrated that removing the chickpea exudates from plants with water prior to inoculation with fungal pathogen (*A. rabiei*) increased the fungal infection on susceptible cultivars 5 weeks after planting (Armstrong and Gossen, 2005). They noted that on the resistant cultivar, the effect of washing the exudates on infection was not visible until late in the early podding stage, when the natural plant's resistance declines. The present study suggests that oxalic acid was more detrimental to *Hear*NPV efficacy, due to its lower pH. However, no explanation could be obtained from the past literature on the mechanism by which oxalic acid affects virus activity. This study also suggests that the effect of oxalic acid on NPV might not necessarily be due the acidic effect alone.

Felton *et al.* (1987) proposed that biological control might not be compatible with host-plant resistance (HPR) where the resistant factor in the plant is based on antibiosis. Because oxalic acid in chickpea has an antibiotic effect on *H. armigera* larvae (Yoshida *et al.,* 1995), this could account for the incompatibility between *Hear*NPV and resistant chickpea cultivars reported previously to be caused by the oxalic acids in the exudates of the resistant cultivars (Rabindra *et al.,* 1992; Cowgill and Bhagwat, 1994). Similarly, use of another insect pathogen (*B.t*) to control insect pests on plants with high tannin content was reported to be ineffective, because the high tannin content of the plant could directly inactivate the δ-endotoxin (Navon *et al.,* 1993), as well as NPV OBs (Young *et al.,* 1995). Tannin was also reported to antagonise *B.t* efficacy when used at high potency concentration, reducing larval mortality by about 2-fold (Bauce *et al.,* 2006). They suggested that in developing *B.t* transgenic host-plant (i.e. spruce trees), this antagonistic effect of tannin on *B.t* toxin should be considered. Other studies demonstrated that as foliar tannin content of host tree increased, the efficacy of *B.t* formulation decreased when used against Spruce budworm (Carisey *et al.,* 2004). Therefore, in the presence of tannin, application of high concentrations of *B.t* formulation against Spruce budworm did not lead to an increase in larval mortality

compared to moderate *B.t* concentrations (Bauce *et al.,* 2006). The present study also demonstrated that even at high lethal concentration of *Hear*NPV, chickpea organic acids was able to significantly reduce the efficacy of *Hear*NPV used against *H. armigera* larvae. This could also explain why increasing the virus concentration application has not enhanced NPV persistence (Grzywacz, 1998). It is surprising that despite all these reports another recent study reported that organic acids of chickpea enhanced the activity of *B.t* against *H. armigera* larvae (Devi *et al.,* 2011; 2013a,b). However it should be noted that the methodology that was used (i.e. incorporating lyophilized leaves and pods of chickpea) in the previous studies (Devi *et al.,* 2011; 2013a,b) does not realistically simulate the leaf surface based inactivation effect that was reported in both the previous and present study (Chapter 7; Stevenson *et al.,* 2010).

It has been demonstrated that calcium-binding protein isolated from the water lettuce plant (*Pista stratiotes*) is involved in calcium-oxalate (CaOx) formation (Li *et al.,* 2003). They proposed that the matrix protein with their calcium-binding activity could serve as agents for CaOx crystal growth. However, another study noted that the CaOx binding proteins of rat and human kidney were only specific for oxalate (Adhirai and Selvam, 1998). They observed that other Ca^{2+} chelators and inhibitors such as ruthenium red, calcium chloride and magnesium chloride do not affect binding activity, which indicates that it is only the oxalate ion moeity that is responsible for specific binding of CaOx and not the calcium ion part. From this it could also be proposed that the mechanism of *Hear*NPV inactivation on chickpea is through oxalate binding to the NPV OBs on chickpea leaf surface which subsequently prevent their release in the insect midgut cells to initiate infection. This idea could be supported from the result of electron microscope (SEM), which shows that NPV inactivation on chickpea was not due to any major change in the morphological structure of the viral particles.

8.2.4 Implication for future development of nucleopolyhedrovirus formulations and other biopesticides on chickpea

Development of formulation for baculovirus has not been a systematic process, usually because the goals required for formulation such as length of persistence in the field

has not been clearly defined (Jones, 1994; Cherry *et al.,* 2000). The extent by which the host-plant affect the persistence of viral insecticides as well as the mechanisms involved has not been clearly understood (Cory and Hoover, 2006). Usually efforts were directed at stabilizing the virus before and after application using different kinds of additives (Jones, 1994; Behle and Birthisel, 2014). Efficient selection of potential adjuvants to reduce the host-plant mediated effect require proper understanding of the mechanism of action of the plant compound(s) on the microbial pesticides (Hoover *et al.,* 2000; Cory and Hoover, 2006). However, less attention was given to this area of research by the scientific community (Behle and Birthisel, 2014).

