NUCLEAR POLYHEDROSIS VIRUS AS BIOLOGICAL CONTROL AGENT OF *SPODOPTERA EXIGUA*

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
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op woensdag 7 januari 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Protinus elucet languentibus aurea pellis
Deinde tumet, turpisque animis ignavia venit
Desidibus, tandem rumpuntur, et omnia tetro
Inficiunt tabo; sanies fluit undique membris

uit: 'De Bombicum', M.H. Vida di Cremona (1527)

CONTENTS

Stellingen

1. Rupsen in het eerste larvale stadium zijn ongeschikt voor het vergelijken van de biologische activiteit van virulente kernpolyedervirussen.

   Hughes, P.R. & H.A. Wood. 1981. J. Invert. Pathol. 37: 154-159.
   Huber, J. & P.R. Hughes. 1984. Bull. Ent. Soc. Am. 30: 31-34.

2. Soortspecifieke kernpolyedervirussen zijn virulenter dan kernpolyedervirussen met een breed gastheerspectrum.

   Dit proefschrift.

3. Op grond van beschikbare gegevens over hun eigenschappen en veiligheid zou de gehele groep van de baculovirussen een toelating als bestrijdingsmiddel voor insekten moeten verkrijgen.

   Harrap, K.A. 1982. Parasitology 84: 288-296.
   Öller, G. 1985. pp. 398-439. In: K. Maramorosch & K.E. Sherman (eds.). Viral insecticides for biological control. Acad. Press, New York.

4. Resistentie bij plaaginsekten tegen pathogenen die gebruikt worden als biologisch bestrijdingsmiddel, zal veel minder frequent optreden dan de resistentie tegen chemische insecticiden.

   Briese, D.T. 1986. pp. 233-256. In: J.M. Franz (ed.). Biological plant and health protection. Gustav Fischer Verlag, Stuttgart.

5. Er is in Nederland een grotere urgentie voor het ontwikkelen van biologische bestrijdingsmethoden tegen aaltjes dan tegen insekten en mijten, hetgeen niet weerspiegeld wordt in het onderzoek dat momenteel plaatsvindt.

6. Bacteriën, virussen en nematoden bieden meer perspectief als biologische bestrijdingsmiddelen dan schimmels en protozoën.

7. Vele zogenaamde 'insect-borne plantviruses' zouden wel eens 'plant-borne insectviruses' kunnen blijken te zijn.

   Harpaz, I. 1986. p. 144. In: R.A. Samson, J.M. Vlak & D. Peters (eds.). Fundamental and applied aspects of invertebrate pathology. Proc. 4th. Int. Col. Invert. Pathol., Veldhoven, the Netherlands.

8. Bij voedselopname van de hozemond, Lophius piscatorius, wordt de kracht geleverd door de epaxiale en hypaxiale lichaamsmusculatuur en hebben de spieren in de kop slechts een sturende functie.

9. Bij de vorming van mesoderm in embryonen van zoogdieren treedt celmigratie over afstanden van meer dan enkele cellengtes niet op.
   Smits-van Prooije, A.E. 1986. Proefschrift RU Leiden.

10. Volgens het motto "de vervuiler betaalt" zou er een progressieve vervuilingsbelasting geheven moeten worden op pesticiden die schadelijk zijn voor het milieu vanwege hun brede werking en persistentie.

11. De depressie die sommige vrouwen krijgen na de geboorte van een kind, dient niet betiteld te worden als een postnatale maar als een postpartale depressie.

12. Het zou een zegen zijn voor het Nederlandse bos als een groot deel van het huidige naaldhoutbestand zou verdwijnen door de zure regen.

13. De nu zo toegejuichte chip is niets anders dan de vroeger zo verguisde snijer.

14. De trekvogels in Nederland hebben gelijk.

Stellingen behorende bij het proefschrift van Peter Hans Smits.
Nuclear polyhedrosis virus as biological control agent of Spodoptera exigua.
Wageningen, 7 januari 1987.

Some chapters of this thesis are similar to the following publications:

Chapter 2: Smits, P.H., M. van de Vrie & J.M. Vlak. 1986. Oviposition of beet armyworm (Lepidoptera: Noctuidae) on greenhouse crops. Environ. Entomol. 15: 680-682.

Chapter 3: Smits, P.H., M.C. van Velden, M. van de Vrie & J.M. Vlak. 1987. Feeding and dispersion of Spodoptera exigua larvae and their relevance for control with a nuclear polyhedrosis virus. Entomologia Exp. & Appl. 43: in press.

Chapter 4: Smits, P.H. & J.M. Vlak. Selection of nuclear polyhedrosis viruses as biological control agents of Spodoptera exigua (Lepidoptera: Noctuidae). Submitted.

Chapter 5: Smits, P.H. & J.M. Vlak. Biological activity of Spodoptera exigua nuclear polyhedrosis virus against Spodoptera exigua (Lepidoptera: Noctuidae) larvae and their progeny. Submitted.

Chapter 6: Smits, P.H. & J.M. Vlak. Qualitative and quantitative aspects of the production of Spodoptera exigua nuclear polyhedrosis virus in beet armyworm larvae. Submitted.

Chapter 7: Smits, P.H., I.P. Rietstra & J.M. Vlak. The influence of application techniques on the control of beet armyworm larvae with Spodoptera exigua nuclear polyhedrosis virus. Submitted.

Chapter 8: Smits, P.H., M. van de Vrie & J.M. Vlak. 1987. Nuclear polyhedrosis virus for the control of Spodoptera exigua larvae on glasshouse crops. Entomologia Exp. & Appl. 43: in press.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The problem

This study was initiated because of increasing problems to control beet armyworm, Spodoptera exigua (Hübner) with chemical insecticides. This noctuid moth species was accidentally introduced in the Netherlands in 1976 from Florida (Van Rossem et al., 1977) and became a serious pest of ornamental and vegetable crops in greenhouses.

From the start the introduced population showed tolerance and resistance to a number of chemical insecticides (Poe et al., 1973), but the use of synthetic pyrethroids was initially successful (Van de Vrie, 1977). Within a year, however, the population became resistant against this group of insecticides (Van de Vrie, 1979). Since then only two chemical insecticides, methomyl (Lannate) and diflubenzuron (Dimilin) provide effective control of S. exigua larvae and only then when they are applied frequently over a period of several weeks (Van de Vrie, 1979). The intensive use of these chemical insecticides may again lead to resistance. In addition these chemicals interfere with biological and integrated control programs and cause environmental pollution.

The development of a biological control method for S. exigua was therefore considered. Predators and parasites seemed less suitable as biological control agents, since they are not compatible with many pesticides used on ornamentals. Pathogens can be used in combination with most chemical pesticides (Jaques & Morris, 1981) and therefore offered greater potential. Bacillus thuringiensis is the most widely used microbial control agent (Payne, 1986) but its activity against beet armyworm larvae is generally low (Poe et al., 1973; Durant, 1979; Dulmage, 1981). Fungi did not seem suitable for control of lepidopteran larvae on plants in Dutch greenhouses, because of their demand for high humidity levels (Payne, 1986). Nematodes showed prospects for the control of beet armyworm pupae in the soil (Kaya & Grieve, 1982), but offer no immediate or short-term control of larvae on plants. Insect viruses, in particular nuclear polyhedrosis viruses (NPVs), however, seemed useful biological control agents because of their virulence and host specificity (Payne, 1986). Several highly virulent NPVs have been isolated from S. exigua larvae (Vlak et al., 1980, 1982; Gelernter & Federici, 1986) and preliminary studies in greenhouses indicated that they

showed promise for control of beet armyworm larvae (Vlak et al., 1982). The work presented in this thesis aims to assess the potential of nuclear polyhedrosis viruses as biological control agents of S. exigua in greenhouse crops in the Netherlands.

1.2 Spodoptera exigua

Taxonomy

Beet armyworm is a lepidopterous species belonging to the family Noctuidae. It was first described as Noctua exigua by Hübner (1808). The species was renamed Laphygma exigua (Hübner) by Hampson (1909) and given its present name Spodoptera exigua (Hübner) by Zimmerman (1958). In older Russian literature the species is sometimes referred to as Caradrina exigua (Hübner) (Steiner, 1936). Todd and Poole (1980) listed various other synonyms that were used between 1830 and 1880. Because of its world-wide pest status (WHO/FAO, 1973; Fig 1.1) it has many local names. In Africa it is called the lesser armyworm, lesser mysteryworm, pigweed caterpillar, swarming caterpillar, false armyworm, asparagus caterpillar, 'luzerne raupe', berseen worm and 'sjalotten uil' (Brown & Dewhurst, 1975). The last name was also used in Indonesia. In Great Britain the species is called small willow moth. The most widely used common name, however, is beet armyworm. Its most recently acquired names originate from Dutch greenhouse growers, who call the species 'Floridamot' or 'koppensneller' (*head-hunter*).

Distribution

S. exigua has a wide distribution in the tropical and subtropical areas of the world (Fig. 1.1). The species originates from South-East Asia. Its first records in the United States date from 1876 (Wilson, 1932). Todd & Poole (1980) reported that it is expanding its territory to Middle- and South-America. As a result of long-distance migration it may sometimes occur in more temperate regions, such as Scandinavia (Mikkola, 1970). These migrations, however, occur only in warm summers and are correlated with long periods of prevailing southern winds (French, 1969; Mitchell, 1979). Although the species may occasionally have one or two generations, it never survives the northern European winters, probably because of the lack of diapause (Cayrol, 1972; Fye & Carranza, 1973).

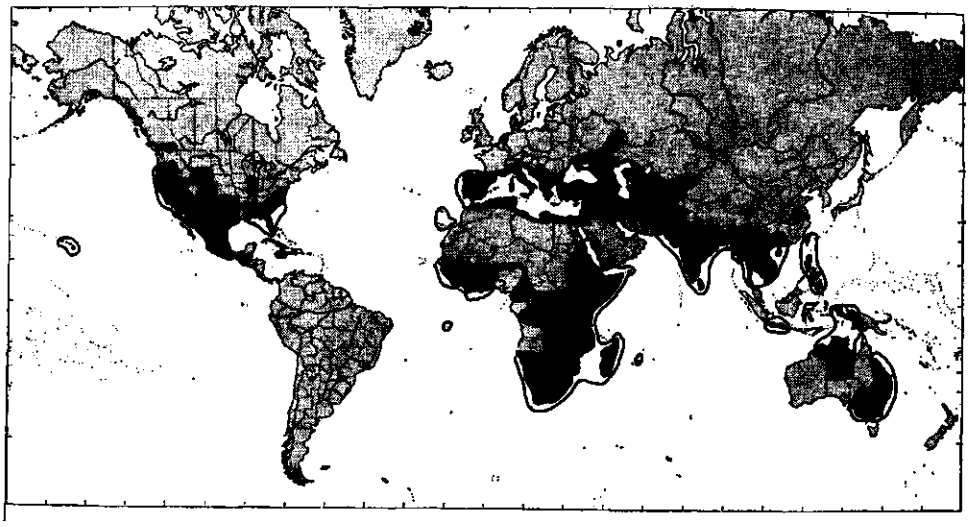

Figure 1.1 Distribution of Spodoptera exigua (after CAB, 1972).

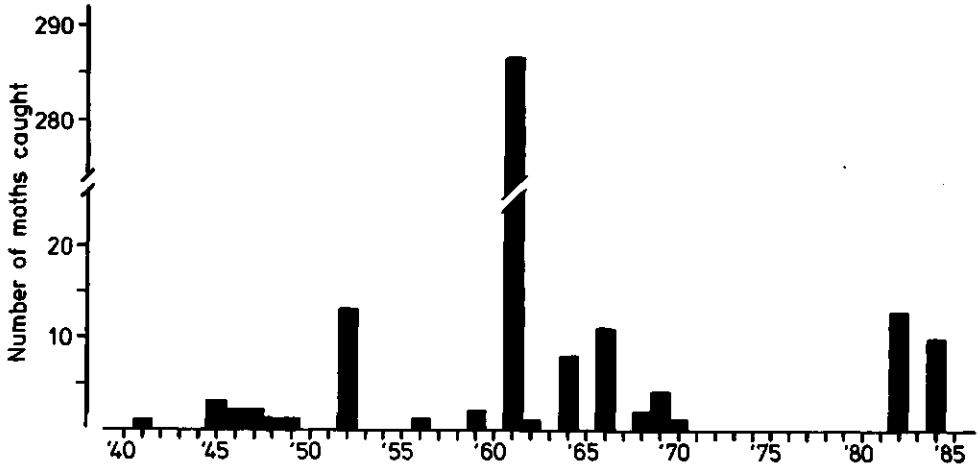

Figure 1.2 Number of Spodoptera exigua adults caught in the Netherlands, outside greenhouses, by means of light-traps in the period between 1940 and 1986 (after Lempke, 1941-1986).

In the Netherlands the species has been reported from the field (Fig. 1.2) but not in the years between 1970 and 1982 (Lempke 1941-1986). In 1976, however, the species was suddenly reported from greenhouses causing damage in chrysanthemums. Almost certainly the species was accidentally introduced with chrysanthemum cuttings, produced in Florida and flown in by plane to the Netherlands. In later shipments from Florida indeed eggs of beet armyworm were found on chrysanthemum cuttings (Van Rossem et al., 1977).

Host plants

Larvae of S. exigua are extremely polyphagous. The total list of host plants exceeds 200 plant species belonging to over 40 different plant families (Steiner, 1936; Brown & Dewhurst, 1975; Hill, 1983). Many economically important crops are among the host plants (Table 1.1) besides various common weeds and grasses.

Table 1.2 gives a list of crops, in which damage by beet armyworm larvae has been reported in the Netherlands since 1976. Infestations are still often found in chrysanthemum, but also in gerbera, saintpaulia and ornamental asparagus the species frequently occurs. From the other crops mentioned in Table 1.2 the species is occasionally reported. However, it is very difficult to get a good perspective of the frequency of occurrence in various crops since no statistical information is available.

Life cycle

The adult S. exigua is a grayish moth with a wing span of 25-30 mm (Fig 1.3). The hindwings are white with dark veins. The only conspicuous marks on the front wings are two orbicular yellow spots, which can easily be distinguished on fresh specimens. Eggs are laid in batches of maximum 150 eggs, and are usually covered with hairs and scales. At first the eggs have a greenish colour, but with age they turn grey and finally black, some hours before the larvae hatch.

Generally, there are five larval instars followed by a prepupal and pupal stage. A proportion of the larvae, 23% at 25°C and 54% at 15°C (Kunneman, 1977), has six instars. The various instars can be distinguished by the width of their head (Table 1.3), or by the length of the body and its colouration. First and second instar larvae, 1.5-2.5 and 2.5-5 mm in size, are pale yellow-green with black head capsules. Third instars, 5-9 mm in size, have brownish head capsules and show two lateral and one dorsal line, in between which also two median lines can be present. Fourth and fifth instars, 9-15 and 15-25 mm in size, also have

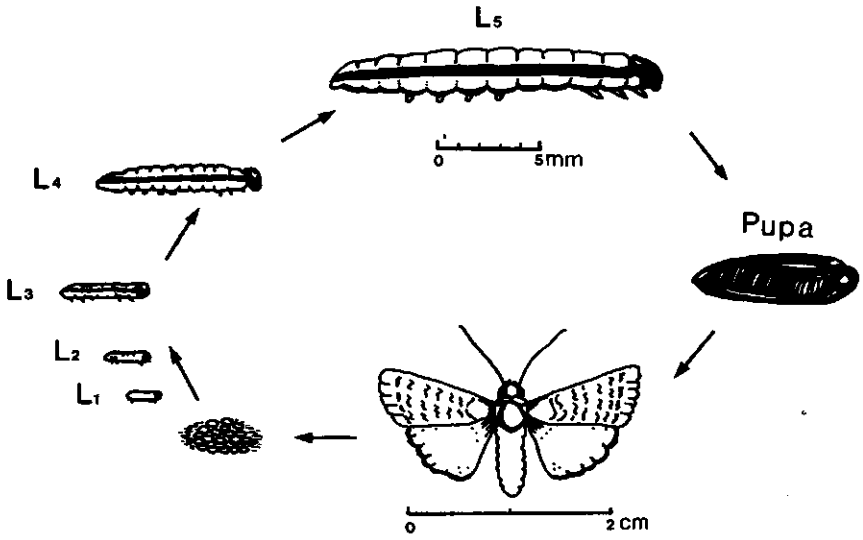

Figure 1.3 Life cycle of Spodoptera exigua.

Table 1.1 Cultivated plants reported to be attacked by Spodoptera exigua larvae, throughout the world.

Alfalfa	Clover	Lentil	Pea	Spinach
Apple	Coffee	Lettuce	Pear	Strawberry
Asparagus	Cotton	Maize	Potato	Sugar beet
Barley	Eggplant	Marigold	Purslane	Sunflower
Bean	Eucalyptus	Millet	Radish	Sweet pepper
Cabbage	Grape	Mint	Rice	Tobacco
Carrot	Hemp	Oat	Safflower	Tomato
Chilli	Indigo	Ochra	Sage	Turnip
Citrus	Jute	Onion	Sesame	Wheat

data: Steiner (1936), Brown & Dewhurst (1975) and Hill (1983).

brownish heads and conspicuous lateral lines. The median and dorsal lines can be obscured by the sometimes dark pigmentation of the back (Steiner, 1936; Cayrol, 1972; Brown & Dewhurst, 1975; Todd & Poole, 1980). The colour of the older larvae ranges from yellow and green to almost black, even if they originate from the same egg batch. High larval density (Faure, 1943) and low temperatures (Wilson, 1932) have been mentioned as factors causing an increase of the pigmentation. In the prepupal stage the larvae shorten and start to make a cocoon in the soil. Pupae are brown and similar to those of many other noctuids.

The rate of development and the relative duration of each stage are strongly temperature dependent (Table 1.4). At a temperature of 30°C a generation cycle is completed in ca. 20 days. The quality and type of food plant may influence the rate of larval development as was shown by Al-Zubaidy & Capinera (1984). Adult emergence is generally followed by a two-day pre-oviposition period. Individual females may lay up to 1600 eggs, but on average between 500-600 eggs are deposited predominantly between the third and eighth day after ecdysis (Fye & McAda, 1972). Adults may live for 10-20 days.

Chemical control

Control of the beet armyworm is mainly based on the use of chemical insecticides. Chemical control of the species, however, has become difficult in particular in North-America, because of the development of resistance against a large number of pesticides.

Early records of resistance of this species against chemical insecticides were given by Wene & Sheets (1961), who reported resistance to DDT and toxaphene. Poe et al. (1973) reported that larvae in floral crops in Florida had a high tolerance to a number of insecticides. This was confirmed by Cobb & Bass (1975) who found that larvae from the Floridan population of S. exigua were less susceptible to a number of chemical insecticides than larvae from the Californian population. Further development of resistance has been reported from South-Carolina (Durant, 1979) and Mexico (Alava & Lagunes, 1976).

Methomyl (Lannate) is nowadays a widely used insecticide to control beet armyworm, but Meinke & Ware (1978) already reported that populations showed tolerance to this chemical and that the potential for resistance was present. The use of diflubenzuron (Dimilin), a chemically-produced insect growth regulator, against beet armyworm larvae currently increases in the Netherlands. Coudriet & Seay (1979) showed that this chemical can be an effective control agent for beet armyworm larvae. Robb & Parrella (1984) screened several insecticides for the control of beet armyworm in chrysanthemums in California

Table 1.2 Greenhouse crops on which Spodoptera exigua occurs in the Netherlands.

Asparagus	Eggplant	Rose
Bouvardia	Geranium	Saintpaulia
Carnation	Gerbera	Sweet pepper
Chrysanthemum	Kalanchoe	Tomato
Cucumber	Lettuce	
Cyclamen	Peperomia	

data: P.H. Smits & M. van de Vrie (pers. observ.)

Table 1.3 Average headwidth (in mm) of Spodoptera exigua larvae with five and six larval instars.

	Larval instar					
	1	2	3	4	5	6
Five moults	0.26	0.38	0.63	1.11	1.71	-
Six moults	0.26	0.38	0.56	0.87	1.29	1.90

(after Kunneman, 1977)

Table 1.4 Rate of development (in days) of Spodoptera exigua larvae reared at different temperatures.

		Larval instar						
	Egg	1	2	3	4	5	Pupa	Total
20°C	5.6	3.6	2.9	2.8	3.3	6.1	10.4	34.7
25°C	2.9	3.2	1.9	1.7	2.1	4.1	7.7	23.6
30°C	2.0	2.6	1.5	1.2	1.5	3.1	5.1	17.0
33°C	1.8	2.0	1.2	1.2	1.3	2.5	5.1	15.1

data: Fye & McAda (1972)

and obtained reasonable to good control efficacy with chlorpyrifos (Dursban 4E) and diflubenzuron.

Natural control

Among the natural control agents of beet armyworm are many parasite and predator species. Steiner (1936) collected and described various ichneumonid and tachinid parasites of S. exigua in Asia Minor. Afify et al. (1970) reported five parasites of beet armyworm in Egypt. Eveleens et al. (1973) showed that predators and parasites are capable of keeping larval populations at relatively low levels in Californian cotton fields. Only after insecticide applications, that killed the natural enemies such as Lygus hesperus, beet armyworm became abundant and caused severe damage. Oatman et al. (1983) collected fourteen species of parasites of beet armyworm, which together caused 25-85% parasitism. Hogg & Gutierrez (1980) estimated that the overall natural mortality of beet armyworm by predators, parasites and pathogens, could be as high as 98.6%.

Among the pathogens a nuclear polyhedrosis virus (SeMNPV) occurs most frequently as a natural disease of S. exigua (Steinhaus, 1949; Hunter & Hall, 1968a; Gelernter & Federici, 1986). Almost yearly this virus causes epizootics in Californian populations of S. exigua, killing off most of the population at the end of the season. A microsporidian protozoa, which does not kill the larvae but causes a more chronic disease, is frequently found in beet armyworm populations in Dutch greenhouses (P.H. Smits, pers. observations). The fungus Nomuraea rileyi has been reported to cause epizootics in beet armyworm populations in Nicaragua (E. den Belder, pers. comm.).

1.3 Nuclear polyhedrosis viruses (NPVs)

Properties of NPVs

Nuclear polyhedrosis viruses (NPVs), together with granulosis viruses and non-occluded baculoviruses, belong to the Baculoviridae, one of the 10 virus families that occur in insects (Tinsley & Kelly, 1985). Unlike members of the other virus families, baculoviruses are restricted to insect species and crustaceans (Burges et al., 1980a). In most cases their host range is limited to a single or a few related species. Over a 1000 baculovirus-insect associations are known (Martignoni & Iwai, 1981). The majority (800) of these involve NPVs. Among the species infected by NPVs many important lepidopteran pests of cultivated crops are found.

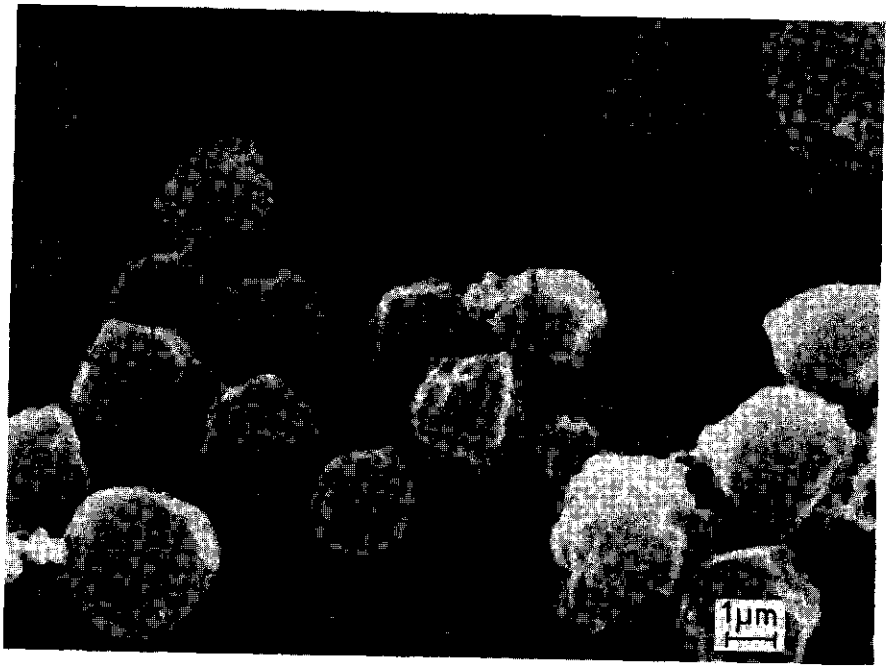

Figure 1.4 Scanning electron micrograph of polyhedra of MbMNPV-NL82. (photo M. Mentink)

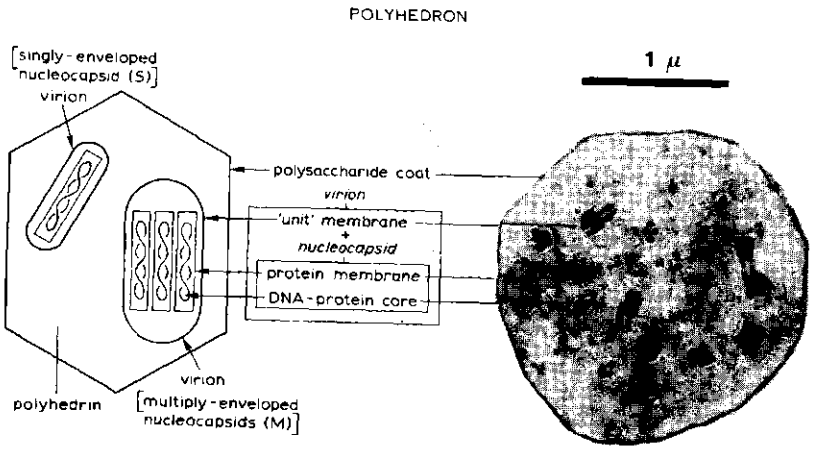

Figure 1.5 Transmission electron micrograph (right) and schematic representation of the structure of a polyhedron.

NPVs are characterized by rod-shaped nucleocapsids (250-400 x 40-70 nm) (Fig. 1.5) containing double-stranded circular DNA (Tinsley & Kelly, 1985). Nucleocapsids are enclosed within an envelope and then called virions. In some NPVs only one nucleocapsid is present per envelope, and they are therefore designated S(ingly-enveloped)NPVs. In others, referred to as M(ultiply enveloped)NPVs, 2-8 nucleocapsids are enclosed in an envelope (Fig.1.4). The virions are occluded in large (1-10 micron) cuboidal or polyhedral proteinacious bodies, referred to as polyhedra, polyhedral inclusion bodies (PIBs) or (polyhedral) occlusion bodies ((P)OBs) (Fig. 1.4 & 1.5). The replication and assembly of these bodies occurs almost exclusively in the nucleus of infected cells.

