UNIVERSITY OF LATVIA

ECOLOGICAL INTERACTIONS BETWEEN BACULOVIRUSES AND PEST POPULATIONS AND THEIR ROLE IN BIOLOGICAL CONTROL

Bakulovīrusu un augu kaitēkļu populāciju ekoloģiskā mijiedarbība un loma bioloģiskajā kontrolē

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KOPSAVILKUMS

Bakulovīrusi ir biocenozēs izplatīta entomopatogēno vīrusu grupa, kas samazina augu kaitēkļu daudzumu dabā un nav patogēna cilvēkiem un dzīvniekiem. Bakulovīrusus saturošie mikrobioloģiskie preparāti izmantojami lauksaimniecībā un mežsaimniecībā, lai samazinātu ķīmisko augu aizsardzības līdzekļu (insekticīdu) pielietošanu, apkārtējās vides un produkcijas piesārņošanu.

Bakulovīrusiem, kā bioloģiskās augu aizsardzības līdzeklim, ir sekojošas priekšrocības: 1) tie ir specifiski noteiktām kukaiņu sugām, 2) vīrusi izplatās biocenozēs un pašreproducējas augu kaitēkļu populācijās, 3) vietējie celmi ir efektīvi Latvijas klimatiskajos apstākļos, augu kaitēkļu masveida savairošanās gadījumos izraisot epiziotijas.

Promocijas darba mērķis bija noskaidrojot bakulovīrusu un augu kaitēkļu mijiedarbības mehānismus, iegūt teorētisko pamatojumu efektīvas stratēģijas iztrādāšanai bakulovīrusu pielietošanai bioloģiskajā augu aizsardzībā pret ābeļu vērpēju (*Malacosoma neustria* L.), priežu rūsgano zāģlapseni (*Neodiprion sertifer* Geoffr.) un kāpostu balteni (*Pieris brassicae* L.).

No kaitēkļu populācijām izdalīti jauni, virulenti vīrusu izolāti. Izmantojot elektonmikroskopiju un citoloģiskās metodes raksturoti izdalītie vīrusu izolāti un noskaidrots, ka vīrusu populācijas nav homogēnas. 1990. g. - 1998. gados veiktie laboratorijas un lauka eksperimenti parādīja, ka pielietotās, Eksperimentālās entomoloģijas laboratorijā izveidotās, preparatīvās formas ir efektīvas un izraisa 86 - 96% kaitēkļu mirstību. Rezultāti parāda, ka pielietotās ekoloģiski nekaitīgās pildvielas palielina vīrusa preparāta efektivitāti un būtiski samazina ārējās vides faktoru kaitīgo ietekmi uz vīrusa aktivitāti. Darba rezultāti parāda, ka bakulovīrusi saglabājas kaitēkļu populācijās un var regulēt kaitēkļu daudzumu vairākus gadus pēc iniciējošās vīrusa pielietošanas.

Pētījumos vīrusu diagnostikai dažādos substrātos, kukaiņu kāpuros un kūniņās, izmantotas un aprobētas jaunas, jūtīgas metodes, tai skaitā, DNS-DNS hibridizācijas metode, un polimerāzes ķēdes reakcija (PCR). Šai nolūkā piemeklētas paraugu apstrādes un DNS izdalīšanas metodes.

Pielietojot izstrādāto uz PCR balstītu un poliedrīna gēnu atpazīstošu diagnostikas metodi, kaitēkļu nākamajās paaudzēs pēc iniciējošās vīrusa pielietošanas tika atrasti kodolu poliedrozes vīrusi un noskaidrota to sastopamība. Izstrādātā metode dod iespēju identificēt atsevišķus vīrusu izolātus bioinsekticīdos, un kukaiņu populācijās un nodrošina iespēju precīzi diagnosticēt augu kaitēkļu saslimstības ierosinātāju. Metodes priekšrocība ir iespēja identificēt vīrusu *in situ* dažādās kukaiņu attīstības stadijās, kad ārējās vīrussaslimšanas pazīmes nav redzamas un izmantot kaitēkļu savairošanās, vai gaidāmās dabiskās epizootijas (kaitēkļu masveida saslimšana) prognozēšanai.

Izmantojot biotestu, DNS-DNS hibridizāciju un PCR noskaidrots, ka bakulovīrusi akumulējas un saglabājas vidē, konstatētais bakulovīrusu daudzums augsnē un uz augu lapām ir pietiekošs, lai atkārtoti izraisītu kāpuru saslimšanu. Apkopotie rezultāti par vīrusinfekciju stimulējošo vielu (augu ekstrakti, preparātu pildvielas un kukaiņu fizioloģiskā stāvokļa pavājnātāji) ietekmi uz vīrusa aktivitāti un noteiktās efektīvās koncentrācijas ļauj uzlabot vīrusu pielietošanas metodes. Noteiktās zemākās efektīvās

vīrusa un pildvielu koncentrācijas ļauj samazināt vīrusa pielietošanas skaitu un izdevumus.

Darbs izstrādāts LU Bioloģijas institūta Eksperimentālās entomoloģijas laboratorijā. Elektronmikroskopiskie pētījumi un vīrusa DNS sekvinēšana veikta sadarbībā ar LU Biomedicīnas pētījumu un studiju centru. Cito un histopatoloģiskie pētījumi veikti Vācijā, sadarbībā Vācijas Federālā Bioloģisko pētījumu centra Bioloģiskās kontroles institūta Virusslimību diagnostikas laboratoriju.

Darba rezultāti ir apkopoti 7 zinātniskajos rakstos un 15 tēzēs. Par zinātnisko pētījumu rezultātiem ziņots Pasaules un Eiropas entomologu kongresos, 6. un 7. Eiropas Starptautiskās Bioloģiskās Kontroles Organizācijas konferencēs Kopenhāgenā un Vīnē un Starptautiskās Bezmukurkaulnieku Patoloģijas Biedrības simpozijā Saporo.

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SUMMARY

Diseases of agricultural pests caused by baculoviruses are a factor that gives the possibilities to regulate pest population. Application of microbial preparation produces a primary infection in direct response to the input of entomopathogens, with subsequent release of new reproduced entomopathogens from larval cadavers. The released microorganisms initiate secondary infection, thus enhancing the efficacy of microbial preparation. Biopreparations based on NPVs may be considered for the following reasons: 1) high host - specificity, 2) multiplication of these viable microbial preparations in pest populations leading to the possibility of control of pest populations several years after initial spraying 3) local virus strains and isolates have high activity in the climatical conditions of Latvia.

The aim of this work was to investigate the interaction between baculoviruses and host pest populations to provide the necessary background for developing an effective biological control's strategy for decreasing populations of *Malacosoma neustria* L., *Neodiprion sertifer* Geoffr., *Pieris brassicae* L. and exploiting pathogens, already presented in the ecosystem.

Investigations were carried out in Institute of Biology, University of Latvia (LU), Laboratory of Experimental Entomology, since 1990. Virulent virus isolates were isolated from insect populations and investigated. The investigations are made in three levels (cell, individ and population). In recent years virus insecticide formulations have been developed and tested using environmentally - friendly additives. Laboratory and field experiments with the new virus insecticide compositions indicated high levels of pests mortality (89-96 %). Results obtained show that additives retain NPV activity in viral preparations.

Using of Polymerase Chine Reaction (PCR) we determine presence of baculoviruses in following generation of pest insects. The priority of elaborated method is it is usable for early *in situ* identification of nuclear polyhedrosis virus in several stages of development of plant pest insects. The PCR procedure was optimized, and used for identification of individual strains. New improved methods for virus identification were used to describe the natural occurrence of NPV in pest populations and the homology of virus population.

The results of this work demonstrate that baculoviruses reduce the amount of insects during the following years after treatment. Baculoviruses accumulate in the environment, determined amount of persisted nuclear polyhedrosis virus can subsequently to initiate an epizootic.

Summarized knowledge's will improve the application methods of baculoviruses. Possibilities to use lowest titer of virus preparation and additions that stimulate infection (plant extracts, additives and substances physiological weakeners) are determined. They would diminish the amount of necessary treatments and expenses of biological plant protection in farming as a result. As we expected the elucidation of the spreading ways of infection in pest population, and transmission of baculoviruses to the next generation is usable in developing models of pest population dynamic and forecasting of insect outbreaks and expected epizootic.

Electron microscopy and DNA sequencing was made in collaboration with LU Biomedical Research and Study Centre. Histological investigations were done in the Laboratory for diagnosis, cyto- and histopathology of insect diseases, Institute for Biological Control (Federal Biological Research Centre for Agriculture and Forestry, Darmstadt, Germany)

Obtained results were published in 7 scientifical papers and 15 abstracts and presented in 5 international conferences and congress of Entomology.

Янкевица, Л. 1999. Изучение экологического взаимодействия между бакуловирусами и популяцией вредителей и его роли в биологической борьбе. Латвийский Университет, Рига, 50 с.

Аннотация

Бакуловирусы являются широко распространенной в биоценозах группой вирусов, которые эффективно регулируют численность вредителей растений в природе и не патогенны для человека и теплокровных животных. Микробиологические препараты, содержащие бакуловирусы используются в сельском и лесном хозяйствах, чтобы снизить кратность применения химических средств защиты растений, а также для защиты от вредного влияния химии на продукцию и окружающую среду.

Бакуловирусы как биологические средства защиты растений имеют ряд преимуществ: 1) они специфичны для определенных видов насекомых; 2) вирусы широко распространены в биоценозах и самопроизводятся в популяциях вредителей; 3) местные штаммы вирусов эффективны в климатических условиях Латвии и в период массового размножения вредителей растений вызывают эпизоотии.

Цель диссертационной работы – выяснить механизм процесса взаимодействия бакуловирусов и вредных насекомых и его роли в ограничении численности вредителей, дать теоретическое обоснование роли вирусных заболеваний в процессе долгосрочного лимитирования количества вредителей в биоценозах.

В Латвийских биоценозах выделены изоляты бакуловирусов, вызывающие заболевания у вредителей растений, отличающиеся своей активностью и патогенностью. В климатических условиях Латвии 1990-1997 г.г. лабораторные и полевые эксперименты показали, что применение вирусных инсектицидов вызывает 86-96% гибель вредителей. В процессе исследования установлено, что инфекционный процесс можно ускорить, добавив в суспензию вирусного препарата препаративные наполнители, растительные экстракты или вещества, ослабляющие физиологическое состояние насекомых.

Опробированы новые методы диагностики вируса в различных субстратах, в том числе метод ДНК-ДНК гибридизации, метод ЦРП, подобраны методы для обработки образцов и выделения ДНК. Используя метод диагностики (ЦРП) появляется возможность идентифицировать вирусы *in situ* на разных стадиях развития вредителя, когда признаки вирусного заболевания еще не проявились, а также прогнозировать природные эпизоотии. Результаты научной работы показали, что бакуловирусы сохраняются в биоценозе и в популяции вредителя на протяжении нескольких лет после одноразового внесения вируса в популяцию и могут регулировать численность вредителя.

Результаты диссертационной работы обобщены в 7 научных статьях и 15 тезисах. О результатах научных исследований сообщено на Европейских и Всемирных Энтомологических конгрессах, на $6_{ой}$ и $7_{ой}$ Европейской Международной конференции Организации Биологического Контроля и на симпозиуме Международного Общества Патологии Безпозвоночных.

ABBREVIATIONS

Ac NPV	*Autographa californica* nucleopolyhedrovirus
Cp GV	*Cydia pomonella* granulovirus
DNA	deoxyribonucleic acid
GV	granulovirus
Ha NPV	*Heliothis armigera* nucleopolyhedrovirus
Hz NPV	*Heliothis zea* nucleopolyhedrovirus
LD_{50}	median lethal dose
LT_{50}	median lethal time
Mn NPV	*Malacosoma neustria* nucleopolyhedrovirus
MNPV	multiple nucleopolyhedrovirus
NPV	nucleopolyhedrovirus
Ns NPV	*Neodiprion sertifer* nucleopolyhedrovirus
OB	occlusion body
Pb GV	*Pieris brassicae* granulovirus
PCR	polymerase chain reaction
REN	restriction endonuclease analysis
SDS	Sodium dodecil sulfate
Tn GV	*Trichoplusia ni* granulovirus
UV	ultraviolet light
wtBV	wild type baculovirus

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INTRODUCTION

Baculoviruses are a large group of DNA viruses capable to infect over 500 species of insects and arthropods. The baculoviruses usually have a very restricted host range, limited to specific insect species, and do not infect vertebrates or plants.

Larvae of lepidopterian insect species often cause severe damage by feeding on agriculturally important crops, in ornamental plants and forest trees. The resistance of many of these insect species to chemical insecticides and a changing public view on the use of those environmentally hazardous chemicals has promoted the development of alternative pest control strategies. Baculoviruses are considered to be safe bio-insecticides and therefore have great potential in integrated pest control. Nowadays, baculoviruses are employed as ecologically safe biological control agents. Many of baculoviruses currently used in biological control were, in fact, isolated from epidemic insect populations. Notable examples are the baculoviruses of the European spruce sawfly, *Gilpinia hercyniae*, which has proved to be an effective, long term regulator of pest populations in Europe and Canada (Cunningham & Entwistle, 1981). More than 20 baculovirus products are now in the market (Entwistle, 1998). Baculoviruses may a successfully used in biological insecticides because of they characteristics: 1) high host - specificity, 2) no evidence of occurrence in non-arthropod hosts; no observations of harmful effects on the rest of the entomofauna including beneficial insects, or on other bioagents in the agroecosystem (GrÖner, 1986), 3) spreading and multiplication of these viable microbial preparations in pest populations leading to the possibility of pest populations control several years after initial spraying (Bird, 1961), 4) diverse virulence and pathogeneity of different viral strains. Therefore attention is paid to development of effective virus insecticide, its formulation and improving the survival of virus in the environment.

Due to some unique features of baculovirus replication and gene expression, baculoviruses are also widely exploited as an expression system for large scale production of recombinant proteins of biotechnological or pharmaceutical importance (Lucknow, 1995). An in-depth analysis of baculovirus genome organization and advancements associated with the generation of expression vectors had a positive feedback on the development of baculoviruses for improved insecticidal properties.

It is now widely appreciated that the safe and effective use of baculoviruses requires a greater knowledge of how these pathogens behave in natural populations of insects.

To exploit the full potential of such pathogens and ensure their safe use, a fuller knowledge of the ecology of the pathogen is required. Until now the ecology of baculoviruses has been relatively unstudied. Recently, molecular and theoretical advances have been used as "ecological tools" to investigate certain aspects of the host-pathogen interactions.

The aim of the project was to investigate the interaction between baculoviruses and host pest populations to provide the necessary background for developing an effective biological control's strategy for exploiting pathogens, already presented in the ecosystem.

The main tasks of the project were;

- to investigate interactions between pest populations and baculoviruses, that decrease density of pest population by:
    - searching for new effective baculovirus isolates, providing the growth of agricultural crops and qualitative yields as an environmental potential,
    - determining virulence, pathogeneity, and the mode of action of isolated baculoviruses,
    - developing and comparison of methods of baculovirus infection's stimulation
- using cytological methods to determine the effect of additives and plant extracts to pathological process caused by baculovirus and to activity of virus;
- to elaborate a new sensitive method of virus detection in pest insects;
- to estimate the persistence and of virus activity in the environment depending on virus insecticide formulation and to estimate the influence of environmental factors to survival of virus pathogeneity;
- to estimate possibilities of baculoviruses to accumulate in the environment and pest population and subsequently initiate an epizootic.

Investigations were carried out in Institute of Biology, University of Latvia (LU), Laboratory of Experimental Entomology, since 1990. Laboratory and field experiments were performed.

Electron microscopy was made in collaboration with chief researcher Dr. habil. biol. Velta Ose, LU Biomedical Research and Study Centre. Histological investigations were done in the Laboratory for diagnosis, cyto- and histopathology of insect diseases, Institute for Biological Control (Federal Biological Research Centre for Agriculture and Forestry, Darmstadt, Germany), DNA sequencing and PCR- analysis were carried out in the Laboratory of Genetic engineering, LU Biomedical Research and Study Centre.

1. LITERATURE REVIEW

1. 1. BACULOVIRUSES

Baculoviruses have been isolated from the *Lepidoptera, Hymenoptera, Diptera, Coleoptera, Crustacea,* and *Arachnida.* By far the majority of baculovirus isolates were from the *Lepidoptera* (Adams & McClintock, 1991). Baculovirus infections, first observed in silkworms regulate the size of host insect populations in nature. The family *Baculoviridae* is characterized by presence of rod-shaped virions (approximately 30-60 x 300 nm) often found occluded into large cuboidal proteinaceous capsules also called occlusion bodies that serve to protect the virus from inactivation by environmental factors. Currently, *Baculoviridae* comprises only two genera, granuloviruses (GVs) and nucleopolyhedroviruses (NPVs), and the terms granulosis viruses and nuclear polyhedrosis viruses have been replaced by nucleopolyhedrovirus and granulovirus (Murphy et al., 1995). NPVs have either multiple nucleocapsids (M) (type species - *Autographa californica* MNPV) in which occluded virions may contain a variable number (1 to 17) of nucleocapsids per virion, or a single nucleocapsid (S) (type species - *Bombyx mori* SNPV) in which only one nucleocapsid is found enveloped into a virion, which in turn is occluded into an occlusion body (Figure 1). GVs, type species *Tn* GV, occlude a single virion per occlusion body (called granulum), which is more irregulary shaped than the occlusion body of NPVs (Murphy et al., 1995). The OB range in size from 0,1 to 10 μm in diameter. These viruses have a large, circular, double-stranded, covalently closed DNA genome varying in size between 50 to 110 million daltons depending on the virus species (Lucknow, 1995)

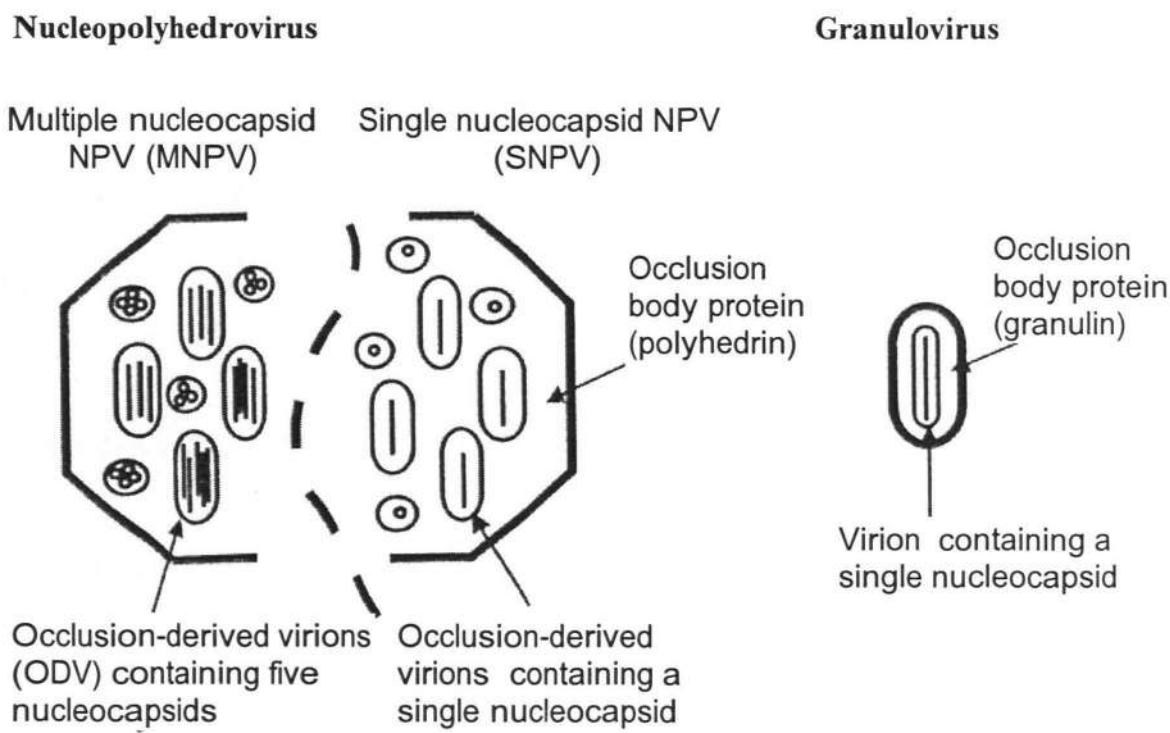

Figure 1. Diagrammic representation of members of the *Baculoviridae* family (Murphy et al., 1995)

Virus identification and characterisation can be achieved by different methods: serology (McCarthy & Lambiase, 1979), microscopy and morphology (Billimoria, 1986; Taniai & Yamakawa, 1992; Chuhrij, 1992), virulence (Hatfield & Entwistle, 1988; Smits & Vlak, 1988) and biochemical characterisation which is particularly accepted as reliable methodology for identification of viruses as well as defining the different isolates of a virus (Maruniak et al., 1984; Kohler et al., 1992).

While the current practice of naming baculoviruses on a basis of their host origin may not be a sound approach, documentation of the relative virulence, host range, and pathology researchers have demonstrated significant differences in the virulence, host range, and pathology of various strains of baculoviruses (Burgess, 1977). The improved in vivo bioassay system developed allows rapid and accurate determination of LD_{50}, LT_{50}, etc. and should be an impetus for further, detailed studies on the biological properties of baculovirus strains.

Morphological criteria may tentatively establish a new baculovirus isolate as a member of a subgenus, further biophysical and biochemical data are required for confirming generic and subgeneric status and for establishing the relationship of the isolate to known viruses (Billimoria, 1992). In this respect, the molecular taxonomy of the *Baculoviridae* is off to a good start. Initial approaches targeted viral structural proteins and utilised polyacrilamide gel electrophoresis or serology. Later, restriction endonuclease digestion, nucleic acid hybridisation, Western blotting, and monoclonal antibody methods were applied to the problem. The general picture emerging from these types of studies is that viruses from related hosts are more similar than those from distant hosts. Many trials were carried out to examine the genotypic and phenotypic relationships of geographically diverse baculovirus isolates (McCarthy et al., 1978; Shapiro, 1991). Molecular genetics of viruses have revealed that heterogeneity of viral isolates is common whether the virus source is one insect or insects from different geographical areas. Variation has been demonstrated within isolates from *Spodoptera frugiperda, Lymantria dispar, Autographa californica* (Maruniak et al., 1984; Shapiro, 1991). Biochemical characterization and comparison of nine isolates of NPVs from *L. dispar* found in different geographical areas in Europe and North America showed that one of them was a mixture of two other genotypic variants (Kohler et al., 1992). It is clear from REN analysis of viral clones separated by plaque purification of field isolates that each field isolate consisted of several strains having different REN profiles but apparently identical structural polypeptides (Gelertner & Frederici, 1990).

Analysis of viral DNAs with the restriction enzymes *Hind*III, and *Bam*HI of two native isolates isolated from geographically diverse populations of *Malacosoma neustria* L. (Sharipo & Eglite, 1990) showed that Mn NPVs Latvian isolate and Armenian isolate were heterogeneous.