The present study has demonstrated that organic acids secretions from chickpea leaf exudates are responsible for the inactivation of *Hear*NPV on chickpea leaf surfaces. Therefore, identifying a low cost additive that could prevent or reduce the pH-mediated effect of the organic acids in the NPV formulation and at the same time maintaining the stability of the viral OBs (i.e. preventing the alkaline-mediated dissolution of OBs) could improve the efficacy of the virus and increase larval mortality. It was reported by Murray *et al.* (2000) that addition of milk powder supplement (Denkavit®) to commercial NPV (Gemstar®) enhanced larval mortality significantly on cotton with more than 77.4% remaining 24 h after application compared to use of Gemstar alone, which recorded significantly low mortality of 34.9% on cotton. However, no clear explanation was given on how the milk additive help to improve viral efficacy, although they suggested that the additive could be acting as feeding stimulant or provide protection against host-plant compounds or as sun screen. Similarly, another preliminary field trial have shown semi-skimmed milk to be the most effective among the low cost additives tested (i.e. robin blue, casein and casein/lactose mixture) in improving *Hear*NPV persistence on chickpea (D'Cunha, 2007). Although all the other additives also proved to enhance field persistence of NPV, however the study noted that none of them was effective compared to unformulated NPV suspension. However, the unformulated virus was reported to lose its stability within 24 h after treatment, and addition of semi-skimmed milk was found to enhance the field persistence of the virus (D'Cunha, 2007). Another field study also reported that the unformulated NPV suspension was no worse than other formulated products of the virus tested (Cherry *et al.,* 2000) although they also observed that the

virus and all other pest control products such as *B.t* and chemical insecticide (endosulfan) had short persistence on chickpea.

Improvement in efficacy of microbial pest control agent could be achieved through stimulating feeding and increasing field persistence (Luttrell *et al.,* 1983). Therefore, application method and use procedures that enhance the ingestion of the lethal dose of the viral pathogen by the target insect have been observed to be consistent in improving the performance and level of control (Bell, 1982). Previosly both laboratory and field studies have also shown that commercial adjuvants (Coax® and Gustol®) could increase the efficacy of viral insecticide against *H. zea* larvae on cotton mainly through stimulating feeding and improving the spray characteristics in terms of coverage and amount of materials deposited (Luttrell *et al.,* 1983). Although high mortality of early instar larvae was reported to be obtained by either increasing the virus rate or the feeding time (Ignoffo *et al.,* 2001), however, on chickpea use of high *Hear*NPV rate did not improve the level of control in the field (Grzywacz, 1998) nor in the laboratory studies (Chapter 7). However, another laboratory study reported that 98% larval mortality (*H. zea*) could be obtained regardless of the viral rate after only 24 h of feeding by the early larval instar on diet surface (Ignoffo *et al.,* 2001). They also noted that after 48 h of feeding all viral rates used (0.1, 1.0, 10, 100 and 1000 OB mm^2) induced > 98% larval mortality. They proposed that provided the virus insecticide is applied uniformly and well protected against solar UV light, this will translate in to more than 50% of early-, medium- or late- stadium larvae that may be anticipated from any viral rate ranging from 10 to 1000 OBs per mm^2. They also proposed that complete protection of applied virus from sunlight inactivation for at least 12 h could provide effective control against damaging effect of field populations of *H. zea* larvae except late instars. In another field study, liquid equivalent of Denkavit (milk powder supplement) additives were compared for their potential to improve the efficacy of commercial viral insecticide (Gemstar) in the field (Grundy and Short, 2002). Their results showed that addition of either Aminofeed® or another similar product with additional solar UV protection ability (Aminofeed UV®) both enhanced the activity of Gemstar in reducing *H. armigera* larvae on cotton. They noted that both additives enhance viral-induced mortality by increasing the persistence of the virus on cotton foliage for up to 24 h, with Aminofeed UV providing more stability to the virus in warm and drier environment and therefore was significantly better in increasing larval

mortality when added to the virus suspension compared to either addition of Aminofeed or use of Gemstar alone (Grundy and Short, 2002).