Infection process

The natural route of infection is by ingestion of polyhedra or in some cases virions by the insect larvae. Usually only the larvae are susceptible. After polyhedra are ingested with the food the proteinaceous occlusion bodies dissolve under the alkaline conditions (pH > 9) in the larval gut. The virions are liberated and infect the midgut epithelial cells. In the nucleus of infected cells new virions are produced which may leave the cell and infect cells of the haemocoel and other tissues, such as the fat body. In these tissues occlusion of virions in polyhedra takes place and this process continues until cell-lysis. One to two days after infection polyhedra can be detected in nuclei of infected cells. The larvae usually die several days later after most tissues have been infected. At death up to 30% of the dry weight of a virus-deceased larva may consist of polyhedra. The larvae completely disintegrate and the polyhedra are spilled on the foliage and soil, available for infection of other larvae. Many predators and parasites feeding on dead and diseased larvae have been shown to transmit and distribute the virus (Evans & Entwistle, 1982). In birds and hemipterans, for instance, polyhedra pass through the digestive track without losing their activity (Entwistle et al., 1977).

Virus and environment

Polyhedra are subject to environmental factors after release from deceased larvae. They are resistent to abiotic factors such as drought, humidity, pressure and acids, but are rapidly inactivated by sunlight. In particular ultra-violet radiation between 280 and 320 nm is detrimental for the virus. Generally, most virus present on foliage is inactivated within a few days after

application. Small numbers of polyhedra situated at protected sites may persist longer (Jaques, 1985).

In soil polyhedra were found to retain their biological activity for periods of more than 10 years (Jaques, 1985). Via splashing rain polyhedra can again contaminate foliage, but it has also been shown that larvae crawling through the soil pick up virus particles which later may contaminate foliage. Particularly with forests pests much attention has been given to the epizootiology of virus diseases (Cunningham, 1982).

Virus as control agent

Viruses have been used to control insect pests in the classical way of biological control by inoculative release of a virus into the population or by inundative application as a biological insecticide (Payne, 1986). In the latter case virus is applied whenever larval populations exceed damage thresholds. Fine examples of the classical method are the control of Gilpinia hercyniae in spruce forests in Canada (Bird & Elgee, 1957) and the control of the Rhinoceros beetle, Oryctes rhinoceros, a pest of palm trees on South Pacific islands by releasing virus infected adults (Bedford, 1981).

Many baculoviruses have been reported from insect pests of economic importance in agriculture and forests (Martignoni & Iwai, 1981). In field trials at least 40 different viruses have given control efficacy comparable to that of chemical insecticides (Yearian & Young, 1982; Cunningham, 1982; Entwistle, 1983). Only seven of them have been developed into commercially available products (Payne, 1986; Cunningham & Kaupp, 1986). Reasons for this are often the relatively high production and registration costs of virus products in combination with a small potential market. Other problems are their short persistence on crops because of UV-inactivation and their relatively slow speed-of-kill. Larvae are not likely to die or stop feeding within 3-4 days after virus application (Payne, 1986).

Most commercial products based on baculoviruses (Gypcheck, TM-Biocontrol-1, Neocheck-S, Lecontvirus) therefore were developed to control insect pests in forests (Cunningham, 1982; Payne, 1986) where economic thresholds are high, cosmetic damage can be tolerated and the use of chemical insectides is not allowed.

The best example of a baculovirus product that has been commercially developed for the use in agriculture is the SNPV of Heliothis, produced and marketed by Sandoz under the name Elcar for use on cotton (Ignoffo, 1973; Ignoffo & Couch, 1981). Despite the fact that it proved to be an effective and

cheap control agent it has not been a commercial success and production has meanwhile been stopped (Cunningham & Kaup, 1986). Recently, Mamestra brassicae MNPV (Mamestrin) and Cydia pomonella granulosis virus (Decyde) (Richards, 1986) have been developed into commercial products (Cunningham & Kaupp, 1986)

Resistance to virus

Development of resistance against chemical insecticides by insect pests nowadays stimulates research on biological control methods, assuming that insects will not develop resistance against these control agents, or at least not as fast as against chemicals. With regard to baculoviruses little is known on the potential of insects to develop resistance. Briese & Podgwaite (1985) recently reviewed the literature and concluded that theoretically the prerequisites for the development of resistance against viruses are present. Variability in response between individuals is often found and selection pressures in some cases have shifted the mean response-level of populations. Insusceptibility, however, has never been induced even by severe selection pressures (Briese, 1985). Possible resistance in insects probably may be overcome by small genetic changes in the virus. These changes occur frequently in nature (Vlak & Rohrman, 1986) and sometimes have a strong influence on virus pathogenicity (Wood et al., 1981).

Production

All NPVs used on a large scale are produced in laboratory-reared larvae. Generally, these larvae are infected by adding virus to their food. After several days the diseased or dead larvae are collected and the virus is liberated from the larval tissues and further processed to a concentrated suspension or dry powder (Shapiro, 1982).

Production in cell culture of some NPVs is nowadays feasible on a small scale, but a major breakthrough has not yet occurred despite years of intensive research. The main problems with the production of viruses in cell culture systems are the relatively high costs of the culture medium and the increase in scale of the production using larger fermentors (Hink, 1982; Tramper & Vlak, 1986).

Processing of the virus can range from grinding dead larvae including the rearing diet to laborious purification procedures (Krieg et al., 1979; Shapiro, 1982). For the efficacy as a control agent purification is not necessary and even may have an adverse effect, as the contaminants often give some protection against ultra-violet radiation. Some purification, however, is generally required to make the virus product suitable for application with spraying

equipment and to meet with the registration demands set by the authorities (Shapiro, 1982).

Registration

Government registration demands for commercial products based on NPVs have been an obstruction in the development of these products and have limited the use of these viruses as insect control agents. Registration of the Heliothis NPV (Elcar) took more than seven years (Ignoffo, 1973). Nowadays authorities have somewhat attenuated their requirements for registration, but the high investments involved in registration still discourage commercial development of pathogen-products with limited markets. Registration demands usually involve toxicity and sensibilisation tests on a number of vertebrates, assessment of the environmental risks, as well as proof of purity and identification of the agent (Burges et al., 1980b; Harrap, 1982).

In the Netherlands pathogens are still covered by the same guidelines for registration as chemical pesticides. New guidelines for registration of biological control agents are momentarily under development.

Safety

It is the general opinion that baculoviruses are absolutely safe and that no risks, concerning public or environmental health, are involved in their use as insect control agents (WHO/FAO, 1973; Summers et al., 1975). Their host range is generally limited to one or at most a few related insect species. Many studies with a large number of different organisms including humans, have never shown a single case in which a baculovirus had any adverse effect on any other species than its host insects (Burges et al., 1980a; Harrap, 1982; Döller, 1985). Furthermore many humans are and have been daily exposed to these viruses as they naturally occur in large quantities (10^6 polyhedra per cm^2) on vegetables such as cabbage (Heimpel et al., 1973), apparently without any negative effects.

1.4 Greenhouses

Conditions

Greenhouses provide a special environment, which has effects on insect pests and the control methods used against these pests. In nearly all Dutch greenhouses crops are grown year-round. This creates an ideal situation for insects pests since a relatively consistent climate and ample food are available throughout

the year. Some insects are especially adapted to this system and have lost their ability for diapause (Van den Bos, 1983a). For introduced tropical and subtropical species that normally do not survive the northern European climatic conditions, except for a few weeks in summer, greenhouses offer an environment to maintain themselves throughout the year. Examples are the greenhouse whitefly, leafminers and beet armyworm. On the other hand the greenhouse situation also offers some advantages for the use of biological control methods (Van Lenteren, 1983). The number of pest species is generally limited and because of the rather constant environmental conditions predator and parasite populations can be maintained over long periods.

The generally glass-roofed greenhouses offer great potential for the use of pathogens because the most detrimental part of sunlight, UV-radiation between 280-320 nm, is absorbed by the glass. It can be expected that the persistence of microbial control agents is enhanced considerably compared to the situation in crops grown in the open, where after a sunny day 90% of the virus may be inactivated (Jaques, 1985).

Crops, acreage and economics

The most important crops grown in Dutch greenhouse industry are tomato, rose, chrysanthemum and cucumber (Table 1.5). The total surface is limited to only ca. 8000 hectares, which provides a small potential market for a specialized control agent. The amount of money earned on this acreage exceeds 5,000 million Ducth guilders (DFl.) a year, which means an average production of over DFl. 600,000 (ca. $480,000) per hectare (LEI/CBS, 1986). Energy to heat the greenhouse contributes 20-30% of the total production costs, pesticides only ca. 5%. For chrysanthemum both cost factors were calculated at DFl. 150,000 and DFl. 25,000 per ha per year (GCRES, 1981). Plant material costs were estimated at DFl. 162,500 per ha per year. Pest control thus is only a minor cost factor. The efficacy of a control agent and the protection of the valuable crop is generally of more concern to the grower than the price of the pesticide.

Pest control

Many growers of vegetable crops nowadays use biological control programs for large parts of the season (Van Lenteren et al., 1980). The use of broad spectrum insecticides against new introduced pests as the beet armyworm and the leafminer Lyriomyza trifolii interferes with these programs and efforts are therefore made to develop control methods compatible with the existing biological control

Table 1.5 Most important crops grown in greenhouses in the Netherlands in 1985.

Crop	Hectares	Yearly value at auction (in million Dutch guilders)
Cutflowers		
Total	3087	2501.0
Rose	758	478.5
Chrysanthemum	518	422.3
Carnation	358	180.0
Freesia	320	149.1
Gerbera	267	143.2
Vegetables and fruits		
Total	4559	2031.0
Tomato	2138	870.4
Cucumber	703	389.7
Sweet peppers	303	100.3
Potplants		
Total	686	855.0
Ficus	-	40.3
Dracaena	-	32.0
Begonia	-	32.0
Bromelia	-	31.0
Saintpaulia	-	27.2

data: LEI/CBS (1986)
-: exact data unknown

systems. NPVs are compatible both with chemical and with biological control methods (Jaques & Morris, 1981).

In ornamental crops grown in greenhouses the situation is more difficult for the introduction of biological control schemes, although the same insect pests may occur. The level of damage tolerated is extremely low, even limited cosmetic damage is inacceptable in many instances. In addition 80% of the flowers are exported to countries that generally only allow import of completely insect-free material. Biological control methods using parasites and predators do not fit well into this situation as they are generally based on keeping both pest insect and control agent present, although at low levels. Pathogens, such as nuclear polyhedrosis viruses, that can be used as pesticides whenever the insect pest occurs, and that are compatible with chemical control agents probably are easier fitted into existing control schemes in ornamentals.

1.5 Introduction to the chapters

Nuclear polyhedrosis viruses (NPVs) are attractive candidates to control S. exigua as they are highly virulent, relatively easy to produce and safe for non-target organisms. Greenhouses provide a stable and protected environment where UV-radiation, a major constraint to the use of NPVs in the field, does not penetrate as it filtered out by the glass coverings. For an assessment of the real potential of NPVs as control of beet armyworm, more knowledge and information on a number of aspects is required.

The temporal and spatial distribution of S. exigua in a greenhouse determines when and where virus applications will give the best control efficacy. The ovipostion (Chapter 2) and the larval feeding and dispersion behaviour (Chapter 3) are therefore studied.

The NPV-isolate most suited for the control of S. exigua is to be selected out of a number of potential candidates (Chapter 4). Since beet armyworm larval populations in greenhouses usually consist of a mixture of developmental stages, the susceptibility of all these stages to the virus, has to be determined to assess its control potential against both young and old larvae (Chapter 5)

Nuclear polyhedrosis viruses are generally used as short-term pest control agents by applying large amounts of virus to a crop. The possibility of mass-producing virus of good quality at reasonable costs thus is an important factor for its practical use (Chapter 6).

Spraying systems may strongly influence the control efficacy of pesticide applications. The efficacy of virus applications with several sprayers that are used in greenhouses is therefore studied (Chapter 7). Finally the rate at which virus has to be applied to give adequate control of beet armyworm populations and to provide crop protection is determined (Chapter 8). Since beet armyworm is an important problem in chrysanthemums, the majority of the experiments were carried out in this crop. When appropriate other crops were also included in the experiments.

The practical situation in greenhouses and various commercial aspects, such as cost of the product, potential market, competition, compatibility, safety and registration requirements are discussed (Chapter 9) before an assessment is made of the potential of nuclear polyhedrosis viruses as a practical means of controlling beet armyworm populations in greenhouse crops in the Netherlands.

CHAPTER 2

OVIPOSITION OF SPODOPTERA EXIGUA IN GREENHOUSE CROPS

2.1 Introduction

Beet armyworm, Spodoptera exigua (Hübner), is a polyphagous pest on many important cultivated crops (Table 1.1). It has a worldwide distribution in tropical and subtropical regions (Steiner, 1936; CAB, 1972; Brown & Dewhurst, 1975; Fig. 1.1). An insecticide-tolerant strain of this species was accidentally introduced in Dutch greenhouses in 1976 with chrysanthemum cuttings from Florida (Van Rossem et al., 1977) and since has spread to many ornamental and vegetable crops in heated greenhouses. Infestations have been recorded in the Netherlands on chrysanthemum, gerbera, asparagus, carnation, rose, bouvardia, cyclamen, saintpaulia, geranium, tomato, sweet pepper, cucumber and lettuce (Table 1.2).

In commercial greenhouses ornamental crops of various ages are often grown simultaneously. In this chapter the distribution of egg batches in chrysanthemums of different ages, and in tomato, gerbera, and geranium crops is studied and the consequences for monitoring and control of beet armyworm are discussed.

2.2 Materials and methods

Insects

The insects used in the experiments were taken from a laboratory colony regularly supplied with freshly collected larvae from greenhouses to prevent extensive inbreeding. Larvae were reared on semi-synthetic diet, modified after Poitout & Bues (1974), at 30°C, 70-80% relative humidity and a 16:8 (L:D) photoperiod. Petridishes and disposable plastic trays covered with paper tissues were used as rearing containers for groups of 30-200 larvae. Vermiculite was added to facilitate pupation. Pupae were collected and transferred to oviposition cylinders. The walls of the cylinder were covered with paper for egg deposition and adult moths provided with a 20% sugar solution. The sex ratio of the pupae was ca. 1:1. Eggs were surface-sterilized with formaldehyde vapor to eliminate external microbial contamination (Bathon & Gröner, 1977).

Experimental setup

The experiments on oviposition were carried out in a greenhouse (4 by 6 m). Temperatures ranged from 23 to 35°C in the daytime, depending on the outside weather conditions, and were kept at 23°C during the night. Relative humidity in the greenhouse varied but was generally ca. 60%. The experiments were conducted one at a time to avoid interaction.

Four oviposition experiments on chrysanthemum were carried out with three different cultivars: 'Super White Spider' (experiment A), 'Horim Milonka' (experiments B and C), and 'Gelac' (experiment D). Rooted cuttings were provided by commercial companies. The plants were grown in six-row plots with 12 cm spacing and regularly supplied with water and fertilizers. The distance between two plots was ca. 50 cm.

Experiment A consisted of nine plots of 102 plants of three different ages. The plots with plants 15, 40, and 75 cm high (2, 5, and 8 weeks old, respectively), were placed in a latin square design. Experiment B was carried out with eight plots of nine plants of two different ages. The plots with plants 10 and 60 cm high (1 and 7 weeks old, respectively) were positioned alternately in a circle with 2 m diameter. Experiment C was carried out in two parallel plots of 250 plants 30 and 130 cm high (4 and 13 weeks old), respectively. Experiment D was carried out with 700 plants 180 cm high (18 weeks old), divided over three separate plots.

Pupae were placed, one per three plants, between the plots, divided evenly over 1-6 release-points. All plants present in the experiments were examined for eggs.

An experiment with gerbera 'Fleur' was conducted with 108 plants in 12 rows of nine plants grown in rockwool substrate. Each plant had 80-100 leaves and one to three flowers. A total of 150 pupae was placed at two central release-points. All leaves of one-third of the plants (randomly chosen) were sampled for eggs.

Distribution of egg batches in tomato 'Moneymaker' was studied on 210 plants that were 150 cm high and grown in soil with 50 cm spacing. The plants were flowering and bearing unripe fruits. In total, 150 pupae were placed at six release-points. All leaves of 66 randomly chosen plants were searched for eggs.

Oviposition in geranium 'Rono' was studied on 210 plants 30 cm high with two to three flowers. The plants were grown in pots placed in soil. A total of 100 pupae was released at four points between the plants. All plants were examined for eggs.

Sampling for eggs was done 3-5 days after the first egg batches were noticed in the crop. Within this period the moths deposit most of their eggs and only a few eggs hatch.

Statistical analysis

The data on the number of egg batches laid in various crops (Table 2.3) were analysed with a χ^2-test ($P < 0.05$). Data on the average size of egg batches between crops were subjected to paired t-tests ($P < 0.05$). Data on the size of egg batches at different heights within crops were grouped in three zones, within 5 cm of the soil surface, between 5 and 10 cm and above 10 cm, respectively. The averages per zone were subjected to a paired t-test ($P < 0.05$).

2.3 Results

Vertical distribution of egg batches

Eggs were predominantly found on the lower leaves of all plants (Table 2.1). On chrysanthemum plants of various heights and cultivars 98%, 79% and 75%, respectively, of the egg batches was deposited within 10 cm of the soil surface on the lowest five or six leaves (Fig. 2.1, Table 2.1).

Table 2.1 Vertical distribution of Spodoptera exigua egg batches on chrysanthemum, tomato and geranium.

Crop	Plant ht(cm)	Total no. of egg batches	% of eggs at different hts (cm) 0-5	6-10	11-15	16-20	21-top
Chrysanthemum:							
'Horim Milonka'	30	90	86	12	2	0	0
'Horim Milonka'	130	72	47	32	6	4	11
'Gelac'	180	65	40	35	6	5	14
Tomato	180	58	41	28	0	5	26
Geranium	30	172	42	41	10	4	3

On tomato and geranium 69% and 83%, respectively, of the egg batches was laid within this zone (Table 2.1, Fig. 2.2 & 2.3). Also, in gerbera most egg batches were laid on foliage near the ground but the growth habit of this plant prohibits a clear separation into foliage layers.

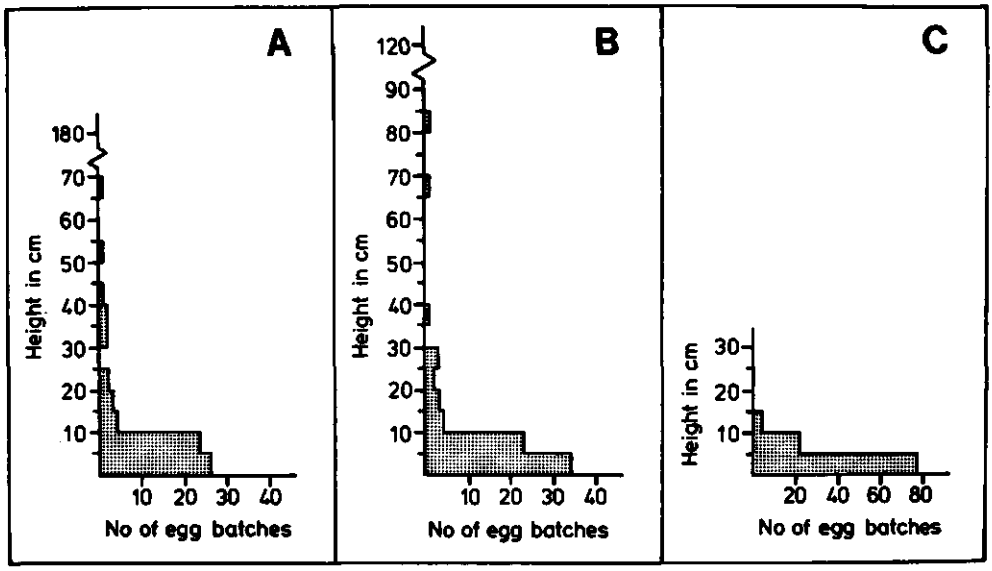

Figure 2.1 Vertical distribution of Spodoptera exigua egg batches on chrysanthemum cultivars of different height. A: 'Gelac' (80 cm), B: 'Milonka' (130 cm), C: 'Milonka' (30 cm).

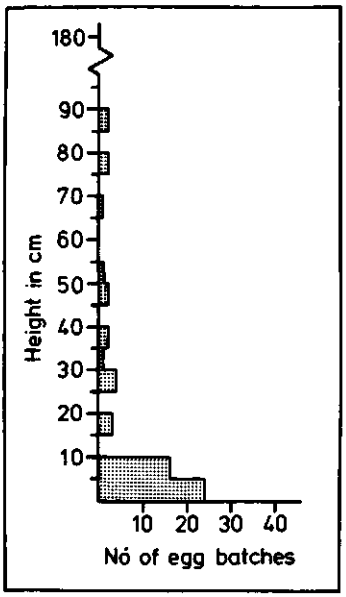

Figure 2.2 Vertical distribution of Spodoptera exigua egg batches on 180 cm high tomato plants.

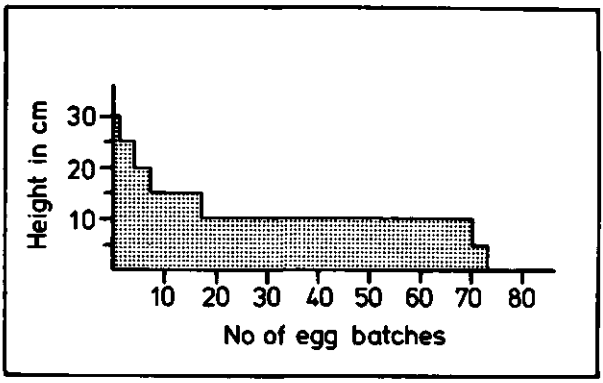

Figure 2.3 Vertical distribution of Spodoptera exigua egg batches on 30 cm high geranium plants.

Oviposition sites

Over 89% of the egg batches was laid on the underside of leaves (Table 2.2). Almost 10% of the egg masses on geranium was laid on the stem. The number of eggs laid on flowers and fruits was very small. One and two egg batches were found on the underside of geranium and gerbera flowers, respectively. No eggs were found on flowers or fruits of tomato.

Table 2.2 Number of Spodoptera exigua egg batches deposited on various parts of plant species studied separately.

Crop	Foliage		Stem	Flowers	Fruits
	Underside	Upperside			
Chrysanthemum°	220	6	1	-	-
Tomato	54	4	0	0	0
Gerbera	68	18	0	2	0
Geranium	146	10	15	1	-

° Combination of three cultivars (see Table 2.1).
- Not present on plants.

Ovipostion on chrysanthemum crops of various ages

When chrysanthemum crops of different age were simultaneously present in the greenhouse, most egg batches were laid in the youngest crop (Table 2.3). In experiments A and B, in which the youngest crops were only 1-2 weeks old, the difference in oviposition was distinct. In experiment C, however, in which the youngest crop was ca. 4 weeks old, there was no significant difference in the number of egg masses on the two ages. This was also true of 5-week-old and 8-week-old crops in experiment A.

Table 2.3 Oviposition by Spodoptera exigua on chrysanthemum crops of different heights.

Expt	Cultivar	Plant ht (cm)	Total no. of egg batches
A	'Super White Spider'	15	241 a°
		40	58 b
		75	44 b
B	'Horim Milonka'	10	67 a
		60	13 b
C	'Horim Milonka'	30	90 a
		130	72 a

°: Numbers followed by the same letter are not significantly different (χ^2-test, $P < 0.05$).

Size of egg batches

The average size of egg batches laid on chrysanthemum ('Milonka' 30 cm and 130 cm high and 'Gelac'), tomato and geranium crops was not significantly different and ranged from 35 ± 19 eggs on 'Gelac' to 39 ± 24 eggs on 'Milonka' 30 cm high. On gerbera, however, egg batches contained an average of 30 ± 15 eggs which was significantly lower than the averages found on the other crops except for 'Gelac' (t-test, $P < 0.05$).

The average size of egg batches laid at three different heights within crops, within 5 cm of the soil surface, between 5 and 10 cm and above 10 cm, did not differ significantly, with the exception of a higher average in the lowest zone as compared to the middle zone on geranium (t-test, $P < 0.05$). The largest egg batches found in the experiments consisted of ca. 150 eggs, but the majority contained 25-50 eggs.

2.4 Discussion

The distribution of oviposition sites on plants appeared to be independent of the plant species, cultivar or age. Within the oviposition zone, the moths discriminated between plants or plant parts, as shown in our experiments by the increased oviposition on young chrysanthemum plants (Table 2.3). In addition, Zalom et al. (1983) reported a correlation between oviposition by beet armyworm and the vicinity of flowers and fruits on tomato. Their observations were made on plants grown lying on the soil surface. We did not find such a correlation. In our situation, however, the tomato plants were grown upright (supported by strings) and flowers and fruits were present from a height of ca. 40 cm, well above the oviposition zone. Although some significant differences were found, in general the average number of eggs per batch seems independent of the position of the egg batch within the crop or the plant species on which the egg batch is deposited.

The oviposition pattern of beet armyworm is different from that of other noctuid moths in greenhouse crops, which showed no particular preference for oviposition in any foliage zone and deposited very few eggs on foliage within 5 cm off the ground (Burges & Jarret, 1976). Oviposition behaviour of noctuid moths shows a great variety of responses to host plants. Noctuids may react to factors as odour, colour, texture and shape of plants or plant parts (Prokopy & Owens, 1983; Ahmad, 1983). Suitability of the host plant for larval development and factors like humidity, temperature and protection from parasites and

predators may determine the oviposition pattern. The concentration of oviposition sites near to the soil surface may be determined by the latter factors as young larvae move upwards shortly after hatching and predominantly feed on the upper leaves of plants. The higher oviposition on very young chrysanthemums and the increased oviposition in the vicinity of both flowers and fruits which was found by Zalom et al. (1983) on tomatoes are possibly directly correlated with host-plant suitability for larval development.

For the development of monitoring systems, our results indicate that sampling for eggs in the crop can be concentrated on the lower leaves of plants. Although most eggs are laid in very young crops when they are present next to older crops, at least 20% of the eggs is deposited on the older plants. For control in situations with crops of various ages, this implies that applications have to be made on all crops present and cannot be limited only to the young crops.

CHAPTER 3

FEEDING AND DISPERSION OF SPODOPTERA EXIGUA LARVAE IN GREENHOUSE CROPS

3.1 Introduction

The feeding behaviour of a target pest organism influences the result of a pesticide application, particularly when the control agent has to be ingested to have effect. Nuclear polyhedrosis viruses are such control agents and the use of these viruses for the control of beet armyworm larvae, Spodoptera exigua (Hübner) (Lep.: Noctuidae), is being considered (Vlak et al., 1982).

Beet armyworm is a polyphagous pest of cultivated crops in tropical and subtropical regions (Steiner, 1936; Brown & Dewhurst, 1975; Chapter 1). The species became a serious pest of many ornamental and vegetable crops in greenhouses in the Netherlands after its accidental introduction from Florida in 1976 (Van Rossem et al., 1977). Eggs are laid at the underside of foliage in batches of 30-40 eggs, generally on the lower leaves of the plant within 10 cm of the soil surface (Chapter 2). At 25°C eggs hatch after three days and the life cycle is completed in about 20 days (Fye & McAda, 1972; Chapter 1).