1.1. Infection cycle

Baculoviruses have an unique biphasic life cycle. Transmission of baculovirus in insect populations in nature occurs either via ingestion of food contaminated with OB by insect larvae or via oviposition of eggs contaminated with OB or infection through spiracles and by parasitism that occurs but is not considered important (Billimoria, 1992). The primary site for virus attachment and entry is considered to be the cells of

the midgut epithelium. Upon ingestion, the OB (polyhedron or granulum) predominantly composed of 29 kDa protein called polyhedrin or granulin, dissolves in the alkaline environment of the larval midgut liberating numerous infectious virions. These virions invade the larval midgut by fusion with the microvilli of the midgut epithelial cells. The virions are uncoated in the cytoplasm and the nucleocapsids are transported to the nucleus, then they enter the nucleus at a nuclear pore. Granados and Lawler (1981) have shown that the NPV nucleocapsid is uncoated after entering the nucleus and the first steps of virus gene expression and genome replication take place. A characteristic feature of baculovirus infection is the existence of two infectious forms of the virus: the occlusion body-derived virus (ODV) form, which is infectious for insects and responsible for the spread of the infection in the population from insect to insect, and the budded virus form (BV) which is responsible for the spread of baculovirus infection through larval bodies (Federici, 1997; Williams & Faulkaner, 1997) (Figure 2).

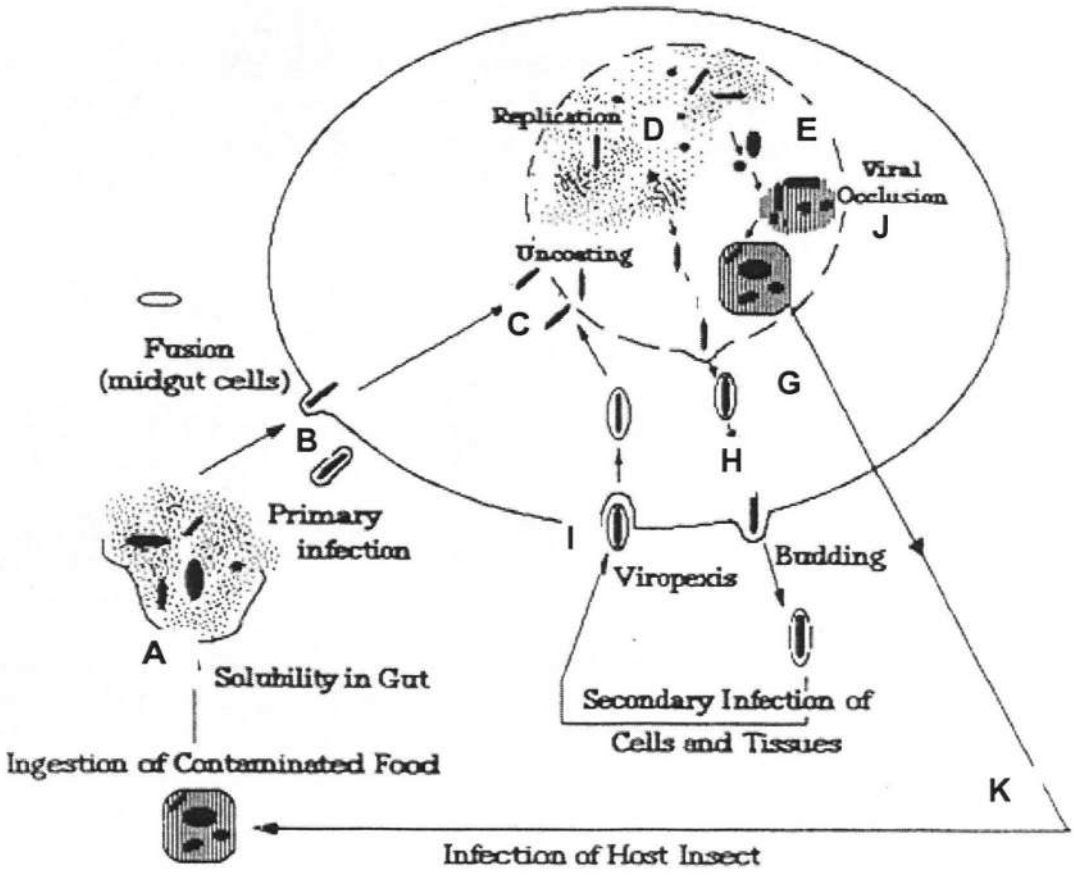

Figure 2. Schematic representation of the baculovirus infection cycle (from Summers, 1982). Ingested polyhedra are solubilized in the midgut and virions are released (A). The envelopes of the virions fuse with plasma membrane of the insect cell (B). After traversing the cytoplasm virions enter the nucleus at a nuclear pore, uncoat, and viral DNA enters the nucleus (C). Progeny viral nucleocapsids (NC) are synthesized in the virogenic stroma (D). The progeny nucleocapsids are initially released by budding (E,F,G). Budded virions infect adjacent cells by endocytosis or viropexis (I). NC produced in later stages of the infection become occluded in polyhedral protein (J). Finally, the OB are released by lysis of the infected cells (K).

Morphologically, these two virus types are largely different (Funk et al., 1997) (Figure 3). The progeny virus produced in the columnar cells of the midgut epithelium buds through the cytoplasmic membrane and basal laminae into haemocel of the host insect acquiring a losse fitting envelope. The virus spreads into all tissues of the insect body, but predominantly targets to the fat body.

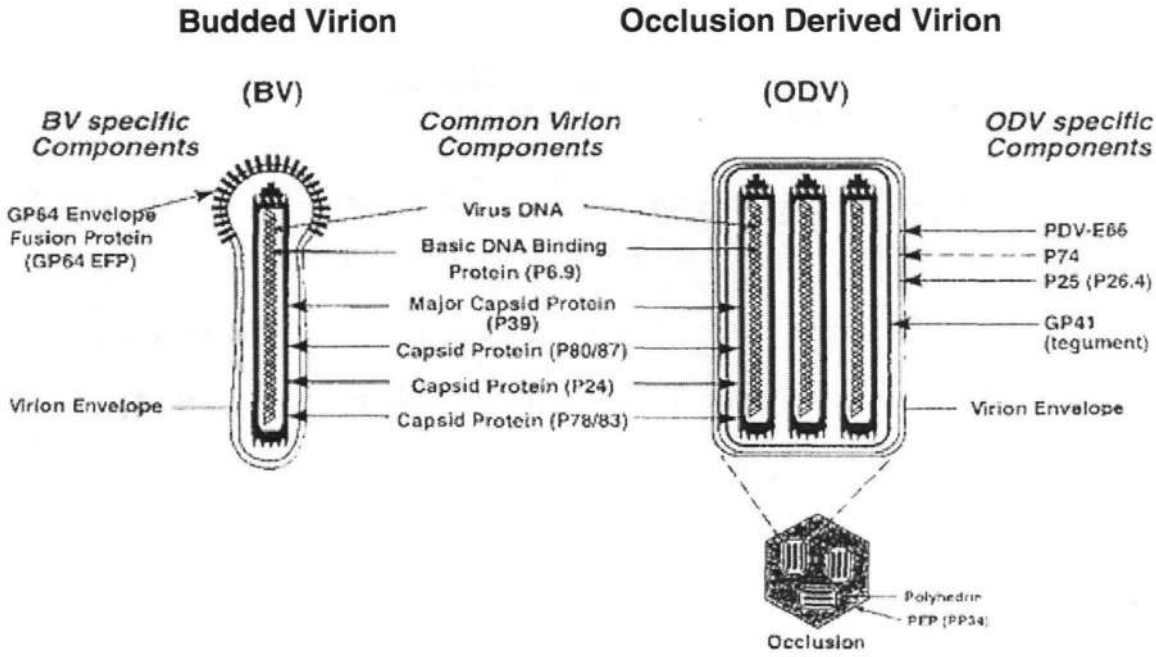

Figure 3. Structural composition of two phenotypes, the budded virion , and the occlusion derived virion (Funk et al., 1997). ODV structure represents NPV subgroup. Proteins specific to both virion types are indicated in the middle of figure. Proteins specific to either budded virion or occlusion derived virion are indicated on the left or right, respectively. The polar nature of baculovirus capsid is indicated in the diagram with the clav lice structure at the bottom and ring-like nipple at the top of the capsid.

The virions produced in the second round of infection 24 h post infection are enveloped in the nucleus in *de novo* formed, tight fitting membrane, followed by OBs (Figures 2, 3). The fat body is the most infected tissue and as a result the main producer of new OBs. NPV infections of the Lepidoptera are generally polyorganotrophic - many target cells (Tanada et al., 1982). OBs have been observed in the nuclei of a large number of tissue types including the hemocytes. Established sequence of infection (based on the presence of OBs) for an MNPV infection. OBs appeared in tissues in the following order: (1) fat body, (2) hypodermis, (3) tracheal matrix. (4) muscular sheath, (5) nerve sheath, (6) muscles, (7) ganglia, and (8) pericardial cells. Eventually the infected cell disintegrates after nuclear and cellular membranes break down realising the OBs in the environment (Federici, 1997).

1. 2. ECOLOGY OF HOST - PATHOGEN INTERACTIONS AND BIOLOGICAL CONTROL

The modern view of baculovirus ecology as a dynamic interaction between the virus and its host requires a rigorous quantitative approach, which would allow predictive modelling. Pest population, its food plants and baculoviruses together make a complex system of interrelation of several levels of organisation, which has to be investigated to control effectively inflectional processes in the population. In this respect, viral infectivity and host specificity, virus availability, and host density are important parameters affecting the ecology of NPV (Kalmakoff & Crafford, 1982; Evans, 1986).

An important task of ecological studies of baculoviruses is to identify the key factors in the population dynamics of the virus and utilise them to optimise the conditions for biopesticidal activity. The use of parameter estimation has been included in control for the principal variables in a given pathogen - host interaction and to optimise application methods for pest management. Developments in pest management employing pathogens have tended to look of processes driving epizootic. Evans (1994) brought all factors that will be taken into account in field use of pathogens together in a concept termed the Control Window (Figure 4.).

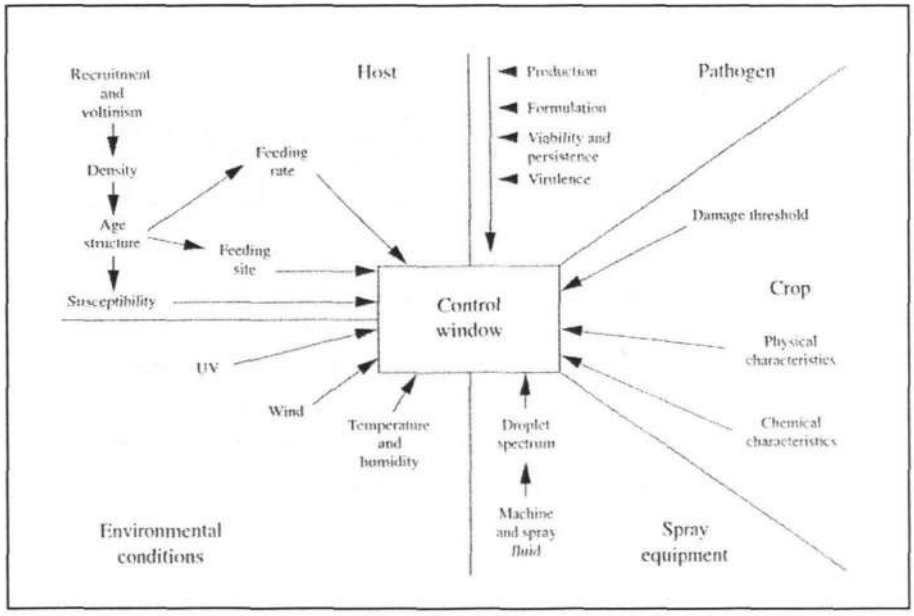

Figure 4. The interactions of host, pathogen, environment and spray technology in defining a Control Window for use of pathogens in pest management (Evans, 1994).

Natural epizootics caused by baculoviruses have been described in a lot of studies of many pest species. In forest ecosystems, where viruses and hosts can co-exist for many years, the investigations are important of the presence and circulation of virulent viruses. The results already obtained show, that viral inclusion bodies spread in a big amount in the environment during epizooties. Dead larvae deliver the disease to the healthy. There are 2 main ways of virus persistence between 2 epizootics: 1) within the host population, 2) accumulated in the environment - in soil, litter, on plant surfaces, etc. Kalmakoff and Crafford (1982) showed an example of effective control of *Wiseana*

population by NPV in pastures in New Zealand. The relative stability of the pasture habitat resulted in accumulation and efficient persistence of NPV in the soil. In future working on the interaction between pest and baculovirus populations, the aim must be improve the quantitative data base on all aspects of virus ecology with particular attention to the study of the ecosystem as a whole. Careful studies of the stability of baculoviruses will be important and virus ecologists will have to determine the degree of stability that is desirable.

Quick and reliable tests for the identification and precise monitoring of baculoviruses in the field will be essential for ecological and biocontrol studies. Improved techniques in detecting, quantifying, and characterising NPVs have resulted in much progress on the quantitative description of host - baculovirus interactions. Evaluation of the distribution of wtBV populations in the soil may allow improved prediction of the fate of recombined baculoviruses following their release and may aid the design of post-release monitoring studies.

The primary factors that are involved in the cause, initiation, and development of infectious diseases in insects are: a pathogen population with its variable virulence and infectivity, efficient means of transmission, and the susceptibility of the host population to the pathogen. Infectivity may vary intrinsically at the individual level or it may vary at the population level. Virulent baculovirus strain may kill larvae in two to five days; less virulent strains may take two to three weeks to kill their host (Tanada & Fuxa, 1987).

The properties of the host population that are most significant in the development of infectious diseases are: (1) susceptibility, including genetic resistance, (2) population parameters such as density, behaviour and association with other insect and animal populations, in relation to the spread of diseases (Young et al., 1987; Young & Yearian, 1988), (3) infection in a host population is also related quantitatively to the amount of virus available for ingestion by larvae (Evans, 1986; Young & Yearian, 1988). It has been elucidated that larvae can infect one other in the same generation during an epizootic. (4) baculovirus persistent infections (Burand et al., 1986). Several investigators have provided tantalising evidence suggesting that NPV might persist in an occult or "latent' state in its host. It has been suggested that acute infection can be induced in such hosts with stimuli such as heat, crowding, a change in diet or ingestion of a heterologous virus (Longworth & Cunningham, 1968; Jurcovicova, 1979; McKinley et al., 1981). (5) Insect host nutrition plays a role in virus infections. The lack of food plants increase the susceptibility. Unfavourable foliage of poor quality or freshness acts as stressors and increases the susceptibility of the insect to virus.

Efforts also need to be made to increase understanding of the ecology of these organisms, and to optimize the impact of these agents by integrating them with other novel crop protection strategies.

1.2.1. Baculoviruses persistence and transmission in the environment

Virus persistence is the single most important factor in the ecology of baculoviruses. Pathogens as baculoviruses, may persist and survive in the abiotic (physical) and biotic environments of the host's habitat. It is obvious that the host population is the major source of survival of the pathogen population, and the pathogen may persist in infected hosts into the next host generation in the same or subsequent year. The common means of virus transmission from one generation to another is through the persistence of the

agent outside the insect host (Evans & Harrap, 1982). In the application of pathogens in microbial control to the host habitat, persistence results from the survival capacity of the applied pathogen and the replication of the pathogen in the initial and subsequent populations of the insect hosts. The persistence of the NPV of the spruce budworm *Choristoneura fumiferana* was high during the second year following field applications. The NPV of the velvet-bean caterpillar *Anticarsia gemmatilis* existed in the environment for three years (Richter & Fuxa, 1984).

Survival and replication of most pathogens occur in "primary hosts" at the initial level of a food chain. The longer the period of infection as in the case of a chronic or less virulent infection, the more advantageous it is for pathogen survival, since the pathogen has a longer period to replicate. In biotic persistence, reservoirs in addition to the adult host would include secondary hosts and alternate hosts -predators, and parasitoids which then serve as carriers. Pathogens with a wide host range would be expected to persist more effectively than those specific or a single host. Studies of such persistence have been reported for the NPV of he tussock moth complex, the granulosis virus of lepidopterous insects in alfalfa (Tanada & Omi, 1974). Although predators have been shown to function as dispersal agents for NPVs (e.e., via bird droppings; Thompson & Scot, 1979), their role in viral persistence, although quite possible, is not proven. Parasitoids appear to disperse virus by external contamination, and the increased dispersal is thought to increase the probability of viral persistence (Evans, 1986).

The main reservoirs for abiotic persistence of NPVs are soil and the host plant. Soil is highly favourable for entomopathogen survival and is a natural reservoir. Its physicochemical properties affect pathogen survival. Viruses accumulate in the litter and soil (Tanada & Omi, 1974). Nuclear polyhedrosis viruses persist overwinter in the soil (Ignoffo et al., 1977; Oloffson, 1987). However, the information on the role and effect of soil on pathogen persistence is limited. In the soil, the temperature, moisture, pH, minerals, humus, and roots of plants may be involved. Factors in the physical environment above the soil surface that most significantly affect the persistence of pathogens are sunlight, moisture, and humidity (Ignoffo et al., 1977). UV radiation is the single most important factor in the inactivation of baculoviruses in the environment (Jones et al., 1993). Next to sunlight, temperature appears to be an important inactivate of viruses (David et al., 1971; Rituma, 1980). Plants are known to contain antimicrobial substances that can inhibit the growth of entomopathogens on plant foliage.

There is probably a better understanding of the capacity of a pathogen population to survive or persist in the host's habitat in insect epizootiology or any other field due to the extensive studies in microbial control. There are three types of such studies: laboratory dosage-mortality or dosage-infection studies (including application in habitat components, such as soil); field microbial control research; and observational field epizootiology that includes estimates of pathogen population density.

1.2.2. Virus insecticides and formulations

A factor limiting the consistent success of viral pesticides is their failure to compare favorable such chemical insecticides in efficiency evaluations on crops. Recently, considerable attention has been proven to enhancement of virus efficacy by increasing

virus persistence and stability, or increasing host susceptibility. Formulations will be designed to retain biological activity of the virus, confers stability during storage. There were different kinds of additives: anti-evaporants, humectants, thickeners, stickers, wetting agents. Extensive lists of formulating materials are given by Entwistle and Evans (1985); Hunter-Fujita et al., (1998). Well known surfactants are Triton –B and Tween –80 (Huber & Dickler, 1977; Bell, 1991).

Considerable progress has been made in the utilization of baculoviruses in microbial control. Some viruses have been applied aerially to several thousand hectares and, in general, with good excellent results. Unfortunately, commercialisation of baculoviruses to be used for pest control has not progressed at the same rate as the cognition of their potential. Entwistle (1998), in one of most recent reviews on the use of baculoviruses lists species of insect pests which have been controlled by baculovirus preparations. NPV from European pine sawfly *Neodiprion sertifer* Geoffr. has been registered and applied in Sweden, UK, and Canada (Huber, 1986). *Spodoptera exigua* MNPV has been registered in the Netherlands and the USA as biological insecticide against beet army worm (Smits & Vlak, 1994). Mn NPV have been successfully used against apple tree pest *M. neustria* (Zarinsh & Kalnina, 1972; Magnoler, 1985; Zariņš & Eglīte, 1993) and regulate their host populations.

Researches on biological control with baculoviruses were carried out in the Institute of Biology since 1986. Several baculoviruses were isolated from pest populations in Latvia (Zariņš & Eglìte, 1993). NPVs and GVs isolated from *M. neustria*, *N. sertifer* and *Pieris brassicae* L. were tested as virus insecticides in this country. Latvian isolates or experimentally improved strains were used as source of virus preparations and applied to control pest populations (Zarinsh, 1989; Rituma, 1989; Eglite & Zariņš, 1993; Zariņš & Eglìte, 1993). High effective experimental strain of Mn NPV was obtained in collaboration with researchers from Belarussian Institute of Plant Protection (Zarinsh et al., 1983). Mn NPV was used as basis of VIRIN-KSH that was the virus preparation registered and produced in Soviet Union. The method of producing the mentioned virus formulation was elaborated in Latvia (Zarinsh et al., 1987). In recent year's Mn NPV, Ns NPV and Pb GV were used as model objects for investigation of ecological host - pathogen interactions and elaboration of virus preparations. Attention was paid to development of virus insecticide formulations, where environmentally safe additives were used.

UV protectants additives

Many substances have been combined with the virus to serve as solar protectants, but none is effective for long periods. The more protenising protectants are activated carbon, dried skimmed milk powder (Schmid, 1974; Zarinsh et al., 1989), sucrose (Schmid, 1974), egg albumin or brewer's yeast (Jaques, 1977), molasses (Stelzer et al., 1975; Eglite & Zarinsh, 1987), bentonite (Eglite & Zarinsh, 1983), lignin sulphate (Young & Yearian, 1974), and Congo red (Shapiro, 1989). The structure, texture and volatile substances emitted by leaves have influenced the denaturing effect of sunlight. When the Ha NPV was applied to leaves of cotton, soybean and tomato, it was inactivated most rapidly on cotton, with little activity remaining after 24 hours (Young & Yearian, 1974).

Various compounds involved in purine catabolism were examined as UV screens. At a 1% concentration, hypoxanthine, urea and xanthine were ineffective, while allantoin and uric acid had some activity. Adenine and guanine were effective protectants of virus. In a subsequent test, uric acid was effective at concentrations ranging from 1-10%. The addition of 10% uric acid to the gypsy moth NPV enhanced viral stability by 3.6 – fold (Shapiro, 1984). Addition of antioxidants -diludine and ionole to virus preparations exert negative effect of UV radiation (Zarinsh & Eglite, 1985).

Stickers

Probably because of the particulate nature of baculoviruses, it has often been perceived that stickers are essential in formulations. may be desirable, the surface of same plants retain baculoviruses OBs very strongly. For instance, despite rain falling immediately after spraying, the biological impact of baculovirus sprays tends often not to be diminished. Formal laboratory demonstrations of very pronounced rain-fastness have made, e.g. of Pb GV on cabbage leaves (David & Gardiner, 1966), Cp GV on apple leaves. From formulation point of view, the matter is further complicated by possible innate differences in adhesion of OBs and particulate formulants such as some UV protectants. Stickers often used in formulations are methylcellulose and hydroxy-methylcellulose (Rituma & Skujane, 1989), milk (skim) (Huber & Dickler, 1977), Polyvinilalchocol (PVA), Triton –B, Agral NN (Smits et al., 1978; Hunter-Fujita et al., 1998).

Enhancins and synergistic factors

Several studies were aimed to increase susceptibility of insects to viruses by mixing the polyhedroviruses with additives of different origins.