The present study has shown that high acidic conditions present on chickpea leaf surface could be responsible for the rapid inactivation of *Hear*NPV, and from the above studies it has been demonstrated that use of milk additives could improve the virus efficacy through increasing the field persistence of the virus (Grundy and Short, 2002; D'Cunha, 2007). Although no explanation could be given on how this could be achieved. The present study has shown through scanning electron microscope that no major physical change was observed after *Hear*NPV OBs were exposed to chickpea leaf surfaces, which suggests that there was no major change in the viral particles morphological structure when compared to the unexposed *Hear*NPV. This could also indicate that the highly acidic condition of the chickpea exudates did not caused any physical toxic effect on the viral OBs. Similarly, the present result has also shown that of the two most abundant organic acids used, oxalic acid seems to be more active in reducing the virus induced mortality which could be attributed to its high acidity compared to that of malic acid or due to its other antibiotic effects (Yoshida *et al.,* 1995). Another possible explanation for the inhibition of *Hear*NPV on chickpea through the action of oxalic acid is the effect of oxalate ion binding on the viral OBs immediately after application, thereby preventing them from initiating infection in the midgut environment. Another related study reported on the leaf factors that were responsible for altering gypsy moth (*Lymantria dispar*) larvae to *Ld*NPV and found out that larval mortality was strongly correlated negatively with leaf tissue pH, hydrolysable tannin content as well as protein binding capacity of the foliage (Keating *et al.,* 1988). The protein binding capacity of foliage extracts was found to correlate negatively with larval mortality, and a significant increase in mortality was observed in larvae fed contaminated foliage after a 40% decline in black oak binding capacity (Keating *et al.,* 1988). Similarly, host plant (oak species) were found to affect the efficacy of commercial *B.t* formulation (Thuricide®), and larval mortality was observed to correlate negatively to concentration of phenolics and protein binding activity in the leaves (Appel and Schultz, 1994). Based on those results, the *Hear*NPV inactivation on chickpea leaf surface reported in this study could have been due to the binding activity of oxalate ion of the oxalic acid as well as the highly acidic effect of the organic acids. Others have also proposed that there are multiple causes and effects

in the inhibition of viral diseases (Hoover *et al.,* 1998b,c), therefore it could be that there are multiple causes of the inactivation of *Hear*NPV on chickpea.

Interestingly, the demonstration by others (D'Cunha, 2007; Murray, 2000; Grundy and Short, 2002) that milk additives was found to improve the effectiveness of viral insecticides by increasing larval mortality is also an indication that the milk additives are either reducing the pH-mediated effect of the leaf exudates (alkaline on cotton and acidic on chickpea) or reducing the binding effect of the oxalate ion or both. In addition, another related study observed that feeding infants with milk before vaccination with attenuated bovine rotavirus (virus that cause severe diarrhoea in infants and young children) was found to neutralize the gastric juice acidity and enhance the stability of the virus (Vesikari *et al.,* 1984). They also showed through *in vitro* test that rotavirus was active at pH 4 but was inactivated at lower pH ≤ 3. This is similar to the pH range that was reported to inactivate viral insecticide (Ignoffo and Garcia, 1966; Gudauskas and Carnerday, 1968), therefore it is possible that use of milk additives in the viral insecticides could act as buffer by neutralising the acidity of viral suspension and /or prevent the binding of oxalate ion to the viral OBs either through forming a coating on the surface of the polyhedral or alternatively providing a substitute for binding by the oxalate, thereby freeing the viral OBs. Interestingly, most of the additives that were reported to enhance viral insecticide efficacy particularly on chickpea or cotton were protein based (i.e. milk, casein, lactose, cotton seed flour etc.), which is in line with the protein binding hypothesis suggested earlier, although other properties of the milk components, such as providing protection against UV light and enhancing feeding, cannot be excluded.

The potential for effective control of *H. armigera* on chickpea using *Hear*NPV is good and this has been demonstrated already by others in the field (Rabindra *et al.,* 1992; Cherry *et al.,* 2000). However, a comprehensive understanding of the tritrophic level of interactions between chickpea, *H. armigera* larvae and the viral insecticides could provide a basis for enhancing the efficiency as well as reducing the variability of control that was often reported on chickpea. Through this knowledge, suitable formulation additives could be selected to enhance the protection of the virus in the field against host-plant factor or UV radiation, before it is eaten by the target larvae. Finally, the selected additive(s) should be compatible with the inherent property of the

virus pathogen and feasible to apply and relatively cheap. All these will only be successful if incorporated into an IPM package through proper laboratory and field research, particularly in choosing the optimum NPV rate that will be suitable for controlling *H. armigera* larvae in the field.

8.4 Conclusions

1. The resent study has demonstrated that chickpea is the most phytochemically antagonistic plant against *Hear*NPV efficacy among all the species tested. Chickpea leaf surfaces leads to the substantial inactivation of *Hear*NPV within a short period of time, which was in agreement with earlier findings.

2. The present result has shown for the first time that the inactivation effect was caused by the organic acids present constitutively on leaf surfaces of chickpea. This is the first study to report on the inactivation of viral insecticide by the highly acidic exudate of chickpea trichomes. The results demonstrated that *H. armigera* larval mortality was positively correlated with pH of the two most abundant acids present in the chickpea exudates.

3. Oxalic acid was shown to be more important compared to malic acid, probably due to it being more acidic compared to malic acid and also because it caused higher larval mortality compared to malic acid at same concentration. This was largely due to the strength of their ionisation (stability); oxalic acid with lower pKa (1.25) value is more acid than malic acid with higher value (pKa 3.40). The inactivation effect of chickpea organic acids was not linked to any major change in the morphological structure of the viral particle based on the results from electron microscopy (SEM). This study has also shown that the two most abundant chickpea organic acids (malic and oxalic) could reduce the viral-induced mortality equal to that of the water control (i.e. produce total inactivation).