The exposure rate of larvae to a control agent is determined by the amount of consumption but also by the position of the feeding sites in the crop. The amount of foliage consumed, the vertical distribution of feeding sites and their position at the under- or upperside of foliage was therefore studied in chrysanthemum (Chrysanthemum morifolium Ramatuelle) and tomato (Solanum lycopersicon L.,) crops. Furthermore, the horizontal dispersion of larvae was observed in chrysanthemum crops in order to assess the number of plants damaged by larvae during their development. The consequences of the larval feeding behaviour for the use of a nuclear polyhedrosis virus as a control agent for beet armyworm are discussed.

3.2 Materials and methods

Insect rearing

The insects used in the experiments were taken from a laboratory culture originating from larvae collected in Dutch greenhouses. Larvae were reared in

groups of 30-200 on semi-synthetic diet modified after Poitout & Bues (1974), at 30°C, 70-80% relative humidity and a 16 hour photoperiod. A more extensive description is given in Chapter 2.

Greenhouse

The experiments were performed in a 4 x 6 m greenhouse. The temperature ranged between 23° and 30°C during daytime, depending on the outside weather conditions, and was kept at 23°C at night. The relative humidity varied around 60%. No pesticides were applied during the experiments.

Vertical dispersion

The experiment on vertical dispersion of larvae was conducted in 6-row-beds of 'Milonka' chrysanthemums, grown in soil at 12 cm spacing. One batch of 35 eggs was fixed to the underside of a leaf within 5 cm of the soil surface on every second plant to ensure an even distribution of larvae. At 3-4 day intervals, corresponding with larval development to the next instar, 10 plants were examined for the presence of feeding marks. Damaged leaves were distinguished as having superficial feeding damage, on the leaf upper- or underside, or having perforations caused by larval feeding.

As the plants within and between the experiments differed in height and number of leaves, each plant was divided into four vertical foliage-strata. The bottom stratum consisted of six leaves that were present when the cutting was planted. The second and third stratum, named lower-middle and upper-middle, were of equal size and consisted of full-grown leaves present in between the bottom and top stratum. The top stratum consisted of the terminal shoot with about six not yet fully expanded leaves.

The average plant height at the start of each of the three experiments was 45, 55 and 80 cm, with an average of 19, 22 and 28 leaves per plant, respectively. During the experiments that lasted 18-21 days, the plants grew about 25 cm in height and formed 8-9 new leaves. Leaf contacts between neighbouring plants were present in the middle and upper foliage strata of all crops.

Experiments with 'Moneymaker' tomato were performed twice on 16 plants of 150 cm height. The plants with flowers and unripe fruits were planted at 50 cm spacing in soil and supported by strings. Eggs were deposited in the crop by released moths. When the larvae were about to pupate the plants were examined

for the presence of feeding marks. The data were grouped per leaf, which were numbered starting from the bottom.

Horizontal dispersion

The experiments on the horizontal dispersion of larvae were carried out on groups of 120 'Milonka' chrysanthemums, planted in 8 rows per bed at 12 cm spacing. In the middle of the plot a batch of 35 eggs was fixed to the underside of a leaf near the soil surface. The plants were examined daily for the presence of feeding marks. The experiments were terminated when the larvae were about to pupate. Four experiments were conducted, two with plants of about 20 cm and two with plants of about 60 cm height. In the low crop only few leaf contacts between plants existed when the experiments started. The experiments lasted 20-23 days in which period the plants grew about 30 cm in height. The experiments in the low crops lasted longer as lower temperatures in the greenhouse had slowed down larval development.

Foliage consumption

The amount of foliage consumption of larvae was estimated in three experiments with leaves of 'Milonka' chrysanthemum. In each experiment five larvae were placed individually, within a few hours of hatching, in a container with excess of foliage picked from the upper-middle stratum (see above). Every two days the leaves were replaced and the area of foliage consumed was determined by tracing the outline of the damaged area on grid paper and measuring the outlined squares.

3.3 Results

Vertical distribution of feeding sites

Figure 3.1 shows that in an 80 cm high chrysanthemum crop the highest proportion of damaged leaves during the first and second larval instar was found in the lower-middle stratum. From the third instar onwards the highest percentage, however, occurred in the top stratum, but also in the lower-middle and upper-middle strata large proportions of the leaves showed feeding damage. In the bottom stratum relatively few leaves were damaged.

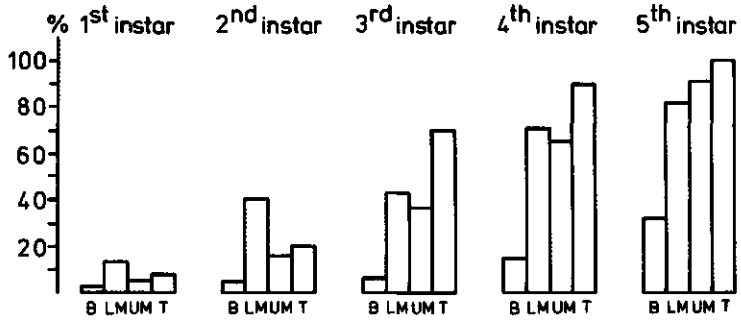

Figure 3.1 Percentages of leaves damaged by Spodoptera exigua larvae in an 80 cm high chrysanthemum crop. Plants were divided into four foliage strata: B = bottom, LM = lower middle, UM = upper middle and T = top stratum.

Experiments on plants of 45 and 55 cm height showed similar patterns although in these crops large proportions of damaged leaves in the top stratum were already found during the second larval instar. During the first instar the number of larvae on the plants diminished by about 50% but the numbers then stabilized at a level of 2-3 larvae per plant.

During the first larval instar 90% of the damaged leaves had only superficial feeding marks whereas only 10% were perforated by larval feeding (Table 3.1). During larval development the proportion of leaves with perforations increased, in particular during the fifth larval instar. Up to the fourth larval instar the perforations were small and found only in the young leaves of the top foliage-stratum. During the fifth instar all the top leaves but also 30% of the leaves in the upper-middle stratum showed large perforations.

Table 3.1 Nature of feeding marks on chrysanthemum foliage of Spodoptera exigua larvae during their development. Cumulative data, combined from experiments in 45, 55 and 80 cm high crops.

Larval instar	% Leaves perforated	% Leaves with superficial damage		
		Total	Underside	Upperside
1	10	90	87	30
2	17	83	86	40
3	30	70	91	37
4	30	70	91	46
5	49	51	92	37

Of the leaves with superficial feeding damage around 90% had feeding marks on the underside of the leaf, whereas around 40% showed feeding marks on the upperside. These proportions did not change much during larval development although the size of the superficial feeding marks increased as the larvae aged.

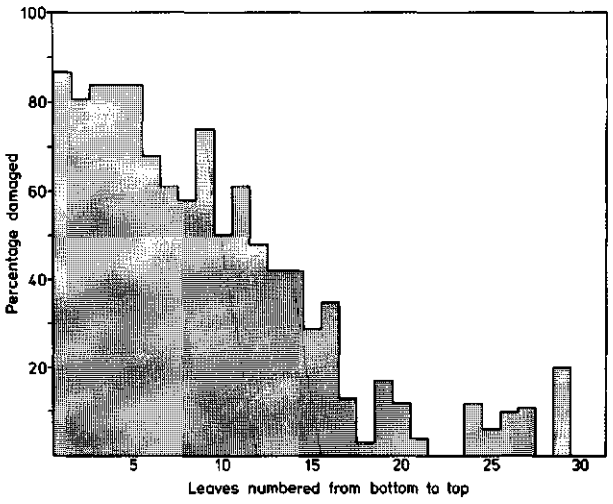

Figure 3.2 Percentage of leaves, numbered from bottom to top, damaged by Spodoptera exigua larvae in a 150 cm high tomato crop.

On tomato the vertical distribution of feeding marks (Fig. 3.2) showed a different pattern from that on chrysanthemum. Most feeding occurred on the lower leaves and the proportion of damaged leaves decreased with increasing height. No feeding on flowers was found and only 0.5% of the fruits showed damage.

First instar larvae mainly fed superficially on the underside of the leaves. Because the tomato foliage is relatively thin compared to that of chrysanthemum, feeding by older larvae generally resulted in perforation of the leaf. As in the experiments on chrysanthemum larval numbers decreased with about 50%, almost entirely during the first larval instar. At the final sampling about 10 fifth-instar larvae were present on each plant.

Horizontal dispersion

The number of plants damaged by larvae originating from a single batch of 35 eggs was larger in low than in tall chrysanthemum crops (Fig. 3.3). The difference occurred mainly during the fourth and fifth larval instar. Around the third larval instar, about 12 days after hatching, an increase in the rate of dispersion occurred in the low but not in the taller crops. In the low crops

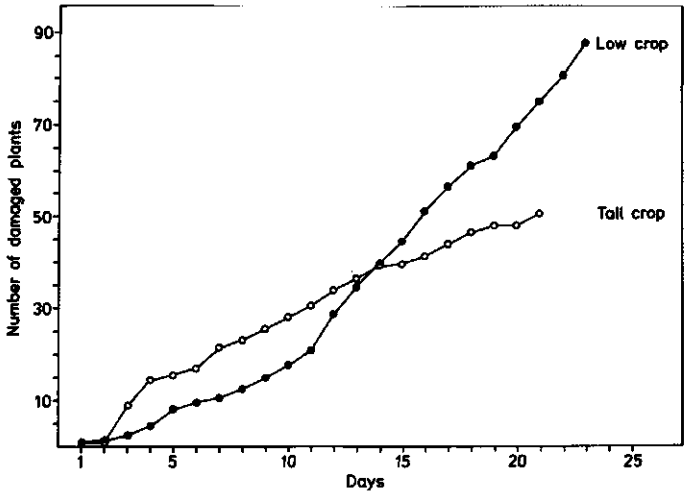

Figure 3.3 Number of plants damaged by Spodoptera exigua larvae originating from a single egg batch (35 eggs) in low (20 cm) and tall (60 cm) chrysanthemum crops.

most damaged plants, in particular those in the center of the plots, showed severe feeding marks on all leaves in the upper foliage layers. In the tall crops the plants were less heavily damaged and even those in the center of the plots had some intact top leaves. Again around 50% larval mortality occurred during the experiments, mainly during the first instar.

Foliage consumption

The total area of chrysanthemum foliage consumed during larval development was 72 cm^2 (Table 3.2), which corresponds with approximately 7 full-grown leaves. Larvae of the first three instars contributed only about 5% to the total amount of foliage consumption, whereas 75% of the feeding occurred during the fifth instar. Until the fourth instar most feeding was superficial either at the leaf under- or upperside. Fifth instar larvae consumed the entire leaf.

Table 3.2 Consumption (in cm^2 leaf area) of chrysanthemum foliage by Spodoptera exigua larval instars.

Larval instar	Cumulative consumption	% Total consumption
1	0.09 ± 0.04	0.12
2	0.48 ± 0.32	0.54
3	3.40 ± 0.41	4.01
4	18.10 ± 4.68	20.19
5	72.80 ± 25.83	75.14

3.4 Discussion

On both chrysanthemum and tomato plants over 90% of the eggs is deposited on the lower leaves within 10 cm of the soil surface (Chapter 2). In chrysanthemum the amount of damage in the upper foliage layers increased as the larvae aged, after a relatively high level of damage in the lower-middle stratum during the first and second instar (Fig. 3.1). This suggests that larvae gradually move upwards in the crop after hatching. This behaviour is in accordance with observations that young beet armyworm larvae show negative geotropic and positive phototropic reactions (Griswold & Trumble, 1985a). The same authors found that second, third and fourth instars did not show strong responses to light and that fifth-instar beet armyworm larvae showed photonegative responses. The vertical distribution of feeding sites observed in our experiments (Fig. 3.1) corresponds rather well with this pattern of phototropic responses. Larvae of other lepidopteran species showed similar behavioural responses on chrysanthemum plants in greenhouses (Jarret & Burges, 1982a).

On tomato, however, larvae predominantly fed on the lower leaves (Fig. 3.2). Although no detailed counts were made, observations indicated that some upward movement occurred but only by fourth- and fifth-instar larvae. Similar feeding behaviour on tomato plants was found in larvae of another noctuid species, Lacanobia oleracea (L.) (Jarret & Burges, 1982a). Jarret & Burges also observed that most young larvae that dropped to the ground failed to reclimb the plants and subsequently died. Possibly the upward movement, in particular of young larvae, is hindered by the large number of glandular hairs present on the stem of tomato plants.

The leaf area consumption of beet armyworm larvae on chrysanthemum was larger than that found on sugarbeet and soybean, of which respectively 32 and 51 cm^2 of leaf area were consumed during larval development (Steiner, 1936; Boldt et al., 1975). The exact amounts of consumption, however, depend strongly on the kind of host plant and the nutritional value or the plant parts offered (Griswold & Trumble, 1985b). The relative amounts of food consumption by larvae of the various developmental stadia seem rather independent of the food plant (Steiner, 1936; Boldt et al., 1975).

On some plants larvae may feed on flowers rather than on foliage. In saintpaulia damage is almost completely restricted to the flowers, where larvae feed mainly on the pollen bearing anthers (P.H. Smits, pers. observations). Feeding on flowers was also often found in gerbera, carnation, kalanchoë and chrysanthemum, although on these plants larvae also feed on foliage (Chapter 3).

The concentration of larval feeding sites on younger leaves in the upper foliage layers and, in some crops, on fruits and flowers may be explained by the presence of relatively high amounts of nitrogen and water (McNeil & Southwood, 1978). According to these authors those parts of plants therefore form a superior food source for lepidopteran larvae as compared to old foliage. The importance of nitrogen in the development of beet armyworm larvae was shown by Al-Zubaidi & Capinera (1983, 1984) and by Griswold & Trumble (1985b).

The toughness of the cuticula of foliage and fruit seems another factor that is of importance for beet armyworm larval development. Juvik & Stevens (1982) showed that mortality of young beet armyworm larvae reared on fruits of various tomato cultivars was correlated with the cuticular toughness. Soo Hoo & Fraenkel (1966a, b) showed that the toughness of foliage was one of the major factors determining the suitability of host plants for the development of a related polyphagous species, Spodoptera eridiana. Larval development was slower and mortality higher when beet armyworm larvae were reared on foliage of chrysanthemum hybrids of the 'Spider'-group as compared to foliage of hybrids of the 'Horim'-group (P.H. Smits, unpubl. results). The foliage of 'Spider'-cultivars does not contain much water and has many dense short haires. In greenhouses in which various chrysanthemum cultivars were grown, beet armyworm infestations were generally concentrated in those cultivars with fresh lush foliage. It seems, therefore, that the nitrogen and water contents as well as cuticular toughness are factors that determine beet armyworm larval feeding behaviour in various crops.

Beet armyworm larval feeding behaviour has some consequences for the use of nuclear polyhedrosis virus and other control agents that have to be ingested with the food. Especially with the virus it is important that larvae ingest a lethal dose as early as possible during their development, as there is generally one moult and 6-10 days between ingestion of a lethal virus dosis and actual death (Chapter 5.3). During most of this period the larvae continue to feed.

The distribution of feeding sites suggests that on tomato virus should be applied to the lower leaves. On chrysanthemum virus applications should be made to the lower-middle foliage stratum when very young larvae are present, but to the top and upper-middle leaves when larvae become older. With the currently used application techniques control agents are generally applied only to the upperside of leaves. Young larvae often feed superficially at the underside of foliage (Table 3.1). Calculations showed that during less than 40% of the time they feed (Smits, 1986), young S. exigua larvae actually ingest virus particles.

Virus application with electrodynamic sprayers may offer some potential in this respect as they also give some deposition on the underside of foliage. On tomato application of virus to the underside of leaves is less important than on chrysanthemum as the young larvae consume the upper epidermis more frequently.

Poe et al. (1973) stated that during their third instar beet armyworm larvae change from gregarious to solitary behaviour. Although the dispersion rate in the low crop indeed increased during the third instar, about 12 days after hatching, in general the dispersion of the larvae in our experiments continued gradually throughout larval development. This means that the earlier virus is applied, the smaller the number of damaged plants will be.

With age, larvae become less susceptible to the virus but their virus intake increases due to a larger area of foliage consumption and an increased consumption of the upper epidermis. Calculations showed that with exception of the fifth larval instar the increased virus intake roughly compensates for the decrease in susceptibility (Smits, 1986). Therefore the same virus dose can be used to control larvae of the first to fourth instar. For control of fifth instars much higher virus dosages will be required.

The general conclusion is that virus and other control agents should be applied as early as possible during larval development, but in any case before larvae reach the third instar, in order to prevent most of the feeding damage to the crop. Preventive or early applications will be most effective when the virus is applied to the underside of the lower leaves both on chrysanthemum and tomato. When older larvae are present in chrysanthemum crops, applications to the upper-middle and top leaves will give the best control results.

CHAPTER 4

SELECTION OF NUCLEAR POLYHEDROSIS VIRUSES FOR CONTROL OF SPODOPTERA EXIGUA

4.1 Introduction

Several nuclear polyhedrosis viruses (NPVs) are potential candidates as biological control agents for beet armyworm. From Californian populations of beet armyworm a multiply-enveloped NPV specific for S. exigua (SeMNPV) has been collected and described (Hunter & Hall, 1968a; Smith & Summers, 1978; Gelernter & Federici, 1986). Another baculovirus, Autographa californica MNPV (AcMNPV) has a broad host range among noctuid pests of agricultural crops and is also infectious for beet armyworm (Vail et al., 1971; Payne, 1986).

In 1980, 1982 and 1983, three MNPVs were collected from beet armyworm larvae that originated from greenhouse populations in the Netherlands. Restriction-endonuclease profiles made from the 1982-isolate (Wiegers & Vlak, 1984) appeared to be similar to the Dutch strain of Mamestra brassicae MNPV (MbMNPV) described previously (Vlak & Gröner, 1980; Wiegers & Vlak, 1984). This particular isolate was therefore named MbMNPV-NL82, although it was isolated from S. exigua larvae. The other MNPVs, isolated in 1980 and 1983, were named MbMNPV accordingly.

This study reports a comparison of the biochemical and biological properties of these five MNPVs of S. exigua. To compare the biological activity of these five MNPVs the droplet-feeding bioassay method of Hughes & Wood (1981) was used, adapted for second instar larvae. Restriction endonuclease profiles of the viral DNA were made to determine and confirm the identity and relationship between these five MNPVs. Finally, the potential of the five MNPVs as biological control agents in greenhouses is discussed with special emphasis on their virulence, host range and perspective as biocontrol agents of S. exigua.

4.2 Materials and methods

Insects

The insects used in the experiments were taken from a laboratory rearing originating from larvae collected in Dutch greenhouses. Larvae were reared in groups of 30-200 on semi-synthetic diet, at 30°C, 70-80% relative humidity and a 16 hour photoperiod. An more extensive description of the rearing is given in

Chapter 2. Mamestra brassicae larvae were obtained from the Department of Entomology, Wageningen and reared on cabbage. Leucoma salicis larvae were reared on willow and poplar leaves (Lameris et al., 1985).

Origin of MNPV isolates

Three virus isolates were obtained from single, deceased beet armyworm larvae which were collected in commercial glasshouses in the Netherlands in 1980, 1982 and 1983, respectively. Since the 1982 isolate appeared to be similar to a Mamestra brassicae MNPV isolate described earlier (Wiegers & Vlak, 1984) and since the other isolates appeared to be related to this isolate (see Results), they were named MbMNPV-NL80, MbMNPV-NL82 and MbMNPV-NL83, respectively.

Autographa californica MNPV (AcMNPV) was originally isolated from the fall armyworm A. californica (Vail et al., 1971); a plaque-purified isolate (E2) was received from Dr. M.D. Summers, Texas A&M University, College Station, Texas, USA. The Spodoptera exigua MNPV (SeMNPV) isolate, described by Hunter & Hall (1968a, b), was also obtained through Dr. M.D. Summers and is identical to the major genotype recently collected from Californian populations of beet armyworm (Gelernter & Federici, 1986).

The five viruses were propagated by infection of fourth instar S. exigua larvae via surface contamination of the semi-synthetic diet. Purification steps included grinding, filtering through cheese-cloth, and two centrifugation steps. The polyhedra were resuspended in phosphate buffered saline (PBS) and stored at -20°C until use. A more detailed description of this procedure is given in Chapter 6.

DNA isolation

Polyhedra were purified from triturated larvae by overnight incubation at room temperature with 0.1% sodium dodecyl sulphate (SDS). The SDS was then removed by sedimentation (7,500 rpm for 15 min) of the polyhedra using a Sorvall SS-34 rotor. The polyhedra were resuspended in a small amount of 10 mM Tris/HCL + 1 mM EDTA, pH 7.5 (TE). Virions were liberated from the polyhedra after incubation with 0.1 M Na_2CO_3, 0.17 M NaCl, 1 mM EDTA, pH 10.9 for 30 min at room temperature. In the case of SeMNPV, 0.4 M sodium carbonate (pH 10.5) was used with an incubation time of only 15 min.

The solution was cleared from undissolved polyhedra by centrifugation at 5000 rpm for 5 min. Virions were purified by centrifugation of the supernatant on a sugar gradient (20-55% w/v) for 20 min at 23,000 rpm in a Beckman SW-27 rotor. The 5-8 virion bands were collected with a syringe, water was added and the

virions were sedimented at 25,000 rpm for 45 min in the same rotor. After incubation with proteinase-K (100 µg/ml) for 2-3 hours at 45-50°C, the DNA was extracted by phenol and chloroform. After dialysis against two changes of 10 mM Tris/HCl (pH 8.1) for 48 hours the DNA-solution was stored at 4°C until use.

Restriction endonuclease analysis

The viral DNA was digested for 2 hr at 37°C with 5 units of restriction endonucleases EcoRI or PstI (Boehringer, Mannheim) per µg of DNA according to the manufactor's instructions. The digests were analysed on a horizontal slab gel of 0.7% agarose containing 0.5 µg/ml ethidium-bromide as a DNA stain. The DNA samples were run for various periods of time at 50 V to allow the separation of high- and low-molecular weight fragments. The DNA bands were visualized by fluorescence over a UV-light source and photographed using a Polaroid film and a Wratten 15 filter. The molecular weigths of the DNA fragments were calculated according to the method described by Southern (1979) using DNA fragments of AcMNPV (Cochran et al., 1982) as a reference.

Bioassay

Bioassays were carried out using the droplet-feeding method (Hughes & Wood, 1981), adapted for second instar S. exigua larvae. One-day-old second instars were starved for 16-20 hr at 25°C and were then allowed to drink from a solution containing 10% sucrose (to stimulate drinking), 0.05% Florella-blue (food colouring) and polyhedra at known concentrations. Suspensions of 10^6, $5x10^5$, 10^5, $5x10^4$, 10^4, $5x10^3$, 10^3 polyhedra per ml were used in such bioassays. The suspension was offered to the larvae as droplets of 5-10 µl which were applied in a circle onto a layer of parafilm on the bottom of a petridish (5 cm). About 40-50 larvae were placed in the center of the circle of droplets. After 15 minutes 32 larvae that had drunk from the suspension and which were recognised by their blue colour, were selected and transferred to diet, each into an individual cell of a 24-well microwell tissue culture plate (Costar). The plate was covered with two layers of paper tissue and by the original lid turned upside down. The construction was held together by rubber bands. Larval mortality was recorded at two or three day intervals during ten days of incubation at 25°C.

LD-50 calculation

The mortality data were processed and analysed using the probit-analysis methods described by Finney (1971). A computer programme on a Hewlett-Packard table calculator (HP-85) was kindly provided by Dr. J. Huber, Darmstadt, W-Germany.

The amount of fluid imbibed by larvae was determined using a suspension with a known concentration of ^{32}P. After drinking, the radioactivity ingested by individual larvae was determined in a scintillation counter (Packard 2450) by measuring the Cerenkov radiation. From the amount of suspension imbibed and the known concentration of polyhedra, the ingested dose (in polyhedra) was calculated.

4.3 Results

Virus identification and characterisation

When digested with EcoRI or PstI restriction endonuclease each DNA of the five MNPVs gave a unique restriction fragment pattern (Fig. 4.1). All virus isolates were thus unequivocally identified. The digestion patterns of MbMNPV-NL82, NL80 and NL83 were very similar. The total molecular weight of the three MbMNPV genomes were close to 150 kbp. Only a few fragments in each MbMNPV isolate, indicated by arrows (Fig. 4.1), were unique for each variant.

The profiles of AcMNPV and SeMNPV DNA were completely different from each other and did not show any resemblance with the DNA fragment patterns of the three related MbMNPVs. The DNA digestion patterns of AcMNPV DNA and SeMNPV DNA were identical to those originally described by Smith & Summers (1978), whereas the MbMNPV-NL82 DNA profile was similar to that described by Vlak & Gröner (1980) and identical to the isolate described by Wiegers & Vlak (1984).

The identification of the five MNPVs indicates that even though these original isolates had been propagated in larvae of the same species, the genetic make-up of the parental virus has been maintained after passage through the alternate host and that no endogenous virus had been induced in S. exigua.

Host range

S. exigua larvae were susceptible to all five MNPVs. The three MbMNPVs and AcMNPV were also infectious for Mamestra brassicae larvae, SeMNPV was not. The Lymantrid L. salicis was not susceptible to any of the five MNPVs.