A synergistic relationship between a GV and NPV was first observed in *Pseudolatia unipuncta* by Tanada (1959). The heat inactivated virus still capable of enhancing the virulence of unheated polyhedrovirus. Heat inactivated NPVs had no effect on the unheated GV, which suggests that the granulosis virus is better synergist. Hukuhura et al. (1987) concluded that degree of enhancement afforded by synergistic factor varied considerably, depending on the host insect and the virus. Uchima et al. (1989) has studied a synergistic factor (SF) enhanced the infection of Ac NPV *in vitro*. The results indicated that SF is a binding molecule in which the phospholipid component is essential for its phagocytosis and enhancement of baculovirus infection. The SF appears to have two different binding sites, one for the cell plasma membrane and other for the enveloped virion.

Enhancement of Ac MNPV infectivity by the viral enhancin factor (VEF) of Tn GV was 1.7 to 4.5 fold (Gallo et al., 1991, Gijzen et al., 1995). VEF is a 104-kDa protein that forms about 5% of the mass of OB of the Tn GV (Hashimoto et al., 1991). Although it has been shown to be present in several GV genomes, it is not present in Ac NPV (Ayres et al., 1994). The enhancin protein is a metallprothease (Lepore et al., 1996), mode of action of Tn GV enhancin is increase permeability of insect pretrophic membrane. It is investigated that, the mixing of polyhedrosis with each other could enhance the virulence. The results shoved that the *P. unipuncta* GV enhanced, *Ha* NPV,

P. separata NPV and *Agrotis segetum* NPV infections in their host insects. Arne and Nordin (1995) reported the larvae of the noctuid *Helicoverpa zea* were simultaneously inoculated with Hz NPV and Ac NPV, respectively, both known to be pathogenic to *H. zea*. In both instar tested, significant reductions were recorded in the LD_{50}, compared with larvae treated with one virus only. Also, viral mixtures significantly reduced the time required between infection and mortality.

Plant extracts

In the 1980s, the use of natural substances, obtained from leaves, fruits, seeds, bulbs and roots of fresh or dried plants, in plant protection has increased (Agrawal & Mall, 1988; Scin-Foon, 1989; Stein & Klingauf, 1990) including insecticides, plant species with hormonal influence on insects, and antifeedants that content alkaloids or alkaloidglycosids. Separate plant substances (tomatine, nicotine) have either insecticidous and antifeedant properties. The properties of plant extracts determine the possibilities to use they in plant protection, for example:

- extract obtained from the neem tree *Azadirachta indica* act as antifeedant, insecticide and insects growth regulator against many insects including lepidopterians (Scin-Foon, 1989, Scmutterer, 1995),
- seed extracts of *C. inophullus* act on the 3rd instar larvae of *Diacrisia obliqua* (Agrawal & Mall, 1988),
- *Derris ssp.* and *Quassia ssp.* extracts against the larvae of plant leaf-cutting beetles (Stoll, 1986),
- extracts of *Aglaia cordata* against the larvae of *Spodoptera frugiperda* (Mikolajczak et al., 1989),
- extracts of garlic extracts against the aphid *Myzus persicae* and moth *Putella xylostella* (Stein & Klingauf, 1990),
- the extract from *Tephorosa vogellii* leaves against the larvae of turnip white butterfly *Pieris napi* and cabbage moth *Barathra brasssicae* (Scin-Foon, 1989). The effects of various concentrations of extracts depend on the stage of insect development.

Few recent records on the effect of the plant extract as synergistic factor for the baculovirus are available. It is shown that extracts of *Meliaceous* plants mixed with NPV work as a synergistic factor (Sarode et al., 1995). Neem oil seemed to contribute the mortality by reducing food consumption and weight increase. If neem oil interferes with NPV- replication, it can be only a minor effect which is overshadowed by synergistic action.

A field evaluation studies of neem seed extract in combination with NPVs gave highest yield and better control of treated pests (Sarode et al., 1995). The disadvantage of mixing the Azadirachtin with the NPV formulation could also results in less virus being produced within the larval cadaver and release into the environment because the affected larvae are smaller in size (Cook et al., 1996).

The successful application of active plant substances in plant protection requires knowledge of their action mechanism. Cytological methods can be used to determine the mechanisms of effect.

2. MATERIALS AND METHODS

2.1. Test insects and viruses

Laboratory populations of *M. neustria,* and also insects *M. neustria, N. sertifer* and *P. brassicae* hatched from eggs collected in field were used in tests.

Insects from laboratory populations grown on artificial diet, were provided by M. Skujane (LAU). Eggs of the insects collected in field were sterilized in 5% Formaline vapor for 5 min. and kept in small boxes (17x15x10 cm) until hatching. Hatched larvae were reared on natural food in special cages (0.5 x 0.35 x 0.35 m).

Virus isolates (Table 1) were obtained from the larvae with symptoms of disease collected in nature or by activation of latent infection.

Table 1. List of the tested viruses.

Virus	Host insect	Origin
NPV		
Mn NPV	European tent caterpillar *Malacosoma neustria* L.	Latvian isolate, 1967 (Zarinsh, Kalnina, 1972)
Mn NPV	*M. neustria*	Latvian isolate, district Saldus, 1995 (Jankevica et al., 1998)
Mn NPV	*M. neustria*	Latvian isolate, district Dobele, 1994 (Jankevica, Zariņš, in press)
Mn NPV	*M. neustria*	Armenian isolate, 1984 (Zarinsh, Eglite, 1986)
Ns NPV	European pine sawfly *Neodiprion sertifer (*Geoffr.)	Latvian isolate, 1970 (Zarinsh, Rituma, 1973)
Eriogaster lanestris NPV	*Eriogaster lanestris* L.	Latvian isolate, 1997 (Jankevica, Jankevics unpublished)
GV		
PbGV	Cabbage white butterfly *Pieris brassicae* L.	Latvian isolate, 1964, (Zarinsh, 1967)
Cp GV	*Cydia pomonella* L.	Kindly provided by Belarussian Inst. of Plant Protection

The dimensions of inclusion bodies of Mn NPV were 0.8 - 1.5 μm, Ns NPV -1.0 - 1.6 μm, and Pb GV -0.3 μm (Figure 5). The virus isolates were multiplied in the Laboratory of Experimental Entomology, Institute of Biology, University of Latvia.

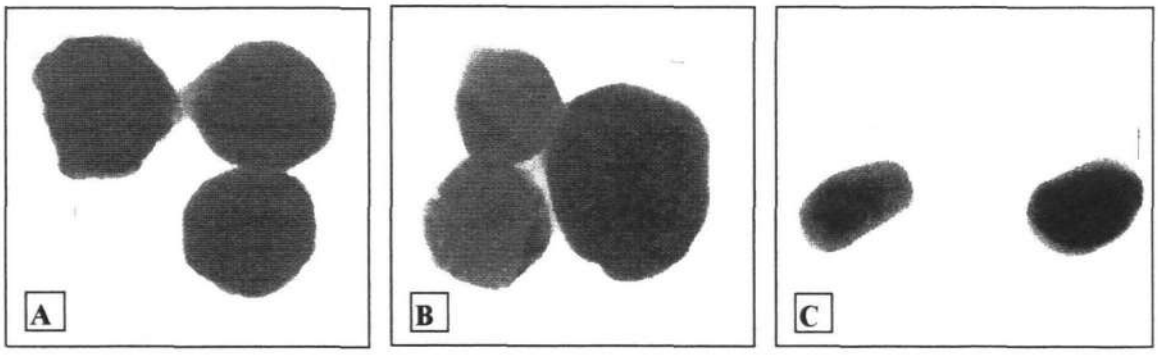

Figure 5. Electron micrographs of *Malacosoma neustria* nucleopolyhedrovirus (A), *Neodiprion sertifer* nucleopolyhedrovirus (B) and *Pieris brassicae* granulovirus (C). Bars represent 0.1μm. Preparations negatively stained with 2% phosphotungstic acid. Author of electron micrographs Dr. Velta Ose.

2. 2. Virus formulations

The method of producing virus formulation developed in our laboratory (Zarinsh et al., 1987) was used. The technical concentrate of NPV - 5x 10^6 or 2x 10^7 polyhedrae/ml was mixed with one of the additives (concentrations 0.5%, 1% or 2%) and stirred for 10-15 min until homogenous suspension was obtained.

2. 3. Additives used in virus formulations

Stickers and UV protectors

The additives used in the formulations were: polyglucine (Factory of Producing Biomedical Preparations - Obolensk, Russia), molasses of peat (experimental product, Institute of Wood Chemistry, Latvia), lysine KKL (Factory for producing lysine, Latvia), a by-product of citric acid production (Institute of Microbiology, Latvia), Belkosine M (Factory -Belcosin, Russia) and bentonite (Sigma). All the tested additives gave good wettability of dispersible dry formulations (Table 2), as well as promoted adhesion to the plants.

Table 2. Physical characteristics of virus preparations containing different additives

Additive in the virus preparation	Stability: concentration of virus suspension, polyhedra/ml		Dispersion, (sec)	Adhesion	Viscosity
	before sedimentation	after 30 min sedimentation			
Polyglucine	$2.8x10^7$	$2.7x10^6$	300-360	good	1.06
Molasses of peat	$6.9\ x10^6$	$6.3x10^6$	15	satisfactory	1.03
Lysine KKL	$2.4x10^6$	$2.4x10^6$	30	satisfactory	1.01
Belkosine M	$4.5x10^5$	$4.0x10^5$	600	very good	1.02
By-product of citric acid production	$5.1x10^6$	$3.2x10^6$	40	satisfactory	1.25

To enhance virus disease we also used substances:

- Commercial preparations - Eim, Dimilin and Nomolt that works as physiological insects' weakeners
- amino acids - lysine and tyrosine
- plant lecithin (ZRA Biolar) and kephalin (ZRA Biolar).

Plant extracts

1. Extract of common marigold (*Calendula officinalis*). Finely cut fresh plant material (80 g) was mixed with one litre of cold water, exposed for 3 days, and then filtered.
2. Extract of French marigold *(Tagetes patula*). Air dried plants (80 g) were mixed with one litre of cold water, exposed for 2 days, and then filtered.
3. Extract of milfoil (*Achillea millefolium*). Air dried plants (80 g) were mixed with one litre of cold water, exposed for 6 days, and then filtered.
4. Broth of tomato (*Lycopersicon esculentum)* leaves. Fresh tomato leaves (400 g) were simmered in one litre of water for 30 minutes, and then filtered. The filtrate was diluted with water in ratio 1:3.

2. 4. Light and electron microscopy

Standard cytological methods and Giemsa staining (Lilli,1969; Adams & Bonami, 1991) was used for observation of presence of NPV in the insects. Virus - infected larvae in different stages of disease (2- 10th day after infection) were fixed, embedded, sliced and stained. Cytological preparations were investigated by a light microscope (Amplival, Germany).

The polyhedrae count in virus formulations and polyhedral yield per larva were determined by a haemocytometer.

For ultrastructural examination, the larvae were fixed in 2.5% glutaraldehyde in pH 7.2 phosphate buffer, postfixed in 1% OsO_4 in the same buffer, enclosed in epon, sliced. Polyhedrae were dissolved with 1% NaOH for 5 min and negatively stained with 2% phosphotungstic deid. The preparations were observed in an electron microscope (JEM-100C, JEOL, JAPAN). Electron microscopy has been done by Dr. Velta Ose (LU BMC, Riga).

2. 5. Virus isolation and purification

Dead larvae were frozen with liquid nitrogen and homogenized in distilled water with 0.1 % SDS. The homogenate was then filtered through cheese-cloth. The filtrate was centrifuged at 6.000 g (NPV) and 11.000 (GV) for 40 min. Polyhedrae and granulas were purified by centrifugation on 30-66 % (w/v) linear sucrose gradient. Inclusion bodies bands were collected, checked by microscope and washed twice with distilled water. The virus pellet was resuspended in a small volume of de-ionized and stored at -18° C (Evans & Shapiro, 1997).

2. 6. Isolation of NPV DNA and digestion with restriction enzymes

DNA was isolated from purified polyhedrae by phenol extraction followed by alkaline disruption of the inclusion bodies as described (Summers, 1988; Sharipo & Eglite, 1990)

The viral DNA was cleaved by *Hind*II, *Hind*III, *Sal*I using the specific assay buffers suggested by the supplier. After 2 h incubation at 37 °C, the digestion was stopped by addition of EDTA till final concentration 20mM. The digest was heated at 70 °C for 5 min. The digested samples were separated on 0.7% agarose gel, stained with ethidium bromide and visualized under short wave UV light (Maniatis et al., 1982).

2. 7. Extraction of virus and host genomic DNA from individual insects

Each individual larva, pupa or imago was homogenized in 600 µl ice-cold TE buffer containing 0.1% SDS using a dounce homogeniser. To 300 µl homogenate 200 µl 0.1M sodium bicarbonate were added. After 10 min at 37 °C, 200 µl lysis buffer and 2.5 µl RNase A (10mg/ml) were added, and the incubation was continued for 30 min. Following the addition of 15 µl Proteinase K (10mg/ml), the incubation was continued for a further 30 min. The mixture was then extracted once with phenol, twice with phenol:chloroform (1:1) and once with chloroform. The DNA was then precipitated

with ethanol, washed in 75% ethanol and the air dried pellets were dissolved in 100 µl distilled water (Saville et al., 1997).

2. 8. Determination of NPV by DNA-DNA hybridization

We used a modified method of DNA-DNA spot-hybridization (Sharipo, 1991) for the detection of Mn NPV in the environment, on plant surfaces and in soil.. We used ^{32} P - labelled DNA probe capable to detect Mn NPV produced in the Institute of Microbiology and Virology or LU BMC.
To lyse virions, 200 µl of 2x SSPE Buffer and 0.4 N NaOH were added to 200 µl of the filtrate of 10% soil extract or leaf disc washing solution. Extracts were vacuum-blotted onto nitrocellulose filters ("Hiju Kallur", Tallinn) (Sharipo, 1991).

2. 9. Determination and identification of viral DNA by PCR

We used a sensitive technique of DNA amplification by the PCR usable for detecting DNA, and developed a PCR-based method that can detect the presence of polyhedrin-specific Mn NPV DNA, in the extracts of *M. neustria* larvae. 1 µg of the insect DNA samples were used in PCR mix. Designed primers correspond to the polyhedrin gene of Mn NPV Latvian isolate.
The PCR reaction mixture contained the following components:
insect or viral DNA - 1µl; 10x PCR buffer - 5µl; $MgCl_2$ - 5µl: BSA (1mg/ml) -4µl; dNTP (10mM) - 1µl; Primer1 (10pM/µl) - 2µl; Primer2 (10pM/µl) - 2µl; Taq polymerase 5U/µl (MBI Fermentas) -1µl; water till 50µl.
The PCR conditions were: 30 cycles on GeneAmp PCR System 2400 (Perkin Elmer)

1) 95 ^{0}C -30 sec.,
2) 56 ^{0}C - for Start1 and End1 primers or 62 ^{0}C for Start2 and End2 30 sec.,
3) 72 ^{0}C -1 min.

The final step of the reaction was followed by extension at 72 ^{0}C for 7 min.
At the end of the reaction the resulting amplification products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under short wave UV light, the size of DNA fragment was detected (Maniatis at al., 1982).

2. 10. DNA sequencing and comparison of virus isolates

PCR fragments were purified from 1% agarose gel by Jetsorb kit (Genomed). DNA fragments were cloned into pUC57T vector (MBI Fermentas) according to the recommendations of supplier. Positive clones were selected by blue/white screening and plasmid DNA was purified by miniprep columns (Qiagen). Positive clones were sequenced using both standard primers as well as primers used for PCR. Sequence analysis was performed by ABI PRISM 310 Genetic Analyser (Perkin Elmer) using BigDye Terminator Mix according to the manual. Obtained data were analysed using the program DNASIS for Windows 2.1 (Hitachi). DNA sequencing was made in cooperation with Laboratory of Genetic Engineering, LU Biomedical Research and Study Centre.

2. 11. Bioassays and field trials

Bioassay

Leaf discs of apple tree leaves (1 cm in diameter) were prepared using a cork borer, measured doses of virus were applied to leaf discs or leaf discs were dipped in the virus suspension (with measured concentration), then dried in room temperature. Using fine forceps, the dried discs were transferred, each in a well of the bioassay plate. A single third instar larva was feed to each disc (Evans, 1981; Evans & Shapiro, 1997). After one day larvae were transferred to fresh, virus free foliage. The bioassay treatments were kept under constant conditions, 25 ^{0}C, 60-70% relative air humidity and 16 hour light and 8 dark. Mortality due to infection was recorded daily and up to 20 days. Experiments were repeated 4 or 5 times (20 -30 larvae in each replica).

Field experiments

The suitability of the additives used in the virus formulations was examined in field trials from 1990 to 1995. Well producing apple-garden located in Jelgava's district infested with second instar of *M. neustria* and a young pine (*Pinus sylvestris* L.) stand located in Talsi district'(4390 larvae of *N. sertifer* per ha^{-1}) were used for investigations. The trees were sprayed with virus preparations (working solution 5x10^6 polyhedra/ml, 50 to 70 litres/ha). Backpack air-blast sprayer (Yamar-10, Japan), nozzle type 1.6 mm, spray angle 50^o, operating pressure 1.6 was used. Mortality due to infection was recorded daily, beginning at 5 day after spraying. Records were repeated 4 or 5 times (5 infested trees with 60-100 larvae were observed in each replica).

In cabbage fields, plants were grown in ten plots, with 15 plants per plot (three rows; five plants). The distance between the rows of plants was 50 cm, adequate to eliminate the spread of the viruses. Cabbages were sprayed at the stage of forming cabbage-heads, without previous picking-off larvae of *P. brassicae*. The sprayed larvae were at the 2nd-4th stages. The concentration of viruses in a working solution was 2x10^7 capsules/ml. A hand-sprayer was used. Water was used as a control. Larval mortality of *P. brassicae* was assessed daily, beginning at 48 hours after spraying. During the field tests, the average daily temperature was 9-13 ^{0}C and relative air humidity - 80-86 %.

2. 12. Determination of persistence of NPV in the environment and in pest populations

Determination of persistence of NPV on plant surface under environmental conditions

An experiment was performed to study the influence of environmental factors on virus viability after the application of viruses. Apple-trees and pines were sprayed with virus preparations (2x10^7 polyhedrae/ml, 50 to 70 litres/ha) using air-blast sprayer, the concentration of tested additives was 2%. The virus was exposed on foliage. Tables 3 and 4 shows some meteorological characteristics. Two hours, 7, 14, 21, 28 and 80 days after virus application and exposure in the environment the leaves and needles were randomly collected for bioassays. 200 discs (10 mm diameter) or 200 needles were cut for DNA-DNA hybridization. Third instar larvae of *M. neustria* reared on artificial diets and larvae of *N. sertifer* reared on natural food were used for bioassay. Virus - sprayed apple tree leaves (1000 mm^2) were homogenized and added to the diets (50 mg). Leaves

sprayed with water were added to diet and used as a control. Determination of persistence of NPV activity on pine needles followed Oloffson (1987).

Table 3. Meteorological conditions during the experiments with *Malacosoma neustria* NPV (Report of Latvian Hidrometeorological Office, unpublished, with permission).

Amount of precipitations per week, mm				Σ precipitation during 80 days, mm	Σ hours of sun shining during 80 days, h	Average daily temperature, °C
Weeks						
I	II	III	IV			
Experiment I						
8.6	12.0	4.9	11.2	176	478	14.4-20.6
Experiment II						
36.3	7.2	3.8	58.3	278	540.9	12.5 - 18.0

Table 4. Meteorological conditions during the experiment with *Neodiprion sertifer* NPV (Report of Latvian Hidrometeorological Office, unpublished, with permission).

Amount of precipitations per week, mm				Σ precipitation during 28 days, mm	Σ hours of sun shining during 28 days, h	Average daily temperature, °C
Weeks						
I	II	III	IV			
17.7	102.6	4.3	1.0	125.6	164	13.6 - 19.1

Determination of the persistence of NPV on plant surface after artificially simulated rainfall

Apple-tree branches were sprayed with the virus preparations ($2x10^7$ polyhedra/ml) using a hand sprayer, concentration of tested additives was 2%. After the application, when foliage had dried, simulated rain was applied to apple-tree branches. An air-blast sprayer was used as water pump. The spray head was held in 2 m distance from the branches. The rain was applied to branches for 10 and 30 minutes. By measuring a rain gauge under the branches we determined that these applications were in equivalent to 20 mm, 100 mm and 300 mm of naturally rainfall, respectively. After simulated rain application branches were dried and leaves were randomly collected for bioassays (4 replicas, 30 larvae in each replica) and 200 discs (10 mm diameter) were cut out for DNA-DNA hybridization (the experiments were repeated 5 times, 40 discs in each replica).

Determination of virus dispersal and accumulation in soil

A model experiment was performed to study the dispersal and accumulation of Mn NPV in soil after simulated rainfall. Sand (pH 6.1), peat moss (pH 5.5) and loamy soil (pH 6.1) were tested.

Glass containers (10 x10 x 50 cm) were filled with soil (height of soil layer 25 cm). Mn NPV suspensions (100 ml) containing $2x10^{11}$ polyhedrae were applied in each container. To determine the influence of rainfall, we used artificially simulated rain, equal to June's average precipitation (69 mm) in Riga's district. The artificial rain was used to leach the polyhedrae every 10 days - 3 times a month. Filtrates that leached

through the soil were collected, filtered through cheesecloth and taken for bioassay, electron microscopy and specific DNA - DNA hybridization.
To determine accumulation of polyhedrae in loamy soil (pH 6.05), soil probes were taken through small holes in the container. 10 g of the soil probe were added to 50 ml of sterilized water and agitated in a shaker for 2 h. Each suspension was filtered through cheesecloth and tested.

Determination of the presence of baculovirus infections in the second generation of insects

To examine the presence of baculovirus infections in the second generation of insects, eggs of *M. neustria* were collected. Hatched larvae were reared on natural food in special cages (0.5 x 0.35 x 0.35 m) under laboratory conditions to determine the presence of pathogens and percentage of mortality. Microscopy (Evans & Shapiro, 1997) and PCR amplification (Saville, 1997) were used for virus identification.

2. 13. Determination of the influence of various additives on viral disease

Substance -- physiological insects weakeners (0.01 -0.10 %), amino acids (0.1% - 1.0%) and plant lecithin and kephalin (0.02 - 0.20%) were added to technical concentrate of NPV 1 x 10^5 polyhedra/ml or GV 1 x 10^6 capsules/ml.
The PbGV preparations were added to the plant extracts (botanical insecticides). The concentration of viruses in a working solution was $2x10^7$ capsules/ml. Water was used as a control. A bioassay was used for determination of the influence of additives to viral disease.

2. 14. Statistical analysis

The efficiency of virus preparations was expressed as the percentage mortality caused by the virus corrected with the mortality in control, LD_{50} and LT_{50} (Abbot, 1925, Lipa and Šližynski, 1973). Statistical analyses of the laboratory and field test results followed Finney (1971).

