Behaviour of wild-type and genetically modified baculoviruses in the *Helicoverpa armigera* – cotton system: a simulation approach

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Behaviour of wild-type and genetically modified baculoviruses in the *Helicoverpa armigera* – cotton system: a simulation approach

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"Not everything that counts can be counted, and not everything that can be counted counts."

(Sign hanging in Albert Einstein’s office at Princeton University)

Abstract

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The bollworm, *Helicoverpa armigera*, is the key pest threatening the production of cotton in China, the world's largest cotton producer. The insect pathogen, *H. armigera* single nucleocapsid nucleopolyhedrovirus (HaSNPV), has been developed as a commercial biopesticide for control of cotton bollworm. To enhance the efficacy of HaSNPV, a recombinant (HaSNPV-AaIT) was constructed by insertion of a gene encoding an insect-selective scorpion neurotoxin (AaIT). Depending on the instar stage this recombinant virus incapacitates bollworm larvae 17-43% quicker than the wild-type virus (HaSNPV-WT). HaSNPV-AaIT also acts quicker than an HaSNPV *egt* (ecdysteroid UDP-glucosyltransferase)-deletion mutant (HaSNPV-EGTD). Field trials indicate that application of HaSNPV-AaIT over an entire cotton season results in a 22% higher cotton lint yield than when HaSNPV-WT is applied. Virus yield per larva inoculated with HaSNPV-AaIT in the 1st to 5th larval stage varied between 23% and 47% of the yield of HaSNPV-WT. Virus production in larvae was studied in the droplet-feeding bioassays. The results indicate that optimal production of recombinant virus for commercial purposes is achieved by inoculating the 4th instar larvae. The optimal dose is indicated. In the field, there was no significant difference in inactivation rate among the two recombinant HaSNPVs and HaSNPV-WT. In field cages and in the open field HaSNPV-AaIT exhibited a significantly lower rate of horizontal transmission than HaSNPV-WT. Vertical transmission of HaSNPV-AaIT was significantly lower than that of HaSNPV-WT, both in laboratory and field tests. On the basis of these data a comprehensive process-based model was developed to simulate the epizootiology of wild-type and genetically modified HaSNPV in cotton. The model integrates ecological information of interactions between HaSNPV, *H. armigera* and the cotton crop. The model is used to study aspects of the biocontrol efficacy of application regimes of HaSNPV variants with differential insecticidal properties and to identify key parameters to assess the biosafety of recombinant HaSNPV when used to control bollworm in cotton. Results show that genetically modified HaSNPV accrues greater benefits to cotton growers than wild-type HaSNPV and that its multiplication in bollworm populations is impaired as compared to HaSNPV-WT. As there are no demonstrable risks to human and environmental health, recombinant HaSNPV has clear potential to deliver effective and safe bollworm control.

Key words: *Helicoverpa armigera*, baculovirus, genetic modification, cotton, transmission, persistence, biological control, modelling.

Preface

At the beginning of 2000 I was invited by Professor Just Vlak to join the collaboration between Wageningen University and the Wuhan Institute of Virology, Chinese Academy of Sciences, on the study of baculoviruses of insects. In the framework of this cooperative project I was involved as a 'sandwich PhD student' sponsored by Wageningen University, which resulted in this dissertation. Here I express my sincere gratitude to all people who helped and supported me to conduct the studies described in this thesis.

First of all, I acknowledge my supervisors, Prof. Just Vlak, Dr. Wopke van der Werf and Prof. Hu Zhihong (Wuhan Institute of Virology). I owe gratitude to Just for inviting me to Wageningen and entrusting me with the PhD project. His insight, guidance and stimulating discussions were indispensable for the successful completion of this thesis. His critical comments and constructive suggestions on the manuscripts of all chapters were very valuable. I thank his wife Ellen Vlak for helping to correct my papers. The hospitality and friendship of the Vlaks made my stay in The Netherlands very enjoyable.

I am indebted to Wopke for his countless discussions over the five years. His professional skills in system analysis and strong background in applied mathematics have contributed to the design and data analysis of the experiments and to the development of the model. I have benefited from him not only in scientific research, but also language, writing skills and life philosophy. I also extend my sincere thanks to Saskia Beverloo for her hospitality.

I am grateful to Zhihong for her guidance, understanding and everything else that, without her, would not have been possible. She took over all my responsibilities in China when I was in The Netherlands.

I thank Dr. Chen Xinwen and Dr. Wang Hualin (both in the Wuhan Institute of Virology) for their contributions to the construction of the recombinant viruses, which were indispensible for my project. I further thank my students Sun Xincheng, Zhou Mingzhe, Bai Bingke, Huang Jinshan, Gao Peijie and Kang Yuan for their hard work. Part of their results are presented in this thesis.

I thank Prof. Rob Goldbach for his inspiring and continued interest in my research, which has been a constant source of inspiration and I thank Dr. Albert Otten (Mathematical and Statistical Methods Group, Wageningen University) for in-depth discussions and valuable advise on statistical data analysis. Further thanks go to Dr. Felix Bianchi (Crop and Weed Ecology Group, Wageningen University) for valuable comments on and suggestions for my papers. I also thank Dr. Jenny Cory (Great Lakes

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The field experiments, reported in this thesis, took place primarily at the China Cotton Research Institute, Chinese Academy of Agricultural Sciences, in Anyang. I wish to thank Ma Yan, Pan Dengming, Xi Jianping and Peng Jun for assisting me in maintaining the plots. My thanks also go to Dr. Cui Jinjie and his team for providing me with numerous experimental insects and equipments. I thank Dr. Zhang Lizhen for providing me with meteorological data for Anyang.

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I am grateful to my home institute for the solid support to my scientific development and my life. Many of my colleagues at the institute helped me, in one way or another. My profound appreciation thus goes to Zhang Guangyu, Jin Feng, Peng Huiying, Zhang Zhongxin, Yuan Li, Wang Hanzhong, Deng Fei, Wu Dong, Shi Zhengli, Chen Shiyun and many other people – too many to mention individually.

I wish to thank my friends in Wageningen, specifically the family of Huang Jiang, the family of Liu Chunming, Tom and Liru, for their help and the enjoyable time.

Above all, I thank my dear family for their love, support and confidence in me. My sincerest gratitude especially goes to my dear wife Yan Hua for her love, understanding and inspiration. During recent years, she made the biggest contribution to the care for and education of our daughter, who has vast interests, such as music, painting, swimming, taekwondo (kick boxing) and English. I am deeply indebted to my mother for her support and encouragement during my studies in China and in The Netherlands.

SUN Xiulian Wageningen, May 2005

Contents

Chapter 1	General introduction	1
Chapter 2	Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* SNPV expressing an insect-selective toxin from a chimeric promoter	17
Chapter 3	Production of polyhedral inclusion bodies from *Helicoverpa armigera* larvae infected with wild-type and recombinant HaSNPV	39
Chapter 4	Field inactivation of wild-type and genetically modified *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus in cotton	57
Chapter 5	Horizontal and vertical transmission of wild-type and recombinant *Helicoverpa armigera* single nucleocapsid nucleopolyhedroviruses	67
Chapter 6	Modelling the behaviour of wild-type and genetically modified baculoviruses in the *Helicoverpa armigera* – cotton system	87
	Appendix 1 Listing of the model	113
Chapter 7	General discussion	127
	References	139
	Summary	159
	Samenvatting	163
	摘 要	167
	List of publications of the author	169
	PE&RC PhD Education Statement Form	170
	Curriculum vitae	171
	Funding	172

CHAPTER 1

General introduction

GENERAL INTRODUCTION

Cotton (*Gossypium hirsutum* L.)

Cotton is an important commodity traded internationally as well as a major employment generator. It is estimated that globally more than 100 million farming units are engaged directly in cotton production, with many more in ancillary activities (Wang, 1996).

China is the largest producer and consumer of cotton in the world (Figure 1). Over the last few years China has 14% of the global cotton growth area and contributes 25% of the global cotton yield (FAS, USDA, 2001). From 1990 to 2003 annual cotton output fluctuated around 4.4 million tons while estimated use of cotton in yarn production grew 25%. This indicates that imports will have to grow unless domestic production can be expanded in coming years (Hsu and Gale, 2001).

Three major cotton production regions are generally distinguished in China: the North China cotton region along the Yellow River, the South China cotton region along the Yangtze River and the Northwest inland cotton region in the Xinjiang Uygur Autonomous Region plus northwestern Gansu province (Figure 2). The weather in the Yellow River region is often dry during spring time and irrigation is needed for cotton production. This region has to adopt early maturing, upland cotton varieties, which are usually double-cropped with winter wheat or intercropped with corn. In contrast with the Yellow River and Northwest regions, rainfall is relatively abundant in the Yangtze region. Annual rainfall averages more than 1,000 mm, of which over 85% comes during the cotton-growing season. With the long growing season in this region, cotton is usually double-cropped with wheat or rapeseed. The climate in the Northwest region is arid, with annual precipitation below 200 mm and wide daily swings in temperature,

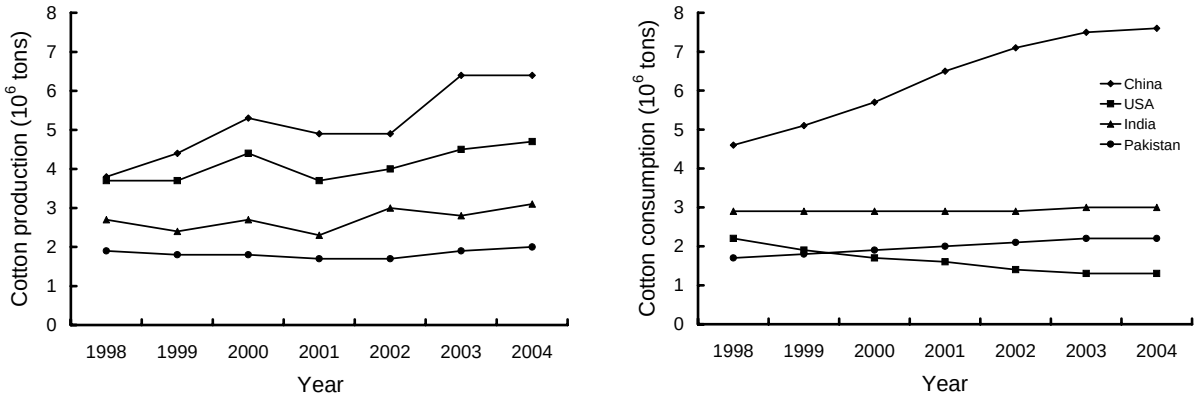

Figure 1. World cotton production and consumption. (Sources: USDA, Monthly Economic Letter, October, 2004).

but the dryness keeps pest and disease problems to a minimum. This region mainly grows upland cotton, with a high quality of colour and fibre length, due to its favourable climate conditions.

In the Yellow River region, the optimum planting date for cotton is between April 15 and April 25. Optimum yields can be achieved with plant populations varying from 52,500 to 60,000 plants per ha with row spacings of 90-100 cm. The cotton-growing season in this region is about 180 days. There are four main stages in cotton life: (i) emergence to first square, (ii) squaring to first flower, (iii) first flower to peak flower, and (iv) boll opening to cutout. A square is the floral bud, enclosed by three bracts, which first appears on the plant when the reproductive growth begins. A typical cotton plant with all fruit stages (squares, blooms and bolls) is shown in Figure 3.

Two periods within a season are important for pest control in cotton. At early-season (from emergence to first square), the entire growth sequence of the plant is upset when the growing point (the terminal bud) is damaged by insects or hail. The branching arrangement of the plant will then be irregular and the growth will be delayed. Another critical period is from early July to mid-August, when pests and diseases can occur. About 85% of the total bolls are set during the first three weeks of blossoming. The cotton plant has a tremendous capacity to make up for square shedding. However, damage of bolls in the blooming period will cause a direct reduction of the final lint production.

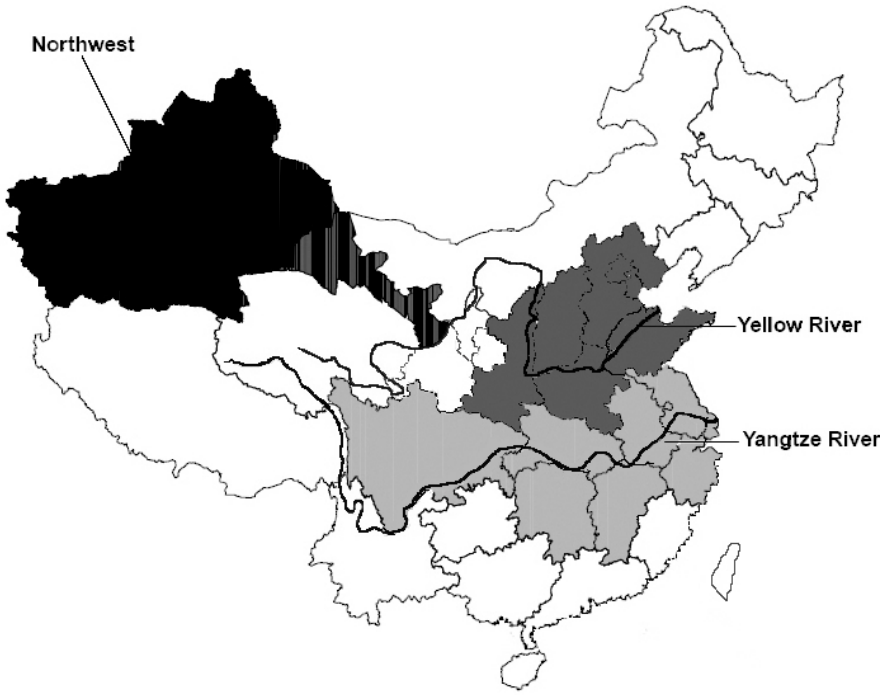

Figure 2. Primary cotton-producing regions in China.

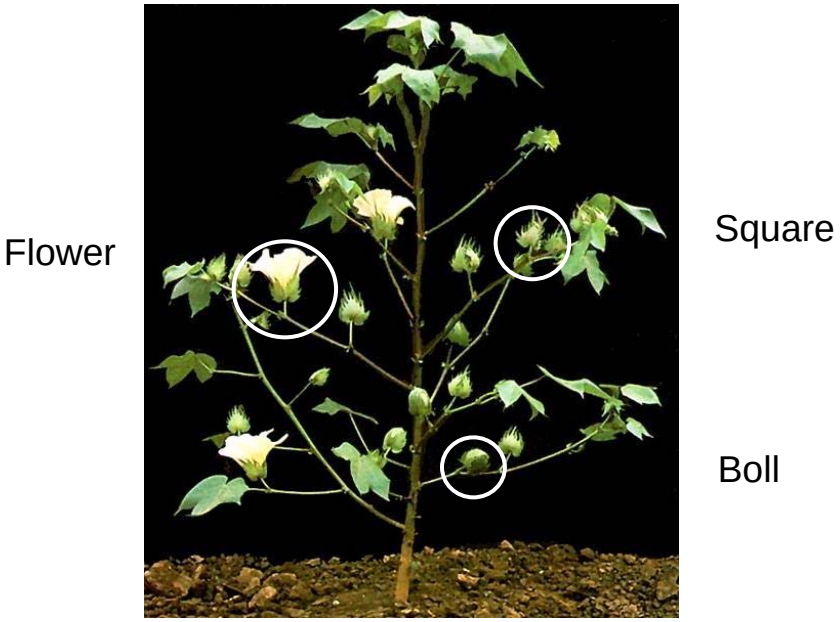

Figure 3. Typical cotton plant showing all fruit stages: squares, flowers and bolls.

The most serious pest insect in cotton in China is the cotton bollworm (*Helicoverpa armigera* Hübner), which causes annual losses up to 20% of the maximum attainable yield (CCRI, 1999). Since 1991 this appeared to be the main reason for the reduced production of cotton in the Yellow River (Xia, 1997a). Other important insect pests on cotton include aphids (*Aphis gossypii* Glover), red spider mites (*Tetranychus urticae* Koch), and pink bollworms (*Pectinophora gossypiella* Saunders) (CCRI, 1999). This thesis is about the bollworm *H. armigera* and the potential of a baculovirus to control this pest insect in the field.

Cotton bollworm (*Helicoverpa armigera* Hübner)

H. armigera is perhaps the most infamous of the heliothine insects (*H. zea* Boddie, *H. punctigera* Wallengren, *H. virescens* Fabricius *and H. assulta* Guenee), which attacks a great variety of agricultural crops such as cotton, pepper, tomato, tobacco, maize, sorghum, sunflower, pigeon pea, chickpea, groundnut, soybean and okra (Fitt, 1989; King, 1994). In cotton damage caused by heliothines (*H. zea* and *H. armigera*) is frequently located on the nitrogen-rich reproductive organs, such as flowers, squares and green bolls, and thus directly influencing yield.

The cotton bollworm is a holometabolous insect. Its developmental stages include egg, five or six larval instars, pupa and adult. Moths normally have a gray-brown colour (Figure 4A). Eggs are white, semi-spherical, with grids on the surface of the shell (Figure 4B). The larvae range in colour from light greenish-yellow to reddish-brown.

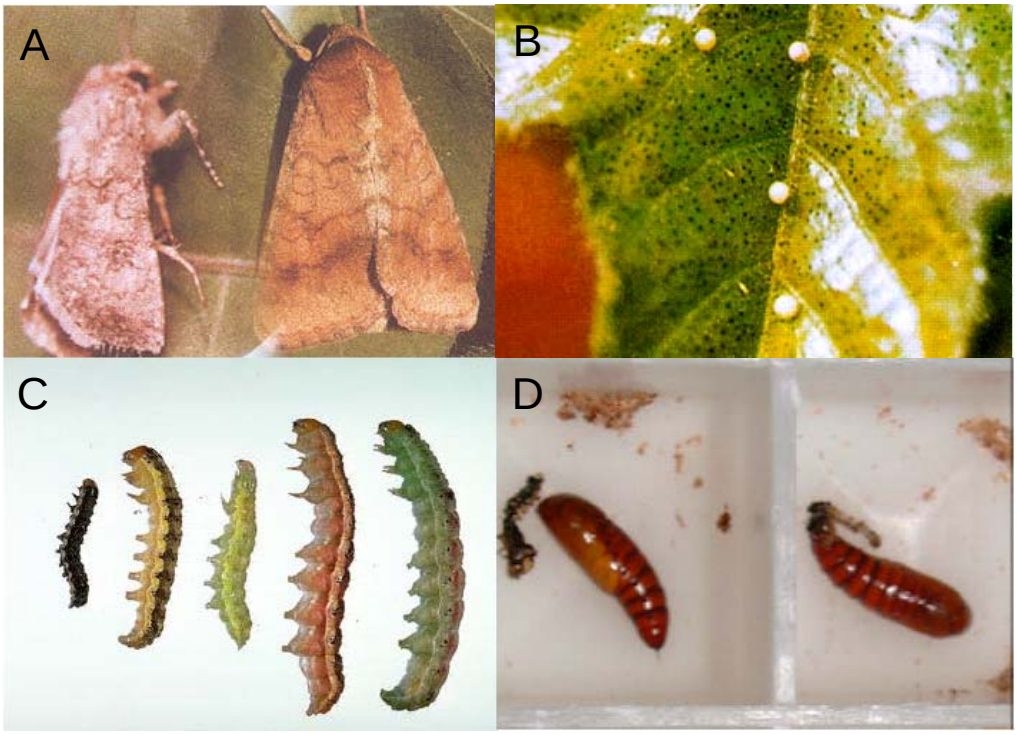

Figure 4. Various stages of *H. armigera*: Adults (A), egg (B), larvae (C) and pupae (D).

Several dark stripes occur on the dorsal side of the larvae (Figure 4C). Pupae are yellow-brown (Figure 4D).

H. armigera is multivoltine. In general there are 4 or 5 generations of bollworm per year in the Yangtze region, while in the Yellow River and Northwest regions, 3 or 4 generations are more common. In the Yellow River region the first generation adults emerge in late April, and the larvae feed on wheat, pea, flax or vegetables. Second generation appears in mid-June and migrates into cotton. Each generation takes 28-35 days, depending on the climate.

In the field, emerged cotton bollworm adults may mate at the same night if they can find partners. Females lay eggs in 2 to 3 days and oviposition may hold 7~8 days. Fecundity ranges from 600 to 1500 eggs per female (Yu *et al*., 1999), but up to 3,000 eggs have been recorded from a single female (Reed, 1965). The eggs hatch in 2 to 4 days into neonate larvae. In the Yellow River region the larval stage lasts 12.5-15.6 days (Yu *et al*., 1999; Ren *et al*., 2001). Neonate larvae normally consume the egg shells. First and second instar larvae feed on tender leaves and terminals. In the third and subsequent instars, larvae feed on floral squares, flowers and bolls. Damage from bollworm normally causes square shed and drop. Bolls fed upon usually rot and do not develop further. Larvae feed on 6 to 7 squares and 2 to 3 bolls during their development period (Sheng *et al*., 2002). By mid-July, damage of 2 to 4 squares or 2

squares plus one green boll per plant will not significantly affect the cotton yield, but loss of 4 squares plus 2 green bolls per plant will cause a 5.2% - 7.8% reduction of the final cotton yield (Sheng *et al*., 2002). Twelve larvae per 100 plants for the third generation of bollworm and 4 larvae per 100 plants for the fourth generation can be used as economic injury level (Fan *et al*., 2000).

Bollworm adults have the ability to undertake local and interregional movements for colonization and exploitation of agro-ecosystems. The movements may be categorized in three types (Farrow and Daly, 1987): (i) Short-range or trivial movements occur within or just above the host canopy and involve appetitive behaviour such as feeding, oviposition, mating and sheltering. These movements are generally confined within habitats over distances of 100-1000 m. (ii) Long-range movements involve a less-frequent response to external stimuli and occur above the canopy at heights of up to 10 m. These flights include movements between crops, normally within 1-10 km. (iii) Migratory movement occurs above the flight boundary layer, where moths take advantage of synoptic-scale wind systems at altitudes of up to 1-2 km. These flights may continue for several hours, allowing downwind displacements of hundreds of kilometres (Guo, 1997) to occur. *H. armigera* moths migrate in response to poor local conditions for reproduction. When plenty of diets are available and conditions are suitable, few *H. armigera* individuals fly a long distance. Field release of bollworm adults indicated that 62.5% of the moths were dispersed within 120 m and 97% within a 720 m range (Lu *et al*., 1999).

Integrated Pest Management (IPM) methodology has been developed in China in 1975, through the establishment of Farmer Field Schools in several provinces by UNDP, FAO and other donor countries to improve the farmer's knowledge. Many farmers were trained for implement of IPM with significant profits (Wang, 2000). However, the agricultural system was gradually reformed in China during the 1980s. Each peasant family managed its fields at a small scale. Farmer practice (FP) is now more frequent than IPM. At this point chemical insecticides are still widely used to control continuous infestations of bollworm in China, despite the high resistance to chemical insecticides, such as pyrethroid and endosulfan (Gunning *et al*., 1984; McCaffery and Walker, 1991). Extensive spraying with chemical insecticides has decimated natural enemies and has reduced natural control of bollworm infestations (Liu *et al*., 2000; Wan *et al*., 2002; Yang *et al*., 2000). Farmers can not cope with serious pest situations when confronted with multi-pest outbreaks, abnormal weather, unusual crop growth and other unfavourable ecological conditions. With the increasing problems of environmental pollution and health hazards caused by overuse of pesticides, as well as biological disasters, China has taken a series of measures to solve these problems by perfecting pesticide regulations and has established the production

of non-pesticide vegetables, non-pesticide fruits production, and ecological demonstration bases to develop green farming and enhanced pest monitoring and implementation of IPM strategies.

Biotechnological approaches have also been introduced for bollworm control. Insect-resistance cotton, based on a genetic modification of cotton through the introduction of toxin genes from *Bacillus thuringiensis* (Bt cotton), became available to Chinese farmers since 1996 and the area planted with GMO cotton rapidly increased over the last years (Figure 5). However, resistance of bollworm to the toxins in the Bt cotton has already been observed (Akhurst *et al*., 2004; Gould *et al*., 1997; Liang *et al*., 2000; Liu *et al*., 1999; Mann, 2001; Ramachandra *et al*., 1998; Shelton *et al*., 2000; Shen *et al*., 1998; Tang *et al*., 1999; Zhao *et al*., 1998) and strategies have been designed to avoid resistance (such as stapling Bt genes in the plant, non Bt-cotton crop refuges, etc.). The general public is worried about the negative effects of such genetically modified varieties on other crop cultures. It is also concerned that the seeds of traditional cultivars would be 'contaminated' by the genetically modified varieties. Farmer research shows that Bt cotton may not contribute substantially to profitability because of increased seed costs, with little seed-cotton yield gain (Röling *et al*., 2004). Therefore, alternative strategies for bollworm control are developed and explored.

There is renewed interest in classical biological control as a strategy in the management and control of *Helicoverpa* pests by introducing natural enemies (King

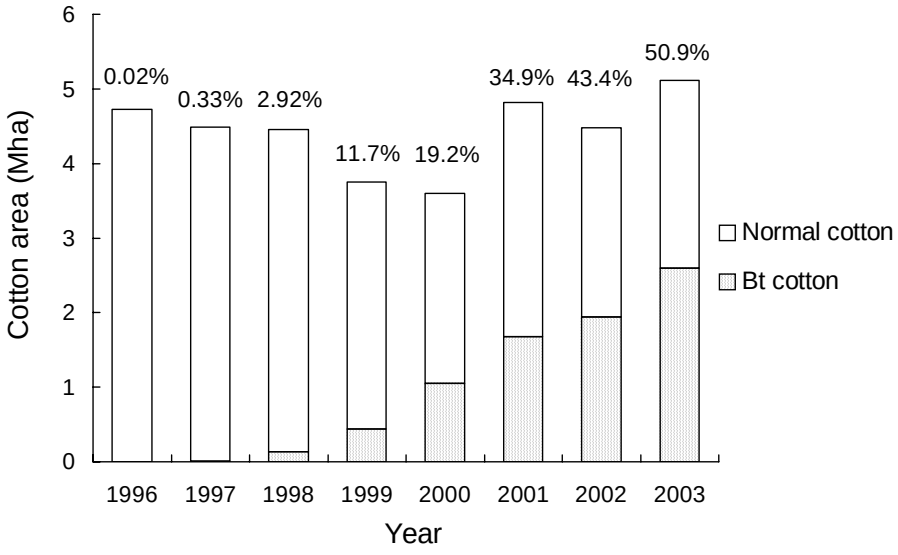

Figure 5. Acreage of Bt cotton and normal cotton in China. Percent of Bt cotton area in total cotton area is shown at top of each bar. Sources: China Chemical Industry News, November 15, 2002; Farmer Daily (China), October 9, 2003; Economic Information Daily (China), January 29, 2004.

and Coleman, 1989). A more frequently tried method to achieve control with natural enemies has been used by augmentative rearing and release of artificially reared parasites or predators (King and Coleman, 1989; Xia, 1997b). While the technical feasibility for controlling heliothine populations in cotton by this method has been demonstrated, the results in the field have not always been consistent (King and Coleman, 1989). Bollworm pathogens such as baculoviruses are attractive alternatives. The latter have been used for more than 20 years with great success in Chinese cotton (Zhang, 1989).

Baculoviruses are pathogens that can cause lethal infections in many insect species. There are several advantages of using baculoviruses for pest control: they are non-pathogenic to vertebrates and plants; they are well-studied systems from both pest management and molecular biology points of view; they leave no undesirable residues and can be used at the 'cottage scale' (FAO/WHO, 1973; Moscardi, 1999). For insect species such as cotton bollworm that have developed resistance to chemical and/or *Bt* insecticides, the application of baculoviruses is one of the few options left for effective bollworm control (Trumble, 1998).

Baculoviruses

Baculoviruses are large DNA viruses that occur only in arthropods. The Baculoviridae comprise two genera, based upon occlusion body (OB) morphology (Blissard *et al*., 2000). Viruses of the genus *Nucleopolyhedrovirus* (NPV) produce large (0.13-15 μm) proteinaceous polyhedron-shaped inclusion bodies (PIBs) (Figure 6A), that contain many virions within each polyhedron (Figure 6B). Viruses of the genus *Granulovirus* (GV) are characterized by smaller (0.3 × 0.5 μm) ovicylindrical-shaped OBs, called granules, that only contain a single virion. The virions of NPVs consist of one (SNPV) or more (MNPV) rod-shaped (30-60 × 250-300 nm) nucleocapsids. OBs enable the virus to survive outside its host for periods as long as four decades (Thompson *et al*., 1981). The OBs are sensitive to alkaline (Figure 6C). The enveloped virons derived from OBs are named occlusion derived viruses (ODV).

In the environment the OBs are taken up orally by the larvae together with plant material. After ingestion the polyhedra dissolve in the alkaline juices in the midgut. ODVs are liberated and pass through the peritrophic membrane lining the gut to infect the midgut epithelial cells. In the nucleus of these cells, mainly columnar cells, the virus replicates and budded viruses (BVs) are produced that leave the infected cells and infect other larval tissues, such as the hemocoel and fat body. In secondarily infected cells new BVs and polyhedra are produced. Finally, the larval body disintegrates and millions of new OBs are released onto the plant or on the soil until picked up by another insect larva (Figure 7).

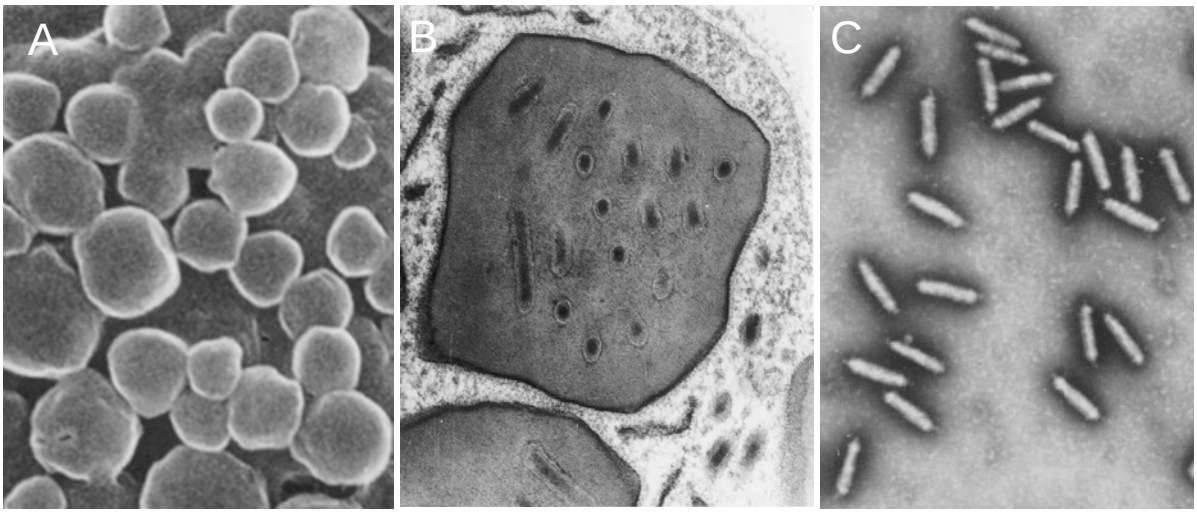

Figure 6. Electron micrographs of HaSNPV forms: (A) scan of occlusion bodies, (B) transmission section of occlusion bodies and (C) ODVs liberated from occlusion bodies by alkaline solution.

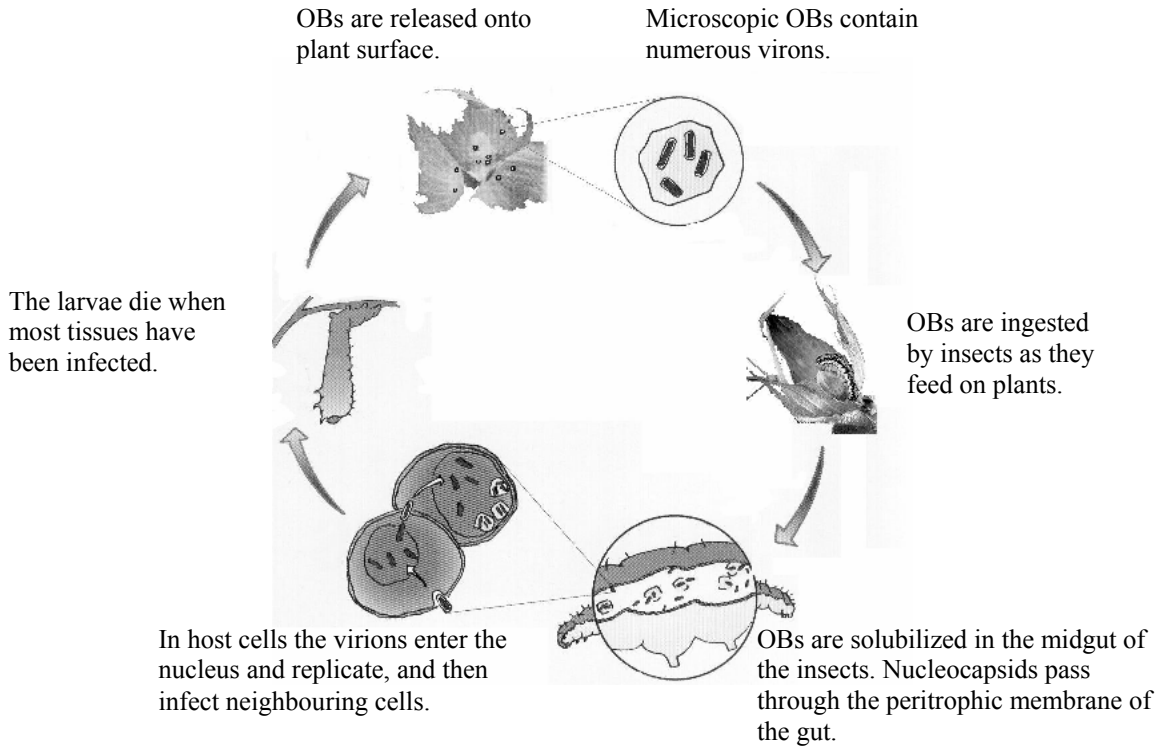

Figure 7. Diagram of the infection cycle of baculoviruses. (Modified from Promotion sheet 'Recombinant Baculoviruses' of DuPont Agricultural Products (2002)).

Infection with a baculovirus frequently alters host behaviour. Infected larvae often tend to move to, and die, in the top of trees giving rise to the term 'Wipfelkrankheit' or tree-top disease (Steinhaus, 1967). Infection of the cabbage moth, *Mamestra brassicae*, by its NPV produces increased movement and upward migration on cabbage plants (Goulson, 1997; Vasconcelos, 1996). It is assumed that this behaviour has evolved to enhance the transmission of the virus by either gravity or rainfall (Goulson, 1997; D'Amico and Elkinton, 1995; Vasconcelos, 1996). Recently, a baculovirus-encoded tyrosine phosphatase (*ptp*) appears to be involved in this process (Kamita *et al*., 2003). Other baculovirus genes, such as the ecdysteroid UDP-glucosyl-transferase (*egt*) gene, chitinase gene and cathepsin gene, were found to have a potential function to manipulate host behaviour. The product of *egt* gene blocks larval molting, thereby increasing the size of the larvae and the quantity of viral progeny produced (O'Reilly and Miller, 1991; Slavicek *et al*., 1999; Wilson *et al*., 2000). The chitinase gene and cathepsin gene appear to act in concert to facilitate the release of the virus OBs from the insect cadaver after death by breaking down the intra cellular protein and chitin, in particular in the insect cuticle (Hawtin *et al*., 1997; Slack *et al*., 1995). Infection by baculoviruses is generally lethal, but survivors can have altered development times and reduced size, fecundity, egg viability and vigour (Duan and Otvos, 2001; Matthews *et al*., 2002; Myers, 2000; Rothman and Myers, 1996).

Baculoviruses isolated from the same species may vary in their pathogenicity (Allaway and Payne, 1983; Ebling and Kaupp, 1995; Hatfield and Entwistle, 1988) and also their speed of kill (Hughes *et al*., 1983). Baculovirus isolates frequently show polymorphisms in their restriction endonuclease profiles (see Cory and Myers (2003), for review), indicating that natural baculovirus isolates often are a mixture of several genotypes or variants. Individual variants from the same virus isolates obtained by *in vitro* or *in vivo* cloning can also vary dramatically in virulence (Hodgson *et al*., 2001; Lynn *et al*., 1993; Ribeiro *et al*., 1997).

The maintenance of baculoviruses in insect populations requires transmission of the virus from infected to uninfected individuals. Transmission in baculoviruses is thought to be primarily horizontal via susceptible larvae ingesting OBs persisting in the environment. The OBs may be further distributed by excrements of infected larvae (Vasconcelos *et al.*, 1996), rain (Kaupp, 1981) and predators, such as birds (Entwistle *et al*., 1993). Baculoviruses can also be released in the environment by human activity, for example by application of baculovirus sprays. Baculoviruses can also be transmitted vertically from adults to their young (Easwaramoorthy and Jayaraj, 1989; Fuxa *et al*., 2002; Kukan, 1999; Smits and Vlak, 1988a). Vertical transmission occurs by surface contamination of eggs (transovum transmission) or virus passing within the egg (transovarian transmission), including transfer of latent infections. Latent virus is a

nonreplicating form of virus that can be reactivated to an infective state by some stressor (Fuxa and Richter, 1991). Possible stressors that activate latent virus could be host crowding, fluctuations in temperature or relative humidity, irradiation, dietary changes, chemical stress, parasitization and inoculation by a second pathogen (Burden *et al*., 2003; Cooper *et al*., 2003; Fuxa *et al*., 1999; Jurkovíčová, 1979; Podgwaite and Mazzone, 1986).

Epizootics of baculoviruses irregularly occur in agricultural, forest and horticultural pests and have the potential to influence host population dynamics (Weiser, 1987). Mathematical models have been used to study and gain insight in these complex systems. The theoretical relationships of host-pathogen dynamics of insects have been widely explored in mathematical models starting with those of Anderson and May (1980, 1981). More recent models have incorporated modifications such as variation in transmission parameters (Getz and Pickering, 1983), vertical transmission (Regniere, 1984), both density dependence and vertical transmission (Vezina and Peterman, 1985), nonlinear transmission (Hochberg, 1991), density dependence (Bonsall *et al*., 1999; Bowers *et al*., 1993; White *et al*., 1996), host stage structure (Briggs and Godfray, 1996), heterogeneity in susceptibility (Dwyer *et al*., 1997), discrete generations and seasonal host reproduction (Dwyer *et al*., 2000), and sublethal infection (Boots and Norman, 2000).

Some numerical process-based models entail a detailed description of the biological system. These models describe the biological system at the process level and integrate information of the different processes (De Kraker, 1996; Van Roermund *et al*., 1997). The usefulness of such a modelling approach has been demonstrated for *Spodoptera exigua* MNPV and AcMNPV in a greenhouse setting, where the control of beet armyworm with baculoviruses in greenhouse crops has been validated (Bianchi, 2001; Bianchi *et al*., 2002a). Using this model, scenario studies have been carried out on the bio-control potential of the introduction of GM baculoviruses in this greenhouse setting (Bianchi *et al*., 2002b, c).

The application of baculoviruses as microbiological agents has not yet met the optimum potential to control the pests. Major impediments include the low infectivity in some cases and the slow 'speed of kill' of host larvae. To improve the insecticidal properties of baculoviruses, one approach is to select the natural variants by *in vitro* cloning with cell culture technique or *in vivo* cloning with larvae. For example, clones of *Anticarsia gemmatalis* NPV, isolated by plaque purification in cell culture, differed in LD_{50} (median lethal dose) by over one-hundredfold (Ribeiro *et al*., 1997). Hodgson *et al*. (2001) found four *Panolis flammea* NPV genotypes differed in LD_{50} by 7 times and also showed significant differences in the speed of action.

Another more recent approach involves the genetic engineering of baculoviruses

either by deletion of viral genes, such as the *egt* gene (O'Reilly and Miller, 1991; Slavicek *et al*., 1999), and/or insertion (expression) of scorpion, mite, and spider neurotoxins (Hughes *et al*., 1997; Lu *et al*., 1996; Popham *et al*., 1997; Prikhod'ko *et al*., 1996; Stewart *et al*., 1991; Tomalski and Miller, 1991, 1992). These modifications yielded a 20~55% reduction in the median time to die or to paralyse (Inceoglu *et al*., 2001; see Van Beek and Hughes, 1998, for review). Further increase in efficacy was achieved by utilizing various promoters for expression of neurotoxin with signal sequences and *hr5* enhancing elements (Van Beek *et al*., 2003) or by combined expression of the excitatory toxin and the depressant toxin that provided an improvement of 40% over the efficacy of wild-type AcMNPV, and of 18% and 22% over baculoviruses that express each of the toxins independently (Regev *et al*., 2003).

Field evaluation of the recombinant baculoviruses is subject to safety regulations and has – thus far – been undertaken at a limited scale in the UK and the USA. In the cotton field, the efficacy of AcMNPV-Δ*egt* against *Heliothis virescens* and *Trichoplusia ni* was only slightly higher than that of wild-type AcMNPV (Treacy *et al*., 1997). In contrast to *egt*-deletion recombinants, toxin expressing AcMNPV recombinants showed notably increased field efficacy and resulted in considerably reduced crop damage (Cory *et al*., 1994; Gard, 1997). Application of a recombinant *H. zea* SNPV expressing a toxin from the scorpion *Leiurius quinquefasciatus* protected cotton better from damage than wild-type virus (Smith *et al*., 2000a).

Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus (HaSNPV)

H. armigera single nucleocapsid nucleopolyhedrovirus (HaSNPV) is a highly infectious and selective pathogen of the cotton bollworm that was isolated from diseased *H. armigera* larvae in Hubei province of China in 1980 (Zhang, 1989). Eight HaSNPV genotypes have been identified and partially purified from wild-type HaSNPV isolation by using an *in vivo* cloning method (Sun *et al*., 1998). One genotype (HaSNPV-G4) is present at the highest frequency and is the predominant genotype of this isolate (Sun, unpublished data). PIBs of HaSNPV are polyhedral, round or irregular shaped with an average diameter of 1.08 μm (0.46-1.82 μm) (Figure 6A; Sun and Zhang, 1994). Each PIB contains 31.2 ± 11.0 virions (Chapter 2). Each viron contains a circular double-stranded DNA molecule in size of 131,403 bp (HaSNPV-G4, Chen *et al*., 2001). Of 135 potential ORFs predicted from the sequence, 115 have a homologue in other baculoviruses, while twenty are unique to HaSNPV and are subject to further investigation. Phylogenly analysis indicated that HaSNPV belongs to the Group II NPVs (Chen *et al*., 2001).

HaSNPV appears to have a host range restricted to members of the genus *Helicoverpa* including *H. zea*, *H. virescens* and *H. assulta* (Herz *et al*., 2003; Ignoffo

and Couch, 1981). *H. zea* single nucleocapsid NPV (HzSNPV) has almost the same host range (Ignoffo *et al*., 1983). There is no significant difference in the infectivity of HaSNPV and HzSNPV in *H. armigera* larvae (Sun and Zhang, 1994) or in *H. zea* (Hughes *et al*., 1982). The restriction enzyme patterns and even the physical maps of HzSNPV DNA and HaSNPV were quite similar (Knell and Summers, 1984; Chen *et al*., 2000a). Sequence analysis of the complete HzSNPV genome revealed that HaSNPV and HzSNPV have a high degree of ORF identity, which is in line with the view that they are two different isolates of the same virus species (Chen *et al*., 2002). HzSNPV was registered as one of the first commercial baculovirus pesticides (Viron-H, Biocontrol-VHZ, Elcar^{TM}) in the 1970s and has been extensively used to control the cotton bollworm in the USA and other countries (Shieh, 1989; Cunningham, 1998).

HaSNPV has been adopted for mass production as a viral pesticide and has been widely used to control the insect pests in China (Zhang *et al*., 1995) and in other countries (Jones, 1994) as well. The virus is produced from a culture of *H. armigera* larvae, infected under controlled conditions. PIBs are extracted from the dead insects and formulated as a wettable powder or a liquid concentrate (Figure 8). The wettable powder product contains 1×10^9 PIBs g^{-1} of virus and the liquid concentrate contains 2×10^9 PIBs ml^{-1} of virus. Additives, such as emulsifiers, enhancing agents and UV-absorbing agents, are incorporated. The quality of the HaSNPV insecticide was controlled both by counting PIBs with a staining procedure (Sun *et al.*, 1999) and by a bioassay. The production costs of one kilogram of wettable powder of HaSNPV insecticide are about $3.75 and about $6.2 for one liter of suspension. Since 1993

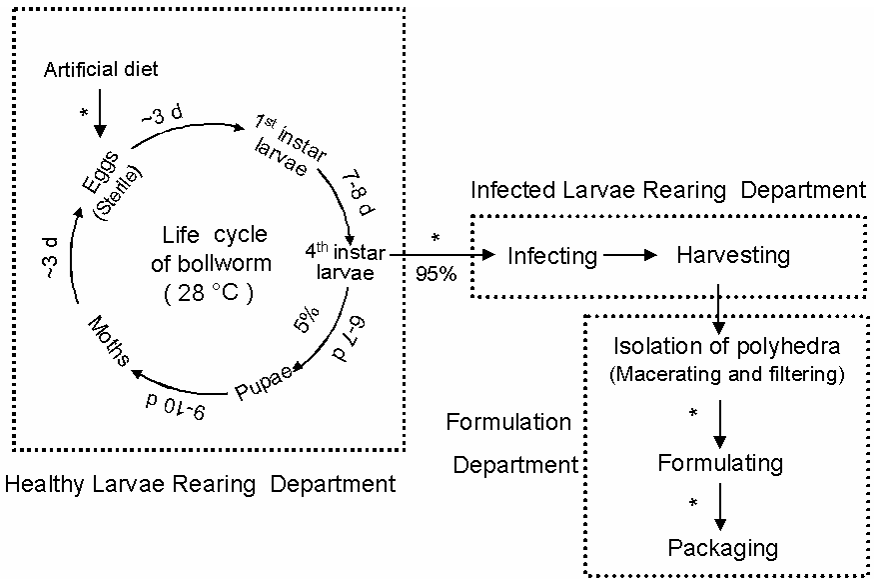

Figure 8. Procedure of mass production of HaSNPV pesticide (steps with asterisk could be done automatically).

the first HaSNPV pesticide was registered in China. It is estimated that in the last ten years, the annual output of HaSNPV was about 200-300 tons, enough to treat about 100,000 ha of cotton.

HaSNPV insecticide is recommended to be applied to control early instars of the pest larvae through either a high volume spray or an ultra low volume spray method. Depending on the size and density of the crop and the age and density of the pest population, the dosage of virus application is suggested to be $1.2\sim2.4 \times 10^{12}$ PIB ha^{-1}, which is 1.2~2.4 kg of wettable powder, or 0.6~1.2 L of liquid concentrate. Normally 2~3 sprays are needed to control one natural infestation with 4~5 days interval. The surviving number of cotton bollworm larvae was reduced by 83.7~91.7 % when cotton was sprayed with this spray regime (Zhang *et al*., 1995).

Like other baculoviruses, HaSNPV suffers the disadvantage of having a relatively slow speed of action when compared to chemical insecticides. To enhance the efficacy of HaSNPV as a pesticide, it has been genetically modified by deletion of the *egt* gene from its genome (recombinant HaCXW1) and insertion of an insect-selective scorpion toxin (*Aa*IT) gene controlled by the HaSNPV *polyhedrin* promoter (recombinant HaCXW2) (Chen *et al*., 2000b). In cotton field plots, artificially released *H. armigera* larvae treated with either HaCXW1 or HaCXW2 were killed faster than larvae in plots treated with wild-type HaSNPV (Sun *et al*., 2002b). However, it is necessary to assess further whether the use of these recombinant viruses can provide better protection of cotton fruits from bollworm damage.

Scope and out line of the thesis

HaSNPV has been successfully used to manage heliothine pests, such as *H. armigera* and *H. assulta,* that are resistant to chemical insecticides and Bt toxin (Zhang, 1989). To enhance the efficacy of HaSNPV as a pesticide, genetically modified HaSNPV variants with improved kill speed have been constructed and their insecticidal properties have been tested in the laboratory (Chen *et al.*, 2000b) and under controlled conditions in the field (Sun *et al.*, 2002b). However, insight in the dynamics of the control of the pest in the field is still limited. Application strategies of HaSNPV were explored by extensive field tests (Zhang *et al.*, 1995; Sun *et al.*, 2002b). In the case of recombinant baculoviruses, the determination of the commercial potential of new formulations or application regimes is subjected to strict regulations of biosafety. In addition to field trials, it is necessary to study in depth the ecological interactions between the host plant, the pest insect, and the recombinant and wild-type viruses.

In this project, I attempted to describe these ecological interactions in detail by studying, at insect level, virus infectivity, incubation time, virus production, virus persistence and vertical and horizontal transmission of the wild-type and genetically

modified HaSNPV variants. At the field level, these interactions are studied through the effect of virus sprays on insect survival and crop production. A detailed model for crop level interactions between *H. armigera* and the NPV pathogens is developed to describe this system and to explore, as an example, the control efficacy of application regimes of wild-type and recombinant HaSNPV variants and their environmental fate after release at a large scale.

The thesis starts with a chapter on the construction of a novel recombinant HaSNPV (HaSNPV-AaIT) expressing the neurotoxin *Aa*IT from a chimeric promoter of the *p6.9* and *polyhedrin* gene of HaSNPV and the assessment of its insecticidal properties (Chapter 2). The infectivity, the killing speed and control efficacy against host larvae of this recombinant was compared with their wild-type parent and an *egt*-deletion mutant (HaSNPV-EGTD) in laboratory or in the field.

Knowledge of virus yield in deceased larvae is not only necessary to plan and optimize virus production in virus 'factories', but also to understand and model the epidemiological dynamics of HaSNPV variants under field conditions. Therefore, the yield of PIBs from 1st to 5th instar of *H. armigera* larvae inoculated with HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT was studied as input for the model (Chapter 3).

Inactivation rate of baculovirus is an important parameter for its epidemiology and application strategy. The field stability of HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT was investigated in cotton in three consecutive years (Chapter 4).

Transmission of baculoviruses plays a central role in the ecology of baculoviruses and the population dynamics of their hosts. Essential parameters describing the horizontal and vertical transmission dynamics of HaSNPV-WT and HaSNPV-AaIT in cotton were quantified (Chapter 5).

To make field assessments of the control efficacy at different spray regimes and of the environmental fate of genetically modified HaSNPV variants relative to wild-type HaNPV, prior to actual releases, a model may be helpful. Based on the collected detailed information on crop–insect–virus interactions, a detailed model for crop level interactions between the cotton bollworm and its wild-type and genetically modified NPV has been developed (Chapter 6).

In Chapter 7, the model is discussed in a wider context and in view of its use to address biosafety issues and devise perspectives for wild-type and GM baculoviruses as commercial insecticides.