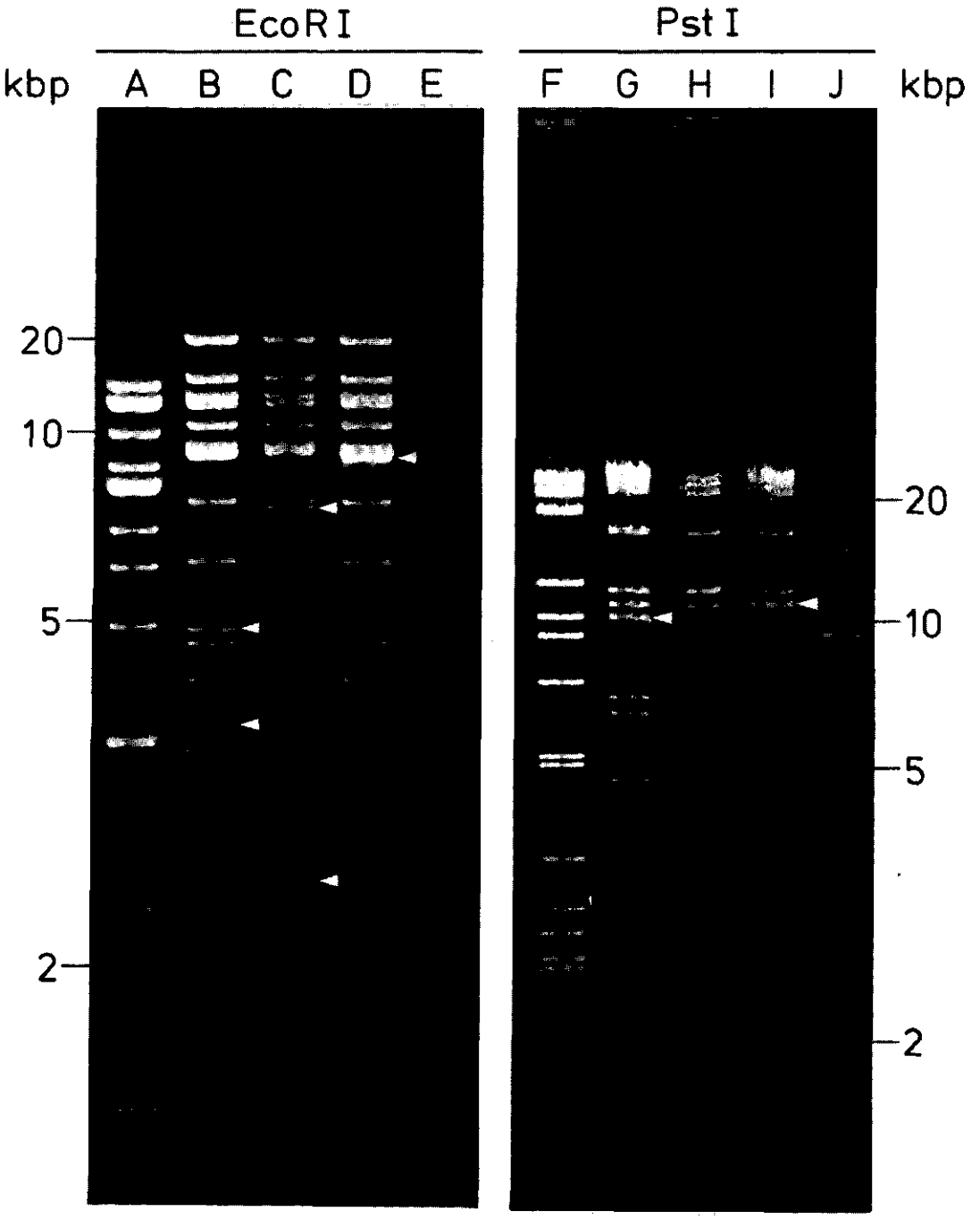

Figure 4.1 Agarose gel (0.7%) electrophoresis of AcMNPV (A,F), MbMNV-NL80 (B,G), MbMNPV-NL82 (E,H), MbMNPV-NL83 (D,I) and SeMNPV (E,J) DNA after digestion with endonucleases EcoRI (A-E) and PstI (F-J). Arrows indicate fragments unique for each of the MbMNPV isolates. Molecular weights of the fragments are indicated in kilo-basepairs (kbp).

Comparison of biological activity

The biological activity of the five MNPVs was measured with the droplet-feeding bioassay of Hughes & Wood (1981), which was adapted for the use second instar larvae. The ingestion of the polyhedra-containing suspensions worked equally well for second instars as for the first instars that were used by Hughes & Wood.

The mean volume of virus suspension ingested by the second instar larvae was 0.33 ± 0.13 µl. This allowed the calculation of LD-50 values based on the dosis-mortality data. Table 4.1 shows that the LD-50 values ranged from 3 to 26 polyhedra for the five MNPVs. The specific SeMNPV had an LD-50 which is ca. five times lower than the LD-50 value for any of the other MNPVs. The other four isolates showed comparable biological activities. Although the slopes of the probit regression lines of the three MbMNPVs and AcMNPV ranged from 0.8 to 1.6 (Table 4.1; Fig 4.2), it appeared that these lines were not significantly different.

4.4 Discussion

Based on the restriction-enzyme patterns of the viral DNAs it is concluded that the five nuclear polyhedrosis viruses studied are unique and that they maintain their genetic make-up after passage in larvae of one and the same insect species. Analysis of the progeny virus is essential here in order to exclude the possibility of induction of an endogenous virus in S. exigua.

Based on the many comigrating fragments of the viral DNAs on agarose gels (Fig. 4.1) it is concluded that the MbMNPV-NL82, -NL80 and -NL83 isolates are closely related variants. The relationship between MbMNPV-NL82 and the original MbMNPV isolate described by Ponsen & De Jong (1964) and Vlak & Gröner (1980) was already established previously (Wiegers & Vlak, 1984). Brown et al. (1981) also described a number of related MbMNPV variants and it is most likely that more MbMNPV variants are to be found. The significance of these small variations in genetic make-up remains an enigma. They may provide a mechanism to adapt to various M. brassicae physiotypes, or to cross the species boundary. This may explain the presence of the three MbMNPVs in S. exigua larvae in the greenhouse. Detailed studies on the DNA structure and host range of the various MbMNPV variants may solidify this hypothesis.

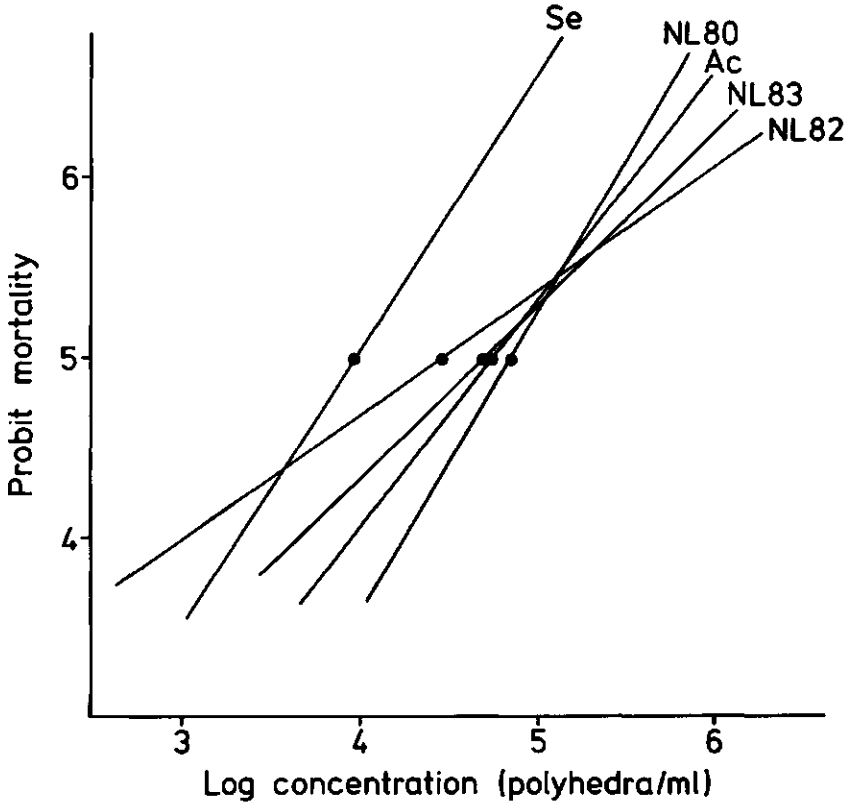

Figure 4.2 Dose-mortality relationship between AcMNPV, MbMNPV-NL80, MbMNPV-NL82, MbMNPV-NL83 and SeMNPV, and second instar Spodoptera exigua larvae.

Table 4.1 LC-50 (polyhedra (pol)/ml), LD-50 (no. of pol) and associated statistics of five MNPVs against second instar Spodoptera exigua larvae.

Virus	LC 50 (pol/ml)	LD-50 (pol)	95% Fiducial limits Lower	Upper	Slope ± SD	Intercept	χ^2/DF
SeMNPV	9.0×10^3	3.2	2.4	4.0	1.64 ± 0.20	-1.49	0.1/2
AcMNPV	5.2×10^4	18.3	13.3	23.8	1.27 ± 0.12	-0.99	2.0/2
MbMNPV-NL80	7.5×10^4	26.4	14.0	31.0	1.53 ± 0.25	-2.46	22.0/2
MbMNPV-NL82	4.1×10^4	14.4	5.3	27.7	0.90 ± 0.09	0.85	4.1/2
MbMNPV-NL83	4.8×10^4	16.9	4.2	36.4	1.06 ± 0.12	0.04	18.5/2

The three MbMNPVs have been isolated from S. exigua. According to the prevailing rules on nomenclature the three MbMNPV viruses should have been named SeMNPVs. However, in order to prevent future confusion and in anticipation of a situation where the genetic relationship becomes a more prominent criterion for baculovirus classification, these viruses were named MbMNPVs.

SeMNPV has a ca. five times lower LD-50 against second instar beet armyworms than AcMNPV and the MbMNPV variants (Fig. 4.2, Table 4.1). Burgerjon et al. (1975) using a bioassay involving diet contamination, arrived at the conclusion that SeMNPV was about six times more active against beet armyworm than a MbMNPV. AcMNPV and the three MbMNPV variants did not show significant differences among each other in their biological activity.

Gelernter & Federici (1986) showed that based on LT-50 values AcMNPV is slower acting on beet armyworm larvae than SeMNPV. Preliminary LT-50 determinations of MbMNPV-NL82 and SeMNPV against second instar beet armyworm larvae also indicated that SeMNPV was superior in the speed-of-kill (P.H. Smits, unpubl. results).

The slopes of the probit regression lines varied and ranged from 0.9 to 1.6. Similar variations are often found in insect-virus bioassays (Hughes & Wood, 1986). This variation can be attributed to physiological heterogeneity of the larvae within each larval stage. In our experiments larvae were used one day after moulting into second instar to minimize this effect. However, this heterogeneity may be intrinsically larger when second instars are used in the droplet-feeding assay as compared to neonates (Hughes & Wood, 1981). Huber & Hughes (1984) suggested that deviation of slopes from the theoretical maximum value of 2 can be either a variation in the performance of the bioassay, a variation in the virulence of the virus or an indication of genetic variability of larval susceptibility. The latter explanation is possible since S. exigua was not an inbred strain. The relatively low values found for the MbMNPV-NL82 and NL83 isolates seem to be caused by experimental variations in the bioassays in particular at the lower dosages.

Both AcMNPV (Vail et al., 1971; Payne, 1986) and MbMNPV (Allaway & Payne, 1984) have a broad host range among noctuid pests. SeMNPV is specific for S. exigua (Gelernter & Federici, 1986). Not only does a broader host range increase the potential market of an eventually developed virus product but it also offers the possibility for virus production in one of the alternate host insects. AcMNPV and MbMNPV can be produced in considerable larger host species such as M. brassicae giving up to five times higher yields per larvae (Shapiro, 1982). As a large proportion of the production costs of a virus can be

contributed to labour involved in rearing and handling host larvae (Shapiro, 1982) the use of alternate hosts, which may be more easy to mass-rear, may lead to considerable lower production costs per quantity of virus. Adding this to the advantage of a larger potential market may well justify the development of these viruses, despite their lower biological activity to the target pests.

The difference in LD-50 values against second instar larvae between SeMNPV and the other MNPVs is ca. fivefold. Preliminary results further indicated that with older instars this difference in biological activity increases considerably in favour of SeMNPV (P.H. Smits, unpubl. results). The speed of kill of SeMNPV is higher than that of the other MNPVs and this is an important property when a high degree of cosmetic damage to the crop cannot be tolerated. For these reasons and on the assumption that its specificity for one single-target insect pest would facilitate registration, it was decided to further develop SeMNPV as biological insecticide to control beet armyworm in greenhouses in the Netherlands.

CHAPTER 5

BIOLOGICAL ACTIVITY OF SPODOPTERA EXIGUA NUCLEAR POLYHEDROSIS VIRUS AGAINST SPODOPTERA EXIGUA LARVAE AND THEIR PROGENY

5.1 Introduction

The studies in Chapter 4 showed that Spodoptera exigua multiply-enveloped nuclear polyhedrosis virus (SeMNPV) showed more potential as a biological control agent of S. exigua than four other NPVs.

SeMNPV has been earlier described by Hunter & Hall (1968a, b) and was biochemically characterized by Smith & Summers (1978). Recently, this virus was reisolated from beet armyworm populations in California (Gelernter & Federici, 1986). The latter authors showed that the virus is highly pathogenic to first instar larvae, but data on the activity against older instars were not presented. Dose-mortality data of SeMNPV against older larvae have been reported (Hunter & Hall, 1968a; Chautani & Rehnborg, 1971), but different bioassay methodologies were used. Since in beet armyworm infestations in greenhouses all larval instars may be present it is important to know the response of the various instars to this virus isolate.

In this chapter we present data on the biological activity of SeMNPV against the five larval instars of beet armyworm. Due to its convenience and its simple dose determination the droplet-bioassay method that Hughes & Wood (1981) described for neonates was used, adapted for each of the five larval instars. The dose-mortality (LD-50) as well as time-mortality (LT-50) relationships were determined for each instar.

In addition to the biological activity, the extent of virus transmission to the next generation was studied in order to evaluate the potential of inoculative release of virus in beet armyworm populations.

5.2 Materials and Methods

Virus

Spodoptera exigua multiply-enveloped nuclear polyhedrosis virus (SeMNPV) originated from the USA (Hunter & Hall, 1968a, b) and was obtained from Dr M.D. Summers, Texas A&M University, College Station, Texas, USA. The isolate proved

to be specific for beet armyworm and to be identical to the major SeMNPV genotype that is still being collected from Californian populations of beet armyworm (Smith & Summers, 1978; Gelernter & Federici, 1986). More details are given in Chapter 4.

Virus was propagated by infecting early fourth instar larvae by contamination of the semi-synthetic diet. Purification of polyhedra included grinding of deceased larvae, filtering the homogenate through cheese-cloth, and two centrifugation steps. The polyhedra were finally resuspended in phosphate buffered saline (PBS) and stored at -20°C. A more detailed description of the purification procedure is documented in Chapter 6.

Insects

The insects used in the experiments were taken from a laboratory rearing originating from larvae collected in a Dutch glasshouse. Larvae were reared in groups of 30-200 on semi-synthetic diet at 30°C, 70-80% relative humidity and a 16 h photoperiod. A more extensive description of the rearing is given in Chapter 2. In order to minimize the physiological heterogeneity (Teakle et al., 1986) in the bioassays larvae were always used two days, including the starvation period, after moulting.

Bioassays

Bioassays were carried out using the droplet-feeding method modified after Hughes & Wood (1981). Larvae were allowed to drink from polyhedra suspensions of known concentrations. Prior to the bioassay the larvae were starved for a period of 16-24 hours at 25°C. Besides virus the suspension also contained 10% sucrose to stimulate drinking and 0.05% Florella-blue (food-colouring) to detect the larvae that had drunk from the suspension. The suspensions were offered to the larvae in the form of small droplets (5-10 µl), applied in a circle onto a layer of parafilm placed on the bottom of a petridish (5 cm). The larvae were placed in the center of the circle of droplets. Those that had drunk from the solution within 15 min were selected and used in the bioassay.

Per virus concentration 32 larvae were taken and transferred to individual wells of a 24-well tissue culture plate (Costar). The wells were covered with two layers of paper tissue and by the original lid in an upside-down position. For fourth and fifth instar larvae larger petridishes, fewer larvae at the same time and larger droplets were used in the procedure. These larvae as well as third instars were further reared in individual wells of 6-well tissue culture

plates (Costar). Larvae were reared at 25°C and mortality was recorded daily until ten days after the assay had begun or until pupation.

In each bioassay suspensions of five different concentrations were used. For first and fifth instars concentrations of 10^3, $3x10^3$, 10^4, $3x10^4$ and 10^5 polyhedra per ml were used. For second, third and fourth instars concentrations of $3x10^4$, 10^5, $3x10^5$, 10^6 and $3x10^6$ polyhedra per ml were used. With each larval instar three bioassays were performed.

LC-50, LD-50 and LT-50 determination

The dosis- and time-mortality data were processed and analysed with a computer program provided by Dr. J. Huber, Darmstadt, W-Germany. The program was based on the probit analysis methods described by Finney (1971). The median lethal times (LT-50) were calculated from truncated data on the proportion of the test population that died of virus infection.

Determination of the ingested dose

The amount of polyhedra ingested by larvae in the bioassays was determined by measuring the volume ingested of a solution of ^{32}P, provided with equal concentrations of sucrose and Florella-blue as the polyhedra suspensions. The radioactivity correlated with a known volume and was measured from individual larvae using a scintillation counter (Packard 2450) recording the Cerenkov radiation of ^{32}P. About 100 larvae per instar were included and the mean value of ingested volume was determined. From this value the actual ingested virus dose could be calculated for each instar.

Virus transmission

Fifth instar larvae surviving three of the dose treatments, 10^5, $3x10^5$ and 10^6 polyhedra/ml, in the bioassays described above, were reared until pupation. The pupae from each dose were sexed, weighed and allowed to eclose in jars covered with paper for egg deposition. The number of eggs laid per jar was recorded as well as the percentage of egg hatch. Neonate larvae, 60 from each jar, were placed in individual wells of a 24-well tissue culture plate (Costar) and reared at 25°C until they reached the fourth instar. The mortality was recorded and deceased insects were checked for polyhedrosis and the instar stage at death. The experiment was performed three times.

5.3 Results

Ingested dose

In order to calculate the LD-50 values using the droplet-feeding bioassay according to Hughes & Wood (1981), it is necessary to know the volumes of polyhedra suspension ingested by the various instars of S. exigua. In Table 5.1 the mean volume imbibed per instar is shown. It is clear that the ingestion volume increases with age. When the ingested volume and the concentration of polyhedra is known the actual dose-intake per concentration could be calculated for each instar. The standard deviation in the ingested volumes ranged from 40-60% and appeared to be independent of instar age.

Table 5.1 Mean volume of virus suspension imbibed by Spodoptera exigua larvae.

Larval instar	Volume ± SD (in µl)
1	0.023 ± 0.015
2	0.33 ± 0.13
3	1.4 ± 0.7
4	3.3 ± 1.4
5	9.0 ± 3.7

The ingested volume of S. exigua neonates (0.023 µl ± 0.015 µl) was well within the range of the values found for other Lepidopterans, such as for Trichoplusia ni (0.013 µl ± 0.003 µl), Heliothis zea (0.014 µl ± 0.004 µl), Estigmene acrea (0.049 µl ± 0.006 µl) and S. frugiperda (0.006 µl ± 0.002 µl) (see Hughes & Wood, 1986), for Leucoma salicis (0.11 µl ± 0.02 µl) (Lameris et al., 1985) and for Lymantria monacha (0.18 µl ± 0.06 µl) (R. Laport, unpubl. results).

Dose-mortality versus instar

The dose-mortality relationship between SeMNPV and the five larval instars of beet armyworm is shown in Fig. 5.1. In Table 5.2 the associated statistics of the regression lines and the LC-50 and LD-50 values are presented. The median lethal dose (LD-50), expressed as number of polyhedra, increases from 4 polyhedra for first instar larvae to more than 11,000 polyhedra for fifth instars. There is no significant difference in LD-50 values between first and second instars. Third and fourth instar larvae have LD-50 values that are respectively about 10 and 30 times higher than those of first and second instar

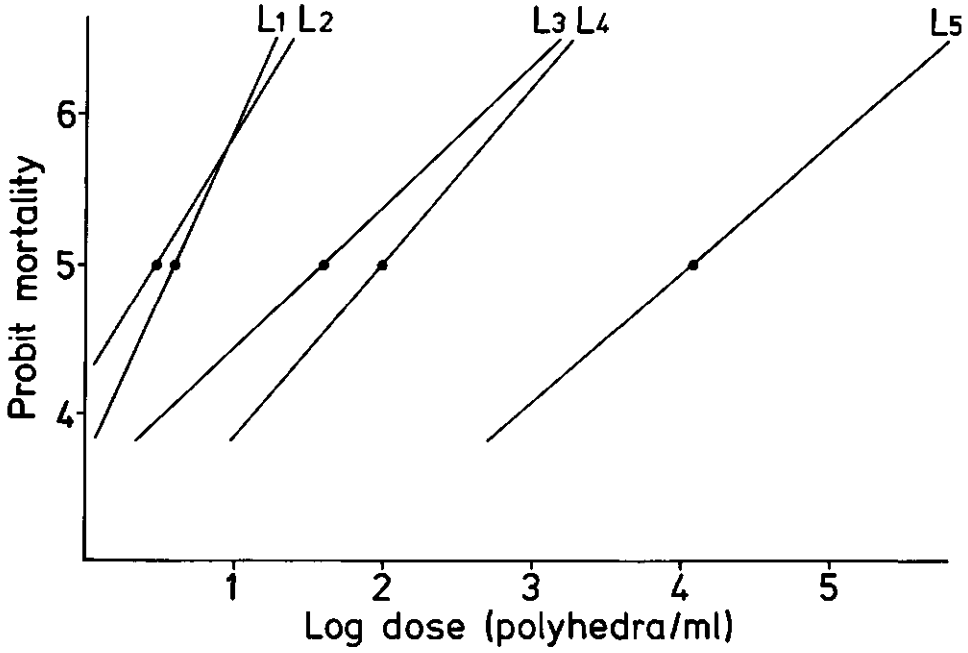

Figure 5.1 Dose-mortality relationship between SeMNPV and the five larval instars (L1-L5) of Spodoptera exigua.

Table 5.2 LC-50 (in polyhedra (pol)/ml), LD-50 (no. of pol) and associated statistics for SeMNPV against Spodoptera exigua larval instars.

Larval instar	LC-50 (pol/ml)	LD-50 (pol)	95% Fiducial limits lower	upper	Slope ± SD	Intercept	χ^2/DF
1	1.9×10^5	4	3	6	2.03 ± 0.39	-5.73	0.25/2
2	9.1×10^3	3	2	4	1.64 ± 0.20	-0.99	2.00/2
3	2.8×10^4	39	21	94	1.02 ± 0.10	0.47	2.68/2
4	3.1×10^4	102	55	180	1.18 ± 0.17	-0.30	0.57/2
5	1.3×10^6	11637	6210	32544	0.98 ± 0.15	-0.99	10.60/2

larvae. Fifth instar larvae are much less susceptible, illustrated by the 100-fold increase in the LD-50 value as compared with the fourth instar stage.

The slopes of the dose-response lines, varying from 0.98 to 2.03, are within the range usually found for insect viruses (Burges & Thomson, 1971). The slopes are lower for older larvae suggesting a decrease in susceptibility to the virus.

Time-mortality versus instar

From the dosis mortality data that were recorded daily also an estimation could be made of the median lethal time (LT-50) for each larval instar at a certain dose (Table 5.3). Only those larvae finally dying from polyhedrosis were included in the calculations. The analysis indicated that the overall LT-50 increases with larval age from 3-4 days for first instars to almost 6 days for fifth instar larvae. As with the dose-mortality relationship the difference between first and second as well as between third and fourth instars is rather small. Between these two groups there is a difference in LT-50 of about one day. Fifth instars on the average die one day later than third and fourth instars. The LT-50 values appeared to be inversely related with dosage.

Table 5.3 LT-50 times (in days) of Spodoptera exigua larval instars, infected with SeMNPV after drinking from suspensions of polyhedra (pol) at various concentrations.

Virus conc. (pol/ml)	Larval instars				
	1	2	3	4	5
1×10^3	-	4.6°	5.6°	4.9°	-
3×10^3	-	3.7	5.9	5.4	-
1×10^4	-	4.1	5.6	4.9	-
3×10^4	5.9°	3.5	5.0	4.9	5.8°
1×10^5	4.0	3.4	4.6	4.9	6.1
3×10^5	3.6	-	-	-	5.9
1×10^6	3.3	-	-	-	5.7
3×10^6	3.2	-	-	-	5.7

-: no data available
°: based on very low numbers of larvae

Virus transmission

The extent of virus transmission to the next generation was studied by recording the mortality caused by virus in the progeny of virus-exposed fifth instar larvae. It appeared that virus transmission to the progeny occurred at a level of 10% to 28% and was not correlated with the dose (Table 5.4). There was no apparent difference in pupal weights, adult emergence, fecundity and percentage egg hatch between the various dosages and as compared to untreated controls (data not shown).

Table 5.4 Mortality in the progeny of fifth instar Spodoptera exigua larvae that drunk from SeMNPV suspensions at three concentrations of polyhedra (pol).

Virus conc. (pol/ml)	% parent mortality	% mortality in progeny
0	6.7 ± 2.9	4.2 ± 3.6
1×10^5	34.1 ± 10.1	17.1 ± 11.5
3×10^5	36.1 ± 17.7	10.0 ± 8.9
1×10^5	54.9 ± 5.2	28.3 ± 16.8

5.4 Discussion

The droplet-feeding bioassay generally has been used to test the virulence of NPVs using neonate larvae (Hughes & Wood, 1981; Hughes et al., 1983; Hughes et al., 1984). In the case of S. exigua it was possible to adapt this technique for other instars and thus to conduct a comparative study of the virulence of SeMNPV for five instars using the same bioassay technique. It is possible that this only applies to Noctuids, as in the case of Leucoma salicis and Lymantria dispar the droplet feeding bioassay could only be used for neonate larvae (A.M.C. Lameris, pers. comm.).

The LD-50 value of 3 polyhedra for first instar larvae of S. exigua indicates that SeMNPV is a highly virulent virus. Similar values have been reported for S. littoralis MNPV (McKinley, 1979), H. zea SNPV (Allen & Ignoffo, 1969), H. armigera SNPV and MNPV, H. virescens SNPV (Hughes et al., 1983) and Cydia pomonella GV (Sheppard & Stairs, 1977) for homologous hosts, and also for Mamestra brassicae MNPV in Plusia gamma (Allaway & Payne, 1984). These low

LD-50 values are close to the minimum theoretical value (Huber & Hughes, 1984). The LD-50 calculation has been performed with conventional computation using the probit model (Finney, 1971), it is possible that at these low values the exponential model is more appropriate (Hughes & Wood, 1984).

The LD-50 values for second, third, fourth and fifth instar larvae are lower than values found for a number of other NPVs with similar virulence against neonate larvae in homologous hosts (Burgerjon et al., 1981; Evans, 1981). For SeMNPV, Chautani & Rehnborg (1971) and Hunter & Hall (1968a) reported LD-50 values of 968 and about 700 polyhedra against third instars of S. exigua, respectively. These values are considerably higher than the value of 39 polyhedra that was found in our experiments for third instars (Table 5.2). This difference could be explained by the fact that these authors used a different bioassay technique, i.e. surface contamination of diet which leads to longer virus acquisition times by the larvae. It is likely that their virus isolate was identical to the one used in our experiments (Gelernter & Federici, 1986).

Generally the LD-50 increases during larval development. Evans (1981) found an increase from 7 to over 900 polyhedra between first and second instar M. brassicae larvae with the corresponding MbMNPV. LD-50 values for third, fourth and fifth instars were 5, 50 and 250 times that of the second instars. Evans (1981, 1983), Allen & Ignoffo (1969) and Teakle et al. (1985) indicated that decrease in larval susceptibility was largely correlated with increase of body weight.

The slope of the computed regression lines (Table 5.2, Fig. 5.1) becomes gradually smaller with age. This could indicate either an increased heterogeneity in the test population, an increased variation in host susceptibility or variability in the bioassay. The latter two possibilities are supposed to be eliminated since the droplet feeding method permits the synchronous treatment of physiologically homogeneous larvae with a uniform dose (Hughes & Wood, 1981, 1986). However, it is possible that despite the strong selection of larvae, the physiological heterogeneity of larvae increases with age.