3 . RESULTS AND DISCUSION

3.1. Characterization of virus isolates

3.1.1. Morphological and biological characterization of virus isolates

In reecent years two new isolates of Mn NPV were obtained from infected larvae with nuclear polyhedrosis symptoms. The infected larvae were collected in well producing apple gardens located in district Saldus and district Dobele. The dimension of polyhedra was 850 to 1400 nm. Polyhedra contained a large number of rod-shaped virions (Figure 6 A). Virions were 400 x 100-200 nm; they contained enveloped, rod-shaped nucleocapsids 360 x 80 nm (Figure 6 B). Based on the variable number of nucleocapsids per virion (Bilimoria, 1986) it was concluded that the type of Mn NPV Latvian isolates is multiple-nucleocapsid nucleopolyhedrovirus, just like as earlier investigated viruses isolated from several *Malacosoma* species - *M. apicolata* MNPV and *M. distria* MNPV (Benz, 1963, Keddie and Erlandson, 1995).

Electron micrographs of Ns NPV Latvian isolate showed that only one rod-shaped nucleocapsid per virion was occluded. Size of polyhedra 1.0 -1.6 μm. Few virions vere found per polyhedra (Figure 6 C).

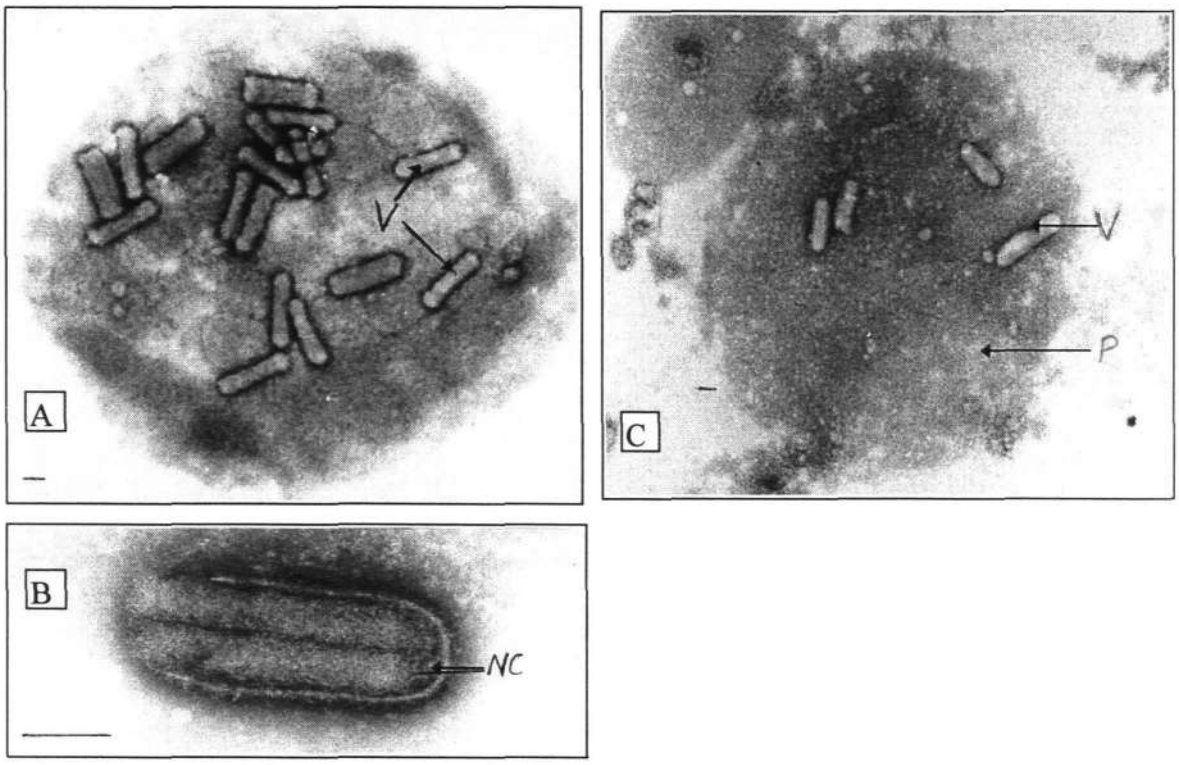

Figure 6. Electron micrographs of *Malacosoma neustria* nuclopolyhedrovirus and *Neodiprion sertifer* nuclopolyhedrovirus. (A) Mn NPV polyhedra dissolved with 1% NaOH for 5 min. (B) Mn NPV virions (V) and nucleocapsids (NC). (C) Ns NPV polyhedra and virions. Bar represents 0.1μm. Author of electron micrographs V. Ose.

In 1997 the infected larvae of *Eriogaster lanestris* L with nuclear polyhedrosis symptoms were observed. Nucleopolyhedrovirus from *E. lanestris* was isolated first time in Latvia. The dimension of polyhedra was 800 to 1600 nm. Virus cause 25.4± 4.1% mortality of *M. neustria* 3rd instar larvae.

3.1.2. Cytological and histological studies of the infection process

The process of virus infection of the European tent caterpillar (*M. neustria*), European pine sawfly (*N. sertifer*) and cabbage white butterfly (*P. brassicae*) were studied.
Fat cells with hypertrofied nucleus are observed at 4th day of Mn NPV postinfection. Necrotic fat cells were visible under light microscope in body cavity of the infected larvae 7 days after infection. Cell-breakage was accompanied by disappearance of fat drops from fat cells as well as by condensation of chromatin. Tissues and cells in the latest stage of the disease contained a large amount of viral polyhedrae.
Electron microscopy shows that nucleocapsids, multiple virions, virogenic stroma and developing polyhedrae were visible in the hypertrophied nuclei of fat cells at the 7th day after infection (Figure 7.).

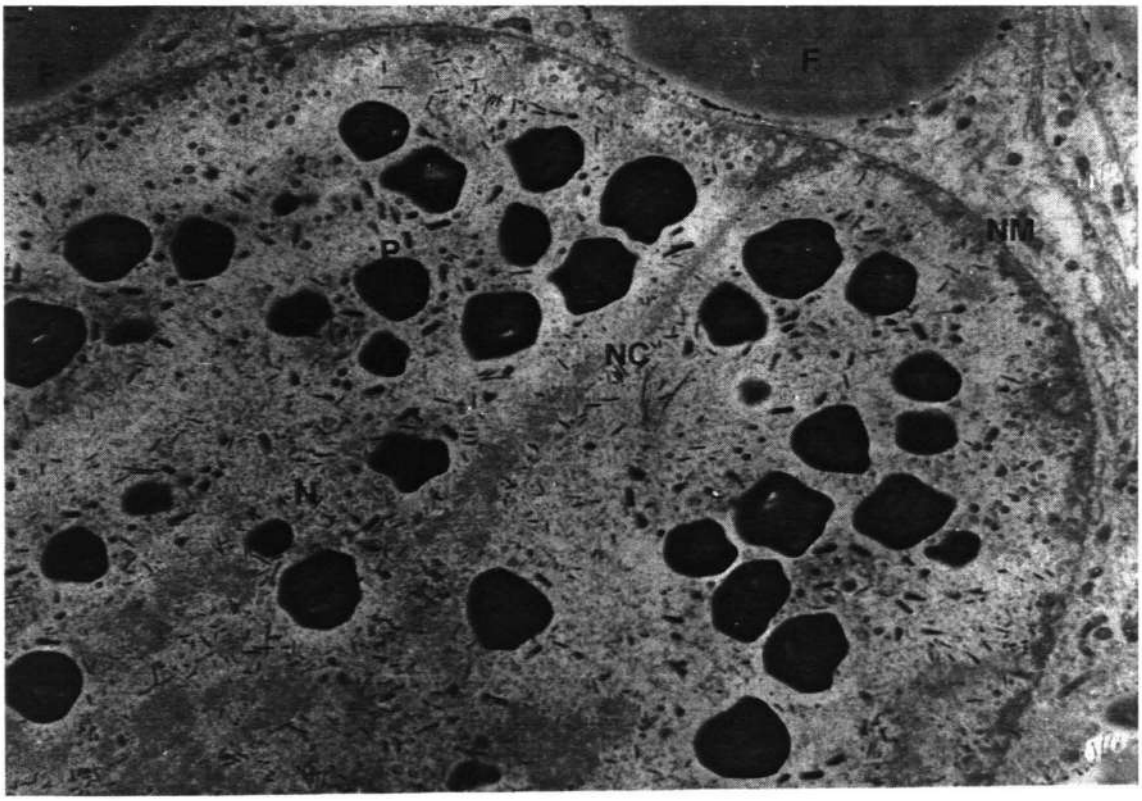

Figure 7. Electron microscopy of *per os* infected *Malacosoma neustria* larve. Ultra thin section through fat cell nucleus. Nucleus of fat cell is increased and filled with developing polyhedrae. N- nucleus, NM - nuclear membrane, P - polyhedrae, NC- nucleocapsids, F - fragment of fat drops. Bar represents 0.1μm. Author of electron micrographs V. Ose.

In a cross section of polyhedrae it is visible that multiple virions contain 1 to 11 nucleocapsids per virion (Figure 8 A). Formation of nucleocapsids took place in nuclei of fat and epithelial cells (Figure 8 B). DNA core are oclluded with capsid and viral envelope. Layers of trilaminar viral envelope are visible (Figure 8 C).
It is well known that NPV infection is polyorganotrophic (Granados and Williams, 1986). Polyhedra of the isolates were observed in nuclei of fat cells, hypodermys, trachea and muscular sheet. It is necessary to emphasize that Mn NPV isolate (Saldus) formed the polyhedra in the nuclei of gut epithelial cells, that was not typical for isolate obtained in 1967 (Zarinsh & Kalnina, 1971).

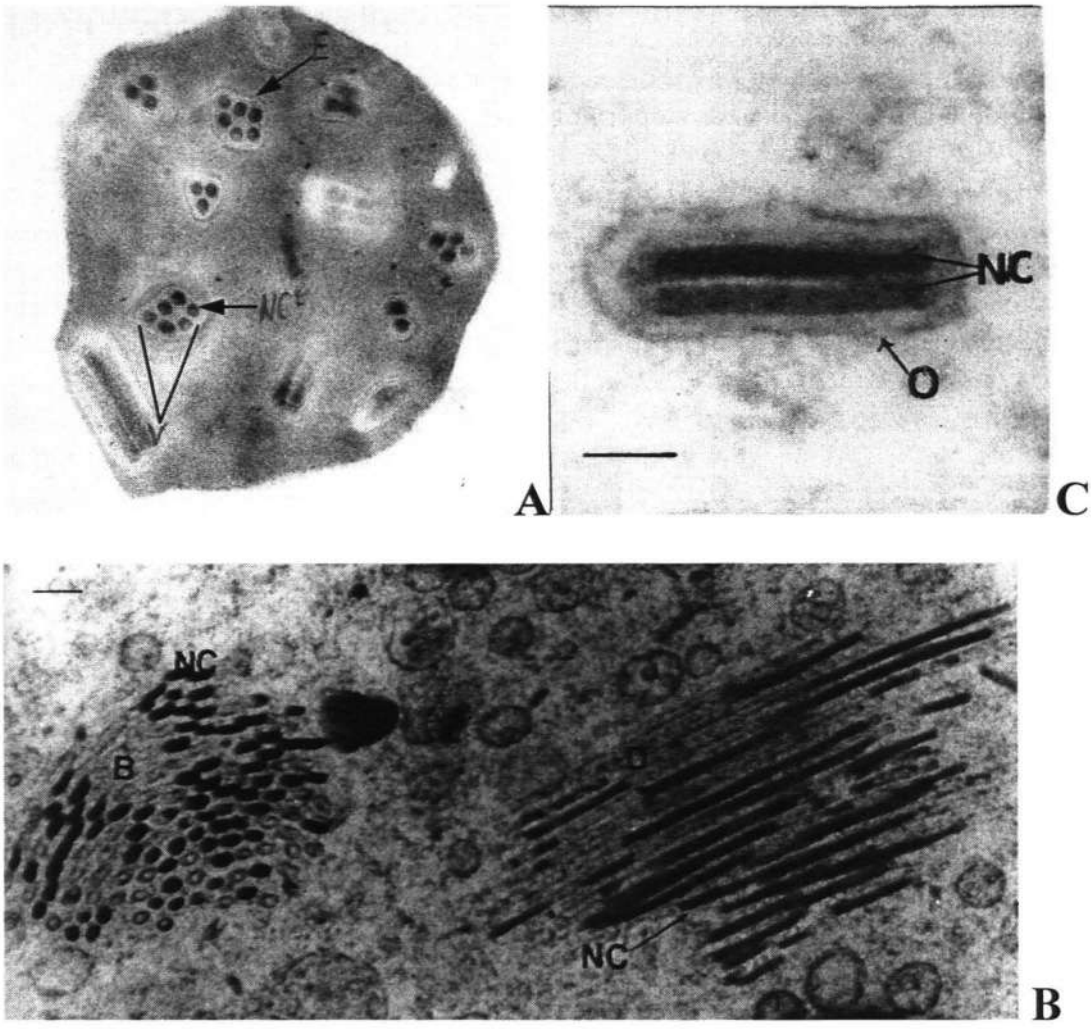

Figure 8. A- Cross section through *Malacosoma neustria* MNPV polyhedra. Virions (V) contain variable number of nucleocapsids (NC). Envelope of virion (E).
B- Ultra thin section through fat cell nucleus. Formation of nucleocapsids are visible. Empty capsids (B), developing (D) and compleate nucleocapsids (NC).
C- Section of multiple virion. Two nucleocapsids (NC) are enveloped in a virion. Layers of viral envelope are visible. Outer layer (protein) (O). Bars represent 0.1 µm. Author of micrographs V. Ose.

Development and replication of Ns NPV were observed in epithelial cells of midgut. It is observed that the Latvian strain of Pb GV causes a first visible changes at 4th day of post infection in fat and hypodermal cells. Condensations of chromatin occurs in nucleus and replication of viruses takes place. Two days later necrotic fat cells are visible in body cavity of the infected larvae. In the infected cells, vacuoles appear and fat drops disappear from fat cells. In places only cellular residues are visible in larvae's body cavity -- fragments of membranes, protoplasm and nuclei. 4- 7 days after the fat body's disintegration the content of its cells mixes with hemolymph. Hypodermal cells increase and thus the layer of hypodermis. Later general cell necrosis takes place, the tissues of muscles also decompose.

3.1.3. Determination of virulence

Biological activity of Mn NPV isolates were characterized by a bioassay. If third instar *M. neustria* larvae were infected, the rate of mortality reached 10%, 27%, 46%, 60%, 69%, 78%, 90%, 100% for the tested doses of $5x10^1$, $1x10^2$, $5x10^2$, $1x10^3$, $5x10^3$, $1x10^4$, $5x10^4$ and $1x10^5$ polyhedrae/larva of MnNPV isolate (Saldus), respectively. LD_{50} values determined were 55 ± 10 and 985 ± 19 polyhedral occlusion bodies (polyhedrae) per larva for second and third instar, respectively. The rate of mortality caused by MnNPV isolate (Dobele) reached 10%, 18%, 37%, 40%, 48%, 64%, 82%, 98% for the tested doses of $5x10^1$, $1x10^2$, $5x10^2$, $1x10^3$, $5x10^3$, $1x10^4$, 5x104 and $1x10^5$ polyhedrae/larva, respectively. The bioasays showed that incubation period of the disease was 5 to 10 days after infection with different doses of Mn NPV. The relationship between time - dose and mortality for the virus isolates is shown in Figure 9 A,B.

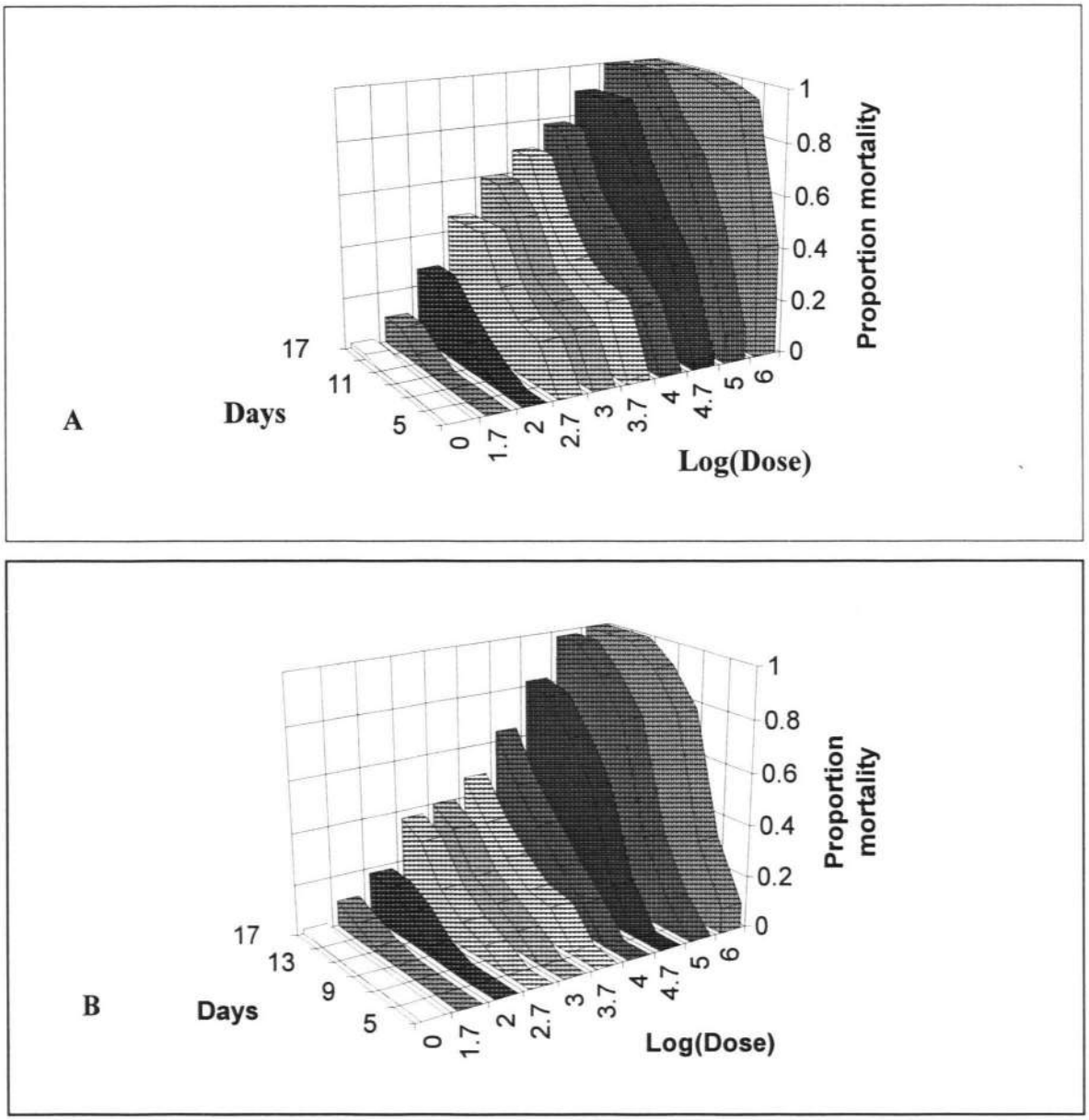

Figure 9. Relationship between cumulative proportion mortality, viral dose, and mortality time of *Malacosoma neustria* third instar larvae. A -Mn NPV isolate (district Saldus), B - Mn NPV isolate (district Dobele).

The results of our experiments showed that tested isolates had different virulence. The isolate obtained in district Saldus had higher virus activity and shorter incubation period of disease. The increasing of virus doses caused decrease of the incubation period of the disease and the time until insects death.

3.1.4. Caracterization of virus productivity

Rituma & Skujane (1983) showed that, to obtain maximum quantity of polyhedrae, it is purposeful to infest third or fourth instar larvae. We used 3rd instar larvae in the experiment performed to evaluate Mn NPV productivity in *M. neustria*. In the case of using the Mn NPV (Saldus) isolate and dose input of $1x10^3$ polyhedrae/larva, the highest yield per larva was obtained with the value of $9.8x10^9$ polyhedrae/larva which decreased to $3.8x10^9$, $2.8x10^9$ and $2.1x10^9$ polyhedra/larva for the tested concentrations of $1x10^4$, $1x10^5$ and $1x10^6$ polyhedra/larva, respectively. The virus yield was maximal on the 8th postinoculation day.

Data in Table 5 show that the amount of NPV polyhedrae produced or virus yield per larva (Mn NPV Saldus) was $2.10x10^9$ to $9.82x10^9$ viruses/larva after feeding insects on leaves containing different virus doses and estimated LT_{50} was 6.1 to 14.5 days.

Table 5. Effect of initial virus concentration on LT_{50} and obtained virus yield of Mn NPV in *Malacosoma neustria* 3rd instar larvae

Virus	Tested doses	Mortality ± SEM, %	$LT_{50} \pm sLT_{50}$, days	Day of yield collection	Average yield/larva
MnNPV (Saldus)	$1x10^3$	62.8±2.8	14.5 ± 0.7	8	$9.82x10^9$
	$1x10^4$	78.6±3.2	11.9 ± 0.45	8	$3.84x10^9$
	$1x10^5$	99.0±1.0	8.4 ± 0.26	8	$2.87x10^9$
	$1x10^6$	99.0±1.0	6.1 ± 0.17	8	$2.12x10^9$
MnNPV (Dobele)	$1x10^3$	40.6±3.8	-	8	$9.47x10^8$
	$1x10^4$	64.0±3.4	15.9 ± 0.56	8	$4.31x10^8$
	$1x10^5$	98.0±2.0	12.7 ± 1.49	8	$5.26x10^8$

Obtained results show that Mn NPV Saldus had highest virulence. Amount of virus yield per larva in variants with MnNPV Dobele was 7 to 10 times lower.

3. 2. Investigation of the influence of additives to viral pathogenicity

I studied the possibilities to stimulate the action of the baculoviruses and increase their efficiency.

A. Influence of additives

Additives used in formulations that increased the stickability of virus preparations, influenced also the process of virus infection. The mortality of the larvae increased till 100% in the variants with by-product of citric acid production and with addition lysine KKL. Additions moolase of peat and belcosine M had no essential effect. The

investigated substances acted similarly to the infection of nucleopolyhedroviruses and to the infection of granuloviruses.

B. Influence of substances -- physiological insects' weakeners.
The effect of the substances Nomolt and Eim, Dimilin in concentration 0.01- 0.10% to the virus disease caused by Ns NPV and Pb GV was studied. These substances in concentration 0.05% gave possibility to reach the efficiency of 30%.

C. Influence of the amino acids and amines
Pathological process in *N. sertifer* caused by NPV (with lowest dose -1x 10^4 polyhedrae per larvae) was stimulated by amino acids lysine and tyrosine in concentration 0.1%. The mortality of the larvae increased in variants with the amino acids where lowest dose of viruses was used. Addition of plant lecithin (conc.1.0%) to low dose of Mn NPV (1x 10^3 polyhedra per larvae)increased larval mortality from 50% to 89%. 0.1% lecithin and 0.02% kephalin did not essentially influence virus activity.