CHAPTER 2

Biological activity and field efficacy of a genetically modified *Helicoverpa armigera* SNPV expressing an insect-selective toxin from a chimeric promoter*

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ABSTRACT

A recombinant baculovirus (HaSNPV-AaIT) with improved insecticidal properties was constructed for the control of cotton bollworm (*Helicoverpa armigera*). A chimeric promoter of the *p6.9* and *polyhedrin* gene of *H. armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV) was used to drive the expression of an insect-selective scorpion toxin (*Aa*IT) at the *egt* gene locus of HaSNPV. This chimeric promoter, denoted ph-p69p, was constructed by directional insertion of the *p6.9* promoter downstream of the *polyhedrin* promoter. Laboratory bioassays indicate that the infectivity (LD_{50}) of this recombinant is unchanged, compared to one wild-type clone of HaSNPV (HaSNPV-WT), and an *egt*-deletion mutant (HaSNPV-EGTD). The median survival times (ST_{50}s) of 1st to 5th instar *H. armigera* larvae were reduced 17 - 34% after infection with HaSNPV-AaIT in comparison to HaSNPV-WT. The median times of feeding cessation (FT_{50}s) were 30 - 43% shorter for HaSNPV-AaIT than for HaSNPV-WT in the 3rd to 5th instar of this species. This virus acts also quicker than HaSNPV-EGTD. Field trials at two research sites in 2000 indicate that the number of larvae and the proportion of damaged squares, flowers and bolls was significantly lower in cotton plots treated with HaSNPV-AaIT than in plots treated with HaSNPV-WT or HaSNPV-EGTD. When HaSNPV-AaIT was applied to control infestations of bollworm over an entire cotton season, yield of cotton lint in plots treated by this recombinant was 22.1% higher than that in HaSNPV-WT treated plots in 2001 ($P < 0.05$) and 20.7% higher in 2002 ($P = 0.251$). These results indicate that this novel HaSNPV recombinant HaSNPV-AaIT is a more effective biocontrol agent than its wild-type relative, at least in the short term, and suggest that it offers potential for practical use.

Key words: HaSNPV, recombinant baculovirus, *p6.9* promoter, *Aa*IT, field efficacy, cotton bollworm.

INTRODUCTION

Baculoviruses are highly selective pathogens for several pest insects. Currently a number of wild-type baculoviruses is used for the protection of crops, vegetables, forests and pastures (Moscardi, 1999). However, wider application of baculoviruses has been limited by their slow speed of action and narrow host specificity. Several different genetic approaches are known to improve the speed of action of baculoviruses. These include the deletion of specific baculovirus genes, e.g. the ecdysteroid UDP-glucosyltransferase (*egt*) gene (O'Reilly and Miller, 1991), or the expression of heterologous genes such as those encoding diuretic hormone, juvenile hormone esterase, a maize mitochondrial product, insect-selective toxins from the scorpion *Androctonus australis* Hector (*Aa*IT), the straw itch mite *Pyemotes tritici* Lagreze-Fossat & Montane (Tox34), the scorpion *Leiurius quinquefasciatus hebraeus* Hemprich & Ehrenberg (*Lqh*IT2), the funnel web spider *Agelenopsis aperta* Gertsch (Aga-IV), the sea anemones *Anemonia sulcata* Pennant and *Stichodactyla helianthus* Ellis (AsII and ShI) and the spiders *Digutia canities* McCook and *Tegenaria agrestis* Walckenaer, etc. (McCutchen *et al*., 1991; Bonning and Hammock, 1996; Black *et al*., 1997).

In laboratory assays, a number of recombinant baculoviruses showed improved insecticidal activities, mainly on the speed of action (Van Beek and Hughes, 1998; Inceoglu *et al*., 2001, for review). In the greenhouse an *egt*-deletion variant of *Autographa californica* multicapsid NPV (AcMNPV) led to improved protection of cotton when compared to wild-type AcMNPV against *Heliothis virescens* Fabricius. However, in a cotton field situation the efficacy of this AcMNPV-Δ*egt* against *H. virescens* and *Trichoplusia ni* Hübner was not significantly different from that of wild-type AcMNPV (Treacy *et al*., 1997). In contrast to AcMNPV-Δ*egt*, AcMNPV-*Aa*IT recombinants showed notably increased field efficacy and resulted in considerably reduced crop damage (Cory *et al*., 1994; Gard, 1997). Application of a recombinant *H. zea* SNPV expressing *Lqh*IT2 toxin protected cotton better from damage of heliothine pests than wild-type virus or the chemical insecticides (Smith *et al*., 2000a).

Cotton is one of the most important cash crops in China. Cotton bollworm (*Helicoverpa armigera* Hübner) is one of most serious pests on cotton. It is a multivoltine species with 3 to 4 generations requiring multiple control interventions per year. A baculovirus, *H. armigera* single nucleocapsid nucleopolyhedrovirus (HaSNPV), has been developed as a commercial biopesticide to control this pest (Zhang, 1989). To improve its speed of action, this virus has been genetically modified by deletion of the *egt* gene from its genome (recombinant HaCXW1) and insertion of an insect-selective scorpion toxin (*Aa*IT) gene controlled by the HaSNPV *polyhedrin*

promoter (recombinant HaCXW2) (Chen *et al*., 2000b). In a cotton field situation, artificially released *H. armigera* larvae treated with either HaCXW1 or HaCXW2 were killed somewhat faster than larvae in plots treated with wild-type HaSNPV (Sun *et al*., 2002a), but crop damage and effect on cotton lint yield was not assessed.

The level and extent of recombinant protein expression in the baculovirus expression system is promoter-dependent (Bonning *et al*., 1999). The efficacy of toxin-expressing baculoviruses could thus be improved by choice of a suitable promoter. For expression of the Tox34, the late 6.9K DNA binding protein (*p6.9*) gene promoter appeared to be the most effective promoter among early, late or very late promoters (Lu *et al*., 1996). *Rachiplusia ou* multicapsid nucleopolyhedrovirus (RoMNPV) expressing *Aa*IT toxins from the *p6.9* promoter killed larvae of *Ostrinia nubilalis* Hübner significantly faster than wild-type virus, but this virus expressing *Aa*IT from the *p10* promoter produced a reduced quantity of polyhedral inclusion bodies (PIBs) and failed to occlude virions efficiently (Harrison and Bonning, 2000). An AcMNPV recombinant expressing cathepsin L of the flesh fly *Sarcophaga peregrina* Robineau-Desvoidy through the *ie-1* promoter killed *H. virescens* only slightly (5.6%) faster than wild-type AcMNPV. However, when this gene was expressed from the AcMNPV *p6.9* promoter, the recombinant virus killed the host 51% faster than the wild-type (Harrison and Bonning, 2001).

In order to further enhance the insecticidal efficacy of HaSNPV by expressing the *Aa*IT toxin also earlier, we constructed a chimeric (dual) promoter by directional insertion of the *p6.9* promoter downstream of the *polyhedrin* promoter and used this element for the expression of *Aa*IT toxin in *egt* locus of HaSNPV. The intrinsic biological activities (LD_{50}s, ST_{50}s and FT_{50}s) for all *H. armigera* instars and the control efficacy of this recombinant in the cotton field were compared with the parent HaSNPV-Δ*egt* (HaSNPV-EGTD) and wild-type HaSNPV.

MATERIALS AND METHODS

Virus, insect cells and insects

The HaSNPV was initially isolated from diseased *H. armigera* larvae collected in Hubei province of China (Zhang *et al*., 1981; Zhang, 1989). A genotype of this wild-type HaSNPV (HaSNPV-G4) was isolated by using an *in vivo* cloning method (Sun *et al*., 1998) and further mentioned as HaSNPV-WT. The recombinant HaSNPV-EGTD (HaCXW1 in Chen *et al*., 2000b; Sun *et al*., 2002b) was generated by deletion of the *egt* gene from HaSNPV-WT by recombination in cell culture. This recombinant was used as parent virus to generate HaSNPV-AaIT reported in this study. HaSNPV viruses were propagated in fourth instar *H. armigera* larvae. Polyhedral inclusion

bodies (PIBs) were purified by sucrose gradient centrifugation and stored in 40% glycerol at 4 °C until use. Viral DNA was isolated from PIBs according to Sun and Zhang (1994). The Hz2e5 cell line (McIntosh and Ignoffo, 1983) was cultured at 27 °C in Grace's medium (Gibco/BRL) supplemented with 10% fatal bovine serum (Gibco/BRL). A laboratory colony of cotton bollworm, *H. armigera*, was reared on artificial diet at 28 ± 1 °C and a 16-h light / 8-h dark photoperiod (Zhang *et al*., 1981).

Construction of transfer vector

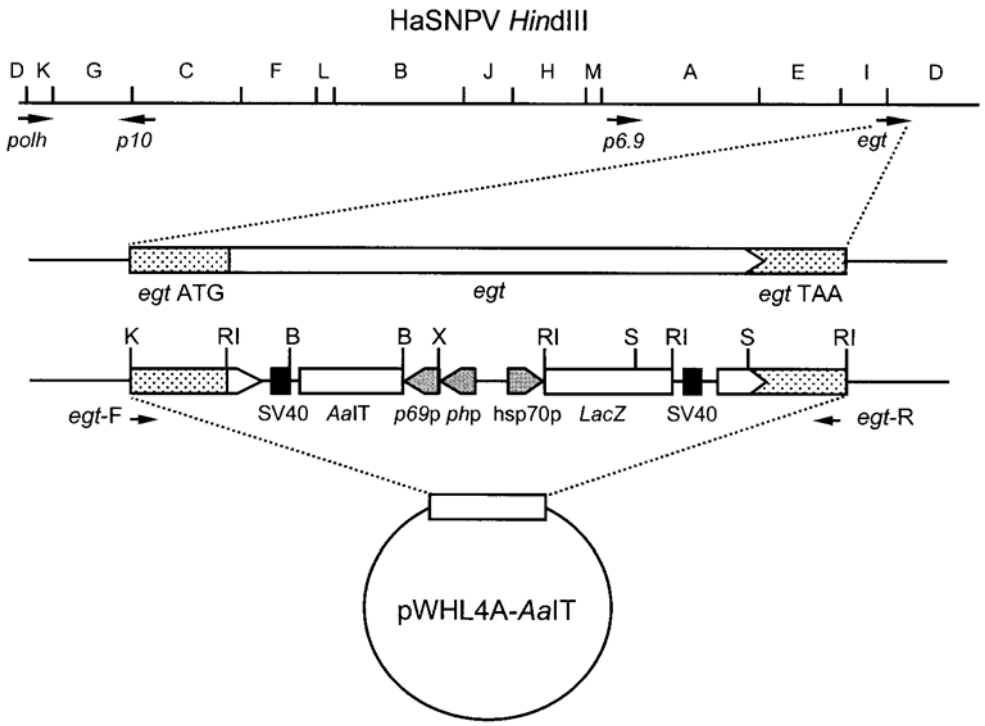
The HaSNPV *p6.9* promoter, p69p, was synthesized with primers *p6.9*F: 5' GGG *TCT AGA* GCA CAA CAA CTG CGT AGC GAT TCG 3' and *p6.9*R: 5' GGG *GGA TCC* CGG GTC GAC GTC TTC GGT ACA TTT TGG 3' on the basis of the *p6.9* gene analysis (Chen *et al*., 2001; Wang *et al*., 2001) using pCXW49 containing HaSNPV *Hin*dIII-K as a template. The PCR product (~150 bp) was cloned into the T-overhang vector pGEM-T (Gibco/BRL) to give pGEM-p6.9p. The promoter p6.9p was released from this plasmid using the new flanking *Xba*I (upstream) and *Bam*HI (downstream) sites from pGEM-T.

The chimeric promoter, ph-p69p promoter, was constructed by directional insertion of the *p6.9* promoter downstream of the *polyhedrin* promoter in *Xba*I / *Bam*HI-cleaved pHaCXW1 (Δ*egt*, see Chen *et al*., 2000b) to give an intermediate plasmid pHaCXW1-14A (Figure 1A). The chimeric promoter is shown in Figure 1B. Plasmid pHaCXW1-14A was linearized by cleavage with *Bsu*36I, which is located upstream of the *polyhedrin* promoter between the upstream fragment of Ha-*egt* gene and the SV40 terminator. A cassette containing *LacZ* gene, flanked by an hsp70 promoter and a SV40 terminator (pAcDZ1; Zuidema *et al*., 1990) was blunted and inserted in the opposite orientation to give intermediate plasmid pWHL4A. LacZ was used as a marker to screen the recombinants. The *AaIT* gene (McCutchen *et al*., 1991) was released from plasmid pXC6 (unpublished data) with *Bam*HI and cloned into the *Bam*HI site downstream of the chimeric ph-p6.9 promoter of pWHL4a to give the transfer vector, pWHL4A-*Aa*IT. The final construct of pWHL4A-*Aa*IT is depicted in Figure 1. All constructs were checked by sequencing new inserts and joining regions.

Generation of recombinant viruses

Hz2e5 cells (2×10^6 per 35-mm Petri dish) were cotransfected with 1 μg HaSNPV-EGTD DNA and 5 μg of transfer vector pWHL4a-*Aa*IT DNA using Lipofectin (GIBCO/BRL) as described (King and Possee, 1992). Five days after cotransfection, the supernatant was used to carry out a plaque assay. Two percent X-gal solution (GIBCO/BRL) was added onto the agarose overlay and the dishes were further incubated overnight at 27 °C. Recombinants were identified as blue colour plaques

A



B

GGATCC(*Bam*HI)CGGGTCGACGTCTTCGGTACATTTTGGAAATAAATTATTTCTATGGCGGAGATTGT
TGTTTTTTTCGTATACACCTTATAAAATAATTATATTCTTCTACGTTTCGACAATGTCGACGTTAATTG
TACATTAAGCGAATCGCTACGCAGTTGTTGTGCTCTAGA(*Xba*I)**GTCGAC**CTGCAG(*Pst*I)AATTATGG
GATATTTGATTTTTCACAATCTTGAACAATAGGAAAAAAATACTTATTTTAAAAATAAAAGTGTATG
TTTAATATTTATATTAAAGGTTTATTTAGTTTTATTACAAATTTTCAACACGACGACAGAAATCCCTC
AGGCTGCAG(*Pst*I)

Figure 1. (A) Location of *egt* on the HaSNPV genome (upper line) and a detailed map of the *egt* region (middle line) and the schematic represent of the transfer vector (pWHL4A-*Aa*IT) used to construct the recombinant HaSNPV-AaIT (lower line). The promoter for driving the expression of *LacZ* was a heat shock protein gene promoter (hsp70) derived from *Drosophila melanogaster* Meigen, and the one for *Aa*IT expression was a chimeric promoter from the *p6.9* (p69p) and *polyhedrin* (php) genes of HaSNPV. Two SV40 (transcription termination sites from Simian Virus 40) were also introduced. Restriction sites are indicated as: K, *Kpn*I; RI, *Eco*RI; B, *Bam*HI; S, *Sst*I and X, *Xba*I. (B) Sequence of the chimeric ph-p6.9p promoter. The nucleotide sequence of the entire (334 bp) chimeric promoter. A 6-nucleotide linker between p69p (left) and php (right) is shown in bold; restriction sites are underlined.

indicating for the insertion of the *LacZ* selection marker and most likely also *Aa*IT. All plaques were polyhedron-positive as the recombination took place at the *egt* locus. The putative recombinants were further purified by three rounds of plaque purification. Viral DNA isolated from the purified polyhedra was analysed by PCR (Figure 1) with the primers *egt*-F: 5' TTA TCA CCA ACA CTA GTA GTC ATC G 3' and *egt*-R: 5' TGC GCA ATA GGA GAC GGG TAG 3' (sequences from the 5' promoter region and 3' end of the HaSNPV *egt* gene, respectively) to confirm the allelic replacement. A PCR product of 4.5 kb is expected when the entire insert is present in a recombinant. The total volume for PCR was 50 µl containing 0.05 nmol L^{-1} *egt*-F and *egt*-R, 0.2 nmol L^{-1} each dNTPs, 1 × PCR buffer, 1 unit DNA polymerase (Expand Long Template PCR system, Roche Diagnostic GmbH) and approximately 5 ng of viral DNA. PCR amplifications were initiated with a denaturation step at 92 °C for 5 min, followed by 35 cycles of 30 sec at 92 °C, 45 sec at 55 °C, and 5 min at 68 °C by a Perkin-Elmer thermal cycler.

Analysis of the virion content within PIBs

Two µl of PIB suspension (1 × 10^9 PIBs ml^{-1}, in 1 × PBS (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 4.3 mmol L^{-1} $Na_2HPO_4 \bullet 7H_2O$, 1.4 mmol L^{-1} KH_2PO_4, pH 7.4)) was loaded onto a copper grid and incubated at room temperature for 10 min. Two µl of 0.5 × dissolution buffer (0.1 mol L^{-1} Na_2CO_3, 0.01 mol L^{-1} EDTA, 0.17 mol L^{-1} NaCl, pH 11.9) was added to dissolve the PIBs for 3~5 min. The remaining solution was removed from the grid with filter paper. The grid was stained with 2% phospho-tungstic acid solution (pH 5.7) for 3 min. The number of virions in an intact envelop of PIBs was counted by using a HITACHI 7000A transmission electron microscope. The difference between virion counts per PIB of two HaSNPV variants was compared by the Kolmogorov-Smirnov Z two-sample test (SPSS Inc., 2000).

Laboratory bioassays

The LD_{50} (median lethal dose), ST_{50} (median survival time) and FT_{50} (median time of feeding cessation) values of the HaSNPV variants against 1^{st} ~ 5^{th} instar *H. armigera* larvae were determined using a modified droplet-feeding bioassay method as described by Hughes *et al*. (1986). First instar larvae were selected 12 - 18 h after hatching and were starved at 28 ± 1 °C for 16 h prior to the bioassay. For 2^{nd} to 5^{th} instars, larvae were selected from those that were at the end of the previous instar, when about 30% of the larvae in the same batch had molted into the next instar. The selected larvae were starved for 16 h at 28 ± 1 °C and were allowed to molt into next aimed instars. Starved larvae were fed on droplets of PIB dilutions in 5% sucrose and 1 mg ml^{-1} Säure-blau blue dye (Waldeck-Gmbh & Co, Germany), which were placed in the

center of a 60 mm diameter glass Petri dish. Larvae that had ingested the PIB suspension within 10 min, as determined by the blue colouration of their midguts, were transferred to fresh diet and maintained at 28 ± 1 °C. The ingestion volume of 1st and 2nd instar larvae was determined by measuring the radioactivity of individual larvae which fed a solution (5% sucrose and 1 mg ml^{-1} Säure-blau blue dye) with a known concentration of ^{32}P-dATP as described by Hughes *et al*. (1984), Smits and Vlak (1988a) and Bianchi *et al*. (2000b). The ingested volume of 3rd to 5th instars of tested larvae was determined by directly weighing the larvae in the control group before and after feeding. The density of the bioassay solution was 1.016 g ml^{-1}.

To determine the LD_{50} values, 1st to 5th instar host larvae were exposed to a set of serial dilutions of each virus (36 - 48 insects per dose) and checked daily for mortality. To determine the ST_{50} and FT_{50} values, tested larvae were fed with a dose giving an LD_{99} for 1st to 3rd instars and an LD_{90} for 4th and 5th instar. The larvae were checked approximately every 8 h for mortality and were transferred onto new diet with smooth surface in 24-wells cell culture plates for FT_{50} determination. Lack of feeding marks within 24 h was considered as feeding cessation.

LD_{50} values were determined by probit analysis using the computer program POLO (Russell *et al*., 1977). Differences of LD_{50}s and the slopes of dose-mortality relationship among treatments against different stages of host larvae were analysed by Univariate Analysis of Variance using SPSS (SPSS Inc., 2000). The LD_{50}s per mg of weight of tested larvae for various HaSNPV variants against newly molted and 1 day-old larvae in 3rd or 4th instar were compared by paired-sample t-tests, respectively. The survival time and time to feeding cessation for each larva were taken as the midpoint of the interval in which they died or stopped feeding. Median survival times (ST_{50}s), median times to feeding cessation (FT_{50}s) and their 95% confidence limits were calculated using a Kaplan-Meier estimator (Kalbfleisch and Prentice, 1980; Collett, 1994) with survivors excluded from the analysis. The differences among the ST_{50} and FT_{50} values of the various HaSNPV variants were compared using of long-rank test (Kalbfleisch and Prentice, 1980). Survival time ratio (STR) and feeding cessation time ratio (FTR) were calculated relative to the wild-type control as described by Bonning and Hammock (1993).

Control efficacy of HaSNPV in field

For field release PIBs were purified by two centrifugation steps and resuspended in tap water containing 0.002% sodium azide and PIB concentrations were determined using a standard hemocytometer. PIB suspensions were formulated as described previously (Sun *et al*., 2002b).

Field trial #1 (Anyang, Henan, 2000) was conducted on ‘Zhongmiansuo #35’ cotton

grown near the China Cotton Research Institute, Anyang (36°5'N, 114°21'E), Henan. HaSNPV-WT, HaSNPV-EGTD, HaSNPV-AaIT at 2.4×10^{12} PIBs ha^{-1} and 2.5% $^{v}/v$ λ-Cyhalothrin emulsifiable concentrate (EC) (Zeneca Agrochemicals, UK) at 2,252 ml ha^{-1} were compared for control efficacy against a natural infestation of *H. armigera*. Formulated materials were diluted in tap water. Treatments were applied to plots (7 rows by 8 m, about 210 plants per plot) of cotton which was planted on April 24, 2000, with 80 cm row spacing. Treatments and non-treated controls were replicated 4 times in a completely randomized design (Sokal and Rohlf, 1995). The first round of treatment applications was made on August 20, when at least 30% of the plants checked had at least one *H. armigera* egg or larva. On August 25, the second round of treatments was made as *H. armigera* eggs still appeared at a high density. There was a slight rainfall (< 1 mm) on August 22. Treatment applications were performed with the appropriate PIB concentration at 600 L ha^{-1} using a backpack sprayer with a 0.4 mm diameter nozzle at a pressure of 10,197.2 kg m^{-2}. In each plot, a total of 25 plants were labelled. Labelled plants were in the central 3 rows of the plots and not in the outer 2 m of each row. On August 25, 28 and 30, the surviving larvae on the labelled plants were counted. On August 30, the total number of squares, flowers and bolls on 25 labelled plants was counted and the number of organs which was damaged because of bollworm feeding was also determined.

Field trial #2 (Jiayu, Hubei, 2000) was conducted on 'Ekangmian #5' cotton grown in Jiayu (29°58'N, 113°58'E), Hubei. The setup of this trial was similar to trial #1, but the recombinant HaSNPV-AaIT, at different concentrations (2.1×10^{12}, 1.5×10^{12} and 9.0×10^{11} PIBs ha^{-1}), was compared with the wild-type HaSNPV at 2.1×10^{12} PIBs ha^{-1} at this trial for efficacy against a natural infestation of *H. armigera*. *Bacillus thuringiensis* Berliner (Bt) wettable powder (WP) (Kenuo Bio-insecticide Corporation, Hubei, China) at 4.8×10^{10} International Units (IU) ha^{-1} was taken as a control as well. Applications were performed once on August 8 with the appropriate concentrations. The spray and investigation methods were the same as in trial #1. There was no rainfall during the experimental period (August 8 to 15). In each plot 25 plants were labelled. On August 8, 11 and 15, larval counts were made on the labelled plants in each plot. On August 15, the total number of squares, flowers and bolls was counted on the labelled plants.

Field trial #3 (Anyang, Henan, 2001) was conducted on 'Zhongmiansuo #38' cotton grown near the China Cotton Research Institute, Anyang, Henan. HaSNPV-WT, HaSNPV-EGTD, HaSNPV-AaIT at 2.4×10^{12} PIBs ha^{-1} and 2.5% λ-Cyhalothrin EC (Zeneca Agrochemicals) at 2,252 ml ha^{-1} were compared for efficacy against two cycles of natural infestation of *H. armigera*. Treatments were applied to plots (11 rows by 8 m, about 330 plants $plot^{-1}$) of cotton which had been planted on April 28, 2001, at

80 cm row spacing. Treatments and non-treated control plots were replicated 4 times in a randomized complete block design (Sokal and Rohlf, 1995). To account for possible effects of heterogeneity of irrigation on cotton yield, blocks were aligned in a series of decreasing levels of water supply by aligning them sequentially down the slope of the field. Plots were sprayed on June 26 and 30, July 3, 16 and 19 with the appropriate concentrations. The spray and investigation methods were the same as in trial #1. The number of surviving larvae and the number of damaged squares, flowers and bolls was determined at 3-4 day intervals. Daily average of rainfall was 8.7 mm from June 26 (date of the initial spray) to July 27 (date of the last measurement). Cotton lint from 150 plants in the central 5 rows of each plot was collected twice, on September 15 and October 10, 2001. The yield of the cotton lint harvest was extrapolated to kilograms of cotton lint per hectare for each treatment.

Field trial #4 (Anyang, Henan, 2002) was a repetition of field trial #3 for lint yield and also conducted in Anyang, Henan. The setup was similar as that in 2001. In trial #4 there were three cycles of bollworm infestation and the density of bollworm was much higher than in 2001. The plots were sprayed 8 times: on June 20, 24 and 28, July 20, 23 and 30, and August 22 and 26. The treatments were the same as in 2001, except that 3 different pesticides, 2.5% λ-Cyhalothrin EC (Zeneca Agrochemicals) at 2,252 ml ha^{-1}, 35% Endosulfan (AgEvo Gmbh) at 900 ml ha^{-1} and 4.5% β-Cypermethrin (Nanjing RedSun Group Co. LTD, China) at 600 ml ha^{-1}, were used in alternation as chemical control to avoid pest resistance. The spray and investigation methods were the same as in 2001. Daily average of rainfall was 1.6 mm from June 20 (the date for initial spray) to August 30 (date of the last measurement). On July 11 and 31, August 2, the entire field, including the 20 research plots, was treated with 40% Monocrotophos (Qindao Pesticide Factory, Shandong, China) at 600 ml ha^{-1} to control an infestation of cotton apid (*Aphis gossypii* Glover) and red mite (*Tetranychus truncatus* Ehara). Moreover, on August 22 and 26, 20% Dicofol (Anyang Pesticide Factory, Henan, China) at 450 ml ha^{-1} was added to tanks for research plots to control *T. truncatus*. Cotton lint was collected three times on September 28, October 20 and 28, 2002. The yield of cotton lint was expressed as kilograms per hectare.

Significant differences among treatments in number of surviving larvae, percentage of damaged squares, flowers and bolls were determined by one way ANOVA. Cotton lint from different collection dates was summed before analysis. Data of cotton yield were analysed with Univariate Analysis of Variance in GLM (SPSS Inc., 2000) with treatment and block as factors. If significant treatment effects were found, Fisher's LSD was used to separate treatment means. Larval counts and yield of cotton were transformed into square roots and percentile data were arcsine-transformed for statistical analysis.

RESULTS

Construction and characterization of HaSNPV recombinant virus

The structure of the transfer vector pHaWHL4A-*Aa*IT, which contains *AaIT* gene under control of the chimeric promoter ph-p69p, and the location of the *egt* gene in the HaSNPV genome are summarized in Figure 1A. The *AaIT* gene was inserted opposite to the orientation of the *egt* gene so that both the *egt* and *AaIT* transcripts terminated in SV40 terminators. The *LacZ* gene was under control of a *Drosophila* hsp70 promoter, known to be active in insect cells (Zuidema *et al*., 1990). The latter cassette was placed in an orientation opposite to the *AaIT* gene to avoid any transcriptional interference. By cotransfecting Hz2e5 cells with HaSNPV-EGTD genomic DNA and the transfer vector DNA, the recombinant HaSNPV-AaIT was generated. After three rounds of plaque purification, viral DNA was isolated from the polyhedra of infected cells and analysed by PCR with appropriate primers. The size of PCR product for HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT was 1.8 kb, 1.3 kb and 4.5 kb, respectively. This PCR test confirmed that the recombinant HaSNPV-AaIT was constructed correctly (Figure 2A).

Number of virions per PIB

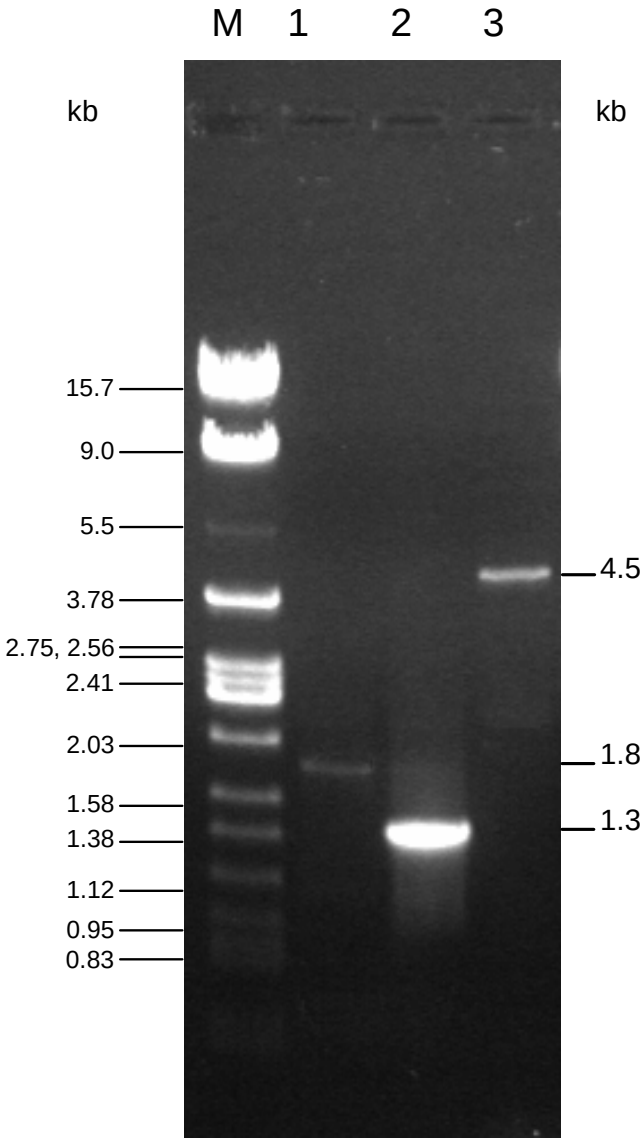
Virions within a polyhedron envelope could be clearly observed by using the method of dissolving the polyhedra on grids (Figure 2B). The average number of virions per PIB for HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT was 31.0 ± 11.1 (mean ± STD, n = 34), 24.9 ± 8.9 (mean ± STD, n = 36) and 27.0 ± 9.5 (mean ± STD, n = 34), respectively. Thus, no significant difference in the number of occluded virions per PIB was observed for these three HaSNPV variants (Asymp. sig. = 0.93, 0.19, and 0.19, respectively).

Biological activities in laboratory bioassays

The ingestion volume of tested larvae for the droplet feeding bioassays were determined by measurement of ingested radioactivity of 1st and 2nd *H. armigera* larvae or weighing the 3rd, 4th and 5th instar larvae before and after feeding. There was an allometric-like relationship between the larval weight and the ingested volume (Figure 3). The relationship of log (ingested volume, µl) and square root of (log(weight, mg) + 1) can be described as: $y = -3.929 + 2.929 \times x$ ($R^2 = 0.974$, n = 19). This relationship can be used to calculate the ingestion dose of this species in future.

Against instars with different sizes (including all five instars) of *H. armigera* larvae, the virulence (LD_{50}s) of these three HaSNPV variants was similar (F = 2.51, d.f. = 2, 18, P = 0.139) (Table 1). In addition, there was no significant difference among the slopes

A



B

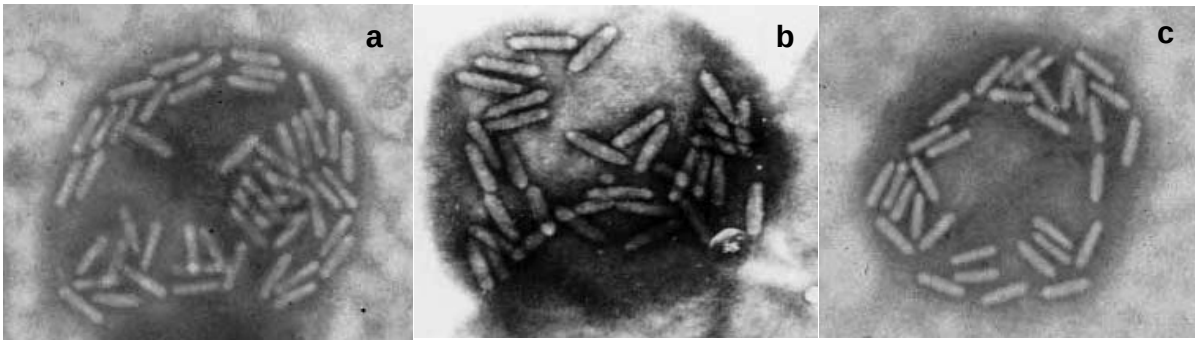


Figure 2. (A) PCR identification of wild-type and recombinant HaSNPV variants. Lane 1, 2 and 3 show amplification products of HaSNPV-WT (1.8 kb), HaSNPV-EGTD (1.3 kb) and HaSNPV-AaIT (4.5 kb), respectively. (B) Electron micrographs of alkali-treated PIBs of HaSNPV-WT (a), HaSNPV-EGTD (b) and HaSNPV-AaIT (c).

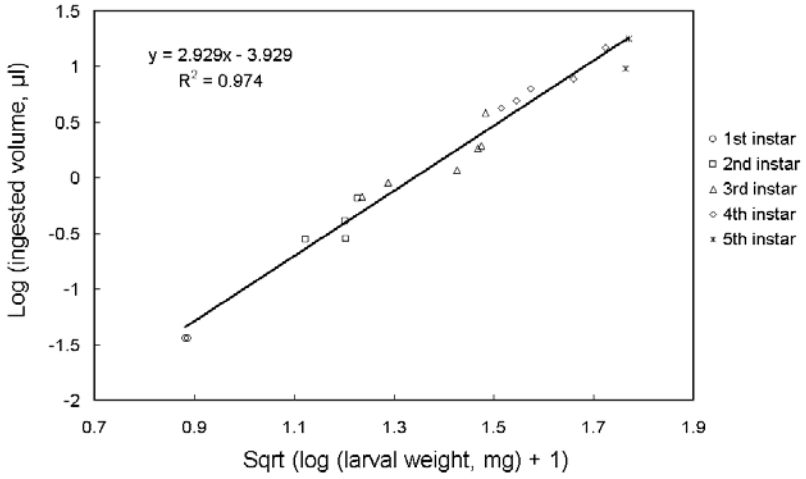


Figure 3. Relationship between larval weight (mg) and ingested volume (in µl, within 10 min) of larvae used for bioassays.

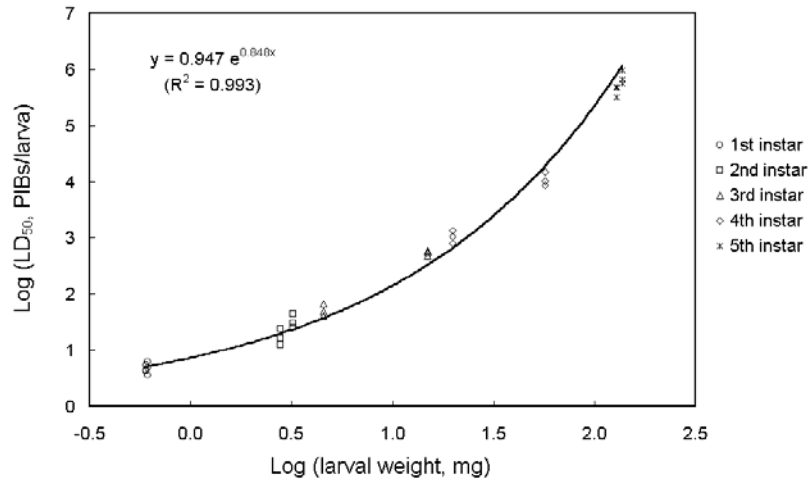


Figure 4. Relationship of larval weight (mg) and LD_{50} values (PIBs per larva).

of the relationship between mortality (probit) and the doses of these viruses against each stage of tested larvae (F = 1.651, d.f. = 2, 18, P = 0.22). In contrast, slopes for different larval stages were significantly different (F = 1.651, d.f. = 9, 18, P < 0.001). The log values of LD_{50}s increased exponentially as the larval weight increased. The relationship for log (weight, mg) and log (LD_{50}, PIBs per larva) can be described by the following equation: $y = 0.947 \times e^{0.848 \times x}$ ($R^2 = 0.993$, n = 10) (Figure 4). This relationship can be used to estimate the expected LD_{50} values at a given larval weight.

Third and 4th instar larvae were tested at an early stage (newly molted) or a late stage (1 day after molt). LD_{50}s of the HaSNPV variants against late 3rd and late 4th instars were 7.3 to 13.4 times higher than that against early 3rd and late 4th instars (Table 1). The LD_{50}s mg^{-1} larval weight ratio of late-stage larvae in 3rd instar or 4th instar was significantly higher than that of newly molted larvae in the respective instar (t = –6.91, d.f. = 2, P = 0.020 for 3rd instar and t = –5.91, d.f. = 2, P = 0.027 for 4th instar).

Table 1. Median lethal doses (LD_{50}) with 95% confidence limits of *Helicoverpa armigera* infected with wild-type and recombinant HaSNPV variants.

Instar	Weight (mg±SEM)	Ingested vol. (µl±SEM)	Conc. sets[1]	Viruses	LD_{50} (PIBs/larva)[2]	95% fiducial limits (lower; upper)	Equation [3]	Heterogeneity[4]
1st	0.60±0.12	0.036±0.009	1	HaSNPV-WT	5.4	(3.4; 9.1)	y = 1.510x +3.894	0.10
				HaSNPV-EGTD	4.2	(2.8; 6.3)	y= 1.302x +4.189	0.45
				HaSNPV-AaIT	4.4	(2.7; 6.3)	y= 1.387x +4.108	0.88
1st	0.61±0.12	0.036±0.009	1	HaSNPV-WT	4.8	(3.3; 7.2)	y= 1.340x +4.085	0.03
				HaSNPV-EGTD	6.1	(3.8; 10.1)	y= 1.103x +4.134	0.45
				HaSNPV-AaIT	3.6	(2.4; 5.1)	y= 1.400x +4.221	0.30
2nd	2.78±0.61	0.41±0.12	1	HaSNPV-WT	12.3	(6.7; 19.5)	y= 1.090x +3.812	0.03
				HaSNPV-EGTD	16.1	(9.8; 24.3)	y= 1.206x +3.545	0.73
				HaSNPV-AaIT	23.4	(14.7; 35.3)	y= 1.172x +3.395	0.57
2nd	3.21±0.27	0.65±0.15	1	HaSNPV-WT	29.9	(16.7; 47.0)	y= 0.854x +3.740	0.93
				HaSNPV-EGTD	25.1	(14.1; 39.7)	y= 0.872x +3.779	0.72
				HaSNPV-AaIT	43.5	(22.8; 80.9)	y= 0.913x +3.504	0.91
3rd	4.55±0.68	0.91±0.24	1	HaSNPV-WT	41.1	(11.8; 93.4)	y= 1.029x +3.494	1.01
				HaSNPV-EGTD	66.1	(39.5; 107.0)	y= 1.212x +2.869	0.56
				HaSNPV-AaIT	49.0	(27.7; 79.8)	y= 0.891x +3.437	0.15
3rd	14.94±4.01	1.93±0.30	3	HaSNPV-WT	582	(293; 961)	y= 0.987x +2.271	0.43
				HaSNPV-EGTD	546	(220; 989)	y= 0.806x +2.794	0.56
				HaSNPV-AaIT	471	(216; 798)	y= 0.996x +2.337	0.01

Table 1. Continued.

4th	19.79±1.86	4.20±0.44	2	HaSNPV-WT	793	(458; | 1,286)	y= 1.240x +1.405	0.11
				HaSNPV-EGTD	1,346	(720; | 2,518)	y= 0.972x +1.959	0.21
				HaSNPV-AaIT	1,059	(560; | 1,899)	y= 1.007x +1.954	0.43
4th	57.0±6.2	7.7±1.5	4	HaSNPV-WT	8,708	(4,084; | 15,112)	y= 1.065x +0.804	0.29
				HaSNPV-EGTD	14,740	(7,202; | 26,914)	y= 0.953x +1.027	0.20
				HaSNPV-AaIT	10,181	(4,896; | 17,788)	y= 1.034x +0.856	0.28
5th	128.6±16.2	9.6±1.9	5	HaSNPV-WT	319,700	(160,969; | 600,105)	y= 0.918x –0.053	0.62
				HaSNPV-EGTD	478,232	(238,918; | 988,507)	y= 0.877x +0.019	0.48
				HaSNPV-AaIT	460,967	(233,779; | 926,304)	y= 0.914x –0.177	0.23
5th	137.0±14.1	17.8±3.5	5	HaSNPV-WT	555,894	(224,314; | 1,293,151)	y= 0.695x +1.007	0.14
				HaSNPV-EGTD	660,954	(286,215; | 1,737,070)	y= 0.626x +1.357	0.53
				HaSNPV-AaIT	968,602	(477,074; | 2,151,131)	y= 0.808x +0.163	0.29

[1] Concentration sets were selected as: set 1: 1.0×10^4, 3.0×10^4, 1.0×10^5, 3.0×10^5 and 1.0×10^6 PIB ml^{-1}; set 2: 3.0×10^4,1.0×10^5, 3.0×10^5, 1.0×10^6 and 3.0×10^6 PIB ml^{-1}; set3: 1.0×10^5, 3.0×10^5, 1.0×10^6, 3.0×10^6 and 1.0×10^7 PIB ml^{-1}; set 4: 3.0×10^5, 1.0×10^6, 3.0×10^6, 1.0×10^7 and 3.0×10^7 PIB ml^{-1}; set 5: 1.6×10^6, 8.0×10^6, 4.0×10^7, 2.0×10^8 and 1.0×10^9 PIB ml^{-1}.

[2] Median Lethal Doses were determined by probit analysis using the program POLO (Russell *et al*., 1977).

[3] The intercepts of the equations of log (dose) – mortality (probit) were calculated based on the slopes and LD_{50}s given by POLO.

[4] Heterogeneity factor = the χ^2 / d.f. (the degrees of freedom) (POLO-PC manual, LeOra software, 1994).

When comparing the speed of action of the various HaSNPV variants, HaSNPV-AaIT had significant lower ST_{50} values than HaSNPV-WT (Table 2). The survival time ratio (STR) of HaSNPV-AaIT against HaSNPV-WT was between 0.66 and 0.83. The *egt*-deletion recombinant (HaSNPV-EGTD) could not always show a significantly quicker speed of action than HaSNPV-WT in these bioassays. For example, for newly hatched 1st instars, there was no significant difference between HaSNPV-WT and HaSNPV-EGTD (Table 2).

A most important agronomic parameter is the cessation time of feeding as a result of baculovirus infection. Compared to STRs, the feeding cessation time ratios (FTRs) of both HaSNPV-AaIT and HaSNPV-EGTD were lower against 3rd, 4th and 5th instars larvae (Table 3). FTRs of HaSNPV-AaIT were 0.61, 0.57 and 0.70, respectively, and for HaSNPV-EGTD they were 0.82, 0.72 and 0.80, respectively.

Control efficacy of HaSNPV in the field

In field trial #1 in Anyang, Henan in 2000, larval counts in HaSNPV-AaIT-treated plots were lower than in HaSNPV-WT-treated plots at 5 days post initial spray ($F = 10.9$, d.f. = 1, 15, $P = 0.219$) (Table 4). The differences were statistically significant at 8 days and 10 days after initial spray ($F = 24.2$, d.f. = 1, 15, $P = 0.029$ and $F = 13.0$, d.f. = 1, 15, $P = 0.001$). At 10 days post initial spray, the percentage of damaged bolls, squares and flowers in the plots treated with HaSNPV-AaIT were significantly lower than those in HaSNPV-WT treated plots ($F = 12.7$, d.f. = 1, 15, $P = 0.020$). The data also showed that the recombinant HaSNPV-AaIT provided control of *H. armigera* equivalent to the chemical standard, 2.5% λ-Cyhalothrin EC, based on the damage counts ($F = 12.7$, d.f. = 1, 15, $P = 0.622$). The *egt*-deletion recombinant HaSNPV-EGTD did not provide a better control of larvae than wild-type HaSNPV in this trial.

In field trial #2 (Jiayu, 2000) the effect of concentration using HaSNPV-AaIT was tested. At 3 days after spraying larval counts in the plots treated with HaSNPV-AaIT at high concentration (2.1×10^{12} PIBs ha^{-1}) started to be significantly lower than that in HaSNPV-WT (at the same high concentration as HaSNPV-AaIT) ($F = 30.1$, d.f. = 1, 18, $P < 0.001$) and Bt-WP treated plots ($F = 30.1$, d.f. = 1, 18, $P < 0.001$) (Table 5). At 7 days after spray, the percentage of damaged bolls, squares and flowers in the plots treated with HaSNPV-AaIT was significantly lower than those in HaSNPV-WT ($F = 71.3$, d.f. = 1, 18, $P = 0.001$) and Bt-WP ($F = 71.3$, d.f. = 1, 18, $P = 0.014$) treated plots. In this trial, HaSNPV-AaIT at a lower dosage did not provide as good a control as at the high concentration.

The field trials #3 and #4 were a repetition of trial #1, but applications were made to control all cycles of bollworm infestation appearing in 2001 and 2002. The final yields of cotton lint were now included in these analyses. In field trial #3 the final lint yield

Table 2. Median survival times (ST_{50}s) of *Helicoverpa armigera* infected with wild-type and recombinant HaSNPV variants.

Instar	Weight (mg ± SEM)	Ingested volume (µl ± SEM)	Dose (PIBs $larva^{-1}$)	Viruses	ST_{50} (h.p.i)[1]	STR[2]	SE	95% fiducial limits (lower; upper)
1st	0.60 ± 0.12	0.036±0.009	360	HaSNPV-WT	85.0 a	1	0.9	(83.2; 86.8)
				HaSNPV-EGTD	76.5 a	0.90	4.2	(68.3; 84.7)
				HaSNPV-AaIT	68.5 b	0.81	3.8	(62.1; 76.9)
1st	0.61 ± 0.12	0.036±0.009	360	HaSNPV-WT	89.5 a	1	1.4	(87.7; 92.3)
				HaSNPV-EGTD	76.5 a	0.85	3.4	(69.9; 83.1)
				HaSNPV-AaIT	66.5 b	0.74	2.3	(62.1; 71.0)
2nd	2.78 ± 0.61	0.41±0.12	4,100	HaSNPV-WT	84.5 a	1	1.2	(82.2; 86.8)
				HaSNPV-EGTD	75.5 b	0.89	1.5	(72.6; 78.4)
				HaSNPV-AaIT	60.5 c	0.72	3.6	(53.4; 67.6)
2nd	3.21 ± 0.27	0.65±0.15	6,500	HaSNPV-WT	96.0 a	1	2.9	(90.4; 102.0)
				HaSNPV-EGTD	87.5 b	0.91	2.4	(82.8; 92.2)
				HaSNPV-AaIT	63.5 c	0.66	3.3	(57.0; 70.0)
3rd	3.37 ± 0.81	0.67±0.19	6,700	HaSNPV-WT	95.0 a	1	2.3	(90.5; 99.5)
				HaSNPV-EGTD	77.5 b	0.82	2.9	(71.8; 83.2)
				HaSNPV-AaIT	71.0 b	0.75	2.9	(65.3; 76.7)
3rd	4.55 ± 0.68	0.91±0.24	9,100	HaSNPV-WT	116.5 a	1	5.1	(106.6; 126.4)
				HaSNPV-EGTD	108.5 b	0.93	4.0	(100.7; 116.3)
				HaSNPV-AaIT	92.5 c	0.79	3.7	(85.2; 99.8)
4th	19.79 ± 1.86	4.20±0.44	42,000	HaSNPV-WT	133.0 a	1	3.7	(125.8; 140.2)
				HaSNPV-EGTD	109.0 b	0.82	1.5	(106.0; 112.0)
				HaSNPV-AaIT	104.6 c	0.79	1.8	(105.4; 112.6)
4th	24.47 ± 3.13	4.93±0.94	148,900	HaSNPV-WT	133.5 a	1	2.0	(129.7; 137.3)
				HaSNPV-EGTD	122.0 b	0.91	2.4	(117.2; 126.8)
				HaSNPV-AaIT	110.0 c	0.83	2.8	(104.5; 115.5)
5th	128.6 ± 16.2	9.60±1.93	9,600,000	HaSNPV-WT	133.0 a	1	2.1	(128.9; 137.1)
				HaSNPV-EGTD	121.0 b	0.91	3.2	(114.8; 127.2)
				HaSNPV-AaIT	108.5 c	0.82	5.3	(98.0; 119.0)

[1] Median survival time was determined by survival analysis (Kaplan Meier Estimator, Kalbfleisch and Prentice, 1980; Collett, 1994). The difference among the ST_{50} values of viruses in the same experiment was compared by log-rank test (Kalbfleisch and Prentice, 1980). Different letters behind ST_{50} values in each trial indicate significant differences between viruses (P = 0.05). h.p.i. = hours post inoculation.

[2] Survival Time Ratio = ST_{50} of recombinant virus / ST_{50} of wild-type virus (Bonning and Hammock, 1993).

Table 3. Median times to feeding cessation (FT_{50}) of *Helicoverpa armigera* infected with wild-type and recombinant HaSNPV variants.

Instar	Weight (mg ± SEM)	Ingested volume (µl ± SEM)	Dose (PIBs/larva)	Viruses	FT_{50} (h.p.i)[1]	FTR[2]	SE	95% fiducial limits (lower; upper)
3rd	4.55 ± 0.68	0.91 ± 0.24	9,100	HaSNPV-WT	84.0 a	1	3.0	(78.1; 89.9)
				HaSNPV-EGTD	68.5 b	0.82	1.5	(65.6; 71.4)
				HaSNPV-AaIT	51.5 c	0.61	2.4	(46.8; 56.2)
4th	19.79 ± 1.86	4.20 ± 0.44	42,000	HaSNPV-WT	117.5 a	1	2.5	(113.0; 122.5)
				HaSNPV-EGTD	85.0 b	0.72	2.1	(80.8; 89.2)
				HaSNPV-AaIT	66.5 c	0.57	2.4	(62.8; 70.5)
5th	128.6 ± 16.2	9.60 ± 1.93	9,600,000	HaSNPV-WT	121.0 a	1	1.9	(117.2; 124.8)
				HaSNPV-EGTD	96.5 a	0.80	3.3	(90.1; 102.9)
				HaSNPV-AaIT	84.5 b	0.70	5.9	(73.0; 96.0)

[1] Median time to feeding cessation was determined by survival analysis (Kaplan Meier Estimator, Kalbfleisch and Prentice, 1980; Collett, 1994). The difference among the FT_{50} values of viruses in same experiment was compared by log-rank test (Kalbfleisch and Prentice). Different letters behind FT_{50} values in each trial indicate significant differences between viruses (P = 0.05). Different letters behind FT_{50} values in each trial indicate significant differences between viruses (P = 0.05). h.p.i. = hours post inoculation.