LT-50 values increased with larval age (Table 5.3), implying a slight increase of the resistance of susceptible larvae with increasing age. The LT-50 values for each instar were dependent of the used virus dose and the level of larval mortality (Table 5.3). This result differs from that of Agrotis segetum and its MNPV and granulosis virus (Allaway & Payne, 1984), where there was evidence for a dose independent LT-50. However, Teakle et al. (1985) reported

that the LT-50 of H. zea MNPV for H. armigera declined with increasing dose for larvae of the same age. Further research is needed to clarify this point.

Gelernter & Federici (1986) also performed time-mortality studies with SeMNPV using the droplet-feeding method, but only with neonate beet armyworm larvae. They found a LT-50 of 2.5 days at 30°C. At 25°C we found an LT-50 for neonates with the same SeMNPV of 3.2 days (Table 5.3), confirming the temperature dependence of the LT-50.

Adults developing from virus-infected insects transmitted the virus to their progeny. However, there was no quantitative relation with the previously inoculated dosages. There have been reports from various insect-virus systems on the effect of baculovirus infection on sex-ratio, pupal weight, adult emergence, egg production, oviposition rate and egg viability (Abul-Nasr et al., 1979; Subrahmanyam & Ramakrishnan, 1980; Young & Yearian, 1982; Vargas-Osuna & Santiago-Alvarez, 1986). In other instances, however, these effects were not observed (Ignoffo, 1964; Vail & Hall, 1969; Luttrell et al., 1981; Perelle & Harper, 1986). From our experiments it is clear that even when high dosages are used, the level of virus transmission to the progeny is too low to warrant sufficient short-term control of the newly hatched generation.

The dosis-mortality data combined with data on the larval feeding behaviour in chrysanthemum crops (Chapter 3) indicate that equal levels of mortality of the first four larval instars can be expected to occur after virus application (Smits, 1986). The decrease in susceptibility with larval age is largely compensated by increased foliage consumption and changes in the feeding behaviour resulting in a higher intake of polyhedra. Similar conclusions were reached by Payne et al. (1981) for Pieris rapae granulosis virus and by Evans (1981) for M. brassicae MNPV. However, the increase in the LD-95 between fourth and fifth instars (Table 5.2) is much larger than the increase of virus acquisition (Chapter 3; Smits, 1986). This means that in order to control first to fourth instar larvae of S. exigua one particular dose is sufficient, but that for control of fifth instars higher virus dosages are required.

CHAPTER 6

PRODUCTION OF SPODOPTERA EXIGUA NUCLEAR POLYHEDROSIS VIRUS

6.1 Introduction

The use of Spodoptera exigua multiply-enveloped nuclear polyhedrosis virus (SeMNPV) (Baculoviridae, subgroup A) as control agent for S. exigua (Hübner) (Lep.: Noctuidae) larvae in greenhouse crops in the Netherlands is being considered (Chapter 1). Besides control efficacy mass production at reasonable costs is a most important factor in assessing the potential for commercial development of the virus into a marketable product.

All baculoviruses developed sofar into commercial products or otherwise used on a large scale were produced in insect larvae (Shapiro, 1982). Virus production in insect cell culture is possible for a few viruses, but only in small fermentors at relatively high costs (Hink, 1982; Tramper & Vlak, 1986).

Both quantitative and qualitative aspects are important in the production of a nuclear polyhedrosis virus (NPV) in insect larvae. Generally, production is carried out in laboratory-reared larvae that are infected with virus in the penultimate instar by contaminating their food. During the infection process the number of polyhedra increases until a maximum is reached, often before the larva actually dies (Shapiro, 1982). The number of produced polyhedra is positively correlated with larval body weight, and maximal virus production is generally obtained with inoculum rates that kill ca. 95% of the larvae. Higher inoculum rates kill the larvae before they reach their maximum weight and therefore lead to lower yields (Shapiro, 1982).

Besides virus also bacteria and fungi may increase in number during the incubation period. Registration requirements set limits to the numbers of these microbial contaminants in the virus product and although removal is possible, it is an expensive step in the production process best avoided (Shapiro, 1982). Virus harvest relatively early during the infection process therefore seems desirable. Ignoffo & Shapiro (1978) and Shapiro & Bell (1981), however, showed that polyhedra harvested from living larvae had a six- to twelvefold lower biological activity than polyhedra harvested from larvae that had died of virus infection.

Often larvae are reared individually during the incubation period in order to avoid cannibalism (Shapiro, 1982). Production in group-reared larvae, however,

would reduce the production costs substantially since ca. 70% of the total costs can be attributed to labour involved in handling larvae (Ignoffo, 1973).

This chapter presents studies on the effect of various inoculum rates on virus yield. Furthermore, the yield of polyhedra and the amount of microbial contaminants produced during the incubation period were determined. The biological activity of virus harvested from living and from dead larvae was compared. Virus production in group-reared larvae was compared to that in individually-reared larvae. Several produced virus batches were tested for the presence of vertebrate pathogens, such as Salmonella spp., Shigella spp., Vibrio spp. and Staphylococcus aureus.

6.2 Materials and methods

Insects

The insects used in the experiments were taken from a laboratory colony regularly supplied with freshly collected larvae from greenhouses to prevent extensive inbreeding. Larvae were reared on semi-synthetic diet modified after Poitout & Bues (1974), at 30°C, 70-80% relative humidity and a 16:8 (L:D) photoperiod. The diet consisted of the following ingredients per 5 liters of water: 140 g agar, 800 g cornmeal, 250 g wheatgerms, 250 g yeastpowder, 40 g ascorbic acid, 10 g sorbic acid, 8 g methyl-hydroxy-benzoate and 0.5 g streptomycin-sulfate. The water was heated and the ingredients were added to the boiling water in the sequence described above. The diet was stored at 5°C for a maximum period of 2 weeks after preparation.

Petridishes and disposable plastic trays covered with paper tissues were used as rearing containers for groups of 30-200 larvae. Vermiculite was added to facilitate pupation. Pupae were collected and transferred to oviposition cylinders. The walls of the cylinder were covered with paper for egg-deposition and adult moths provided with a 20% sugar solution. The sex ratio of the pupae was ca. 1:1. Eggs were surface-sterilized with formaldehyde vapor to eliminate external microbial contamination (Bathon & Gröner, 1977).

Virus

Spodoptera exigua multiply-enveloped nuclear polyhedrosis virus (SeMNPV) originates from the USA and the isolate is identical to the major genotype described by Gelernter & Federici (1986). The virus was propagated in laboratory-reared beet armyworm larvae as described in this chapter. Virus was

stored at -20°C in phosphate buffered saline (PBS). Concentrations were determined by three independant counts of polyhedra with a haemocytometer under a phase-contrast microscope (x400).

Virus production in individually-reared larvae

The experiments on virus production in individually-reared larvae were carried out with late fourth instar larvae (2-3 days after moulting) that were placed in rectangular compartments (2.5 x 2.5 cm) covered with gauze. Compartments were formed by pressing a plastic grid into a 0.5 cm thick layer of diet (Poitout & Bues, 1974). The inoculum was applied on top of the diet with a micropipette, in a volume of 50 µl PBS per compartment. The trays with infected larvae were placed in an incubator at 30°C and 70% relative humidity, in complete darkness. After harvest of infected larvae, the virus was liberated from the larval tissues in a Sorvall mixer (30 sec). The suspension was then forced through cheese-cloth and finally centrifuged (7500 rpm/min for 15 minutes) twice in a Sorvall SS-34 rotor. The virus pellets were resuspended in PBS and stored at -20°C.

Inoculum rate

The effect on virus production of five inoculum rates, 0.15, 0.75, 1.5, 7.5 and 15 x 10^4 polyhedra/cm^2 diet surface, was studied in three experiments with groups of 25 individually-reared larvae per treatment. The inoculum rates were chosen in the range 10^3-10^5 polyhedra per cm^2 after preliminary experiments had shown that they would give 70-100% larval mortality. The inoculum was applied on top of the diet as described above. After seven days of incubation virus was harvested by placing the tray, including larvae and diet, at -20°C. Frozen larvae were collected with a pair of tweezers. The virus was further processed as described above and the total number of polyhedra produced by each group of larvae was determined.

Virus production during the incubation period

The production of virus during the incubation period was studied with an inoculum of 7.5 x 10^4 polyhedra/cm^2 diet surface. To reduce the loss of virus at harvest larvae were separated from the diet after three days of incubation, and further incubated individually in cells of a 24 microwell dish (Costar) without diet. Every 24 hours for a total period of ten days a group of 24 larvae was harvested and processed as described above. The experiment was carried out three times.

Virus production in group-reared larvae

In the group-production experiment 400, 600 and 800 late fourth instar larvae (2-3 days after moulting) were placed in a container (44 x 30 x 10 cm) provided with a 1 cm thick layer of diet. On top of the diet an inoculum was applied of 7.5×10^4 polyhedra/cm^2 in a total volume of 10 ml PBS per container. After three days larvae were separated from the diet by flooding the container with water. All larvae were collected in a sieve, placed in a clean container and returned to the incubator for another four days. After this period the deceased and desintegrated larvae were collected by rinsing the container with water. The virus was further processed as described above. The experiment was carried out twice.

Biological activity

The biological activity, in terms of LC-50 and LD-50 values, of virus harvested from living and dead larvae was compared in bioassays in which second instar larvae were allowed to drink from droplets of virus suspension (Hughes & Wood, 1981). The mean volume of virus suspension imbibed by second instar larvae is 0.33 ± 0.13 µl (Chapter 5). The larvae were starved for 18 hours at 25°C prior to the experiment and readily drank from the virus suspension, to which 10% sucrose and 0.05% Florella blue (blue food colouring) was added. Per virus concentration 32 larvae, that had drunk from the virus suspension and could be recognised by their blue colour, were selected and placed individually in cells of 24-well tissue culture dishes (Costar) provided with diet. After 10 days of incubation at 25°C, larval mortality was recorded. Larvae that survived for ten days generally reached pupation. Bioassay were performed with virus from living larvae harvested four days after infection, and from dead larvae harvested seven days after infection, at three concentrations, 0.5, 1 and 2 x 10^4 polyhedra/ml, respectively. The bioassays were carried out three times at each concentration. The mortality data were analysed using the probit analysis method described by Finney (1971) (see Chapter 5).

Microbial contamination

The overall microbial contamination of produced virus was determined by applying series of tenfold dilutions up to 10^{-8}, from virus suspensions containing 1 x 10^9 polyhedra/ml, to plate-count agar (PCA, Merck). For the separate counts of fungi, oxytetracycline-glucose-yeastextract agar (OGY, Merck) was used. For each virus sample, two independent dilution series were started from 10 ml of virus suspension. At each dilution step 9 ml of sterilised PBS was added to 1 ml of

virus suspension. A ml out of each of these diluted suspensions was mixed with a thin layer of warm agar (ca. 50°C) and incubated at 30°C. After three days the number of colonies was counted, at the appropiate concentration of ca. 200 colonies per dish. The number of microbial contaminants was determined for all virus batches produced in the experiment on virus production during a 10-day incubation period, which was described earlier.

The presence of Salmonella spp., Shigella spp., Vibrio spp. and Staphylococcus aureus in various virus batches was tested with methods that are routinely used for food products. For the tests on Salmonella spp. both short term and long term accumulation in Muller-Kaufman medium and buffered-peptone water, respectively, was carried out. Tests on the presence of Shigella spp. were performed on SS-agar. Tests for the presence of Vibrio spp. were performed on TCBS-agar. The presence of Staphylococcus aureus in the samples was tested after accumulation in Giolitti-Cantoni broth to which potassium-tellurite was added. Detailed descriptions of all these tests can be found in Buchanan & Gibbons (1974) and Mossel & Tamminga (1980). All tests were performed twice on 1 ml samples from seven different virus production batches. The virus concentration in all tested suspensions was 1×10^8 polyhedra/ml.

6.3 Results

Inoculum rate

The influence of five inoculum rates on the virus production, measured in number of polyhedra produced per larva, is shown in Table 6.1. The average virus yield ranged from 6.1×10^8 to almost 1×10^8 polyhedra per larva. The inoculum rate of 7.5×10^4 polyhedra/cm^2 gave the highest virus production in each of the three experiments.

The proportion of larvae that died of virus infection increased together with increase of the inoculum rate, from 83% to 99% (Table 6.1). The highest average yield was obtained at the inoculum rate that caused 95% larval mortality. Surviving larvae generally pupated, but some of the pupae showed severe malformations. Virus-killed larvae turned black and liquified, sometimes spilling part of the virus onto the diet surface. This may have lead to some loss of virus and underestimation of the actual virus production.

Table 6.1 The influence of the inoculum rate on the yield of SeMNPV polyhedra (pol) in fourth instar Spodoptera exigua larvae, incubated for seven days at 30°C.

Inoculum (pol/cm²)	Avg. no. of polyhedra produced/larva (± SD)	% Larval mortality
1.5×10^3	$6.1 \pm 1.2 \times 10^8$	83
7.5×10^3	$5.8 \pm 0.6 \times 10^8$	89
1.5×10^4	$8.3 \pm 2.1 \times 10^8$	92
7.5×10^4	$9.0 \pm 2.0 \times 10^8$	95
1.5×10^5	$6.9 \pm 1.3 \times 10^8$	99

Virus production during the incubation period

In a separate experiment the production of polyhedra in time was followed. Figure 6.1 shows that the production of polyhedra started 1-2 days after the larvae were placed on the virus-contaminated diet. The number of produced polyhedra increased rapidly from 10^7 to 10^9 per larva between the second and fourth day of incubation and then more or less stabilized at a level of 1-2 x 10^9 polyhedra per larva.

The first external symptoms of virus infection became visible around the second day of incubation, when larvae became sluggish. At the third day they became puffy and lighter of colour and at day five or six they turned black and their tissues except for the skin liquified. After about seven days of incubation the skin also liquified.

Biological activity

The biological activity of virus harvested after four days of incubation from infected larvae that were still alive, and after seven days of incubation from larvae that had died of virosis, was compared in bioassays against second instars. Table 6.2 shows that the LD-50 of the virus harvested from dead larvae is slighty lower than that of virus harvested from living larvae. Because the LD-50 fiducial limits show considerable overlap, the differences however are not significant. This is in contrast with studies by Ignoffo & Shapiro (1978) and Shapiro & Bell (1981) who found a six- to twelvefold higher biological activity in virus harvested from dead larvae.

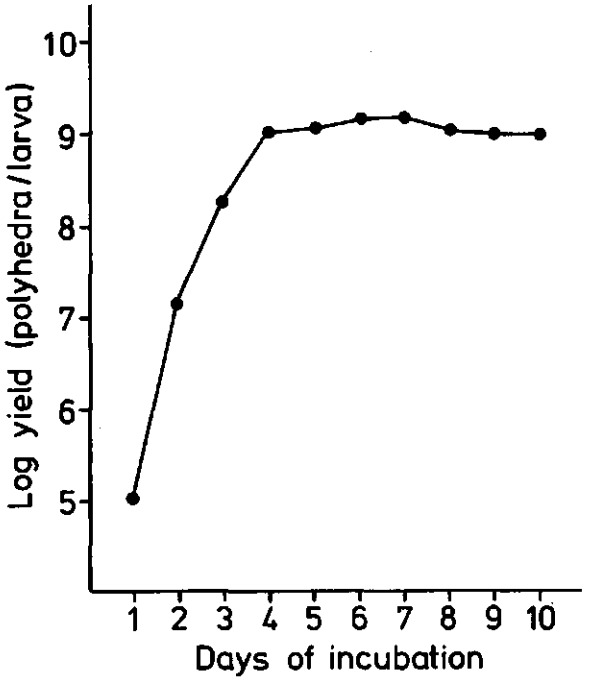

Figure 6.1 Production of SeMNPV polyhedra in fourth instar Spodoptera exigua larvae during incubation at 30°C. Inoculum 7.5 x 10^4 polyhedra/cm^2 of diet surface.

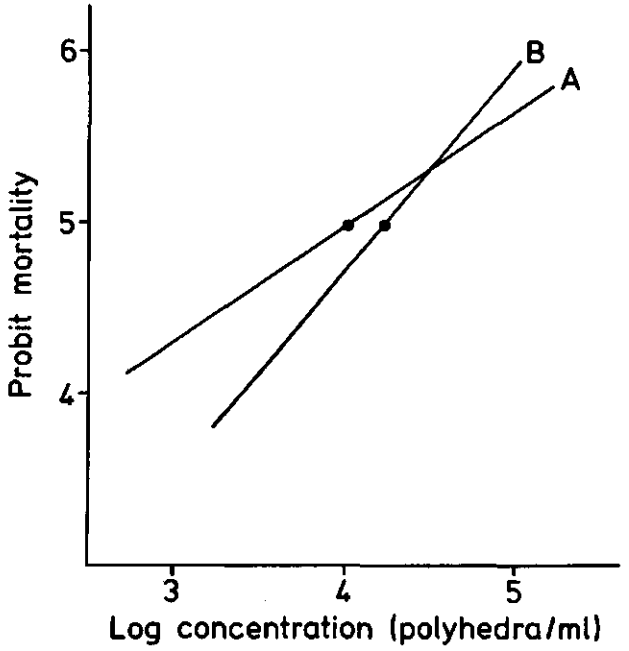

Figure 6.2 Dose-mortality relationship between SeMNPV and second instar Spodoptera exigua larvae. A: polyhedra harvested from dead larvae after seven days of incubation at 30°C. B: polyhedra harvested from living larvae after 4 days of incubation at 30°C.

Table 6.2 Biological activity against second instar Spodoptera exigua larvae of SeMNPV polyhedra harvested from living larvae, after 4 days of incubation, or from deceased larvae, after 7 days of incubation at 30°C. Calculations made with combined data from three series of bioassays.

Virus Source	LC-50 (pol/ml)	LD-50 (pol)	95% Fiducial Limits Lower	Upper	Slope	Intercept	χ^2/DF
Living larvae	1.7×10^4	5.6	4.2	11.1	1.25	-0.12	1.2/7
Dead larvae	1.1×10^4	3.5	1.5	12.4	0.70	2.18	1.1/7

The slope of the probit transformed dose-mortality response curve (Table 6.2, Fig. 6.2) is higher for the virus harvested from living larvae. Both slopes, however, are considerably lower than the value of 1.64 found in earlier studies with virus harvested from dead larvae. The latter bioassays were performed with a larger range of virus concentrations (Chapter 5).

Microbial contamination

The level of microbial contamination of virus harvested at daily intervals during the incubation period is presented in Table 6.3. The data show that the number of colonies (or number of viable spores) per harvested larva increased until the fourth day of incubation and then more or less stabilized. The relatively high number of microbial contaminants present in the virus after one day of incubation, which was found in two out of three experiments, was probably caused by bacteria and fungi present on larvae that originated from rather dirty rearing containers. The number of these contaminants decreased after two days of incubation, probably because of the antibiotics in the diet. The presence of fresh faeces and the death of the larvae around the fourth incubation day offered new opportunities for bacteria and fungi to multiply. The number of contaminants did not increase further when the larvae died and liquified, after ca. six days of incubation.

The ratio of microbial contaminants and polyhedra did not change considerably between the third and the tenth day of incubation and the contaminants numbered 1-5% of the amount of polyhedra produced. The contaminants consisted mainly of bacteria. Fungi and yeasts contributed only between 0.3% and 4% to the total number of microbial contaminants.

The presence of vertebrate pathogens was tested for seven different virus batches all produced in fourth instar larvae at 30°C and harvested after seven

days of incubation. In none of the samples tested any positive reaction as described in Mossel & Tamminga (1980) or Buchanan & Gibbons (1974) was found. We can therefore conclude that Staphylococcus aureus, Vibrio spp. Shigella spp. and Salmonella spp. were not present in the tested SeMNPV batches.

Table 6.3 Microbial contamination (no. of viable spores) of polyhedra (pol) harvested at daily intervals from fourth instar Spodoptera exigua larvae infected with SeMNPV and incubated at 30°C.

Days of incubation	Avg. no. of viable spores per larva (min.- max.)	Avg. no. of viable spores per 100 pol. (min.- max.)
1	6.4×10^6 (5.0 - 7.2)	9569 (23800 - 7.8)
2	1.7×10^4 (0.9 - 2.8)	0.2 (0.05 - 0.4)
3	2.5×10^6 (0.3 - 5.8)	1.6 (0.06 - 1.2)
4	4.6×10^7 (0.9 - 7.7)	3.9 (1.8 - 5.8)
5	5.0×10^7 (3.5 - 7.7)	4.3 (2.5 - 5.3)
6	9.8×10^7 (20.0 - 2.5)	5.7 (2.8 - 10.1)
7	4.6×10^7 (1.4 - 8.8)	3.2 (0.8 - 7.0)
8	6.1×10^7 (2.1 - 9.1)	4.9 (2.6 - 6.7)
9	3.1×10^7 (0.9 - 4.6)	2.7 (1.4 - 3.8)
10	2.8×10^7 (0.5 - 4.4)	2.5 (3.3 - 3.9)

Group production

Virus production in larvae reared in isolation resulted in yields of ca. 1×10^9 polyhedra per larva (Fig. 6.1). Since the larvae can be rather cannibalistic, certainly if some are diseased and weak, it was anticipated that the virus yield per larva would decrease when the larvae were incubated together in larger groups. Table 6.4 shows that the average yield of virus per larva indeed was lower than found in the experiments with individually-reared larvae. Densities of 400 and 600 larvae per container gave average yields of about 9×10^8 polyhedra per larva. At the highest density of 800 larvae per container the average production per larva decreased to 7.5×10^8 polyhedra. The total production per container thereby was only marginally higher than in containers

with 600 larvae. The latter larval density therefore seems most appropriate for large scale virus production. Preliminary tests showed that the microbial contamination of virus produced in groups of larvae was in the same order of magnitude as found in virus produced in isolated larvae.

Table 6.4 Number of SeMNPV polyhedra (pol) harvested from fourth instar Spodoptera exigua larvae, that were incubated in large groups per container (44 x 30 x 10 cm).

No. of larvae per container	Avg. no. of pol. per larva ± SD	Avg. no. of pol. per container ± SD
400	8.8×10^{8}	3.5×10^{11}
600	9.0×10^{8}	5.4×10^{11}
800	7.5×10^{8}	6.0×10^{11}

6.4 Discussion

The inoculum rate of 7.5×10^{4} polyhedra per cm^2 that gave the highest yields in our experiments, is slightly lower than that found for the production of a Mamestra brassicae NPV in larvae of S. exigua (Smits et al., 1984) and a number of other NPVs produced in various noctuid larvae (Shapiro, 1982). The virus inoculum was applied on top of the medium because this method seems more convenient than the alternative of mixing the virus with the diet when it is prepared. In the latter case two separate diet-preparation equipments and sites have to be used in order to prevent contamination of the rearing colony, one of the major problems in virus production in larvae. Also beet armyworm larvae tend to feed mainly on the diet surface and do not bore into the diet as some other noctuids. The maximal amount of virus produced per larvae lies around 2×10^{9} polyhedra, which is comparable to yields reported by Gelernter et al. (1986). Shapiro (1982) reported that a production of 6×10^{8} polyhedra per beet armyworm larva could be reached.

Late fourth instar larvae appear to be the optimal stage to infect, since they will succumb of virus infection just before pupation. If early fifth instars are used, a proportion will reach pupation, leading to a reduction in

yield. Younger larvae can be used in combination with a lower inoculum rate but the incubation time will be unnecessarily longer. Also with most other insect virus productions penultimate larval instars were used (Shapiro, 1982).

Since the production of polyhedra does not increase anymore after the seventh day of incubation and even slightly decreases because of loss of yield at harvest, this day seems the optimal day for harvest. The experiments on the biological activity and the microbial contamination of the virus do not give reasons to advance or delay the moment of harvest.

The microbial contamination of virus produced in our experiments ranges between 1 and 10 contaminants per 100 polyhedra and thereby approaches the values tolerated by the EPA in two baculovirus products that were registered as control agents in the USA, Elcar and Gypcheck (Shapiro, 1982; see also Chapter 9). The microbial contamination in the end product can be further reduced by better sanitary conditions during rearing and virus production and by use of a highly purified inoculum, preferably produced in cell culture. Further removal of microbial contaminants from virus preparations is possible by centrifugation over sugar gradients (Van der Geest, 1968) or by addition of antibiotics (Krieg et al., 1979). These procedures, however, add extra steps in the production process and increase the production costs. The microbial contamination of a virus product exists mainly of harmless species belonging to the normal gut flora of the larvae (Krieg et al, 1979; Podgwaite et al., 1983). Until now pathogens for vertebrates have not been found in other baculovirus production systems (Krieg, 1981; Shapiro, 1982).

The comparison of the biological activity of virus harvested from living and of dead larvae showed that although there may be a difference in activity this is not as large as the six- to twelvefold difference found by Ignoffo & Shapiro (1978) and by Shapiro & Bell (1981). The slightly lower activity of virus harvested from living larvae at the lower concentrations could well be related with lower numbers of virions in the relatively small polyhedra that are present at this stage of the virus infection (P.H. Smits, unpubl. results).

The separation of the larvae from the diet after three days of incubation is a new approach to avoid the difficulty of harvesting liquifying larvae. After three days of incubation most of the larvae become inactive and already show symptoms of viris infection. Furthermore an amount of faeces is removed, which reduces the microbial contamination. There are, however, usually some larvae that are still active and will not hesitate to feed upon relatives. The amount of virus lost hereby is difficult to assess, but it is probably not very high since those larvae will soon ingest lethal quantities of virus and die. Since

the larvae are deprived of food at a moment when at least some of them still were feeding on the diet, the average weight of larvae will be somewhat reduced, which also may lead to a reduction of the virus yield.

The experiments showed that a average yield of 9×10^8 polyhedra per larva is possible in group production, whereas production in isolated larvae can give yields up to almost 2×10^9 polyhedra per larva. The reduction in yield per larva therefore is ca. 50%, but considering the amount of labour that is saved, group production seems far more attractive for large scale production than production in individually-reared larvae.

SeMNPV mass production in laboratory-reared beet armyworm larvae appeared to be possible. Nonetheless the rearing and processing of large numbers of larvae remains a tedious and labourious process. Therefore studies now have to be focussed on automatization of the various steps involved in virus production in insect larvae. Another option is to improve virus production in cell culture. SeMNPV can be produced in a S. exigua cell line (Gelernter & Federici, in prep.), but in general virus production in cell culture is relatively expensive and not yet feasible on a large scale (Tramper & Vlak, 1986). Therefore for the time being the virus has to be produced in larvae. Cost calculations, including registration, have to be made to assess the economic feasibility of SeMNPV mass production in this way at the present moment (see Chapter 9).

CHAPTER 7

APPLICATION TECHNIQUES

7.1 Introduction

Various spraying systems can be used to apply nuclear polyhedrosis viruses to crops (Smith & Bouse, 1981). Dutch greenhouse growers often use conventional high volume spraying systems and ca. 2000 l of spray volume per hectare for pesticide applications. Recent developments in spraying techniques in greenhouses focus on reducing the spray volume in order to reduce the waste of pesticides on the soil, to avoid high humidity levels which may provoke bacterial and fungal diseases and to allow the development of computerized spraying systems (De Heer et al., 1984).