3.2.1. Influence of plant extracts

The applied antifeedant extracts from common marigolds, French marigolds and milfoil, resulted in, respectively, 55.9 %, 46.1 % and 12.0 % mortality of *P. brassicae* 18 days after spraying. The extracts, together with PbGV, caused mortality ranging from 51 - 90 % (Figure 10). Extract from common marigolds caused very low mortality of the larvae

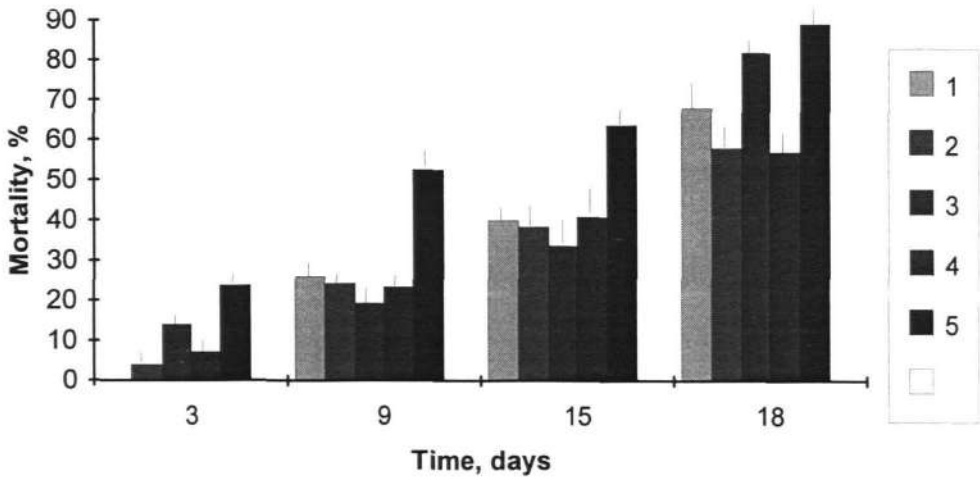

Figure 10. Comulative mortality of *P. brassicae* 3rd instar larvae after spraying with Pb GV water suspension, and with Pb GV plus various plant extracts (virus titre in working suspension was 2x10^7 capsules/ml).

1 -- Pb GV
2 -- extract from milfoil + Pb GV
3 -- extract from marigolds + Pb GV
4 -- extract from French marigolds + Pb GV
5 -- tomato leaf broth + Pb GV

after 3 days, but it increased to 26.3±3.8 % after 15 days, probably due to weakening of the larvae after starvation and increased susceptibility to diseases (in many cases, the dead larvae contained viruses, bacteria and fungi). Common marigold extracts with

added Pb GV caused higher mortality. Larval mortality was very low, when extract from milfoil were used, but it increased with addition of Pb GV. However, the mortality due to extracts from milfoil together with Pb GV did not significantly differ after 15 days from that caused by Pb GV suspensions alone. Therefore, milfoil extracts do not diminish the activity of viruses, but only prolong the development period of the viral disease by antifeedant activity.

Tomato leaf broth caused 25.7 % mortality of the larvae at 3 days after spraying, likely due to a toxic effect. About 70 % of the larvae that were sprayed with tomato leaf broth had died from granulosis after 15 days, suggesting the activation of latent viruses. The addition of Pb GV to tomato leaf broth did not increase the larval mortality during first few days. After 9 days, the mortality caused by the viruses had increased and at the 18th day it had exceeded that caused by the Pb GV suspension in water. The toxic effect of tomato leaf broth is likely to be summed with the activity of the granuloviruses, thereby increasing larval mortality. The investigated substances acted commonly to infection of NPV and to infection of granuloviruses.

3. 2.2. Enhancing of NPV by addition of GV

Granuloviruses isolated from *Cydia pomonella* were used as an additive to Mn NPV. Data presented in Table 6 show that, NPV - mortality rate increased significantly with increasing the concentration of Cp GV additive. Mortality was enhanced in most instances.

Table 6. Effect of *Cydia pomonella* granulovirus as a synergistic factor (SF) to *Malacosoma neustria* NPV, on the NPV - caused mortality among test larvae.

NPV concentration (polyhedra/ml)	NPV -mortality ± SEM ,%		
	Concentration of GV additive (capsules/ml)		
	0	1x 10^5	1x 10^7
1x 10^2	27. 8± 3.6	23. 4± 3.1	33.2± 4.8
1x 10^3	60.2± 2.2	66.8± 3.6	68.4± 2.7
5x 10^3	69.3± 2.4	78.1± 2.5	81.1± 3.0
1x 10^4	78.2± 1.8	94.4± 2.2	97.2± 2.6
5x 10^4	90.1± 1.6	98.3± 1.7	98.8± 0.8

We determined detectable synergistic effect in the case of combining Cp GV with Mn NPV.

3.3. Efficiency of developed virus formulations

Field trials with the developed virus insecticide formulations indicated that high levels of mortality 89 to 96 % of second and third instar larvae could be achieved 15 days after spraying (Table 7).

Table 7. Efficiency of developed virus formulations with different additives.

Virus	Additive in the virus preparation (1%)	Mortality ±SEM, %
Ns NPV	Polyglucine	95.2±1.8 (a)
	Molasses of peat	92.7±2.3 (a)
	Lysine KKL	96.0±1.3 (a)
	By-product of citric acid production	86.7±4.5 (b)
	Belkosine M	85.2±3.5 (b)
	Bentonite	93.2±2.6 (a)
	Control - virus-water suspension	84.1±2.6 (b)
Mn NPV	Polyglucine	98.2±1.8 (a)
	Molasses of peat	92.7±2.7 (b)
	Lysine KKL	96.8±1.7 (a)
	By-product of citric acid production	85.3±3.5 (c)
	Bentonite	95.2±3.5 (a)
	Control - virus-water suspension	81.4±3.6 (c)

* Means followed by the same letter are not significantly different at P=0.05

Best efficiency showed virus preparations where additives; polyglucine, molasses of peat and lysine KKL were used.

3. 4. Persistence and accumulation of NPV

3. 4. 1. Persistence of NPV on plant surface after artificially simulated rainfall

The persistence, distribution and accumulation of NPVs after artificial rainfall were determined to optimise successful biological control of *M. neustria* population.

Amount of Mn NPV polyhedrae on sprayed apple tree leaves after exposing them in artificial rain depends on used additives and intensity of rain. Loss of the polyhedrae on apple-tree leaves after simulated rain was 70-93% in variants with additives and 99% in the control (virus-water suspension without additives).

The bioassay shows that the larvae fed on the leaves exposed to the artificially simulated rain died after 10-15 days. The efficiency of virus preparations containing different additives was 90 to 96% before and 76 to 84% after exposing the leaves to the artificial rain equal to 20 mm (Figure 11). After exposing the branches for 30 min to artificial rain equal to precipitation 300 mm the efficiency of the preparations containing additives was 52 to 60%, but in the control without additives - only 18%. All of the tested additives gave good wettability of dispersible dry formulations as well as promoted adhesion to the plants.

Used additives lysine KKL, the by-product of citric acid production, molasses of peat, polyglucine, and Bentonite increased the persistence of the polyhedrae 20, 10, 13, 3 and 3 times, respectively in comparison with control. The results showed that directly after spraying the new tested additives increased adhesion of the virus polyhedrae to apple-tree leaves approximately 2 times in comparison with Bentonite. The mortality of *M. neustria* larvae fed on the leaves exposed to artificial rain did not show significant differences among the preparations with additives Lysine KKL, Polyglucine, and molasses of peat.

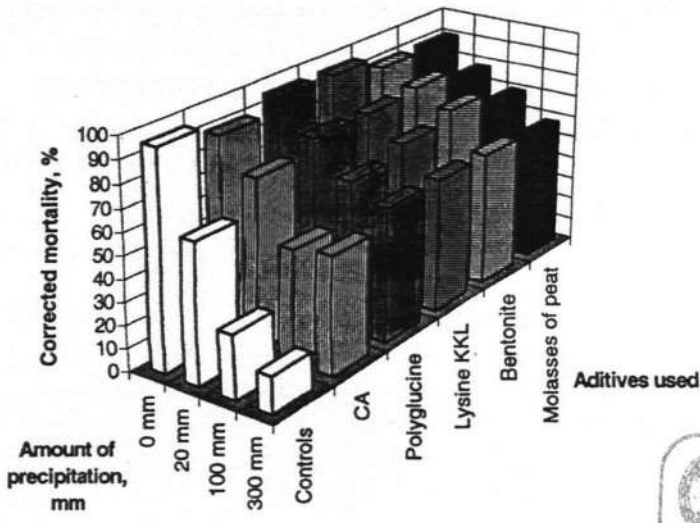

Figure 11. *Malacosoma neustria* larval mortality caused by different virus preparations after exposing to artificial rain.

The efficiency of the preparations with the by product of citric acid production was lower. Application of additives exerted the influence of rainfall on Mn NPV viability.

3.4.2. Accumulation, dispersal and persistence of NPV in soil

The distribution and accumulation of NPVs in soil after artificial rainfall were determined to optimize biological control strategies. Results of our investigations show that rainfall washed out the virus. We determined that polyhedra leached through 25 cm layer of tested soils, if they were sprayed and washed with water amount equal to average precipitations in June. The least leaching efficiency was established for loamy soil, because loam can adsorb the polyhedrae (Table 8). The bioassay showed that the larvae

Table 8. Leaching efficiency of *Mn* NPV leached through 25 cm soil layer determined by DNA-DNA hybridization and bioassay.

Soil	Number of artificial rainfall	Intensity of DNA-DNA hybridization	Larval mortality ± SEM, %	$LT_{50} \pm sLT_{50}$, days
Loamy	I	+	33.6± 5.6	*
soil	II	++	60.4± 4.6	15.6±1.6
	III	+	18.9± 3.8	*
Peat	I	+++	98.1± 1.0	7.8±0.8
moss	II	++	98.4± 1.6	11.7±1.0
	III	++	76.6± 3.0	11.7±0.8
Sand	I	++	95.0± 2.5	9.1±0.7
	II	++	98.3± 1.6	10.7±0.5
	III	++	90.0± 3.0	11.1±0.7

\+ virus conc. more than 1000 polyhedra per 1 ml, ++ 10000 - 90000 polyhedra per 1 ml, +++ more than 100000 polyhedra per 1 ml, * larval mortality did not exceed 50%, in controls larval mortality 1.5 to 4% and no hybridization was observed.

fed on the leaves sprayed with soil filtrates died after 6 -17 days. The viruses leached through peat and sand had high activity and caused larval mortality 95 to 99% after first and second water spraying. Determined LT_{50} showed that the development of disease is faster after the first artificial rainfall, it means that after two weeks a part of the viruses is inactivated or adsorbed in soil. Leaching efficiency could be decreased, if the additives were used in the virus preparations.

Artificial rain spread viruses into loamy soil; DNA-DNA hybridization showed that viruses were accumulated in the soil layers (Table 9).

Table 9. Persistence of Mn NPV in loamy soil after artificial rainfall (precipitation 3 x 23 mm).

Depth of soil layer, cm	Intensity of DNA-DNA hybridization	Larval mortality ±SEM, %
0-5	+++	67.1± 3.0
5-10	+++	73.0± 2.6
10-15	++	12.1± 4.6

+ + virus conc. 10^5 - 10^6 polyhedra per 1 g, +++ more than 10^7 polyhedra per 1 g, in control no hybridization and larval mortality 1.8 % was observed.

The bioassay showed that after three applications of artificial rain the amount of viruses in tested soil was high, because third instar larvae of *M. neustria* got a sufficient dose of viruses by eating up 5-10 cm^2 of the leaves sprayed with soil filtrates. The results showed that most of the polyhedrae were near the soil surface.

The viruses which were kept in soil suspension maintained their activity for 1 year. In the variant with humus content 6% virus activity was significantly lower, than in the variants with humus content 3.0 % and 3.5 %. Probably the humus acids play some role in virus inactivation. This study shows that the Mn NPV Latvian isolate accumulates and survives in the soil for 1 year and maintains the ability to reproduce in larvae of *M. neustria.*

3.4.3. Persistence of NPV on plant surface in the environment

Experiments were performed to study the influence of environmental factors on virus viability after its application. The results of first experiment demonstrated that tested Mn NPV virus insecticide formulations secure the persistence of virus activity 22 days after spraying (Jankevica & Zarins, 1997).

In general, percentage of larval mortality of the *M. neustria* and *N. sertifer* larvae fed on the leaves or needles exposed 14 and 21 days was 2.5 to 3.5 times higher than that in the control (Figure 12). Efficiency of virus preparations containing different additives was 84 to 96% before, and 60 to 80 % and 15 to 51% after exposing the leaves in environment for 14 and 28 days, respectively. The calculated amount of polyhedrae that after spraying sticks to the treated apple tree leaves was 5000- 7000 polyhedrae/cm^2. The loss of polyhedrae during the first week determined by the specific DNA-DNA hybridization on apple leaves varied between 20 and 65% in variants with additives; in the control (virus in water suspension without additives) the loss was 81%. All

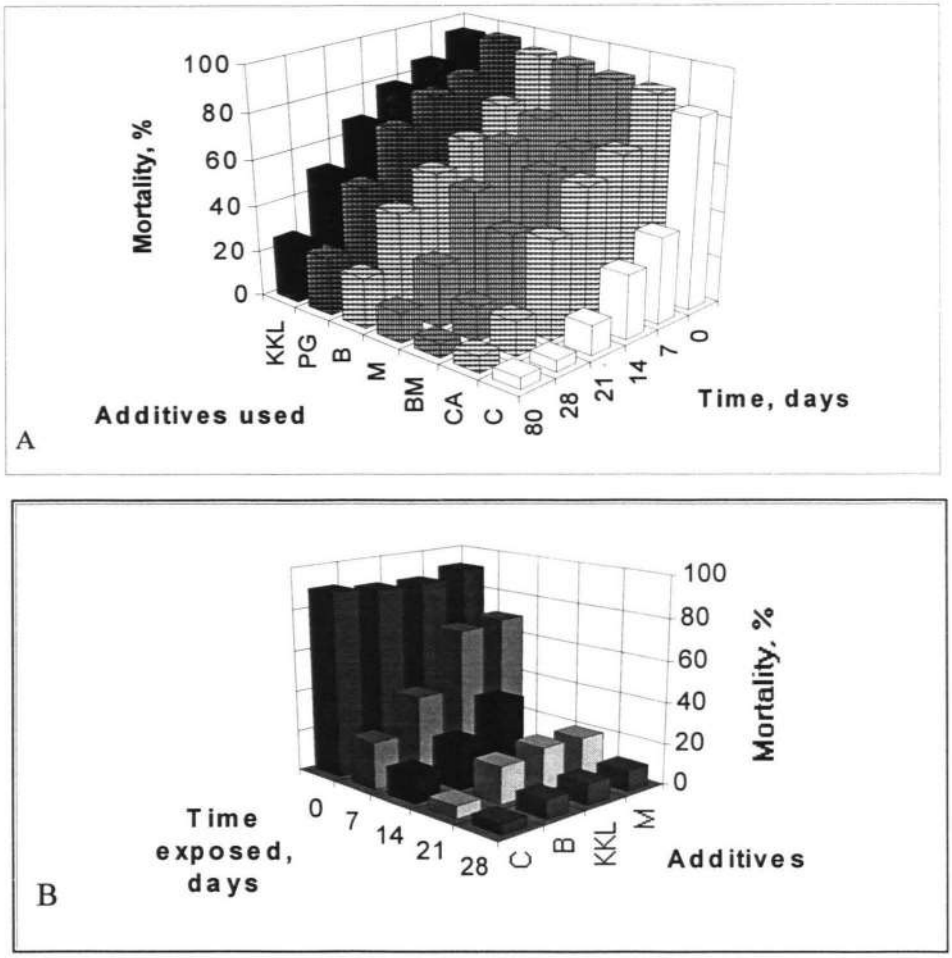

Figure 12. Efficiency of Mn NPV and Ns NPV virus preparations depending on additives used and exposure time in environment. A - Mn NPV; B- Ns NPV. PG- polyglucine, M- molasses of peat, KKL- lysine KKL, CA- by-product of citric acid production, BM - Belkosine M and B- bentonite, C- control- virus water suspension.

experiments showed, that the loss of polyhedrae in the control depends on the amount of precipitation per week. We determined that in the control 99.8% of polyhedrae were lost during 28 days. The use of the additives: lysine KKL, polyglucine, by-product of citric acid production and molasses of peat increased the persistence of the polyhedra on leaves 7, 6, 4, and 3 times, respectively.

Application of additives exerted the influence of environmental factors. This was similar to the results obtained in laboratory experiments with simulated rain, where tested additives exerted the influence of rainfall (Jankevica et al., 1998b). After 28 days exposure the amount of polyhedra - more than 240 polyhedrae on 1cm^2 leaf surface exceeded determined LD_{50} - 55 polyhedra/larvae for 2nd instar *M. neustria* larvae (Jankevica et al., 1998a) and is enough to infect insects in the population. Actively feeding 3rd and 4th instar larvae got a sufficient dose of viruses (LD_{50} of 985 polyhedrae, Jankevica et al, 1998a), when eating up 5-10 cm^2 of the leaves, treated and exposed for 28 days.

The results presented show that additives used retain Mn NPV and Ns NPV activity in viral preparations used to control pest populations.

3.5. Detection of virus DNA by the PCR

3.5. 1. Elaboration of sensitive method of virus DNA determination by the PCR

Microscopic examination of larval smears to ascertain the presence of virus polyhedral inclusion bodies is too labor intensive. We have elaborated a sensitive PCR-based method usable for detection the presence of viral DNA in the extracts of *M. neustria* larvae. Sequence analysis of the fragment (approximately-1000 bp) of polyhedrin gene of Mn NPV Latvian isolate was done and two sets of primers (20-mers) corresponding to polyhedrin gene were designed and synthesized.

The first set of primers (START1- 5' CGTTTATAAGTCATCGCCGC 3' and END1- 5' CACACTGGGCCGCACCTATG 3') amplified 985 bp, and the second set (START2 -5' CACACTGGGCCGCACCTATG 3' and END2 -5' GCGGGTCCCGTGTATAGAGG 3') amplified 708 bp of the polyhedrin gene. We used nested PCR to increase the sensitivity of determination (Figure 13).

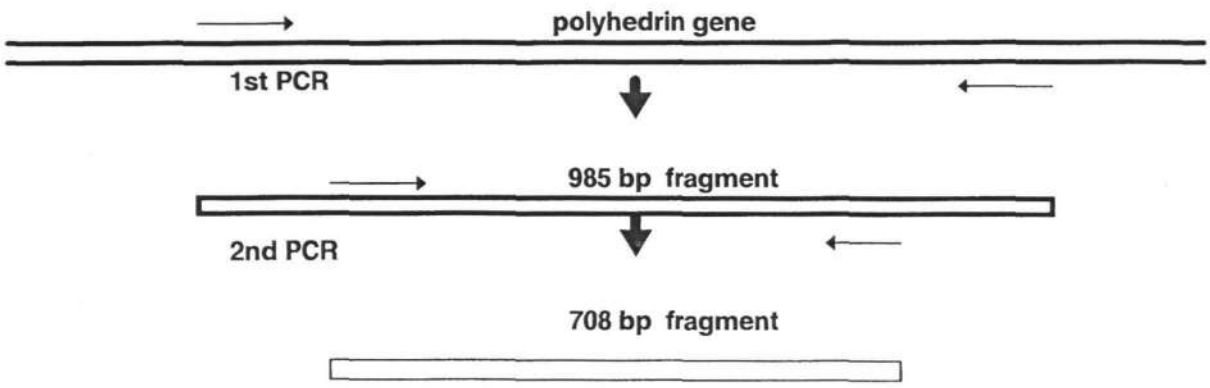

Figure 13. Optimized two step PCR procedure for detection of Mn NPV.

The elaborated method showed high sensitivity and specificity. 2nd PCR had a less unspecified amplifications, only amplifications of 708 bp fragment of polyhedrin gene are detectable. Designed primers amplified DNA of different Mn NPV isolates (Figure 14). No amplification were detected in tests with NPV, that are isolated from sawflies *N. sertifer* and belong to distinguish type of NPV with different histopathology.

No polyhedrin-specific sequences were detected in DNA of GVthat was used as negative controls.

The DNA extraction and PCR detection method described here was used to evaluate the survey and transmission of baculovirus infections in natural insect populations.

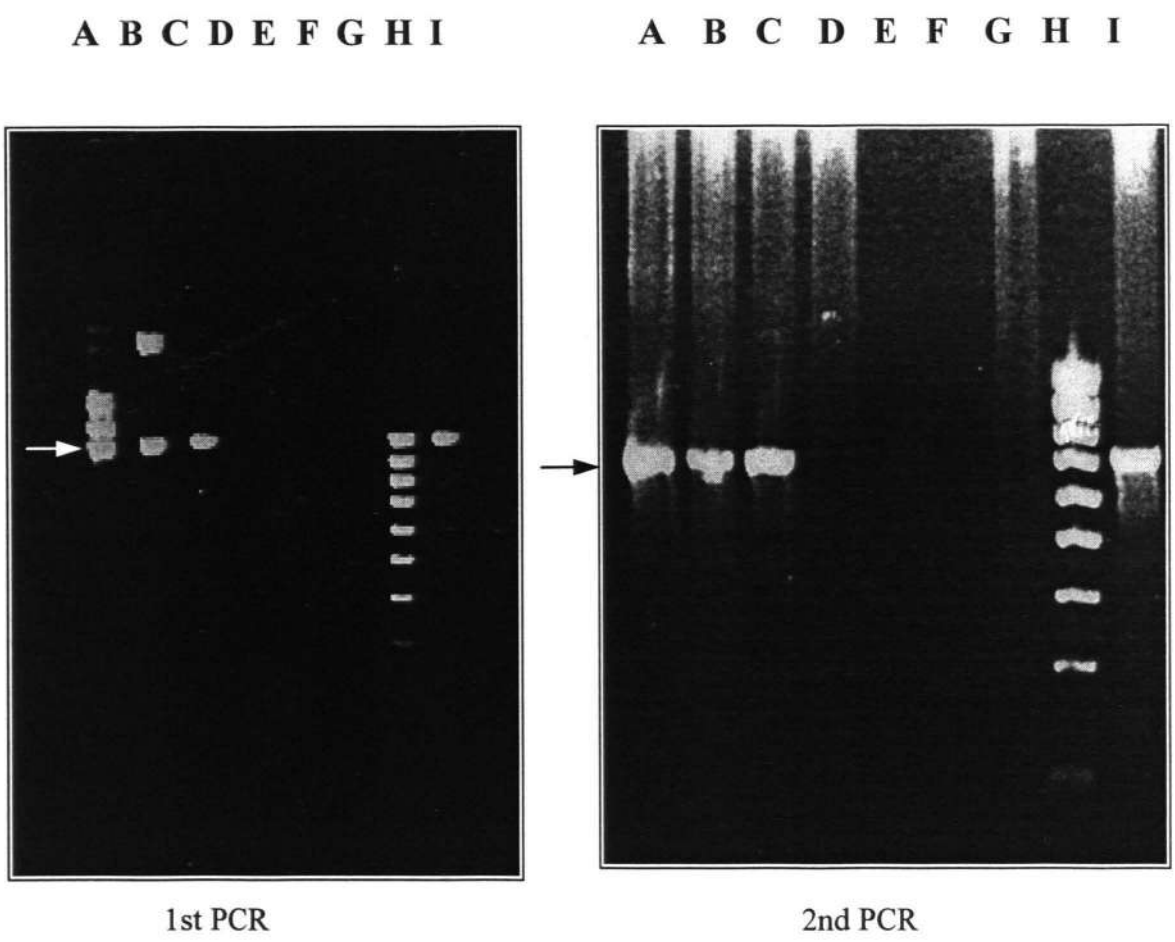

Figure 14. Determination of polyhedrin-specific DNA in different virus isolates. Electrophoresis of PCR products in 1% agarose gel. Arrows indicate 985 bp product in 1st PCR and 708 bp product in 2nd PCR.