[2] Feeding cessation Time Ratio = FT_{50} of recombinant virus / FT_{50} of wild-type virus.

Table 4. Control of bollworm *Helicoverpa armigera* in cotton (Anyang, Henan, 2000, trial #1).

Treatments	Conc. per ha	Surviving larvae (mean ± SEM) / 25 plants[1]				% damage (mean ± SEM)[1, 3]
		20 August[2]	25 August[2]	28 August	30 August	
HaSNPV-WT	2.4×10^{12} PIBs	48.2±6.7 a	15.8±5.3 bc	13.5±1.5 c	17.5±4.7 b	10.3±3.1 b
HaSNPV-EGTD	2.4×10^{12} PIBs	39.6±3.1 a	30.0±5.6 c	17.3±3.4 cd	21.0±3.5 b	15.1±0.4 b
HaSNPV-AaIT	2.4×10^{12} PIBs	50.2±7.0 a	8.8±2.5 ab	7.8±1.8 b	3.8±1.3 a	4.1±1.4 a
λ-Cyhalothrin	56.3 g	34.4±2.7 a	3.5±1.2 a	2.5±0.3 a	3.3±0.9 a	4.7±1.2 a
Control[4]	-	39.2±3.3 a	34.5±3.8 c	25.8±3.1 d	22.3±3.9 b	18.4±0.8 c

[1] Different letters indicate significant differences between treatments (P = 0.05).

[2] Applications were made on August 20 and 25. Larvae were counted before spray.

[3] Number of squares, flowers and bolls on 25 plants in each plot which were damaged or undamaged due to bollworm feeding was counted on August 30. % damage was calculated as counts of damaged fruits / (counts of undamaged fruits + counts of damaged fruits).

[4] Control plots were treated with formulation only.

Table 5. Control of bollworm *Helicoverpa armigera* in cotton (Jiayu, Hubei, 2000, trial #2).

Treatments	Concentration per ha	Surviving larvae (mean ± SEM) / 25 plants[1]			% damage (mean ± SEM)[1, 3]
		8 August [2]	11 August	15 August	
HaSNPV-WT	2.1×10^{12} PIBs	20.8±0.6 a	13.8±0.5 b	9.8±0.4 c	11.2±0.8 b
HaSNPV-AaIT	2.1×10^{12} PIBs	19.0±1.0 a	4.8±0.5 a	2.8±0.5 a	7.6±0.7 a
HaSNPV-AaIT	1.5×10^{12} PIBs	22.5±2.1 a	12.0±1.7 b	6.8±0.9 b	12.5±0.5 bc
HaSNPV-AaIT	9.0×10^{11} PIBs	19.3±1.5 a	11.8±0.7 b	8.3±0.5 bc	14.3±0.6 c
B. thuringiensis WP	4.8×10^{10} IU[4]	19.0±1.5 a	12.3±0.9 b	8.0±0.6 bc	10.7±0.7 b
Control [5]	-	20.8±1.5 a	19.5±1.3 c	18.5±1.3 d	17.5±0.6 d

[1] Different letters indicate significant differences between treatments (P = 0.05).

[2] Applications were made on August 8. Larvae were counted before spray.

[3] Number of squares, flowers and bolls on 25 plants in each plot which were damaged or undamaged due to feeding by bollworm was counted on August 15. Percentage damage was calculated as counts of damaged fruits / (counts of undamaged fruits + counts of damaged fruits).

[4] IU = International Units.

[5] Control plots were treated with formulation only.

in the plots treated with HaSNPV-AaIT was 21.1% and 31.3% higher than that in HaSNPV-WT (F = 3.3, d.f. = 1, 12, P = 0.027) and HaSNPV-EGTD (F = 3.3, d.f. = 1, 12, P = 0.006) treated plots, respectively, and not significantly different from that in λ-Cyhalothrin treated plots (F = 3.3, d.f. = 1, 12, P = 0.09) (Table 6). In field trial #4 the final lint yield in the plots treated with HaSNPV-AaIT was 20.7% higher than that in HaSNPV-WT treated plots, but this difference was not significant (F = 11.9, d.f. = 1, 12, P = 0.251). There was also no significant difference between the lint yields in plots treated with standard chemicals and in HaSNPV-AaIT (F =11.9, d.f. = 1, 12, P = 0.402) or HaSNPV-EGTD (F =11.9, d.f. = 1, 12, P = 0.305) treated plots (Table 6).

DISCUSSION

Expression of the insect-specific toxin *Aa*IT from the North African scorpion *Androctonus australis* along with baculovirus infection is among the most effective approaches to enhance the efficacy of baculovirus insecticides (Inceoglu *et al*., 2001). Usually, toxins are expressed under a very late (e.g. the *polyhedrin*) promoter. Several groups have reported that the baculovirus *p6.9* promoter is one of best promoters for expressing toxin or other genes at very high levels late after infection but prior to *polyhedrin* (Bonning *et al*., 1999; Popham *et al*., 1997; Harrison and Bonning, 2000,

Table 6. Yield of cotton lint after application of wild-type and recombinant HaSNPV variants or standard chemical(s) to control of *Helicoverpa armigera* over one season (Anyang, Henan, 2001, trial #3 and 2002, trial #4)

Treatments	Concentration per ha	Yield of cotton lint (mean ± SEM, kg ha^{-1}) [1]	
		2001 [2]	2002 [3]
HaSNPV-WT	2.4 ×10^{12} PIBs	1023.0 ± 33.7 b	1474.0 ± 199.6 b
HaSNPV-EGTD	2.4 ×10^{12} PIBs	951.5 ± 36.8 b	1750.0 ± 305.5 ab
HaSNPV-AaIT	2.4 ×10^{12} PIBs	1249.5 ± 68.0 a	1799.5 ± 140.1 ab
Standard chemicals (s)	Depending	1083.6 ± 49.5 ab	1993.8 ± 387.2 a
Control	-	931.0 ±109.5 b	732.3 ± 276.9 c

[1] Lint yields were extrapolated from hand-harvested 5 rows (8 m) from each plot (4 plots per treatment). Different letters indicate significant differences between treatments (P = 0.05).

[2] Plots were sprayed on June 26 and 30, July 3, 16 and 19, 2001, respectively. Standard chemical was 2.5% λ-Cyhalothrin EC at 2,252 ml ha^{-1}.

[3] Plots were sprayed on June 20, 24 and 28, July 20, 23 and 30, and August 22 and 26, 2002, respectively. Standard chemicals were used as 2.5% λ-Cyhalothrin EC at 2,252 ml ha^{-1} (June 20 and 28, July 20 and August 26), 35% Endosulfan at 900 ml ha^{-1} (June 24 and August 22) and 4.5% β-Cypermethrin at 600 ml ha^{-1} (July 23 and 30).

2001). Therefore, we have generated and tested a recombinant HaSNPV, HaSNPV-AaIT, which expressed *Aa*IT toxin under the control of a chimeric *p6.9* and *polyhedrin* promoters. This HaSNPV recombinant showed a significant improvement in insecticidal properties, both in laboratory bioassays and in the field, compared to a wild-type HaSNPV and an *egt*-deletion recombinant, HaSNPV-EGTD. The laboratory bioassays provide a base for the development of a process-based model for the control of the cotton bollworm in the field as has been done for the beet armyworm (Bianchi *et al*., 2002b).

The question arises whether any of the effects presented in this study can be attributed to the marker genes. Both genes are generally considered to be neutral, but counter evidence has also been collected. For instance, HaSNPV-EGTD contained *GFP* and HaSNPV-AaIT contained *LacZ* as marker genes, whereas wild-type HaSNPV did not contain these markers. For the *GFP* gene no differences in insecticidal properties have been recorded for other baculoviruses with and without GFP (Chen *et al*., 2000b; Bischoff and Slavicek, 1999; Dai *et al*., 2000). LacZ is generally neutral (Bianchi *et al*., 2000b), although in one instance an AcMNPV recombinant containing *LacZ* gene had a 7 times higher LD_{50} value as the wild-type AcMNPV in *Heliothis virescens* larvae (Bonning *et al*., 1992). In our case HaSNPV-AaIT, which

expresses *Aa*IT toxin as well as LacZ, did not differ in infectivity (LD_{50}) as compared to wild-type virus (Table 1). Wood *et al*. (1993) found that a polyhedrin-negative AcMNPV recombinant expressing LacZ had a 17 h increase in survival time against neonate *T. ni* larvae as compared to a polyhedrin-negative AcMNPV recombinant without LacZ. On the other hand, based on a comparative bioassay using an AcMNPV-Δ*egt* mutant expressing LacZ (Flipsen *et al*., 1995) and an AcMNPV-Δ*egt* mutant without LacZ showing similar LT_{50} values, Bianchi *et al*. (2000b) concluded that the absence/presence of LacZ in recombinants of this virus is not responsible for difference of the speed of action between these viruses. Thus, no convincing evidence is present for effects of markers on the virulence and aggressiveness of baculoviruses. Accordingly, we attribute the differences in bollworm response in the field between wild-type and recombinant HaSNPV variants as a consequence of the *egt* deletion or the *Aa*IT insertion.

When *H. armigera* larvae were dosed at late stage in a certain instar, they were 7.3 to 13.4 times more resistant to the virus infection compared to a very early stage. This phenomenon has also been observed when *T. ni* larvae was infected by AcMNPV at 2nd and 4th instars (Washburn *et al*., 1995). Another explanation for this phenomenon is that late-stage larvae have a much thicker peritrophic membrane that makes it difficult for virions to penetrate and to access the midgut epithelium cells. The midgut epithelium is sloughed off when the instars molt into the next instar. The new epithelial cells are generated after the molt and the epithelium membrane is very thin. Therefore it is easy to be penetrated by virions.

Another widely used nucleopolyhedrovirus from a heliothine pest insect, *H. zea* SNPV, which is genetically and biologically closely-related to HaSNPV (Gettig and McCarthy, 1982; Hughes *et al*., 1983; Sun and Zhang, 1994; Chen *et al*., 2002b), was also genetically modified by both deletion of the *egt* gene and insertion of an insect-selective neurotoxin gene (*tox34*) in the *egt* locus (Popham *et al*., 1997). The toxin-expression recombinant HzSNPV significantly decreased the median effective time (ET_{50}) of this virus. However, the *egt* deletion mutation of this virus did not shorten the ET_{50} when tested against neonate *H. zea*. In our present study, the *egt* deletion mutation of HaSNPV (HaSNPV-EGTD) also did not show a significant improvement of the killing speed of 1st instar *H. armigera* larvae relative to HaSNPV-WT. HaSNPV-EGTD, however, killed 2nd to 5th instar *H. armigera* faster than HaSNPV-WT (Table 2). When dosed on 3rd to 5th instars of this species, HaSNPV-EGTD clearly reduced the FT_{50} as compared to HaSNPV-WT (Table 3). Possibly, the deletion of the *egt* gene affects the speed of action of the virus in the later instars more strongly than in earlier instars. This situation also seems to occur in *Lymantria dispar* MNPV and its gypsy moth host as well (Slavicek *et al*., 1999). In our field study, HaSNPV-EGTD

only provided slightly better control efficacy than wild-type HaSNPV in trial #4. It might be that the effect of the *egt* deletion (7 - 18% reduction in ST_{50}) is too subtle to obtain an improved efficacy in a complex field situation.

Larvae infected with recombinant baculoviruses encoding a scorpion toxin (*Aa*IT) would exhibit signs of neurotoxicity and fall from the plant about 3 days after consuming the viral inoculum (Hoover *et al*., 1995). In one case (field trial #2), the larval counts in the plots treated with HaSNPV-AaIT were significantly lower than those in HaSNPV-WT or Bt-WP treated plots at 3 days after treatment application (Table 5). In three other cases, significant differences between larval counts in HaSNPV-AaIT and HaSNPV-WT treated plots were occasionally found at a later stage (Table 6 and data not shown). This situation also appeared in the experiments conducted by Smith *et al*. (2000a). When recombinant AcMNPV and HzSNPV expressing *Lqh*IT2 toxin were applied to control two heliothine flights in 1997 and one flight in 1998, the number of larvae in plots treated with the recombinant viruses was similar to that in the wild-type viruses treatment in all cases except one. So, larval counts did not prove to be a reliable measure of viral control efficacy. On the other hand, final cotton yield integrated overall effects of virus application in an entire season and seems a more reliable measure of baculovirus efficacy.

In summary we concluded that baculoviruses expressing an insect-selective toxin (*Aa*IT) from a chimeric, late-very late baculovirus promoter have superior properties in the control of *H. armigera* in the field as compared to wild-type and *egt*-deletion recombinant. Such a recombinant has a potential to be developed as a commercial biopesticide to control cotton bollworm in the future.

CHAPTER 3

Production of polyhedral inclusion bodies from *Helicoverpa armigera* larvae infected with wild-type and recombinant HaSNPV*

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ABSTRACT

We report on the yield of Polyhedral Inclusion Bodies (PIBs) from 1st to 5th instar *Helicoverpa armigera* larvae inoculated with wild-type *Helicoverpa armigera* nucleopolyhedrovirus (HaSNPV-WT) or with one of two HaSNPV recombinants, one in which the ecdysteroid UDP-glucosyltransferase (*egt*) gene was deleted (HaSNPV-EGTD) and a second in which the *egt* gene was replaced by a scorpion toxin (*Aa*IT) gene (HaSNPV-AaIT). A significant linear relationship between the logarithm of cadaver weight and the logarithm of the number of PIBs per cadaver was observed for all three HaSNPV variants. The increase of the number of PIBs with larval weight was significantly greater for HaSNPV-WT than for the recombinant viruses. For each of the three HaSNPV variants, PIB yield per cadaver was significantly affected by larval instar at death and by time to death, with later instars and longer surviving larvae producing a greater number of PIBs. As both recombinants caused host larvae to die at earlier instars than HaSNPV-WT, their virus yields were significantly reduced. Virus yield per larva, inoculated with HaSNPV-AaIT in the 1st, 2nd, 3rd, 4th or 5th larval stage was 23%, 32%, 41%, 44% and 47% of the yield of HaSNPV-WT, respectively. For HaSNPV-EGTD, virus yield per larva inoculated in 1st through 5th instar, respectively, was 41%, 55%, 63%, 54% and 82% of the yield of HaSNPV-WT. These results provide a basis for optimizing the production regime of recombinant HaSNPV variants in larvae and for modelling the behaviour of these viruses in agro-ecosystems.

Key words: HaSNPV, *Helicoverpa armigera*, recombinants, PIB production, larval instars.

INTRODUCTION

To improve the efficacy of baculoviruses as insect biocontrol agents, two main genetic strategies have been followed (Black *et al*., 1997; Van Beek and Hughes, 1998). These are the deletion of genes, e.g. the ecdysteroid UDP-glucosyltransferase (*egt*) gene (O'Reilly and Miller, 1991), or the insertion of insecticidal genes from mites and scorpions encoding, e.g. insect-selective toxins (Inceoglu *et al*., 2001). Laboratory studies have shown that the insertion of insect-selective toxin genes into baculovirus genomes can result in a quicker cessation of feeding and onset of death in infected insect larvae (Van Beek and Hughes, 1998; Inceoglu *et al*., 2001). However, rapid larval paralysis or accelerated death also results in reduced yield of polyhedral inclusion bodies (PIBs) from diseased larvae (Burden *et al*., 2000; Cory *et al*., 1994; Hernández-Crespo *et al*., 2001; Ignoffo and Garcia, 1996; O'Reilly and Miller, 1991; Tomalski and Miller, 1992). Because industrial production of viruses relies on the use of larvae, reduction in yield of PIBs of a recombinant can result in an increase in the cost of a commercial product (Ignoffo and Couch, 1981). An ecological factor to be considered is that a reduction in PIB yield of a recombinant in its host might affect the dynamics of the virus in the field, e.g. by reducing horizontal transmission or limiting the amount of residual virus in the soil reservoir. This might be detrimental for control efficacy of the insect pest in the field. On the other hand, such limiting effects might be valued as an advantage in achieving ecological safety, due to decreased likelihood for spread and persistence in host populations.

Cotton bollworm, *Helicoverpa armigera* Hübner, is one of the most serious pests on cotton around the world (Fitt, 1989). *H. armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV) is a baculovirus specific for this and related species (Gettig and McCarthy, 1982) and has been developed and applied as a commercial biopesticide to control this pest in China since the 1980s (Zhang, 1989). To improve the insecticidal properties HaSNPV has been genetically engineered by deleting the ecdysteroid UDP-glucosyltransferase gene (*egt*) from its genome (recombinant HaSNPV-EGTD) (Chen *et al*., 2000b) and incorporating the toxin gene (*AaIT*) from the scorpion *Androctonus australis* Hector (recombinant HaSNPV-AaIT) (Chapter 2). In the field, this recombinant exhibits improved efficacy in reducing pest insect populations and crop damage, compared to wild-type HaSNPV (Sun *et al*., 2002b; Chapter 2). The objective of the present paper is to determine to what extent the deletion of the *egt* gene or the insertion of an *Aa*IT toxin gene in HaSNPV affect the yield of PIBs in the 5 larval instars of *H. armigera*. This is not only important for evaluating the logistics and economics of virus production in virus 'factories', but also for modelling the quantitative epidemiology of wild-type and recombinant HaSNPV infections in the (cotton) field.

MATERIALS AND METHODS

Viruses and insects

HaSNPV was initially isolated from diseased *H. armigera* larvae collected in Hubei province of China (Zhang *et al*., 1981). A genotype of this wild-type HaSNPV (HaSNPV-G4) was isolated with an *in vivo* cloning method (Sun *et al*., 1998) and is designated here as HaSNPV-WT. The recombinant HaSNPV-EGTD (HaCXW1 in Chen *et al*., 2000b; Sun *et al*., 2002b) was generated by deletion of the *egt* gene from HaSNPV-WT by recombination in cell culture with a plasmid with the flanking regions of *egt* and the green fluorescent protein (GFP) gene inserted. The recombinant HaSNPV-AaIT was generated by insertion of the insect-selective scorpion toxin *Aa*IT downstream of a chimeric promoter of the *p6.9* and *polyhedrin* genes of HaSNPV at the *egt* gene locus using the same recombination strategy (Chapter 2). Viruses were propagated in fourth instar *H. armigera* larvae as described by Sun *et al*. (1998). PIBs were purified by sucrose gradient centrifugation and stored in 40% glycerol at 4 °C until use. A laboratory colony of cotton bollworm was reared on artificial diet at 28 ± 1 °C and a 16 h : 8 h (light:dark) photoperiod (Zhang *et al*., 1981).

Inoculation and yield measurement

First to fifth instar *H. armigera* larvae were inoculated with HaSNPV using a modified droplet-feeding bioassay method as described previously (Chapter 2). First instar larvae were selected 12 - 18 h after hatching and were starved at 28 ± 1 °C for 16 h prior to infection. Second to fifth instar larvae were selected from a near-synchronous rearing. Selected larvae were taken at the end of the previous instar when about 10 ~ 30% of the larvae in the same batch had already molted into the instar to be tested. The selected larvae were starved for 16 h at 28 ± 1 °C and allowed to molt into the required instar. Starved larvae were fed on droplets of PIB dilutions in 5% w/v sucrose and 1 mg ml^{-1} Säure-blau blue dye (Waldeck-Gmbh & Co). Larvae that had ingested the PIB suspension within 10 min, as shown by the blue colouration of their midguts, were transferred to fresh diet and further reared individually at 28 ± 1 °C. The following serial dilutions of each virus were used: for first and second instars: 1×10^4, 3×10^4, 1×10^5, 3×10^5 and 1×10^6 PIBs ml^{-1}; for third and forth instars: 1×10^5, 3×10^5, 1×10^6, 3×10^6 and 1×10^7 PIBs ml^{-1}; for fifth instars: 1.6×10^6, 8×10^6, 4×10^7, 2×10^8 and 1×10^9 PIBs ml^{-1}. At each concentration, 36 ~ 48 larvae were inoculated. The larvae were incubated at 28 ± 1 °C and 16 h : 8 h (light : dark) photoperiod and inspected daily for larval stage and disease status. Individuals showing terminal disease symptoms (discolouration, no movement) were transferred to microtubes and weighed prior to death. The whole experiment was conducted twice. The averaged

weights of the inoculating larvae in the two replicates were 0.60 mg and 0.61 mg, 2.78 mg and 3.21 mg, 4.55 mg and 14.9 mg, 19.8 mg and 57.0 mg, 128.6 mg and 137 mg, for the 1st, 2nd, 3rd, 4th or 5th larval stage, respectively. The averaged volumes of PIB solution ingested by 1st through 5th instar larvae in the two replicates were 0.036 µl and 0.036 µl, 0.41 µl and 0.65 µl, 0.91 µl and 1.93 µl, 4.20 µl and 7.7 µl, 9.6 µl and 17.8 µl, respectively (Chapter 2). The data of larval mortality and yield observed in the two replicates were pooled for statistical analysis.

After collection, cadavers were stored at −20 °C. For yield measurement, 1st and 2nd instar larvae were individually homogenized with 0.1 ml of sterile water, while 3rd, 4th and 5th instar larvae were individually homogenized with 1 ml of water. The homogenate was sonicated for 2 min, after which the PIBs were counted using a hemocytometer (Neubauer improved). Yield was not estimated from larvae that died and lysed before collection, because in this case accurate determination of larval weight and virus yield is not possible. In the calculation of yield per inoculated larvae, it is assumed that the collected larvae are a representative sample from the population of virus-infected larvae, that is, we assume that there is no difference in virus yield between those larvae that were collected before they lysed and those larvae that could not be collected because they had lysed before they could be collected.

Statistical analysis

The relationship between the number of PIBs per cadaver and cadaver weight (both log-transformed) was analysed by linear regression with SPSS (SPSS Inc., 2000). The slopes of the regression lines were compared with a two sides z-test (Snedecor and Cochran, 1989). The effects of four influencing factors on virus yield per cadaver was analysed by Analysis of Variance, separately for each instar of inoculation, using the General Linear Models procedure in SPSS (SPSS Inc., 2000). Virus type, inoculum concentration and instar at death were introduced as fixed factors (i.e. as class variables) in the analysis, and time to death as a covariate, i.e. an influencing factor that is measured at a continuous scale (days). Initially, all explanatory variables and the interactions among them were fitted to the data and the contribution of each term was tested for significance. Nonsignificant interaction terms were subsequently removed, resulting in a minimal adequate model which is presented here.

RESULTS

There was a linear relationship between the logarithm of the number of PIBs per cadaver and the logarithm of cadaver weight after inoculation with HaSNPV-WT (Figure 1A), HaSNPV-EGTD (Figure 1B) or HaSNPV-AaIT (Figure 1C) (data from

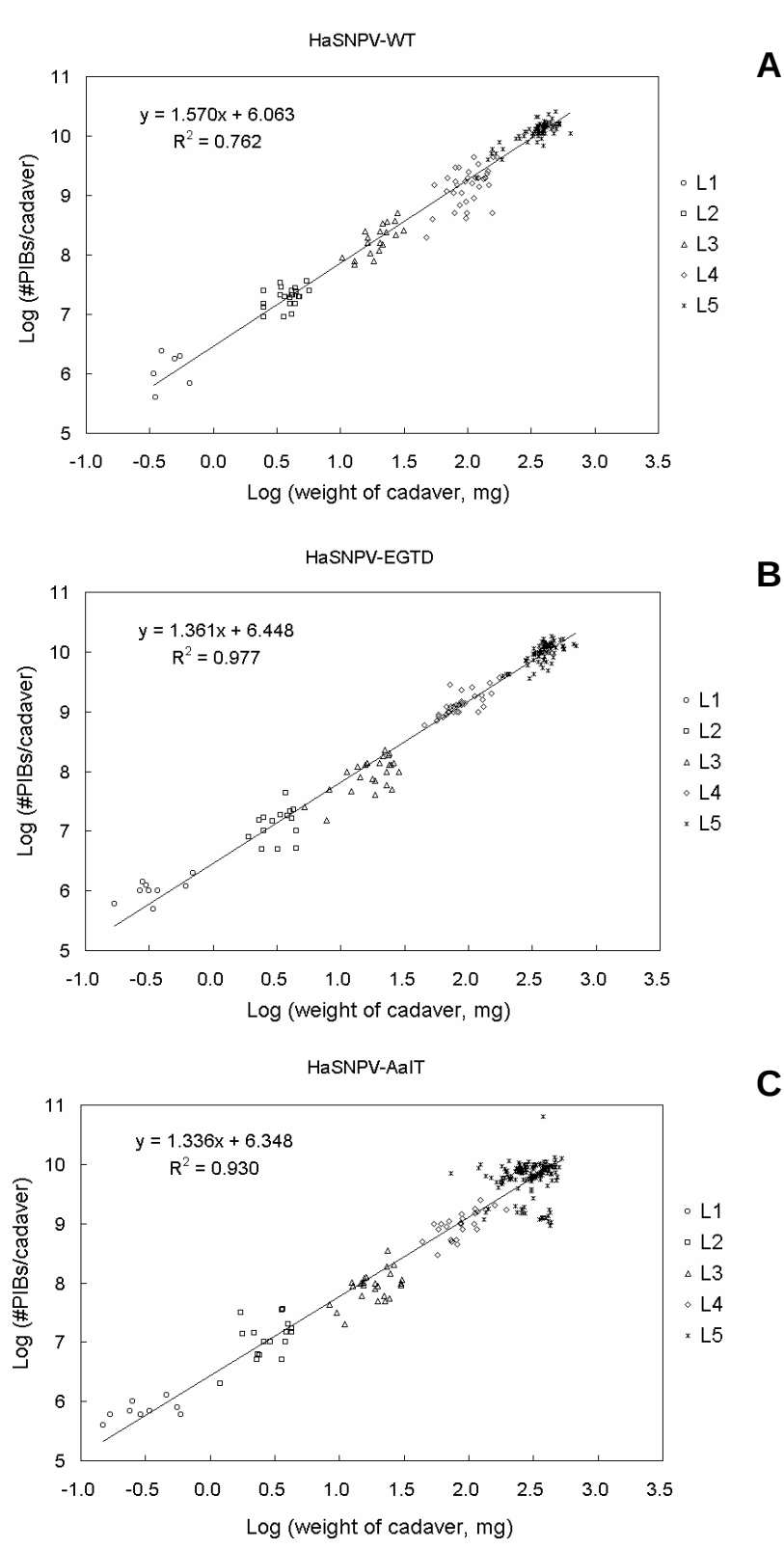

Figure 1. Relationship between the log-transformed PIB yield per cadaver and the log-transformed weight of cadaver at instars of death following inoculation of 1st to 5th instar larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD and (C) HaSNPV-AaIT. Data from inoculations in different instars and at five concentrations were pooled.

Table 1. Overview of the statistics of the log-transformed PIB yield per cadaver at a certain instar at death for three HaSNPV variants inoculated at the 1st to 5th instar (five separate ANOVA, one for each larval stage of inoculation).

Stage at inoculation	Mean effects of factors*			
	Variants	Inoculum concentration	Instar at death	Time to death
1st instar	$F_{2, 44} = 5.48$	$F_{4, 44} = 1.32$	$F_{2, 44} = 112.75$	$F_{1, 44} = 12.08$
	$P = 0.008$	$P = 0.279$	$P < 0.001$	$P = 0.001$
2nd instar	$F_{2, 60} = 7.45$	$F_{4, 60} = 1.977$	$F_{2, 60} = 88.93$	$F_{1, 60} = 8.80$
	$P = 0.003$	$P = 0.110$	$P < 0.001$	$P < 0.004$
3rd instar	$F_{2, 103} = 12.03$	$F_{4, 103} = 1.40$	$F_{2, 103} = 342.04$	$F_{2, 103} = 6.19$
	$P < 0.001$	$P = 0.240$	$P < 0.001$	$P < 0.014$
4th instar	$F_{2, 181} = 27.82$	$F_{4,181} = 2.69$	$F_{1, 181} = 353.04$	$F_{1, 181} = 14.61$
	$P < 0.001$	$P = 0.033$	$P < 0.001$	$P < 0.001$
5th instar	$F_{2, 109} = 20.44$	$F_{4, 109} = 2.225$	Not applicable	$F_{1, 109} = 10.37$
	$P < 0.001$	$P = 0.225$		$P = 0.002$

* F is Fisher's variance ratio statistic (Snedecor and Cochran, 1989) and the figures subscripted to F are the attendant degrees of freedom for numerator and denominator, respectively. F and P values for virus type, inoculum concentration, instar at death and time to death are reported for the model after nonsignificant interaction terms were dropped from the full model. The main factor 'inoculum concentration', which was not significant except in the 4th instar, was kept in the model for all instars.

inoculations in different instars and at five concentrations were pooled). The slopes of the regression lines for these three viruses were 1.570 ± 0.073, 1.362 ± 0.017 and 1.336 ± 0.020, respectively. A significant difference between the slopes for HaSNPV-WT (Figure 1A) on the one hand and HaSNPV-EGTD (Figure 1B) or HaSNPV-AaIT (Figure 1C) on the other hand was observed (z = 2.75 and 3.05, $P < 0.05$). No significant difference between the slopes for HaSNPV-EGTD and HaSNPV-AaIT (z = 0.85, $P > 0.05$) was found.

The relationship between virus yield per cadaver and four influencing factors was studied for each of the five instars of *H. armigera* separately by 4-way ANOVA. Two of the influencing factors are imposed by the experimental design, i.e. virus type and virus dose, while two others are response variables, measured in the experiment: larval instar at death and time to death in days after inoculation. Three of the four influencing factors significantly affected virus yield in all five instars, as shown by F-statistics and P-values in Table 1. An influencing factor is significant when the associated value of

the F-statistic (resulting from ANOVA) is significantly greater than 1 (e.g. Snedecor and Cochran, 1989). The influencing factors, significantly affecting virus yield, are virus type, larval instar at death and time to death ($P < 0.05$ for all these factors in all larval instars; Table 1). The wild-type virus had the highest yield per inoculated larva, and yields of the EGTD and *Aa*IT recombinants were reduced by, respectively, 41 and 63%, based on relative yields, averaged over the five instars.

Details on virus production in the five larval instars of *H. armigera* and the influences of the instar dying and time to death on yield are provided in Figures 2-6. Each of these 3 × 3 composite figures, represents aspects of yield of the wild-type virus (A-C), the EGTD recombinant (D-F), and the *Aa*IT recombinant (G-I). The left most column of graphs in each 3 × 3 composite figure represents yield as a function of the instar dying and the time to death. The middle column of graphs represents percentage mortality and the instar dying as a function of virus dose. The right most column of graphs in each 3 × 3 composite figure represents the relationship between virus dosage and total virus production from a batch of 100 larvae. Here the contribution of different instars dying to the total virus production is indicated. PIB yield per cadaver increased with a longer time to death for all three viruses and in all five instars (Figures 2-6, A, B and C; Table 1). Larvae dying at later instars at the same 'time to death' produced a significantly greater number of PIBs than larvae dying at less advanced instars (Figures 2-6, A, B and C; Table 1). Mortality, naturally, increased with virus concentration (Figures 2-6, D, E and F), while the larval instar of death tended towards a decrease (i.e. an earlier instar dying) at higher virus concentrations when the 1st, 2nd or 3rd instar was inoculated or when the 4th instar was inoculated with one of the recombinant viruses. However, no effect of virus concentration on the instar dying was demonstrated when the 4th instar was inoculated with the wild-type virus or in the 5th instar with any of the virus types. As mortality *increases* with virus concentration, while instar of death (and yield per cadaver) *decreases* with virus concentration in the 1st, 2nd and 3rd instar, and in the 4th instar inoculated with one of the recombinants, there is – for these cases – an optimum concentration at which the greatest yield per larva is obtained (Figures 2-6, Table 2). In the fifth instar, however, the highest yield was obtained at the highest inoculum concentration (Figure 6). There were no significant differences in the number of PIBs per cadaver among different inoculum concentrations in 1st, 2nd, 3rd and 5th instar larvae ($P > 0.05$; Table 1), whereas such a difference was found in the 4th instar ($P = 0.033$; Table 1). Overall, the mean number of PIBs per cadaver, dying in the 1st to 5th instars after inoculation with HaSNPV-AaIT, and pooled for different instars of inoculation, was 52%, 43%, 48%, 60% and 45% of the yield of HaSNPV-WT, while the relative yield of HaSNPV-EGTD was 76%, 82%, 64%, 81% and 82% compared to

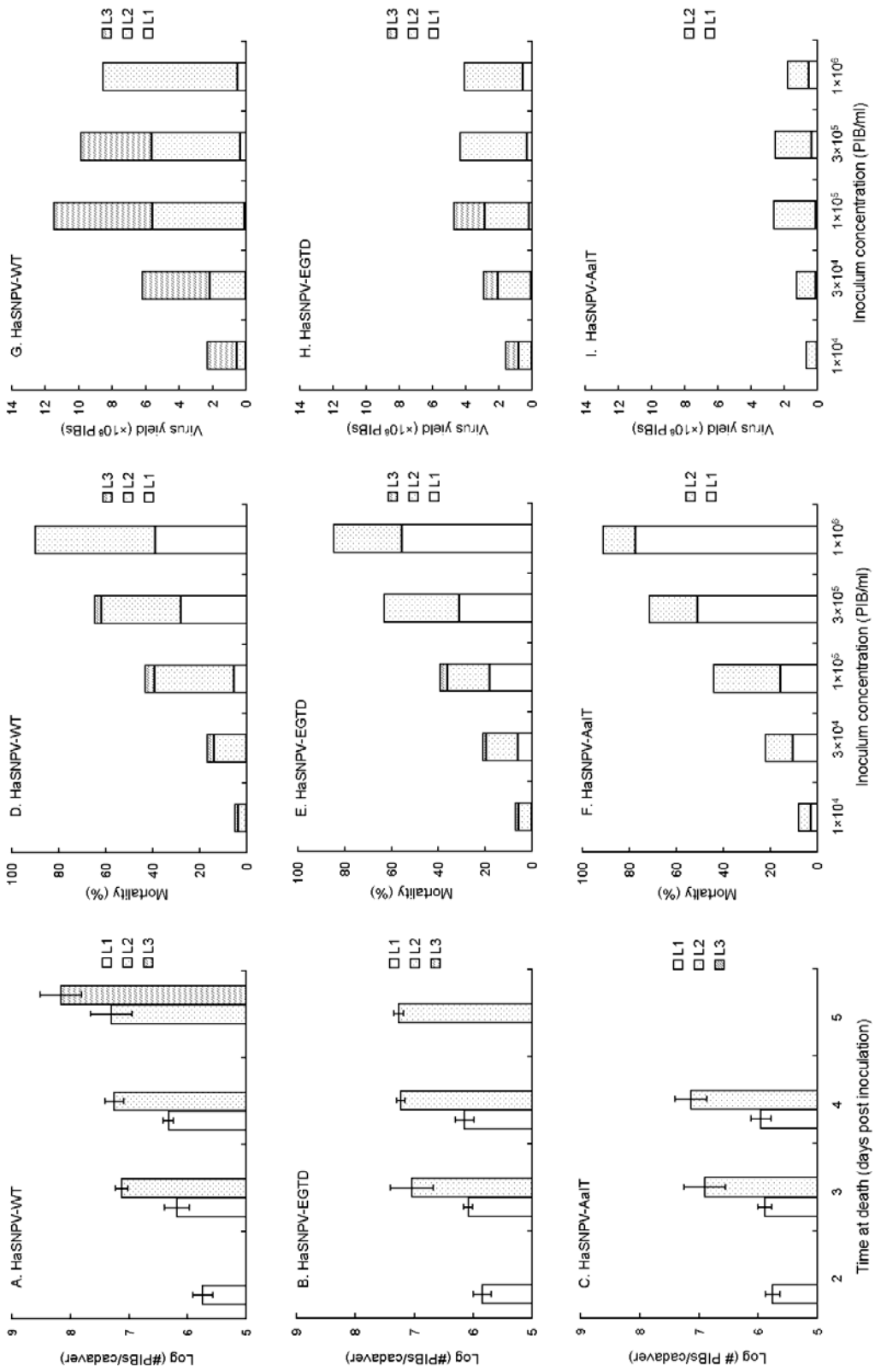

Figure 2. PIB yield per larva (± SEM) as influenced by the instar dying and the time to death following inoculation of 1st instar *H. armigera* larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD or (C) HaSNPV-AaIT; D, E and F: effect of virus dose on mortality and instar dying; and G, H and I: effect of virus dose on total PIB production from a batch of 100 inoculated larvae.

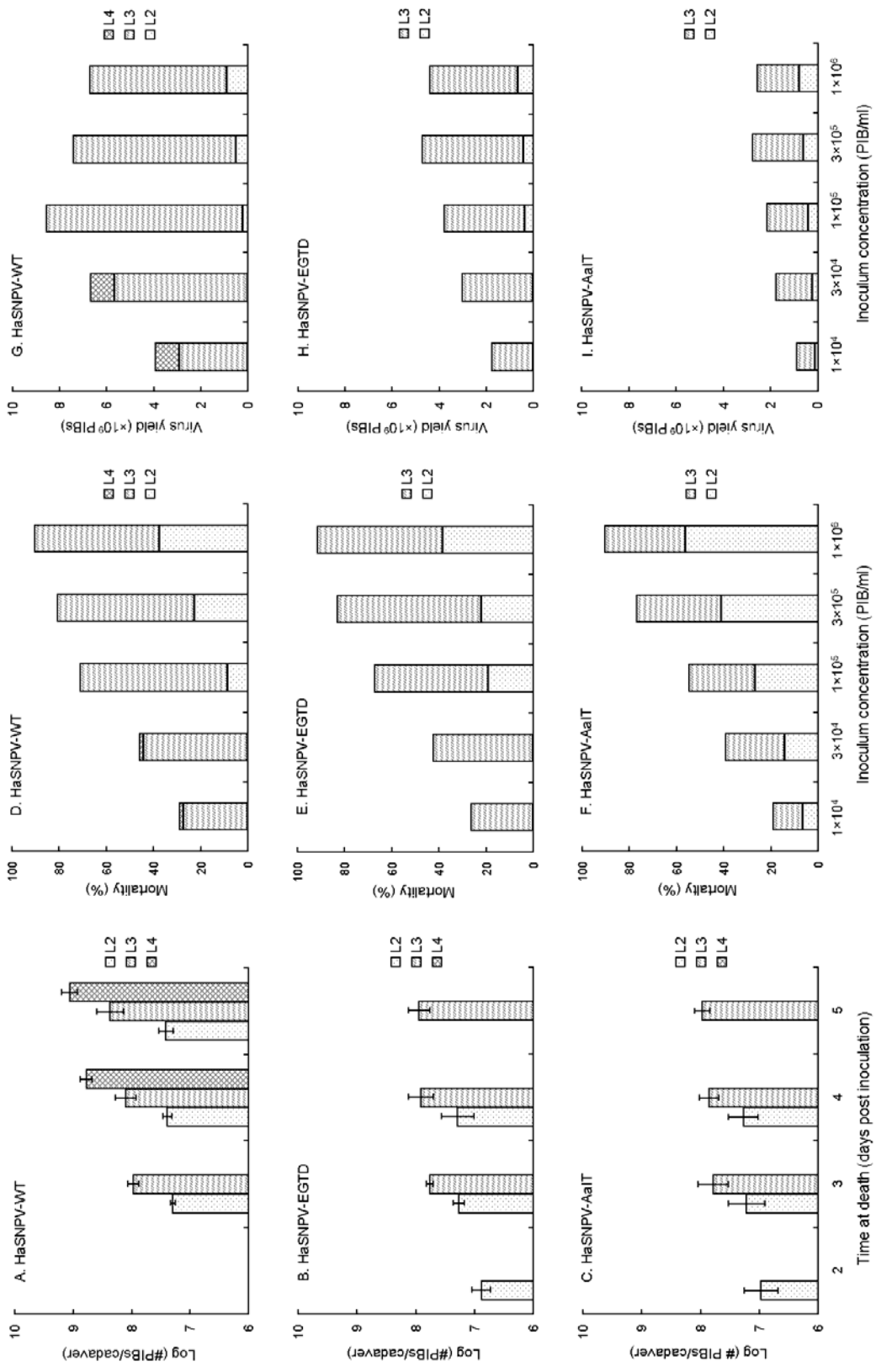

Figure 3. PIB yield per larva (± SEM) as influenced by the instar dying and the time to death following inoculation of 2nd instar *H. armigera* larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD or (C) HaSNPV-AaIT; D, E and F: effect of virus dose on mortality and instar dying; and G, H and I: effect of virus dose on total PIB production from a batch of 100 inoculated larvae.

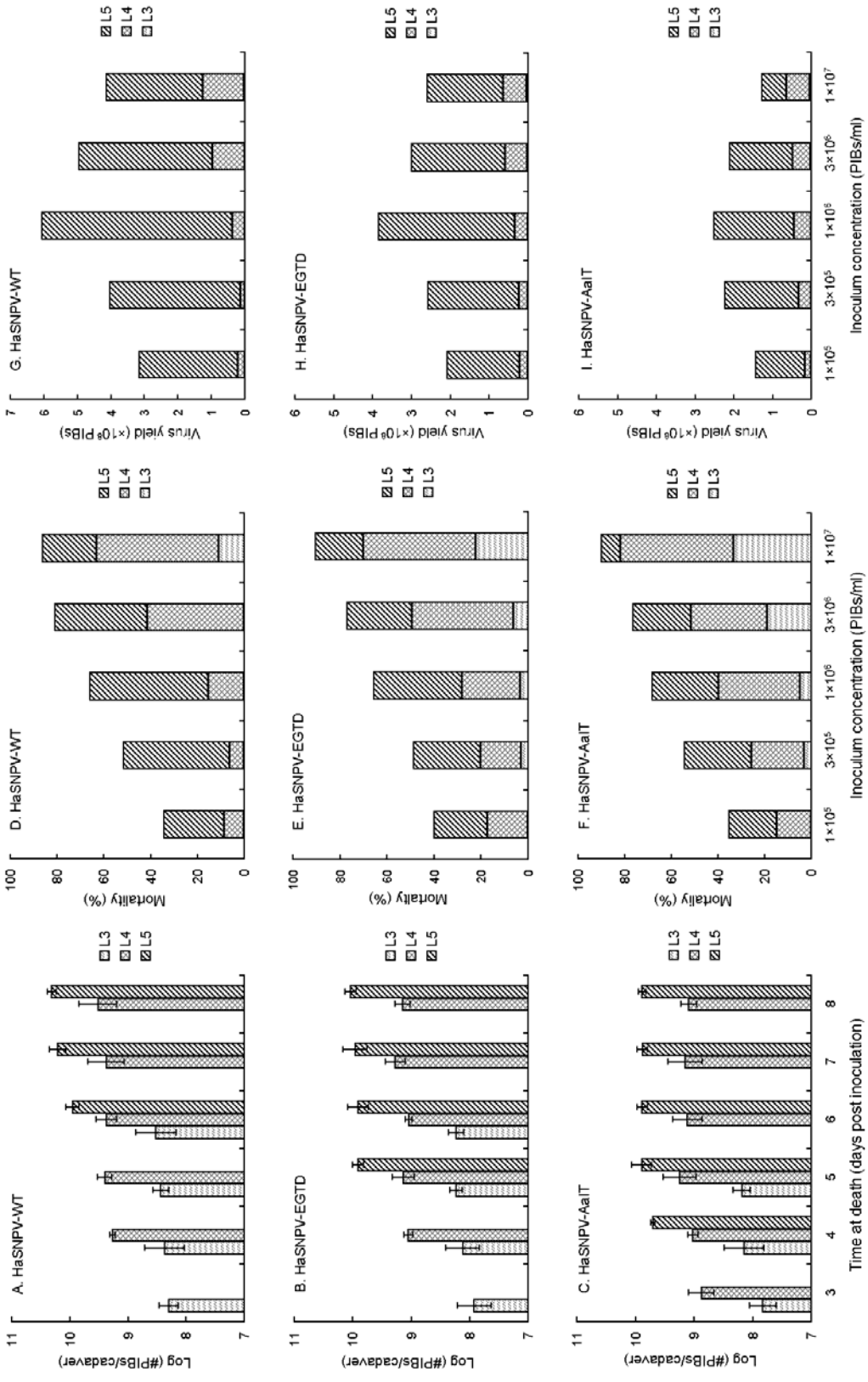

Figure 4. PIB yield per larva (± SEM) as influenced by the instar dying and the time to death following inoculation of 3rd instar *H. armigera* larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD or (C) HaSNPV-AaIT; D, E and F: effect of virus dose on mortality and instar dying; and G, H and I: effect of virus dose on total PIB production from a batch of 100 inoculated larvae.

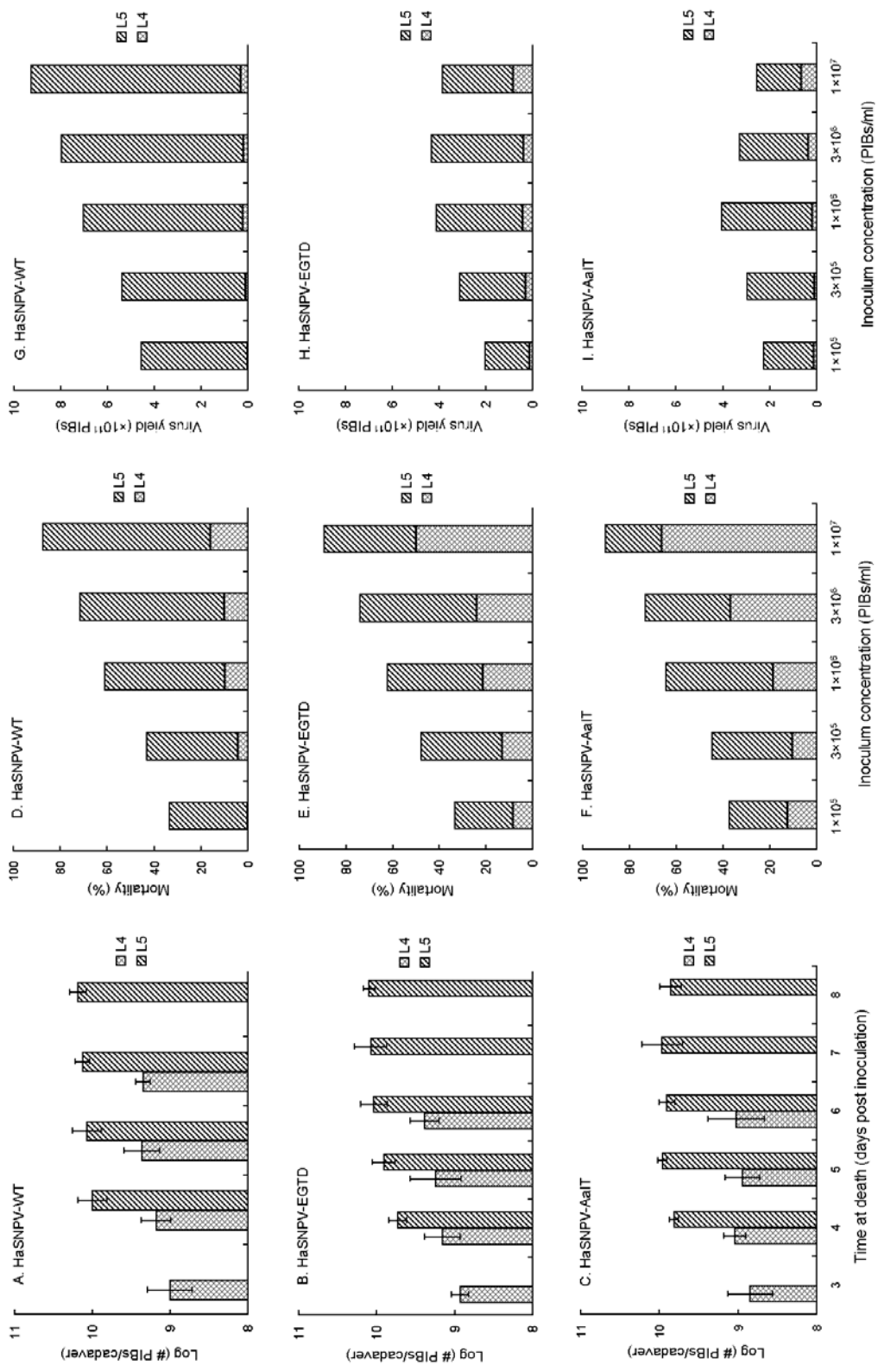

Figure 5. PIB yield per larva (± SEM) as influenced by the instar dying and the time to death following inoculation of 4th instar *H. armigera* larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD or (C) HaSNPV-AaIT; D, E and F: effect of virus dose on mortality and instar dying; and G, H and I: effect of virus dose on total PIB production from a batch of 100 inoculated larvae.

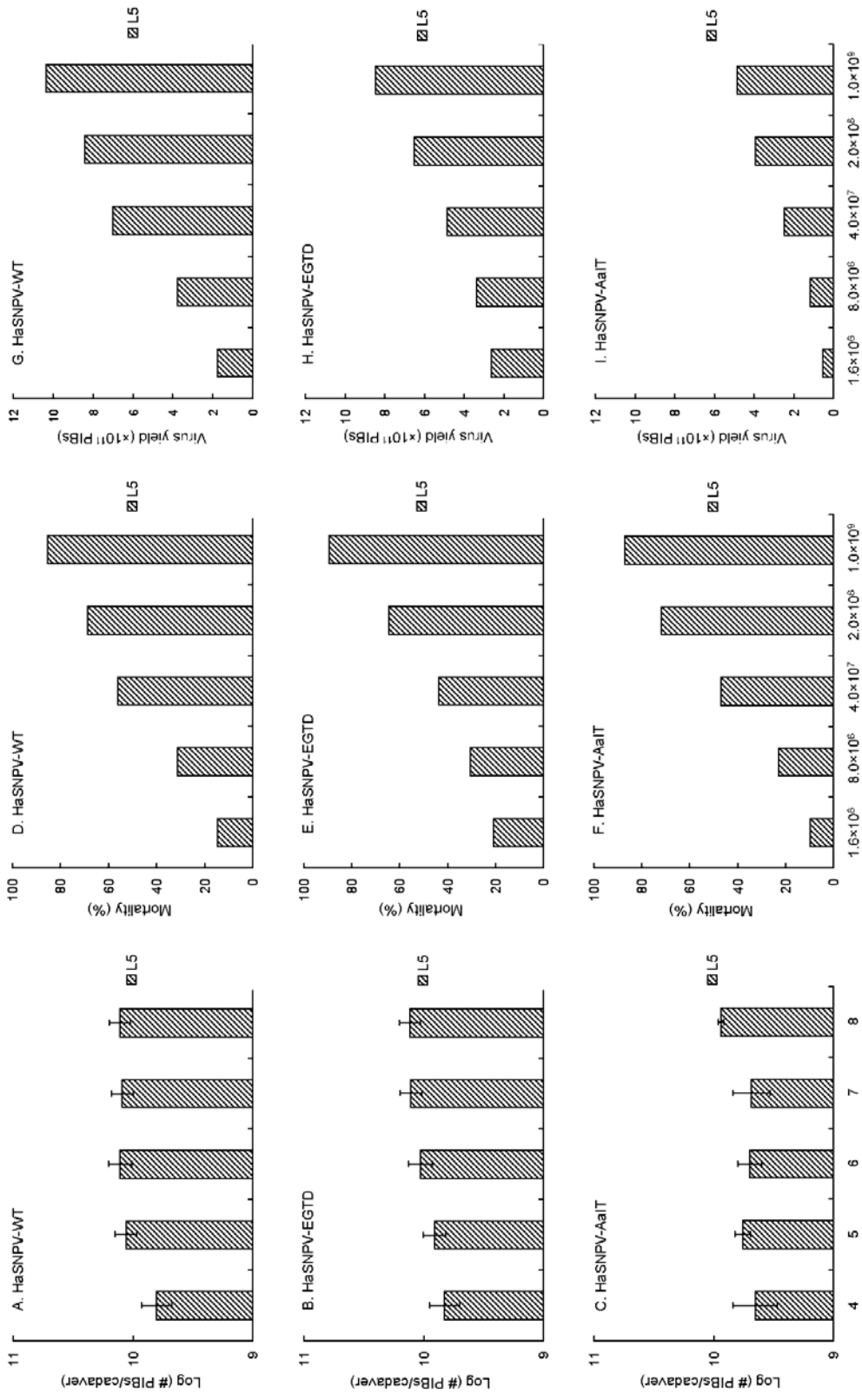

Figure 6. PIB yield per larva (± SEM) as influenced by the instar dying and the time to death following inoculation of 5th instar *H. armigera* larvae with (A) HaSNPV-WT, (B) HaSNPV-EGTD or (C) HaSNPV-AaIT; D, E and F: effect of virus dose on mortality and instar dying; and G, H and I: effect of virus dose on total PIB production from a batch of 100 inoculated larvae.