A method to meet these goals is controlled droplet application with specific nozzles such as spinning discs, which generate many small droplets in a narrow size spectrum. The conventional high-volume sprayers produce much lower numbers of droplets of various sizes. Another type of low-volume application system is the electrodynamic sprayer, which generates charged droplets and thereby provides coverage of both upper- and underside of leaves (Coffee, 1979; Morton, 1982). This is important since beet armyworm larvae tend to feed predominantly on the underside of leaves (Chapter 3). Another advantage of virus deposition at the underside of leaves as compared to the upperside, is the lower rate of inactivation by ultra-violet radiation (Jaques, 1985).

In this chapter the control of beet armyworm larvae in chrysanthemum crops of different heights by applications of comparable virus rates with low-volume spinning disc (LV-SD), ultra-low-volume electrodynamic (ULV-ED) and high-volume handsprayer (HV) application systems is compared.

7.2 Materials and Methods

Virus

Spodoptera exigua MNPV (Baculoviridae, subgroup A) originated from the USA (Chapter 4) and proved to be identical to the major genotype described by Gelernter & Federici (1986). Virus was propagated in laboratory-reared fourth instar beet armyworm larvae. Purification of polyhedra included grinding of

deceased larvae, filtering through cheesecloth and two centrifugation steps (Chapter 6). Prior to use the virus was stored in phosphate buffered saline at -20°C. Virus concentrations were determined by three independent counts of polyhedra made with a haemocytometer under a phase contrast microscope (x400).

Insects

Insects were taken from a laboratory culture originating from larvae collected in Dutch greenhouses. Larvae were reared in groups of 30-200 on semi-synthetic diet at 30°C, 70-80% relative humidity and a L:D = 16:8 photoperiod (Chapter 2).

Greenhouse

The experiments were performed in a 20 x 5 m greenhouse, which was heated to 23°C at night. During daytime temperatures varied between 23 and 35°C, depending on the outside weather conditions. Relative humidity varied around 60%.

Application systems

High-volume (HV) applications were made with an AZO-propane handsprayer using a Birchmeier helicon safier nozzle 100, pressure 4 bar. The sprayer was held by hand ca. 50 cm above the crop. The virus suspension with 0.05% Agral NN (ICI) spreader-sticker was applied at a rate of 200 ml per m^2. The volume median diameter (VMD) of the droplets was ca. 300 µm.

Low-volume spinning disc applications (LV-SD) were made with a Micron Ulva 8 spinning disc. The disc was positioned 75 cm above the crop and the droplets were blown into the crop at an angle of 50° by two fans. A volume of 3 ml virus suspension in water with 20% mineral oil (Ulvapron, BP) was applied per m^2. The VMD of the droplets was ca. 70 µm.

ultra-low-volume electodynamic (ULV-ED) applications were made with an 'electrodyn' sprayer provided by ICI (Coffee, 1979). The nozzle was positioned 50 cm above the crop. The formulation consisted of ULV-oil (Hoechst AG) to which freeze-dried and ground virus powder was added. The ULV-oil contained a fluorescent dye which allowed observations of the deposition patterns under an UV-lamp. The applied volume, except in the fourth experiment, was 0.6 ml/m^2. The VMD of the droplets was ca. 30 µm.

Both the LV-SD and the ULV-ED sprayer were mounted on a motorized chart developed by the Institute of Agricultural Engineering (IMAG) in Wageningen. A computer controlled mechanism regulated both the running speed and spray flow to the nozzle, allowing very accurate deposition. The chart with spraying equipment passed over the containers with plants twice, from opposite directions.

Experiments

To compare the effect of virus applications with the three spraying systems experiments were carried out on 40 cm high chrysanthemums cv. 'Milonka', at a virus rate of 1×10^8 polyhedra/m^2. Plots consisted of a group of 45 plants that were grown in an aluminium container (120 x 80 x 30 cm) filled with soil. The outside rim of each container was provided with sticky tape to prevent the escape of larvae. Within 24 hours after spraying, per plot six batches of 30-40 eggs were attached to the underside of foliage near the soil surface, the natural oviposition sites (Chapter 2). The experiment was carried out three times.

The influence of crop height on virus applications with the three spraying systems was studied in plots consisting of 24 chrysanthemum plants grown in groups of 12 in small containers (50 x 30 x 20 cm). Crops of two heights, 20 and 60 cm at the moment of virus application, were used. The experiment was performed once.

The effect of HV and ULV-ED virus applications, at rate of 1×10^9 polyhedra/m^2, was studied on a 30 cm high chrysanthemum crop. Plots were identical to those in the first experiment. Per plot 200 second instar larvae were released within 4 hours after virus application. The experiment was carried out three times.

The influence of rate and spray volume on virus applications with the ULV-ED system was studied on plots of 36 plants, 30-35 cm high and grown in groups of 12 per container (50 x 30 x 20 cm). Three virus rates, 0.25, 0.5 and 1×10^8 polyhedra/m^2 were applied each in two spray volumes, 5 and 7.5 l/ha. Nine egg batches were placed in each plot, at the underside of bottom leaves, within 24 hours after virus application. The experiment was carried out once.

Check plots in each of the experiments described in this paragraph remained untreated.

Sampling

In all experiments plots were sampled 14 days after egg hatch or larval release. Damage to the crops was determined by sampling all leaves of one-third of the plants in each plot. Each leaf was classified on a scale of 0-2 for the amount of feeding damage (0): no feeding marks; (1): superficial feeding marks on less than one-third of the surface or a few small perforations; (2) more damage than in (1).

All other plants were sampled for the presence of living larvae. Larval mortality was calculated by dividing the number of living larvae in the treated plots by the number of living larvae in the untreated check plots.

Statistical analysis

Differences in the distribution of leaves in damage classes between treatments were analysed with paired χ^2 tests with significance level $P < 0.05$.

7.3 Results

HV, LV-SD and ULV-ED applications on a 40 cm high crop

Preventive virus applications with HV and LV-SD systems resulted in averages of 99 ± 1% and 100% larval mortality, respectively. ULV-ED applications gave much lower and rather variable results with 85%, 74% and 24% larval mortality in the three experiments. An average of 87 ± 18 living fifth instar larvae were recovered from the untreated plots.

In all instances virus applications lead to statistically significant reductions (χ^2 test, $P < 0.05$) in the amount of feeding damage as compared to the untreated check plots (Fig. 7.1). LV-SD applications lead to the smallest amount of damage, followed by HV and ULV-ED treatments. Despite the reduction

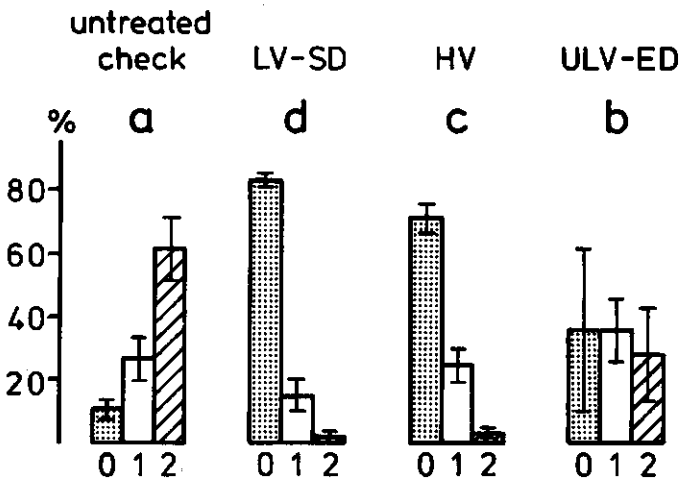

Figure 7.1 Damage caused by beet armyworm larvae in 40 cm high chrysanthemums, sprayed with SeMNPV, before egg-hatch, at a rate of 1×10^8 polyhedra/m^2 with low-volume spinning disc (LV-SD), high-volume (HV) handsprayer and ultra-low-volume electrodynamic (ULV-ED) application systems. Percentages of leaves are given in three damage classes: 0 = none, 1 = light and 2 = severe. Different letters (a,b,c,d) indicate statistically significant differences (X^2-tested, $P < 0.05$) between treatments.

of damage in the ULV-ED plots, most plants were severely damaged and not marketable. In the LV-SD and HV plots only those plants on which the egg batches had been placed had some damaged leaves. In the LV-SD plots most larvae died as first instars, generally on the lower leaves near the site of the egg batch. In the HV plots some larvae developed to second instars before they died of virus infection. In the ULV-ED plots, however, most larvae did not die of virus infection before they reached the fourth or fifth instar.

Table 7.1 Percentage Spodoptera exigua larval mortality in chrysanthemum crops of two heights sprayed with SeMNPV at a rate of 1×10^8 polyhedra/m^2 with three different application systems.

Application system	Crop height 20 cm		Crop height 60 cm	
	No. surviving larvae	% Larval mortality	No. surviving larvae	% Larval mortality
Untreated check	70	(0)	70	(0)
LV spinning disc	3	96	2	97
HV handsprayer	5	93	7	90
ULV electrodyn	3	96	21	70

HV, LV-SD and ULV-ED applications on crops of two heights

Larval control by virus applications with the three spraying systems was comparable on the 20 cm high crop, with 93-96% larval mortality (Table 7.1). The ULV-ED treatment thus resulted in much higher, and the LV-SD and HV in slightly lower levels of larval control as compared to the first experiment. On the 60 cm crop, however, in the ULV-ED plots only 70% larval mortality was found. The crop height did not seem to greatly influence the level of larval control achieved with HV and LV-SD applications (Table 7.1).

The amount of damage (Fig. 7.2) followed the same pattern as the mortality data. In the low crop damage in the ULV-ED plot was not significantly different from that in the LV-SD plot (χ^2 test, $P < 0.05$), whereas the HV-treated plot showed significantly more damage. In the higher crop the results were similar to those of the previous experiment (on 40 cm high chrysanthemums), with the LV-SD applications causing the largest reduction of damage, followed by the HV and the ULV-ED treatments.

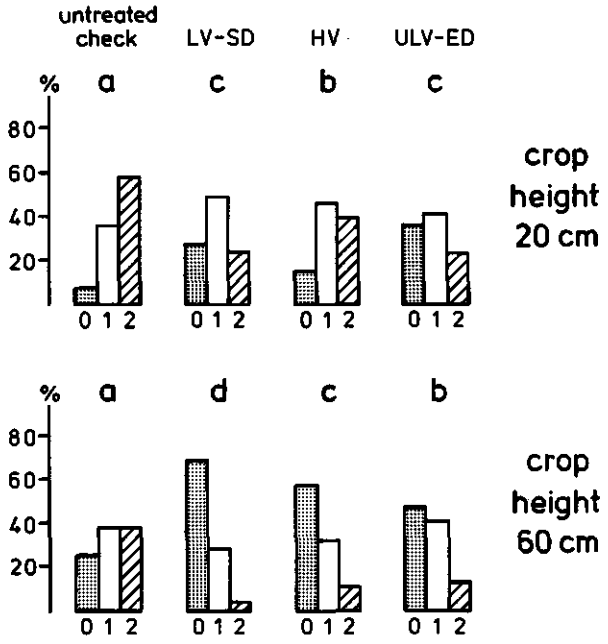

Figure 7.2 Damage caused by Spodoptera exigua larvae in low (20 cm) and high (60 cm) chrysanthemum crops, sprayed with SeMNPV, before egg-hatch, at a rate of 1×10^8 polyhedra/m² with low-volume spinning disc (LV-SD), high-volume handsprayer (HV) and ultra-low-volume electrodynamic (ULV-ED) application systems. Data presented as in Fig. 7.1.

HV and ULV-ED applications against second instar larvae

In contrast with both previous experiments in which virus was applied prior to egg hatch, applications were now made against second instar larvae. The ULV-ED applications caused an average of 88 ± 4% larval mortality and in the HV plots 99 ± 1% mortality was found.

Corresponding with the obtained levels of larval mortality, the amount of damage to the crops (Fig. 7.3) was lower in the HV plots than in the ULV-ED plots. Despite the high levels of larval mortality the reduction in the amount of damage compared to the untreated check plots, although statistically significant (χ^2 test, $P < 0.05$), was considerably less pronounced as compared to the previous experiments. This is not surprising since most larvae died of virus infection as fourth instars, after they had already severely damaged the plants.

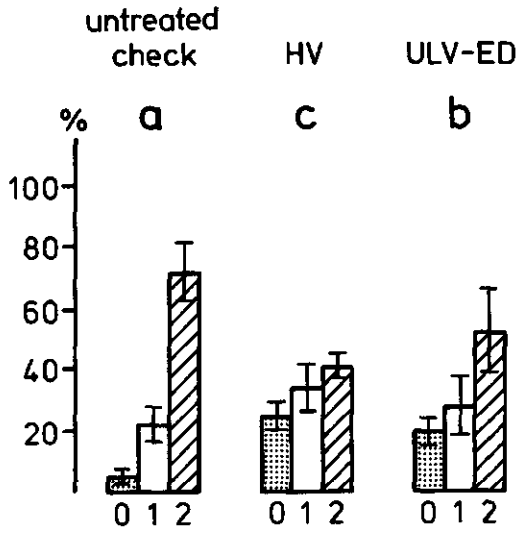

Figure 7.3 Damage caused by second instar Spodoptera exigua larvae in a 30 cm high chrysanthemum crop, sprayed with SeMNPV at rate of 1 x 10^8 polyhedra/m^2 with high-volume handsprayer (HV) and ultra-low-volume electrodynamic (ULV-ED) application systems. Data presented as in Fig. 7.1.

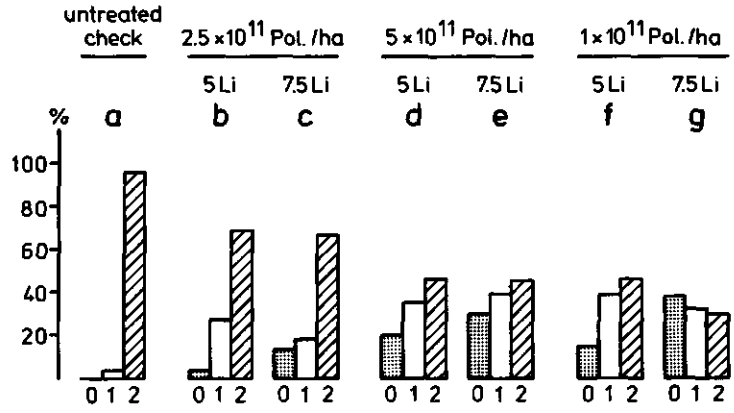

Figure 7.4 Damage caused by Spodoptera exigua larvae in a 30 cm high chrysanthemum crop, sprayed with SeMNPV before egg-hatch, at three virus rates (polyhedra/m^2) in two spray volumes (5 and 7.5 liters) with an ultra-low-volume electrodynamic (ULV-ED) application system. Data presented as in Fig. 7.1.

ULV-ED applications at various rates and spray volumes

To further assess the potential of the ULV-ED system for virus applications, different virus rates were applied in spray volumes of 5 and 7.5 l per ha, on a 35 cm high chrysanthemum crop. The results showed that larval mortality increased from ca. 70% to ca. 95% with increasing virus rate (Table 7.2, Fig. 7.4). In all cases a slightly higher larval mortality was found in the plots sprayed with the 5 l spray volume. An average of 188 ± 11 living fifth instar larvae were recovered from the untreated check plots.

The amount of damage to the crop at comparable virus rates, however, was in all cases significantly lower (χ^2 test, $P < 0.05$) in the crops sprayed with the 7.5 liter spray volume. Increase of the virus rate in all cases gave statistically significant reductions in the amount of damage.

Table 7.2 Percentage Spodoptera exigua larval mortality in 35 cm high chrysanthemum crops sprayed with SeMNPV at three rates in two spray volumes with an ultra-low-volume electrodynamic application system.

Virus rate (polyhedra/m²)	Spray volume 5 liter/ha		Spray volume 7.5 liter/ha	
	No. surviving larvae	% Larval mortality	No. surviving larvae	% Larval mortality
Untreated check	158	(0)	158	(0)
2.5×10^7	41	74	56	65
5×10^7	21	87	31	80
1×10^8	5	97	11	93

7.4 Discussion

Virus applications with the LV-SD system resulted in lower amounts of damage than HV applications. Levels of larval mortality were, however, in most cases comparable. ULV-ED applications generally gave poorer results than the two other systems, both in the level of larval mortality and in the amount of damage to the crop. Nonetheless the experiments showed that control of beet armyworm with virus using the ULV-ED system is possible, but that this technique still has some limitations.

Comparing the effects of ULV-ED applications in the various experiments there is a tendency of poorer control efficacy with increasing crop height. Observations on the deposition patterns under ultra-violet light showed that droplets were predominantly deposited on the top 5-6 leaves, with very little deposition on lower leaves. The underside coverage of upper leaves was rather good, although the number of droplets on the underside was lower than on the upperside of leaves. Penetration of charged droplets into the crop seems to be the main problem with this application technique (Matthews, 1982). Top leaves, in particular those of plants that are slightly higher than their surrounders, tend to 'catch' a relatively large proportion of the charged droplets (Morton, 1982). Deposition of virus on lower parts of the plant where the younger larvae reside (Chapter 3) was limited, which explains the poor control results. Addition of air support may help to improve the efficacy of virus applications with this spraying system.

HV spraying is the most common used application method for pesticides in greenhouses. Virus applications with this system generally gave good control of beet armyworm larvae. A main disadvantage of this system is the large waste of the control agent. Jarret et al. (1978) showed that 67% of a Bacillus thuringiensis spray was wasted on the soil surface. Furthermore the large size of the spray droplets leads to a patchy and suboptimal distribution of the control agent on foliage.

Virus applications with the LV-SD spraying system showed a slightly better control efficacy than the HV applications in all the experiments. The LV-SD application system, in particular when provided with air support, generally gives high deposition rates and the doplets with a VMD of ca. 70 µm provide excellent coverage of the crop (De Heer et al., 1984). Falcon & Sorenson (1976) showed that applications with such systems gave superior crop coverage as comparable HV sprayings. They applied B. thuringiensis, four different nuclear polyhedrosis viruses and an entomopathogenic fungus. Smith et al. (1977) also used a spinning disc to apply various pathogens and concluded that a combination of small droplet size and high concentration of droplets per cm^2 would give optimal results. Smith & Bouse (1981) reviewed available literature on spraying techniques for pathogens and suggested that a droplet size of 100-150 µm probably would be most effective, but that increasing the number of droplets would improve coverage and the chance that larvae would ingest virus.

Larval feeding behaviour probably is the most important factor determining the optimal deposition pattern of a control agent in a particular crop. S. exigua deposits its eggs on chrysanthemum leaves near to the soil surface

(Chapter 2). After hatching larvae first feed on leaves in the lower parts of the crop but then gradually move upwards and predominantly feed on the upper foliage layers (Chapter 3). This explains why in the first experiment in the ULV-ED plots many larvae died of virosis as fourth instars, whereas in the other plots larvae were killed as first or second instars. ULV-ED applications give deposition only on the top leaves and larvae do not feed on top leaves before they have developed to second or third instars (Chapter 3). After ingesting a lethal virus dosis it generally takes about 6 days or one moult (Chapter 5) before the larvae actually die of the virus infection. In the LV-SD and HV treated plots apparently the larvae ingested virus at a much earlier stage while still feeding on the lower leaves.

De Heer et al. (1984) showed that on ca. 60 cm high chrysanthemums, LV-SD applications gave 30-50% depositon of the control agent on leaves in the upper foliage stratum and only ca. 10% deposition on leaves in the lower part of the crop. Deposition patterns of HV applications were comparable, although at a slightly lower level. Their results thereby show that also with these spraying systems good coverage of the lower leaves in high chrysanthemum crops is difficult, if not impossible. Nonetheless the relatively low deposition rates on the lower leaves appeared to be sufficient to infect a large proportion of the larvae at a relatively early stage.

From studies on larval feeding behaviour in chrysanthemum (Chapter 3) it was concluded that deposition of the virus at the underside of leaves would be desirable since young larvae predominantly feed superficially at this side of leaves. Another argument favouring virus application to the underside of leaves is the reduction of virus inactivation by ultra-violet radiation (Biache & Chaufaux, 1982; Jaques, 1985). The only spraying system that gives underside deposition is the ULV-ED system. The results of this study showed, however, that in practice the ULV-ED system did not live up to its expectations, for reasons discussed above.

Summerizing we can conclude that both the high-volume handspraying and low-volume spinning disc application systems are suitable for applying nuclear polyhedrosis virus to control S. exigua in greenhouse chrysanthemums, with the latter system giving slightly better results. Application of virus with the ultra-low-volume electrodynamic application system showed some promise, but in particular on higher crops did not give satisfying results.

CHAPTER 8

CONTROL OF SPODOPTERA EXIGUA LARVAE WITH NUCLEAR POLYHEDROSIS VIRUS

8.1 Introduction

The results of a previous study on the application of Spodoptera exigua multiply-enveloped nuclear polyhedrosis virus (SeMNPV) with various spraying systems (Chapter 7) showed that good control efficacy of beet armyworm larvae in chrysanthemums was achieved at a rate of 1×10^8 polyhedra per m^2. The experiments also showed that low-volume applications with a spinning disc sprayer gave slightly better results than high-volume applications with a handsprayer. Nonetheless the latter spraying system is used in the study presented in this chapter, because most growers still use it, whereas spinning disc sprayers are only scarsely used.

In this chapter the effect of virus applications at various rates on larval mortality and feeding damage in several greenhouse crops is described. Experiments were carried out mainly on chrysanthemums (Chrysanthemum morifolium Ramatuelle), but also on gerbera (Gerbera jamesonii Bolus), kalanchoe (Kalanchoe blossfeldiana v. Poelnitz) and tomato (Solanum lycopersicon L), infested with larval populations of various ages. In addition, the effect of virus applications was compared with that of two currently used chemical insecticides, methomyl and diflubenzuron.

8.2 Materials and methods

Virus

The isolate of SeNPV (Baculoviridae, subgroup A) originated from the USA (Chapter 4) and is identical to the major genotype described by Gelernter & Federici (1986). The virus was propagated in laboratory-reared larvae as described in Chapter 6. Prior to use the virus was stored in phosphate buffered saline (PBS) at -20°C. Virus concentrations were determined by three independent counts of polyhedra made with a haemocytometer under a phase-contrast microscope (x400).

Concentrated virus suspensions were diluted with water and 0.05% Agrall NN (ICI) as a spreader-sticker was added. A total volume of 200 ml was applied per

m^2 of soil surface, with a AZO-propane handsprayer (AZO) using a Birchmeier helicon safier nozzle 100 or 120, at a pressure of 4 bar. The rates at which the virus was applied are given in numbers of polyhedra per area of soil surface. The control plots were sprayed with the equal amounts of water with spreader-sticker.

Insects

Insects were taken from a laboratory culture originating from larvae collected in Dutch greenhouses. Larvae were reared in groups of 30-200 on semi-synthetic diet at 30°C, 70-80% relative humidity and a L:D = 16:8 photoperiod (Chapter 2).

Greenhouse

The experiments were carried out in a heated greenhouse (4 x 6 x 4 m). The temperature ranged between 23 and 35°C during daytime, depending on the outside weather conditions and was kept at 23°C at night. The relative humidity varied around 60%.

Experiments on chrysanthemum

The experiments on chrysanthemum were carried out on 30 cm high (3-4 weeks old) and 90 cm high (8-9 weeks old) plants of the cultivar 'Milonka', that were planted as small cuttings in soil. The plants were not flowering. Each plot of 1 x 1 m consisted of 36 plants, planted in six rows with 12 cm spacing. The plots were surrounded by a 20 cm high plastic screen covered with a band of sticky tape to prevent larvae from escaping.

Larvae were released several hours after virus applications in order to prevent larvae from drowning in the water droplets. Per plot 144 larvae, either 72 first and 72 second or 72 third and 72 fourth instar larvae were released. Virus was applied at four different rates: 2.5×10^7, 5×10^7, 1×10^8 and 2×10^8 polyhedra per m^2. All experiments were carried out three times.

Plots were sampled 14 days after virus application. Because pupae buried in the soil are hard to find, the sampling was carried out a few days earlier when larvae started to pupate within that 14-day period. In such cases all retrieved larvae were further reared in the laboratory on semi-synthetic diet at 25°C until pupation. All plants were sampled for the presence of living and dead larvae. The quantity and intensity of the feeding marks on plants was determined by sampling the individual leaves of one-third of the plants in each plot. Three arbitrary classes of damaged leaves were distinguished to quantify the amount of feeding damage (0): leaves with no feeding marks; (1): leaves with superficial

feeding damage on less than one-third of the surface or with a few small perforations; (2): leaves with superficial feeding marks on more than one-third of the surface or with large perforations.

The percentage of larval mortality was calculated by dividing the number of surviving larvae in the treated plot by the number of living larvae found in the control plots. Virus-killed larvae were recorded but not included in the calculations as the chance of finding them is strongly influenced by their size at the moment of death.

Experiments on gerbera

Gerbera 'Fleur' plants were grown on rockwool provided with a nutritional solution. Each plot of 2 x 2 m consisted of three rows of nine plants, about one year old with 80-100 leaves each. 150 Moths were allowed to oviposit on the plants. One plot (plot A) was sprayed with virus, at a rate of 1×10^8 polyhedra per m^2, just before the eggs hatched. A second plot (plot B) was sprayed when the larvae had developed to third or fourth instars. A third plot (plot C) served as control. The experiment was carried out twice.

At several day intervals all 30 flowers present in each plot were sampled for living and dead larvae as well as for feeding marks. The flowers were then replaced by fresh ones obtained from an identical crop in another greenhouse. The flowers were provided with small tubes filled with water that were pressed into the rockwool in between the foliage. Living larvae were returned to the plots after sampling. The foliage was sampled for larvae only at the end of the experiment, 30 days after the first virus application, by shaking the leaves above white paper sheets.

Experiments on tomato

Tomato 'Moneymaker' plants were 150 cm high and grown in soil. Each plot of 4 x 2 m consisted of 30 plants bearing flowers and unripe fruit. As in the gerbera experiment 150 adults were allowed to oviposist in the crop. Virus applications were performed at a rate of 1×10^8 polyhedra per m^2, just before egg hatch in one plot and when larvae had developed to third or fourth instars in the second plot. All individual leaves of all plants were sampled for feeding marks and the presence of living and dead larvae when the larvae in the control plots started to pupate, about 30 days after egg hatch. The experiment was carried out twice.

Experiments on kalanchoe

Kalanchoe 'Singapore' plants were grown in pots on tables in the greenhouse. The plants were flowering and ca. 30 cm high. Both the treated and the control plot consisted of 44 flowering plants, placed in a 1 x 1 m compartment. Three first instar larvae were released per plant after the plots were sprayed. A virus rate of 1×10^8 polyhedra/m^2 was used. The percentages of flowers and foliage with feeding marks were recorded, as well as the numbers of living and dead larvae, 14 days after the virus application. The experiment was performed only once.