4. It is hypothesised that apart from the acidic effect, oxalic acid might also be affecting the *Hear*NPV OBs through its oxalate ion binding effect on the viral

protein matrix thereby affecting their solubility and subsequently preventing the virions from initiating primary infection in the insect midgut cells.

5. This study has shown that chickpea leaf isoflavonoids (formononetin and biochanin A) are relatively less important in *Hear*NPV inactivation than the chickpea organic acids (malic and oxalic). Previous studies Stevenson *et al.* (2010) have also shown that although both sissotrin and biochanin A also significantly affect the efficacy of NPV however, the effect of both isoflavonoids could not completely account for the total inactivation that was recorded on chickpea leaf surfaces

6. Even though, the present work has shown that isoflavonoids might not be important against NPV on chickpea, those compounds could be important in other legumes such as cowpea and pigeonpea.

8.5 Suggestions for future work

The following studies are recommended as future work because they could not be carried out in this study either due to lack of time or resources.

1. Determine the effect of each of two acids (malic and oxalic) individually using the *in vitro* model used in this study to identify conclusively which is more active against *Hear*NPV. For example, oxalic acid should be validated alone at $\geq$ 12 mg ml^{-1}, the concentration at which it was found to inactivate the NPV in present work. In addition, future work should also determine the role of oxalate ion binding effect on the viral protein matrix on NPV efficacy using a suitable laboratory experiments.

2. Determine the suitability of low-cost additives that have been tested earlier by others (Murray, 2000, Grundy and Short, 2002; D'Cunha, 2007), using the *in vitro* model that was developed in this study and subsequently in the field. Such additives should take into consideration their potential to act as pH buffer (neutralising the acidic condition of chickpea leaf exudates), and possibly prevent or reduce the oxalate binding effect on the NPV OBs. The additives

selected should also be compatible with the special requirements of the virus particle. Because previous studies have mentioned that additives selected to enhance the physical properties of the formulation could also cause reduction in the efficacy of the viral insecticides (Hostetter *et al.,* 1982; Matthews, 2001) or affect the physiology of the plant (Ballard *et al.,* 2000; Goulson *et al.,* 2003), the additives selected should be compatible with inherent biological properties of the virus.

3. Further studies are needed to determine the factors in the unpurified NPV which causes it to be more effective than other virus formulations, particularly whether this has anything to do with the insect protein and other metabolites in the insect cadaver. Because chickpea organic acids have been reported to vary depending on the location, growth stage of the plant and weather conditions (i.e. temperature) (Rembold, 1981; Kaudal and Sinha, 1981; Rembold *et al.,* 1990a,b), further field studies are required to determine the optimum field application rate to use on chickpea at different locations to reduce the pH-dependent inactivation.

4. Develop a simple technique of testing the pH of the chickpea leaf exudates (i.e. using pH paper) in the field so as to determine the strength of the buffer required to neutralise the acidic conditions on chickpea leaves.

5. Further field studies should also be carried out to determine whether the inactivating effect demonstrated in this study on pigeonpea and cowpea will manifest in the field. This is particularly important in Africa, where efforts are underway to use biological control against cowpea insect pests, such *Maruca vitrata*. Field studies are also needed to determine quantitatively the NPV inactivation effect of solar UV in relation to that caused by chickpea organic acids.

6. As previous studies have shown a possible link between phenolics binding to viral protein coat and their ability to initiate infection in the insect midgut cells (Felton *et al.,* 1987; Felton and Duffey, 1990), and this study has also suggested that such possible mechanism could also be occurring with the oxalate ion and the viral OBs. Further studies are needed to verify this possibility, and if both

mechanisms were found to be the same, then both inactivation effects could be prevented using one single adjuvant which will help to reduce cost, expand the use of viral insecticides to other potential crops as well as increase adoption of this novel pest control technology.

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Appendices

Appendix 1: Data Analysis

1.1 Analysis for Chapter 3

Analysed data comparing the median LC_{50} of *Hear*NPV exposed to chickpea and other treatments using Kruskal-Wallis one way ANOVA on Ranks.

Group	N	Missing	Median	25%	75%
Log NPV	7	0	3.343	3.250	3.437
Log tomato	7	0	3.167	2.885	3.523
Log Pigeon	5	0	3.857	3.556	3.962
Log Cowpea	5	0	3.931	3.794	4.049
Log Chickpea	5	0	8.903	8.555	8.990

H = 21.619 with 4 degrees of freedom. (P = <0.001)

ANOVA for LC_{50} of *Hear*NPV exposed to cowpea, pigeonpea and unexposed *Hear*NPV

Response: val

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
trt	3	182173780	60724593	12.089	9.796e-05 ***

Residuals 20 100459488 5022974

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Tukey multiple comparisons of means