Table 2. Inoculum concentration (PIBs ml^{-1}) with which the maximum virus yield was obtained and the maximum PIB yield of wild-type and recombinant HaSNPV variants from 100 inoculated larvae.

Stage at inoculation	HaSNPV-WT		HaSNPV-EGTD		HaSNPV-AaIT	
	Inoculum conc.	PIB yield	Inoculum conc.	PIB yield	Inoculum conc.	PIB yield
1st instar	1×10^5	11.5×10^8	1×10^5	4.7×10^8	1×10^5	2.6×10^8
2nd instar	1×10^5	8.6×10^9	3×10^5	4.7×10^9	3×10^5	2.8×10^9
3rd instar	1×10^6	6.1×10^{11}	1×10^6	3.9×10^{11}	1×10^6	2.5×10^{11}
4th instar	1×10^7	9.3×10^{11}	3×10^6	4.3×10^{11}	1×10^6	4.1×10^{11}
5th instar	1×10^9	10.4×10^{11}	1×10^9	8.5×10^{11}	1×10^9	4.9×10^{11}

Table 3. Mean number of PIBs of wild-type and recombinant HaSNPV present in cadavers at different stages at death (PIBs ± SEM).

Viruses	PIB yield per cadaver*				
	1st instar	2nd instar	3rd instar	4th instar	5th instar
HaSNPV-WT	$1.43 \pm 0.31\times10^6$ a	$1.96 \pm 0.19\times10^7$ a	$2.11 \pm 0.27\times10^8$ a	$1.85 \pm 0.22\times10^9$ a	$1.28 \pm 0.56\times10^{10}$ a
HaSNPV-EGTD	$1.10 \pm 0.15\times10^6$ ab	$1.53 \pm 0.25\times10^7$ b	$1.36 \pm 0.12\times10^8$ b	$1.50 \pm 0.13\times10^9$ a	$1.05 \pm 0.40\times10^{10}$ b
HaSNPV-AaIT	$0.74 \pm 0.88\times10^6$ b	$1.31 \pm 0.21\times10^7$ b	$1.02 \pm 0.13\times10^8$ b	$1.11 \pm 0.11\times10^9$ b	$0.71 \pm 0.44\times10^{10}$ c

* Data from different inoculated instars and five concentrations were pooled for the same virus. Means followed by the same letter in each column are not significantly different according to LSD at P = 0.05.

the yield of HaSNPV-WT (Table 3). The number of PIBs per cadaver in a certain instar was 6.8 to 15.8 times higher than that in the preceding instar (Table 3).

In all cases recombinant HaSNPV variants yield a lower number of PIBs than the wild-type HaSNPV. First, in all of the instars dying of virus infection, the yields per cadaver were lower for the recombinants than for the wild-type virus. Secondly, when inoculating a given instar, the recombinant viruses in all larval instars except the fifth caused an earlier death (i.e. an earlier instar dying and/or an earlier time of dying) than the wild-type virus, with an attendant decrease in virus yield. These effects are shown in detail in Figures 2-6 and summarized in Figure 7. Yield per cadaver of HaSNPV-EGTD varied from 64 to 82% of the wild-type yield, with no clear increasing or

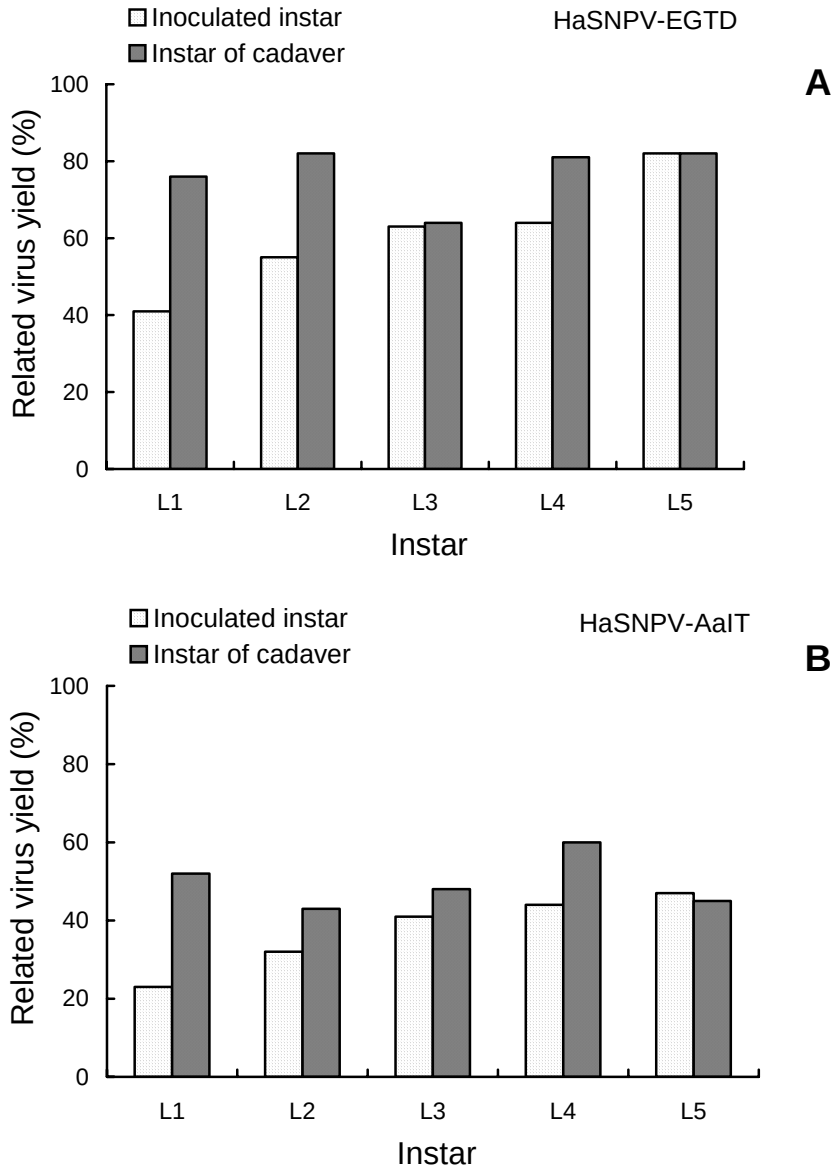

Figure 7. Relative yields of (A) HaSNPV-EGTD and (B) HaSNPV-AaIT, compared to yields of HaSNPV-WT, in different instars of *H. armigera* inoculated or dying.

decreasing trend with larval instar. However, the relative yield, compared to wild-type virus, increased from 41% when a 1st instar larva was inoculated to 82% when a 5th instar larva was inoculated (Figure 7A). In the case of HaSNPV-AaIT, relative yield compared to wild type varied from 43 to 60% in different instars *dying*, with no clear increasing or decreasing trend with larval instar, whereas the relative yield when *inoculating* different instars showed a clear increase: from 23% when the 1st instar was inoculated to 41% when the 5th instar was inoculated (Figure 7B).

DISCUSSION

Variable results are found in the literature regarding the level of production of toxin-expressing recombinant baculoviruses compared to their wild-type parents. (i) A recombinant *Autographa californica* multicapsid NPV (AcMNPV), which expressed a neurotoxin (Tox 34) and killed *Trichoplusia ni* Hübner larvae 60% quicker than the wild-type virus, had a 95% reduced yield of progeny virus compared to the wild-type AcMNPV in 2nd instar *T. ni* larvae (Burden *et al*., 2000). (ii) *Helicoverpa zea* SNPV (HzSNPV) expressing the scorpion toxin LqhIT2 produced an approximately 4 to 10-fold lower number of PIBs in 3rd instar larvae of *Helicoverpa zea* Boddie and *Heliothis virescens* Fabricius than the wild-type parent of this virus (Ignoffo *et al*., 2000). (iii) The yield of a recombinant AcMNPV, expressing the toxin ST3, was 78% lower than that of wild-type AcMNPV in 2nd instar of the susceptible host *T. ni*, whereas the reduction was 52% for a less-susceptible species, *Mamestra brassicae* (Hernández-Crespo *et al*., 2001). (iv) The PIB yield of an AcMNPV recombinant expressing the predatory mite toxin vSP-TOX-34, when fed to 5th instar *T. ni* larvae, was about 40% less than that of the wild strain (Tomalski and Miller, 1992). (v) The mean number of PIBs produced per larva inoculated with an *Aa*IT-expressing AcMNPV was ca. 20% of that produced by 3rd to 5th instar larvae inoculated with wild-type AcMNPV (Kunimi *et al*., 1996). (vi) In another experiment, mid-instar and late-instar larvae infected with wild-type AcMNPV produced 1.25 to 2.42 times more PIBs than those infected with the recombinant expressing toxin *Aa*IT (Ignoffo and Garcia, 1996). In the experiments we describe here, when 1st to 5th instar larvae were inoculated, the maximum virus yields per inoculated larva of HaSNPV-AaIT were 23%, 32%, 41%, 44% and 47% of those of HaSNPV-WT, respectively. Therefore, the reduction in yield due to expression of the toxin genes seems to depend on the type of toxin expressed or its timing of expression, the host species involved and the larval instar inoculated. The reduction of the yield of the *Aa*IT-expressing recombinant compared to wild-type HaSNPV, found here, is moderate compared to the values reported for toxin-expressing NPVs in the literature (Burden *et al*., 2000; Ignoffo *et al*., 2000).

In our experiments the yield of recombinants in early host instars was substantially reduced compared to wild-type HaSNPV (Table 3). Since in a pest control context, baculovirus insecticides are targeted at early (1st and 2nd) instars of heliothines (Smith *et al*., 2000a; Chapter 2), the reduced progeny yields of HaSNPV-AaIT in early instars of larvae may result in less horizontal transmission and a smaller persisting population of PIBs in the agro-ecosystem. Reduced transmission and persistence may affect control of bollworms by recombinants of HaSNPV, and necessitate a greater number of sprays or higher dose. On the other hand, reduced progeny yields of recombinants

should reduce the risk of exposure to non-target organisms. Better understanding of these trade-offs may be obtained by quantifying and analysing the horizontal transmission dynamics and developing a process-based model (cf. Bianchi *et al*., 2002a, b, c) of the epidemiology of HaSNPV recombinants in the cotton – cotton bollworm ecosystem.

When HaSNPV-AaIT was inoculated in late (3^{rd} to 5^{th}) instars, the yield reductions as compared to the wild-type virus were not as great as in early instars (Table 3). The yields of HaSNPV-AaIT from 4^{th} and 5^{th} instars are high enough to make commercial production of this virus economically feasible. The yield of recombinant HaSNPV-AaIT was highest (4.9×10^{11} PIBs per 100 inoculated larvae; Figure 6I and Table 2) when the highest concentration was used for inoculation of the 5^{th} larval instar (1×10^9 PIBs ml^{-1}). This yield per inoculated larva was 11.1% higher than the maximum yield when 4^{th} instar larvae were inoculated at an intermediate concentration (1×10^6 PIBs ml^{-1}). However, the ratio of virus yield to the dose of inoculum (as calculated from the average consumed volume of PIB solution and the PIB concentration) was lower in 5^{th} instars (358 times) than in 4^{th} instars (6.9×10^5 times). Thus, production of HaSNPV appears much more profitable in 4^{th} instar than in 5^{th} instar. Moreover, it is more difficult to maintain healthy larvae for inoculation in the 5^{th} instar than in the 4^{th} instar under factory conditions. We therefore conclude that inoculation of 4^{th} instar larvae at a concentration of 1×10^6 PIBs ml^{-1} is the optimal regime for production of this recombinant in larvae.

In this research the intrinsic production of wild-type and recombinant HaSNPV viruses in various instars of *H. armigera* larvae was investigated with a well-controlled droplet feeding inoculation procedure. However, in the field as well as in a production facility, larvae ingest virus gradually from surface-contaminated diet. The replication dynamics of the baculoviruses might be different following such a different inoculation procedure, given that the diet contamination method delivers inoculum over a longer period of time rather than as a pulse. Further investigation is needed to determine if our results hold when viruses are ingested using the diet surface contamination method.

The observed reduction of 56% of the maximal virus yield of HaSNPV-AaIT compared to HaSNPV-WT might double the cost of a commercial product of HaSNPV-AaIT. The question arises whether a better field efficacy of HaSNPV-AaIT can offset this higher production cost. Field experiments (Chapter 2) showed that the yield of cotton lint in plots treated by HaSNPV-AaIT was 21.4% higher than in plots treated with HaSNPV-WT. This production increase, in terms of the value of the extra cotton harvested, is more than enough to recover the higher costs of production of HaSNPV-AaIT compared to HaSNPV-WT.

CHAPTER 4

Field inactivation of wild-type and genetically modified *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus in cotton*

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ABSTRACT

Cotton bollworm (*Helicoverpa armigera*) is a serious pest on cotton in China. A specific baculovirus, *H. armigera* nucleopolyhedroviruses (HaSNPV), is used as a commercial biopesticide to control this pest. To improve the pesticidal properties, HaSNPV has been genetically engineered by both deleting the ecdysteroid UDP-glucosyltransferase (*egt*) gene from its genome (recombinant HaSNPV-EGTD) and incorporating an insect-selective toxin gene from the scorpion *Androctonus australis* (*Aa*IT) (recombinant HaSNPV-AaIT). In the field, there was no significant difference among the inactivation rates of the two recombinant HaSNPV variants and their parent wild-type, HaSNPV-WT. The inactivation rate of these viruses was significantly different in different years. The average half-life of HaSNPV was 0.57, 0.90 and 0.39 days in 2000, 2001 and 2002, respectively. Inactivation rates correlated well with solar radiation over these years.

Key words: HaSNPV, genetically modified virus, cotton bollworm, field inactivation.

INTRODUCTION

Baculoviruses are highly specific and often virulent viruses that infect insects, mainly lepidopteran species (Hunter-Fujita *et al*., 1998). Currently a number of wild-type baculoviruses are used for the protection of crops, vegetables, forests and pastures (Moscardi, 1999). However, lack of field stability after application has long been considered a primary factor limiting commercialization of baculoviruses as microbial control agents (Bull *et al*., 1976; Jaques, 1977). In cotton, low persistence of baculoviruses can be caused by several factors. First of all, solar irradiation has been recognized as a major environmental factor accountable for the quick inactivation of baculoviruses (Ignoffo, 1973; Ignoffo *et al*., 1997a, b; Shapiro and Robertson, 1992; Teakle *et al*., 1986). Secondly, activity of NPV can be inhibited by secondary chemicals secreted by plants (exudates) and by plant-mediated peroxidase activity which generates free radicals (Elleman and Entwistle, 1985; Hoover *et al*., 1998). Thirdly, pH and concentrations of ions in cotton dew affect the activity of NPVs (Andrews and Sikorowski, 1973; Tuan *et al*., 1989; Young and Yearian, 1974).

Cotton bollworm (*Helicoverpa armigera* Hübner) is one of the most serious pests on cotton around the world (Gary, 1989). *H. armigera* single nucleocapsid nucleopolyhedrovirus (HaSNPV) is a baculovirus specific for this species and has been used as a biopesticide to control this pest in China since the 1980s (Zhang, 1989). To improve the pesticidal properties, HaSNPV has recently been genetically engineered by both deleting the ecdysteroid UDP-glucosyltransferase (*egt*) gene from its genome (recombinant HaSNPV-EGTD) (Chen *et al*., 2000b) and incorporating an insect-selective toxin gene from the scorpion *Androctonus australis* (*Aa*IT) (recombinant HaSNPV-AaIT) (Chapter 2). In the laboratory and in the field, these recombinant HaSNPV variants show improved control efficacy compared to the wild-type virus (Sun *et al*., 2002b; Chapter 2).

It has been reported that insertion of an juvenile hormone esterase gene or scorpion toxin (*Aa*IT) gene did not result in recombinant *Autographa californica* multicapsid NPV (AcMNPV) being more or less sensitive to simulated sunlight-UV than the parental wild-type AcMNPV in the laboratory (Ignoffo *et al*., 1997a). Furthermore, there was no difference between the inactivation patterns of wild-type AcMNPV and a recombinant which expresses an insect-selective toxin (*Aa*HIT) on cabbages in the field (Hernández-Crespo *et al*., 1999). Nevertheless, the possibility exists that the persistence of recombinants might be different from that of the wild-type virus in the case of HaSNPV on cotton. Here we report on the field stability of HaSNPV-WT and an *egt*-deletion mutant (HaSNPV-EGTD) and an *Aa*IT-expressing recombinant (HaSNPV-AaIT) in cotton in three consecutive years of field application.

MATERIALS AND METHODS

Viruses and insects

HaSNPV was initially isolated from diseased *H. armigera* larvae collected in the Hubei province of China (Zhang *et al*., 1981; Zhang, 1989). A genotype of this wild-type HaSNPV (HaSNPV-G4) was isolated by *in vivo* cloning (Sun *et al*., 1998) and designated here as HaSNPV-WT. The recombinant HaSNPV-EGTD (HaCXW1 in Chen *et al*., 2000b; Sun *et al*., 2002b) was generated by deletion of the *egt* gene from HaSNPV-WT by recombination in cell culture. The recombinant HaSNPV-AaIT was generated by insertion of the insect-selective scorpion toxin *Aa*IT downstream of a chimeric promoter of *p6.9* and *polyhedrin* genes of HaSNPV at the *egt* gene locus (Chapter 2). HaSNPV viruses were propagated in fourth instar *H. armigera* larvae. PIBs were purified by sucrose gradient centrifugation and stored in 40% v/v glycerol at 4 °C until use. Cotton bollworm, *H. armigera*, larvae were collected from cotton fields in August, each year, near the China Cotton Research Institute (CCRI), Anyang, Henan, China, and individually reared on artificial diet at 28 ± 1 °C and a 16-h photoperiod for several cycles and used for laboratory bioassays in the following year.

Treatment of cotton, sampling and bioassays

HaSNPV-WT, HaSNPV-EGTD, HaSNPV-AaIT were sprayed onto three rows of cotton (Zhongmiansuo #35) at 4×10^6 PIB ml^{-1} in 0.5% v/v Tween 20 on August 23, 26, 28, 29, 30 and 31, in 2000, near the CCRI, Anyang, Henan, China. Each row contained 30 plants. There was an unsprayed guard row between every two treatments. Sprays were made at 0.06 L m^{-2} using a backpack sprayer with a 0.4 mm diameter nozzle between 7:00 and 8:00 h. One hour after the last spray on August 31, 48 squares were randomly sampled from the upper 30 cm of the cotton canopy in each of the time-treatment plots. The sampled squares were of a standardized stage, approximately 10 days before opening of the flower. The droplets of sprayed suspension were only on the outer surface of the bracts as the squares had not opened. Squares were put in 12-well tissue culture plates containing 0.5 ml of 1% agar in each well. One 2nd instar *H. armigera* larva was subsequently introduced into each well. The larvae were reared at ambient temperature (24 - 28 °C) for 48 h and were then transferred onto fresh artificial medium (Zhang *et al*., 1981). The larvae were checked daily for mortality until all larvae had either died or pupated. The inactivation experiment was repeated in 2001 and 2002. The sprays were made on 23, 24, 25, 26 and 27 August in 2001, and on 9, 10, 11, 12, 13, 14 and 15 August in 2002, respectively. There was no rainfall during any of the three experiments.

To calibrate known virus concentration with observed mortality of bollworm fed on

cotton squares, serially diluted suspensions of HaSNPV-WT in 0.5% v/v Tween 20 were sprayed on cotton on August 31, 2000, August 27, 2001 and August 15, 2002, respectively. In 2000 and 2001, serial dilutions at concentrations of 4×10^6, 2×10^6, 1×10^6, 5×10^5 and 2.5×10^5 PIBs ml^{-1} were used, while in 2002 these concentrations were changed to 4×10^6, 1.3×10^6, 4×10^5, 1.3×10^5 and 4×10^4 PIBs ml^{-1}. Methods for spraying and sampling of squares were the same as in the time-treatment plots. Standard mortality response regression lines (probit mortality versus $\log^{10}$ virus concentration; Finney, 1978) were generated for HaSNPV-WT separately for each year's data, using the probit analysis procedure in SPSS for Windows v10.0 (SPSS Inc., 2000). Differences in slope between years were compared with two sided z-tests (Snedecor and Cochran, 1980). The standard mortality response regression line was made for HaSNPV-WT only, as the recombinants and wild type HaSNPV do not differ in LD_{50} and slopes of the regression (Chapter 2).

The volume of virus suspension deposited on the surface of the squares was measured on five potted cotton plants in the laboratory. The cotton plants were sprayed with virus suspension at the same rate as in the field. Before spraying, 20 squares were separated from the plants at the middle of the petioles. The squares were labelled with a marker pen, weighed individually at an accuracy of 0.1 mg, and reattached to the plants by linking the two half petioles with a thin iron thread. Immediately after spray, the labelled squares were collected and weighed again. All three bracts of each square were taken and their width and length were measured. The area of bracts was estimated by multiplying the width and length by a factor of 0.7 (CCRI, 1999). The density of PIBs on the surface of the squares (PIBs cm^{-2}) was calculated by multiplying the deposited volume of virus suspension per unit of area of squares by the virus concentration applied.

Density of infectious PIBs on the surface of the squares at different days after exposure was calculated from observed mortality of 2nd instar larvae fed on the squares according to the standard mortality response regression line. In calculating virus inactivation, allowance was first made for the 'dilution' of virus density on the bracts, as a result of bract growth. At 1 to 8 days post spray, the bracts had increased in area by 3.9%, 7.8%, 12.3%, 19.4, 25.4%, 34.5%, 46.6% and 58.6%, respectively. Virus density estimated from the bioassay was corrected upward by the respective growth percentages of the bracts. Virus persistence, thus calculated, was then expressed in percentage of original activity remaining (%OAR) at various sample times. %OAR was calculated as:

$$\%\mathrm{OAR} = \frac{\text{density of infectious PIBs at the sampled time (PIBs cm}^{-2})}{\text{density of infectious PIBs at 1 h post spray (PIBs cm}^{-2})} \times 100.$$

Negative exponential decay curves were fitted by nonlinear regression with SPSS (SPSS Inc., 2000) for each of the viruses in each of the years to characterize the time course of inactivation:

$$\%\mathrm{OAR} = 100 \times \mathrm{e}^{(-r \times t)}$$

where r is the relative rate of inactivation (d^{-1}) and t is time post exposure (d). The resulting nine values of r were compared in a two way ANOVA with years and virus as factors, each with two degrees of freedom, and the interaction (d.f. = 4) as error term (SPSS Inc., 2000). The half-life of the virus was calculated as

$$\tau_{1/2} = \ln(2)/r$$

with a standard error of $\ln(2)/r^2$ times the standard error of r.

RESULTS AND DISCUSSION

Relationship between virus density and mortality of tested larvae

The mean deposited volume of virus suspension per unit of area of square surface within the top 30 cm of cotton canopy was 2.36 ± 1.04 µl cm^{-2} (mean ± SD). There was a typical linear relationship between log^{10} estimated virus density and mortality (probit) of 2nd instar *H. armigera* larvae fed on the squares (Figure 1). There were no significant pairwise differences in slope of the regression between years (z = 0.14, 0.84 and 1.44, P > 0.05). Therefore, the data obtained in 2000, 2001 and 2002 were described with a common regression:

$$y = 0.939\,x + 2.484 \quad (R^2 = 0.918)$$

where y is mortality (probit), x is log^{10} (virus density, PIBs cm^{-2}).

Inactivation rate of PIBs of HaSNPV variants on cotton squares

PIBs inactivation of wild-type and recombinant HaSNPV variants on the square surface followed a typical exponential decay pattern in three years 2000, 2001 and 2002 (Figures 2A, B and C). The fitted relative inactivation rates (r) for the various viruses are listed in Table 1. All data fitted this equation very well as shown in Figure 2. No difference in inactivation rates was demonstrated among the three HaSNPV variants ($F_{2,\,4}$ = 2.183, P = 0.229). This confirms previous results for wild-type and recombinant AcMNPV on cabbage (Hernández-Crespo *et al*., 1999). However, the inactivation rates for HaSNPV in different years were significantly different ($F_{2,\,4}$ = 20.088, P = 0.008).

The average half-lives of these HaSNPV variants were 0.57, 0.90 and 0.39 days in

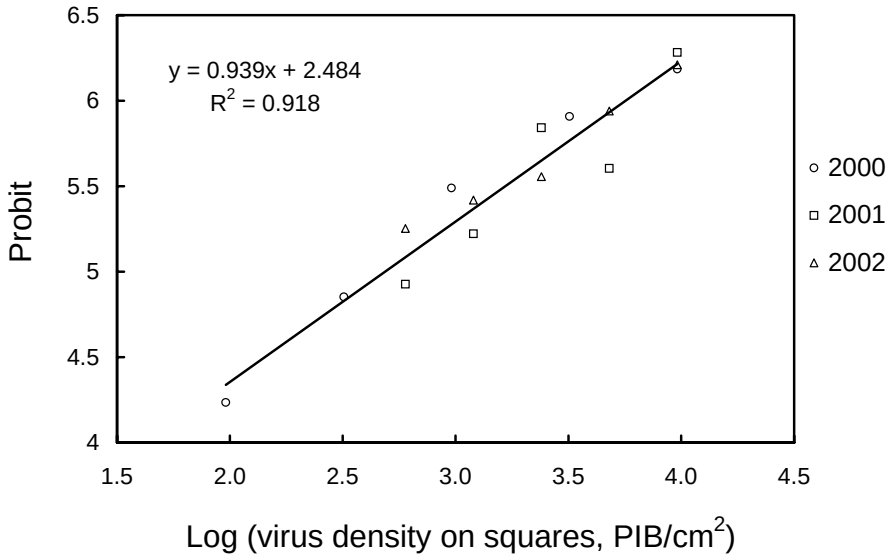

Figure 1. Calibration of known virus density (PIBs cm^{-2}) applied on cotton squares with observed mortality of 2nd instar *H. armigera* larvae fed on the squares.

Table 1. Inactivation rates (r, d^{-1}) and half-life ($\tau_{1/2}$, d) of wild-type and recombinant HaSNPV variants on cotton squares in different years.

Viruses	2000		2001		2002	
	r (± SE*, d^{-1})	$\tau_{1/2}$ (± SE, d)	r (± SE*, d^{-1})	$\tau_{1/2}$ (± SE, d)	r (± SE*, d^{-1})	$\tau_{1/2}$ (± SE, d)
HaSNPV-WT	1.249 ± 0.037	0.55 ± 0.02	0.688 ± 0.036	1.01 ± 0.05	1.780 ± 0.078	0.39 ± 0.02
HaSNPV-EGTD	1.056 ± 0.039	0.66 ± 0.02	0.837 ± 0.025	0.83 ± 0.02	1.472 ± 0.046	0.47 ± 0.01
HaSNPV-AaIT	1.366 ± 0.099	0.51 ± 0.04	0.804 ± 0.035	0.86 ± 0.04	2.219 ± 0.016	0.31 ± 0.01
Average	1.224	0.57	0.776	0.90	1.824	0.39

* These SE (standard error) values are obtained from the separate regression curves, not used in ANOVA.

2000, 2001 and 2002 respectively (Table 1). The half-lives of HaSNPV variants were higher than the 4.9 h reported by Ignoffo *et al*. (1997b) for *H. zea* SNPV exposed to natural sunlight on soybean, but were much shorter than the 7 days reported by Tuan *et al*. (1989) for *H. zea* SNPV on corn silks in June or July in Taiwan. In the latter case, virus was applied as a suspension from a commercial product (Elcar®) which contained UV-protectants. A similar half-life value (7 days) was observed for AcMNPV in UV-protected glasshouse chrysanthemum (Bianchi *et al*., 1999) underscoring the importance of UV inactivation. In order to provide a sustained efficacy of control of bollworm, it thus remains important to use suitable formulations

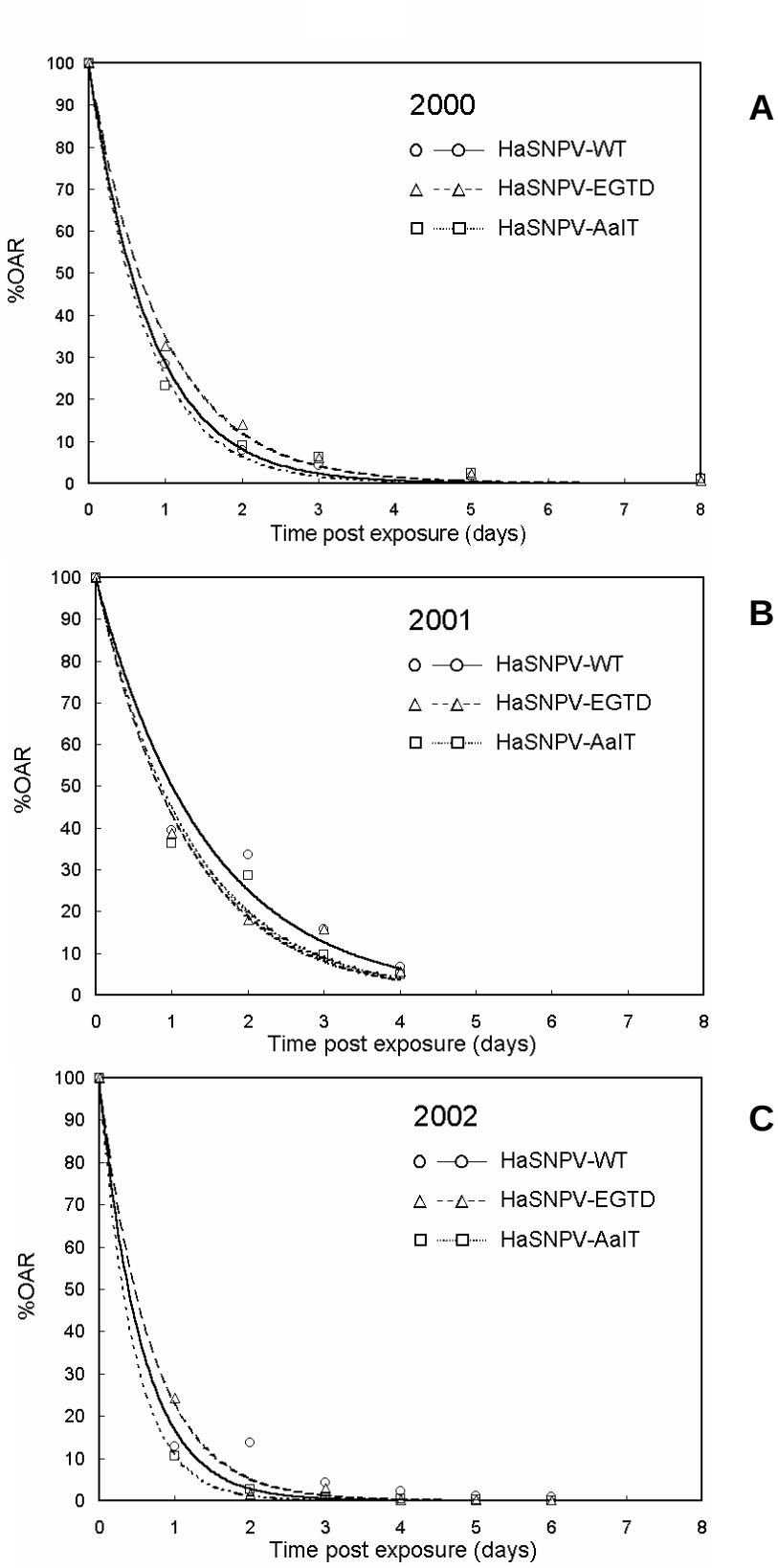

Figure 2. Inactivation curves of wild-type and recombinant HaSNPV variants on cotton squares in 2000 (A), 2001 (B) and 2002 (C). Measured percentage OAR (symbols) and fitted exponential curves (lines) of HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT are represented.

and UV-protectants for wild-type and recombinant HaSNPV insecticides.

The average values of daily global radiation during the experimental period in 2000, 2001 and 2002 in Anyang (36°5'N, 114°21'E) were 1.92×10^7, 1.64×10^7 and 2.12×10^7 J m^{-2} d^{-1}, respectively (CCRI, Anyang). There was a positive relationship between the average inactivation rates of HaSNPV variants (Table 1) and the average daily global radiation ($R^2 = 0.968$) over these years. Bianchi *et al*. (1999), as well as Jones *et al*. (1993) and Brassel and Benz (1979), found a residual PIB fraction that appeared to be more resistant to UV radiation. In our experiments reported here, the decaying rate slowed down as time progressed. However, such resistant PIBs could not be found on cotton in the field. For example, the %OAR was less than 0.1% at 6 days after exposure in 2002 (Figure 2C). This observation might be explained by the fact that inactivation of NPVs in the field cotton is not only caused by irradiation during daytime, but also by alkaline dew on the cotton surface at night (Andrews and Sikorowski, 1973; Young *et al*., 1977; Tuan *et al*. 1989). In addition, the exudates and secondary chemicals secreted by cotton gland and plant-mediated peroxidase activity, which generates free radicals, affect their activity (Elleman and Entwistle, 1985; Hoover *et al*., 1998). It may be possible to lessen the effect of plant-mediated peroxidase activity by incorporating additives, such as mannitol (a hydroxyl radical scavenger), into a suitable formulation (Hoover *et al*., 1998). Further investigation is needed to determine the relative importance of each of these factors and to help to select suitable additives to formulate HaSNPV insecticides. Finally, the data on the field inactivation of HaSNPV are important in the development of a process-based model to describe the behaviour of HaSNPV and its recombinants in the cotton – cotton bollworm eco-system.

CHAPTER 5

Horizontal and vertical transmission of wild-type and recombinant *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus*

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ABSTRACT

Transmission plays a central role in the ecology of baculoviruses and the population dynamics of their hosts. Here we report on the horizontal and vertical transmission dynamics of wild-type *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV-WT) and a genetically modified variant (HaSNPV-AaIT) with enhanced speed of action through the expression of an insect-selective scorpion toxin (*Aa*IT). In field cages, horizontal transmission of both HaSNPV variants was greatest when inoculated 3rd instar larvae were used as infectors, transmission was intermediate with 2nd instar infectors and lowest with 1st instar infectors. Transmission was greater at a higher density of infectors (1 per plant) than at a lower density (1 per 4 plants); however, the transmission coefficient (number of new infections per initial infector) was lower at the higher density of infectors than at the lower density. HaSNPV-AaIT exhibited a significantly lower rate of transmission than HaSNPV-WT in the field cages. This was also the case in open field experiments. In the laboratory, the vertical transmission of HaSNPV-AaIT from infected females to offspring of 16.7 ± 2.1%, was significantly lower than that of HaSNPV-WT (30.9 ± 2.9%). Likewise, in the field, vertical transmission of HaSNPV-AaIT (8.4 ± 1.1%) was significantly lower than that of HaSNPV-WT (12.6 ± 2.0%). The results indicate that the recombinant virus will be transmitted at lower rates in *H. armigera* populations than the wild-type virus. This may potentially affect negatively its long term efficacy as compared to wild-type virus, but contributing positively to its biosafety.

Key words: *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus, genetic modification, horizontal transmission, vertical transmission.

INTRODUCTION

Baculoviruses are double-stranded DNA viruses that are pathogenic to insects, especially Lepidoptera. The polyhedral inclusion body (PIB) is the characteristic phenotypic appearance of baculoviruses and, in case of a nucleopolyhedrovirus (NPV) typically comprised of a proteinaceous matrix with a large of number of embedded virus particles. PIBs reduce degradation of the viral particles and allow the virus to remain active in the environment for several years. Baculoviruses have a long history as effective and environmentally benign insect control agents in field crops, vegetables, forests and pastures (Moscardi, 1999). Although much is known about the genetics of baculoviruses (Herniou *et al*., 2003), very little is known about their ecology and epidemiology in particular their transmission. Transmission is also key to the persistence of baculoviruses in the environment (Cory and Myers, 2003).

Distinction is made between horizontal transmission (from infected individuals to the healthy ones) and vertical transmission (from parents to offspring) of the baculoviruses (Fine, 1984; Andreadis, 1987). Horizontal transmission occurs primarily when a NPV-infected larva dies and lyses, releasing a massive number of PIBs onto foliage and soil. Susceptible hosts become infected when they ingest PIBs while feeding. Defecation and regurgitation by infected larvae have been reported as additional routes of contamination of host plants with viruses, resulting in horizontal transmission (Ali *et al*., 1987; Vasconcelos *et al*., 1996; Young, 1998). Moreover, some studies suggest that cannibalism and predation may also be routes of virus transmission (Evans, 1986; Dhandapani *et al*., 1993). Environmental factors such as rainfall, wind transport (Fuxa *et al.*, 2001) and contaminated ovipositors of parasitic hymenopterans (Hamm *et al*., 1988) could contribute to NPV transmission as well.

Vertical transmission includes transovum transmission on the egg surface, transovarian transmission within the eggs, or passage as a latent infection (Cory and Myers, 2003; Fuxa, 2004, for review). Kukan (1999) published a detailed review of vertical transmission of NPV in insects. In the laboratory, the rate of vertical transmission of NPVs in eight species of *Mythimna*, *Pseudoplusia*, *Spodoptera*, *Lymantria* and *Trichoplusia* varied from 0.5 to 57.1%. In the field, egg masses produce infected larvae at rates ranging from 2 to 80%, whereas decontaminated egg masses yield only 0.1 - 9% infected larvae. This indicates that transovum transmission (which can be nullified by decontamination) is the most important factor in vertical transmission, while transovarian transmission is a minor but significant factor.

Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus (HaSNPV) is a specific viral pathogen with high virulence to its host, and has been developed as a commercial bioinsecticide to control cotton bollworm (*Helicoverpa armigera* Hübner)

in China (Zhang, 1989). To improve its relative slow speed of kill as compared to chemical pesticides, a recombinant HaSNPV (HaSNPV-AaIT) with improved biocontrol properties has been constructed (Chapter 2). This recombinant lacks the ecdysteroid UDP-glucosyltransferase (*egt*) gene and expresses an insect-selective toxin (*Aa*IT) from the scorpion *Androctonus australis* Hector. In the laboratory and in the field, the recombinant HaSNPV shows a significantly higher speed of kill of insect larvae than the wild-type HaSNPV and provided a better protection of cotton fruits from the damage by the cotton bollworm (Sun *et al*., 2002b; Chapter 2).

A quicker speed of action in recombinants results in a reduced production of PIBs in diseased larvae (Burden *et al*., 2000; Hernández-Crespo *et al*., 1999; Chapter 3). Due to the lower virus yield per cadaver, such faster recombinants may have a reduced rate of transmission in the field. This disadvantage in epidemiological terms might affect the effectiveness of recombinant viruses in control programs for pests, especially if the longer term effects of NPVs on insect populations are considered. On the other hand, a reduced rate of transmission might be considered an asset from the biosafety point of view of using recombinant viruses. Insight in the epidemiological dynamics, when wild-type and genetically modified baculoviruses are used, is necessary to predict optimal timing, frequency, and dosage of virus application and to assess the long term persistence of recombinant NPVs in insect populations and the environment. Ultimately, it may be possible to build epidemiological models that predict the behaviour of recombinant baculovirus-host dynamics under field conditions. The purpose of this study is to quantify horizontal and vertical transmission parameters of wild-type and recombinant HaSNPV variants, which are essential to parameterize and validate such a model.

MATERIALS AND METHODS

Viruses and insects

HaSNPV was initially isolated from diseased *H. armigera* larvae collected in the Hubei Province of Southern China (Zhang, 1989). The genotype HaSNPV-G4 used in these studies was isolated by using an *in vivo* cloning method (Sun *et al*., 1998) and is further referred to here as HaSNPV-WT. The recombinant HaSNPV-AaIT was generated by deletion of the *egt* gene from the genome of HaSNPV-WT and insertion of an *Aa*IT toxin gene using a chimeric promoter of the *p6.9* and *polyhedrin* promoters of HaSNPV to achieve gene expression (Chapter 2). Virus to be used in the experiments was produced in 4th instar of *H. armigera* larvae. PIBs were purified from the cadavers by two centrifugation steps alternated by a washing step and resuspended in a sterile 40% glycerol solution. PIB stocks were stored at 4 °C. Cotton bollworm, *H.*

armigera, larvae were collected from cotton fields in August near the China Cotton Research Institute (CCRI), Anyang (36°5'N, 114°21'E), Henan, China, and individually reared on artificial diet (Zhang *et al*., 1981) at 28 ± 1 °C and a 16-h photoperiod for several cycles and used for experiments in the following year.

Inoculation of infector larvae

First, 2nd and 3rd instar larvae (L1, L2 and L3) were infected with virus by feeding them a small cubic plug of artificial diet permeated with 10 µl of PIB suspension of virus at concentrations of 1×10^7, 1×10^7 and 1×10^8 PIBs ml^{-1}, respectively, which caused near 100% infection (Chapter 2). Inoculated larvae were reared individually at room temperature (24 - 28 °C) for 1 d before release. Time is expressed in days after larval inoculation (d.a.i.). Controls consisted of larvae fed on artificial diet treated with sterile water.

Experiment #1: Movement of healthy and virus-infected *H. armigera* larvae treated with wild-type or recombinant HaSNPV variants

Horizontal transmission is the result of an encounter process between healthy insects and infected larvae or cadavers and the surrounding PIBs. The more an infected larva moves, the more it can spread PIBs, e.g. by regurgitation, defecation and leakage of body fluids. Likewise, the chance of uptake of virus by healthy larvae increases as they visit a great number of leaves. Therefore, the movement of infected and healthy larvae on cotton plants was studied.

Two HaSNPV variants, a wild-type and a recombinant, were included to study the effect of genetic modification of the virus on the movement of the host. Field cages (3 m × 3 m × 3 m) were set up in a cotton field near the CCRI, Anyang, Henan, China. The cages had polythene gauze (pore size, 0.4 mm) on the sides and plastic covering on top. The sides were buried 20 cm into the soil. In each cage there were 4 rows of cotton (cultivar: Zhongmiansuo #35), each with 12 to 14 plants. The plants were 13 weeks old and approximately 1.15 m high when the cages were set up. Leaves from adjacent plant were in contact. Before the experiment started, all insects (predators, parasites, scarabids etc.) in the cages were removed by hand.

On July 19, 2002, one day after larval inoculation, one infector larva (L2) was used for every four cotton plants. The location of each larva was monitored daily, until it died or abandoned the plants. Number of nodes that the larvae traversed is used as a measure of distance moved. For example, if a larva was introduced at position A (Figure 1), found at point B one day later, and found dead with symptoms of viral infection at point C during the following day, it was estimated to have visited 7 nodes. If a larva was found to have moved to an adjacent plant, we assumed it had taken

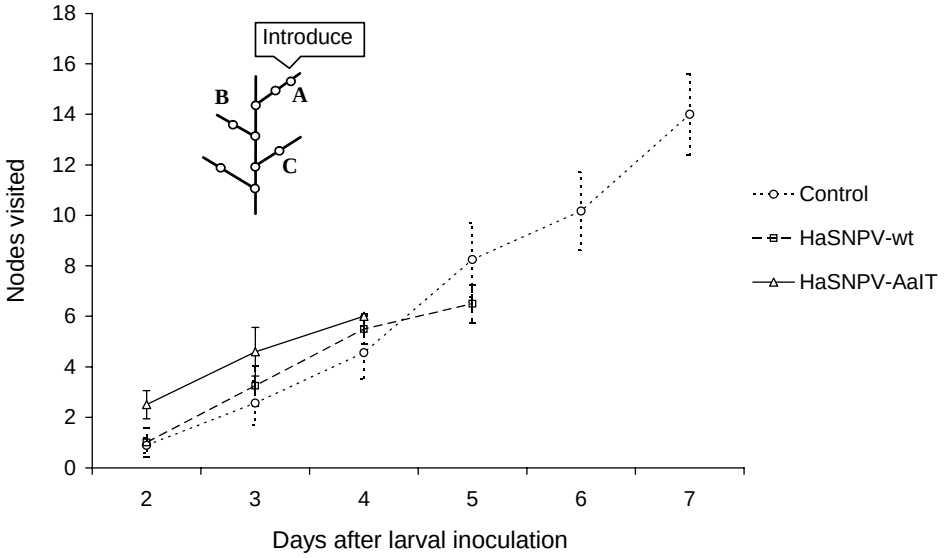

Figure 1. Number of nodes visited by larvae treated with HaSNPV-WT, HaSNPV-AaIT and water (Experiment 1). Inset: schematic representation of cotton plant showing the calculation of number of the visited nodes.

the shortest way to move and took the number of nodes between the two points where it was found as the distance moved. The transition from one plant to another was thereby counted as one more node.

The daily movement of the larvae (number of nodes visited) in 3 d after the release of inoculated larvae was analysed with the Kruskal-Wallis parameter-free ANOVA (SPSS Inc., 2000). The total number of nodes visited by larvae in different treatments was compared by pair wise t-tests (SPSS Inc., 2000).

Experiment #2: Horizontal transmission of HaSNPV variants in field cages

This experiment measured horizontal transmission from L1, L2 or L3 infector larvae inoculated with HaSNPV-WT or HaSNPV-AaIT. The infected hosts were introduced either at a lower density (one per 4 plants, Experiment #2a) or at a higher density (1 per plant, Experiment #2b) into field cages as described for Experiment #1. Experiment #2a was started on July 21, 2003 and Experiment #2b on August 12, 2003 in Anyang, Henan, China. Both experiments were set up as a 3 × 3 factorial with 9 field cages as experimental units. The experimental factors were: (1) instar of infectors (L1, L2 or L3) and (2) virus (HaSNPV-WT, HaSNPV-AaIT or water control). To measure virus transmission, three batches of healthy L1 (2 d old, hereafter referred to as recipients) were released in the cages at 3, 5 and 7 d.a.i.. At each time, 4 healthy recipients were introduced onto each plant. The recipients were recovered after 2 days

(before the next batch was introduced). They were reared individually on artificial diet in the laboratory until death or pupation to determine the presence of viral infection. Stained samples from cadavers were checked by phase contrast microscopy to confirm viral infection.

The proportion of virus infected recipients (p) at different sampling time in each cage was fitted by logistic regression (SPSS Inc., 2000). Mean values of ln(p/(1−p)) and the regression coefficients (slopes) were further analysed with 3-way ANOVA with virus, instar of infectors and density of infectors as factors combining the results of Experiment #2a and #2b. Interactions among infectors were initially included in the model, whereas nonsignificant interaction terms were subsequently removed, resulting in a minimal adequate model. Data in control cages were not included in the analysis, as very few infections were found.

The transmission coefficient for a host-pathogen system can be estimated using a protocol developed by Dwyer and Elkinton (1993), which is a simplification of the host-pathogen model developed by Anderson and May (1980, 1981). For short-term experiments in which the numbers of infected and susceptible host individuals are determined and only a single transmission cycle is observed, the model can be reduced. The rate of change of susceptible host density due to virus infection is given by

$$\frac{\mathrm{d}S_\mathrm{t}}{\mathrm{d}t} = -\nu W_0 S_\mathrm{t}\,, \tag{1}$$

where S_t is the density of susceptible hosts at time t. ν is the transmission coefficient (proportion of new infections per initially infected caterpillar per day). W_0 is the density of infectors at time zero (it stays constant in the short term). The rate of change of infected host density is

$$\frac{\mathrm{d}I_\mathrm{t}}{\mathrm{d}t} = \nu W_0 S_\mathrm{t} \tag{2}$$

where I_t is the density of infected hosts at time t. This simpler model can be solved to calculate ν values for the experimental results

$$\nu = \frac{-1}{tW_0} \ln\left(\frac{S_\mathrm{t}}{S_0}\right) \tag{3}$$

Differences among treatments in the values of transmission coefficient, ν, were analysed by using Univariate ANOVA (SPSS Inc., 2000) with virus, density of infectors and instar of infectors as factors, and sampling time as covariate. Initially, all explanatory variables and the interactions among the factors and sampling time were

included in the regression and the contribution of each term was tested for significance. Nonsignificant interaction terms were then removed, leaving a minimal adequate model which is reported here.

Experiment #3: Horizontal transmission of HaSNPV variants in the field

As the effects of rainfall, wind and biota on the transmission of virus are different in cages as compared to the field, we carried out an open field experiment (Experiment #3a) on horizontal transmission. In the middle of a 1.5 ha field near Anyang, Henan, China, horizontal transmission of HaSNPV-WT and HaSNPV-AaIT was compared in 2 plots consisting of 6 rows × 15 plants each. The two plots were separated by a free plot which contained same amount of rows and plants. On August 12, 2003, L1, L2 and L3 infectors were inoculated with HaSNPV-WT and HaSNPV-AaIT, respectively. One day later, these infectors were released onto each of the 90 plants at a rate of one infector per plant. Instars of infectors were rotated in subsequent plants. Four recipients (L1) were released as test insects onto each cotton plant in the plot at 3, 5 and 7 d.a.i.. The larvae were recovered as in Experiment #2 and individually reared in the laboratory. Dead larvae were checked for viral infection by phase contrast microscopy. It rained twice during the experiment, 11.8 mm on August 15 and 0.1 mm on August 18, 2003.