Comparing control with virus with that of chemical insecticides

The comparison of virus with chemical insecticides was studied on plots of 48 chrysanthemum 'Milonka' plants grown in soil in aluminium containers (125 x 80 x 40 cm). Commercial wettable powder preparations of Methomyl (Lannate) and diflubenzuron (Dimilin) were used at concentrations of 0.1% in 200 ml of water/m^2. Second instar larvae were released at a rate of four per plant. The plots were sprayed several hours after release of the larvae. The virus plot was sprayed only once at a rate of 1×10^8 polyhedra/m^2, whereas the methomyl and diflubenzuron plots were sprayed at the same time the virus plots were treated and then again 7 days later. The plots were sampled as described for the other experiments on chrysanthemum 14 days after the first applications. The experiment was performed three times.

Statistics

Differences between treatments in the distribution of leaves in the three damage classes were statistically analysed using a χ^2 test with significance at $P < 0.05$.

8.3 Results

Chrysanthemums

High levels of larval mortality were achieved by all the virus applications (Table 8.1). Treatments against young larvae in the shorter chrysanthemums even at the lowest rate of application resulted in almost complete larval control. Control of third and fourth instar larvae on the small plants was also effective although the lowest rate gave only 91% larval mortality. On tall chrysanthemums larval control was less effective and to achieve a larval mortality level of 95%, needed for practical use, at least a rate of 1×10^8 polyhedra was

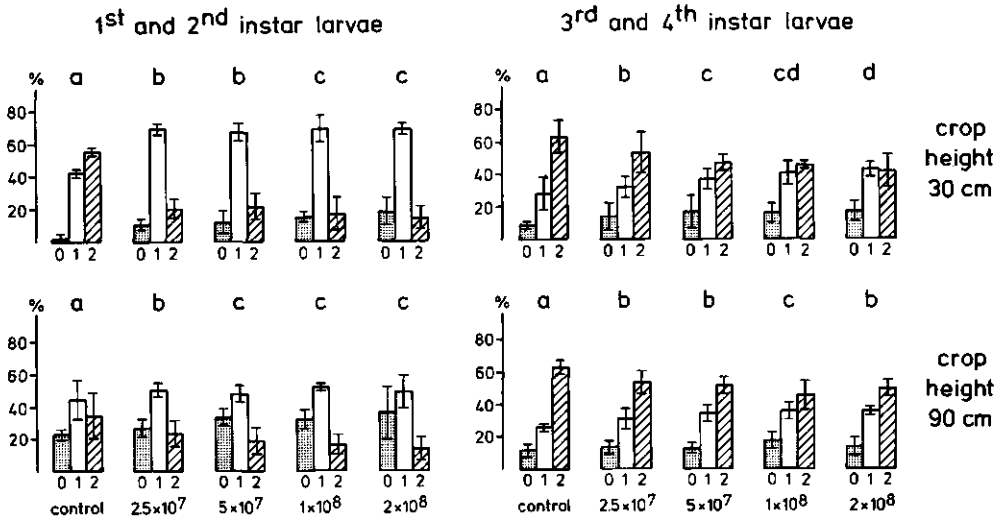

Figure 8.1 Damage caused by various Spodoptera exigua larval instars in chrysanthemum crops of two different heights sprayed with four rates (polyhedra/m²) of SeMNPV. Average percentages of leaves are given in three damage classes: 0 = none, 1 = light and 2 = severe. Different letters (within each combination of crop height and larval instars) indicate statistically significant differences (χ^2-tested, $P < 0.05$).

Table 8.1 Mean percentage mortality ± SD of Spodoptera exigua larval instars in chrysanthemum crops of two heights after application of SeMNPV at four different rates (polyhedra/m²).

Crop height	Larval instars	Virus rate in polyhedra/m²			
		2.5×10^7	5×10^7	1×10^8	2×10^8
30 cm	1 & 2	99.5 ± 0.9	99.5 ± 0.9	100 ± 0	100 ± 0
	3 & 4	91.5 ± 5.3	100 ± 0	100 ± 0	100 ± 0
90 cm	1 & 2	72.9 ± 24.4	85.8 ± 13.7	97.1 ± 2.6	100 ± 0
	3 & 4	94.8 ± 1.5	90.9 ± 6.6	91.5 ± 3.5	98.1 ± 2.1

required. Again the control of young larvae was more effective than that of older instars.

The number of living larvae retrieved from the control plots at sampling ranged from 50-70 larvae per plot, which means about 35-50% survival of the numbers released. Between 10 and 20% of the larvae were found trapped in the sticky tape between the plots. In the virus-treated plots the number of retrieved virus-killed larvae together with the survivors generally added up to about 40% of the number of larvae released originally.

All virus applications resulted in significant (χ^2 tested, $P < 0.05$) reductions of the amount of feeding damage as compared to the control plots (Fig. 8.1). Despite these reductions the plants in the virus-treated plots often were severely damaged. Increasing the rate of virus application reduced feeding damage only slightly. In most cases a fourfold increase in the rate was necessary to obtain a statistically significant decrease of damage (Fig. 8.1). The reduction in the amount of damage was most pronounced in the shorter crops with virus applications against young larvae. Most of these larvae died during the second or the third instar. In the tall crops the released first and second instars often developed to fourth instars before they were killed by the virus. Therefore relatively more damage occurred in these crops, despite effective larval control. In both the short and tall chrysanthemums in which third and fourth instar larvae were released, most larvae developed to the fifth instar before they were killed by the virus. Therefore, the reduction in the amount of damage as compared to the control plots was relatively small.

Gerbera

A rate of 1×10^8 polyhedra per m^2 was chosen, as it had given good control of both early and late instar larvae in chrysanthemums. Data presented in Fig. 8.2 originate from one of the two experiments. As the sampling data and the temperature were slightly different it was difficult to combine the data from both experiments, although the results were similar.

Fig. 8.2 shows that shortly after egg hatch most of the flowers were damaged by larval feeding. Sometimes up to 40 larvae were found in a single flower. In plot A, which was treated with virus around egg hatch, the proportion of damaged flowers began to decrease about 12 days after virus application. Most larvae were killed by virus during the first or second instar and large numbers of virus-killed larvae were observed on flowers and foliage 7-10 days after the virus application. Larval mortality increased from 80% on day 12 to 99% on days 22 and 29.

In the plots that were sprayed with virus when the larvae had developed to third and fourth instars (plot B), the proportion of damaged flowers decreased after day 22, 10 days after the virus was applied. Most larvae in these plots died of virus infection 8-12 days after virus application and larval mortality increased from 80% at day 22 to 90% at day 29. In the control plots the proportion of damaged flowers remained high throughout the experiment. Larval numbers in the control plots at the end of the experiments were 4 and 10 larvae per plant, respectively, in both experiments.

The results of the second experiment were comparable to those of the first, with a decrease in the proportion of damaged flowers in plots A and B, 12 and 14 days after virus application, respectively. Larval mortality reached levels of 99% and 96% in plots A and B, respectively.

In addition to the decrease in the number of damaged flowers in the virus-treated plots, the intensity of feeding damage to the foliage was also reduced. In both plots A and B almost all leaves showed feeding marks, but in the early-sprayed plots the damage was limited to small superficial marks, whereas in the late-sprayed and the control plots all leaves showed large perforations.

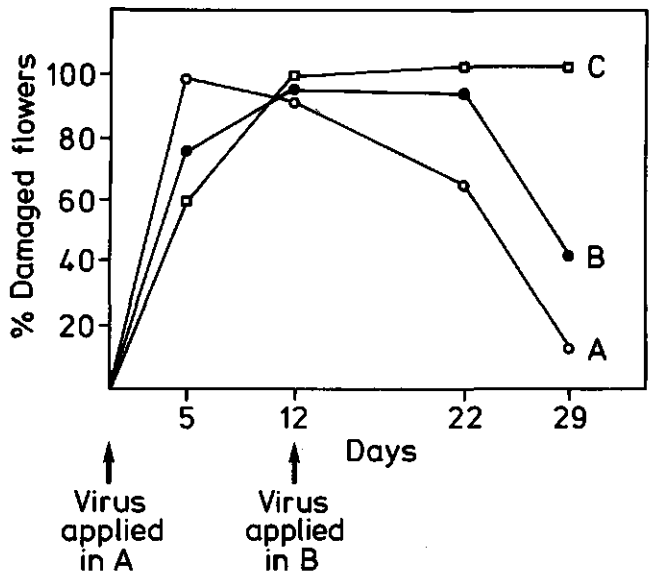

Figure 8.2 Proportion of flowers damaged by Spodoptera exigua larvae in gerbera crops sprayed with SeMNPV at a rate of 1 x 10^8 polyhedra/m^3. A: crop sprayed with virus at egg hatch. B: crop sprayed with virus when larvae were third instars. C: control plot.

Tomato

Early virus applications, around the moment of egg hatch, at a rate of 1×10^8 polyhedra/m^2 resulted in 100% larval mortality in both experiments on tomato. Late applications, when larvae had developed to third or fourth instars, resulted in an average of 98% mortality. An average number of 257 larvae was retrieved alive from both control plots.

The average proportion of damaged leaves was 19%, 42% and 46% in the early virus-treated, late virus-treated and control plots, respectively. Damage to fruit was not found in the early virus-treated plots. In the late virus-treated and control plots an average of 2% and 1% of the fruit showed feeding marks, respectively. No feeding marks on flowers were observed in either of the plots. Most feeding took place on the lower leaves of the plants (see also Chapter 3). Larval feeding generally resulted in perforations of the leaves, and the typical superficial feeding marks found on chrysanthemum and gerbera foliage were not encountered often.

Kalanchoe

On kalanchoe 99% larval mortality was achieved by application of virus at a rate of 1×10^8 polyhedra/m^2. The proportion of flowers and the proportion of leaves with feeding marks was reduced from 93% and 84% in the control plot to 69% and 22% in the virus-treated plot. The reduction in feeding marks on the foliage was therefore much more evident than that on the flowers. In the virus-treated plot most larvae died during the second and third instar, between 8 and 10 days after virus application. In the control plot, 70 fifth instar larvae were retrieved at sampling.

Virus versus chemical insecticides

As shown above, virus applications at a rate of 1×10^8 polyhedra/m^2 were effective against beet armyworm larvae. Greenhouse growers currently use methomyl (Lannate) or diflubenzuron (Dimilin) to control beet armyworm. Multiple applications of these chemical insecticides are generally necessary to eliminate the insect from the greenhouse.

Comparison of the efficacy of the three control agents against second instar larvae in chrysanthemums showed that a single virus application caused 99 ± 1% larvae mortality, whereas two successive treatments with methomyl and diflubenzuron resulted in 81 ± 3% and 80 ± 15% mortality, respectively. The large variation in the results with diflubenzuron is caused by the relatively

low level of 62% mortality in the first experiment. In the two other experiments larval mortality levels of 87% and 90% were found.

The amount of feeding damage, perhaps a more important aspect for growers, was reduced significantly (X^2-tested, $P < 0.05$) as compared to the control plots by all three treatments (Fig. 8.3). In the plots treated with diflubenzuron significantly less damage was found than in the plots treated with virus, which on their turn showed significantly less damage than the plots treated with methomyl. Despite the reduction of damage in all treated plots, most plants had several damaged leaves and were not marketable.

The virus and diflubenzuron killed most larvae during the fourth and fifth instar 8-12 days after the first application. In the methomyl-treated plot the larvae that were killed generally died within days after the first application, as second or third instars. The second methomyl application killed only a few larvae. An average number of 188 living larvae were retrieved from the control plots.

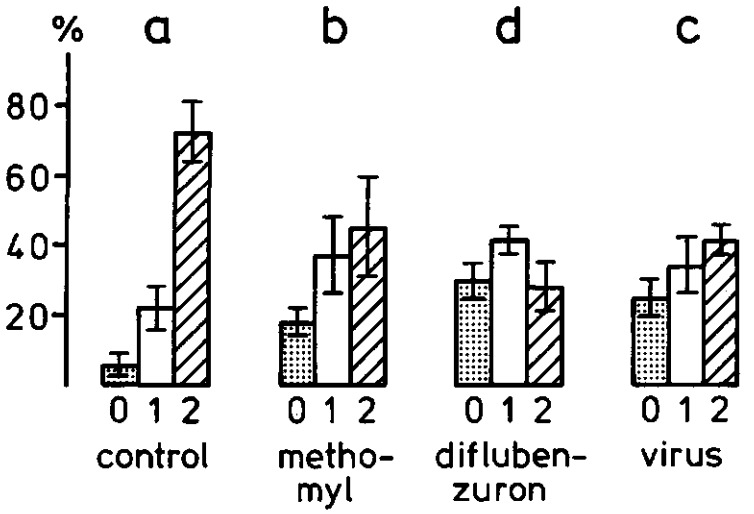

Figure 8.3 Comparison of the amount of feeding damage on chrysanthemum foliage by second instar Spodoptera exigua larvae after application of methomyl (0.1%), diflubenzuron (0.1%) and SeMNPV (1×10^8 polyhedra/m²). Data presented as in Fig. 8.1.

8.4 Discussion

Virus applications at a rate of 1×10^8 polyhedra per m^2 of soil surface generally resulted in over 95% larval mortality in all crops. Despite these high levels of mortality and statistically significant reductions in the amount of feeding marks severe damage to treated crops often occurred, particularly when the virus was applied against late instar larvae. Virus applications resulted in only slightly lower mortality levels, when applied against late as compared to early instars. Early instar larvae, however, are much more susceptible to the virus (Chapter 5). Increased feeding on virus-treated surfaces probably compensates the lower susceptibility of older larvae by a higher virus intake (Smits, 1986).

Gelernter et al. (1986) applied SeMNPV at rates of 2.8, 5.5 and 11×10^7 polyhedra/m^2 on outside grown lettuce. The highest rate gave 50-80% reduction in the number of larvae about two weeks after application. Falcon (1971) states that multiple applications at a rate of about 3.6×10^7 polyhedra/m^2 provided effective suppression of beet armyworm populations in cotton. Mcleod et al. (1978) found that rates up to 6×10^7 polyhedra/m^2 caused a 50% reduction of larvae populations in soybeans but no reduction of damage.

'Natural mortality' in our experiments was consistent at a level of about 50% in nearly all experiments. Only in the experiments carried out in aluminium containers over 95% of the larvae were retrieved. Factors involved in the natural mortality are the change from artificial diet to plants, the presence of predators like ants in the greenhouse and cannibalism. Mortality in the control plots due to virus contamination generally was at a level lower than 5% of the number of retrieved larvae.

Timing of the application as early as possible during larval development is important especially on crops such as chrysanthemums and kalanchoe, because almost any feeding mark on flowers and foliage results in economic loss. On most ornamentals there is almost a null-tolerance for insect feeding damage. In tomato and other vegetable crops feeding marks on foliage are less important, as long as the production of fruit is not influenced by a severe reduction in the foliage surface.

Growers generally detect the damage to the crop when the larvae are already in their third instar. Methomyl is not very effective against larvae of this age. Virus or diflubenzuron can control the larvae and prevent future generations but cannot avoid severe damage to the crop inflicted by the generation present at that moment. A monitoring system based on light-trap

catches may provide sufficient information on the presence of adults in the greenhouse and the moment eggs and young larvae can be expected to occur. Pheromones of beet armyworm are also available for monitoring (Tingle & Mitchell, 1975) but do not perform well under greenhouse conditions (Van den Bos, 1983b).

Preventive virus applications showed variable results (P.H. Smits, unpubl. results). Although virus inactivation in greenhouses proceeds less rapidly than under field conditions, due to the filtering of short-wave ultra-violet radiation by the glass coverings, the remaining light in combination with environmental factors such as temperature probably still inactivates a large proportion of the virus within one or two weeks after application (Biache & Chaufaux, 1982; Jaques, 1985).

Application to the underside of foliage will probably increase the efficacy of the virus as the young larvae mainly feed at the underside of leaves, without consuming the upper epidermis (Chapter 3). Spraying from underneath with conventional spraying systems is feasible on tomatoes. Many ornamentals, however, can only be sprayed from above, as they are grown in dense multi-row beds, like chrysanthemums, or have their foliage close to the soil surface, like gerbera and most potted plants.

It can be concluded that SeMNPV shows promise as a control agent for beet armyworm larvae in Dutch greenhouses. A virus rate of 1×10^8 polyhedra/m^2 performed well in various crops, also against late instars. Virus applications resulted in higher larval mortality levels than the only two currently available chemical insecticides. The damage inflicted to the crop in the relatively long period between virus application and larval death causes problems for the practical use. If, however, the development of resistance against chemical insecticides in beet armyworm continues as it has done and no better alternative control methods become available, the virus will be an useful control agent and its development into a commercial product should therefore be considered.

Timing of virus application

S. exigua adults lay most of their eggs between 5 and 10 days after emergence (Fye & McAda, 1972) and eggs hatch within 3-5 days (Table 1.1). This means that between 3 and 10 days after adults are caught, young larvae can be expected to occur in the crops. Because adults may not be caught on the first day of their life-span or on the first day of their presence in the greenhouse this period may even be shorter than three days. When virus is applied immediately or within one or two days after adults are caught a good control efficacy therefore seems ensured (Chapter 8).

In most situations, however, the grower will not become aware of the pest before the larvae have developed to third or fourth instars. Feeding marks caused by younger larvae are difficult to spot since they are small and situated on the lower leaves of plants (Chapter 3). Even individual larvae from the same egg batch may show considerable differences in their rate of development and in most situations therefore a mixture of various developmental stages will be present in the greenhouse by the time the grower applies the virus.

Young instars already present and those hatching from eggs within a few days will be killed without severely damaging the crop (Chapter 8). Although third and fourth instars will also be killed by the virus, they may cause severe damage to the crop before they die (Chapter 8). Part of the population of fifth instars will be killed by the virus, but another part will certainly survive, pupate and give rise to a new generation (Chapter 5). Between 10% and 30% of the progeny of those fifth instar larvae that were infected with virus but managed to survive and pupate, can be infected with the virus by transovarial transmission (Chapter 5). Pupae, adults and eggs present at the moment of application are not affected by the virus. Because of the short life cycle (Table 1.4) the surviving part of the population will produce a new generation of larvae within 2-3 weeks.

It is doubtful whether enough virus persists on the crop during this period to effectively control the larvae of this new generation. On fast growing crops certainly a second application 10-14 days after the first appears to be necessary, in order to cover the new-grown leaves with virus. For this second application probably a lower rate can be used as it is aimed at young instars (Chapter 8).

Where to apply virus?

Virus has to be ingested with the food by larvae to cause infection. Therefore virus will show the best control efficacy when it is applied to those parts of the crop or sites within the crop, where most of the larval feeding occurs.

Eggs are laid on the bottom leaves of plants (Chapter 2). Young larvae first feed in the vicinity of the egg batch and then gradually move upwards to the top of plants (Chapter 3). Predominantly they feed superficially on the underside of foliage. The underside of the bottom leaves of plants therefore seems the optimal site for virus application. The only spraying system that gives spray deposition at the underside of leaves, the electrodynamic sprayer, did not give good control efficacy on chrysanthemums because the deposition of spray droplets on the lower leaves was poor (Chapter 7). High-volume handspayers and low-volume spinning disc systems gave better deposition on leaves in the lower part of the crop but not at the underside of foliage (De Heer et al., 1984). In dense crops that are grown in multiple-row beds, such as chrysanthemums and many other cutflowers, there seems no alternative for high- or low-volume applications with sprayers positioned above the crop. Potplants are generally grown on tables and not very tall, but treatment of the underside of leaves also seems difficult in many cases. On tomato, cucumber and sweet pepper more ideal situations for the application of virus exist, as the crops are very open, tall (2 m) and grown in only single or double rows. Virus application at the underside of leaves both in the lower and upper regions of plants therefore is relatively easy. Many growers already have spraying booms with upwards directed nozzles. An experiment on sweet peppers showed that virus application with such a spraying system gave good control of beet armyworm larvae (P.H. Smits, unpubl. results).

Oviposition by beet armyworm can be concentrated on crops of certain age or cultivar, but about 20% of eggs may still be deposited on other plants (Chapter 2). Surprisingly often egg batches were also found on the glass walls of greenhouses or on the plastic used for isolation. Observations in various commercial greenhouses further showed that beet armyworm larvae were often present on weeds. It seems therefore, that all plants in a greenhouse have to be treated with virus to eliminate a beet armyworm population. Growers who used chemical insecticides against beet armyworm larvae and treated only that part of the crop where most of the larvae were found with intervals of several weeks, often had continuing problems with beet armyworm, sometimes over periods of several years. Growers who conscientiously applied insecticides at regular intervals during a several week period, who treated all the crops in the greenhouse and that removed the weeds, generally managed to eliminate beet

armyworm from their greenhouses. In this respect the use of virus will not differ much from the use of chemical insecticides, although two treatments should be sufficient to eliminate the population (see previous paragraph).

Persistence of virus on foliage

Despite the fact that a large proportion of the UV-radiation is absorbed by the glass coverings the persistence of virus on foliage in the greenhouse appears to be rather limited. Studies by Jaques (1967, 1972) and Biache & Chaufaux (1979) showed that the persistence of virus on plants in greenhouses is about twice as long as on similar plants exposed to direct sunlight. Most virus present on the upper leaves of plants in greenhouses is therefore probably inactivated within a week after application. On lower leaves that are less exposed to sunlight and at the underside of foliage virus persistence may be considerably longer (Jones & McKinley, 1986).

The part of the UV-radiation that is most detrimental to NPVs lies between 320 and 280 nm (Jaques, 1985). Radiation of these wavelenghts does not pass through the glass that is generally used for greenhouses (J.A. Stoffers, unpubl. results) and the rate of virus inactivation would be expected to be much lower than is actually observed. There are, however, also other factors that influence the persistence of virus on foliage. Light of longer wavelengths, temperature and the pH of dew on the leaf surface have all been mentioned as factors that alone or in combination may inactivate virus (Jaques, 1985). Many different compounds have been added to NPV formulations in order to protect the virus from UV-radiation. A number of these compounds indeed gave some protection, but until now no real effective UV-protectant has been reported (Jaques, 1985). In the soil virus may persist for several years without losing its activity (Jaques, 1985).

Inoculative versus inundative virus application

After a single application a certain amount of virus may persist for longer periods in the greenhouse, on plants or in the soil. This inoculum can be sufficient to cause epizootics in future beet armyworm generations entering the greenhouse. It was shown that virus present in the soil can again contaminate foliage by splashing rain or via crawling larvae (Cunningham, 1982). A small proportion of larvae may be infected in this way and the disease may then spread rapidly through the population. This happens in California where yearly at the end of the season the beet armyworm population crashes by a SeMNPV epizootic (B.A. Federici, pers. comm.). Such epizootics may also occur in Dutch

greenhouses but probably not before the population in a greenhouse has had time to build up to high numbers. Considering the low damage thresholds for ornamentals and vegetables grown in Dutch greenhouses inundative virus applications therefore appear to be more suited than inoculative applications.

Compatibility with application systems, additives and other control agents

SeMNPV can be effectively applied with high-volume handsprayer and low-volume spinning disc application systems. Applications with the ultra-low-volume electrodynamic sprayer did not give good control results (Chapter 7).

In general, nuclear polyhedrosis viruses (NPVs) can be applied with most spraying equipment, even foggers (Jarret & Burges, 1982b), without losing activity as long they are not exposed to temperatures above 60-70°C (Smith & Bouse, 1981). The biological activity of NPVs is not affected by oils, emulsifiers and most other chemicals that are used as spray and formulation additives (Couch & Ignoffo, 1981). NPVs are, however, sensitive to pH-values higher than 9 or lower than 4, and to desinfectants as formaldehyde and hypochlorite. Prolonged exposure to temperatures above 40°C may also cause inactivation (Jaques, 1985).

With a few exceptions NPVs are compatible with other chemical and biological control agents (Jaques & Morris, 1981). Sometimes they are even used in spray mixtures with chemical and microbial insecticides because of additive, synergistic or potentiating effects. NPVs may compete for hosts with other pathogens but also with parasites and predators. In larvae that are infected with NPV before or shortly after parasitization, the parasite is generally killed when the host larvae dies of virus infection (Kaya & Tanada, 1972). Since SeMNPV is specific for beet armyworm larvae no adverse effects are likely to occur on the parasites and predators that are used in Dutch greenhouses as biological control agents for other insect pests.

Predators like birds, beetles and hemipterans in most cases will feed readily on virus-diseased and virus-killed larvae but are themselves not affected by the virus. They are often reported as major factors in the spreading of insect virus diseases to other host populations, since the virus passes their alimentary canal without losing activity and is excreted with the faeces (Entwistle, 1983). Recent experiments with Podisus sagitta, showed that this hemipteran predator readily consumed virus-killed beet armyworm larvae. SeMNPV polyhedra passed the digestive tract of the bugs and was excreted with the faeces without losing their biological activity (Smits et al., in prep.). The bugs were also able to distribute SeMNPV to plants by walking over virus-sprayed surfaces.

Combinations of predator release and SeMNPV applications gave better control of beet armyworm populations, than the separate use of both control agents. Unfortunately, however, the predator is difficult to rear in large numbers and therefore probably not a good candidate for biological control programs in combination with the virus.

9.3 Commercialization

Virus production

Virus production in itself is straightforward and relatively easy (Chapter 6), illustrated by the input/output ratio of SeMNPV in fourth instar S. exigua larvae of ca. 1/10,000. One of the most vulnerable parts in the virus production process is the maintenance of a disease-free rearing colony of beet armyworm larvae. The best way to minimize problems is to have separate personnel and separate units for insect rearing and virus production. Furthermore clean rooms with over-pressure and extremely strict sanitary conditions are required (Shapiro, 1982). This is possible, as was shown in the large facilities of the USDA for production of Lymantria dispar MNPV (Doane & McManus, 1981), but would not be feasible for small companies that may want to produce SeMNPV for the Dutch market.

In this situation it seems best to use cheap and simple facilities and to carry out the production during a only few months of the year, e.g. in periods with low levels of other activities. The rest of the year the insect colony can be kept at low numbers and possible contaminations can be removed by individual rearing, selection and desinfection. The short life cycle of S. exigua in combination with its high fertility (avg. of 600 eggs per female) allows extention of the basic rearing colony to high numbers in a rather short period of time.

Production costs

The actual production costs for a SeMNPV product are difficult to assess but a rough estimation can be made based on the data that are available for other NPVs. The calculations are made based on the rearing costs per larva and not on the costs per quantity of virus. Larvae of the various species differ considerably in size and therefore produce various amounts of virus (Shapiro, 1982). Since the major proportion of the costs are those involved in handling

and rearing of larvae (Ignoffo, 1973), production costs per larva probably give the best estimation of the virus production costs.

Ignoffo (1973) made calculations for the production costs of Heliothis MNPV, that was later developed into the commercial product Elcar by Sandoz. Production costs, excluding overhead, were estimated at $0.07 per larva, for a small unit that produced 'only' 50.000 larvae a month. Over 70% of the costs were attributed to labour. Food and container costs were of minor importance. The costs were reduced to $0.05 per larva in a larger production unit, rearing a million larvae a month. These are 1973 prices. In 1981 Elcar was commercially sold for $3.12 per acre treatment. The product contained 2.4×10^{11} polyhedra produced in 25 larvae (Ignoffo & Couch, 1981). The costs, including overhead, were thus ca. $0.12 per larva.

The product Gypcheck contains Lymantria dispar MNPV and was produced in L. dispar larvae, at a cost of $0.02 per larva (Doane & McManus, 1981). The costs involved in processing of the virus were calculated at $0.015 per larva. Overhead costs were probably not included.