95% family-wise confidence level

Trt

	diff	lwr	upr	p adj
NPV-Cowpea	-6556.5429	-10229.6202	-2883.4655	0.0003738
Pigeonpea-Cowpea	-2191.2000	-6158.5770	1776.1770	0.4303797
Tomato-Cowpea	-6435.2571	-10108.3345	-2762.1798	0.0004610
Pigeonpea-NPV	4365.3429	692.2655	8038.4202	0.0163197
Tomato-NPV	121.2857	-3231.7598	3474.3312	0.9996152
Tomato-Pigeonpea	-4244.0571	-7917.1345	-570.9798	0.0199593

1.2 Analysis for Chapter 4

Analysed data comparing formononetin induced on chickpea leaf surfaces after spraying water at different time interval with unsprayed control leaves as determined by LC-MS using ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.105)

Equal Variance Test: Passed (P = 0.827)

Group Name	N	Missing	Mean	Std Dev	SEM
Control 0hr	5	0	7173.000	4223.491	1888.803
Control 48hr	4	0	3883.000	2701.117	1350.559
Water 24 hr	3	0	12681.333	3593.735	2074.844

Source of Variation	DF	SS	MS	F	P
Between Groups	2	133378006.250	66689003.125	5.041	0.034
Residual	9	119069472.667	13229941.407		
Total	11	252447478.917			

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Water 24 hr vs. Control 48hr	8798.333	3.167	0.034	Yes
Water 24 hr vs. Control 0hr	5508.333	2.074	0.131	No
Control 0hr vs. Control 48hr	3290.000	1.348	0.210	No

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Analysed data comparing formononetin induced on chickpea leaf surfaces after spraying with water at different time interval with unsprayed control leaves as determined by LC-MS using Kruskal-Wallis one way ANOVA on Ranks

Group	N	Missing	Median	25%	75%
Control 0hr	5	0	6379.000	4324.500	10418.500
Water 2 hr	5	0	3996.000	3336.500	7036.500
Water 24 hr	3	0	12025.000	9461.000	16558.000
Water 48hr	3	0	3717.000	1808.000	4574.000

H = 8.041 with 3 degrees of freedom. (P = 0.045)

All Pairwise Multiple Comparison Procedures (Dunn's Method):

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
Water 24 hr vs Water 48hr	10.000	2.572	No
Water 24 hr vs Water 2 hr	7.933	2.282	Do Not Test
Water 24 hr vs Control 0hr	4.733	1.361	Do Not Test
Control 0hr vs Water 48hr	5.267	1.515	Do Not Test
Control 0hr vs Water 2 hr	3.200	1.063	Do Not Test
Water 2 hr vs Water 48hr	2.067	0.594	Do Not Test

Analysed data comparing formononetin level induced on chickpea leaf surfaces after spraying with Triton surfactant at different time interval with unsprayed control leaves as determined by LC-MS using ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.447)

Equal Variance Test: Passed (P = 0.609)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Control 48hr	4	0	3883.000	2701.117	1350.559
Triton 2 hr	5	0	15129.200	7742.350	3462.484
Triton 8 hr	5	0	35482.400	8043.112	3596.989
Triton 24 hr	5	0	15066.400	12127.495	5423.581

Triton 48 hr	5	0	24573.000	7815.199	3495.063

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	4	2575167150.758	643791787.690	9.040	<0.001
Residual	19	1353044485.200	71212867.642		
Total	23	3928211635.958			

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Triton 8 hr vs. Control 48hr	31599.400	5.582	<0.001	Yes
Triton 8 hr vs. Triton 24 hr	20416.000	3.825	0.010	Yes
Triton 8 hr vs. Triton 2 hr	20353.200	3.813	0.009	Yes
Triton 48 hr vs. Control 48hr	20690.000	3.655	0.012	Yes

Analysed data comparing formononetin level induced on chickpea leaf surfaces after spraying with *Hear*NPV with unsprayed control leaves as determined by LC-MS using Kruskal-Waalis one way Analysis of Variance on Ranks.

Group	**N**	**Missing**	**Median**	**25%**	**75%**

Control 0hr	5	0	6379.000	4324.500	10418.500
NPV 2 hr	5	0	23349.600	10632.600	28802.900
NPV 8 hr	5	0	27530.000	17936.200	43773.750
NPV 24 hr	4	0	31912.200	26050.850	65023.075
NPV 48 hr	5	0	19872.900	13464.050	26603.900

H = 13.697 with 4 degrees of freedom. (P = 0.008)

Multiple Comparisons versus Control Group (Dunn's Method):

Comparison	**Diff of Ranks**	**Q**	**P<0.05**
NPV 24 hr vs Control 0hr	16.150	3.405	Yes
NPV 8 hr vs Control 0hr	12.800	2.862	Yes
NPV 2 hr vs Control 0hr	8.800	1.968	No
NPV 48 hr vs Control 0hr	8.200	1.834	Do Not Test

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Analysed data of biochanin A level recorded on chickpea leaf surfaces after treatment with water, Triton surfactant and HearNPV with unsprayed control leaves as determined by LC-MS using Kruskal-Wallis one way ANOVA on Ranks.