A second field experiment was conducted in 2004 (Experiment #3b). The only significant difference with Experiment #3a was the experimental design. In Experiment #3a, interplot interference was minimized by using two large plots, one for each treatment. In Experiment #3b, a randomized complete block design was used with 3 treatments (HaSNPV-WT, HaSNPV-AaIT and 'no virus' control) and 3 blocks. Plots measured 3 rows × 15 plants and were separated by 3 cotton rows or 1 m row distance each other. The same cotton variety was used as in Experiment #3a, and the infectors were inoculated on July 24, 2004. Details of inoculation of the infectors and release of the recipient larvae are identical as in Experiment #3a. It rained 11.0 mm on July 29 and 15.1 mm on August 2.

The proportion of virus infected recipients (p) at different sampling time in each plot was fitted by logistic regression (SPSS Inc., 2000). For Experiment #3a, mean effects of virus and sampling time on proportion of virus infection was analysed by binary logistic regression (measured by Wald statistics) (SPSS Inc., 2000). For Experiment #3b, mean values of $\ln(p/(1-p))$ and the regression coefficients (slopes) were analysed with one-way ANOVA (SPSS Inc., 2000).

Prediction of horizontal transmission rate in fields based on the parameters in the field cages

We further studied the reasonability of using the parameters obtained in the cages to predict horizontal transmission rate of HaSNPV variants in stage-structured *H. armigera* populations in the field, based on the Dwyer and Elkinton's model (1993). Eqn 2 can be revised as

$$\frac{1}{S_t}\left(-\frac{dS_t}{dt}\right) = \nu W_0 \tag{4}$$

here, νW_0 is relative rate of horizontal transmission (η) of a certain instar of infectors. In the open field experiments reported here, recipients were exposed to a mixture of L1, L2 and L3 infectors. As a time t (here it is 2 d) has elapsed, the expected proportion of the recipients getting infection is

$$\frac{I_t}{S_0} = 1 - e^{-(\eta_1+\eta_2+\eta_3)\times t} \tag{5}$$

where η_1, η_2 and η_3 are relative horizontal transmission rates for L1, L2 and L3 infectors at a certain density. This prediction was to be compared with the results obtained in Experiment #3a and #3b.

Experiment #4: Vertical transmission in the laboratory

While horizontal transmission accounts for virus transmission within a generation, vertical transmission accounts for the passage of virus to the next generation. Vertical transmission was studied by inoculating 300 of L3 *H. armigera* larvae with HaSNPV-WT, HaSNPV-AaIT or water in the laboratory. Larvae were infected by the droplet feeding method using a virus solution of 1×10^7 PIB ml^{-1} which would result in approximately 90% mortality (Chapter 2). The surviving larvae developed were cultured to pupae. To determine whether males or females were responsible for transmission, a mating schedule was devised. For each virus variant, four mating groups were set up: (1) control ♀ × infected ♂, (2) infected ♀ × control ♂, (3) infected ♀ × infected ♂, (4) control ♀ × control ♂. For each mating group and virus variants combination, 4 to 11 pairs of pupae which pupated at a similar time were incubated separately in a 1 L beaker. When moths emerged, 10% honey solution was supplied and 3M paper (Beckman) was put around the inner wall of the beakers. We collected all eggs laid beginning the 3rd day following the onset of oviposition. Progeny larvae were reared individually on artificial diet (Zhang *et al*., 1981) without formaldehyde at 28 ± 1 °C. Dead larvae were checked for viral infection by examining stained samples from cadavers by phase contrast microscopy for the presence of polyhedra.

Mortality of the F1 larvae was analysed by one-way ANOVA (SPSS Inc., 2000) with mating group as factor, while pair wise differences were further evaluated with t-tests.

Experiment #5: Vertical transmission in the field

Conditions (such as food, temperature, irradiation etc.) of laboratory experiments were different from those in the fields, therefore, the vertical transmission of HaSNPV variants was also studied in the field situation. On August 15, 2003, a field population of *H. armigera* consisting of 67% L1, 20% L2 and 13% L3 in a cotton field of 1.5 ha near Anyang, Henan, China was sprayed with HaSNPV-WT or HaSNPV-AaIT. Each virus variant was applied on 0.5 ha at a rate of 2.4×10^{12} PIBs ha^{-1} with a 50 m wide untreated strip between the two virus-treated zones. Size and density of cotton was the same as in Experiment #3. Eight days after spraying, 100 larvae (L5) were collected from both treated areas. The larvae were individually reared on artificial diet in laboratory. DNA samples were isolated from 10 larvae collected from each treatment area and PCR was conducted (Chapter 2) to determine the proportion of larvae that had contracted infection with the virus variant applied. The remaining 90 larvae were allowed to pupate. The survived pupae were cultivated in 2 L beakers (1 beaker per treatment) in an insect rearing room (28 ± 1 °C, 16-h photoperiod and 90% of humidity). When moths emerged, 10% honey solution was supplied and 3M paper (Beckman) was put around the inner wall of the beakers. When a moth started to oviposit, it was individually transferred to a 1 L beaker containing 3M paper for egg deposition. For each treatment, eggs from 3 females were separately collected and further cultivated. From each collection of eggs, 96 offspring larvae were reared individually and dead ones were checked for viral infection by phase contrast microscopy. Differences in mortality of the F1 larvae in two virus treatments were compared by t-test after arcsine-square root transformation (SPSS Inc., 2000).

RESULTS

Experiment #1: Movement of healthy and virus-infected *H. armigera* larvae treated with wild-type or recombinant HaSNPV variants

Horizontal transmission depends on the encounter chance between healthy insects and infectious larvae or cadavers and the surrounding PIBs. The movement of *H. armigera* larvae infected with HaSNPV-WT and HaSNPV-AaIT, compared to the healthy larvae, on cotton plants was studied in the field cages. Movement data were collected from 8 larvae treated with HaSNPV-WT, HaSNPV-AaIT or water. From 2 to 4 d.a.i., larvae treated with HaSNPV-AaIT visited more nodes than those treated with

HaSNPV-WT or water, and a significant difference occurred at 2 d.a.i. (Kruskal-Wallis ANOVA, $\chi^2 = 6.189$, P = 0.045) (Figure 1). The total number of visited nodes was strongly affected by the residence time on the cotton plants: healthy larvae had residence time of 5.4 ± 0.9 days and visited ca. 11.0 ± 2.0 nodes, whereas larvae infected with HaSNPV-WT had residence time of 3.3 ± 0.5 days (t = 6.07, d.f. = 7, P = 0.001) and visited 6.1 ± 1.6 nodes (t = 2.39, d.f. = 7, P = 0.048), those infected with HaSNPV-AaIT only had residence time 1.8 ± 0.9 days (t = 9.67, d.f. = 7, P < 0.001) and visited 4.8 ± 2.6 nodes (t = 3.13, d.f. = 7, P = 0.017). The residence time of the HaSNPV-AaIT infected larvae also differed from that of the HaSNPV-WT infected larvae (t = 3.97, d.f. = 7, P = 0.005). The total number of nodes visited by larvae infected with either of these two variants did not differ from each other (t = 1.21, d.f. = 7, P = 0.264).

Experiment #2: Horizontal transmission in the field cages

In this experiment, horizontal transmission from L1, L2 or L3 infector larvae inoculated with HaSNPV-WT or HaSNPV-AaIT was determined in field cages. In Experiment #2a (1 infector per 4 plants), a total of 2302 recipient larvae were recovered from 9 cages (49.4% recovery) (Table 1). Only 1 of 870 larvae (0.12%) died of viral infection in the 3 control cages, indicating a negligible background level of virus infection. In experiment #2b (1 infector per plant), a total of 1649 larvae were recovered from 9 cages (33.7% recovery) (Table 1). Ten virus deaths occurred in the 3 control cages (2.1%) again indicating a low level of natural infection.

Proportion of virus infection in recipients was strongly affected by the instar of infectors (F = 71.4, d.f. = 2, 7, P < 0.001) and the density of infectors (F = 196.6, d.f. = 1, 7, P < 0.001). L3 infectors gave the highest horizontal transmission, L1 infectors gave the lowest (Table 1). Transmission of HaSNPV-AaIT was significantly lower than that of HaSNPV-WT (F = 63.7, d.f. = 1, 7, P < 0.001). Transmission did not vary significantly with time, as there were no significant effects of virus (F = 1.17, d.f. = 1, 7, P = 0.315), instar of infectors (F = 0.35, d.f. = 2, 5, P = 0.72) on the slope of the relationship between proportion of infection of the recipients and the sampling time.

The transmission coefficient, *v*, significantly varied with virus variants (F = 28.66, d.f. = 1, 30, P < 0.001), density of infectors (F = 29.99, d.f. = 1, 30, P < 0.001), and instar of infectors (F = 31.03, d.f. = 2, 30, P < 0.001), but did not vary with sampling time (F = 0.31, d.f. = 1, 30, P = 0.582) (Table 2). The *v* value was higher when the density of infectors was lower.

Experiment #3: Horizontal transmission in the field

The horizontal transmission of virus in fields might be different from in cages,

Table 1. Mortality of recipients in the field cages in which L1, L2 or L3 infector larvae were introduced at two densities (Experiments #2a and #2b, Henan, Anyang, China, 2003).

Densities of infectors	Instar of infectors	Treatments	Sampling time of recipients								
			5 d.a.i.*			7 d.a.i.			9 d.a.i.		
			# released recipients	# collected recipients	mortality of recipients (%)	# released recipients	# collected recipients	mortality of recipients (%)	# released recipients	# collected recipients	mortality of recipients (%)
1 infector per 4 plants	L1	HaSNPV-WT	220	106	4.7	220	102	4.9	240	117	5.1
		HaSNPV-AaIT	200	83	3.6	200	105	1.9	210	106	3.8
		Control	150	70	0	100	48	0	100	78	0
	L2	HaSNPV-WT	150	72	5.6	150	73	11.0	150	74	9.5
		HaSNPV-AaIT	200	101	6.0	200	91	3.3	200	82	6.1
		Control	200	167	0.6	110	56	0	100	41	0
	L3	HaSNPV-WT	220	106	13.3	220	104	11.6	200	74	8.1
		HaSNPV-AaIT	200	97	8.2	200	85	8.2	200	109	8.3
		Control	120	70	0	100	42	0	100	43	0
1 infector per plant	L1	HaSNPV-WT	220	66	12.2	200	44	11.3	220	64	9.5
		HaSNPV-AaIT	210	66	9.1	210	52	9.7	200	48	4.2
		Control	150	47	2.1	120	49	0.0	120	63	1.6
	L2	HaSNPV-WT	190	55	20.0	200	86	25.5	190	50	21.9
		HaSNPV-AaIT	200	87	15.0	220	66	10.6	190	44	9.1
		Control	150	46	4.3	130	54	0	130	45	4.4
	L3	HaSNPV-WT	220	57	24.4	200	142	28.3	180	77	26.1
		HaSNPV-AaIT	180	48	16.7	190	79	17.7	170	55	14.5
		Control	180	81	3.7	180	35	2.9	150	43	0

* d.a.i is days after larval inoculation.

Table 2. Calculated transmission coefficient (v) at two levels of infector density, for L1 to L3 infectors and two virus variants (Experiment #2a and #2b).

Density of infectors	Instar of infectors	Virus	v (± SD)
1 infector/ 4 plants	L1	HaSNPV-WT	0.101 ± 0.004
		HaSNPV-AaIT	0.063 ± 0.021
	L2	HaSNPV-WT	0.183 ± 0.061
		HaSNPV-AaIT	0.106 ± 0.033
	L3	HaSNPV-WT	0.234 ± 0.059
		HaSNPV-AaIT	0.172 ± 0.001
1 infector/ 1 plant	L1	HaSNPV-WT	0.058 ± 0.008
		HaSNPV-AaIT	0.040 ± 0.016
	L2	HaSNPV-WT	0.127 ± 0.018
		HaSNPV-AaIT	0.062 ± 0.017
	L3	HaSNPV-WT	0.152 ± 0.013
		HaSNPV-AaIT	0.089 ± 0.010

therefore experiments were conducted to measure the horizontal transmission in the open fields. For Experiment #3a, a total of 2160 recipient larvae were released, 301 of which were recovered during the 3 samples. The recovery rate (13.9%) was lower than in the cages. Horizontal transmission of HaSNPV-AaIT was significantly lower than that of HaSNPV-WT (W = 3.946, P = 0.047) (W is Wald statistic, d.f. = 1) (Figure 2A). In Experiment #3b, on average, 30% of released recipients were recovered from plots. There was 6.7%, 7.7% and 9.3% of virus infection in recovered larvae at 5, 7 and 9 d.a.i. in the control plots. Transmission of HaSNPV-AaIT was again significantly lower than that of HaSNPV-WT (F = 37.1, d.f. = 1, 4, P = 0.004). Transmission of viruses did not vary significantly with time (F = 3.67, d.f. = 1, 4, P = 0.128) (Figure 2B).

Prediction of horizontal transmission rate in the field based on the parameters in the field cages

To check if the parameters obtained in the cages could be used to predict the horizontal transmission rate of HaSNPV variants in stage-structured *H. armigera* populations in the field, we fitted the data in the cage experiments into Dwyer and Elkinton's model (1993). We further plotted η at 1 infector per 4 plants and 1 infector per plant for L1, L2 and L3 infectors obtained in the cage fields against the infector densities and further fitted the data with exponential equations to estimate η at different densities of infectors as a function of infector instar (Figure 3). According to

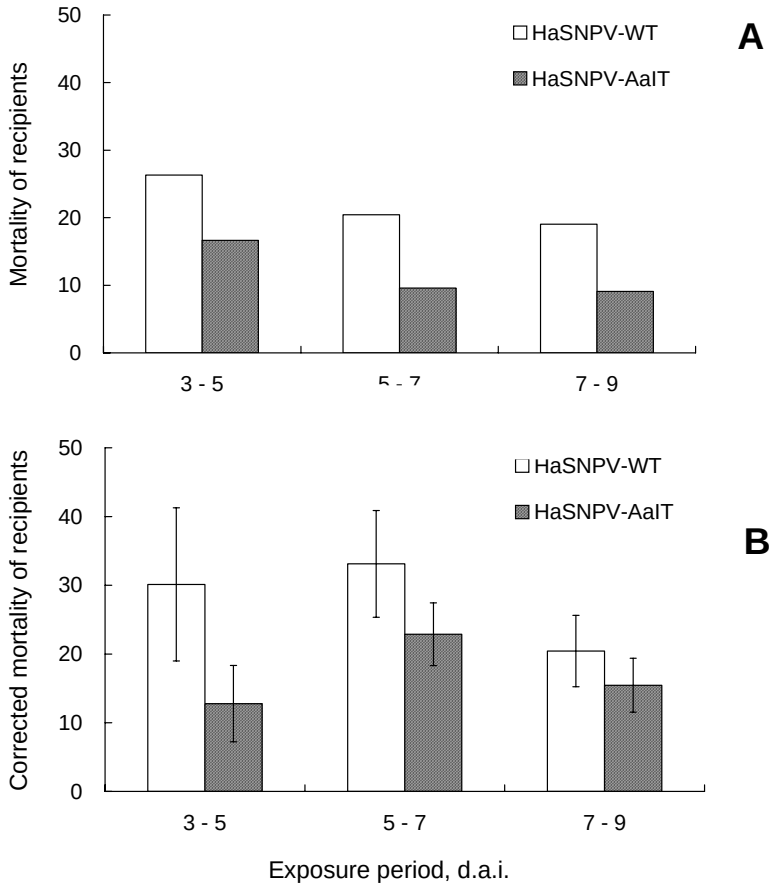

Figure 2. Mortality of recipients recovered from open field plots infested with infector larvae (L1+L2+L3) inoculated with HaSNPV-WT or HaSNPV-AaIT in (A) 2003 (Experiment #3a) and (B) 2004 (Experiment #3b). In Figure 2B, mortality of recipients was calculated by using Abbott's correction (Matsumura, 1985). d.a.i. = days after larval inoculation.

the equations in the legends of Figure 3, in Experiment #3a and #3b at an infector density of 0.333 per plant for each instar, η was 0.030, 0.056 and 0.071 d^{-1} for L1, L2 and L3 infectors treated with HaSNPV-WT, respectively, and 0.019, 0.032 and 0.050 d^{-1} for L1, L2 and L3 infectors treated with HaSNPV-AaIT, respectively. Applying these values to Eqn 5, mortality of recipients collected from field plots after a 2 days exposure period would be 27.0% in the case HaSNPV-WT treated infectors were used and 18.2% for the HaSNPV-AaIT treated infectors over three continuous exposure periods (6 d). This prediction is in line with the measured proportion of infections in the open field experiments (Figures 2A and 2B), except for the transmission rate at last two exposure periods was obviously lower than the predicted values in 2003 (Figure 2A). This result indicates that the parameters of horizontal transmission obtained in the cages can be used to predict the horizontal transmission rate of HaSNPV variants in field situations.

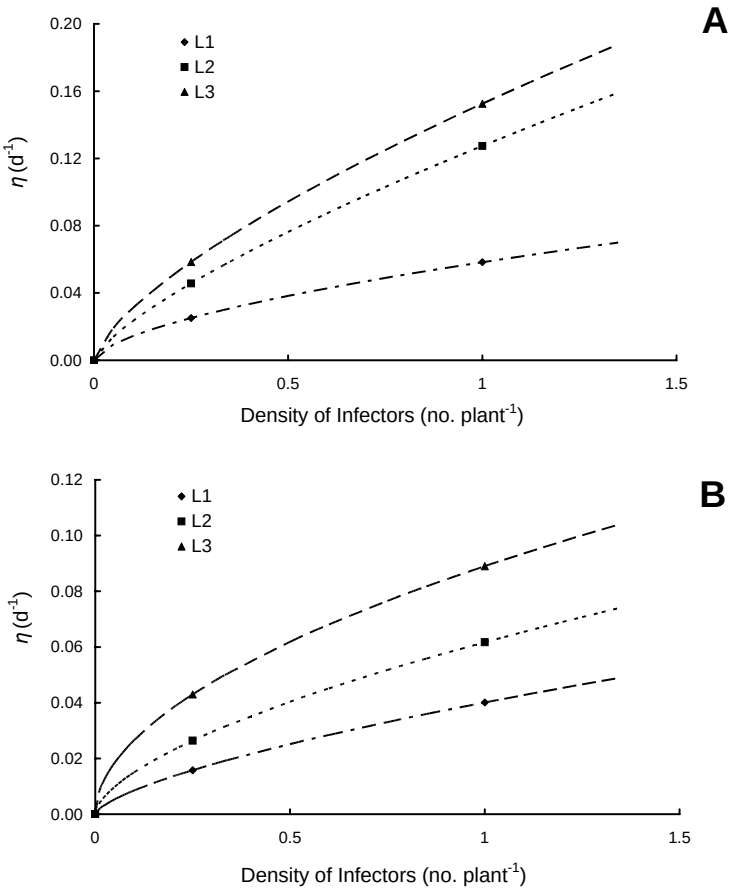

Figure 3. Estimation of the relative rate of horizontal transmission (η). (A) When recipients exposed to L1, L2 and L3 HaSNPV-WT treated infectors, $\eta_1 = 0.0583 \times W(0)_1^{0.6079}$, $\eta_2 = 0.1274 \times W(0)_2^{0.7395}$, and $\eta_3 = 0.1525 \times W(0)_3^{0.6924}$, respectively. (B) When recipients exposed to L1, L2 and L3 HaSNPV-WT treated infectors, $\eta_1 = 0.0401 \times W(0)_1^{0.6718}$, $\eta_2 = 0.0617 \times W(0)_2^{0.6124}$, and $\eta_3 = 0.089 \times W(0)_3^{0.5247}$, respectively. For both A and B, $W(0)_1$, $W(0)_2$ and $W(0)_3$ are the initial densities of L1, L2 and L3 infectors, respectively.

Experiment #4: Vertical transmission in the laboratory

Not only horizontal transmission but also vertical transmission plays important roles in baculovirus epidemiology. Vertical transmission of wild-type and recombinant HaSNPV variants in *H. armigera* was studied here as well. Inoculation of L3 larvae with HaSNPV-WT and HaSNPV-AaIT at a dose of 1×10^7 PIBs ml^{-1} caused mortality of 86.2% and 90.1%, respectively. When female moths, developing from the larvae treated with one of the two virus variants, either mated with a virus-treated male moth or a healthy moth, the F1 larvae had a significantly greater total viral mortality (17.2% to 32.0%) than the control group (3.6%) ($F = 21.96$, d.f. = 6, 35, $P < 0.05$). For both virus variants, the vertical transmission rate did not differ significantly whether the

treated female moth had mated with a virus treated male or with a control male (t = 0.37, d.f. = 6, P = 0.72 for HaSNPV-WT; t = 0.22, d.f. = 6, P = 0.84 for HaSNPV-AaIT). Vertical transmission of HaSNPV-AaIT was significantly lower than that of HaSNPV-WT (t = 4.06, d.f. = 14, P = 0.001) (Table 3). When a male moth, developing from L3 treated with one of the two virus variants, mated with a healthy female moth, the mortality of the next generation (F1) larvae (6.3% and 5.9%) did not significantly differ from that of F1 larvae originating from pairs of control males and control female moths (3.6%) (t = 1.48, d.f. = 13, P = 0.16 for HaSNPV-WT; t = 1.61, d.f. = 14, P = 0.13 for HaSNPV-AaIT) (Table 3).

Experiment #5: Vertical transmission in the field

The vertical transmission of virus in fields might be different from in the laboratory, experiments were also conducted in the open fields. In experiment #5, PCR results showed that all larvae that were collected from the plots treated with HaSNPV-WT or HaSNPV-AaIT had contracted the applied viruses (data not shown). Thirteen out of 90 larvae survived the challenge of HaSNPV-WT treatment and 6 out of 90 larvae survived from the HaSNPV-AaIT treatment. Twelve (7♀ and 5♂) of the 13 surviving larvae treated with HaSNPV-WT molted into moths. The six HaSNPV-AaIT treated larvae (3♀ and 3♂) all molted into moths. The proportion of viral infection of F1 larvae was 8.4 ± 1.1% in the case of HaSNPV-AaIT, which was significantly lower than in the case of HaSNPV-WT (12.6 ± 2.0%) (t = 3.310, d.f. = 4, P = 0.03).

Table 3. Virus-induced mortality among F1 progeny from paired bollworm adults with either both parents or 1 parent treated with HaSNPV-WT and HaSNPV-AaIT as L3 larvae in the laboratory (Experiment #4).

Treatments	Mating types	# moth pairs	# larvae observed	Mortality of F1 larvae*	
HaSNPV-WT	Control ♀ × infected ♂	4	144	6.3 ± 2.1	a
	Infected ♀ × control ♂	4	159	32.0 ± 4.2	c
	Infected ♀ × infected ♂	4	108	29.8 ± 4.5	c
HaSNPV-AaIT	Control ♀ × infected ♂	5	238	5.9 ± 1.1	a
	Infected ♀ × control ♂	4	186	16.2 ± 2.8	b
	Infected ♀ × infected ♂	4	192	17.2 ± 3.4	c
Control	Control ♀ × control ♂	11	455	3.6 ± 0.6	a

* Different letters indicate significant differences between treatments (P = 0.05).

DISCUSSION

Studies on transmission of genetically modified baculoviruses in the environment are essential for planning an application regime (such as timing, frequency and dosage) and for the risk assessment preceding their use as biocontrol agents in a certain crop-pest complex. In this paper, the horizontal and vertical transmission dynamics of an economically important virus pathogen of *Helicoverpa armigera*, HaSNPV, and a derived recombinant, HaSNPV-AaIT, with improved speed of action were studied in semi-controlled conditions (field cages and laboratory) and in an open field situation. Our results demonstrated that (1) the recombinant HaSNPV with quicker speed of kill exhibits less horizontal transmission than the wild-type virus, (2) later instar infectors resulted in a greater transmission rate, (3) the horizontal transmission rate of both recombinant and wild-type was higher at higher density of infectors, and (4) the recombinant HaSNPV has a lower vertical transmission rate than the wild-type.

The reduced horizontal transmission rate of toxin-expressing virus may have several reasons, primarily the reduced virus yield per host cadaver (Burden *et al.*, 2000; Hernández-Crespo *et al.*, 1999; Chapter 4) and the altered behaviour of infected larvae (Cory *et al*., 1994; Hoover *et al*., 1995). Hails *et al*. (2002) investigated the transmission of wild-type and toxin-expressing *Autographa californica* nucleopolyhedrovirus (AcMNPV) in a highly susceptible host, *Trichoplusia ni*, and a semipermissive host, *Mamestra brassicae*. They concluded that the number of cadavers remaining on the foliage has a greater influence on transmission than the yield of virus from those cadavers. The genetically modified virus always resulted in a lower rate of transmission in the field compared to the wild-type, most likely because the recombinant virus caused paralysis and the cadaver falls off the plant prior to death and virus release (Cory *et al*., 1994; Hoover *et al*., 1995). In our experiments, the larvae infected with HaSNPV-AaIT have a shorter residence time observation than those infected with HaSNPV-WT on the cotton plants. However, due to the observation that the HaSNPV-AaIT infected larvae visited more nodes in the crop in a given time than HaSNPV-WT infected larvae (Figure 1), the former visited in total a similar number of nodes than the HaSNPV-WT infected larvae before they died or abandoned the plant. Therefore, in the HaSNPV-bollworm-cotton system, the lower yield of recombinant virus plays also an important role in the reduced horizontal transmission. This conclusion is also supported by the observation that the later instar of infectors has a higher transmission rate (Table 1).

In our experiments, the transmission rate in the open field was close to that in cages (Figure 2). In the field, rain splash, wind and biotics (such as predators, parasitic arthropods and scavengers) can contribute to NPVs transmission and cause a greater

transmission rate than in the cages. On the other hand, predators and parasitic arthropods can also reduce the density of infectors before they spread viruses on the plant annihilating this effect. Furthermore, the inactivation rate of 'free' virus in the crop may also be faster in the fields than in the cages. These factors, which increase or decrease the transmission rate of HaSNPV, may cancel each other out in the field. Thus, the parameters of horizontal transmission obtained and quantified in the cages can be used to predict the transmission of HaSNPV variants in field situations.

The vertical transmission rate of HaSNPV-AaIT was significantly lower than that of wild-type HaSNPV, both in the laboratory and in the field (Table 3). This might be attributed to fact that less PIBs are produced when the host is infected with the recombinant virus than for the wild-type virus (Chapter 3). A similar phenomenon has been found for AcMNPV and its host, *Trichoplusia ni* Hübner (Fuxa *et al*., 2002). In our field experiment (Experiment #5), where we collected L5 larvae from fields treated by wild-type or recombinant HaSNPV variants, vertical transmission rates (12.6% for HaSNPV-WT and 8.4% for HaSNPV-AaIT) were obviously lower than those observed when we dosed the L3 larvae in the laboratory (30.9% for HaSNPV-WT and 16.7% for HaSNPV-AaIT). This might be due to the lower activity of virus persisting in bollworms fed on cotton leaves than on artificial diet, because exudates and secondary chemicals secreted by cotton gland (Elleman and Entwistle, 1985) and free radicals mediated by peroxidase in cotton tissues (Hoover *et al*., 1998) diminish the virus activity and hence virus transmission. A similar phenomenon as seen for HaSNPV as above has been observed in the *Spodoptera exigua*-tomato-SeMNPV system (Bianchi *et al*., 2001). In the field situation, where bollworm eggs are exposed to sunlight and other environmental factors, vertical transmission (primarily transovum) would be even lower than the values we measured by collecting the virus-treated larvae in the field and rear them out in the laboratory.

Data obtained in this study and those in other systems (Hails *et al*., 2002; Fuxa *et al*., 2002) indicate that transmission rate of genetically modified baculoviruses is lower than that of wild-type viruses. For migratory pests, such as *H. armigera*, lower vertical transmission of the recombinant virus would reduce their spread from field to field and the risk to expose the nontarget populations. Undoubtedly, the reduced transmission of the recombinant baculoviruses is beneficial for their biosafety profile. A full assessment of the risk of a genetically modified baculovirus needs to include all components of their fitness, such as the virus yield, transmissibility and persistence in the environment. It has been reported the recombinant HaSNPV variants have a similar inactivation rate on cotton tissues as the wild-type strain (Chapter 4). However, their fate in other compartments, such as soil, predators, parasitic arthropods, scavengers, birds and mammals, where they may persist, is not easy to predict and

therefore needs further investigation.

On the other hand, the reduced transmission rate of the recombinant baculoviruses is a disadvantage for the long-term control efficacy of the insect. For HaSNPV, this disadvantage is relatively unimportant because HaSNPV inactivates quickly on the cotton (Chapter 4) and thus is required to be applied frequently (e.g. in 3 to 4 d intervals) (Zhang *et al*., 1995). Due to the quick speed of action, the recombinant HaSNPV expressing an *Aa*IT toxin incapacitates the host earlier and thus causes less damage of cotton bolls and give higher yield of cotton lint in comparison with the wild-type virus (Chapter 2).

CHAPTER 6

Modelling the behaviour of wild-type and genetically modified baculoviruses in the *Helicoverpa armigera* – cotton system*

* **Submitted to *Ecological Modelling***

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ABSTRACT

A comprehensive process-based model is developed to simulate the epizootiology of wild-type and genetically modified (GM) *Helicoverpa armigera* single nucleocapsid nucleopolyhedroviruses (HaSNPVs). The model integrates ecological information on interactions between variants of HaSNPV, cotton bollworm (*H. armigera*), and cotton. The model is used to assess and predict the biocontrol efficacy of application regimes of the wild-type and GM HaSNPV variants and the ecological behaviour of HaSNPV variants when used to control cotton bollworm in cotton, viz. at different spraying regimes, dosages, UV-protection agents, as affected by rates of horizontal and vertical transmission and transport of virus between soil and plants. In simulations on dosage and timing, good control efficacy against a common density of *H. armigera* of 12 larvae m^{-2} soil was obtained when cotton was sprayed 3 times at 1.2×10^8 Polyhedral Inclusion Bodies (PIBs) m^{-2} soil in June, 3 times at 2.4×10^8 PIBs m^{-2} soil in July and 4 times at 3.0×10^8 PIBs m^{-2} soil in August. An early timing of virus applications, soon after egg deposition, with a short, 3 days, spray interval appeared to be optimal for effective control. Recombinant viruses, with a lower mean time to kill, gave better short term protection than wild-type virus. However, due to reduced horizontal transmission, use of recombinant virus caused more pupae to survive at the end of the growing season. These pupae may then form the initial population for the next season. The density of recombinant virus persisting in soil was lower than of wild-type virus, mainly due to the lower virus yield per cadaver for the recombinant virus. UV-protection agents extending the half-life of HaSNPV on the canopy from 0.5 to 12 days contributed significantly to the effectiveness of control of bollworm under field conditions. A minimum half-life of 3 days was essential for effective control of bollworm with HaSNPV insecticides. Whereas horizontal transmission appeared to contribute significantly to control efficacy, this was not the case for vertical transmission or for transport of HaSNPV from soil to cotton plants at the low rates characteristic for this system. The results of this study indicate that recombinant HaSNPV with faster speed of kill truly provides better control of *H. armigera* infestations in the short term, but not necessarily in the long term.

Key words: *Helicoverpa armigera*, baculovirus, genetically modification, cotton, modelling.

INTRODUCTION

The cotton crop is host to several species of heliothine moths, whose larvae can cause major damage and devastating crop losses. Considerable investments in crop protection in the form of crop breeding (Bt-based molecular resistance), frequent pesticide sprays, and the application of biologicals are often required. A particularly effective biological control option is the use of insect-specific nucleopolyhedroviruses, but these viruses, albeit highly virulent and effective, have one important limitation: they are slow acting (Black *et al.*, 1997; Moscardi, 1999). Lethally infected caterpillars continue to feed for almost one week after infection at ambient temperature, causing continued crop damage (Moscardi, 1999). Genetic modification has been successfully employed to develop faster killing, highly specific nucleopolyhedroviruses that have high potential for practical pest control (Van Beek and Hughes, 1998; Inceoglu *et al.,* 2001, for review).

Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus (HaSNPV) has been developed as a commercial biopesticide to control cotton bollworm, *H. armigera* (Zhang, 1989). Several recombinants of HaSNPV with an increased speed of action have been constructed for cotton bollworm control (Chen *et al*., 2000b; Chapter 2). These recombinants lack the *egt* gene or/and contain a neurotoxin gene (*AaIT*) from the scorpion species *Androctonus australis*. Laboratory bioassays showed that these recombinants kill second instar larvae of *H. armigera* faster than the wild-type (Chen *et al*., 2000b; Chapter 2). In field trials, fast killing HaSNPV recombinants provided better larval control and cotton protection than wild-type HaSNPV (Sun *et al*., 2002b; Chapter 2). Recombinant viruses gave rise to reduced virus production and transmission in its host populations than the wild-type (Chapters 3 and 5). These characteristics of the recombinant viruses suggest that they may be disadvantaged in the long-term control as compared to wild-type virus. On the other hand, the reduced production and transmission of recombinant viruses might be considered an asset from the biosafety point of view because their spread and persistence in the environment may be impaired. Furthermore, insect behaviour and crop ecology add to the complexity of the cotton-bollworm ecosystem.

Field experiments with recombinant baculoviruses are subjected to strict regulations of biosafety and therefore costly. Thus far, these have been undertaken at a limited scale in the UK, the USA and in China (Cory *et al.*, 1994; Cory, 2000; Sun *et al.*, 2002b). Nevertheless, our insight in the ecological behaviour of GM baculoviruses is limited. Mathematic simulation models are effective tools to understand the ecological process of control pests with baculoviruses and to evaluate and optimize the spraying regimes of baculoviruses (De Moed *et al.*, 1990; Van der Werf *et al.*, 1991; Bianchi *et*

al., 2002a, b, c). Some analytical models have been developed to study epizootics of baculoviruses in insect populations (Anderson and May, 1981; Dwyer, 1992; Dwyer and Elkinton, 1993; Dwyer *et al.,* 1997), and these are suited for addressing theoretical issues related to the long-term dynamics and stability of the virus in the insect-plant ecosystems.

However, these models are less suitable for answering most of the short-term questions that emanate from the practical application of baculoviruses as biocontrol agents, e.g. the optimal timing and dosages of baculovirus applications, because they lack the degree of detail that is required for obtaining quantitatively realistic answers to such questions. In contrast, simulation models that describe a system at a detailed process level and can be numerically solved, offer good perspectives for practically useful analyses as shown in studies in insect–parasitoid and insect–predator systems (De Kraker, 1996; Bianchi *et al.*, 2002a, b, c; Xia *et al*., 2003).

We developed a process-based model for crop level interactions between the cotton bollworm, *H. armigera*, and its wild-type and genetically modified nucleopolyhedroviruses (HaSNPV). The model integrates information on virus infectivity, incubation time, virus inactivation, vertical and horizontal transmission, virus production and the movement of virus between the soil and crop compartments. The purpose of the model is to integrate and verify our insights in the functioning of insect-virus interactions at the population level under field conditions, and to make predictions and scenario studies prior and complementary to field releases. In this chapter, we describe the conceptual basis of the model, its parameterization by quantitative descriptions of model components, a sensitivity analysis and scenario studies with the model.

MODEL DESCRIPTION

The model was developed in the Fortran Simulation Translator (FST, version 2.0) (Rappoldt and Van Kraalingen, 1996). It consists of a main program and two FORTRAN subroutines (Appendix 1). FST includes a system for weather data input and utilities for specific tasks such as initialization, integration and sorting.

Development and population dynamics of *H. armigera*

H. armigera has an egg stage, five larval stages (L1 to L5), a pupal stage, and an adult stage. Insect development is modelled by keeping track of numbers in state variables (so called ‘boxcar trains’) that represent different stages of development and virus infection. The network of state variables that is used to represent insect development, virus infection, and attrition (background mortality in field) is analogous to the structure of the insect life cycle and infection processes (Figure 1).

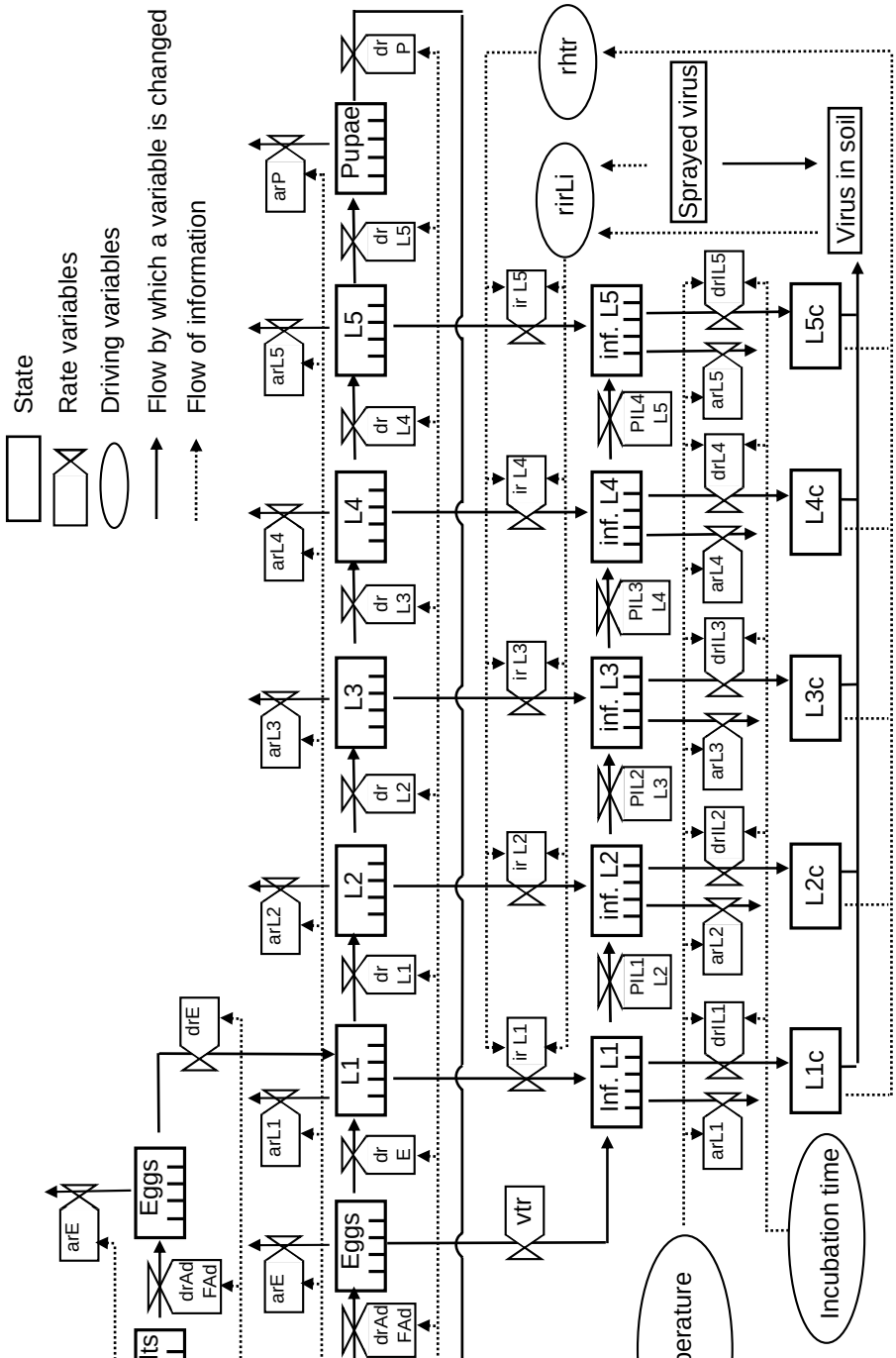

Figure 1. Relational diagram of interaction of *H. armigera* and HaSNPV in cotton field. The diagram consists of a flow of healthy insects (upper), infected larvae (middle) and cadavers (lower). Legends: IMrAd: Immigraant adults. FAd: Fecundity rate of adults; drA: developmental rate of adults; drE: developmental rate of eggs; drLi: developmental rate of Li larvae; drP: developmental rate of pupae; arA: attrition rate of adults; arE: attrition rate of eggs; arLi: attrition rate of Li larvae; arP: attrition rate of pupae; irLi: infection rate of Li larvae; rirLi: relative infection rate of Li larvae; inf. Li: infected Li larvae; rhtr: relative rate of horizontal transmission; vtr: vertical transmission rate; PIL1L2: proportion of infected L1 developed to L2; PIL2L3: proportion of infected L2 developed to L3; PIL3L4: proportion of infected L3 developed to L4; PIL4L5: proportion of infected L4 developed to L5; drILi: developmental rate of infected Li larvae; Lic: cadavers at stage i.

Individuals of a certain stage are distributed over 'boxcars' in accordance with their state of development within that stage, while the total number in a stage is represented by the sum of numbers in the whole sequence of boxcars, i.e. a 'boxcar train'. If a stage is represented by n boxcars, then each of those represents $1/n$ th of the development duration of the stage. Development is simulated by shifting individuals from one boxcar to the next one. The time between shifts is determined by the rate of development of individuals as depending upon temperature. The number of boxcars of each boxcar train was chosen according to the variability in developmental time among individuals in the stage (Leffelaar, 1993). At the end of a boxcar train, individuals can be transferred to a subsequent boxcar train, representing the next development stage. Virus infection is represented by lateral outflow from the boxcar trains representing healthy larvae to boxcar trains representing infected individuals (Figure 1). Uninfected larvae develop either into uninfected next instar or into infected current instar (due to horizontal transmission), or they may die due to attrition.

H. armigera may have four generations in one cotton growing season in the Yellow River region in China. The first generation of bollworms feeds on wheat, pea or vegetables. After mid-June, the adults migrate into cotton fields forming three bollworm generations that generally need to be controlled. At the beginning of September, *H. armigera* hibernates as pupae, which form the source of *H. armigera* infestation in the following year.

Simulation of the infection process

Infection by ingestion of sprayed polyhedra

The dose-mortality response of larvae to ingested virus particles is modelled on the basis of laboratory data, using a linearized logistic equation with the logarithm of virus dose as independent variable

$$\ln(\frac{m}{1-m}) = a + b \times \ln(dose)\,, \qquad (1)$$

where m is the proportion of virus-killed larvae, and $dose$ is number of PIBs ingested per larva. Equation 1 can be rewritten as:

$$m = \frac{dose^{\mathrm{b}}}{\mathrm{e}^{-\mathrm{a}} + dose^{\mathrm{b}}} \qquad (2)$$

Under field conditions, larvae become infected as they accumulate PIBs while feeding. To represent this process in time, the number of PIBs ingested per larva is kept track in boxcar trains that are parallel to the boxcar trains that do the bookkeeping of the

number of healthy larvae. For each boxcar in the boxcar train for ingested dose, the rate of change is formulated as the resultant of two components, one expressing ingestion of polyhedra as the product of feeding rate (mm^2 leaf d^{-1}) and PIB density on the leaf surface (PIBs mm^{-2} leaf), and a second component mimicking the shifting of virus dose from one boxcar to the next, in exactly the same fashion as the development process of larvae is formulated (Appendix 1). Thus, the accumulation of virus dose and insect development through time are intimately coupled.

The logistic relationship given in Eqns 1 and 2 can be used to predict the mortality response at a given dose of virus, ingested at a given moment. The infection chance per time step, which depends on the rate of uptake of virus, is described as

$$p(dose(t)) = 1 - \frac{1 - m(dose(t + \Delta t))}{1 - m(dose(t))} = \frac{dose(t + \Delta t)^{b} - dose(t)^{b}}{e^{-a} + dose(t + \Delta t)^{b}}, \quad (3)$$

where the parameters a and b are the same as in Eqn 1 and 2, Δt is the simulation time step, $dose(t+\Delta t)$ is the virus dose ingested per larva in a boxcar at time $t+\Delta t$, while $dose(t)$ is the virus dose ingested by one larva in the same boxcar at time t. The calculation of accumulated dose is re-initialized at zero after caterpillars recruit to a subsequent instar, i.e. the infection process in a subsequent instar is considered independent.

The relative infection rate (*rir*) for larvae in each boxcar is:

$$rir = \frac{p(dose(t))}{\Delta t}. \quad (4)$$

Deposition of virus spray

In practice, virus suspension is sprayed from the top and both sides of the cotton canopy. It was considered unnecessary to include the vertical profile of virus density in the crop in the model, because the majority of the eggs are laid in the top canopy of the plant on leaf surfaces, stems, the bracts of fruiting structures, and the plant terminals (Lu and Yin, 1999; Duffield and Chapple, 2001), where they are exposed to virus treatment applications, while earlier instar larvae also feed in this canopy layer (Parrott *et al.,* 1978; King, 1994). The density of virus on cotton (PIBs mm^{-2} leaf) after a spray of dosage D (PIBs m^{-2} soil) is calculated as

$$PIB_0 = \frac{k \times D}{2 \times L \times 10^6}, \quad (5)$$

where k is the proportion of virus intercepted by the crop, and L is the leaf area index (LAI, m^2 m^{-2}). It is assumed that the density of virus is equal on both sides of the leaves, while larvae feed on one leaf side only, thus a factor of 2 is used here.

Virus inactivation on cotton surface

Polyhedra that are sprayed on the cotton canopy may be inactivated by UV radiation, secondary chemicals secreted by plants (exudates) or plant-mediated peroxidase activity which generates free radicals (Chapter 4). We simulate this inactivation as an exponential decay.

$$PIB_t = PIB_0 \times e^{-rirv \times t}, \quad (6)$$

where PIB_t is the density of infectious polyhedra per unit of cotton leaf or fruit area (PIBs mm^{-2} leaf) at time t, PIB_0 is as defined in Eqn 5, *rirv* is the relative inactivation rate of PIBs (d^{-1}) and t is time (d).

Development of infected larvae

The numbers of larvae contracting a lethal virus dose as determined by Eqn 3 are moved into the boxcar trains representing infected larval stages (Figure 1). Development rates of infected larvae reflect the incubation time of virus disease.

Infection by horizontal transmission

Infected larvae excrete viruses by regurgitation, defecation and leakage of body fluid while still alive or by spilling polyhedra on cotton plants after liquefaction of the cadavers (Chapter 5). The relative rate of susceptible hosts contracting infection due to horizontal transmission (η) is determined by the density of all 5 stages of infectors (W_0) and the relative transmission rate (v, proportion of new infections per initially infected caterpillar per day) (Dwyer and Elkinton, 1993).

$$\eta = \frac{1}{S_t}\left(-\frac{dS_t}{dt}\right) = vW_0, \quad (7)$$

where, S_t is the density of susceptible hosts at time t. The relationship between η and the density of virus sources is a power function (Chapter 5). Bodies of infected larvae that die by attrition are considered not to contribute horizontal transmission.

Infection by vertical transmission

Vertical transmission is the direct transfer of virus from sublethally infected insects to their offspring. A certain proportion of the eggs laid by female moths sublethally infected with HaSNPV is contaminated. L1 larvae hatching from contaminated eggs can be infected by consuming the chorion (Figure 1). Contaminated eggs develop into infected first instar larvae that can transmit the virus horizontally when they die. Immigrant moths are assumed to be free from virus contamination.

Virus persistence in soil and movement from soil to plants

Soil is a major reservoir for NPV, particularly for long-term persistence. The model takes into account that part of the virus sprayed or produced by virus-killed larvae in the crop during the growing season is deposited to the soil and can be transported back to the foliage, e.g. by rain, wind, insects, etc. (Fuxa and Richter, 2001), and cause infection of the host larvae (Figure 1). Based upon measurements of PIB density resided in soil (X. Sun, unpublished data) and of virus density on the crop along the course of a season, a function was calculated that expresses the proportion of virus density in soil out of the amount of virus sprayed or produced by virus-killed larvae in the field since the start time of simulation (Table 3). A proportionality factor of 10^{-5} is assumed between the virus density on leaves, as a result as transport from soil, and the amount of virus in the soil (Fuxa and Richter, 2001).

Cotton injury

L1 and most of L2 larvae feed on tender leaves. As they do not affect the fruits, their feeding usually does not cause damage. In the third and subsequent instars, larvae feed on floral squares, flowers and bolls. On average, an L3 larva injures 2 fruits per day, while L4 and L5 larvae injure 2.5 fruits per day (Sheng *et al*., 2002). Larvae getting infection of virus are able to injure cotton fruits until the time of feeding cessation, which occurs before they die (Chapter 2).

MODEL PARAMETERIZATION

H. armigera developmental rate, fecundity and attrition rate

Development rates of *H. armigera* stages depend on temperature and were derived from literature (Pest Forecasting Station of Ministry of Agriculture of China, 1983) (Table 1). Fecundity of female moths was measured by Tian *et al.* (2002) in incubators at 28 ± 1 °C. One fertilized female moth oviposits on average 809 eggs. Rates of egg hatch are high (> 80%) for the first 4 batches and then decrease to less than 10% (Table 2). Sex ratio of adults is set to 0.5. Attrition rates of bollworm life stages in the field are based on experimental life tables (Wu *et al.*, 1978; Yang *et al.*, 2000; Xia *et al.*, 2000) and the field experiments described in Chapter 2 (Table 1). *H. armigera* population has multiplication factors of 3.3 from the 2nd to 3rd generation and of 3.6 from the 3rd to 4th generation in the cotton field, using these parameters (Figure 2).

HaSNPV infectivity

The dose-mortality relationship was determined for wild-type HaSNPV (HaSNPV-WT) and two recombinant HaSNPVs. One recombinant (HaSNPV-EGTD) lacked the

Table 1. Parameters for *H. armigera* and wild-type and recombinant HaSNPV variants.

Process	Parameter	Eggs	L1	L2	L3	L4	L5	Pupae	Moths	Unit	Reference
H. armigera	Temperature threshold	11.6	13.6	12.8	11.5	6.4	10.8	12.1	8.7	°C	Pest Forecasting
	Tmax*	34	34	34	34	34	34	34	34	°C	Station, China
	Tstop*	40.3	38.4	39.2	39.3	40.8	41.2	43.5	43.0	°C	
	Relative developmental rate	0.501	0.585	0.795	0.807	0.606	0.260	0.162	0.140	d^{-1}	
	Developmental time at 28 °C	2.7±0.3	2.5±0.3	1.8±0.3	1.7±0.3	2.0±0.3	5.2±0.8	8.4±1.2	9.4±1.3	d	
	Attrition rate$	0.566	0.266	0.109	0.074	0. 068	0.058	0.096	0.073	d^{-1}	
	Feeding rate		2.9±1.6	4.5±1.9	3.4±2.0	12.3±3.8	20.0±6.0			$mm^2\ d^{-1}$	X. Sun, unpubl.
HaSNPV-WT &	Dose-mortality regression †										Chapter 2
HaSNPV-AaIT	Intercept *a*		−2.755	−3.223	−4.061	−6.693	−8.682			-	
	Slope *b*		0.882	0.758	0.724	0.757	0.618			-	
HaSNPV-WT	Incubation time at 28 °C		3.64±0.37	3.77±0.55	4.32±0.80	5.56±0.54	5.74±0.64			d	Chapter 2
	Feeding cessation time at 28 °C				3.64±0.37	4.84±0.61	4.94±0.53			d	
	Horizontal transmission										Chapter 5
	Parameter *k* ‡		0.058	0.127	0.153	0.190	0.229			-	
	Parameter *p* ‡		0.608	0.740	0.692	0.747	0.754			-	
HaSNPV-AaIT	Incubation time at 28 °C		2.81±0.61	2.59±0.51	3.45±0.71	4.51±0.72	4.63±0.49			d	Chapter 2
	Feeding cessation time at 28 °C				2.27±0.44	2.78±0.48	3.88±0.58			d	
	Horizontal transmission										Chapter 5
	Parameter *k* ‡		0.040	0.062	0.089	0.113	0.137			-	
	Parameter *p* ‡		0.672	0.612	0.525	0.510	0.492			-	

* Tmax: Temperature at which the developmental rate arrives maximum, Tstop: Temperature above which insect stops to develop.