TM-BioControl-1 contains Orgyia pseudotsugata MNPV and was produced in O. pseudotsugata larvae, which are rather small and produce 7×10^{8} polyhedra per larva. Insect rearing costs in 1977 were ca. $0.02 per larva and the virus processing costs were $0.005 per larva. The total costs, including a small overhead, were estimated at $0.033 per larvae or $13.27 per 2.5×10^{11} polyhedra, which is the rate used per hectare (Brookes et al., 1978).

The rearing of beet armyworm larvae does not seem to be more complicated or expensive than the rearing of the above-mentioned species. Furthermore production is possible in large groups of larvae (Chapter 6), which should give a considerably reduction of the labour costs. All above-mentioned species were kept individually or in small groups during and prior to virus production. The production costs, including virus processing and overhead, of SeMNPV in beet armyworm larvae are estimated to be in the order of $0.20 (Dfl. 0.50) per larva. The production costs of a hectare equivalent of 10^{12} polyhedra, which can be produced in about a 1000 larvae, can thus be roughly estimated at $200 (Dfl. 500).

Quality control

It is beyond discussion that a virus product should meet certain quality standards with respect to the identity of the produced virus, its biological activity and the level of microbial contamination.

The standards used by the Environmetal Protection Agency (EPA) in the USA tolerate 10^7 viable spores per gram for the Heliothis NPV product (Ignoffo & Shapiro, 1978) and 10^9 for the Orgyia NPV (Brookes et al., 1978). These numbers correspond with 0.1 and 10 viable spores per 100 polyhedra. It seems that the levels of microbial contamination that are tolerated have been chosen rather arbitrarily. Krieg (1981) recommended to use the criterium that the microbial contamination of a virus product should not increase the naturally occurring microbial population on foliage with more than a factor 10. In nature often 10^6-10^7 bacteria are present per m^2 of foliage (Krieg, 1981). In the present study the proposed rate for SeMNPV applications is 1×10^8 polyhedra per m^2 (Chapter 6). The number of microbial contaminants in the SeMNPV product should therefore not be higher than ca. 10 contaminants per 100 polyhedra. This criterium was satisfied by almost all the SeMNPV batches that were produced in beet armyworm larvae during this study (Chapter 6). Bacteria present in NPV products generally are common non-pathogenic species like Streptococcus faecalis, that occur naturally in guts of lepidopteran larvae. Aspergillus niger is the most frequently encountered fungal contaminant (Krieg, 1981; Podgwaite et al., 1983).

Vertebrate pathogens like Salmonella spp., Shigella spp., Vibrio spp. and Staphylococcus aureus are not tolerated in virus products (WHO, 1973; EPA, 1982) and were not found in our production batches nor in other virus products (Krieg, 1981; Podgwaite et al., 1983). Nonetheless it seems wise to routinely perform at least a simple feeding test with an excessive amount of virus on mice for each produced virus batch. In case mice die or show other suspect symptoms, further tests can be performed to establish the identity of the contaminant.

In order to minimize microbial contamination and reduce the chance of other pathogenic organisms entering the production system, the inoculum should be taken from a standard stock of virus that has been purified as much as possible, e.g. by sucrose-gradient centrifugation or desinfectants, and of which the identity and biological activity are well-known.

An unequivocal identification of the virus is obtained with restriction endonuclease patterns of the viral DNA (Chapter 4). The DNA fragment patterns obtained by electrophoresis of the digest also allow tracing of possible contamination with other DNA viruses or any genetic variability.

The biological activity of the virus product should also meet certain quality standards. Apart from polyhedra counts to determine the virus concentration, also bioassays (Chapter 5) should routinely be carried out to test the biological activity of produced virus batches. Differences in biological

activity as compared to the virus stock may indicate contaminations with other NPVs or genetic changes in the virus. The droplet-feeding technique (Chapter 5) seems the most suitable bioassay system for routine use. It is relatively easy to perform and can be much better standardized and is more accurate than other bioassay systems such the leaf-disc method (Hughes & Wood, 1986). First instar S. exigua larvae are very susceptible to SeMNPV (Chapter 5) and the chance of ingesting a polyhedron rather than the quality of the ingested virus determines the dose-response relationship (Huber & Hughes, 1984). In addition first instars are difficult to handle. Second or third instar S. exigua larvae are less susceptible and easier to handle and therefore seem more suitable for testing the biological activity of SeMNPV.

The following quality control measurements for each produced virus batch are proposed:

- Determination of the polyhedra concentration by multiple counts with a haemocytometer under a phase-contrast microscope. The marketed product should contain at least the number of polyhedra per gram or milliliter which is indicated on the label.
- A viable-spore count on a general plate count agar (Chapter 6). The level of microbial contaminants should not be higher than 10 colonies per 100 polyhedra.
- A simple mouse feeding test in which a high dose of polyhedra is added to the food (EPA, 1982). No death, growth reduction or any deviation from the normal behaviour should occur. If this does occur a sample of the virus batch should be analysed for the presence of vertebrate pathogens or toxic ingredients.
- Bioassays testing the biological activity (LC-50 or LD-50) of the virus against S. exigua larvae. The biological activity should not show significant deviations from the activity of the virus stock from which the inoculum was taken. The marketed product should have at least the biological activity per gram or milliliter that is indicated on the label.

Shelf life

Two factors are of main importance in the storage of virus. The number of microbial contaminants should not increase and the virus should maintain its biological activity. Jaques (1985) gives a number of examples that show that both as dried powder and as a liquid suspension NPVs can be stored for periods as long as 4 years, without losing more then 10% of their biological activity. Storage was generally better at 5°C and lower temperatures than at room temperature.

Increase of the microbial contamination is a greater problem in storage as a liquid suspension than as a dried powder. Powders, however, are more difficult to formulate in such a way that the product has good tank-mix qualities. In this respect liquid formulations are easier to handle. Preliminary experiments indicated that storage and formulation of SeMNPV as a concentrated suspension in 75% glycerine may be a good option, since the number of microbial contaminants even decreased in time and the suspension readily mixed with water.

SeMNPV versus chemical control agents

When commercially produced and marketed, the virus product will have to compete with the existing chemical insecticides and with new chemical and biological control agents that may be developed in the near future. There are two chemical insecticides that are currently used in Dutch greenhouses to control beet armyworm larvae and both have some disadvantages. Older larvae are tolerant to methomyl (Lannate), which has to be applied twice a week for a period of 4 weeks to effectively control beet armyworm populations (M. van de Vrie, pers. comm.; Chapter 8). Diflubenzuron (Dimilin), applied once a week during a similar period, will give reasonable to good control of beet armyworm larvae, but is a rather slow acting chemical. It takes several days or one to two moults before the larvae actually die. The frequent application of both chemicals often leads to phytotoxic effects on plants and to clearly visible residues on foliage, which in particular on ornamentals may reduce the value of the crop. For the grower and his personnel, that have to work in the greenhouse every day, the constant exposure to chemical control agents, especially methomyl, may involve health risks.

SeMNPV gives good control of both young and old larvae except perhaps for fifth instars (Chapter 8), but these are not controlled by both chemical insecticides either. A main advantage of the virus is that one or two applications are sufficient to control the larval population, whereas four to eight applications of both chemical insecticides are required. The use of virus therefore means a considerable saving of labour costs for the grower (Table 9.1). The labour time involved in spraying a hectare of chrysanthemums was estimated at ca. 8 hours (GCRES, 1981). Furthermore the virus is compatible with biological control systems that are frequently used in vegetable crops such as tomato, sweet pepper and cucumber (Van Lenteren et al., 1980). The safety period between pesticide application and harvest is also an important factor for growers. For the virus this period can be much shorter than for both chemical insecticides. The reduction of environmental pollution should also be mentioned

as a factor in favour of the virus. Residues of insecticides that are applied in greenhouses often end up in the surface water.

The main shortcoming of the virus remains the relatively long period between virus application and cessation of feeding and actual death of the larvae. In vegetable crops this does not have to be a major concern but in ornamentals with an extremely low tolerance also for cosmetic damage, this can be a major constraint to the use of virus.

Costs of control with SeMNPV and chemical control agents

Although greenhouse growers may be more concerned with the efficacy of a control agent (Chapter 1), costs also play an important role in the choice of the product that is used. Assuming that methomyl (Lannate) is applied at the advised rate of 0.1% in 2000 l of water per hectare twice a week during a four-week period, the total cost for control of beet armyworm will be almost DFl. 2900 (Table 9.1). Comparable calculations for Dimilin applied once a week during a four-week period, add up to ca. DFl. 1600. The production costs of SeMNPV were estimated at DFl. 500 for a single hectare treatment (this Chapter) and the total costs for a double application, including labour costs involved in application, therefore can be estimated at ca. DFl. 1400.

The use of virus as a control agent for beet armyworm therefore may even be cheaper for a grower than the use of chemical insecticides. Of course the price of the virus product may be higher than estimated and often the chemical insecticides are used less frequently than advised. Nonetheless it can be concluded that the total costs involved in control of S. exigua with SeMNPV will be of the same order of magnitude as the costs involved in chemical control.

Table 9.1 Costs (in DFl.) involved in control of beet armyworm with SeMNPV and two chemical insecticides, methomyl (Lannate) and diflubenzuron (Dimilin) based on product costs and labour costs.

Product	Rate of application per ha	Product cost per application	Labour cost per application°	No. of required applications	Total costs
methomyl	2 kg	150,-	210,-	8	2880,-
diflubenzuron	2 kg	200,-	210,-	4	1640,-
SeMNPV	10^{12} pol	500,-	210,-	2	1420,-

°: data LEI/CBS (1986) and GCRES (1981)

Market

The initial Dutch market for a SeMNPV-product will be rather small, probably somewhere between 10 and 100 hectares a year. The potential international market, however, is much larger. Beet armyworm is an important pest in the United States where it occurs in chrysanthemum, celery, tomato, strawberry, cotton and alfalfa (Griswold & Trumble, 1985b) and occasionally in a large number of other crops (Table 1.1). Africa and Asia are other potential markets (Fig.1.1) although beet armyworm populations in these regions can still be controlled with relatively cheap chemical insecticides, such as synthetic pyrethroids (Hill, 1983).

In these countries SeMNPV, however, can also be used to control beet armyworm when the virus is produced in field-collected larvae by local farming communities. Deceased larvae can again be collected from virus-treated crops and stored until the next growing season (Van der Geest & Wassink, 1986). A small amount of virus to start the production with and a simple manual are the only items that have to be provided for.

Insect resistance to virus

One of the main reasons for carrying out this study was the increasing resistance of S. exigua larvae to chemical insecticides (Chapter 1). Naturally the question then rises whether S. exigua can also develop resistance against the virus. It has been suggested that the potential for development of resistance against viruses is present in Lepidoptera (Briese & Podgwaite 1985), but that this resistance is less likely to develop, or at least at a much lower speed than the resistance against chemical insecticides. This expectation is based on the fact that like the insect the virus may also change genetically. Vlak & Rohrman (1985) showed that NPVs and their insect hosts have closely co-evolved for millions of years.

Evidence that the potential for resistance against viruses is present in insects originates from studies by Briese (1982). He found thirtyfold higher LD-50 values for a granulosis virus against a laboratory inbred strain of the potato tuber moth, Phthorimaea operculella, as against larvae that were collected from a field population. Briese also showed that this difference in susceptibility was inheritable. He suggested that the laboratory strain had been under selection pressure over a long period and had developed resistance to the virus. In what respect the 'fitness' of the laboratory strain was still comparable to that of the field population is not known. Differences in susceptibility to a specific virus between various populations of an insect

species have been shown to occur more often (Briese, 1981), but are no proof for the development of resistance, only perhaps that the potential is present. The definite proof has to be given by long term use of viruses under practical conditions and no virus up to now has been used as intensive and regular as chemical insecticides.

Safety

Despite the results of an overwhelming number of safety tests that were carried out on all sorts of animals and plants (Summers et al., 1975; Burges et al., 1980a; Döller, 1985), some people still fear that nuclear polyhedrosis viruses may mutate by chance in such a way that they become pathogenic for non-target organisms such as plants, vertebrates or even human beings.

Vlak & Rohrman (1985) showed by studies on the base sequences of a variety of polyhedrin genes, that NPVs and their insect host have co-evolved for millions of years. They also concluded that mankind must have been exposed to NPVs during its entire evolution. Also now many people are daily exposed to these viruses as they are present in large quantities on many vegetables (Heimpel et al., 1973; Thomas, 1975). Apparently this has never given any problems. Furthermore these viruses have been thoroughly tested on many different living organisms and not a single record is available indicating that a nuclear polyhedrosis virus ever infected any living organism, other than its natural insect host(s).
Furthermore, the amount of virus we ever hope to produce in laboratories is only a minute fraction of the amounts of virus that are yearly produced in nature. Kaupp (1983) estimated for Neodiprion sertifer NPV that as much as 2×10^{15} polyhedra were produced in a single hectare of forest. Such an amount of SeMNPV would be sufficient for treatment of 2000 hectares of chrysanthemums. The chance that a dangerous mutation would occur in a laboratory production of a NPV, thus is only a fractional proportion of the chance that such a mutation would occur in nature.

It seems, therefore, that there are no safety risks involved in the use of SeNPV and other baculoviruses as insect control agents on ornamental and vegetable crops, certainly if the producer lives up to the quality control standards that were proposed in a previous paragraph.

Registration procedures in the Netherlands

To date microbial pesticides fall under the same registration guidelines as chemical pesticides. New guidelines for the registration of biological control agents, with special emphasis on pathogens, are momentarily being formulated. It

is hoped that the new guidelines will respect the completely different and unique mode of action of microbial control agents. It is further hoped that the safety test requirements will be based on the 'Tier'-system such as used by the EPA in the United states (EPA, 1982). With the Tier-system the principle is used that if no negative effects are found in a first screening with extremely high exposure of a number of different test animals to a product (Tier I), further and more elaborate tests (Tiers II and III) will not be necessary. The proposed Dutch registration guidelines list more or less the same safety tests that are mentioned in the EPA guidelines (EPA, 1982) but exact requirements for each group of pathogens are not yet set and the Tier-system is not mentioned. One sentence in the proposed guidelines gives both hope and fear for the future as it states: 'depending on the nature of the product to be registered will be decided which safety tests are required.'

The 'safe nature' of NPVs is sufficiently documented in literature, as discussed in the previous paragraph. NPVs are a homogeneous group of pathogens (Maramorosch & Sherman, 1985) restricted to insects, and therefore it seems pointless to repeat the same safety tests for every new NPV that is submitted for registration. Acceptance of data from safety tests with other NPVs for the registration would be a step towards the desired group-registration for nuclear polyhedrosis viruses and granulosis viruses in the Netherlands. In that case SeMNPV could soon be the first baculovirus registered as an insect control agent in the Netherlands.

9.4 Final conclusion

The final conclusion of this study is that SeMNPV shows good potential as a control agent of beet armyworm in Dutch greenhouses and perhaps also in other parts of the world. The decision as to develop the virus into a marketable product is, however, beyond the scope of this thesis and has to be put into the hands of commercial companies that should further judge the economic feasibility.

Chapter 10

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SUMMARY

Several aspects of the control of the beet armyworm, Spodoptera exigua (Hübner) (Lepidoptera: Noctuidae) in greenhouse crops with nuclear polyhedrosis viruses (NPVs) (Baculoviridae, subgroup A) were studied.

Beet armyworm behaviour was observed in various crops. The distribution of egg batches (Chapter 2) was found to be similar in chrysanthemum, tomato, gerbera and geranium. Most eggs were laid on the underside of leaves within 10 cm of the soil surface. In chrysanthemum moths deposited more eggs on very young than on older plants. No correlation was found between size of egg batches and plant species, plant age or position within in a crop.

After hatching beet armyworm larvae gradually moved upwards to the top of chrysanthemum plants (Chapter 3) and then predominantly fed on the upper foliage layers. On tomato, however, larvae did not move upwards and mainly fed on the lower leaves. On chrysanthemums most feeding of the younger larvae occurred at the underside of foliage while the upper epidermis remained intact. Larvae dispersing from a single egg batch of 35 eggs damaged about 90 small and 50 tall chrysanthemum plants during their development. The five successive larval stages contributed 0.1%, 0.4%, 4%, 20% and 75% to the total foliage consumption, respectively.

Five nuclear polyhedrosis viruses infectious for beet armyworm larvae were compared for their potential as biological control agent (Chapter 4). Restriction endonuclease patterns of the DNA showed that three of the isolates, collected from deceased beet armyworm larvae in the Netherlands, are closely related with Mamestra brassicae nuclear polyhedrosis virus (MbMNPV). Therefore they were named MbMNPV-NL80, MbMNPV-NL82 and MbMNPV-NL83. These isolates are not closely related with Autographa californica MNPV (AcMNPV) and Spodoptera exigua MNPV (SeMNPV), both originating from the USA.

Comparison of the biological activity of these five MNPVs (Chapter 4) showed that the SeMNPV has a greater biological activity against beet armyworm larvae than MbMNPV-NL80, -NL82, -NL83 and AcMNPV. The LD-50 values of the five MNPVs against second instar larvae were 3, 26, 14, 17 and 18 polyhedra, respectively. SeMNPV, therefore, seemed to be the most suitable candidate for biological control of the beet armyworm.

Bioassays carried out with the droplet-feeding method showed that larvae became less susceptible to SeMNPV with increasing age (Chapter 5). The LD-50

values for the five subsequent larval instars were 4, 3, 39, 132 and 11610 polyhedra. The LT-50 values increased from ca. 3.5 days for first instars to almost 6 days for fifth instar larvae.

Production of SeMNPV (Chapter 6) was carried out in late fourth instar larvae reared on semi-synthetic diet. A maximum amount of virus, 1 to 2 x 10^9 polyhedra/larva, was produced in individually-reared larvae, after seven days of incubation at 30°C, with an inoculum of 7.5 x 10^4 polyhedra/cm^2 diet surface. Virus yield was reduced to ca. 9 x 10^8 polyhedra/larva when production was carried out in groups of 400 and 600 larvae per container.

Biological activity of virus harvested from living and from deceased larvae was similar. Microbial contaminants, predominantly bacteria, in the produced virus batches numbered 1-6% of the number of polyhedra. Vertebrate pathogens were not present in any of the produced virus batches.

The effect of high-volume handspraying (HV), low-volume spinning disc (LV-SD) and ultra-low-volume electrodynamic (ULV-ED) application systems on the efficacy of larval control with SeMNPV was studied on chrysanthemum crops (Chapter 7). Virus applications with the LV-SD system generally showed a slightly better control efficacy than was achieved with the HV system. The efficacy of ULV-ED applications was much lower except on a very short crop, where control comparable with that of the LV-SD system was obtained.

The efficacy of SeMNPV for control of beet armyworm larval populations was studied in chrysanthemum, gerbera, kalanchoe and tomato crops (Chapter 8). Applications of 1 x 10^8 polyhedra/m^2 resulted in 95-100% larval mortality on each of the crops. Virus applications caused comparable levels of mortality in populations of early and late instar larvae. Reduction in feeding damage to the crops, however, was more pronounced when the larvae were early instars at the time of virus application. Single applications with the virus resulted in higher levels of larval mortality as double applications with 0.1% methomyl or 0.1% diflubenzuron.

In the general discussion (Chapter 9) the results from the various studies, commercial aspects and the current situation in Dutch greenhouse industry were evaluated. This lead to the final conclusion that there is good potential for the practical use and commercial development of SeMNPV as a control agent of beet armyworm larvae in Dutch greenhouses.

SAMENVATTING

Verscheidene aspecten van de bestrijding van de Floridamot, Spodoptera exigua (Hübner) (Noctuidae), een in de sier- en groenteteelt onder glas voorkomende vlindersoort, met voor insekten specifieke kernpolyedervirussen (Baculoviridae, subgroep A) zijn onderzocht.

Het gedrag van de Floridamot in kassen werd onderzocht in verscheidene gewassen. De verticale verdeling van eipakketten in chrysant, tomaat, gerbera en geranium was vrijwel identiek (Hoofdstuk 2). Verreweg de meeste eipaketten werden gevonden op de onderste bladeren, op minder dan 10 cm hoogte. Als zowel zeer jonge als oudere chrysanten in de kas aanwezig waren werden er significant meer eieren gelegd op de jonge planten. Eipakketten bestonden meestal uit zo'n 35 eieren. De grootte van eipakketten werd niet beinvloed door de positie in het gewas of door de plantesoort.

Jonge rupsen hadden de neiging zich geleidelijk aan naar boven te bewegen tot in de top van planten. Oudere rupsen voedden zich voornamelijk met blad in de bovenste regionen van het gewas (Hoofdstuk 3). Dit was echter niet het geval op tomaat waar de rupsen zich bleven voeden met bladeren onderin het gewas.

Tot en met het vierde larvale stadium vond de meeste vraat plaats aan de onderzijde van het blad, zonder dat de rups daarbij de opperhuid van het blad consumeerde. Dit is van belang voor de toepassing van bestrijdingsmiddelen en dus ook van virus, omdat die met de meeste spuittechnieken juist aangebracht worden op de bovenkant van het blad. De opeenvolgende vijf larvale stadia droegen respectievelijk 0,1%, 0,4%, 4%, 20% en 75% bij aan de totale vraat tijdens de larvale ontwikkeling. Rupsen die afkomstig waren van een enkel eipakket, bestaande uit 35 eieren, konden zo'n 90 jonge chrysanten beschadigen. In een oud en hoog gewas liep dit aantal terug tot zo'n 50 planten.

Vijf kernpolyedervirussen, die infectieus waren voor larven van de Floridamot, werden vergeleken op hun potentie als biologische bestrijdingsmiddel (Hoofdstuk 4). Restrictie-enzym patronen van het virale DNA toonden aan dat drie van de isolaten, diegene die verzameld waren uit Floridamot-rupsen in Nederland, zeer nauw verwant waren aan het kernpolydervirus van de kooluil, Mamestra brassicae, (MbMNPV). De drie isolaten werden daarom MbMNPV-NL80, MbMNPV-NL82 en MbMNPV-NL83 genoemd, hoewel ze geisoleerd waren uit S. exigua rupsen. De twee andere virussen die vergeleken werden waren het Autographa californica (AcMNPV) en Spodoptera exigua kernpolydervirus (SeMNPV), beide

afkomstig uit de Verenigde Staten. Dit laatste virus, dat specifiek is voor de Floridamot, bleek een grotere biologische activiteit t.o.v. Floridamot-rupsen te bezitten dan MbMNPV-NL80, -NL82, -NL83 en AcMNPV, die in dit opzicht onderling weinig verschillen vertoonden. De LD-50 waarden (dosis waarbij 50% mortaliteit optreedt) t.o.v tweede stadium rupsen was respectievelijk 3, 26, 14, 17 en 18 polyeders voor elk van de virus-isolaten. SeMNPV leek daarom het meest geschikt voor de biologische bestrijding van de Floridamot.

Verdere studies naar de biologische activiteit van SeMNPV ten opzichte van de overige larvale stadia toonden aan dat de rupsen minder gevoelig werden voor het virus naarmate hun leeftijd toenam (Hoofdstuk 5). De LD-50 waarden voor de vijf larvale stadia bedroegen respectievelijk 4, 3, 39, 132 and 11.610 polyeders. De LT-50 waarden (periode waarin 50% van de mortaliteit optreedt) steeg van ca. 3½ dag voor eerste stadium rupsen tot bijna 6 dagen voor vijfde stadium rupsen.

De produktie van virus (Hoofdstuk 6) vond plaats door infectie van rupsen van het vierde stadium, die in het laboratorium werden opgekweekt op een semi-kunstmatig dieet. De maximale virusopbrengst per rups was 1 tot 2 x 10^9 polyeders. Dit werd bereikt in individueel gekweekte rupsen, die geinfecteerd werden door een inoculum van 7,5 x 10^4 polyeders per cm^2 dieet-oppervlakte aan te brengen. De rupsen werden geoogst na 7 dagen van incubatie bij 30°C. De oogst aan virus liep terug tot 9 x 10^8 polyeders per larve toen de produktie werd uitgevoerd in groepen van 400 and 600 rupsen per container. Deze methode van virusproduktie verdient echter wel de voorkeur aangezien het een aanzienlijke reductie geeft van de arbeidskosten. De biologische activiteit van virus geoogst uit nog levende of uit reeds dode rupsen toonde geen significante verschillen. Het geproduceerde virus was verontreinigd met 1-6% aan bacteriën en schimmels, gemeten ten opzichte van het aantal polyeders. Pathogenen van vertebraten werden niet aangetroffen.

Uit vergelijking van de effectiviteit van virus bespuitingen met verschillende spuittechnieken (Hoofdstuk 7) bleek dat in het algemeen laag-volume toepassingen met schijfvernevelaars wat betere resultaten gaven dan de conventioneel toegepaste groot-volume applicaties met een handspuit. Bespuitingen met het virus bleken ook uitvoerbaar met behulp van een electrodynamisch spuitsysteem, waarbij electrisch geladen druppeltjes verspoten worden. De resultaten waren echter slechter dan met beide eerder genoemde spuitsystemen, behalve op een zeer laag gewas, waar vergelijkbare resultaten werden behaald.

De bestrijding van rupsen met het virus werd voornamelijk bestudeerd in chrysant maar ook in gerbera, kalanchoë en tomaat (Hoofdstuk 8). Het bleek dat

bespuiting van deze gewassen met een dosis van 1 x 10^8 polyeders per m^2 grondoppervlak resulteerde in 95-100% mortaliteit van de rupsen. De bestrijding van oude en van jonge rupsen was vergelijkbaar. De schade aan het gewas was echter veel geringer wanneer de bespuiting werd uitgevoerd tegen jonge rupsen. Eenmalige bespuiting van chrysanten met virus resulteerde in betere bestrijding van rupsen dan de tweemalige toepassing van de chemische insekticiden methomyl en diflubenzuron in de gangbare concentratie van 0.1%.

In de algemene discussie (Hoofdstuk 9) werden de resultaten van de verscheidene onderwerpen samengevoegd en bekeken in het licht van huidige praktijksituatie in de Nederlandse glastuinbouw. Uiteindelijk leidde dit tot de conclusie dat er voor het virus goede mogelijkheden bestaan voor verdere ontwikkeling tot een commercieel geproduceerd biologisch insekticide voor de bestrijding de Floridamot in de Nederlandse kassen.

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CURRICULUM VITAE

Peter Hans Smits werd op 5 januari 1957 geboren te Velsen. Na in 1975 het eindexamen Gymnasium β aan het Gymnasium Felisenum te Velsen behaald te hebben werd in hetzelfde jaar begonnen met de studie Biologie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen werd behaald in 1978, het doctoraalexamen, met als vakken dierenoecologie en dierenmorfologie, werd afgelegd in mei 1982.

Tussen juli 1982 en oktober 1985 is de promovendus verbonden geweest aan de vakgroep Virologie van de Landbouwuniversiteit te Wageningen en het Proefstation voor de Bloemisterij in Nederland te Aalsmeer, als wetenschappelijk onderzoeker in tijdelijke dienst.

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