A DNA isolated from Latvian isolate (Saldus) of Mn NPV
B DNA isolated from Armenian isolate of Mn NPV
C DNA isolated from Latvian isolate (Dobele) of Mn NPV
D DNA isolated from *Eriogaster lanestris* NPV
E DNA isolated from Latvian isolate of Ns NPV
F negative control DNA isolated from of Pb GV
G negative control distilled water
H 100 bp ladder
I positive control (plasmid containing polyhedrin gene)

3. 5. 2. Comparison of virus isolates by sequencing of PCR products

Our purpose was to compare different isolates of the *M. neustria* NPV. In collaboration with Laboratory of Genetic Engineering, Biomedical Research and Study Centre we analysed and compared the primare structure of the polyhedrin gene of Mn NPV isolated from diverse populations of *M. neustria.* Two native Latvian isolates and Armenian isolate were analysed and compared. Restriction analysis of viral DNAs showed differences between *M. neustria* NPVs Latvian isolates and Armenian isolate. Sequence analysis of a fragments of polyhedrin gene of two Mn NPV Latvian isolates (987 bp) and Armenian isolate (985 bp) was done. DNA fragments were cloned into pUC57T. Positive clones were sequenced using both standard primers as well as primers used for PCR.

The comparison of sequences of Latvian isolates (Saldus) and (Dobele) revealed a number of nucleotide exchanges. There were 5 exchanges in the coding region of polyhedrine gene, but only one of them leads to amino acid exchange in position 222. Leucine was substituted with phenylalanine.

After the comparison of polyhedrin gene DNA sequences of Armenian and Latvian isolate (Saldus), it was found that there are 1 nucleotide exchange in 5' non coding region, 8 exchanges in coding region of polyhedrin gene and 11 exchanges in 3' non coding region. These exchanges were sinonimic and did not cause any exchanges of amino acids in polyhedrin.

The comparison of sequences of Latvian isolates with polyhedrin genes publiced before were done (Figure 15).

- Analyzed sequence of Latvian isolate (Saldus) were comprised with sequence of Mn NPV polyhedrin gene published before by Sharipo (1991). It was found that there are no nucleotide exchange in 5' non coding region, 10 exchanges in coding region of polyhedrine gene and 4 exchanges in 3' non coding region. Detected exchanges cause 6 exchange of amino acids in polyhedrin.
- Sequence of Latvian isolate (Saldus) were comprised with sequence of Mn NPV polyhedrin gene found in EMBL genebank [X55658]. It was found that there are no nucleotide exchange in 5' non coding region, 4 exchanges and 3 insertions in coding region of polyhedrin gene and no exchanges in 3' non coding region. Detected exchanges cause deletion with following exchange of two amino acids in polyhedrin (Figure 16).

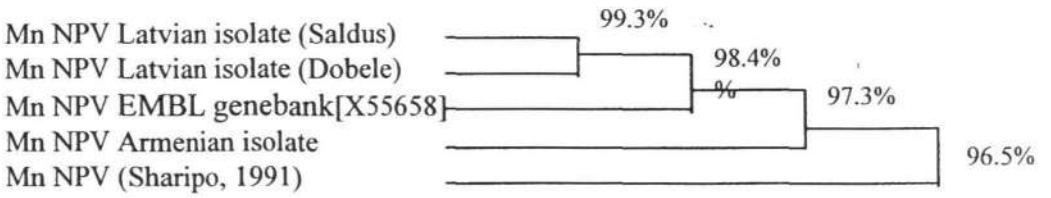

Figure 15. Phylogenetic tree of Mn NPV polyhedrin gene sequences.

```
                          10         20         30         40         50
ARMEN1.AMI          1 MYTRYSYNPT LGRTYVYDNK YYKNLGHVIK NAKRKKNAAE HELEERNLDP
DOBELE1.AMI         1 MYTRYSYNPT LGRTYVYDNK YYKNLGHVIK NAKRKKNAAE HELEERNLDP
EMBLX55A.AMI        1 MYTRYSYNPT LGRTYVYDNK YYKNLGHVIK NAKRKKNAAE HELEERNLDP
MNNPVPOL.AMI        1 MYTRYSYNPT LGRTYVYDNK YYKNLGHVNK NAKRKKNAAE HELEERNLDP
SALDUS1.AMI         1 MYTRYSYNPT LGRTYVYDNK YYKNLGHVIK NAKRKKNAAE HELEERNLDP
                             60         70         80         90        100
ARMEN1.AMI         51 LDKYLVAEDP FLGPGKNQKL TLFKEIRNVK PDTMKLIVNW SGKEFLRETW
DOBELE1.AMI        51 LDKYLVAEDP FLGPGKNQKL TLFKEIRNVK PDTMKLIVNW SGKEFLRETW
EMBLX55A.AMI       51 LDKYLVAEDP FLGPGKNQKL TLFKEIRNVK PDTMKLIVNW SGKEFLRETW
MNNPVPOL.AMI       51 LDRYLVAEDP FLGPGKNQKL TLFKEIRNVK PDTMKLIVNW SGKEFLRETW
SALDUS1.AMI        51 LDKYLVAEDP FLGPGKNQKL TLFKEIRNVK PDTMKLIVNW SGKEFLRETW
                            110        120        130        140        150
ARMEN1.AMI        101 TRFMEDSFPI VNDQEIMDVF LVVNMRPTKP NRCFRFLAQH ALRCDSDYVP
DOBELE1.AMI       101 TRFMEDSFPI VNDQEIMDVF LVVNMRPTKP NRCFRFLAQH ALRCDSDYVP
EMBLX55A.AMI      101 TRFMEDSFPI VNDQEIMDVF LVVNMRPTKP NRC-SVLAQH ALRCDSDYVP
MNNPVPOL.AMI      101 TRFREDSFPI VNDQEIMDVF LVVNMRPTEA DRCFRFLAQH ALRCDSDYVP
SALDUS1.AMI       101 TRFMEDSFPI VNDQEIMDVF LVVNMRPTKP NRCFRFLAQH ALRCDSDYVP
                            160        170        180        190        200
ARMEN1.AMI        151 HEVIRIVEPS YVGSNNEYRI SLGKRYNGCP VMNLHSEYTN SFEDFINRVI
DOBELE1.AMI       151 HEVIRIVEPS YVGSNNEYRI SLGKRYNGCP VMNLHSEYTN SFEDFINRVI
EMBLX55A.AMI      151 HEVIRIVKPS YVGSNNEYRI SLGKRYNGCP VMNLHSEYTN SFEDFINRVI
MNNPVPOL.AMI      151 HEVIRIVEPS YVGSNNEYRI SLGKRYNGCP VMNLHSEYTN SFEDFINRVI
SALDUS1.AMI       151 HEVIRIVEPS YVGSNNEYRI SLGKRYNGCP VMNLHSEYTN SFEDFINRVI
                            210        220        230        240        250
ARMEN1.AMI        201 WENFYKPLVY IGTDSAEEEE ILLEVSLVFK IKEFAPDAPL YTGPAY*...
DOBELE1.AMI       201 WENFYKPLVY IGTDSAEEEE IELEVSLVFK IKEFAPDAPL YTGPAY*...
EMBLX55A.AMI      201 WENFYKPLVY IGTDSAEEEE ILLEVSLVFK IKEFAPDAPL YTGPAY*...
MNNPVPOL.AMI      201 WENFYKPLVY IGTDSAEEEE ILLEVSLVFK IKEFAPDAPL YTGPAY*...
SALDUS1.AMI       201 WENFYKPLVY IGTDSAEEEE ILLEVSLVFK IKEFAPDAPL YTGPAY*...
```

Figure 16. Alignment of the analysed polyhedrine sequences. Mismatched amino acids are framed.

ARMEN1.ami - Mn NPV Armenian isolate
DOBELE1.ami -Mn NPV Latvian isolate (Dobele)
EMBLX55A.ami - Mn NPV polyhedrine found in EMBL genebank [X55658]
MNNPVPOL.ami - Mn NPV isolate sequenced by Sharipo (1991)
SALDUS1.ami - Mn NPV Latvian isolate (Saldus)

Obtained results demonstrate that characterised Latvian isolates has a high DNA homology -99.3%.

3.5.3. Detection of virus DNA in virus-infected insects

Using PCR analysis, we determined Mn NPV in all instar *per os* infected *M. neustria* larvae. The developed and optimized method showed the presence of Mn NPV in second and third instar larvae just a day after treatment (laboratory and field experiments) with the virus preparation. The virus yield and amount of viral DNA in virus-infected insects increase from 1st to 7th day post infection (Figure 17). Results obtained with PCR analysis are similar to results obtained by light microscopy.

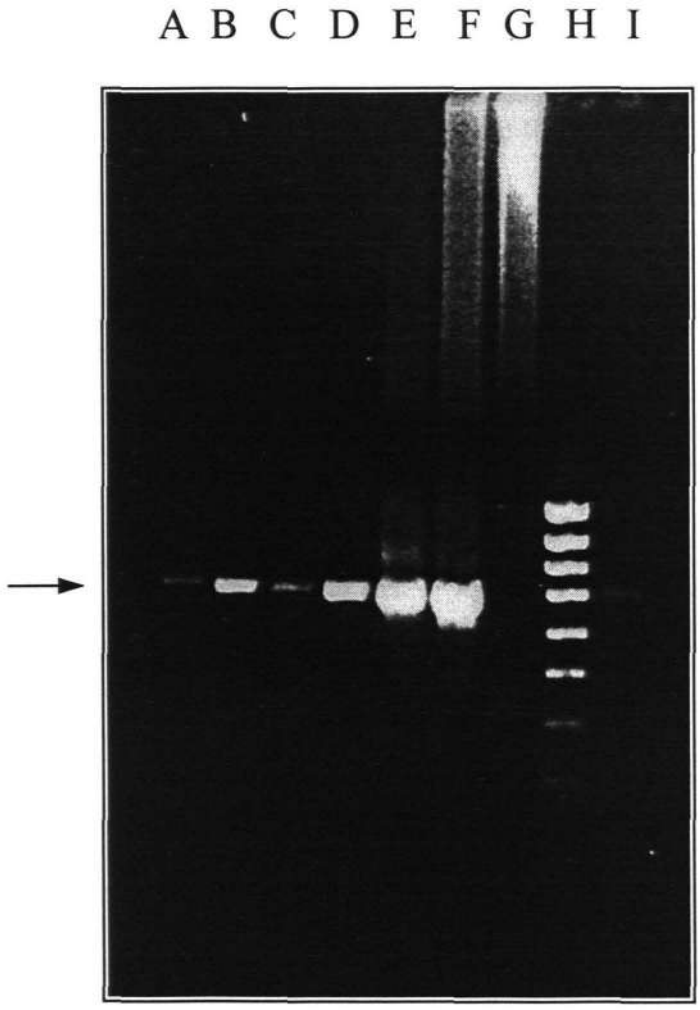

Figure 17. Presence of NPV DNA in virus-infected insects at different moments of post infection. Electrophoresis of 2nd PCR products in 1% agarose gel. Arrow indicated 708 bp product.

A DNA isolated from *Malacosoma neustria* (1 day post infection)
B DNA isolated from *M. neustria* (2 day post infection)
C DNA isolated from *M. neustria* (4 day post infection)
D DNA isolated from *M. neustria* (5 day post infection)
E DNA isolated from *M. neustria* (7 day post infection)
F positive control (plasmid containing polyhedrin gene)
G negative control distilled water
H 100 bp ladder
I DNA isolated from *M. neustria* larvae

Presence of Mn NPV was determined also in the second generation of treated *M. neustria*.

3.5.4. Presence of baculovirus infections in followed generations of virus treated insects

Virus disease and mortality of pests was observed also in following generations (F_1, F_2) of *M. neustria* and *P. brassicae* after virus application (Table 10). Survival of insects in F_1 generation was 35.2- 58.7%, in F_2 generation 41.6 % (in untreated population 85.3 -91.7%). In comparison with an untreated population introduced baculoviruses increased the mortality of insects in different stages of their development and diminished the pest population density. Comparative loss of the fecundity, average egg production and egg hatchability was relatively higher in F_1 generation.

Table 10. Insect mortality from the polyhedrosis and granulosis in different stages of their development after application of virus preparation.

Virus	Host insect	Generation	Mortality of insects in different stages of their development, %			
			Egg	Larvae	Pupae	Imago
NPV	*M. neustria*	S_1	-	38.0	5.3	9.9
		F_1	12.5	15.3	9.0	4.5
Control (Without virus)		F_1	4.0	4.0	1.3	0
GV	*P. brassicae*	S_1	-	30.3	18.3	2.8
		F_1	16.2	44.6	9.7	4.3
		F_2	12.7	20.7	16.5	8.5
Control (Without virus)		F_1	2.5	5.0	4.7	2.5
		F_2	3.0	4.5	4.0	2.0

S_1 - treated generation, $F_{1,2}$ - filial generation

Investigations in a forest ecosystem (Talsi's district) with the European pine sawfly *N. sertifer* population (continuation of monitoring of pest population) show that the larval population was slightly reduced in the virus-treated plot in the second year. Population density in the untreated (control) plot was 6 times higher. Larval mortality began earlier than in previous year and reached 72.6%.
The results of this work demonstrate that Mn NPV, Ns NPV and Pb GV can reduce the amount of pest insects during the following years after treatment.

3.5.5. Presence of NPV in untreated *M. neustria* populations

In Jelgava's district a population of *M. neustria* was observed in a well producing apple-garden where no insecticides had been used before. We determined that 27 % of *M. neustria* collected there were infested by the parasitic flies (*Tachinidae*), 11 % by cocoon parasites. Approximately 10 % of the larvae were infected by *M. neustria* NPV (*Baculoviridae*) and 6% by microsporidia (*Microsporidia*). Mixed infections were found in 9 % of the dead larvae.

In second year nested PCR analysis showed the presence of Mn NPV in approximately 21 % of the 2nd and 4th instar larvae collected in the plots in Jelgava's district. Results obtained by microscopy showed that 10 % of examined larvae were infected with Mn NPV. Comparison of the results obtained by both methods showed that the new method was most sensitive.

CONCLUSIONS

- After morphological and cytological studies (light and electron microscopy) it was concluded that Latvian isolates of Mn NPV are multiple NPV. Latvian populations of baculoviruses are not homogenous, viral isolates had different virulence and pathogenesis.

- Investigations of the enhancing of virus disease showed that addition of amino acids, plant lecithin and substances physiological insects weakeners added to virus preparations increase larval mortality and fasten the infection process.

  Plant extracts from marigolds *(Calendula officinalis)*, French marigolds *(Tagetes patula)* and milfoil (*Achillea millefolium)* show antifeedant properties, suppress the feeding activity of the cabbage white butterflies' larvae. Mentioned plant extracts are not advisable for using in plant protection together with granulosis viruses, as in most cases they prolong development period of the virus disease.

  A synergistic relationship between a Cp GV and Mn NPV was observed. The addition of GV enhancing the virulence of Mn NPV.

- Sensitive method of virus diagnostic were elaborated. The method for the detection of polyhedrin-specific DNA sequences using PCR has been proved to have good possibilities for the determination of presence viruses in pest populations and can be a useful tool for the risk assessment of the releasing of baculoviruses in the environment.

- In our laboratory developed virus formulations strongly reduce the harmful effect of weather conditions on the persistence of Mn NPV and Ns NPV viral activity. They retain persistence of Mn NPV and Ns NPV on plants after the rainfall. The best efficiency was shown by the formulations with lysine KKL and polyglucine.

- After initial spraying high amount of virulent polyhedra accumulate and persist in soil. Artificial rain transferred Mn NPV and NsNPV into soil, virulent Mn NPV viruses leached through 25 cm of soil.

- The results of this work demonstrate that Mn NPV and Ns NPV can reduce the amount of host pest during the following years after treatment. Virus persist within the pest population after the treatment of the virus preparation.

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LIST OF PUBLICATIONS ON WHICH THESIS IS BASED

1. Čudare Z., Jankevica L. 1996. Augu ekstraktvielu un granulozes vīrusa izmantošana kāpostu balteņa *Pieris brassicae* L. apkarošanā. *Latvijas Entomologs*, 35, 28-34.
2. Čudare Z., Jankevica L. 1997. The role of granulosis viruses and active plant extracts in regulation of pest population. *Proceedings of the Latvian Academy of Sciences*, 51, 3/4, 170-176.
3. Jankevica L., Zarins I. 1997. Biological control of *Malacosoma neustria* L. population with Latvian isolate of nuclear polyhedrosis virus. In: Proceedings of International Symposium "*Microbial Insecticides: Novelty or Necesity?*", UK, 284-288.
4. Čudare Z., Jankevica L. 1998. Accumulation and vertical migration of *Malacosoma neustria* nuclear polyhedrosis virus in soil. *IOBC Bulletin*, Vol.21(4), 245-248.
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18. Jankevica L., Čudare Z., Jankevics E. 1998. Use of PCR in detection of *Malacosoma neustria* nuclear polyhedrosis virus in pest populations after virus application. In: VIIth International Colloquium on Invertebrate Pathology and Microbial Control, Sapporo, August 23-28, 62.

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21. Zarins I., Jankevica L. 1998. Efficiency of *Pieris rapae* granuloses viruses isolated in China under environmental conditions of Baltic region. In: International Conference of Integrated Pest Management (IPM) - theory and practice, developing sustainable agriculture, Guangzhou, China, June 15-20, 255.

22. Jankevica L. 1999. Persistence and accumulation of *Malacosoma neustria* nuclear polyhedrosis virus in the ecosystem and pest population after virus application. In: 7th Europeann Meeting in the IOBC/WPRS Working Group: Capturing the Potential of Biological Control, Abstracts, Vienna, Austria, March 22-26.

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Čudare Z., Jankevica L. 1996. Augu ekstraktvielu un granulozes vīrusa izmantošana kāpostu balteņa** Pieris brassicae L. **apkarošanā. Latvijas Entomologs. 35, 28-34.

Augu ekstraktvielu un granulozes vīrusa izmantošana kāpostu baltena (*Pieris brassicae* L.) apkarošanā

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Abstract

Z. Čudare, L. Jankevica 1996. Control of cabbage white butterfly *Pieris brassicae* L. by active plant extracts and granulosis virus. - Latv. Entomol., 35: 9-16.

The aim of the present study was to investigate the effect of *Pieris brassicae* granulosis virus (PbGV) and active plant extracts on the larvae of the cabbage white butterfly (*Pieris brassicae*). Antifeedant characteristics of extracts from milfoil (*Achillea millefolium*). French marigolds (*Tagetes patula*) and marigolds (*Calendula officinalis*) was found with the mortality of *P. brassicae* larvae of 12.0%, 46.1% and 55.9%, respectively. Tomato (*Lycopersicon esculentum*) leaf broth extract caused 25% mortality of larvae at the third day after spraying and showed toxic activity. Inspected substances affected viral pathogenicity negatively by diminishing the feeding activity of insects. In natural populations of *P. brassicae* sprayed with granulosis virus suspension 82.3% of the mortality of larvae was caused by granulosis viruses, 9,9 % of that - by bacteriosis, 0,3 % - by mycosis and the rest - by parasitic insects.

Key words: active plant extracts, granulosis virus, *Pieris brassicae* .

Ievads

Augu kaitēkļu savairošanās procesam raksturīga populācijas blīvuma palielināšanās jeb progradācija līdz maksimālam līmenim ar sekojošu blīvuma samazināšanos. Augu aizsardzībā galvenā uzmanība tiek veltīta progradācijas perioda aizkavēšanai vai likvidēšanai, jo šajā periodā augu bojājumi ir vislielākie. Augu kaitēkļu populācijas blīvumu un struktūru ietekmē abiotiskie un biotiskie (konkurence, sugu mijiedarbība u. c.) faktori. Faktoru iedarbība dažādās kombinācijās un intensitātē var sekmēt kaitēkļu savairošanos vai samazināt to. Viens no paņēmieniem kā aizkavēt kaitēkļu savairošanos ir ķīmisko un bioloģisko augu aizsardzības līdzekļu izmantošana.

Mūsu gadsimta 80-tajos gados pastiprinājās tendence izmantot augu aizsardzībā vielas, ko iegūst no augiem (Agraval, Mall, 1988; Banerji et al., 1985; Barnerjee, Choudhuri, 1986; Brauer, Devkota, 1990). Šīm vielām piemīt dažādas īpašības, jo savā evolūcijas gaitā augi radījuši visdažādākos pašaizsardzības mehānismus. Dažas vielas

darbojas kā insekticīdi. Dažos augos ir vielas ar hormonālu iedarbību uz kukaiņiem. Dažas vielas - antifidanti inhibē kukaiņu barošanos. Pie pēdējām pieder alkaloīdi, alkaloīdglikozīdi u. c. Tomatīnam, nikotīnam, taniniem piemīt kā antifidantas, tā toksiskas īpašības (Swinger, 1986).

Vielu īpašības nosaka to izmantošanu augu aizsardzībā. Piemēram, eļļu, ko iegūst no auga *Calophyllus inophyllus* var izmantot kā insekticīdu (Banerji et al., 1985).