$ Attrition rate at average temperature 28 °C.

† Parameters for the linear relationship between virus dose and larval mortality (see Eqn 1).

‡ Parameters for calculation of relative horizontal transmission rate (η) according equation $\eta = k \times W_0^p$, where W_0 is density of infectious larvae of each stage (number $plant^{-1}$). Values of k for L4 and L5 cadavers were calculated from extrapolation of v values of L4 and L5 cadavers from L1 to L3 cadavers.

Table 2. Fecundity (with SD) of female *H. armigera* moths at 28 ± 1 °C (Tian *et al.*, 2002).

Time*	2	3	4	5	6	7	8	9	10	Total
Number of eggs	66 ± 5	116 ± 7	168 ± 57	171 ± 61	115 ± 24	86 ± 28	58 ± 15	21 ± 10	9 ± 8	809
Hatch rate (%)	82 ± 2	94 ± 3	92 ± 5	86 ± 5	76 ± 14	65 ± 13	30 ± 7	17 ± 2	7 ± 4	

* Days after moth emergence.

Table 3. The relationship between the virus density in soil and the cumulative virus yield in cotton field after multiple applications of HaSNPV to control of *H. armigera* infestations (Anyang, Henan, China, 2002).

Items	Time				
	July 8	August 3	August 25	September 25	November 12
Virus density in 0.2 cm depth in soil ($\times 10^6$ PIBs m^{-2})*	6.6	33.5	162	2520	257
Cumulative virus yield in field ($\times 10^6$ PIBs m^{-2})**	1590	4850	7370	12900	12900
Proportion	0.004	0.007	0.022	0.195	0.020

* Virus density in soil was measured by a bioassay method following Fuxa *et al.* (2001) (X. Sun, unpublished data). Two generations of *H. armigera* infestations (at approx. 12 larvae m^{-2} soil) were treated 8 times with HaSNPV-WT at 2.4×10^8 PIBs m^{-2} soil on June 20, 24 and 28, July 20, 23 and 30, and August 22 and 26, 2002.

** Result from a simulation in accordance with the situation mentioned above.

ecdysteroid UDP-glucosyltransferase (*egt*) gene and in another recombinant (HaSNPV-AaIT), an insect-selective scorpion toxin (*Aa*IT) gene replaced the *egt* gene. L1 through L5 *H. armigera* larvae were fed with five different concentrations of polyhedra each by droplet-feeding method and further rearing on artificial diet as described in Chapter 2. There was no significant difference between the median lethal doses (LD_{50}s) of wild-type HaSNPV and two recombinant HaSNPVs, the infectivity data were therefore pooled for all three virus variants. Susceptibility of heliothine species to NPVs can be affected by the food plant (Ali *et al*., 1998). We observed that the LD_{50}s of HaSNPV against L1 and L2 *H. armigera* larvae on cotton leaves were five times higher than on artificial diet, while LD_{50}s against L3 to L5 larvae on cotton reproductive organs (squares, flowers and bolls) were about three times higher than on artificial diet (X. Sun, unpublished data). The laboratory observed data were thus fitted

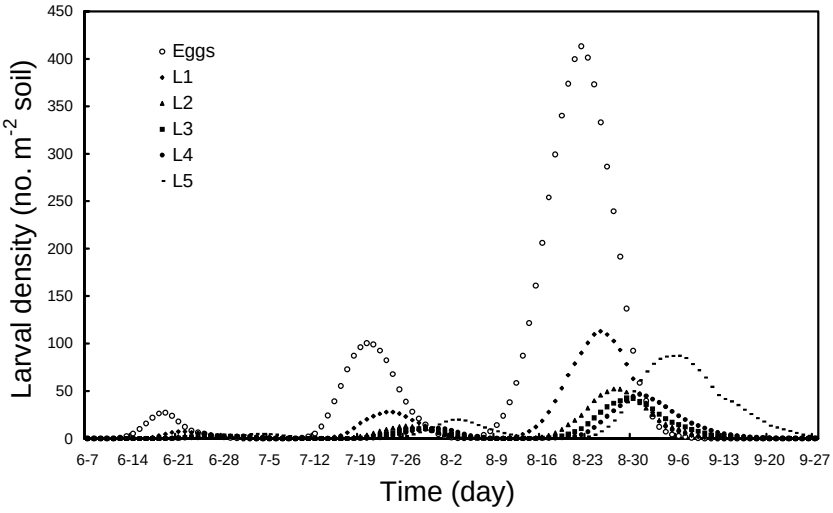

Figure 2. Simulated *H. armigera* population in cotton in Anyang, Henan. *H. armigera* populations are initialized with 0.1 moth m^{-2} soil out of cotton field on May 2. Moths immigrate into cotton fields in the middle of June. Simulated eggs and larvae cohorts in three generations in cotton fields are presented. Parameters of the development and attrition rates of *H. armigera* stages refer to Table 1.

to Eqn 1 after correcting the LD_{50}s as mentioned above, while keeping the slopes of the ln(dose) - logit(mortality) relationships unchanged. The calculated intercept (*a*) and slope (*b*) parameters for HaSNPV-WT and HaSNPV-AaIT against all 5 instars of *H. armigera* larvae are listed in Table 1.

Incubation time and feeding cessation time of HaSNPV variants

Time-mortality responses of HaSNPV-WT and HaSNPV-AaIT in the five larval instars of *H. armigera* were determined after infection by droplet-feeding (Chapter 2). Values of the mean incubation time and its standard deviation are listed in Table 1. Speed of kill by baculoviruses is affected by temperature (Van Beek and Hughes, 1998). We use the mean incubation time observed in the laboratory at 28 °C for the simulation model without adjustment, because the average temperature in Anyang, Henan, China is also about 28 °C during the bollworm infesting season (from mid-June to early September). Similarly, the feeding-cessation time of L3 to L5 larvae observed in Chapter 2 are used as measured (Table 1).

The density of virus on cotton

The density of virus on cotton is determined by the spray rate, the proportion of virus intercepted by cotton crop and by the LAI (Eqn 5). The proportion of virus intercepted

Table 4. Measured LAI of cotton variety Zhongmiansuo #35 in 2001.

Time	LAI	Time	LAI	Time	LAI
June 11	1.3	July 11	3.2	August 11	5.0
June 16	1.5	July 16	3.6	August 16	5.0
June 21	1.8	July 21	4.0	August 21	5.0
June 26	2.0	July 26	4.3	August 26	5.0
July 1	2.3	July 31	4.5	August 31	5.0
July 6	2.8	August 5	4.7	September 5	5.0

by the crop is set at 0.7. LAI for the cotton variety Zhongmiansuo #35 is derived from empirical data collected in 2002 in Anyang, Henan, China (Table 4) (L. Zhang, China Cotton Research Institute, Anyang, China, personal communication, 2004).

Polyhedron inactivation

In the cotton field, there was no significant difference in inactivation rate between the wild-type and recombinant HaSNPV variants (Chapter 4). Polyhedra had an average half-life of 0.62 day on the cotton foliage if no UV-protectant was included in the spray formulation (Chapter 4). In practice, UV-protection agents always are incorporated in the virus formulations (Tuan *et al.*, 1989; Zhang *et al.*, 1995). In this situation, a half-life of 3 d for the virus polyhedra is observed (X. Sun, unpublished data). Thus, the relative polyhedron inactivation rate used in Eqn 6 is 0.231 d^{-1}.

Feeding rate of H. armigera larvae

The areas consumed in 24 h by *H. armigera* L1 and L2 larvae were measured on leaf discs: 2.9 ± 1.6 and 4.5 ± 1.9 mm^2, respectively (X. Sun, unpublished data). L3 - L5 larvae feed on squares, flowers or bolls. On average, they consumed an area of 3.4 ± 2.0 mm^2, 12.3 ± 3.8 mm^2, 20.0 ± 6.0 mm^2 in 24 h, respectively (X. Sun, unpublished data). Feeding rates of *H. armigera* larvae are listed in Table 1.

Horizontal transmission

Horizontal transmission rate has been observed in field cages by using L1 to L3 *H. armigera* larvae inoculated with of HaSNPV-WT or HaSNPV-AaIT at two different densities (Chapter 5). The relative rate by horizontal transmission (η) was separately calculated for each instar of infectors according to following equation:

$$\eta_i = k_i \times W_{0i}^{\,p_i}, \qquad \text{(Eqn 8)}$$

where W_{0i} is density of infectious larvae of each instar (number plant^{-1}) and k_i and p_i are the fitted parameters for infectors in each instar. Infected larvae are considered infectious for horizontal transmission 3 days after infection (Chapter 5). In the cotton field, polyhedra released by cadavers are diluted by rain and dew and exposed to sunlight. Based on the observations in Chapter 5, we assume that cadavers are infectious for horizontal transmission for ten days after death.

Vertical transmission

Under field conditions, where bollworm eggs are exposed to sunlight and other environmental factors, vertical transmission (primarily transovum) would be lower than measured by collecting the virus-treated larvae in the field and rearing them in the laboratory (that is 12.4% for HaSNPV-WT and 8.6% for HaSNPV-AaIT) (Chapter 5). Assuming one-fourth of the virus-contaminated eggs is containing infectious virus at the moment of eggs hatch, the vertical transmission rate of HaSNPV-WT and HaSNPV-AaIT is estimated as 3% and 2%, respectively.

Virus movement between the soil and crop compartments

Cumulative virus yield produced by the virus killed cadavers depends on the density of the cadavers of each instar per m^2 field area and the average virus yield per cadaver at the stage they die. Mean virus yields of HaSNPV-WT and HaSNPV-AaIT present in cadavers of 5 instars are listed in Table 3 of Chapter 3. Proportions of infectious virus m^{-2} soil down to 0.2 cm depth out of the accumulated virus in the field (including cadaver released virus and sprayed virus) are listed in Table 3 (X. Sun, unpublished data).

MODEL INITIALIZATION AND SENSITIVITY ANALYSIS

Scenario studies apply to the 2nd, 3rd and 4th generation of *H. armigera,* in June, July and August, respectively, in Anyang, Henan, China (36°5'N, 114°21'E). These generations occur on cotton. Individuals of the first generation, which occurs mainly on other crops, are not considered as variables in the simulations. Cotton plant density is set at 6 m^{-2}. During the 2nd, 3rd and 4th generation, the model takes into account immigration of adults from outside the field. The temporal profile of immigration is based on a simulation of reproductive output from a first generation population, starting at an initial density of 0.1 moth m^{-2} soil on May 2. The number of the immigrant moths of the 2nd, 3rd and 4th generation is thereby set at a value that could produce in each generation peak densities of 12 larvae m^{-2} soil if no control measures were applied, and assuming there is no carry-over from previous generations in the

same field. This larval density of 12 larvae m^{-2} soil is referred to as the potential population density to be controlled with HaSNPV.

A sensitivity analysis of a selection of parameters is presented in Table 5. Simulations were conducted using standard parameter values of HaSNPV-WT (Table 1) and all three *H. armigera* infestations with a potential population density of 12 larvae m^{-2} soil. This corresponds with the observations in the field (Chapter 2). The cotton was treated with virus 3 times at 1.2×10^8 PIBs m^{-2} soil in June, 3 times at 2.4×10^8 PIBs m^{-2} soil in July, and 4 times at 3.0×10^8 PIBs m^{-2} soil in August. The first virus application in each generation takes place at the beginning of egg hatch (i.e. on June 16, July 16 and August 16) with 3 days intervals between sprays. The simulation is terminated on September 30 after all larvae have pupated. The following model parameters are included in the sensitivity analysis: infectivity (LD_{50}s), incubation time (mean lethal time and mean feeding cessation time of infected larvae), half-life of polyhedra on cotton, horizontal and vertical transmission rate, and *H. armigera* attrition rates. Effects of deviations of 10% of these parameter values on the total

Table 5. Sensitivity analysis of a selection of parameters on the number of fruit injured by bollworm, the peak virus density in soil and the number of surviving pupae at the end of the season.

Parameter	Deviation	Deviation in injured fruits (%)	Deviation in virus density (%)	Deviation in surviving pupae (%)
Incubation time*	+ 10%	+ 8.0	+ 2.5	+ 4.7
	– 10%	– 7.1	– 2.5	– 5.8
Infectivity (LD_{50} values)	+ 10%	+ 6.2	+ 5.2	+ 4.7
	– 10%	– 6.5	– 5.4	– 5.1
Half-life of polyhedra	+ 10%	– 8.3	– 8.4	– 11.6
	– 10%	+ 9.7	+ 8.1	+ 14.5
η**	+ 10%	– 3.4	+ 0.1	– 11.9
	– 10%	+ 3.7	– 0.3	+ 14.0
Vertical transmission rate	+ 10%	– 0.1	– 0.1	– 0.1
	– 10%	+ 0.1	+ 0.1	+ 0.1
H. armigera attrition rate	+ 10%	– 12.0	– 3.6	– 3.9
	– 10%	+ 13.4	+ 3.9	+ 2.9

* Both mean incubation time and feeding cessation time are changed.

** η: relative horizontal transmission rate.

number of fruits injured by *H. armigera* larvae, the peak virus density in soil (on September 25) and the number of surviving pupae in the end of September are determined (Table 5).

Variation in parameter values of virus infectivity, incubation time, half-life on cotton, relative horizontal transmission rate and *H. armigera* attrition rate had different impacts on the three output variables. Ten percent deviations of mean incubation time and feeding cessation time caused approx. 7.5% change in the number of injured fruits. Change of 10% of the LD_{50} values of the virus caused 6.4% variation of the number of injured fruits. The number of surviving pupae was more sensitive to relative horizontal transmission rate (av. 13% variation). Half-life of virus on cotton was the most influential parameter both to the number of injured fruits and to the number of surviving pupae (9% and 13% variation, respectively). Deviations in the vertical transmission rate had a minimal impact on all of these three output variables (about 0.1%).

SCENARIO STUDIES

Application strategies of HaSNPV variants

Dosage

Dosages of HaSNPV applications vary in June, July and August to give similar virus density on cotton leaves. During the periods of application of viruses in June, July and August, the average LAI is 2.0, 4.0 and 5.0, respectively. Thus, 5 dosage sets are used to evaluate the control efficacy of wild-type and recombinant HaSNPV variants (HaSNPV-WT and HaSNPV-AaIT) against 3 generations of *H. armigera* infestations at various densities in cotton (Table 6).

For both HaSNPV-WT and HaSNPV-AaIT, three times at 1.2×10^8 PIBs m^{-2} soil in June, 3 times at 2.4×10^8 PIBs m^{-2} soil in July, and 4 times at 3.0×10^8 PIBs m^{-2} soil in August (Dosage #3) gave satisfactory control of bollworm (e.g. total number of injured fruits < 50 m^{-2} soil, Sheng *et al.*, 2002) when the potential population density is less than 12 larvae m^{-2} soil (Figures 3A and B). However, if the potential pest density is extremely high (e.g. > 30 larvae m^{-2} soil), higher dosages/more sprays or other control methods (e.g. chemical insecticides) are needed. At the same dosage and spray frequency, HaSNPV-AaIT always gives a better protection of cotton fruits from bollworm than HaSNPV-WT. The number of injured fruits increases less than linearly with the density of pest population, as horizontal transmission rate depends on the density of infected or virus-killed larvae. Thus, at higher pest infestation level, there is a greater contribution to total mortality from horizontal transmission. Furthermore, the

Table 6. Dosage sets used to evaluate the control efficacy of HaSNPV on three *H. armigre* generations at various densities in cotton.

Dosage #	Dosage ($\times 10^8$ PIBs m^{-2} soil)		
	June	July	August
1	0.6	1.2	1.50
2	0.9	1.8	2.25
3	1.2	2.4	3.00
4	1.5	3.0	3.75
5	1.8	3.6	4.50

number of surviving pupae at the end of the season does not increase when the pest population increases (Figures 3C and D), due to the higher transmission rate at higher density of infectors (Chapter 5).

Timing of virus applications

The optimal timing of applications of HaSNPV-WT and HaSNPV-AaIT are evaluated for 3 generations of *H. armigera* infestations in cotton. The potential population density of 3 generations are all set as 12 larvae m^{-2} soil, while the stage structures of the 3 generations are kept the same as in Figure 2. Virus applications take place in June (1.2×10^8 PIBs m^{-2} soil, 3 times), July (2.4×10^8 PIBs m^{-2} soil, 3 times) and August (3.0×10^8 PIBs m^{-2} soil, 4 times). At first, the optimal time of the first spray and the spray time intervals for each infestation are studied by keeping the spray regimes on the other 2 generations fixed at an expected optimal spray regime. The results indicate that in all 3 generations, spraying at an early time, soon after egg deposition, with 3 days intervals (spraying once per 4 days) provides optimal crop protection. The overall effect of different moments and time intervals of applications of HaSNPV variants on the 3 generations of *H. armigera* on the total number of injured fruits, the peak virus yield in soil and the number of surviving pupae at the end of the season are shown in Figure 4. The optimal days for the first spray are June 16, July 16 and August 16 against the 2nd, 3rd and 4th generation of bollworm infestation, respectively. On average, application of HaSNPV-AaIT reduces fruit damage by 24% from the values obtained when applying HaSNPV-WT (Figures 4A and B). However, control with HaSNPV-AaIT results in about 10% greater pupal survival at the end of the cotton season than the case of control with HaSNPV-WT (Figures 4C and D). This is because the horizontal transmission rate of the recombinant is significantly lower than of the wild-type virus (Zhou *et al*., 2005). The peak density of HaSNPV-AaIT

polyhera residing in soil (on September 25) is about 48% lower than that of HaSNPV-WT during the cotton growing season (Figures 4E and F), mainly due to the lower virus yield per cadaver for the recombinant virus.

Effect of inactivation rate of polyhera on the control efficacy of HaSNPV variants

Under field conditions, a long residence time of baculoviruses on the crop is essential for effective control. The effect of half-time of HaSNPV formulations and time intervals between applications on the number of injured fruits is shown in Figure 5. A total 10 applications (3 times at 1.2×10^8 PIBs m^{-2} soil in June, 3 times at 2.4×10^8 PIBs m^{-2} soil in July, and 4 times at 3.0×10^8 PIBs m^{-2} soil in August) are used to control

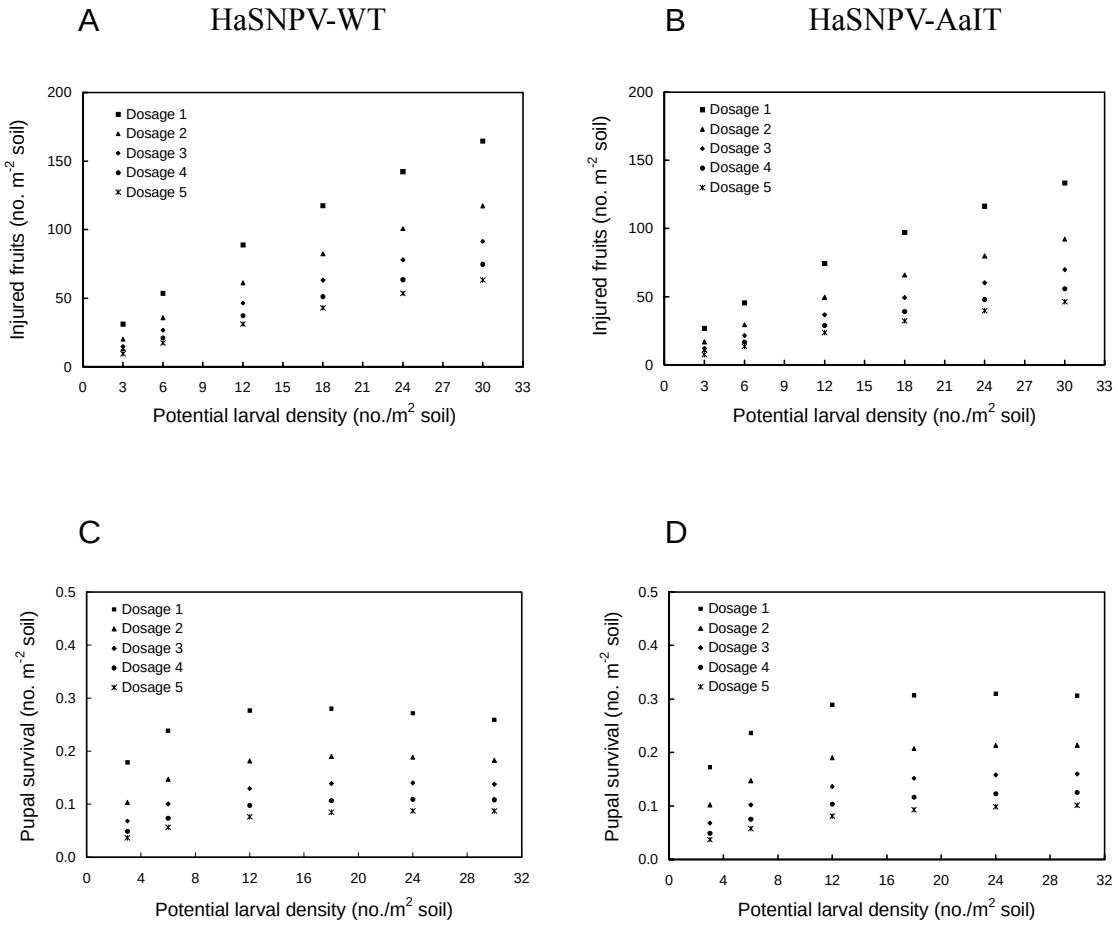

Figure 3. Simulation of control of *H. armigera* larvae at different densities with wild-type and recombinant HaSNPV variants. Three generations of *H. armigera* with same potential density are sprayed with viruses 3 times in June and July, 4 times in August at 5 dosage sets (see Table 6). The 1st sprays during each generation are made on June 16, July 16 and August 16, respectively, and there are intervals of 3 days between sprays. Half-life of PIBs on cotton is 3 days.

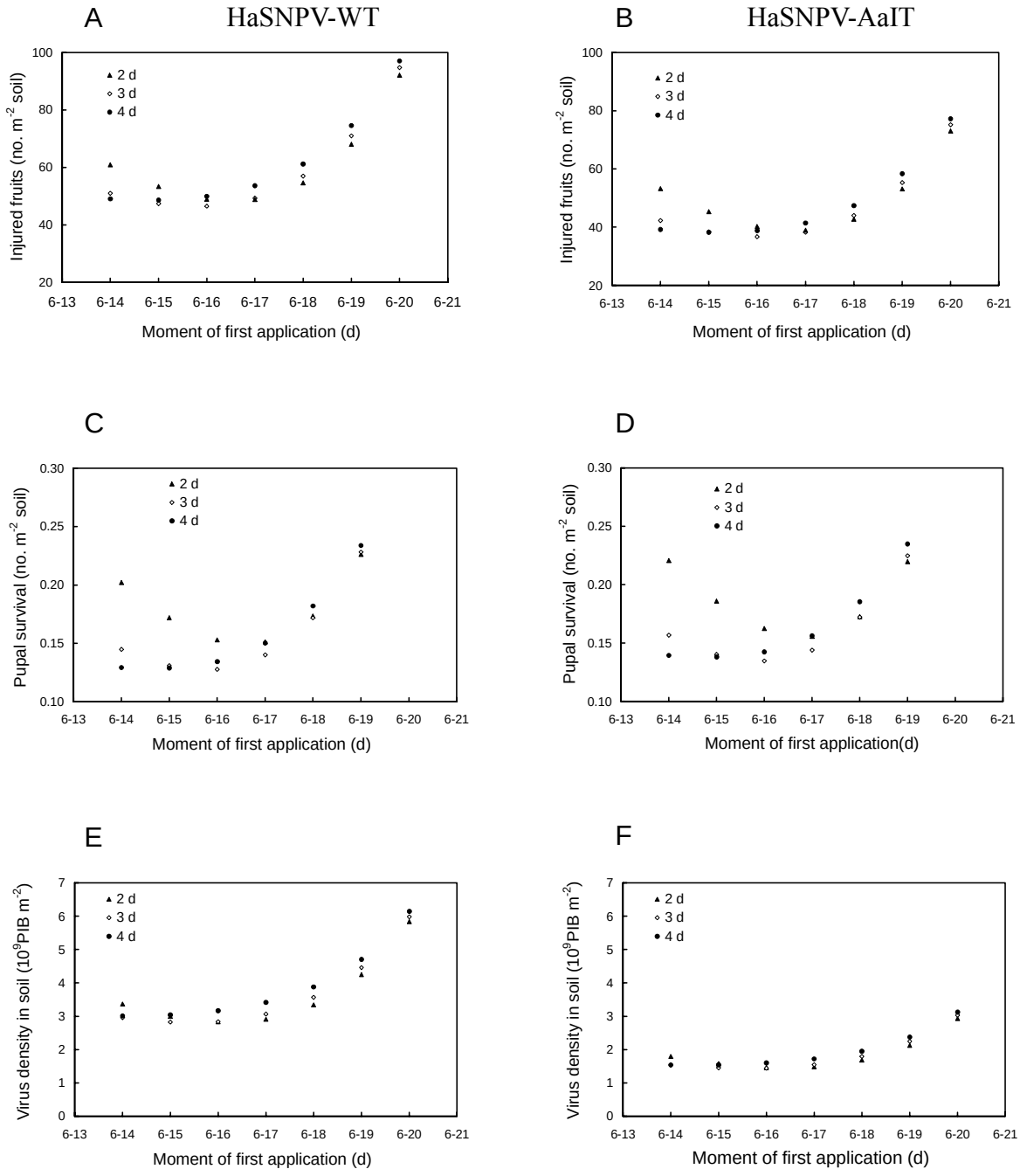

Figure 4. Simulation of the optimal spraying moment and time intervals for controlling *H. armigera* with wild-type and recombinant HaSNPV. Three generations of *H. armigera* at 12 larvae m^{-2} soil are sprayed 3 times at 1.2×10^8 PIBs m^{-2} in June, 3 times at 2.4×10^8 PIBs m^{-2} in July, and 4 times at 3.0×10^8 PIBs m^{-2} in August. First sprays are started on June 14, July 14 and August 14, respectively. Three spray intervals (2 d, 3 d and 4 d) are tested with subsequent moments of the first sprays. Half-life of PIBS is 3 days.

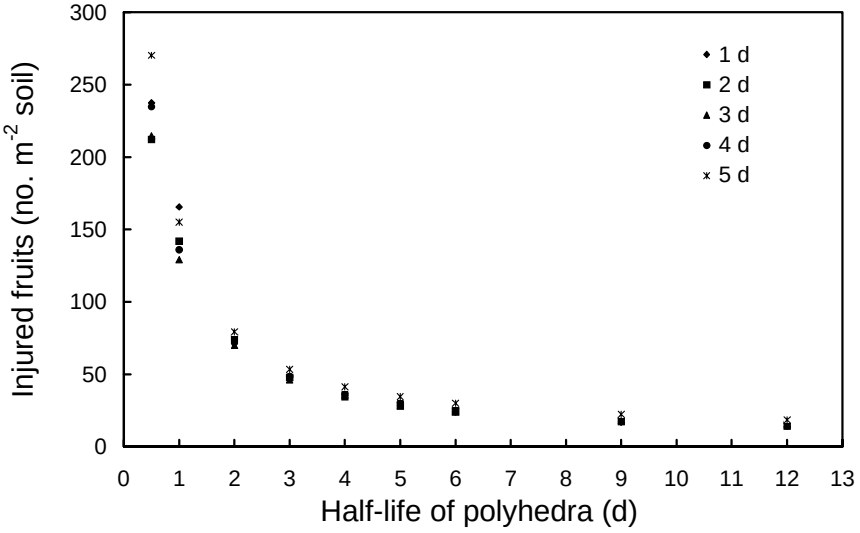

Figure 5. Simulation of the effect of half-life values of polyhedra on control efficay. Three generations of *H. armigera* with a potential density of 12 larvae m^{-2} soil are sprayed 3 times at 1.2×10^8 PIBs m^{-2} in June, 3 times at 2.4×10^8 PIBs m^{-2} in July, and 4 times at 3.0×10^8 PIBs m^{-2} in August. Five spray intervals (1 d, 2 d, 3 d, 4 d and 5 d) are tested with different moments of the first sprays. The number of injured fruits of the optimal timing of the initial applications in each generation for each combination of half-life and time interval is showed.

three generations of bollworm with a potential density of 12 larvae m^{-2} soil. Optimal timing of virus applications is tested for nine different half-lifes of the HaSNPV formulation. When half-time of viruses is short (i.e. < 3 d), both the timing of the initial application in each generation as well as the time intervals between applications have strong effects on control efficacy. The number of injured fruits decreases strongly with a longer half-life. A HaSNPV formulation exhibiting a half-life of 3 days may provide a satisfactory control of a common density of *H. armigera* infestation (e.g. 12 larvae m^{-2} soil). A further extension of the half-life results only in a marginal increase in control efficacy. The effect of inactivation rate on control efficacy is similar for wild-type and recombinant viruses.

Effect of vertical transmission on the control efficacy of HaSNPV

Vertical transmission may be an important route for a baculovirus to maintain itself in host populations. Different vertical transmission rates of HaSNPV are compared with respect to their effect on the control of cotton bollworm. Simulations are done for a potential density of 12 larvae m^{-2} soil in June and there is no further immigration of moths in July and August. The 2nd generation of infestation (June) is treated 2 times with HaSNPV at a rate of 1.2×10^8 PIBs m^{-2} on 16 and 20 June. Here, the assumption

is made that the virus is not transmitted from the 3rd to the 4th generation because the 3rd generation is subject only to vertical and horizontal transmission, and there is no evidence that these infection routes can lead to sublethal infection and further trans-generational vertical transfer of virus. When the vertical transmission rate is less than 10%, it has a limited effect on either the survival of *H. armigera* larvae or on the number of injured fruits (Figures 6A and B). If there would be immigrations of *H. armigera* in July and August, the effect of vertical transmission would be even smaller either with or without virus treatments (data not shown). Only at very high vertical transmission rates (e.g. > 25%) would *H. armigera* populations and damage be reduced. However, such high rates of vertical transmission are beyond the range

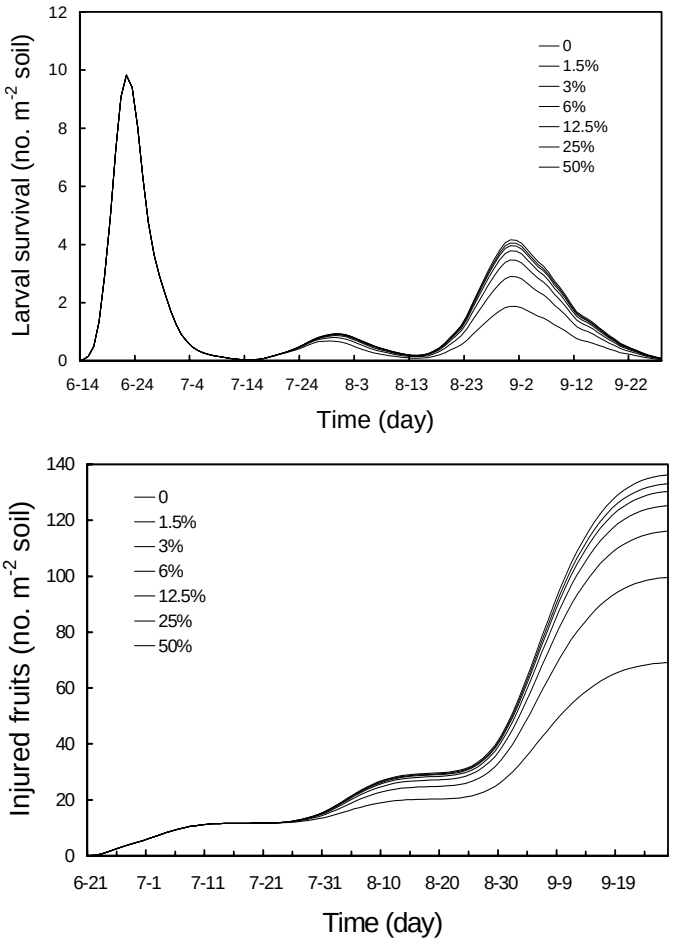

Figure 6. Simulation of the effects of vertical transmission rates of HaSNPV on the larval survival and number of injured fruits. Simulations are initiated with a potential density of 12 larvae m^{-2} soil in June and there is no immigration of moths in July and August. The 2nd generation of infestation is treated 2 times with HaSNPV-WT at 1.2×10^8 PIBs m^{-2} soil at June 16 and 20. For both A and B, from top to bottom, vertical transmission rates are 0, 1.5%, 3%, 6%, 12.5%, 25% and 50%, respectively.

measured in experiments (Chapter 5). Thus, although vertical transmission is – in principle – an important process for maintenance and persistence of baculovirus in insect populations, the rates of vertical transmission of HaSNPV in *H. armigera* in cotton are not high enough to ensure persistence.

Effect of flux rate of virus from soil to plant and the persistence of virus in soil

Movement of polyhedra from the soil reservoir to the crop is another route of maintenance of virus in the host populations. Different flux rates of HaSNPV variants from soil to plants as characterized by a proportionality factor between the virus density on leaves and the amount of virus in the soil are tested for the control efficacy against bollworm. Potential densities of three generations of *H. armigera* infestations in cotton are all 12 larvae m^{-2} soil. Cotton is sprayed 3 times in June and July and 4 times in August with HaSNPV-WT or HaSNPV-AaIT at the moderate dosages (Dosage #3) at optimal moments and with 3 d intervals. Proportionality factors ranging from 10^{-6} to 10^{-3} have a very limited effect on the control efficacy of HaSNPV against *H. armigera* infestations (Figure 7A). However, when this factor increases to values of 10^{-2} or 10^{-1}, the virus reservoir in the soil may strongly reduce crop injury (Figure 7A). However, when this factor increases to values of 10^{-2} or 10^{-1}, the virus reservoir in the soil may strongly reduce crop injury (Figure 7A). However, no evidence is (yet) able to suggest that such high transfers from soil to crop can be practically achieved (Fuxa *et al*., 2001).

In the situation mentioned above, for all 6 proportionality factors of virus density in soil and plants, the maximum virus density in soil down to 0.2 cm depth is similar (2.83×10^9 and 1.44×10^9 PIBs m^{-2} for HaSNPV-WT and HaSNPV-AaIT, respectively). In the next season, the residual activity of the polyhedra in soil is about 1% (Fuxa and Richter, 2001b; X. Sun, unpublished data). Even if the flux rate from soil to the plants is assumed to be as high as 10^{-3}, the infection chance of 1st instar larvae at the beginning of the next season is only about 0.6% and 0.4% for the cases of HaSNPV-WT and HaSNPV-AaIT, respectively (Figure 7B). When this proportionality factor increases to 10^{-2} or 10^{-1}, the virus reservoir in the soil is sufficient to affect the control of bollworm in the next season (Figure 7B).

DISCUSSION

In this chapter, we described a process-based model to study the epidemiology of wild-type and genetically modified HaSNPV variants in the cotton-bollworm system in the field. The model integrates the major biological characteristics, such as virus infectivity, incubation time, inactivation, transmission, virus production and the

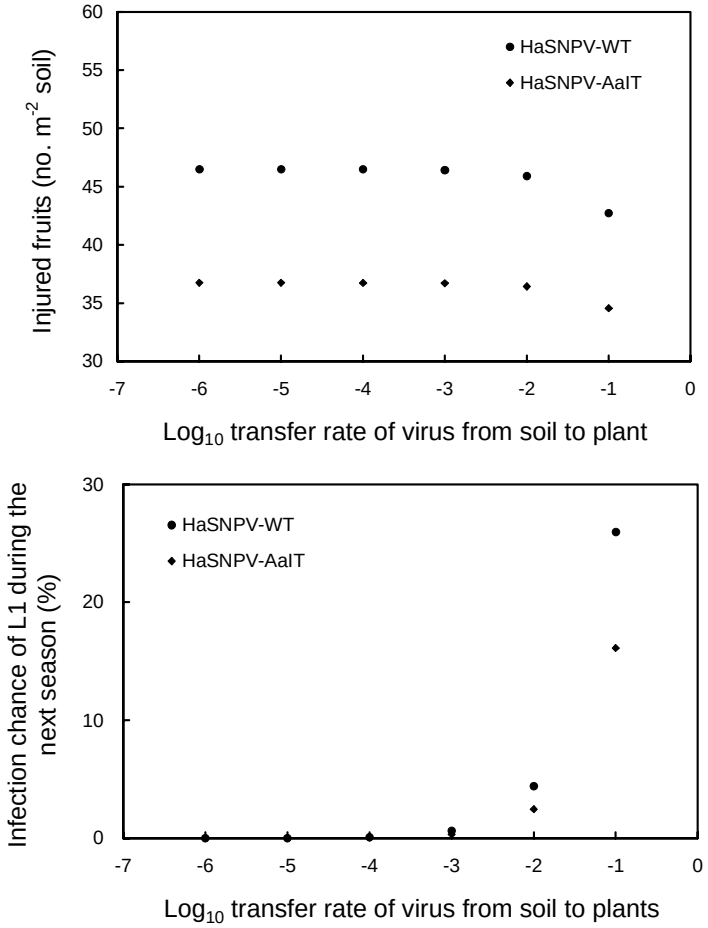

Figure 7. Simulation of the effects of the transfer of virus from soil to plants on the control efficacy against *H. armigera* larvae (A) and the infection chance of L1 *H. armigera* in the next season (B). Potential densities of 3 generations of *H. armigera* infestations are all 12 larvae m^{-2} soil. Cotton is sprayed 3 times at 1.2×10^8 PIBs m^{-2} in June, 3 times at 2.4×10^8 PIBs m^{-2} in July, and 4 times at 3.0×10^8 PIBs m^{-2} in August with 3 d intervals.

movement of the virus between soil and plants. The effect of the application regimes of the wild-type and recombinant baculoviruses on the control efficacy against *H. armigera* and the persistence of HaSNPV variants in the cotton ecosystem have been assessed.

The model used in this study is a numerically solved, comprehensive process-based model. This kind of model contains more detailed biological information than analytical models (De Kraker, 1996; Van Roermund, 1997). The usefulness of such a modeling approach has been demonstrated for *Spodoptera exigua* MNPV (SeMNPV) and *Autographa californica* MNPV (AcMNPV) in a greenhouse setting, where the control of beet armyworm with the baculoviruses in greenhouse crops has been validated (Bianchi *et al*., 2001; 2002a, b, c). All of the above models deal with foliage-

feeding insects. Because fruit feeders, such as *H. armigera*, directly damage the harvested product, application of bacoluviruses to control such direct pests must be very precise. So far, no model has been developed to study the complex relationship between application of baculoviruses and the control efficacy on fruit-feeding pests.

H. armigera development, dose-mortality and time-mortality relationships between *H. armigera* and HaSNPV variants are simulated using the boxcar train technique (Goudriaan and Van Roermund, 1993) and it provides a satisfactory description of the development processes. The boxcar formalism is among others used to keep track of the number of PIBs consumed by caterpillars in a certain boxcar. The number of PIBs consumed per caterpillar is calculated as an input to the calculation of the infection chance per newly ingested PIB. According to the logistic infection model, infection probability decreases as more PIBs are ingested. The simulation results are in line with calculations according to the equations (Eqns 1 and 2). This is logical because the model is based on the equation. To our knowledge, this adaptation of the logistic dose-response model to simulate a dynamic process, is new and has not been previously used.

The translation of the dose-response trials in the laboratory to the dynamic approach in the simulation model requires several assumptions to be made. One of those is that the dose-response of *H. armigera* to HaSNPV is the same, whether the dose is administered all at once in a laboratory trial (droplet feeding, Van Beek *et al*., 1998), or where it is accumulated over time as larvae feed on virus contaminated plant material in the field. We have not experimentally tested this assumption. A further assumption is that the dose response in a certain stage is independent of the virus exposure history of the insects in preceding stages. In dose-response trials, larvae have not been previously exposed to virus. However, in model calculations, larvae surviving exposure to virus in a certain instar may be exposed again in the subsequent instar. This is likely to occur at dosages lower than LD_{50}, remaining on the plant foliage after inactivation has reduced the number of infectious PIBs, following a spray. It is possible that exposure to virus in a previous instar may affect the dose-response in a later instar, for instance due to carry over of infectious virus to the next stage, or due to selection of the most resistant genotypes from a population of exposed insects. The assumption that there is no after-effect of survival of virus exposure therefore needs further testing.

Some of the parameters (such as virus infectivity, horizontal transmission, inactivation, persistence, etc.) have been validated by the field experiments. However, some parameters (e.g., vertical transmission, attrition rates) are very difficult to quantify under field conditions. Thus, this model is preferably used to address general strategic questions on spray regimes, dosages, etc., but not for prediction of control

success in very specific cases, taking into account of weather factors, such as rain and solar irradiation, or the effects of natural enemies. In the current version of this model, the total number of cotton fruits injured by bollworm is used as an index of control efficacy of the viruses. However, the final objective of application of the virus is to protect cotton products from damage from bollworm. In some periods of cotton growing, the cotton plant has a capacity to make up for square shedding. However, in some periods, such as the blooming period, damage of bolls will cause a direct reduction of the final lint production. Linkage with a physiologically-based mechanistic potential simulation model for cotton, such as COTCROS (Zhang *et al*., in preparation), will make the prediction of cotton production with different spray regimes and viruses possible.

The model built here can be used for a variety of purposes. One application of the model is to assist in the determination of effective spray regimes for HaSNPV formulations to control *H. armigera*. Simulations indicate that the dosages of 1.2×10^8, 2.4×10^8 and 3.0×10^8 PIBs m^{-2} or higher in June, July and August provide good control of a common density of *H. armigera* infestations, provided that the application is properly timed (Figures 3 and 4). This is in agreement with the results of field experiments, where similar dosages of HaSNPV formulations provided 80-90% mortality of *H. armigera* populations in the field experiments literature (Zhang *et al*., 1995; Sun *et al*., 2002b; Chapter 2). The time of application of NPVs is critical for a fruit feeder, such as *H. armigera*, that feeds in protected locations because the virus must be ingested while the insect feeds on the surface of the crop before it burrows into the fruits. Therefore HaSNPV sprays should always be applied as soon as possible after egg hatch.

Another application of this model is to evaluate the commercial potential of the recombinant viruses. Simulation results indicate that recombinant virus, with a lower mean time to kill or feeding cessation, is better to protect the damage of fruits from the bollworm than wild-type viruses. Further studies indicate that using 'faster' recombinant viruses with enhanced speed of action in combination with enhanced infectivity would dramatically improve the control efficacy (Figure 8). The model is useful to assist in the evaluation of the commercial potential of a new recombinant virus by incorporating economic parameters, such as production cost and determining the difference in economic result between controlling pests with a recombinant virus or with the wild-type virus.

Furthermore, the model provides insight in the ecological behaviour of baculovirus after releasing to control the pest insects. For example, the persistence of wild-type and recombinant variants in the fields can be anticipated. This parameter is important for the assessment of the risk of release of a genetically modified baculovirus as

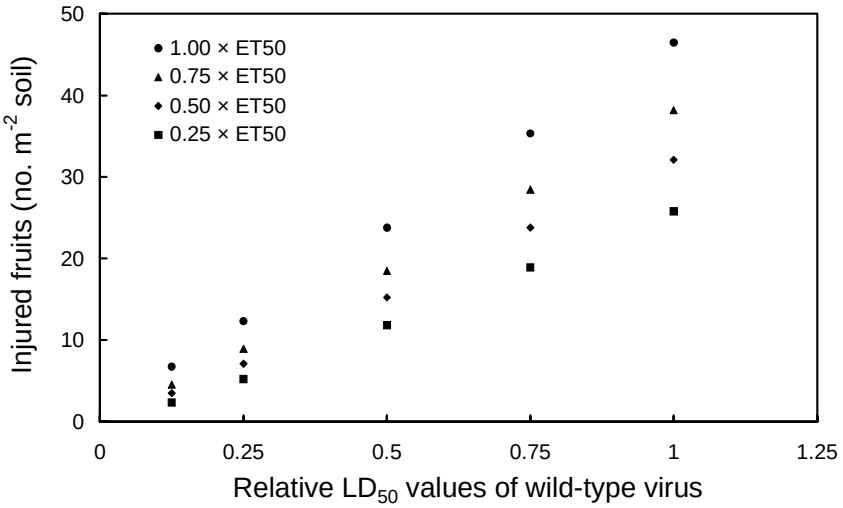

Figure 8. Control efficacy of genetically modified HaSNPV with enhanced speed of action in combination with enhanced infectivity. Enhanced speed of action is expressed by a reduced ET_{50} (both median lethal time and median feeding cessation time are reduced by same factor). Other parameters, such as inactivation rate, transmission rate and virus yield, of recombinants with different speed of action are assumed equal to those of HaSNPV-AaIT.

bioinsecticide. The simulation results and empirical data indicate that a recombinant HaSNPV expressing a selective neurotoxin has less persistence in the cotton ecosystem. Simulation results indicate that it is not possible to use either the wild-type and recombinant HaSNPV variants to control its host pests by an inoculative application or introduction–establishment approach. For recombinant HaSNPV, this is an *a priori* objective, i.e. to act as a dead-end virus.

The results in this study demonstrate that HaSNPV can be applied to control of *H. armigera* infestation as a microbial insecticide and a precise spray regime is very important for the successful control. Recombinant HaSNPV variants with faster speed of kill truly provide better control of *H. armigera* infestations and have a potential to be commercially applied.

Appendix 1

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TITLE Process-based model of control of bollworm with HaSNPV

* Authors: Xiulian Sun and Wopke van der Werf
* 16 January 2005

* This model is used to study the control efficacy of wild-type and
* recombinant HaSNPV and to anticipate their environmental fate in
* cotton. The model includes H. armigera development and virus infection
* processes (from sprayed virus, horizontal and vertical transmission,
* and movement of virus between the crop and soil reservoir).
* Cumulative number of injured fruits, virus density in soil and number
* of surviving pupae at the end of season are used as outputs.

* Abbreviations for the different bollworm stages
* EG = eggs
* L1 = first instar larvae
* L2 = second instar larvae
* L3 = third instar larvae
* L4 = forth instar larvae
* L5 = fifth instar larvae
* PU = pupae
* MO = moths (both female and male)

* H. armigera population and virus density are expressed per m2
* of soil area. Plant density is 6/m2.

* Two subroutines are used, one for bookkeeping of larvae and
* ingested virus in boxcar trains (see below), and a second for
* bookkeeping of virus density on the canopy as a result of virus sprays
* and inactivation.

  DEFINE_CALL BOXRAT(INTEGER_INPUT, INPUT, INPUT, INPUT, INPUT_ARRAY,...
                  OUTPUT_ARRAY, OUTPUT)
  DEFINE_CALL VIRUS(OUTPUT, INPUT, INTEGER_INPUT, INPUT_ARRAY,...
                 INPUT_ARRAY, INPUT, INPUT,INPUT)

* Declaration initial number of individuals in boxcars (#/m2)
   ARRAY MOI(1:NM), EGGI(1:N0)

*** Virus spray array
* Spray rate (PIB/m2 soil area)
   ARRAY SPRAYR(1:NSPRAY)
* Virus density on cotton resulting from one spray (PIB/mm2 leaf area)
   ARRAY VDENS(1:NSPRAY)
* Spray day
   ARRAY SPRDAY(1:NSPRAY)

* Boxcar trains: Insect dynamics is modeled by keeping track of numbers
* in state variables that represent different states of development and
* virus infection (Leffelaar, 1993).

*** Arrays of healthy insect boxcar trains
* Number of healthy individuals in boxcars (#/m2 soil)
   ARRAY AEGG(1:N0),  AL1(1:N1),  AL2(1:N2),  AL3(1:N3),  AL4(1:N4), ...
         AL5(1:N5),   APU(1:NP),  AMO(1:NM)
* Rate of change of individuals in boxcars (#/m2 soil/day)
   ARRAY REGG(1:N0),  RL1(1:N1),  RL2(1:N2),  RL3(1:N3),  RL4(1:N4), ...
         RL5(1:N5),   RPU(1:NP),  RMO(1:NM)
* Netflow rates in of individuals boxcars (#/m2 soil/day)
   ARRAY NTEGG(1:N0), NTL1(1:N1), NTL2(1:N2), NTL3(1:N3), NTL4(1:N4),...
         NTL5(1:N5),  NTPU(1:NP), NTMO(1:NM)

*** Virus uptake arrays
* The boxcar concept is used to keep track of the number of PIBs
* consumed by caterpillars in a certain boxcar. The number of PIBs
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* consumed per caterpillar is calculated as an input to the calculation
* of the infection chance per newly ingested PIB. According to the
* logistic infection model, infection probability decreases as more PIBs
* are ingested.

* In the arrays "ADOS**", the total amount of PIBs ingested by the
* corresponding developmental cohort of larvae is stored.

* Netflow rate of ingested PIBs in boxcars (# PIB/m2 soil/day)
  ARRAY NTVL1(1:N1), NTVL2(1:N2), NTVL3(1:N3), NTVL4(1:N4), NTVL5(1:N5)
* Rate of change of ingested PIBs in boxcars (# PIB/m2 soil/day)
  ARRAY RDOSL1(1:N1),RDOSL2(1:N2),RDOSL3(1:N3),RDOSL4(1:N4),RDOSL5(1:N5)
* Ingested virus dose in each boxcar (# PIB/m2 soil)
  ARRAY ADOSL1(1:N1),ADOSL2(1:N2),ADOSL3(1:N3),ADOSL4(1:N4),ADOSL5(1:N5)
* Accumulated virus dose per larva(PIB/larva)
  ARRAY DOSEL1(1:N1),DOSEL2(1:N2),DOSEL3(1:N3),DOSEL4(1:N4),DOSEL5(1:N5)
* Relative infection rate (/day)
  ARRAY RIRL1(1:N1), RIRL2(1:N2), RIRL3(1:N3), RIRL4(1:N4), RIRL5(1:N5)
* Infection rate of larvae (#/m2 soil/day)
  ARRAY INFRL1(1:N1),INFRL2(1:N2),INFRL3(1:N3),INFRL4(1:N4),INFRL5(1:N5)

* Infection rate due to sprayed virus (#/m2 soil/day)
  ARRAY INFSL1(1:N1),INFSL2(1:N2),INFSL3(1:N3),INFSL4(1:N4),INFSL5(1:N5)
* Average dose that newly infected larvae consumed (PIB/larva)
  ARRAY DOSIL1(1:N1),DOSIL2(1:N2),DOSIL3(1:N3),DOSIL4(1:N4),DOSIL5(1:N5)

* (average dose consumed by newly infected larvae is used to calculate
* whether they will die due to virus infection in the same or in later
* stages.)