Group	N	Missing	Median	25%	75%
Control 0hr	5	0	32811.000	19007.500	63371.000
Control 48hr	5	1	30491.500	17128.750	96002.500
Water 2 hr	5	0	15082.000	11609.000	25470.000
Water 24 hr	5	0	72356.000	43518.000	106978.000
Water 48hr	5	0	32341.000	20251.500	44430.000
Triton 2 hr	5	0	28471.000	11325.000	53589.500
Triton 8 hr	5	0	58060.000	27210.000	125811.000
Triton 24 hr	5	0	24169.000	21586.500	44683.500
Triton 48 hr	5	0	35589.000	31054.500	60831.000
NPV 2 hr	5	1	35074.500	27210.500	64895.500
NPV 8 hr	5	0	36729.000	30602.500	86796.000
NPV 24 hr	5	1	31868.500	20336.250	90158.000
NPV 48 hr	5	0	28399.000	17262.500	40424.000

H = 16.202 with 12 degrees of freedom. (P = 0.182)

Analysed data of formononetin level induced on chickpea whole leaves after spraying with water, Triton surfactant or *Hear*NPV with unsprayed control leaves as determined by LC-MS using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.479)

Equal Variance Test: Passed (P = 0.725)

Group Name	N	Missing	Mean	Std Dev	SEM
log10(col(1))	5	0	5.843	0.163	0.0730
log10(col(2))	5	0	5.676	0.162	0.0725
log10(col(3))	5	0	5.649	0.101	0.0450
log10(col(4))	5	0	5.795	0.174	0.0779
log10(col(5))	5	0	5.779	0.117	0.0523
log10(col(6))	5	1	5.812	0.292	0.146
log10(col(7))	5	0	5.831	0.144	0.0646
log10(col(8))	5	0	5.786	0.121	0.0540
log10(col(9))	5	0	5.965	0.121	0.0542
log10(col(10))	5	0	5.947	0.172	0.0769
log10(col(11))	5	0	6.321	0.177	0.0791
log10(col(12))	5	0	6.352	0.336	0.150
log10(col(13))	5	1	6.248	0.169	0.0846

Source of Variation	DF	SS	MS	F	P
Between Groups	12	3.228	0.269	8.072	<0.001
Residual	50	1.666	0.0333		
Total	62	4.894			

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
log10(col(1) vs. log10(col(12	0.509	4.406	<0.001	Yes
log10(col(1) vs. log10(col(11	0.477	4.135	0.001	Yes
log10(col(1) vs. log10(col(13	0.404	3.303	0.018	Yes

Analysed data of biochanin A level recorded on chickpea whole leaves after spraying with water, Triton surfactant or *Hear*NPV as determined by LC-MS using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.382)

Equal Variance Test: Passed (P = 0.518)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Control 0hr	5	0	3069479.800	1234462.865	552068.577

Control 48hr	5	0	2106849.800	1069341.577	478224.091
Water 2 hr	5	0	2736990.800	627930.736	280819.162
Water 24 hr	5	0	3379507.000	551046.317	246435.405
Water 48hr	5	0	2721573.600	505243.480	225951.753
Triton 2 hr	5	1	2521338.500	422454.576	211227.288
Triton 8 hr	5	0	2973322.000	1082023.492	483895.616
Triton 24 hr	5	0	2513939.400	911882.002	407806.029
Triton 24 hr	5	0	2513939.400	911882.002	407806.029
Triton 48 hr	5	0	3437874.600	973112.594	435189.182
NPV 2 hr	5	0	3969835.200	1193265.773	533644.677
NPV 8 hr	5	0	3554998.400	885910.322	396191.140
NPV 24 hr	5	0	2212957.600	1203815.749	538362.770
NPV 48 hr	5	1	2754786.500	1182962.732	591481.366

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	13	1.858E+013	1.429E+012	1.576	0.121
Residual	54	4.897E+013	906865534230.785		
Total	67	6.755E+013			

Analysed data comparing water, Triton surfactant and *Hear*NPV induction of formononetin on chickpea leaf surfaces using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.468)

Equal Variance Test: Passed (P = 0.406)

Group Name	N	Missing	Mean	Std Dev	SEM
Water log	16	0	3.757	0.263	0.0656
Triton log	20	0	4.273	0.302	0.0674
NPV log	19	1	4.398	0.216	0.0510

Source of Variation	DF	SS	MS	F	P
Between Groups	2	3.875	1.938	27.790	<0.001
Residual	51	3.556	0.0697		
Total	53	7.431			

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Water log vs. NPV log	0.641	7.067	<0.001	Yes
Water log vs. Triton log	0.516	5.827	<0.001	Yes