Ekstraktus gatavo no augu lapām, augļiem, saknēm un sīpoliem (Luthria et al, 1989; Verma et al., 1986). Izmanto gan svaigus, gan žāvētus augus, nosaka to ķīmisko sastāvu un nepieciešamo koncentrāciju. Ļoti bieži ekstraktu iegūšanai un izpētei izmanto tropiskos augus. Pētīta auga *C. inophyllus* sēklu ekstrakta insekticīdā un antifidantā iedarbība uz tauriņu *Diacrisia obligue* kāpuriem (Agrawal, Mall, 1988); auga *Ocimus sanotus* ekstrakta insekticīdā iedarbība uz laputīm (Stein, Klingav , 1980), augu *Derris* spp. un *Quassia* spp. ekstraktu - uz lapgraužu kāpuriem (Stoll, 1986). Vielu iedarbība un koncentrācija atkarīga no kukaiņu attīstības stadijas. Tikko izšķīlušies tauriņa *Spodoptera litura* kāpuri nobeidzas, uzņemot 0,0001 % alkaloīda šķīdumu, kas ekstraģēti no auga *Tylophora astahanitica*. Turpretīm V vecuma kāpuri uzņemot 0,01-0,0001% alkaloīda šķīdumu nenobeidzas. Ja apmiglotiem kāpuriem pēc 48 stundām ļauj baroties ar neapmiglotu barību, tad zūd aizkavētā augšana, kas radusies alkoloīdu ietekmes rezultātā un tie attīstās normāli (Verma et al., 1986). Ekstraktu toksiskums atkarīgs arī no tā, kāda auga daļa izmantota ekstrakta iegūšanai un kāda ekstraģējošā viela izmantota. Visbiežāk ekstraģēšanai izmanto ūdeni, spirtu vai acetonu. Ekstrakts no auga *Melia azedarch* sēklām 1% koncentrācijā inhibē *Mythimna separata* I un II vecuma kāpuru barošanos un, sasniedzot IV vecumu, tie nobeidzas. Ļoti augsts toksiskums raksturīgs tazenolamīnam, ko iegūst no auga *M. toosendam* mizas (Shin-Foon, 1980). Heksānu un etanolu ekstrakti, kas iegūti no 10 dažādiem augiem, pielietoti dažādās koncentrācijās, izraisīja tauriņa *Spodoptera frugiperda* kāpuru nobeigšanos, aizkavēja to augšanu un iekūņošanos. Ekstrakti, kas iegūti izmantojot etanolu, sevišķi no auga *Aglaia cordata* iegūtie, izrādījās efektīvāki, nekā izmantojot citus extraģentus (Mikalajczak at al., 1989). Kāļu balteņa *Pieris napi* kāpuru augšanu aizkavē ekstrakti, kas iegūti ekstraģējot auga *Tephorosa vogellii* lapas ar acetonu. Pielietojot šos ekstraktus, nobeidzās līdz 89% balteņa kāpuru, dzīvotspēju zaudēja 98,2% olu. Ekstrakts efektīvs arī pret kāpostu pūcītes *Barathra brassicae* kāpuriem (Shin-Foon, 1980).

Ļoti plašus pētījumus par aktīvo augu ekstraktvielu izmantošanu veic Indijā (Lutria et al., 1989; Raghunatha Rao et al., 1988). Luknovas nacionālajā botānikas institūtā publicēts saraksts, kurā minēta 51 augu suga, piederoša pie 28 dzimtām, kam piemīt insekticīdas vai repelentu īpašības (Banerji et al., 1985). Augos ietilpstošo vielu analogi tiek sintezēti arī laboratorijās. Augu aizsardzībā plaši izmanto piretroīdus un retroīdus (Raghunatha Rao et al., 1988).

Lai sekmīgi izmantotu aktīvās augu vielas augu aizsardzībā, ļoti svarīgi ir noteikt augu ekstraktvielu iedarbības mehānismu. Viens no drošākajiem paņēmieniem vielu iedarbības noteikšanai ir citoloģiskā metode. Ja termīti *Reticulitermes seperatus* barojas ar auga *Phellodendronus amurense* mizas ekstraktu tie pārtauc barošanos un nomirst badā. Ekstrakts darbojas kā antifidants (Kawaguchi et al., 1989).

Atsevišķos gadījumos no augiem iegūtās vielas var izmantot augu aizsardzībā kopā ar mikroorganismiem, piemēram, bakulovīrusiem. Sojas, kokvilnas, riekstu eļļas, niedru cukurs darbojas kā pievilinātāji vai barošanās stimulatori. Šīm vielām nepiemīt insekticīdas vai antifidantas īpašības (Filho, Nilson, 1987).

Arī Latvijā pēdējos gados bieži rekomendē augu aizsardzībā pret kaitēkļiem izmantot no augiem iegūtās vielas - ekstraktus. Lai izstrādātu precīzas augu ekstraktvielu izmantošanas metodes, jānoskaidro pie kādas grupas pieder darbīgā viela, jāzin tās iedarbības spektrs un iedarbības mehānisms. Mūsu eksperimentu mērķis bija noskaidrot atsevišķu augu ekstraktu iedarbību uz kāpostu balteņa kāpuriem un iespējas ekstraktus pielietot kopā ar granulozes vīrusu.

Materiāls un metodika

Eksperimentos izmantoti kāpostu balteņu (*Pieris brassicae*) I - IV vecuma kāpuri, *P. brassicae* granulozes vīrusa (PbGV) suspensija ar titru $2x10^7$ granulas/ml (vīruss izdalīts LLU Augu un kukaiņu vīrusslimību problēmu laboratorijā), kliņģerīšu, tomātu lapu, pelašķu un samteņu ekstrakti.

Ekstraktu sagatavošana. Kliņģerīšu (*Calendula officinalis* L.) izvilkumam sasmalcināja 80 g zaļo augu, pārlēja ar 1 l ūdens, uzglabāja 3 dienas, tad filtrēja. Tomātu (*Lycopersicon esculentum* Mill.) lapu novārījumam 400 g svaigas tomātu lapas vārīja 1 l ūdens 30 minūtes, tad filtrēja, filtrātu atšķaidīja ar ūdeni attiecībā 1 : 3. Pelašķu (*Achillea millefolium* L.) izvilkumam 80 g kaltētas augu virszemes daļas pārlēja ar 1 l ūdens, uzglabāja 6 dienas, tad filtrēja. Samteņu (*Tagetes patula* L.) izvilkumam 80 g kaltētas augu virszemes daļas pārlēja ar 1 l ūdens, glabāja 2 dienas, tad filtrēja.

Laboratorijas eksperimentā kāpostu lapas vispirms migloja ar PbGV ūdens suspensiju (titrs $1x10^7$ granulu/ml), tad nožāvēja un izbaroja kāpuriem. Pirmā vecuma kāpurus ievāca dabā, vēlāk audzēja izolatoros. Atkārtojumu skaits eksperimentā bija 3, kāpuru skaits atkārtojumos 30. Kontrolē kāpostu lapas apmigloja ar ūdeni.

Lauka izmēģinājumos Bioloģijas institūta teritorijā iekārtoja 10 variantus. Katrā variantā izmantoja 15 kāpostu stādus (t. i. 5 stādi 3 rindās). Attālums starp stādiem 45 cm. Starp variantiem, lai izslēgtu vīrusu izplatību, bija 3 rindas ar citiem augiem (attālums starp rindām 50 cm). Kāpostu stādus apmigloja galviņu veidošanās stadijā, nenolasot kāpostu balteņa kāpurus. Uz augiem bija I-IV vecuma kāpuri. Miglošanai izmantots rokas miglotājs.

Lai noskaidrotu, kā augu ekstrakti ietekmē kāpurus, augus apmigloja ar iepriekš minētajiem augu ekstraktiem (4 varianti). Lai noskaidrotu, kā vīrusslimības attīstību ietekmē augu ekstrakti, tiem pievienoja granulozes vīrusus (4 varianti). Vīrusu koncentrācija darba šķīdumā bija $2x10^7$ granulu/ml. Vīrusa aktivitātes noteikšanai kāpostu stādus apmigloja ar PbGV ūdens suspensiju (vīrusa koncentrācija $2x10^7$ granulu/ml). Kontrolē augus migloja ar ūdeni.

Kāpostu balteņu kāpuru mirstības kontroli uzsāka 48 stundas pec auga apmiglošanas un veica katru otro dienu. Lauka izmēģinājumu laikā vidējā diennakts temperatūra bija 9-13°C, relatīvais gaisa mitrums bija 80-86 %.

Iedarbības mehānismu noskaidrošanai, pagatavoja kāpuru citoloģiskos preparātus. Citoloģiskiem pētījumiem kāpurus ar raksturīgam saslimšanas pazīmēm fiksēja Šafera, Karnuā fiksatoros vai 10% formalīnā, tad tos krāsoja, izmantojot Romanovska metodiku, un sudānā III, IV (Lilli, 1969). Citoloģiskie preparati pētīti, izmantojot gaismas mikroskopu Amplival.

Aprēķināts koriģētās mirstības procents un noteikts vidējais mirstības laiks LT_{50} (Lipa, Sližynski, 1973).

Rezultāti

Laboratorijas eksperimentos ar PbGV ūdens suspensiju apmiglotās kāpostu lapas izbaroja III vecuma kāpuriem. Kāpostu balteņu kāpuru mirstība pēc 15 dienām bija 48.3%, kontrolē - 5,7%. Jāpiezīmē, ka daļa kāpuru (56,4%), kas tika uzlikti uz apmiglotām kāpostu lapām, 24 stundas nebarojās un uzturējās uz izolatoru sienām. Visos laboratorijas izmēģinājuma variantos augstākā mirstība novērota kāpostu balteņu IV vecuma kāpuriem: attiecīgi 42,5% un 79,3%. Tikai daļa kāpuru - attiecīgi 29,3% un 6,9% izdzīvoja un sasniedza piekto vecumu. Saslimšanu ar mikozi vai bakteriozi vizuāli konstatēja 6,2% kāpuru.

Lauka izmēģinājumos kāpostu balteņu mirstība pēc apmiglošanas ar PbGV ūdens suspensiju sasniedza 68.8% 18 dienu laikā. LT_{50} PbGV ūdens suspensijai bija 16 dienas. Pielietojot augu ekstraktus, kāpostu balteņa kāpuru mirstība bija 12-87%, pielietojot augu ekstraktus kopā ar PbGV - 51-90% (1. tabula).

1. tabula

Augu ekstraktu un vīrusa suspensiju efektivitāte un iedarbības laiks, apkarojot P. brassicae kāpurus

Varianti	Kāpuru mirstība pēc 9 dienām, %	Kāpuru mirstība pēc 18 dienām, %	LT_{50}	sLT_{50}
Pelašķu izvilkums	10.0± 2.1	18.0± 3.0	*	-
Kliņģerīšu izvilkums	5.0± 2.4	55.9± 4.4	15.7	0.4
Samteņu izvilkums	37.0± 3.3	46.1± 3.5	*	-
Tomātu lapu novārījums	51.1± 4.9	87.2± 2.1	8.9	0.7
Pelašķu izvilkums + PbGV ūdens suspensija	25.0± 3.5	54.0± 3.7	18.4	1.5
Kliņģerīšu izvilkums + PbGV ūdens suspensija	20.0± 3.8	78.2± 4.6	13.3	1.6
Samteņu izvilkums + PbGV ūdens suspensija	24.6± 3.8	51.1± 3.3	22.4	1.2
Tomātu lapu novārījums + PbGV	53.0± 4.7	90.0± 1.7	9.2	0.6
PbGV ūdens suspensija	25.0± 2.2	68.8± 4.7	16.0	0.9
Kontrole - ūdens	1.2± 1.0	6.9± 2.3	*	-

* - variantos 50% mirstība netika novērota

Rezultātu analīze

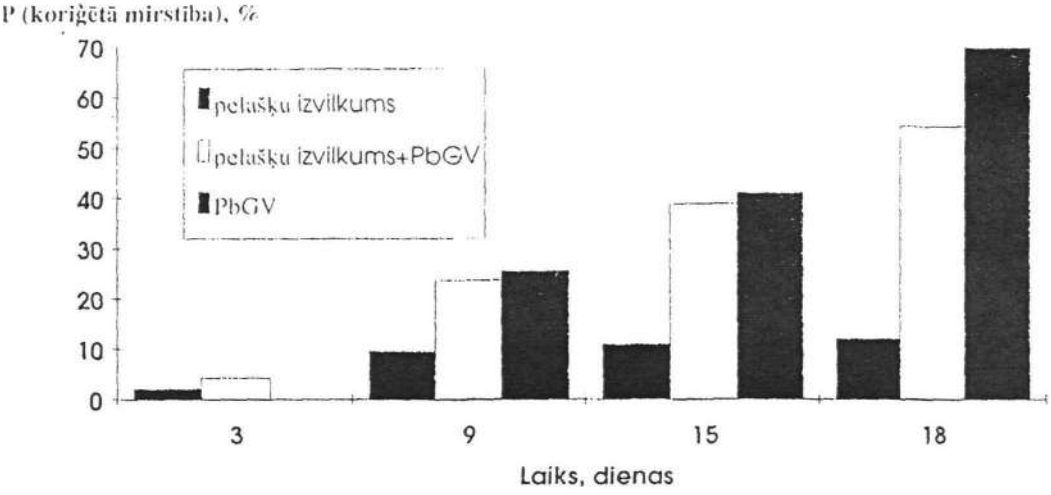
Laboratorijas eksperimentos ar PbGV ūdens suspensiju apmiglotu kāpuru mirstība bija par 20-27% zemāka neka pielietojot laboratorijā izstrādatās vīrusa preparatīvās formas, kas satur pievilinātājus un pielipinātājus (Zariņš, Eglīte, 1993).

Kliņģerīšu izvilkums pēc 9 dienām izraisīja ļoti zemu kāpostu balteņu mirstību, pēc 15 dienām mirstība pieauga. Tas izskaidrojams ar kāpuru novājināšanos pēc badošanās un uzņemības pret slimībām palielināšanos. Izvilkums, kam pievienoja PbGV, izraisīja augstāku mirstību.

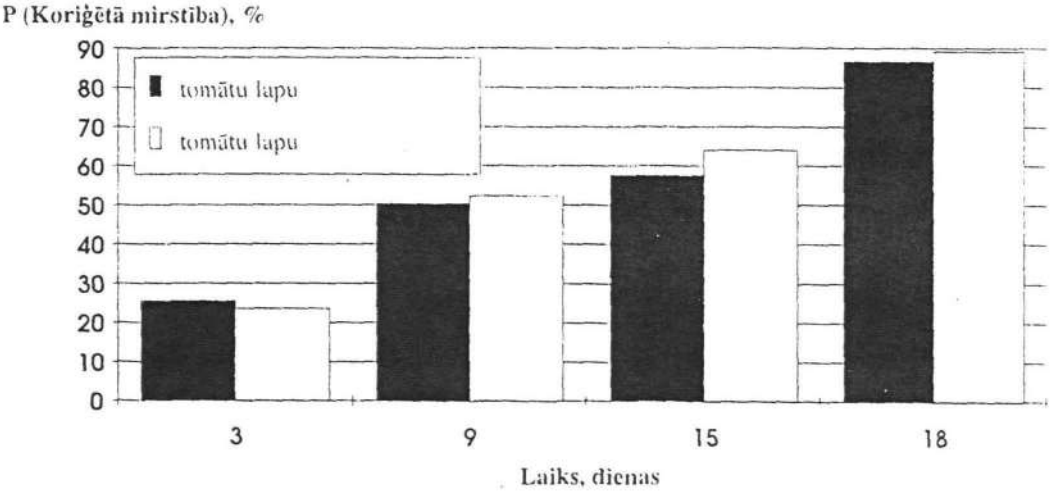
Izmantojot pelašķu izvilkumu kāpuru mirstība bija ļoti zema. PbGV pievienošana izvilkumam būtiski paaugstināja mirstību (1. attēls). Pelašķu izvilkuma, kam pievienots PbGV, izraisītā kāpuru mirstība un PbGV ūdens suspensijas izraisītā kāpuru mirstība 15 dienas pēc pielietošanas būtiski neatšķīrās. Tas norāda, ka pelašķu izvilkums nesamazina vīrusa aktivitāti, bet tikai paildzina vīrusslimības attīstības periodu. Vizuālie novērojumi parādīja, ka pelašķu izvilkumam piemīt antifidantas īpašības. Samteņu izvilkums iedarbojās kā antifidants. Kāpuri uzsāka baroties pēc 1-3 dienu badošanās perioda. Kāpuru mirstība nesasniedza 50%. PbGV pievienošana mirstību pirmās 15 dienas būtiski nepalielināja, pēc tam vīrusa izsauktā mirstība palielinājās.

Izmantojot kāpostu apmiglošanai tomātu lapu novārījumu, 3. dienā novērota augsta - 25,7% kāpuru mirstība (2. attēls). Tas izskaidrojams ar tomātu novārījuma toksisko iedarbību, rezultāti saskan ar literatūras datiem (Swinger, 1986) par tomatīna iedarbību. Veicot citoloģisko analīzi, konstatēts, ka 70% ar tomātu lapu novārījumu apmigloto kāpuru bija inficēti ar granulozes vīrusu. Tas norāda, ka tomatu lapu novārījums aktivizē latento vīrusu kāpostu balteņa kāpuros. PbGV pievienošana tomātu novārījumam nepalielināja kāpuru mirstību pirmajās 3 dienās. Zināms, vīrusslimības attīstības process ilgst 6-12 dienas (Zariņš, Eglīte, 1993). Pēc 9 dienām vīrusa izraisītā mirstība pieauga un 18. dienā pārsniedza mirstību, ko izraisīja PbGV ūdens suspensija. Iegūtie rezultāti parāda, ka tomātu lapu novārījuma toksiskā iedarbība, summējoties ar vīrusa granulozes vīrusa aktivitāti, palielina kāpuru mirstību. LT_{50} tomātu lapu novārījumam bija 8,9 dienas, bet granulozes vīrusa suspensijai kopā ar tomātu lapu novārījumu - 9,2 dienas.

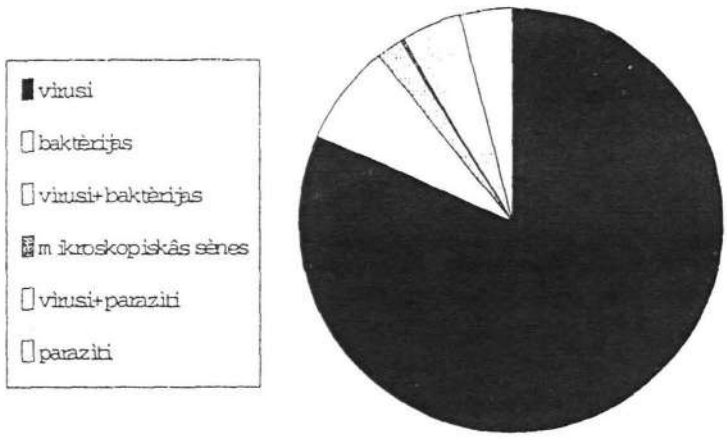
Mikroskopiski pārbaudīti 667 mirušie kāpuri, kas bija apmigloti ar PbGV ūdens suspensiju vai augu ekstraktu, kam pievienots PbGV. Pētījumi parādīja, ka 82,5±1,5% kāpuru miruši tikai no vīrusu infekcijas, 9,9±1,2% kāpuru konstatētas baktērijas, 0,3±0,2% - mikroskopiskās sēnes, 8,5±1,0% kāpuru bija parazitēti (3. attēls). Citoloģiskajos griezumos 7,7±0,9% gadījumu konstatētas dažādas formas un lieluma baktērijas un vīrusu granulas, 2,1±0,5% gadījumu konstatētas tikai baktērijas, sēņu hifas - samērā reti. Patoloģiskais process zarnu traktā un konstatētie patogēni parādīja, ka dabiskajās kaitēkļu populācijās kāpuru mirstības iemesls bieži bija jauktā infekcija.



1. attēls. *P. brassicae* kāpuru mirstības dinamika pēc apmiglošanas ar pelašķu izvilkumu, ar pelašķu izvilkumu, kam pievienots *P. brassicae* granulozes vīruss (PbGV), un ar PbGV ūdens suspensiju. Granulozes vīrusa titrs darba šķīdumā - $2x10^7$ granulas/ml.



2. attēls. *P. brassicae* kāpuru mirstības dinamika pēc apmiglošanas ar tomātu lapu novārījumu un ar tomātu lapu novārījumu, kam pievienots *P.brassicae* granulozes vīruss (PbGV). Granulozes vīrusa titrs darba šķīdumā - $2x10^7$ granulas/ml.



3. attēls. Patogēno mikroorganismu un parazītu sastopamība ar PbGV suspensiju apmiglotos un vēlāk mirušos *P. brassicae* kāpuros.

Secinājumi

1. Pārbaudītie augu ekstrakti - klinģerīšu, samteņu un pelašķu izvilkumi nomāc kāpostu balteņa kāpuru barošanos un 18 dienās izsauc attiecīgi 55,9%, 46,1% un 12% lielu to mirstību. Tomātu lapu novārījums ir izteikti toksisks kāpostu baltenim, tas izraisa 25% mirstību jau 3. dienā pēc miglošanas.

2. Pārbaudītie augu ekstrakti samazina kāpuru inficēšanos ar granulozes vīrusu, jo samazina kāpostu balteņu kāpuru barošanās aktivitāti. Minētie augu izvilkumi nav ieteicami lietošanai augu aizsardzībā kopā ar granulozes vīrusiem, jo vairumā gadījumu tie inhibē vīrusinfekciju.

3. Kāpostu balteņa kāpuros, kas lauka izmēģinājumos tika apmigloti ar granulozes vīrusa suspensijām, sastopamas jauktās infekcijas. Baktērijas konstatētas 9,9% gadījumu, parazīti - 8,5% , mikroskopiskās sēnes - 0,3% gadījumu.