*** Arrays of infected larval boxcars
* Number of infected larvae in the boxcar (#/m2)
   ARRAY AIL1(1:N1I), AIL2(1:N2I), AIL3(1:N3I), AIL4(1:N4I), AIL5(1:N5I)
* Rate of change of infected larvae in boxcars (#/m2/day)
   ARRAY RIL1(1:N1I), RIL2(1:N2I), RIL3(1:N3I), RIL4(1:N4I), RIL5(1:N5I)
* Netflow rate of infected larvae in boxcars (#/m2/day)
   ARRAY NTIL1(1:N1I),NTIL2(1:N2I),NTIL3(1:N3I),NTIL4(1:N4I), ...
         NTIL5(1:N5I)

* Sublethally infected moths can lay virus-contaminated eggs, which
* cause virus infection in L1 (vertical transmission)

*** Arrays of virus-contaminated moths and eggs
* Number of individuals in boxcars (#/m2 soil)
   ARRAY AVMO(1:NM), AVEG(1:N0)
* Rate of change of individuals in boxcars (#/m2 soil/day)
   ARRAY RVMO(1:NM), RVEG(1:N0)
* Netflow rate of individuals in boxcars (#/m2 soil/day)
   ARRAY NTVMO(1:NM),NTVEG(1:N0)

*** Arrays of the 'feeding' larvae boxcar trains
* Infected larvae stop feeding before they die. To calculate fruit
* damage, separate boxcars are used to simulate the number of infected
* larvae that are still feeding. "FC" in the variable names stands for
* "feeding cessation".
* Number of feeding larvae in the boxcar (#/m2 soil)
   ARRAY AFCL3(1:N3FC), AFCL4(1:N4FC), AFCL5(1:N5FC)
* Rate of change of feeding larvae in boxcars (#/m2 soil/day)
   ARRAY RFCL3(1:N3FC), RFCL4(1:N4FC), RFCL5(1:N5FC)
* Netflow rate of feeding larvae in boxcars (#/m2 soil/day)
   ARRAY NTFCL3(1:N3FC), NTFCL4(1:N4FC), NTFCL5(1:N5FC)

* Cadaver density and residence time on cotton is calculated for
* determination of horizontal transmission rate

*** Arrays for cadaver boxcar trains
* Number of cadavers in the boxcars (#/m2 soil)
   ARRAY ACL1(1:NC),  ACL2(1:NC), ACL3(1:NC), ACL4(1:NC), ACL5(1:NC)
```

```
* Rate of change of cadavers in boxcars (#/m2 soil/day)
   ARRAY RCL1(1:NC),  RCL2(1:NC), RCL3(1:NC), RCL4(1:NC), RCL5(1:NC)
* Netflow rate of cadavers in boxcars (#/m2 soil/day)
   ARRAY NTCL1(1:NC), NTCL2(1:NC), NTCL3(1:NC), NTCL4(1:NC), NTCL5(1:NC)

INITIAL

* Initial condition: # in all stages of bollworm (#/m2 soil)
   INCON MOI(1:NM) =0.
   INCON EGGI(1:N0)=0.

* Number of boxcars is determined by the developmental time and its
* variability of individuals in healthy or infected boxcar trains.

   ARRAY_SIZE N0=68; N1=66; N2=30; N3=32; N4=52; N5=48; NP=48; NM=50
   ARRAY_SIZE N1I=45; N2I=40; N3I=32; N4I=40; N5I=40
   ARRAY_SIZE N3FC=58; N4FC=45; N5FC=59; NC=40
   ARRAY_SIZE NSPRAY = 13
   INCON ZERO = 0.

* Feeding rate of larval stages (mm2 leaf/day)
   PARAM FEEDL1=2.9; FEEDL2=4.5; FEEDL3=3.8; FEEDL4=12.3; FEEDL5=20.

** HaSNPV-WT parameters
* Developmental rate of infected larvae (/day)
   PARAM DVIL1=0.2746;DVIL2=0.2649;DVIL3=0.2317;DVIL4=0.1798;  ...
         DVIL5=0.1743

* Development rate for L3 to L5 to stop feeding (/day),
   PARAM DVFCL3=0.2750; DVFCL4=0.2068; DVFCL5=0.2026

* Horizontal transmission (HT) parameters for the equation:
* RHTR = HTKi*(W0i)**HTPi. (Eqn 6, Chapter 6)
* W0i: density of infectious larvae (#/plant)
   PARAM HTK1=0.0583; HTK2=0.1274; HTK3=0.1525; HTK4=0.1896; HTK5=0.2290
   PARAM HTP1=0.6079; HTP2=0.7395; HTP3=0.6924; HTP4=0.7465; HTP5=0.7541

* Virus yield per cadaver (PIB/DLi)
   PARAM YNPVL1=1.43E6; YNPVL2=1.96E7; YNPVL3=2.1E8; YNPVL4=1.85E9; ...
         YNPVL5=1.28E10

* Vertical transmission happens when the previous generation of bollworm
* is sprayed with virus (#/m2 soil)/(#/m2 soil)
   PARAM RVT = 0.03

* Infectivity parameters for ln(Dose)-logit(Mortality) relationships,
* for both HaSNPV-WT and HaSNPV-AaIT against all 5 instars.
* (Equation 1 - 4) in Chapter 6.
   PARAM L1A=-2.755; L2A=-3.223; L3A=-4.061; L4A=-6.693; L5A=-8.682
   PARAM L1B= 0.882; L2B= 0.758; L3B= 0.724; L4B= 0.757; L5B= 0.618

* Relative inactivation rate of virus (/day), assuming a half-life of 3d
   PARAM RIRNPV=0.231

* Weather data in Anyang, Henan, China.
   WEATHER WTRDIR='D:\WEATHER\'; CNTR='AY'; ISTN=1; IYEAR=2002

  PRINT DAMAGE, VSOIL, HIBEPU
  PRINT TOTEGG, TOTL1, TOTL2, TOTL3, TOTL4, TOTL5, PU, TOTMO, SLARV

  TIMER STTIME = 158.; FINTIM = 273.; PRDEL = 1.0; DELT = 0.01
  TRANSLATION_GENERAL DRIVER='EUDRIV'

DYNAMIC

*(1)$$$$$$$$$$$$$$$$$$$$$$$ Virus spray process $$$$$$$$$$$$$$$$$$$$$*

* Spray rate of virus (PIB/m2 soil)
PARAM SPRAYR(1)=1.2E8;SPRAYR(2)=1.2E8; SPRAYR(3)=1.2E8; SPRAYR(4)=0.;...
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      SPRAYR(5)=2.4E8;SPRAYR(6)=2.4E8; SPRAYR(7)=2.4E8; SPRAYR(8)=0.;...
      SPRAYR(9)=3.0E8;SPRAYR(10)=3.0E8;SPRAYR(11)=3.0E8; ...
      SPRAYR(12)=3.0E8; SPRAYR(13:NSPRAY)=0.

* Spray time (day)
PARAM SPRDAY(1)=167.;  SPRDAY(2)=171.; SPRDAY(3)=175.;SPRDAY(4)=182.;...
        SPRDAY(5)=197.;SPRDAY(6)=201.; SPRDAY(7)=205.;SPRDAY(8)=209.;...
        SPRDAY(9)=228.;SPRDAY(10)=232.;SPRDAY(11)=236.;...
        SPRDAY(12)=240.;               SPRDAY(13:NSPRAY)= 244.

* Spray efficiency. Spray deposition can be on 2 leaf sides when
* spraying by hand, while larvae feed on one leaf side only.
   PARAM KSPRAY = 0.5

* Interception coefficient of spray in cotton.
   PARAM KDEPOS = 0.7

* Leaf Area Index (LAI, m2 leaf area/m2 soil area).
   LAI = AFGEN(LAITB, TIME)
   FUNCTION LAITB= 113., 0.01, 158.,1.3,...
                   163.,1.6, 168.,1.8, 173.,2.0, 178.,2.3, 183.,2.8, ...
                   188.,3.2, 193.,3.6, 198.,4.0, 203.,4.3, 208.,4.5, ...
                   213.,4.7, 218.,5.0, 223.,5.0, 228.,5.0, 233.,5.0, ...
                   238.,5.0, 253.,5.0, 273.,5.0, 290.,5.0, 330.,0.
* Virus density on cotton surface (PIB/mm2 leaf) after spray,
* (1 m2=1.0E6 mm2).
   VDENS = SPRAYR*KSPRAY*KDEPOS/LAI/1.0E6

* Calculation of virus spray and inactivation
   CALL VIRUS(IDENS, RIRNPV, NSPRAY, SPRDAY, VDENS, TIME, STTIME, DELT)
* IDENS  infectious virus density on cotton from sprayed virus (PIB/mm2)
* RIRNPV relative rate of virus inactivation (/day)
* NSPRAY number of sprays
* SPRDAY spray time (day)
* VDENS  density of sprayed virus on cotton (PIB/mm2 leaf area)

* The model takes into account that part of the virus sprayed or
* produced in the crop during the growing season is transported to the
* soil and can be transported back to the foliage and cause infection.
* Based upon measurements of PIB density in soil and calculations of
* virus density on the crop in the course of a season, a function
* RINFVS is introduced that specifies the ratio between the amount of
* virus in the soil per m2 soil area, and the amount of virus sprayed
* or produced in the field since the start time of simulation. A ratio
* of 10^(-5) is assumed as proportionality factor between the virus
* density on leaves, as a result as transport from soil, and the amount
* of virus in the soil.

* VSOIL: virus density in soil (PIB/m2 soil)
* VDENSF: cumulative amount of virus sprayed or produced in the field
* PIB/m2 soil)
   VSOIL = VYIELF*AFGEN(RINFVS,TIME)

* RINFVS: proportion of infectious virus in soil per 1 m2 of soil
* in 0.2 cm depth out of the accumulated virus in the system (including
* cadaver released virus and sprayed virus).
      FUNCTION RINFVS =120.,0.,189.,0.004,215.,0.007,237.,0.022, ...
                       268.,0.195, 295.,0.020,360.,0.0006

* Proportion of virus in soil (1m2 with 0.2 cm depth) transported to
* 1 cotton plant (LAI=1)(PIB/m2 leaf)/(PIB/m2 soil)(Fuxa et al., 2001)
   PARAM RTRANS = 1.0E-5

* Transport from soil to plant depends on LAI in such a way that
* for low LAI, VSOILP -> VSOIL * RTRANS * KDEPOS / 1.0E6, while
* for large LAI, VSOILP -> VSOIL * RTRANS / LAI / 1.0E6
* Virus density on cotton transported from soil (PIB/mm2 leaf)
   VSOILP = VSOIL*RTRANS*(1.-EXP(-KDEPOS*LAI))/LAI/1.0E6
```

```
* Infectious virus density on cotton leaf surface (IVDENS; PIB/mm2
* leaf area) includes infectious sprayed virus (IDENS) and virus
* transported from soil (VSOILP).
   IVDENS = IDENS + VSOILP

* Calculation of relative rate of horizontal transmission (/day).
* Relative rates of horizontal transmission are power functions of
* the density of infectors (data in thesis). Contributions of different
* instars are summed. It is assumed that different instars of recipient
* larvae have the same rate of horizontal transmission. The division by
* 6 is to convert density to a per plant basis.
   RHTR = (HTK1*(INFDL1/6.)**HTP1 + ...
           HTK2*(INFDL2/6.)**HTP2 + HTK3*(INFDL3/6.)**HTP3+ ...
           HTK4*(INFDL4/6.)**HTP4 + HTK5*(INFDL5/6.)**HTP5)

*(2)$$$$$$$$$$$$$$ Bollworm development and virus infection $$$$$$$$$$$*

* Circadian temperature process
   AVTMP  = (TMMX + TMMN)/2.
   AMPTMP = (TMMX - TMMN)/2.
   TEMP   = AVTMP + AMPTMP*(-COS(2.*PI*TIME))
   PI = 3.141592654

* Relative development rate of life stages of bollworm (/day).
   DVREG = AFGEN(DVEGTB, TEMP)
   DVRL1 = AFGEN(DVL1TB, TEMP)
   DVRL2 = AFGEN(DVL2TB, TEMP)
   DVRL3 = AFGEN(DVL3TB, TEMP)
   DVRL4 = AFGEN(DVL4TB, TEMP)
   DVRL5 = AFGEN(DVL5TB, TEMP)
   DVRPU = AFGEN(DVPUTB, TEMP)
   DVRMO = AFGEN(DVMOTB, TEMP)
   FUNCTION DVEGTB = 0.0,0., 11.6,0., 34.,0.5013, 40.3,0., 50.0,0.
   FUNCTION DVL1TB = 0.0,0., 13.6,0., 34.,0.5845, 38.4,0., 50.0,0.
   FUNCTION DVL2TB = 0.0,0., 12.8,0., 34.,0.7949, 39.2,0., 50.0,0.
   FUNCTION DVL3TB = 0.0,0., 11.5,0., 34.,0.8070, 39.3,0., 50.0,0.
   FUNCTION DVL4TB = 0.0,0., 6.40,0., 34.,0.6058, 40.8,0., 50.0,0.
   FUNCTION DVL5TB = 0.0,0., 10.8,0., 34.,0.2601, 41.2,0., 50.0,0.
   FUNCTION DVPUTB = 0.0,0., 12.1,0., 34.,0.1616, 43.5,0., 50.0,0.
   FUNCTION DVMOTB = 0.0,0., 8.70,0., 34.,0.1396, 43.0,0., 50.0,0.

* Relative attrition rate during each stage at 28 C (proportion/day)
PARAM RAREGG = 0.586; RARL1G = 0.266; RARL2G = 0.109; RARL3G = 0.074;...
      RARL4G = 0.068; RARL5G = 0.058; RARPUG = 0.096; RARMOG = 0.073

* RAR is corrected by effects of temperature on developmental rate.
* Denominators in each statement are developmental rates at 28 C.
   RAREG = RAREGG*DVREG/0.370
   RARL1 = RARL1G*DVRL1/0.400
   RARL2 = RARL2G*DVRL2/0.556
   RARL3 = RARL3G*DVRL3/0.588
   RARL4 = RARL4G*DVRL4/0.500
   RARL5 = RARL5G*DVRL5/0.192
   RARPU = RARPUG*DVRPU/0.116
   RARMO = RARMOG*DVRMO/0.106

* ======================== Adults boxcar trains ======================*

******************** Healthy adults boxcar train **********************

* Final value of the physiological age of stage g (gf)
   PARAM GF = 1.
* Width of the boxcars
   GAMMAF = GF/REAL(NM)

   CALL BOXRAT(NM, GAMMAF, IFLRMO, DVRMO, AMO, NTMO, OFMO)
* Subroutine BOXRAT(N, GAMMA, INFLR, DEVR, A, NETFLR, OUTFLR)
* N      number of boxcars for each stage
* GAMMA  width of the boxcars
```

```
* INFLR  inflow rate of individuals in each stage(#/m2 soil/day)
* DEVR   development rate of each stage (/day)
* A      number of individuals in each boxcar (#/m2 soil)
* NETFLR netflow rate between adjacent boxcars (#/m2 soil/day)
* OUTFLR outflow rate of individuals from a boxcar train (#/m2 soil/day)

* Rate of change of moths (#/m2 soil/day) is calculated from netflow
* rate and mortality of individuals in the boxcar.
   RMO    = NTMO - AMO*RARMO

* Integrate the rate of change to get moth number in each boxcar
* (#/m2 soil).
   AMO    = INTGRL(MOI, RMO)

* Total number of moths (#/m2 soil) equals the sum of individuals
* in each boxcar.
   MO     = ARSUMM(AMO, 1,NM)

**Immigration rate of moths

* The distribution patterns of immigrant moths in June, July and August
* are generated by initializing 0.1 moth/m2 soil on 2, May. The density
* of larvae is adjusted by factors DENFi according to the requests of
* simulations.

* At middle of June, July and August, moths immigrate.
* DENFi: density factor are used to initialize deferent densities of
* bollworm population),
  PARAM DENF1 = 1.0; DENF2 = 1.0; DENF3 = 1.0

* Immigration rate of moths (#/m2 soil/DELT)
  IFLRMO = IMRAT1*DENF1 + IMRAT2*DENF2 + IMRAT3*DENF3

* Immigration rate of moths in June (#/m2 soil/DELT)
  IMRAT1 = AFGEN(INFLT1, TIME)
  FUNCTION INFLT1=150.,0.   , 158.,0.,    159.,0.002, 160.,0.010, ...
                 161.,0.021, 162.,0.044, 163.,0.067, 164.,0.090, ...
                 165.,0.120, 166.,0.090, 167.,0.065, 168.,0.034, ...
                 169.,0.015, 170.,0.007, 171.,0.005, 172.,0.002, ...
                 173.,0.,    300.,0.

* Immigration rate of moths in July (#/m2 soil/DELT)
  IMRAT2 = AFGEN(INFLT2, TIME)
  FUNCTION INFLT2=150.,0.   , 187.,0.   , 188.,0.006, 189.,0.012, ...
                 190.,0.022, 191.,0.036, 192.,0.045, 193.,0.048, ...
                 194.,0.056, 195.,0.065, 196.,0.062, 197.,0.072, ...
                 198.,0.062, 199.,0.052, 200.,0.036, 201.,0.018, ...
                 202.,0.010, 203.,0.005, 204.,0.002, 205.,0., 300.,0.

* Immigration rate of moths in August (#/m2 soil/DELT)
 IMRAT3 = AFGEN(INFLT3, TIME)
 FUNCTION INFLT3= 150., 0.  , 217.,0.   , 218.,0.002, 219.,0.005, ...
                 220.,0.009, 221.,0.016, 222.,0.028, 223.,0.040, ...
                 224.,0.042, 225.,0.045, 226.,0.050, 227.,0.056, ...
                 228.,0.065, 229.,0.056, 230.,0.053, 231.,0.045, ...
                 232.,0.042, 233.,0.029, 234.,0.025, 235.,0.021, ...
                 236.,0.010, 237.,0.003, 238.,0.002, 239.,0.001, ...
                 240.,0.000, 300.,0.

*************** Boxcar train for virus contaminated adults *************

* Moths developed from virus treated larvae may lay virus-free eggs
* or virus-contaminated eggs (vertical transmission)(Figure 1)
* The character V is used to indicate vertical transmission.

   CALL BOXRAT(NM, GAMMAF,OFPU,DVRMO, AVMO, NTVMO, OFVMO)
* Rate of change of density of contaminated moths (#/m2 soil/day)
   RVMO    = NTVMO - AVMO*RARMO
```

```
* Moth number in each boxcar (#/m2 soil)
   AVMO    = INTGRL(ZERO, RVMO)
* Total number of virus contaminated moths (#/m2 soil)
   VMO     = ARSUMM(AVMO, 1, NM)
* Total number of moths (#/m2 soil)
   TOTMO = MO + VMO

*======================== Eggs boxcar trains ========================

********************** Healthy eggs boxcar train **********************
* Width of the boxcars
   GAMMA0 = GF/REAL(N0)

* The rate of egg laying depends on the development rate
* (Fecundity was measured at 28C, with relative developmental rate
* of moths 0.106/day)
    FDVFEC = DVRMO/0.106

* Fecundity of female moths (sex ratio=0.5) depends on their age.
* Fertilized female moths begin to deposit eggs on the 3rd day after
* emergence. The eggs laid by females of different age are summed.
* No egg laying occurs after August 31 in Anyang, China

* Inflow rate of eggs laid by immigrant moths (#/m2 soil)
   INFLEG=(ARSUMM(AMO, 6,10)*27.1 + ARSUMM(AMO,11,15)*54.2 + ...
           ARSUMM(AMO,16,20)*77.2 + ARSUMM(AMO,21,25)*72.3 + ...
           ARSUMM(AMO,26,30)*43.5 + ARSUMM(AMO,31,35)*27.9 + ...
           ARSUMM(AMO,36,40)* 8.8 + ARSUMM(AMO,41,45)* 1.8 + ...
           ARSUMM(AMO,46,NM)*0.3)*FDVFEC*INSW(TIME-250.,1.,0.)

   CALL BOXRAT(N0, GAMMA0, INFLEG, DVREG, AEGG, NTEGG, OFEGG)
   REGG   = NTEGG - AEGG*RAREG
   AEGG   = INTGRL(EGGI, REGG)
   EGG    = ARSUMM(AEGG, 1,N0)

****************Boxcar train for virus contaminated eggs****************

* Inflow rate of eggs laid by virus contaminated moths (#/m2 soil)
   IFVEG=(ARSUMM(AVMO, 6,10)*27.1 + ARSUMM(AVMO,11,15)*54.2 + ...
          ARSUMM(AVMO,16,20)*77.2 + ARSUMM(AVMO,21,25)*72.3 + ...
          ARSUMM(AVMO,26,30)*43.5 + ARSUMM(AVMO,31,35)*27.9 + ...
          ARSUMM(AVMO,36,40)* 8.8 + ARSUMM(AVMO,41,45)* 1.8 + ...
          ARSUMM(AVMO,46,NM)* 0.3)*FDVFEC*INSW(TIME-250.,1.,0.)
   CALL BOXRAT(N0, GAMMA0, IFVEG, DVREG, AVEG, NTVEG, OFVEG)
   RVEG   = NTVEG - AVEG*RAREG
   AVEG   = INTGRL(ZERO, RVEG)
   VEGG   = ARSUMM(AVEG, 1, N0)

* Total number of eggs (#/m2 soil)
   TOTEGG = EGG + VEGG

* ====================== L1 boxcar trains ============================

********************** Healthy L1 boxcar train ***********************

* Width of the boxcars
   GAMMA1 = GF/REAL(N1)

* Inflow rate of healthy L1 boxcar train includes outflow rate of
* all the eggs laid by immigrant moths (OFEGG) plus the virus-free
* portion of the eggs laid by "local" moths (OFEGG).
* RVT = rate of vertical transmission (#/#).
   INFL1 = OFEGG + (1. - RVT)*OFVEG

   CALL BOXRAT(N1, GAMMA1, INFL1, DVRL1, AL1, NTL1, OFL1)
   RL1    = NTL1 - AL1*RARL1 - AL1*RIRL1 - AL1*RHTR
   AL1    = INTGRL(ZERO, RL1)
   L1     = ARSUMM(AL1, 1,N1)
```

```
* Infection rate of L1 (#/m2 soil/day) is the sum of infection by sprayed
* virus and horizontal transmission
   INFRL1 = AL1*RIRL1 + AL1*RHTR

* Total rate of L1 getting infection (#/m2 soil/day)
   SINRL1 = ARSUMM(INFRL1,1,N1)

******************** L1 uptake virus boxcar train **********************

   CALL BOXRAT(N1, GAMMA1, 0., DVRL1, ADOSL1, NTVL1, OFVL1)

* RDOSL1: rate of change of the total amount of ingested virus per larval
* cohort in the boxcar train AL1 (# PIB/m2 soil/day).
* NTVL1: Net flow rate of virus dose (# PIB/m2 soil/day).
   RDOSL1 = NTVL1 + AL1*(IVDENS*FEEDL1 - (RARL1 + RIRL1 + RHTR)*DOSEL1)

* Total amount of virus (# PIB/m2 soil)
   ADOSL1 = INTGRL(ZERO, RDOSL1)

* Accumulated virus dose per larva (PIB/#)
   DOSEL1 = ADOSL1/NOTNUL(AL1)

* Relative infection rate of L1 at each boxcar (Equation 3 and 4);
* Infection chance of L1 per time step is depends on the rate of
* uptake of virus and on the already accumulated dose.
   RIRL1  = ((DOSEL1 + FEEDL1*IVDENS*DELT)**L1B - DOSEL1**L1B)/...
             (EXP(-L1A) + (DOSEL1 + FEEDL1*IVDENS*DELT)**L1B)/DELT

********************** Infected L1 boxcar train **********************

* Dynamics of infected larvae is simulated to calculate the contribution
* to the amount of virus in the field after their death. When L1s are
* infected, they may die as L1, L2 or L3.
* The last part of the infected L1 stage is a source for horizontal
* transmission, i.e. infected larvae become a source for horizontal
* transmission even before the turn into cadavers.

* Width of the boxcars for infected L1 is different from healthy L1.
   GAMA1I = GF/REAL(N1I)

* Inflow rate of infected L1 (#/m2 soil/day) includes rate of healthy
* L1 getting infection from sprayed virus or horizontal transmission
* (SINRL1) plus vertical transmission (OFVEG*RVT)
   INFIL1 = SINRL1 + OFVEG*RVT

* The rate of development in the "infected" condition (DVIL1) is
* the reciprocal of the mean time to death. The outflow
* pours out into a boxcar for cadavers (see below).
   CALL BOXRAT(N1I,GAMA1I,INFIL1, DVIL1, AIL1, NTIL1, OFIL1)

* Rate of change of density of infected L1 (#/m2 soil/day)
   RIL1   = NTIL1 - AIL1*RARL1

* Infected L1 in each boxcar (#/m2 soil)
   AIL1   = INTGRL(ZERO, RIL1)

* Total infected L1 (#/m2 soil)
   IL1    = ARSUMM(AIL1, 1,N1I)

* Total number of L1 (#/m2 soil)
   TOTL1  = L1 + IL1


********************** L1 cadavers boxcar trains **********************

* Horizontal transmission rate is calculated from the density of
* infectors which get infection at a certain instar (do not mater which
* instar they die). A boxcar is used to track the cadavers on cotton
* which get infection at L1.
```

```
   GAMMAC = GF/REAL(NC)
   CALL BOXRAT(NC, GAMMAC, OFIL1, DVRCAD, ACL1, NTCL1, OFCL1)
* Time coefficient of cadavers as sources for horizontal transmission
* (/day), assuming cadavers are "infectious" for 14 days.
   PARAM DVRCAD = 0.0714

* Relative "decay" rate of cadavers infectivity (/day), derived from
* Chapter 5, figure 2, field experiments
   PARAM RDRCAD = 0.061

* Rate of change of density of cadavers (#/m2/day)
   RCL1   = NTCL1 - ACL1*RDRCAD

* Number of cadavers in each boxcar (#/m2)
   ACL1   = INTGRL(ZERO, RCL1)

* Total cadavers density (#/m2 soil) after L1 were infected
   CL1    = ARSUMM(ACL1, 1,NC)

* L1 Infectors includes the last phase of infected L1 and cadavers which
* get infection at L1.
   INFDL1 = CL1+ ARSUMM(AIL1, 37, N1I)

* ======================== L2 boxcar trains ============================

********************** Healthy L2 boxcar train ************************

   GAMMA2 = GF/REAL(N2)
* Inflow rate of L2 (#/m2/day)
   INFLL2 =  OFL1
   CALL BOXRAT(N2, GAMMA2,INFLL2, DVRL2, AL2, NTL2, OFL2)
   RL2    = NTL2 - AL2*RARL2- AL2*RIRL2- AL2*RHTR
   AL2    = INTGRL(ZERO, RL2)
   L2     = ARSUMM(AL2, 1,N2)
   INFRL2 = AL2*RIRL2 + AL2*RHTR
   SINRL2 = ARSUMM(INFRL2,1,N2)

* L2 uptake virus boxcar train
   CALL BOXRAT(N2, GAMMA2, 0., DVRL2, ADOSL2, NTVL2, OFVL2)
   RDOSL2 = NTVL2 + AL2*(FEEDL2*IVDENS - (RARL2 + RIRL2 + RHTR)*DOSEL2)
   ADOSL2 = INTGRL(ZERO, RDOSL2)
   DOSEL2 = ADOSL2/NOTNUL(AL2)
   RIRL2  = ((DOSEL2 + FEEDL2*IVDENS*DELT)**L2B - DOSEL2**L2B)/...
            (EXP(-L2A)+(DOSEL2 + FEEDL2*IVDENS*DELT)**L2B)/DELT

* Infected L2 boxcar train
   GAMA2I = GF/REAL(N2I)
* Inflow rate of infected L2 (#/m2/day)
   IFLIL2 = SINRL2
   CALL BOXRAT(N2I, GAMA2I, IFLIL2, DVIL2, AIL2, NTIL2, OFIL2)
   RIL2   = NTIL2 - AIL2*RARL2
   AIL2   = INTGRL(ZERO, RIL2)
   IL2    = ARSUMM(AIL2, 1, N2I)
   INFDL2 = CL2 + ARSUMM(AIL2, 32, N2I)
   TOTL2  = L2+IL2

* L2 cadavers boxcar train
   CALL BOXRAT(NC,GAMMAC,OFIL2, DVRCAD, ACL2, NTCL2, OFCL2)
   RCL2   = NTCL2 - ACL2*RDRCAD
   ACL2   = INTGRL(ZERO, RCL2)
   CL2    = ARSUMM(ACL2, 1,NC)

* ======================== L3 boxcar trains ============================

* Healthy L3 boxcar train
   GAMMA3 = GF/REAL(N3)
   CALL BOXRAT(N3, GAMMA3, OFL2, DVRL3, AL3, NTL3, OFL3)
   RL3    = NTL3 - AL3*RARL3 - AL3*RIRL3 - AL3*RHTR
   AL3    = INTGRL(ZERO, RL3)
```

```
   L3     = ARSUMM(AL3, 1,N3)
   INFRL3 = AL3*RIRL3 + AL3*RHTR
   SINRL3 = ARSUMM(INFRL3,1,N3)
* L3 uptake virus boxcar train
   CALL BOXRAT(N3, GAMMA3, 0., DVRL3, ADOSL3, NTVL3, OFVL3)
   RDOSL3 = NTVL3 + AL3*(FEEDL3*IVDENS- (RARL3 + RIRL3+ RHTR)*DOSEL3)
   ADOSL3 = INTGRL(ZERO, RDOSL3)
   DOSEL3 = ADOSL3/NOTNUL(AL3)
   RIRL3  = ((DOSEL3 + FEEDL3*IVDENS*DELT)**L3B - DOSEL3**L3B)/...
            (EXP(-L3A)+(DOSEL3 + FEEDL3*IVDENS*DELT)**L3B)/DELT

* Infected L3 boxcar train
   GAMA3I = GF/REAL(N3I)
   IFLIL3 = SINRL3
   CALL BOXRAT(N3I,GAMA3I,IFLIL3, DVIL3, AIL3, NTIL3, OFIL3)
   RIL3   = NTIL3 - AIL3*RARL3
   AIL3   = INTGRL(ZERO, RIL3)
   IL3    = ARSUMM(AIL3, 1,N3I)
   INFDL3 = CL3 + ARSUMM(AIL3, 21, N3I)
   TOTL3  = L3 + IL3
* L3 cadavers boxcar train (for horizontal transmission)
   CALL BOXRAT(NC,GAMMAC,OFIL3, DVRCAD, ACL3, NTCL3, OFCL3)
   RCL3   = NTCL3 - ACL3*RDRCAD
   ACL3   = INTGRL(ZERO, RCL3)
   CL3    = ARSUMM(ACL3, 1,NC)

* Feeding cessation L3 boxcar train. This boxcar keeps track of the
* number of infected L3 that are feeding and cause damage. In L3, L4
* and L5, feeding stops before the larvae die. Hence, a distinction
* is made between "infected" larvae (AIL3) and those infected L3 that
* feed (AFCL3)
   GAMA3F  = GF/REAL(N3FC)
   IFLFL3  = SINRL3
   CALL BOXRAT(N3FC,GAMA3F,IFLFL3, DVFCL3, AFCL3, NTFCL3, OFFCL3)
   RFCL3   = NTFCL3 - AFCL3*RARL3
   AFCL3   = INTGRL(ZERO, RFCL3)
   FCL3    = ARSUMM(AFCL3, 1, N3FC)

* ====================== L4 boxcar trains ============================

* Healthy L4 boxcar train
   GAMMA4 = GF/REAL(N4)
   CALL BOXRAT(N4, GAMMA4, OFL3, DVRL4, AL4, NTL4, OFL4)
   RL4    = NTL4- AL4*RARL4 - AL4*RIRL4- AL4*RHTR
   AL4    = INTGRL(ZERO, RL4)
   L4     = ARSUMM(AL4, 1,N4)
   INFRL4 = AL4*RIRL4+ AL4*RHTR
   SINRL4 = ARSUMM(INFRL4,1,N4)

* L4 uptake virus boxcar train
   CALL BOXRAT(N4, GAMMA4, 0., DVRL4, ADOSL4, NTVL4, OFVL4)
   RDOSL4 = NTVL4 + AL4*(FEEDL4*IVDENS-(RARL4 + RIRL4 + RHTR)*DOSEL4)
   ADOSL4 = INTGRL(ZERO, RDOSL4)
   DOSEL4 = ADOSL4/NOTNUL(AL4)
   RIRL4  = ((DOSEL4 + FEEDL4*IVDENS*DELT)**L4B - DOSEL4**L4B)/...
            (EXP(-L4A)+(DOSEL4 + FEEDL4*IVDENS*DELT)**L4B)/DELT

* Infected L4 boxcar train
   GAMA4I = GF/REAL(N4I)
   IFLIL4 = SINRL4
   CALL BOXRAT(N4I,GAMA4I,IFLIL4, DVIL4, AIL4, NTIL4, OFIL4)
   RIL4   = NTIL4 - AIL4*RARL4
   AIL4   = INTGRL(ZERO, RIL4)
   IL4    = ARSUMM(AIL4, 1,N4I)
   INFDL4 = CL4 + ARSUMM(AIL4, 22, N4I)
   TOTL4  = L4 + IL4

* L4 cadavers boxcar train
   CALL BOXRAT(NC,GAMMAC,OFIL4, DVRCAD, ACL4, NTCL4, OFCL4)
```

```
   RCL4   = NTCL4 - ACL4*RDRCAD
   ACL4   = INTGRL(ZERO, RCL4)
   CL4    = ARSUMM(ACL4, 1,NC)
* Feeding cessation L4 boxcar train
   GAMA4F = GF/REAL(N4FC)
   IFLFL4 = SINRL4
   CALL BOXRAT(N4FC, GAMA4F, IFLFL4, DVFCL4, AFCL4, NTFCL4, OFFCL4)
   RFCL4   = NTFCL4 - AFCL4*RARL4
   AFCL4   = INTGRL(ZERO, RFCL4)
   FCL4    = ARSUMM(AFCL4, 1,N4FC)

* ====================== L5 boxcar trains ============================

* Healthy L5 boxcar train
   GAMMA5 = GF/REAL(N5)
   CALL BOXRAT(N5, GAMMA5, OFL4, DVRL5, AL5, NTL5, OFL5)
   RL5    = NTL5- AL5*RARL5 - AL5*RIRL5 -AL5*RHTR
   AL5    = INTGRL(ZERO, RL5)
   L5     = ARSUMM(AL5, 1,N5)
   INFRL5 = AL5*RIRL5 + AL5*RHTR
   SINRL5 = ARSUMM(INFRL5,1,N5)

* L5 uptake virus boxcar train
   CALL BOXRAT(N5, GAMMA5, 0., DVRL5, ADOSL5, NTVL5, OFVL5)
   RDOSL5 = NTVL5 + AL5*(FEEDL5*IVDENS- (RARL5 + RIRL5 + RHTR)*DOSEL5)
   ADOSL5 = INTGRL(ZERO, RDOSL5)
   DOSEL5 = ADOSL5/NOTNUL(AL5)
   RIRL5  = ((DOSEL5 + FEEDL5*IVDENS*DELT)**L5B - DOSEL5**L5B)/...
            (EXP(-L5A)+(DOSEL5 + FEEDL5*IVDENS*DELT)**L5B)/DELT

* Infected L5 boxcar train
   GAMA5I = GF/REAL(N5I)
   IFLIL5 = SINRL5
   CALL BOXRAT(N5I, GAMA5I, IFLIL5, DVIL5, AIL5, NTIL5, OFIL5)
   RIL5   = NTIL5 - AIL5*RARL5
   AIL5   = INTGRL(ZERO, RIL5)
   IL5    = ARSUMM(AIL5, 1,N5I)
   INFDL5 = CL5 + ARSUMM(AIL5, 21, N5I)
   TOTL5  = L5 + IL5

* L5 cadaver boxcar train
   CALL BOXRAT(NC,GAMMAC,OFIL5, DVRCAD, ACL5, NTCL5, OFCL5)
   RCL5   = NTCL5 - ACL5*RDRCAD
   ACL5   = INTGRL(ZERO, RCL5)
   CL5    = ARSUMM(ACL5, 1,NC)

* Feeding cessation L5 boxcar train
   GAMA5F = GF/REAL(N5FC)
   IFLFL5 = SINRL5
   CALL BOXRAT(N5FC, GAMA5F, IFLFL5, DVFCL5, AFCL5, NTFCL5, OFFCL5)
   RFCL5  = NTFCL5 - AFCL5*RARL5
   AFCL5  = INTGRL(ZERO, RFCL5)
   FCL5   = ARSUMM(AFCL5, 1, N5FC)

* Total larval population
  SLARV   = TOTL1 + TOTL2 + TOTL3 + TOTL4 + TOTL5

* ====================== Pupae boxcar trains =========================

* Pupae boxcar train
   GAMMAP = GF/REAL(NP)

* Outflow rate of pupae (OFPU) is to produce virus contaminated moths.
   INFLPU = OFL5*INSW(TIME - 230.,1.,0.)
   CALL BOXRAT(NP, GAMMAP, INFLPU, DVRPU, APU, NTPU, OFPU)
   RPU    = NTPU - APU * RARPU
   APU    = INTGRL(ZERO, RPU)
   PU     = ARSUMM(APU, 1, NP)
```

```
* In the 3rd generation in August, pupae hibernate.
   RHIBPU = OFL5*INSW(TIME - 230.,0.,1.)
   HIBEPU = INTGRL(ZERO, RHIBPU)

*(3)$$$$$$$$$$$$$$$$$$$ Virus density in cotton field $$$$$$$$$$$$$$$$$*

* The amount of virus produced by infected caterpillars is calculated
* and accumulated over the season to assess virus production in the
* field and estimate the recycling of infection from soil to crop.
* Virus production per caterpillars depends on the stage in which they
* die, while the stage in which a caterpillar dies depends on the
* infected instar and the virus dose. The average dose ingested by the
* caterpillars dying each time step is determined.

* Infection rate of larvae due to sprayed virus (#/m2 soil/day)
* This infection rate is an array variable. It is used below as a
* weight factor in the calculation of average dose ingested.
   INFSL1=AL1*RIRL1
   INFSL2=AL2*RIRL2
   INFSL3=AL3*RIRL3
   INFSL4=AL4*RIRL4
   INFSL5=AL5*RIRL5

* Intermediate variable (Infection rate* Virus dose/larva)
   DOSIL1= INFSL1*DOSEL1
   DOSIL2= INFSL2*DOSEL2
   DOSIL3= INFSL3*DOSEL3
   DOSIL4= INFSL4*DOSEL4
   DOSIL5= INFSL5*DOSEL5

* Weighted average of the accumulated virus dose until the moment of
* infection.
   AVDIL1=ARSUMM(DOSIL1,1,N1)/NOTNUL(ARSUMM(INFSL1,1,N1))
   AVDIL2=ARSUMM(DOSIL2,1,N2)/NOTNUL(ARSUMM(INFSL2,1,N2))
   AVDIL3=ARSUMM(DOSIL3,1,N3)/NOTNUL(ARSUMM(INFSL3,1,N3))
   AVDIL4=ARSUMM(DOSIL4,1,N4)/NOTNUL(ARSUMM(INFSL4,1,N4))
   AVDIL5=ARSUMM(DOSIL5,1,N5)/NOTNUL(ARSUMM(INFSL5,1,N5))

* Calculation of virus killed larvae in field.
* Proportion of infected larvae developing into subsequent instars
* before dying.
   RIL1L1 = AFGEN(RL1L1T, AVDIL1)
   RIL1L2 = AFGEN(RL1L2T, AVDIL1)
   RIL1L3 = AFGEN(RL1L3T, AVDIL1)
   RIL2L2 = AFGEN(RL2L2T, AVDIL2)
   RIL2L3 = AFGEN(RL2L3T, AVDIL2)
   RIL2L4 = AFGEN(RL2L4T, AVDIL2)
   RIL3L3 = AFGEN(RL3L3T, AVDIL3)
   RIL3L4 = AFGEN(RL3L4T, AVDIL3)
   RIL3L5 = AFGEN(RL3L5T, AVDIL3)
   RIL4L4 = AFGEN(RL4L4T, AVDIL4)
   RIL4L5 = AFGEN(RL4L5T, AVDIL4)

* Tables of ratio of infected larvae developing into subsequent instars
   FUNCTION RL1L1T = 0.0,0.,    1.0,0.,     15.79,0.0, 15.8,0.13,  ...
                     50.7,0.43, 313.,0.43,  500.,0.5,  1000.,1.
   FUNCTION RL1L2T = 0.0,1.,    1.0,0.75,   4.1,0.83,  15.8,0.78,  ...
                     50.7,0.52, 313.,0.57,  500.,0.4,  500.1,0.
   FUNCTION RL1L3T = 0.0,0.0,   0.99,0.,    1.0,0.25,  4.1,0.17,   ...
                     15.8,0.09, 50.7,0.05,  313.,0.0,  500.,0.
   FUNCTION RL2L2T = 0.0,0.,    15.4,0.,    142.19,0., 142.2,0.12, ...
                     405.,0.28, 1281.,0.42, 10000.,1.
   FUNCTION RL2L3T = 0.0,1.,    15.4,1.0,   48.5,1.0, 142.2,0.88,  ...
                     405.,0.72, 1281.,0.59, 1281.1,0., 10000.,0.
   FUNCTION RL2L4T = 0.0,0.,1000.,0.
   FUNCTION RL3L3T = 0.0,0.,    4265.9,0.,  4266.,0.13, 10000.,0.
   FUNCTION RL3L4T = 0.0,0.1,   114.,0.12,  320.,0.25,  724.,0.23, ...
                     1697.,0.51,4266.,0.61, 10000.,1.
   FUNCTION RL3L5T = 0.0,0.9,   114.,0.88,  320.,0.75,  724.,0.77, ...
```

```
                    1697.,0.49,4266.,0.27, 4266.1,0.,  10000.,0.
   FUNCTION RL4L4T = 0.0,0.,    7144.9,0.,  7145.,0.1, 19730.,0.16,...
                    35552.,0.14, 152263.,  0.18, 10000000.,0.
   FUNCTION RL4L5T = 0.0,1.,    4463.,1.,   7145.,0.9, 19730.,0.84,...
                    35552.,0.86,152263.,   0.82,152263.1,0.,1000000.,0.
* Total outflow rate of infected larvae in each instar (#/m2 soil/day),
   TOFIL1 = OFIL1*RIL1L1
   TOFIL2 = OFIL1*RIL1L2 + OFIL2*RIL2L2
   TOFIL3 = OFIL1*RIL1L3 + OFIL2*RIL2L3 + OFIL3*RIL3L3
   TOFIL4 = OFIL2*RIL2L4 + OFIL3*RIL3L4 + OFIL4*RIL4L4
   TOFIL5 = OFIL3*RIL3L5 + OFIL4*RIL4L5 + OFIL5

* Total virus killed larvae in each instar (#/m2 soil)
   DL1   = INTGRL(ZERO, TOFIL1)
   DL2   = INTGRL(ZERO, TOFIL2)
   DL3   = INTGRL(ZERO, TOFIL3)
   DL4   = INTGRL(ZERO, TOFIL4)
   DL5   = INTGRL(ZERO, TOFIL5)

* Virus yield in cotton field from virus-killed larvae (PIB/m2 soil)
* YNPVLi: Virus yield (PIBs) per cadaver
   VYIELL= DL1*YNPVL1+ DL2*YNPVL2+ DL3*YNPVL3+ DL4*YNPVL4+ DL5*YNPVL5

* Virus yield in cotton field from sprayed virus (PIB/m2 soil), 70% of
* sprayed virus is intercepted by crop and 30% is deposited on soil
   VYIELS=(INSW(TIME-SPRDAY(1),0.,1.) *SPRAYR(1)  + ...
           INSW(TIME-SPRDAY(2),0.,1.) *SPRAYR(2)  + ...
           INSW(TIME-SPRDAY(3),0.,1.) *SPRAYR(3)  + ...
           INSW(TIME-SPRDAY(4),0.,1.) *SPRAYR(4)  + ...
           INSW(TIME-SPRDAY(5),0.,1.) *SPRAYR(5)  + ...
           INSW(TIME-SPRDAY(6),0.,1.) *SPRAYR(6)  + ...
           INSW(TIME-SPRDAY(7),0.,1.) *SPRAYR(7)  + ...
           INSW(TIME-SPRDAY(8),0.,1.) *SPRAYR(8)  + ...
           INSW(TIME-SPRDAY(9),0.,1.) *SPRAYR(9)  + ...
           INSW(TIME-SPRDAY(10),0.,1.)*SPRAYR(10) + ...
           INSW(TIME-SPRDAY(11),0.,1.)*SPRAYR(11) + ...
           INSW(TIME-SPRDAY(12),0.,1.)*SPRAYR(12) + ...
           INSW(TIME-SPRDAY(13),0.,1.)*SPRAYR(13))* 0.3

* Cumulative virus yield in field including sprayed virus and
* cadaver-released virus (PIB/m2 soil)
   VYIELF= VYIELS + VYIELL


*(4)$$$$$$$$$$$$$$$$$$$ Calculation of Crop injury $$$$$$$$$$$$$$$$$$*

* L1 and L2 feed on tender leaves, their feeding normally
* does not cause loss of cotton yield. L3, L4 and L5 feed on floral
* squares, flowers or bolls. Injured squares shed and drop.
* Injured bolls rot and do not develop further.