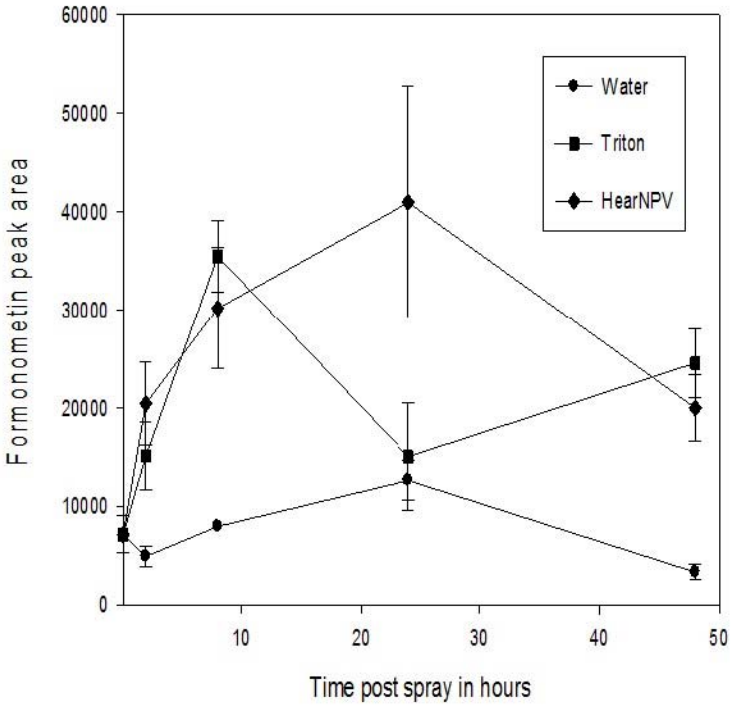
Analysed data to determine the interaction between spray and time on induction of formononetin levels on chickpea leaf surface using two way ANOVA.

Dependent Variable: log10(col(3))

Normality Test (Shapiro-Wilk) Passed (P = 0.812)

Equal Variance Test: Passed (P = 0.266)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Spray	2	0.149	0.0746	1.594	0.222
Time	3	0.606	0.202	4.318	0.013
Spray x Time	6	0.911	0.152	3.246	0.016
Residual	27	1.263	0.0468		
Total	38	2.773	0.0730		



Graph of formoonetin induced on chickpea leaf surfaces over time after spraying with water, Triton surfactant and *Hear*NPV

1.3 Analysis for Chapter 5

Analysed data to determine the difference between water and acetone treated diet on *H. armigera* larval mortality using t-test.

Normality Test (Shapiro-Wilk) Passed (P = 0.753)

Equal Variance Test: Passed (P = 0.515)

Group Name	N	Missing	Mean	Std Dev	SEM
H2O	4	0	16.725	14.219	7.109
Acetone	4	0	11.700	9.520	4.760

Difference 5.025

t = 0.587 with 6 degrees of freedom. (P = 0.578)

Analysed data to determine the effect of formononetin on *Hear*NPV efficacy used against *H. armigera* larvae using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.437)

Equal Variance Test: Passed (P = 0.579)

Group Name	N	Missing	Mean	Std Dev	SEM

HearNPV	6	0	4015.000	4524.968	1847.311
F 5000ppm	8	0	9604.000	3485.434	1232.287
F50 ppm	7	0	8871.714	2182.043	824.735
F5 ppm	5	0	6524.000	4425.045	1978.940

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	3	126204007.187	42068002.396	3.145	0.046
Residual	22	294306397.429	13377563.563.519		
Total	25	420510404.615			

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
HearNPV vs. F 5000ppm	9123.750	3.925	0.017	Yes
HearNPV vs. F50 ppm	7481.250	2.838	0.050	Yes
HearNPV vs. F5 ppm	6033.250	2.289	0.056	No

Analysed data to determine the effect of biochanin A on *Hear*NPV efficacy used against *H. armigera* larvae using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.265)

Equal Variance Test: Passed (P = 0.684)

Group Name	N	Missing	Mean	Std Dev	SEM
Log Hear	4	0	3.176	0.205	0.102
log Bio500	4	0	3.957	0.220	0.110
Log Bio 50	4	0	3.644	0.213	0.107

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.234	0.617	13.637	0.002
Residual	9	0.407	0.0453		
Total	11	1.641			

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Log Hear vs. log Bio500	0.780	5.188	0.001	Yes
Log Hear vs. Log Bio 50	0.468	3.109	0.013	Yes

1.4 Analysis for Chapter 6

Analysed data to determine the effect of isoflavonoids in combination with chickpea organic acids on *Hear*NPV (LC_{25}) efficacy used against *H. armigera* larvae using one way ANOVA.