* Number of infected L2 developing to infected L3 (#/m2 soil), which
* cause damage, is calculated.
* DAMIL3 is the density of infected L3s that were infected in the L2
* stage. They are calculated as the total density of larvae in the
* last 20 boxcars of the boxcar train of infected L2.
* Number of boxcars for infected L2 (N2I)=40.
* RIL2L3: ratio of L2 developing to L3 (see below)
   DAMIL3 = ARSUMM(AIL2, 20, N2I)*RIL2L3

* Rate of fruit injury by later instars (#Fruit/m2 soil/day).
* Both L3, L4 and L5 in "feeding cessation" boxcar trains and those
* in the healthy boxcar trains injure cotton fruits.
   RDAM = (DAMIL3 + FCL3 + L3)*RL3DAM + ...
          (FCL4 + L4)*RL4DAM + (FCL5 + L5)*RL5DAM
* Number of fruits injured daily by a single L3, L4 or L5
* (#fruit/larva/day)
   PARAM RL3DAM = 2.0; RL4DAM = 2.5; RL5DAM =2.5
```

```
* Cumulative injured fruits (#Fruit/m2 soil)
   DAMAGE = INTGRL(ZERO, RDAM)

END

* HaSNPV-AaIT parameters
   PARAM DVIL1 =0.3557; DVIL2 =0.3861; DVIL3 =0.2896; DVIL4=0.2219; ...
         DVIL5 =0.216
   PARAM DVFCL3=0.4405; DVFCL4=0.3601; DVFCL5=0.2578
   PARAM HTP1  =0.6718; HTP2  =0.6124; HTP3  =0.5247; HTP4=0.5097;  ...
         HTP5  =0.492
   PARAM HTK1  =0.0401; HTK2  =0.0617; HTK3  =0.0890; HTK4=0.1125;  ...
         HTK5  =0.137
   PARAM RVT   =0.02
   PARAM YNPVL1=7.4E5; YNPVL2=1.31E7; YNPVL3=1.02E8; YNPVL4=1.1E9;  ...
         YNPVL5=7.1E9
   FUNCTION RL1L1T = 0.0,0., 0.99,0.,1.0,0.33, 4.1,0.47, 15.8,0.35, ...
                     50.7,0.71,  313.,0.85, 500.,0.95, 1000.,1.0
   FUNCTION RL1L2T = 0.0,1.0,    1.0,0.67,  4.1,0.53,  15.8,0.65,   ...
                     50.7,0.29,  313.,0.15, 500.,0.0,  1000.,0.
   FUNCTION RL1L3T = 0.0,0.0,    1000.,0.
   FUNCTION RL2L2T = 0.0,0.,     15.39,0.,    15.4,0.34, 48.5,0.36, ...
                     142.2,0.49, 404.,0.53,  1281.,0.62, 10000.,1.0
   FUNCTION RL2L3T = 0.0,1.0,    15.4,0.66,  48.5,0.64,  142.2,0.51,...
                     404.,0.47,  1281.,0.38, 1281.1, 0., 10000.,0.
   FUNCTION RL2L4T = 0.0,0.,1000.,0.
   FUNCTION RL3L3T = 0.0,0.,     319.9,0.,   320.,0.05,  724.,0.07,...
                     1697.,0.24, 4266.,0.37, 100000.,1.
   FUNCTION RL3L4T = 0.0,0.3,     114.,0.42,  320.,0.42, ...
                     724.,0.51,  1697.,0.43, 4266.,0.54, 4266.1,0.
   FUNCTION RL3L5T = 0.0,0.7,    114.,0.58,  320.,0.53,  724.,0.42,...
                     1697.,0.33, 4266.,0.09, 4266.1,0.,  100000.,0.
   FUNCTION RL4L4T = 0.0,0.,     4462.,0.,   4463.,0.33, 7145.,0.23,...
                     19730.,0.29,35552.,0.5, 152263.,0.73,10000000.,1.0
   FUNCTION RL4L5T = 0.0,1.0,    4463.,0.67, 7145.,0.78, 19730.,0.71,...
                     35552.,0.5, 152263.,0.27, 152263.1,0., 10000000.,0.
   END

   STOP

       SUBROUTINE BOXRAT(N, GAMMA, INFLR, DEVR, A, NETFLR, OUTFLR)
           IMPLICIT REAL(A-Z)
           INTEGER N,I
           REAL CONCEN(N), NETFLR(N), FLOWR(N+1), A(N)
           FLOWR(1) = INFLR
           DO 10 I=1,N
              FLOWR(I+1)= DEVR*A(I)/GAMMA
              NETFLR(I)= FLOWR(I)-FLOWR(I+1)
              OUTFLR = FLOWR(I+1)
10         CONTINUE
           RETURN
           END

       SUBROUTINE VIRUS(IDENS,RIR,NSPRAY,SPRDAY,VDENS,TIME,STTIME,DELT)
           IMPLICIT REAL(A-Z)
           INTEGER I,NSPRAY
           REAL VDENS(NSPRAY), SPRDAY(NSPRAY)
           SAVE
           IF (TIME.EQ.STTIME) IDENS=0.
               IDENS = IDENS*EXP(-RIR*DELT)
           DO 20 I=1, NSPRAY
           IF(TIME.GE.SPRDAY(I).AND.TIME.LT.SPRDAY(I)+0.1*DELT) THEN
               IDENS = IDENS + VDENS(I)
           ENDIF
20         CONTINUE
           RETURN
           END
   ENDJOB
```

CHAPTER 7

General discussion

GENERAL DISCUSSION

Baculoviruses have been developed as microbial insecticides against a range of lepidopteran pests of crops, vegetables, forests and pastures. For insect species such as cotton bollworm that have developed resistance to chemical and/or *Bacillus thuringiensis* insecticides, the application of baculoviruses is one of the few options left for effective control. The development of recombinant baculoviruses with improved insecticidal properties provides the potential to successfully compete with chemical insecticides in a commercial setting. To support and promote the biosafe and biorational use of recombinant baculoviruses, it is important to understand in detail their biological properties, their agronomic efficacy of insect control, and their biosafety to human and environmental health.

In this thesis, the construction of a recombinant *H. armigera* single nucleocapsid nucleopolyhedrovirus expressing a neurotoxin *Aa*IT (HaSNPV-AaIT) is described. The infectivity, incubation time, inactivation rate, virus production, rates of vertical and horizontal transmission, control efficacy of both wild-type and recombinant HaSNPV variants were quantified in the laboratory and/or in the field. The collected information on the crop-insect-virus interactions is integrated in a process-based model for crop level interactions between the cotton bollworm and its wild-type or genetically modified (GM) NPV variants. This model is used to study the efficacy of application regimes of wild-type and recombinant HaSNPV in a cotton crop and to anticipate their control efficacy and environmental fate. In this Chapter the application of strategies, the biosafety issues and the perspective of using the wild-type and GM baculoviruses as commercial insecticides are further discussed.

Application of wild-type and GM baculovirus for bollworm control

Application strategies

For using NPVs in pest management there are four basic strategies: (1) The short-term ‘microbial insecticide’ approach, in which large numbers of viral polyhera are released for quick suppression of the pest infestation, (2) The ‘recycling microbial insecticide’ or ‘seasonal colonization’ approach, in which the NPV release results in viral replication and suppression of more than one pest generation, (3) The ‘introduction–establishment’ approach, which results in permanent pest population suppression; the NPV becomes a permanent part of an ecosystem in which it was not previously indigenous, (4) Environmental manipulation approach, which is the enhancement of viral suppression of pest populations by means other than the release of NPV into the environment; instead, the usual agricultural or resource management practices are

altered to conserve or enhance the activity of a viral population against a pest insect (Fuxa, 2004).

There are many factors involved in the selection of a strategy for using a baculovirus for insect pest control. In the case of using *H. armigera* SNPV to control cotton bollworm, the short-term microbial insecticide approach is the most appropriate one. There are several reasons: First, young larvae are the most likely stage to consume viral OBs from the surface of cotton plants. The first and second instar *H. armigera* larvae are more sensitive to virus infection than later larval instars (Chapter 2). After the second instar larvae hide in a protected structure such as unfurled leaves, squares, flowers or bolls (Sheng *et al.*, 2002; King, 1994). Later instar larvae therefore have little chance to contract virus. Second, polyhedra of viruses inactivate quickly on cotton plants (Chapter 4). Third, *H. armigera* has a high fecundity, which combined with a short life span, gives this species multiple generations in one cotton growing season and a high capacity for population increase. Forth, unlike foliage feeder, damage of bolls from bollworm causes a direct reduction of the final lint production.

These characteristics of the HaSNPV – *H. armigera* – cotton system require a very precise regime to obtain effective control of the cotton bollworm. Results from the experiments and scenario studies in Chapter 6 clearly indicate that timing and dosage are key factors for the efficacy of baculovirus application against *H. armigera*. HaSNPV formulations should always be applied as early as possible, preferably at egg hatch. Polyhedra doses should be high enough to kill 90% of the larvae. As a consequence monitoring of *H. armigera* flight is essential for the successful control of bollworm with baculoviruses. The simulation model provides quantitative guidance for the application of HaSNPV insecticides.

Feasibility of commercialization of GM baculovirus

Over the past 10 years a lot of field trials have been conducted at geographically distant sites using several types of GM baculoviruses and under different cropping situations (Cory, 2000). These trials show that in general recombinant baculoviruses are better to control their host pests. The question for the commercial application of the GM viruses widely is whether the benefits obtained by using GM baculoviruses outweigh the risks that they pose. To answer this question, we need to know how much benefit producers and growers can get by using the GM virus products and what kinds and levels of risks exist (Bonning and Hammock, 1996; Cory, 2000).

Production of GM HaSNPV insecticide

As a trade-off of the increase of killing speed of a GM baculovirus, its progeny yield in the infected host decreases. The virus kills the host more rapidly, the progeny yield

is less. This makes it more difficult to economically produce recombinant virus than the wild-type strain by the traditional method *in vivo.*

One way to solve this problem is to produce such recombinant viruses in insect cell culture. A 'bottle-neck' for *in vitro* production of baculoviruses has been the so-called passage effect. This effect is notable as a significant drop in production after repeated virus passaging in insect cell culture (Krell, 1996) and is a result of the accumulation of defective interfering particles (DIs) (Kool *et al.,* 1991). These DIs are rapidly generated in cell culture and become predominant after repeated passaging, meanwhile interfering with the replication of intact helper virus. Recently, it was found that the non-homologous region origin of DNA replication (non-*hr ori*) is involved in the mechanism of the generation of DIs, and that mutants packing this non-*hr ori* sequence can be stably passaged in insect cells (Pijlman *et al.,* 2002). This finding provides a chance to solve problems associated with large-scale production in insect cells.

The feasibility still exists to produce the toxin-expressing baculoviruses following the traditional *in vivo* method. To produce the recombinant HaSNPVs in its host larvae, a combination of optimal virus dose and a specific larval stage was selected to compensate as much as possible for the yield reduction of recombinant HaSNPV variants as compared to wild-type HaSNPV (Chapter 3). Pilot production of HaSNPV-AaIT demonstrated that it is feasible to economically produce the recombinant virus *in vivo* in a laboratory scale plant in Wuhan, China (Sun *et al.,* 2002a).

To further decrease the production costs of GM baculoviruses, a novel strategy has been developed by generating recombinant viruses, in which the insecticidal genes are expressed by inducible/suppressible promoters, such as temperature sensitive, antibiotic, metal- or hormone inducible/suppressible promoters. A successful approach has been the 'Tet' transactivator system (Dupont, 1996). Here the toxin gene is under control of a promoter that is activated by the tetracycline transactivator protein, which is also expressed by the recombinant baculovirus. During the production tetracycline is added to the diet and binds to the transactivator protein. As a result the toxin gene transcription and its translation do not occur. The yield of recombinant virus thus will be approximately the same as with wild-type virus. In the field, in the absence of tetracycline, the toxin protein will be produced resulting in faster death of target insects. Also much less PIBs are produced, reducing possible risks. This system has been successfully used to produce a recombinant HzSNPV with toxin and appeared to be safe and efficacious in the field (Dupont, 1996).

Profits of application of genetically modified baculovirus

Using recombinant HaSNPV to control *H. armigera* in cotton provides yield benefits, e.g. the yield of cotton lint of HaSNPV-AaIT treatment was 20% higher than the wild-

Table 1. Estimation of profit of using recombinant HaSNPV insecticides in comparison with wild-type virus and chemical insecticide.

Treatment	Yield of cotton lint (kg ha^{-1})*	Value ($ ha^{-1}) §	Costs of sprays ($ ha^{-1}) †	Economic returns ($/ha) ‡
Control	832	832	-	-
HaSNPV-WT	1249	1249	40	377
HaSNPV-EGTD	1351	1351	53	466
HaSNPV-AaIT	1525	1525	70	623
Chemicals	1539	1539	45	662

* Average yield of cotton lint in 2001 and 2002 (see Table 6 in Chapter 2).

§ Average price of cotton was $1.00 kg^{-1} lint in 2001 and 2002.

† Costs of sprays include production costs, dealing costs and manpower costs of spraying.

‡ Economic returns are calculated by subtracting the values of cotton lint in treatments by the values of control and the costs of sprays.

type treatment (Chapter 2). However, the virus yield of HaSNPV-AaIT in host larvae reduced to 40% of the wild-type virus at an optimal production regime (Chapter 3). Assuming production of viruses comprises half of the final costs of virus application (including costs of other components in the virus product, handling and labour costs), the total costs of a GM HaSNPV treatment is about 1.5 times higher than the wild-type virus treatment. The return of using HaSNPV-AaIT is about 1.7 times higher than that of HaSNPV-WT (Table 1). In this case study the higher costs of production of GM baculovirus can be easily recovered by the value of the extra cotton lint harvested compared to wild-type strain.

A further practical advantage of the use of viruses for pest control is that the growers take much less risk of poisoning by handling chemical insecticides. GM baculoviruses can be used in combination with other control strategies. It has been reported that the action of the neuro-toxins such as *Aa*IT is compatible and even synergizes with pyrethroid insecticides (McCutchen *et al.*, 1997; Popham *et al.*, 1998). The GM baculoviruses can be used effectively in crops expressing endotoxins of *Bacillus thuringiensis* (Inceoglu *et al.*, 2001). The compatibility and complementary nature of GM baculoviruses expressing neurotoxins provide us an extraordinary tool to manage pests that are resistant to chemical insecticides, provided their ecological safety is ascertained.

Biosafety of GM baculovirus

The risk of releasing GM baculoviruses is determined by their hazard and exposure to

nontarget species. The potential negative effects of GM baculoviruses on nontarget species and their fitness in the ecosystem are extensively studied.

Potential effects of GM baculoviruses on nontarget species

Numerous studies indicated there was no deleterious effect on humans and invertebrates under constant exposure to baculoviruses (Black *et al.*, 1997). Our experiments indicate that HaSNPV-AaIT is not pathogenic for bees, silkworms, birds, fish and that they do not have any adverse effect on the developmental parameters of these vertebrate species (Sun *et al.*, 2003). Neither is there an effect of a GM AcMNPV on aquatic microbial communities in microbial decomposition or respiration activity, bacterial abundance, or average metabolic responses (Kreutzweiser *et al.*, 2001).

Another aspect of their biosafety is the host range of GM viruses relative to wild-type virus. The host range of a baculovirus is generally limited to the lepidopteran insect species from which it was originally isolated (Gröner, 1986). It has been found seven different recombinant NPVs (based on AcMNPV, BmNPV, LdMNPV, or OpMNPV), carrying reporter genes, had an unaltered host range compared with the wild-type viruses (Huang *et al.*, 1997). Natural enemies of lepidopterans such as parasitoids, scavengers and predators were not found to be adversely affected with respect to parameters such as survival rate, developmental rate, reproductive rate etc., when preying upon larvae that are infected with recombinant baculoviruses expressing toxin or protease (Boughton *et al.*, 2003; Heinz *et al.*, 1995; Li *et al.*, 1999; McCutchen *et al.*, 1996; McNitt *et al.*, 1995; Smith *et al.*, 2000b). In the field experiments presented in Chapter 2 of this thesis, densities and diversity of beneficial parasitoids and predators were similar between recombinant and wild-type HaSNPVs treated plots over two growing seasons (Sun *et al.*, 2003). Survival time and fecundity of ladybeetles *Propylaea japonica* Thunberg, which constantly fed on HaSNPV-AaIT infected *H. armigera* larvae, did not differ from those fed on HaSNPV-WT infected or healthy larvae (Sun *et al.*, 2002c).

Fitness of GM baculovirus

Although they do not seem to have detrimental effects on non-target species, we can not completely exclude the unseen and unpredictable risks to the environment and humans associated with the release of GM baculoviruses into the environment where they compete with wild-type relatives. The fitness of GM viruses in comparison to the wild-type relatives has been widely studied. Fitness is a term that describes the ability of an organism to produce progeny that survives and contributes to the following generation (Cory, 2000). To assess the fitness of a GM virus the following components

of baculovirus needed to be considered: virus productivity, transmission and persistence.

It has been widely reported that yields of recombinant viruses in infected host are lower than those of wild-type virus (e.g. Burden *et al.*, 2000; Hernández-Crespo *et al.*, 2001; Chapter 3). Further more, the transmission rate of genetically modified baculoviruses is usually lower than that of wild-type viruses in several NPV - insect systems (e.g. Hails *et al.*, 2002; Fuxa *et al.*, 2002; Chapter 5).

In general polyhedra of baculoviruses inactivate quickly on host plants (e.g. Ignoffo *et al.*, 1997a, b; Chapter 4). In contrast, soil can protect the virus from sunlight and subsequently increase its persistence (e.g. Peng *et al.*, 1999). As infection of toxin-expressing viruses cause paralysis of the host larvae, making them drop as intact moribund larvae onto the ground (Cory *et al.*, 1994; Hoover *et al.*, 1995), while the cadavers killed by recombinant virus normally take longer time to disintegrate in comparison to wild-type virus killed ones (Fuxa *et al.*, 1998), it has been proposed that the recombinant virus may increase their relative concentrations in the soil such that the recombinant's persistence is increased. However, Fuxa *et al.* (2001) found that wild-type HzSNPV accumulated 2.3 times as many OBs in soil as a recombinant expressing LqhIT2 (HzSNPV.LqhIT2) after multiple applications for control of heliothine complex over one cotton growing season. It also was found that OBs of HzSNPV.LqhIT2 in field plots were not more clumped among soil samples than OBs of wild-type HzSNPV (Fuxa *et al.*, 2001). Furthermore, when wild-type AcMNPV and two recombinant AcMNPV which respectively express *Aa*IT and juvenile hormone esterase were introduced into soil microcosms, the number of viable OBs remaining did not differ among the treatments after 17 months (Fuxa *et al.*, 2001). The simulation model described in Chapter 6 indicates that HaSNPV-WT might accumulate two times higher than HaSNPV-AaIT in soil after applications at the same regime given a certain density of *H. armigera* population (Chapter 6), mainly due to the higher virus yield per cadaver (Chapter 3). In the case of the field experiments presented in Chapter 2, only wild-type HaSNPV can be detected by the bioassay method one year after the last spray (X. Sun, unpublished data). Current results further indicate that recombinant baculoviruses show reduced fitness in a competitive setting in comparison to the wild-type.

Potential movement of the foreign gene to other organisms

Another concern associated with recombinant baculovirus safety is the potential of the foreign gene (e.g. the *Aa*IT gene) 'jumping' from the recombinant baculovirus to another organism. Several key factors exclude or limit the occurrence of genetic recombination between donor and recipient DNAs, including physical proximity (i.e.

localization within the same compartment within a single cell), similar modes of replication and degree of DNA sequence homology (Black *et al.*, 1997). However, if a recombinant baculovirus pesticide is used long enough and at high enough concentrations in the field, it is expected that genetic recombination may eventually occur (Inceoglu *et al.*, 2001). It has been shown that the *Aa*IT gene in HaSNPV-AaIT has not been transferred to a common pathogen, *Verticillium dahliae* Lleb., on cotton or to the ladybeetles, *Propylaea japonica* Thunberg, after up to 3 months contact (Sun *et al.*, 2005). Moreover, it has been concluded that the detrimental effect on the recipients is minimal, even though this gene 'jumping' is in theory possible (Inceoglu *et al.*, 2001).

So far, there is no evidence that GM baculoviruses provide increased threats to human or environmental health in comparison to natural baculoviruses. It is not possible to commercially use a genetically modified organism without any risk, but the risks are up until now minimal. It is believed that the benefits from the use of GM baculoviruses far outweigh the unseen or unpredicted risks that they pose, especially in comparison to the risks associated with the use of chemical pesticides or genetically modified plants.

Further improvement of the insecticidal properties of baculoviruses

Up-to-date studies indicate that GM baculoviruses accrue greater benefits than wild-type viruses and their risks are low. To make them more competitive with chemical insecticides, it is necessary to further improve their insecticidal efficacy and UV sensitivity. As with any pesticide, there is a whole range of factors affecting control efficacy of baculoviruses. In the case of using HaSNPV to control *H. armigera*, pest larvae only have a chance to contract virus infection at early stages, while only late stages cause crop damage. Well-organized spraying regimes can somehow alleviate this problem. To further improve its competency to other agents, genetically engineering methods may be used in combination with some traditional methods.

Further improvement of the killing speed

Among the insect-selective toxins that have been used for improving the killing speed of baculoviruses, *Aa*IT is not the most effective one (Inceoglu *et al.*, 2001). The *Aa*IT encoding gene is often used because its mode of action is well known (Zlotkin *et al.*, 1995; Loret and Hammock, 1993). Besides selection of more insecticidal toxins or other proteases, faster kill speed can also be achieved by utilizing various promoters for expression of neurotoxins with signal sequences and enhancing elements (Van Beek *et al.*, 2003; Chapter 2) or by combined expression of the excitatory toxin and

the depressant toxin (Regev *et al.*, 2003). Chang *et al.* (2003) reported on the construction of a recombinant AcMNPV that produces novel polyhedra co-occluded with the *Bacillus thuringensis* Cry1Ac toxin and can kill *Plutella xylostella* larvae 63% faster than the wild-type AcMNPV.

Enhancing infectivity

Modelling results indicate that using recombinant HaSNPV with enhanced speed of action in combination with enhanced infectivity would dramatically improve its control efficacy (Chapter 6). To enhance the infectivity of baculoviruses one approach is genetic modification. Chang *et al.* (2003) showed that the LD_{50} of the recombinant AcMNPV which occludes Bt-Cry1Ac toxin in its polyhedra was reduced by 100-fold in comparison to wild-type AcMNPV. Another approach is to select naturally aggressive variants by *in vitro* cloning with cell culture technique or *in vivo* cloning with larvae (Ribeiro *et al.*, 1997; Hodgson *et al.*, 2001). Enhanced infectivity can also be achieved by incorporation with optical brighteners in a virus spray. Vail *et al.*, (1996) found that Calcofluor white M2R can reduce the LC_{50} of AfMNPV against its host *Anagrapha falcifera* by a factor of 2.9 to 13.6. Okuno *et al.* (2003) reported that the LD_{50} of *Spodoptera litura* NPV was reduced about 2200-fold by the addition of 1% Tinopal UNPA-GX to the virus suspension. The presence of fluorescent brighteners in some cases also shortened the LT_{50} values of baculoviruses (Vail *et al.*, 1996; Shapiro and Argauer, 2001).

Prolonging residual time of polyhedra on crop

Modelling results in Chapter 6 indicated residence time of baculoviruses contribute significantly to effective control in field situations. In an attempt to reduce UV inactivation Petrik *et al.* (2003) generated a recombinant AcMNPV that expresses an algal virus pyrimidine dimer specific glycosylase, cv-PDG (Furuta *et al.*, 1997), which is involved in the first steps of the repair of UV damaged DNA. Budded virus of this recombinant AcMNPV was threefold more resistant to UV inactivation. Recently, Van Oers *et al.* (2004) identified a novel DNA photolyase gene in *Chrysodeixis chalcites* NPV and this gene might be used to improve persistence when introduced into HaSNPV. More popularly UV-protection agents, such as lignosulfates, barbon, gelatin, titanium dioxide and stilbene optical brighteners etc., are added into the virus formulation to protect polyhedra from solar irradiation (Black *et al.*, 1997).

Quick inactivation of baculoviruses, such as HaSNPV, on cotton is not only caused by solar irradiation, but also by secondary chemicals secreted by plants (exudates) and by plant-mediated peroxidase activity, which generates free radicals (Chapter 4). The effect of plant-mediated peroxidase on virus activity may be lessened by incorporating

additives, such as mannitol (a hydroxyl radical scavenger) or superoxide dismutase (SOD), into a suitable formulation (Hoover *et al.*, 1998; Zhou *et al.*, 2004). It is also possible to co-occlude SOD into the polyhedra of HaSNPV to alleviate the effects on virus particles from the free radicals mediated by peroxidase on cotton (X. Sun, unpublished data).

Use of simulation models in baculovirus ecology and application

Determination of the commercial potential of new recombinant baculovirus formulations or application regimes is subjected to strict regulations and therefore costly. To assess the risk of the release of GM baculoviruses, it is important to understand in detail their behaviour in agro-ecosystems. Mathematical models have been used to study and gain insight in epizootics of baculoviruses in insect populations (see Cory, 2003, for review). In this study, we developed a process-based simulation model for the population dynamics of cotton bollworm, *H. armigera*, and baculoviruses in cotton under field conditions.

H. armigera is a fruit-feeder that is protected against virus sprays as soon as it has bored into bolls or squares. It can only be controlled with baculoviruses before they hide in a protected structure. Thus the window of opportunity for delivering the viral pathogen to its target is narrow and the timing of sprays becomes crucial. The required timing of sprays is intimately related to the phenology of the subsequent stages of the insect. Analytical models are not suitable to address questions on spraying regimes because they lack a detailed representation of insect phenology and life stages. The model developed in this study is suitable for addressing issues such as optimum spray regimes because it uses a detailed representation of life stages and phenology, based on the boxcar methodology. Furthermore, it includes detailed biological information on interactions in the HaSNPV – *H. armigera* – cotton system, such as virus infectivity, incubation time, inactivation, virus production, vertical and horizontal transmission, movements of virus between soil and crop compartments over a cotton growing season with three generations of pest infestation in cotton.

Although it is primarily used to understand and predict the behaviour of HaSNPV when used as insecticides, the model could be run for multiple seasons and could thus be used to assess the long-term dynamics of virus variants in the cotton agro-ecosystem. Additional experimental information will then be required on long-term virus persistence in the soil (years) and the movement of virus between crop and soil. The current model uses initial results to represent those processes, and needs to be further tested and developed in this respect. As the model is able to represent the whole of the virus life cycle in the crop-soil compartment over multiple generations in

multiple seasons, it could be used to understand the importance of the movement of the virus in the agro-ecosystem for pest control and virus persistence in the environment. The model could be used to study the competition between two baculoviruses, e.g. one wild-type virus and one GM virus, in a single host population. For this purpose, quantitative experimental data on viral replication in mixed infections of the two virus types are required. Such results may be combined with the results on horizontal and vertical transmission of HaSNPV variants, presented in this study and included in the model. At the moment, studies on viral competition and fitness in insect hosts are ongoing (PhD study L. Georgievska, Wageningen University). The model may be further developed to include competition between virus genotypes and can then be used for scenario studies on the competition and persistence of virus genotypes under field conditions and to assist in the assessment of environmental risks of the use of genetically modified HaSNPV for pest control.

Perspective of use of natural and GM baculovirus insecticides

Chemical pesticides have been used for more than 50 years. However, abuse of chemical insecticides has led to pest resistance, resurgence and environmental problems. These problems are often serious in developing countries because of weak governmental regulation and poor training of the appliers. Nowadays, society demands for safer pesticides and less-toxic chemical residues in food and water. Effective management of pests requires diverse and flexible tools. Among classical biocontrol agents, baculovirus is the most attractive tool which appears to be the supplement or replacement for synthetic chemical pesticides in IPM schemes.

Despite their high potential and successful cases (Moscardi, 1999), use of baculoviruses has been sometimes disappointing. The problem is mainly derived from their relative slow speed of action and low intrinsic virulence (especially against the later instar pest larvae). Most of the disadvantages of baculovirus have been or can be addressed by molecular biology and well-designed ecological studies. The studies in this thesis and elsewhere (Inceoglu *et al.*, 2001; Bonning and Hammock, 1996) indicate that GM baculoviruses are highly selective and effective for insect pest control, and that they can be used without disrupting biological control. Furthermore, they can be produced industrially but also by village industries, indicating that they have the potential to deliver sustainable pest control, especially in developing countries. Two key issues are involved in the greater use of natural and GM baculoviruses worldwide. First, growers need to be aware of the adverse effects of chemical pesticides on both human health and the environment and the necessity to adopt IPM instead of relying on chemical control. Second, greater efforts are needed to

make governments and the general public aware of the benefits of GM baculoviruses for insect pest control and to take away psychological fears due to perceived risks that are scientifically unproven. Application of the principles of precaution and familiarity will require a careful approach to practical implementation. Models like the one presented in this thesis may assist in these ventures.

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Summary

The cotton bollworm *Helicoverpa armigera* Hübner is one of most serious pest insect threatening the production of cotton in China, the world's largest producer of cotton. The insect pathogen, *H. armigera* single nucleocapsid nucleopolyhedrovirus (HaSNPV), has been developed and used as a commercial biopesticide for cotton bollworm control. To make it more competitive with chemical insecticides, HaSNPV has been genetically engineered by both deleting the ecdysteroid UDP-glucosyltransferase (*egt*) gene from its genome and incorporating an insect-selective toxin gene from the scorpion *Androctonus australis* (*Aa*IT). For the biosafe and biorational use of the recombinant baculoviruses it is necessary to understand in detail their biological properties, their agronomic efficacy as pest control agent and their biosafety to human and environmental health.

Since the level and extent of recombinant protein expression in baculoviruses is promoter-dependent, a chimeric promoter of the *p6.9* and *polyhedrin* gene of HaSNPV was designed to regulate high-level AaIT expression. A new recombinant baculovirus (HaSNPV-AaIT) was built by inserting the *Aa*IT gene at the *egt* gene locus (HaSNPV-AaIT), thereby deleting the *egt* gene. In the laboratory assays the infectivity (LD_{50}s) of this recombinant is unchanged, compared to wild-type HaSNPV (HaSNPV-WT) and an *egt*-deletion mutant (HaSNPV-EGTD). The median survival times (ST_{50}s) of 1st to 5th instar *H. armigera* larvae were reduced 17 - 34% after infection with HaSNPV-AaIT in comparison to HaSNPV-WT. For the 3rd to 5th instar of this insect the median times of feeding cessation (FT_{50}s) were 30 - 43% shorter for HaSNPV-AaIT than for HaSNPV-WT. HaSNPV-AaIT also acts quicker than HaSNPV-EGTD. Field trials at two research sites in 2000 indicated that the number of larvae and the proportion of damaged fruits were significantly lower in the plots treated with HaSNPV-AaIT than in plots treated with HaSNPV-WT or HaSNPV-EGTD. When HaSNPV-AaIT was applied to control infestations of bollworm over an entire cotton season, the yield of cotton lint in plots treated by this recombinant was 22.1% higher in 2001 than that in HaSNPV-WT treated plots and 20.7% higher in 2002 (Chapter 2).

Virus yield in deceased larvae is important for both virus production in a commercial setting as well as for understanding the consequences for baculovirus and insect dynamics and epidemics under field conditions. In Chapter 3, the yield of polyhedral inclusion bodies (PIBs) from 1st to 5th instar *H. armigera* larvae inoculated with HaSNPV-WT, HaSNPV-EGTD and HaSNPV-AaIT was studied. For each of the three types of HaSNPV the PIB yield per cadaver was significantly affected by larval instar at death and by time to death, with older instars and longer surviving larvae

producing a greater number of PIBs. As both recombinants caused bollworm larvae to die at earlier instars than HaSNPV-WT, their virus yields were significantly reduced. Virus yield per larva, infected with HaSNPV-AaIT in the 1st, 2nd, 3rd, 4th or 5th larval stage was 23%, 32%, 41%, 44% and 47% of the yield of HaSNPV-WT, respectively. For HaSNPV-EGTD, virus yield per larva inoculated in 1st through 5th instar, respectively, was 41%, 55%, 63%, 54% and 82% of the yield of HaSNPV-WT. These results provide a basis for optimizing the production regime of recombinant HaSNPVs in larvae and for modelling the behaviour of these viruses in agro-ecosystems.

The inactivation rate of baculoviruses is another important parameter for its epidemiology. In the field there was no difference between the inactivation rates of the two recombinant HaSNPVs and their parent HaSNPV-WT. The average half-life of HaSNPV was 0.57, 0.90 and 0.39 days in 2000, 2001 and 2002, respectively. The inactivation rate of these viruses was significantly different in different years, but correlated well with solar radiation over these years (Chapter 4).

Transmission plays a central role in the ecology of baculoviruses and the population dynamics of their hosts. In Chapter 5, the horizontal and vertical transmission dynamics of HaSNPV-WT and HaSNPV-AaIT were investigated. In field cages horizontal transmission of both HaSNPV variants was greatest when inoculated 3rd instar larvae were used as infectors, transmission was intermediate with 2nd instar infectors and lowest with 1st instar infectors. The transmission was greater at a higher density of infectors (1 per plant) than at a lower density (1 per 4 plants); however, the transmission coefficient (number of new infections per initial infector) was lower at the higher density of infectors than at the lower density. HaSNPV-AaIT exhibited a significantly lower rate of transmission than HaSNPV-WT in the field cages. This was also the case in open field experiments. In the laboratory the vertical transmission of HaSNPV-AaIT from infected females to offspring (16.7 ± 2.1%) was lower than that of HaSNPV-WT (30.9 ± 2.9%). Likewise, in the field vertical transmission of HaSNPV-AaIT (8.4 ± 1.1%) was lower than of HaSNPV-WT (12.6 ± 2.0%). The results indicate that the recombinant virus will be transmitted at lower rates in *H. armigera* populations than the wild-type virus. As a result, the recombinant HaSNPV may be impaired in its ability to maintain itself over multiple insect generations. This may potentially affect its long term efficacy in pest suppression negatively as compared to wild-type virus, but supports its use as an ‘insecticide’ and contributing positively to its biosafety.

Chapter 6 describes a comprehensive process-based model based on quantitative information of processes associated with the HaSNPV-bollworm-cotton system. This model can simulate the epizootiology of wild-type and genetically modified (GM) HaSNPV variants and integrates ecological information on interactions between

variants of HaSNPV, *H. armigera* and cotton. The model is used to assess and predict the biocontrol efficacy of application regimes of the wild-type and GM HaSNPV variants and the ecological behaviour of HaSNPV variants when used to control cotton bollworm in cotton, viz. at different spraying regimes, dosages, UV-protection agents, as affected by rates of horizontal and vertical transmission and transport of virus between soil and plants. In simulations on dosage and timing, good control efficacy against a common density of *H. armigera* of 12 larvae m^{-2} soil was obtained, when cotton was sprayed 3 times at 1.2×10^8 PIBs m^{-2} soil in June, 3 times at 2.4×10^8 PIBs m^{-2} soil in July and 4 times at 3.0×10^8 PIBs m^{-2} soil in August. An early timing of virus applications, soon after egg deposition, with a short, 3 days, spray interval appeared to be optimal for effective control. These results confirm farmers' practices which have evolved through experience.

Simulations further indicated that recombinant viruses with a lower mean time to kill gave better short term protection than wild-type virus. However, due to reduced horizontal transmission, use of recombinant virus caused more pupae to survive at the end of the growing season. The concentration of recombinant baculovirus persisting in soil was lower than of wild-type virus. UV-protection agents extending the half-life of HaSNPV on the canopy from 0.5 to 12 days contributed significantly to the effectiveness of control of bollworm under field conditions. A minimum half-life of 3 days was essential for effective control of bollworm with HaSNPV insecticides. Whereas horizontal transmission appeared to contribute significantly to control efficacy, this was not the case for vertical transmission or for transport of HaSNPV from soil to cotton plants at the low rates characteristic for this system.

The results presented in this thesis show that genetically modified HaSNPV accrues greater benefits to cotton growers than wild-type HaSNPV and that their multiplication in bollworm populations is impaired, compared to wild-type HaSNPV. As there are no demonstrable risks to human and environmental health up to now, GM baculoviruses have clear potential to provide effective and safe insect pest control (Chapter 7).

Samenvatting

De katoenrups *Helicoverpa armigera* Hübner is een van meest bedreigende plaaginsecten voor de katoenproductie van China, de grootste producent van katoen in de wereld. Het insectenpathogeen *H. armigera* kernpolyedervirus (HaSNPV) is ontwikkeld en in gebruik als een biologisch bestrijdingsmiddel tegen de katoenrups en commercieel verkrijgbaar. Om concurrentiepositie van dit middel te verbeteren ten opzichte van chemische bestrijdingsmiddelen is HaSNPV genetisch gemodificeerd door het ecdysteroid UDP-glucosyl transferase-gen (*egt*) uit het virusgenoom te verwijderen en een insectspecifiek toxine-gen van de schorpioen *Androctonus australis* (*Aa*IT) in te bouwen. Om dergelijke recombinante baculovirussen veilig en verantwoord te kunnen gebruiken is het noodzakelijk hun biologische eigenschappen nauwkeurig te bestuderen en goed te begrijpen. Ook moeten de effectiviteit als biologisch bestrijdingsmiddel en de veiligheid voor mens en milieu worden vastgesteld.

Omdat de mate en hoogte van expressie van recombinante eiwitten bij baculovirussen afhankelijk is van de promoter en de expressie in eerdergenoemd geval sterk moet zijn, werd een 'chimere' promoter op basis van het HaSNPV *p6.9* en het *polyhedrine*-gen ontworpen om de expressie van het *Aa*IT gen aan te sturen. Op basis van deze promoter werd een nieuw recombinant baculovirus (HaSNPV-AaIT) gemaakt en werd het *Aa*IT gen ter vervanging van het *egt* gen in het virus geplaatst. Uit laboratoriumtesten bleek, zoals verwacht, dat de mate van besmettelijkheid van deze recombinant onveranderd was vergeleken met het wildtype HaSNPV (HaSNPV-WT) en een *egt* deletie mutant zonder *Aa*IT (HaSNPV-EGTD). Wel werd na infectie met HaSNPV-AaIT de overleving van 1^e tot 5^e stadium *H. armigera* larven met 17-34% gereduceerd in vergelijking met HaSNPV-WT. Voor het 3^e t/m 5^e stadium van dit insect werd de gemiddelde tijd, waarbij larven stoppen met eten als gevolg van de toxinewerking, met 30-43% bekort wanneer HaSNPV-AaIT werd gebruikt in plaats van HaSNPV-WT. HaSNPV-AaIT werkt dus sneller dan HaSNPV-AaIT. Een vergelijking van de drie virusvarianten HaSNPV-WT, HaSNPV-EGTD en HaSNPV-WT tijdens veldexperimenten in 2000 op twee onderzoekslocaties in China, gaf aan dat het aantal larven en het aantal beschadigde katoenbollen het laagst waren in met HaSNPV-AaIT behandelde katoen. Wanneer HaSNPV-AaIT werd toegepast om katoenrupsen een seizoen lang te bestrijden, was de katoenopbrengst in proefvelden waarbij deze recombinant in 2001 gebruikt was 22,1% hoger ten opzicht van HaSNPV-WT en in 2002, 20,7% hoger.

De virusopbrengst uit dode larven is niet alleen belangrijk voor efficiënte virusproductie in een productiefaciliteit, maar ook voor het begrijpen van de gevolgen voor

de dynamica van virus en insecten onder praktijkomstandigheden in het veld. In Hoofdstuk 3 werd de opbrengst aan polyeders uit 1e t/m 5e stadium *H. armigera* larven, die besmet waren met respectievelijk HaSNPV-WT, HaSNPV-EGTD en HaSNPV-AaIT, bestudeerd. Voor ieder van de drie typen HaSNPV bleek de productie van polyeders per larve bepaald te worden door het stadium waarin de larven zich bevonden bij overlijden en door de overlevingsduur, waarbij oudere larven en larven die langer overleefden een groter aantal polyeders produceerden. Aangezien beide recombinanten ervoor zorgden dat *H. armigera* larven in een jonger stadium stierven dan die welke geïnfecteerd waren door HaSNPV-WT, was hun virusopbrengst duidelijk lager. De virusopbrengst per larve, geïnfecteerd met HaSNPV-AaIT, in het 1e t/m 5e larvale stadium bedroeg resp. 23%, 32%, 41%, 44%, en 47% van de opbrengst van HaSNPV-WT. Voor HaSNPV-EGTD was de virusopbrengst per larve, geïnfecteerd in het 1e t/m 5e stadium, 41%, 55%, 63%, 54% en 82% van de opbrengst van HaSNPV-WT. Deze gegevens vormen de basis voor de optimalisering van het productieproces van recombinante HaSNPV varianten in *H. armigera* larven en voor de modellering van het gedrag van deze virussen in agro-ecosystemen.

De inactivering van baculovirussen door zonlicht speelt een belangrijke rol in de ecologie en epidemiologie van virussen en insecten. In het veld werd geen verschil gevonden in de inactiveringssnelheid van de twee recombinante HaSNPV varianten en HaSNPV-WT. De gemiddelde tijd die verliep om 50% van de HaSNPV activiteit over te houden was 0,57, 0,90 en 0,39 dagen in respectievelijk 2000, 2001 en 2002. De inactiveringssnelheid van deze virussen was duidelijk verschillend in de diverse jaren, maar staat in verhouding tot de (zonne)straling die in deze jaren is gemeten (Hoofdstuk 4).

Overdracht speelt een centrale rol in de ecologie van baculovirussen en de populatiedynamica van hun gastheren (insecten). In Hoofdstuk 5 werd de dynamiek van de horizontale (van larve naar larve) en verticale (van ouder op kind) overdracht van HaSNPV-WT en HaSNPV-AaIT onderzocht. In veldkooien was de horizontale overdracht van beide HaSNPV varianten het grootst wanneer 3e stadium-larven werden gebruikt als infectiebron; de overdracht was gemiddeld bij 2e stadium-larven en het laagst wanneer larven van het 1e stadium als infectiebron werden gebruikt. De overdracht was groter bij een hoge dichtheid van infectiebronnen (1 per plant) dan bij een lagere dichtheid (1 per 4 planten). Echter, de overdrachtscoëfficiënt (het aantal nieuwe infecties per oorspronkelijke infectiebron) was lager bij een hogere dichtheid van infectiebronnen dan bij een lage dichtheid. In veldkooien vertoonde HaSNPV-AaIT een lagere overdracht dan HaSNPV-WT. Dit was ook het geval bij experimenten in het open veld. In het laboratorium was de verticale overdracht van HaSNPV-AaIT van geïnfecteerde vrouwtjes naar nakomelingen (16,7 $\pm$ 2,1%) lager dan bij HaSNPV-

WT (30,9 ± 9%). Evenzo was de verticale overdracht van HaSNPV-AaIT (8,4 ± 1,1%) significant lager dan in het geval van HaSNPV-WT (12,6 ± 2,0%). De resultaten geven aan dat recombinante HaSNPV-varianten met een lagere frequentie worden doorgegeven in *H. armigera* populaties dan het wildtype virus. Het recombinante HaSNPV wordt daardoor zeer waarschijnlijk beperkt in zijn vermogen zichzelf in meerdere insectengeneraties te handhaven. Dit effect zou in principe de effectiviteit van HaSNPV op langere termijn negatief kunnen beïnvloeden in vergelijking tot het wildtype virus, maar is gunstig bij het gebruik van HaSNPV als insecticide en draagt in positieve zin bij tot zijn biologische veiligheid.

Hoofdstuk 6 beschrijft een uitgewerkt model, gebaseerd op kwantitatieve informatie over processen die van belang zijn voor het HaSNPV–katoenrups–katoen systeem. Het model integreert ecologische informatie van interacties tussen varianten van HaSNPV, *H. armigera* en van katoen. Het kan epidemieën van wildtype en genetisch gemodificeerde HaSNPVs onder diverse omstandigheden simuleren. Het model kan worden gebruikt om de effectiviteit van de bestrijding van wildtype en HaSNPV recombinanten te voorspellen en om het ecologische gedrag van diverse HaSNPVs bij de katoenrupsbestrijding bij diverse scenario's te begrijpen en te evalueren. De scenario's betreffen de vergelijking van diverse bespuitingregimes, doseringen, UV-beschermingsmiddelen, effecten van horizontale en verticale overdracht en transport van virus tussen bodem en planten. Bij simulaties met diverse doseringen en met verschillende tijdsduur werd een goed bestrijdingsresultaat bereikt bij een gebruikelijke dichtheid van *H. armigera* van 12 larven per m^2 grond, wanneer het gewas tien keer met virus werd behandeld (drie keer bij $1{,}2 \times 10^8$ PIBs m^{-2} grondoppervlak in juni, 3 keer bij 2.4×10^8 PIBs m^{-2} in juli en vier keer bij 3×10^8 PIBs m^{-2} grondoppervlak in augustus). Virustoepassing kort na de eerste ovipositie en bij een kort bespuitinginterval (3 dagen), bleek optimaal te zijn voor de bestrijding van de katoenrups. Dit schema is vergelijkbaar met wat in de praktijk gebruikelijk is.

Simulaties met het model lieten verder zien dat recombinante HaSNPV-varianten met een snellere werking op korte termijn een betere bescherming tegen insectenaantasting geven dan wildtype virus. Echter, als gevolg van de afgenomen horizontale overdracht zorgde het gebruik van een recombinant virus ervoor dat er aan het eind van het groeiseizoen meer insecten in het popstadium overleefden. De concentratie van recombinant HaSNPV, dat in de grond overblijft, was voor HaSNPV-recombinanten lager dan voor wildtype virus. UV-beschermingsmiddelen, die de 50% overleving van HaSNPV in de top van het gewas van een half tot 12 dagen verlengen, dragen aantoonbaar bij aan de effectiviteit van katoenrupsbestrijding van onder veldomstandigheden. Een minimale 50% overleving van drie dagen was essentieel om nog een effectieve katoenrupsbestrijding met HaSNPV te verkrijgen. Daar waar horizontale overdracht

van HaSNPV een belangrijke rol speelt bij de effectiviteit van de bestrijding, was dit niet het geval bij verticale overdracht of bij transport van HaSNPV van grond naar gewas.

De resultaten die in dit proefschrift zijn beschreven tonen aan dat het gebruik van genetisch gemodificeerd HaSNPV voordelen heeft voor katoentelers boven wildtype HaSNPV en dat een vermeerdering van recombinant HaSNPV in katoenrupspopulaties achterblijft in vergelijking met wildtype HaSNPV. Aangezien er tot nu toe geen aantoonbare risico's voor mens en milieu zijn geïdentificeerd, hebben recombinante baculovirussen een duidelijke toekomst om een effectieve en veilige bestrijding van insectenplagen mogelijk te maken (Hoofdstuk 7).

摘　要

中国是世界上最大的棉花生产国。棉铃虫（*Helicoverpa armigera*）是对棉花产业可持续发展最具威胁的害虫之一。棉铃虫单核衣壳核型多角体病毒 (HaSNPV) 是棉铃虫的专一性病原物，已经被成功开发为一种商业杀虫剂。为了提高 HaSNPV 与化学农药的竞争力，我们前期成功地构建了缺失蜕皮激素 UDP-葡萄糖基转移酶基因（*egt*）或插入北非蝎子昆虫选择性毒素（*Aa*IT）基因的改良型重组棉铃虫病毒。安全、合理地使用重组病毒杀虫剂需要全面评价其生物学特性、杀虫效果以及对人类与环境的安全性。

本论文的第二章构建了一个新的重组棉铃虫病毒。鉴于在杆状病毒中重组蛋白的表达水平与启动子的效率相关，我们设计了一个新的来源于 HaSNPV 的 *p6.9* 基因和 *polyhedrin* 基因的双启动子，并利用这个启动子构建了在 *egt* 基因位点高效表达 *Aa*IT 基因的重组病毒 HaSNPV-AaIT。生物活性测定结果表明，该重组病毒的半致死剂量（LD_{50}）与野生型病毒（HaSNPV-WT）以及单一缺失 *egt* 基因的重组病毒（HaSNPV-EGTD）相比没有显著差异。但与 HaSNPV-WT 相比，HaSNPV-AaIT 感染的 1~5 龄棉铃虫幼虫的半数存活时间（LT_{50}）缩短了 17% - 34%，3~5 龄被感染幼虫的半数停食时间（FT_{50}）缩短了 30% - 43%。同时，HaSNPV-AaIT 的杀虫速度也比 HaSNPV-EGTD 快。2000 年在中国两个地点的田间试验表明，HaSNPV-AaIT 处理的棉花小区中棉铃虫的数量和蕾铃被害率都显著低于 HaSNPV-WT 和 HaSNPV-EGTD 处理的小区。2001 年和 2002 年利用 HaSNPV 全季节防治棉花棉铃虫的试验表明，HaSNPV-AaIT 处理的小区皮棉产量较野生型病毒处理的小区分别高 22.1%和 20.7%。

病毒在致死幼虫中的产量对病毒杀虫剂的工厂化生产以及了解病毒的田间流行病学都非常重要。第三章描述了 HaSNPV-WT，HaSNPV-EGTD 和 HaSNPV-AaIT 感染 1~5 龄棉铃虫幼虫时的病毒多角体产量。这三种病毒的单虫病毒产量都受到死亡龄期和死亡时间的影响，即龄期较高的幼虫以及死亡较晚的幼虫中病毒的产量较高。因为重组病毒感染的幼虫死亡龄期较野生型病毒早，子代病毒的产量也显著下降。感染 1~5 龄棉铃虫幼虫时，HaSNPV-AaIT 的产量分别为 HaSNPV-WT 的 23%，32%，41%，44% 和 47%，而 HaSNPV-EGTD 的产量分别为 HaSNPV-WT 的 41%，55%，63%，54%和 82%。这些结果为制定重组病毒的工厂化生产方案以及为构建病毒在生态系统中的流行病学模型提供了依据。

杆状病毒在自然环境中的失活速度是其流行病学的另一个重要参数。第四章的研究结果表明，在田间条件下，两种重组病毒（HaSNPV-EGTD 和 HaSNPV-AaIT）及野生型病毒的失活速度之间没有显著差异。在 2000 年，2001 年和 2002 年，HaSNPV 在棉花植株上的半数存活时间分别为 0.57，0.90 和 0.39 天。

在这三年中，病毒的失活速度有显著差异，而且跟太阳的辐射强度相关。

杆状病毒的传播能力在它们的生态学以及它们与宿主种群动态的关系中占据重要的地位。第五章描述了 HaSNPV-WT 和 HaSNPV-AaIT 在棉铃虫种群中的水平传播和垂直传播动态。在田间罩笼实验中，两种 HaSNPV 的水平传播率随着感染幼虫龄期的增加而增加。在感染幼虫密度较高时，病毒传播率更高。无论是在田间罩笼实验中还是田间开放实验中，HaSNPV-AaIT 的水平传播率都显著性于野生型病毒。在实验室中，HaSNPV-AaIT 在亚致死感染棉铃虫的垂直传播率（16.7%）极显著低于野生型病毒（30.9%）。同样，在田间重组病毒的垂直传播率（8.4%）也低于野生型病毒（12.6%）。实验结果说明，重组病毒在宿主种群中的传代能力低于野生型病毒，这一特性可能会影响它长期抑制害虫种群效力，但是对其作为杀虫剂的生物安全性有利。

第六章描述了一个基于病毒－棉铃虫－棉花生态系统中定量化信息的综合性模型。该模型模拟野生型和重组型 HaSNPV 的流行病学动态，并且综合了不同 HaSNPV 基因型、棉铃虫和棉花相互作用的生态学信息，用于评价和预测野生型和重组型 HaSNPV 在不同应用方案、使用剂量以及光保护剂等条件下防治棉铃虫的效果以及生态习性。在一般的棉铃虫密度下，在第二代发生期以 1.2×10^8 PIBs/m^2 防治 3 次，在第三代发生期以每 2.4×10^8 PIBs/m^2 防治 3 次及在第四代发生期以每 3.0×10^8 PIBs/m^2 防治 4 次，可以达到较好的防治效果。在每一代棉铃虫发生期第一次施药的时机应该选择在卵刚出现时，然后每间隔 3 天施药一次。这些模拟结果进一步证实了棉农经过长期实践得到的施药方案。

第六章的模拟结果还表明，应用具有较快杀虫速度的重组病毒能够得到比野生型病毒更好的短期防治效果。但是，由于重组病毒的水平传播率较低，单一使用这种病毒在一个防治季节结束时，有更多的棉铃虫蛹存活；病毒多角体在土壤中的滞留量也比野生型病毒低。延长 HaSNPV 在棉花植株上的存活时间极显著地增加其对棉铃虫的防治效果，HaSNPV 杀虫剂至少具有 3 天的半数存活期才能达到对棉铃虫的有效防治。相对于水平传播对 HaSNPV 防治效果的重要性，垂直传播率以及病毒多角体从土壤向植株的转移率对其防治效果的影响则不大。

本论文的结果表明，棉农应用重组棉铃虫病毒能够得到比野生型更好的利益，而重组病毒在宿主种群中的增殖能力相对下降。迄今为止的研究结果表明，重组杆状病毒对人类和环境都没有不利影响，它们具有发展为安全、高效的商品化生物杀虫剂的潜力。

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* in Chinese.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (ECTS 4)
- Control of cotton bollworm, *Helicoverpa armigera* with HaSNPV (2002)
- Horizontal and vertical transmission of wild-type and recombinant HaSNPV (2004)

Writing of project proposal (ECTS 5)
- Control of cotton bollworm, *Helicoverpa armigera* with nucleopolyhedroviruses in cotton fields in China: A Simulation Study (2001)

Post-Graduate Courses (ECTS 3)
- Spatio-temporal models in ecology (2001)
- Advanced Statistics (2002)
- Survival Analysis (2003)

Deficiency, Refresh, Brush-up and General Courses (ECTS 2)
- System Analysis and System Design (2000)

PhD discussion groups (ECTS 2)
- Plant-insect interaction (2002)
- Virology (2003)

PE&RC annual meetings, seminars and introduction days (ECTS 1.5)
- Winter school “Functional Biodiversity of Sustainable Crop Protection” (2001)
- PE&RC Theme Day “Agriculture and Nature”(2001)
- PE&RC Day “Food Insecurity” (2001)

International symposia, workshops and conferences (ECTS 9)
- 37th Annual meeting of the Society of Invertebrate Pathology (SIP, 2004)
- 15th International Plant Protection Conference (IAPPS, 2004)
- 36th Annual meeting of the Society of Invertebrate Pathology (SIP, 2003)
- 7th International Symposium on the Biosafety of GMOs (ISBGMO, 2003)
- British Crop Protection Conference (BCPC, 2003)
- 8th Conference of Cotton of China (CSC, 2001)
- 8th Conference of Microbiology of China (CSM, 2001)

Laboratory training and working visits (ECTS 1)
- SLFA für Landwirtschaft, Weinbau und Gartenbau, Neustadt. Baculovirus Ecology (2002)

Curriculum vitae

Xiulian SUN was born on August 27, 1968 in Anlu City, Hubei Province, P. R. China. He finished his BSc study at the Department of Plant Protection, Huazhong Agricultural University, Wuhan, China, in 1990 and completed his MSc study at the Wuhan Institute of Virology, Chinese Academy of Sciences, where he received his MSc degree in Virology in 1993. After graduation he has been working as a research assistant, research associate and associate professor at the Wuhan Institute of Virology. Since 2000, he joined the cooperation between the Royal Academy of Sciences of the Netherlands and the Chinese Academy of Sciences and worked in the Laboratory of Virology and the Crop and Weed Ecology Group, Wageningen University, the Netherlands, to carry out a PhD study. Since January 2002, he has been involved in the 'sandwich PhD program' of Wageningen University, under supervision of Professor Just Vlak, Dr. Wopke van der Werf and Professor Hu Zhihong. The results of the PhD research are described in this thesis.

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