Normality Test (Shapiro-Wilk) Passed (P = 0.162)

Equal Variance Test: Passed (P = 0.182)

Group Name	N	Missing	Mean	Std Dev	SEM
Acid	7	0	7.071	9.048	3.420
NPV	7	0	31.857	13.471	5.092
NPV+acid	7	0	16.286	9.447	3.570
NPV + F+B+Acid	7	0	17.429	9.641	3.644
NPV+F+acid	7	0	16.714	13.450	5.084
NPV+B+acid	7	0	5.571	8.443	3.191

Source of Variation	DF	SS	MS	F	P
Between Groups	5	3096.554	619.311	5.326	<0.001
Residual	36	4186.357	116.288		
Total	41	7282.911			

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
NPV vs. NPV+B+acid	26.286	4.560	<0.001	Yes
NPV vs. Acid	24.786	4.300	<0.001	Yes
NPV vs. NPV+acid	15.571	2.701	0.031	Yes
NPV vs. NPV+F+acid	15.143	2.627	0.025	Yes
NPV vs. NPV + F+B+Acid	14.429	2.503	0.017	Yes

Analysed data to determine the effect of isoflavonoids in combination with chickpea organic acids on *Hear*NPV (LC_{75}) efficacy against *H. armigera* larvae using one way ANOVA (first bioassay).

Normality Test (Shapiro-Wilk) Passed (P = 0.833)

Equal Variance Test: Passed (P = 0.818)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Control	5	0	16.800	7.294	3.262

NPV	5	0	79.600	10.040	4.490
100 F+B	5	0	23.600	6.841	3.059
F100	5	0	19.200	5.215	2.332
B100	5	0	22.800	10.354	4.630
F500	5	1	14.000	7.303	3.651
B500	5	0	11.600	3.286	1.470

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	6	16664.941	2777.490	48.570	<0.001
Residual	27	1544.000	57.185		
Total	33	18208.941			

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
NPV vs. Control	62.800	12.424	<0.001	Yes
NPV vs. F500	65.600	12.236	<0.001	Yes
NPV vs. F500	65.600	12.236	<0.001	Yes
NPV vs. F100	60.400	11.949	<0.001	Yes

NPV vs. B100	56.800	11.237	<0.001	Yes
NPV vs. 100 F+B	56.000	11.079	<0.001	Yes

Analysed data to determine the effect of isoflavonoids in combination with chickpea organic acids on *Hear*NPV (LC75) efficacy used against *H. armigera* using one way ANOVA (second bioassay)

Normality Test (Shapiro-Wilk) Passed (P = 0.052)

Equal Variance Test: Passed (P = 0.242)

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
Control	5	0	11.000	4.416	1.975
LC75	5	0	87.000	7.483	3.347
F+B 100	5	0	24.600	17.170	7.679
F100ppm	5	0	21.200	11.735	5.248
B 100 ppm	5	0	15.200	8.408	3.760
B 500ppm	5	0	18.600	4.336	1.939
F500 ppm	5	0	16.000	9.192	4.111

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	6	21115.886	3519.314	36.122	<0.001

Residual	28	2728.000	97.429
Total	34	23843.886	

Multiple Comparisons versus Control Group (Holm-Sidak method):

Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
LC75 vs. control	76.000	12.174	<0.001	Yes
LC75 vs. B 100 ppm	71.800	11.501	<0.001	Yes
LC75 vs. F500 ppm	71.000	11.373	<0.001	Yes
LC75 vs. B 500ppm	68.400	10.957	<0.001	Yes
LC75 vs. F100ppm	65.800	10.540	<0.001	Yes
LC75 vs. F+B 100	62.400	9.996	<0.001	Yes

1.5 Analysis for Chapter 7

Analysed data to determine the role of chickpea organic acids on *Hear*NPV efficacy used against *H. armigera* larvae using one way ANOVA.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatments	5	3964	792.9	25.57	1.38e-07 ***
Residuals	18	558	31.0		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Tukey multiple comparisons of means

95% family-wise confidence level

Treat.	diff	lwr	upr	p adj
NPV-c	28.75	16.235261	41.264739	0.0000114
a-c	16.00	3.485261	28.514739	0.0080767
NPV-b	35.25	22.735261	47.764739	0.0000006
d-a	-17.75	-30.264739	-5.235261	0.0031510
NPV-a	19.25	6.735261	31.764739	0.0014077
Water-a	-14.25	-26.764739	-1.735261	0.0204714

NPV-d	37.00	24.485261	49.514739	0.0000003
Water-NPV	-33.50	-46.014739	-20.985261	0.0000013

Analysed data to determine the effect of pH on *H. armigera* larval survival using Analysis of deviance analysis.

	Df	**Deviance**	**Resid. Df**	**Resid. Dev**	**p-value**
NULL			15	122.415	
pH	1	75.983	14	46.432	< 0.0001***

Analysed data of relationship between pH and *H. armigera* larval survival obtained from logit regression analysis.

> cor (yy,pH)

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Alive (-0.7784179)

Dead (0.7784179)

Licence to import, move and keep prohibited invertebrates

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