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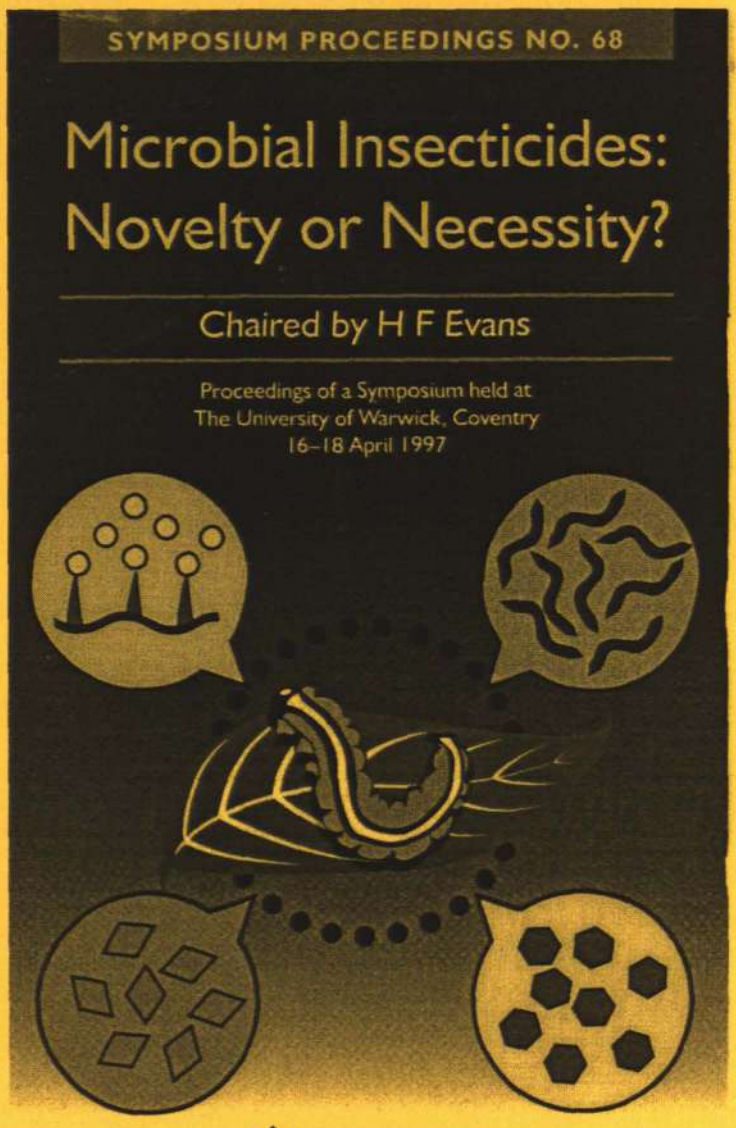

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THE ROLE OF ACTIVE PLANT EXTRACTS AND GRANULOSIS VIRUSES IN REGULATION OF PEST POPULATIONS

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The aim of the present study was to investigate the effect of Pieris brassicae *granulosis virus (PbGV), active plant extracts, and mixed formulations on the larvae of the cabbage white butterfly* Pieris brassicae. *Antifeedant extracts from marigolds (*Calendula officinalis*), French marigolds (*Tagetes patula*), and milfoil (*Achillea millefolium*) were tested. Tomato (*Lycopersicon esculentum*) leaf broth caused 25% mortality of larvae three days after spraying. The use of active plant extracts, together with the granulosis virus, against the pest was investigated. The action mechanism of granulosis virus and the effects of the plant extracts on the virus infection process were cytologically studied. The investigated substances negatively affected viral pathogenicity by diminishing the feeding activity of insects and prolonging the period of development of the viral disease.*

Key words: *active plant extracts, granulosis virus,* Pieris brassicae.

INTRODUCTION

Population growth of a plant pest is characterised by an increase of population density (progradation) and its subsequent decrease. In plant protection, the main attention is paid to inhibition of the pest during the progradation phase, when the greatest plant damage occurs, using chemical or biological plant protection. Active plant substances, beneficial insects and microorganisms (bacteria, virus, fungi) are widely used in biological plant protection. In the 1980's, the use of natural substances, obtained from leaves, fruits, seeds, bulbs and roots of fresh or dried plants, has increased in plant protection (Banerjie et al., 1985; Barnerjee and Choudhuri, 1986; Stoll, 1986; Schwinger, 1986; Verma et al., 1986; Agrawal and Mall, 1988; Raghunatha Rao et al., 1988; Болгарь и Ковалев, 1988; Kawaguchi et al., 1989; Luthria et al., 1989; Mauchamp, 1989; Mikolajczak et al., 1989; Scin-Foon, 1989; Breuer and Devkota, 1990; Stein and Klingauf, 1990), including the use of plant species with hormonal influence on insects, and antifeedants that contain alkaloids or alkaloid glycosides. Some plant substances (tomatine, nicotine) have both insecticidous and antifeedant properties. Various solvents are used to extract active plant substances, for example, water, alcohol and acetone; and the obtained substances can be preserved at normal temperature or they may be frozen, dried or ground (Mikolajczak et al., 1989; Scin-Foon, 1989; Stein and Klingauf, 1990). The properties of plant extracts determine their suitability for use in plant protection, for example:

Melia azedarach — effective antifeedant (Breuer and Devkota, 1990; Scin-Foon, 1989; Schwinger, 1986);

oil extracted from *Calophyllus inophyllus* — insecticide against cockroaches (Banerjie et al., 1985);

seed extracts of *C. inophyllus* — act on the 3rd instar larvae of *Diacrisia obliqua* (Agrawal and Mall, 1988);

Ocimum sanctum extract — against aphids (Stein and Klingauf, 1990);

Derris ssp. and *Quassia ssp.* extracts — against the larvae of plant leaf-cutting beetles (Stoll, 1986);

extracts of *Melia azedarach* seeds (1% concentration) — inhibit the feeding of *Mythimna separata* larvae of the 1st and 2nd instar (Scin-Foon, 1989);

hexane and ethanol extracts of *Aglaia cordata* — against the larvae of *Spodoptera frugiperda* (Mikolajczak et al., 1989);

ethanol and water extracts of garlic extracts — against the aphid *Myzus persicae* and moth *Plutella xylostella (Stein and Klingauf, 1990)*;

the extract from *Tephrosia vogelii* leaves in acetone — against the larvae of turnip white butterfly *Pieris napi* and cabbage moth *Barathra brassicae* (Scin-Foon, 1989).

The effects of various concentrations of extracts depend on the stage of insect development. Freshly hatched larvae of *Spodoptera litura* die when sprayed with a 0.0001 % alkaloid solution extracted from *Tylophora asthmatica*, but larvae of the 5th instar survive when sprayed with the extract at concentrations of 0.01–0.0001 %. If unsprayed food is given to the larvae after 18 hours of previously inhibited growth caused by the alkaloids, the larvae begin to develop normally (Verma et al., 1986).

The successful application of active plant substances in plant protection requires knowledge of their action mechanism. Cytological methods can be used to determine the mechanisms of effect. For example, by the use of a dye to indicate accumulation of food in the digestion tract, extracts of *Phellodendron amurense* were found to have an antifeedant effect on the termite *Reticulitermes seperatus* (Kawaguchi et al., 1989). In a study of the grasshopper *Schizodactylus monstrosus*, it was determined that nicotine acts on the central nervous system of the insects as a neurotoxin (Barnerjee and Choudhuri, 1986). Substances obtained from plants (for example, oils of soy bean, cotton, nuts, and sugar-cane) may be used together with microorganisms (for example, baculoviruses) in plant protection, acting as adhesion promoters and feeding stimulators, but with no insecticidous or antifeedant properties (Filho and Nilson, 1987). In nature, baculoviruses, for example granulosis virus, which are frequently associated with declining populations of *Lepidoptera*, cause disease in insects and can control the population size of their hosts. Baculoviruses are considered to be safe biological insecticides and have great potential use in pest control (Huber, 1986).

The aims of our experiments were: 1) to use cytological methods to determine the effects of various plant extracts on the larvae of white cabbage butterfly, and 2) to determine the effect of plant extracts on the pathology caused by granulosis virus and on granuloses virus activity.

MATERIAL AND METHODS

Larvae of the cabbage white butterfly *Pieris brassicae* L. (2nd–4th instar) were used as a test pest. For pest control, the following virus preparations were used: suspension of *Pieris brassicae* granulosis virus (PbGV), Latvian isolate, with titre 1×10^7 capsules/ml (Laboratory of Plant and Insect Viral Diseases, Latvian Agricultural University), and two PrGV preparations produced in China (Laboratory of Virology, University of Uhan) with titre 2×10^7 capsules/ml. The PrGV preparations were fine green-gray powder that easily dissolved in water. Active plant substances extracted from common marigold, French marigold, milfoil and tomato leaves were tested. Extracts were obtained as follows: 1. Extract of common marigold (*Calendula officinalis*). Finely cut fresh plant material (80 g) was mixed with one litre of cold water, left exposed for 3 days, and then filtered. 2. Extract of French marigold (*Tagetes patula*). Air dried plants (80 g) were mixed with one litre of cold water, left exposed for 2 days, and then filtered. 3. Extract of milfoil (*Achillea millefolium*). Air dried plants (80 g) were mixed with one litre of cold water, left exposed for 6 days, and then filtered. 4. Broth of tomato (*Lycopersicon esculentum*) leaves. Fresh tomato leaves (400 g) were simmered in one litre of water for 30 minutes, and then filtered. The filtrate was 1/3 diluted with water.

In laboratory tests, cabbage leaves were sprayed with viral suspensions, then dried and used for the feeding of larvae. First instar larvae were collected in nature, and reared in growth chambers. Three replications (30 larvae in each replication) were used. Control cabbage leaves were sprayed with water.

In field tests, plants were grown in ten plots, with 15 plants per plot (three rows of five plants). The distance between the rows of plants was 50 cm, adequate to eliminate the spread of the viruses. Cabbage was sprayed at the stage of forming cabbage-heads, without previous picking-off of larvae of *P. brassicae*. The sprayed larvae were at the 2nd–4th stages. A hand-sprayer was used.

To determine how the extracts affected the infection process of viral disease, PbGV preparations were added to the extracts. The concentration of viruses in a working solution was 2×10^7 capsules per ml. Water was used as a control. Larval mortality of *P. brassicae* was assessed daily, beginning at 48 hours after spraying. During field tests, the average daily temperature was 9–13 °C and the relative air humidity was 80–86 %.

The statistical analyses of laboratory and field test results followed Lipa and Šližynski (1973). Cytological preparations were made by fixing larvae of *P. brassicae* that showed characteristic symptoms of illness. For light microscopy, larvae were fixed in Schaffer's or Carnua's fixators, or in a 10 % solution of formalin, and coloured by the method of Romanovski, and with Sudan III and Sudan IV (Лилли, 1969). For ultrastructural examination, the larvae were fixed in 3 % glutaric dialdehyde, then in 1% osmic acid, and enclosed in Epon.

RESULTS AND DISCUSSION

Under natural conditions, the infection of insects with viral diseases occurs when they feed on plants that are infected with baculoviruses. The infection process is slower when insects are infected with viral suspension *per os*.

In laboratory experiments, after the feeding of 3rd instar larvae of *P. brassicae* on cabbage leaves sprayed with the PbGV Latvian isolate water suspension, the mortality level of the larvae after 15 days reached 48.3 %, compared to 5.7 % in the control. It was observed that 56.4 % of the larvae on sprayed cabbage leaves did not

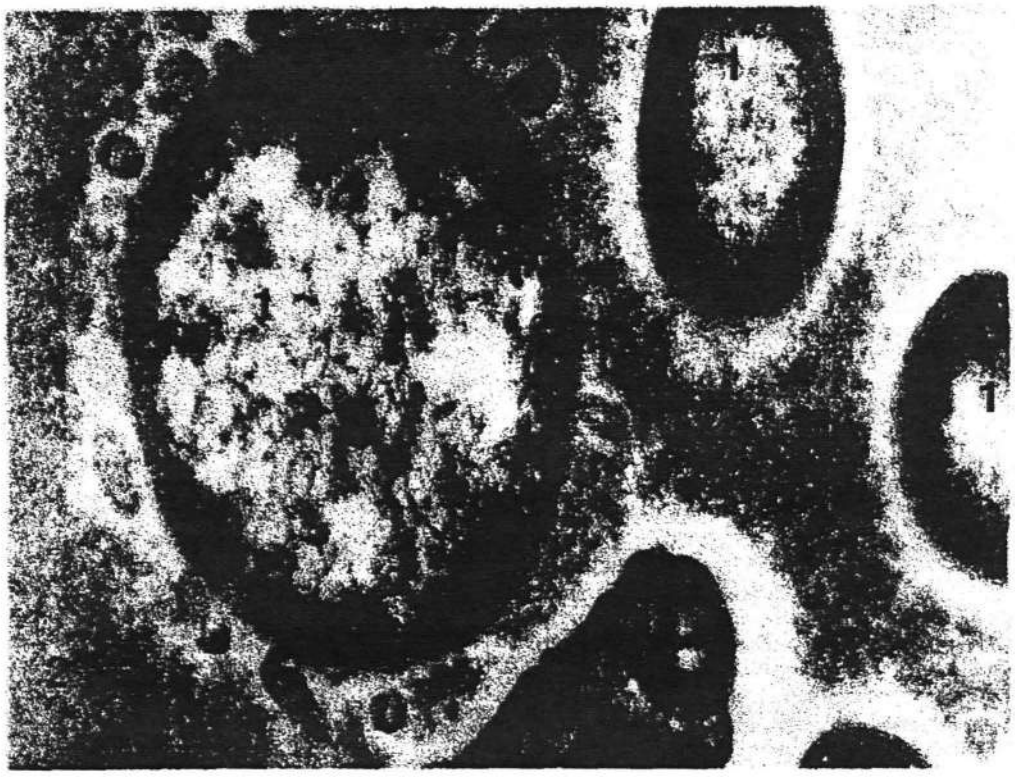

Fig. 1. Bacteria found in the body cavity of virus-infected and paralysed *P. brassicae* larvae. x 82 500
1, bacteria; 2, remains of cells

feed for 24 hours, and they remained on the walls of the chambers. The PrGV suspensions of preparations produced in China had low adhesion and ran down the cabbage leaves. Using the Chinese preparations of PrGV, the mortality of the larvae of *P. brassicae* was 58.8 %–64.4 %, 15 days after spraying. In the laboratory tests, the highest larval mortality was observed at the end of the 4th instar stage: 37.5 % – 76.3 %. After spraying with the Latvian preparation, only 29.3 % of the larvae reached the 5th instar.

Microscopic examination showed that 3.1 % of the larvae were infected with bacteria (Fig. 1.), and 3.0 % with fungi. This may be because the larvae of white cabbage butterflies collected in nature were used.

Light microscopy showed that control larvae of the white cabbage butterflies feeding on unsprayed plants were developing normally and that fat cells filled the cavities among tissues and organs. The size of the fat cells was $16.3\pm0.48 \times 18.4\pm0.9\ \mu m$ and they contained abundant reserve substances (lipids, protein and glycogen).

When larvae were fed on cabbage leaves sprayed with the suspensions of virus, necrotic fat cells were evident in the body cavities of the infected larvae (Fig. 2). Fat globules gradually disappeared, and condensation of chromatin had occurred. In some locations, only residues of cells (fragments of membranes, protoplasm and nuclei) were visible in the larval body cavities. Bacteria, and sometimes fungi, were observed in histological preparations of infected larvae, suggesting that mixed infection is often a mortality factor in natural populations. It was estimated that about 82.5 ± 1.5 % of dead previously infected *P. brassicae* larvae died from granulosis, 2.2 ± 0.5 % from bacteriosis, and 3.8 ± 0.5 % were infected with parasitical insects. Mixed infections were found in 12.4 ± 1.0 % of the dead larvae.

In field experiments, larval mortality 18 days after the spraying with PbGV suspension reached 68.8 ± 4.7 %, compared to 6.9 ± 2.3 % in the control. The applied antifeedant extracts from common marigolds, French marigolds and milfoil resulted in, respectively, 3.8 %, 11.5 % and 4.0 % mortality of *P. brassicae* larvae at the 3rd day after spraying and 55.9 %, 46.1 % and 12.0 % after 18 days. The extracts, together with PbGV, caused mortality ranging from 51–90 % (Fig. 3). Extract from common marigolds caused very low mortality of the larvae after 3 days, but it increased to 26.3 ± 3.8 % after 15 days, probably due to weakening of the larvae after starvation and increased susceptibility to diseases (in many cases, the dead larvae contained viruses, bacteria and

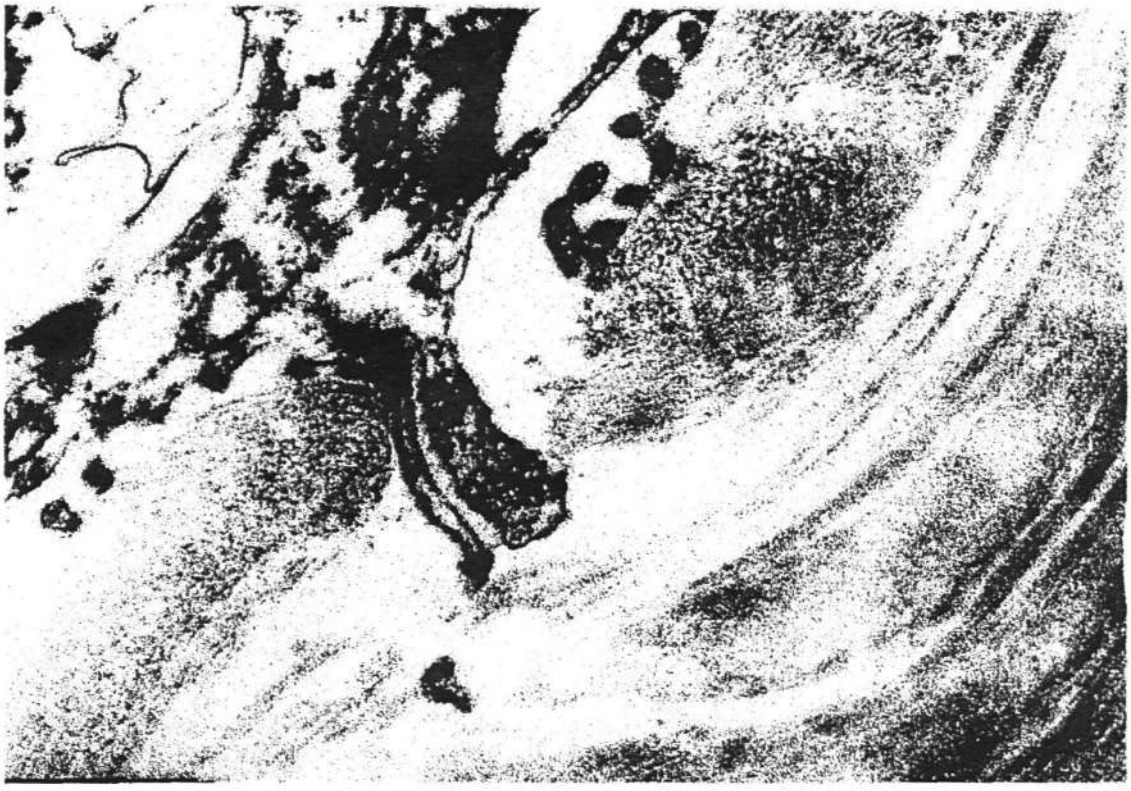

Fig. 2. Necrosis of virus-infected cells of *P. brassicae* 3rd instar larvae: necrotic muscle cells, fragments of membranes, and remains of protoplasm. x 60 000

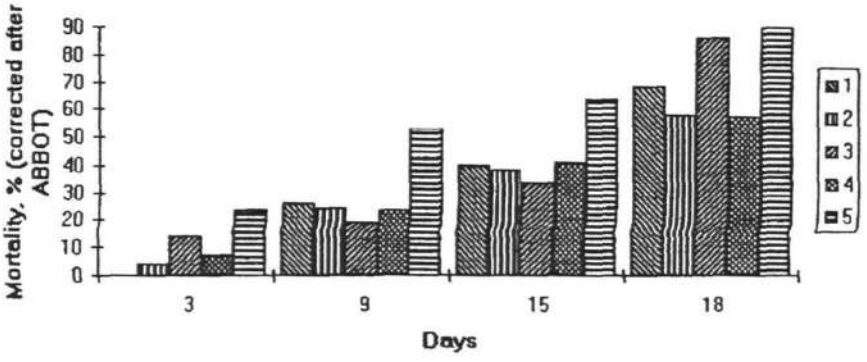

Fig. 3. Comulative mortality of 3rd instar larvae of *P. brassicae* after spraying with a water suspension of *Pieris brassicae* granulosis virus (PbGV) (1), and with PbGV plus extracts of various plants (2,3,4,5) (virus titre in working suspension was $2x10^7$ *capsules /ml*).

1, PbGV; 2, PbGV + extract of milfoil; 3, PbGV + extract of marigolds; 4, PbGV + extract of French marigolds; 5, PbGV + tomato leaf broth

fungi). Common marigold extracts with added PbGV caused higher mortality. Larval mortality was very low when extract from milfoil were used, but it increased with addition of PbGV. However, the mortality due to extracts from milfoil together with PbGV did not significantly differ after 15 days from that caused by PbGV suspensions alone. Therefore, milfoil extracts do not diminish the activity of viruses, but only prolong the development period of the viral disease by antifeedant activity. The PbGV suspensions did not have any additional effect on mortality when added to French marigold extracts.

Tomato leaf broth caused 25.7 % mortality of larvae at 3 days after spraying, likely due to a toxic effect (Schwinger, 1986). About 70 % of the larvae that were sprayed with tomato leaf broth had died from granulosis after 15 days, suggesting the activation of latent viruses. The addition of PbGV to tomato leaf broth did not increase the larval mortality during first few days, since the

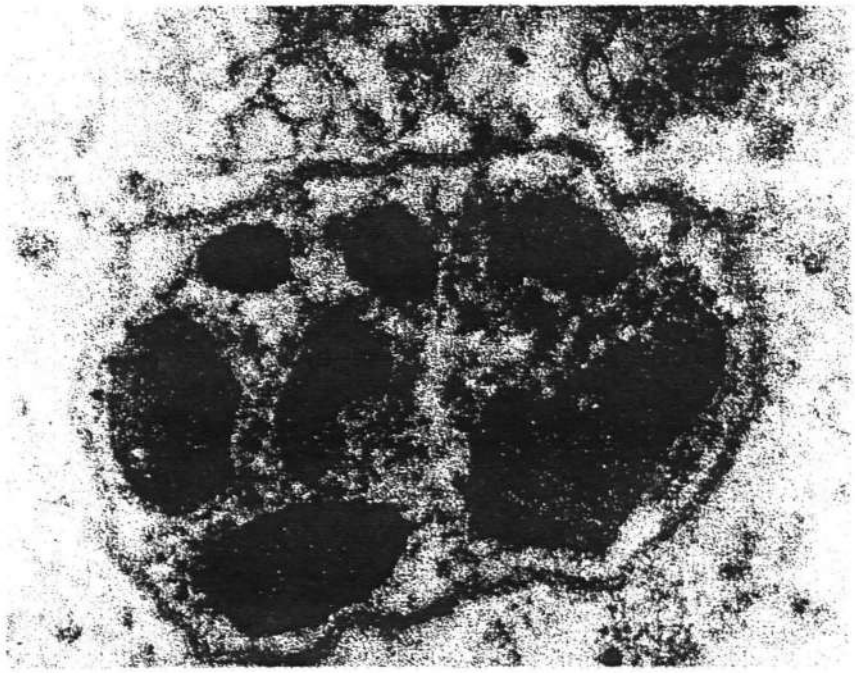

Fig. 4. Lysed remains of cabbage leaf in the digestive tract of *P. brassicae* after spraying with tomato leaf broth. A cross-section of plant cell with a deformed cell wall and partially dissolved protoplasm. x 25000

development of viral diseases usually takes 6–12 days (Zariņš and Eglīte, 1993). After 9 days, the mortality caused by the viruses had increased and at the 18th day it had exceeded that caused by the PbGV suspension in water. The toxic effect of tomato leaf broth is likely summed with the activity of the granulosis viruses, thereby increasing larval mortality. A breakdown of larval fat bodies begins on the 3rd day after the spraying of cabbages with tomato leaf broth. In the digestive tract, remains of cabbage leaves with lysed cells were observed (Fig. 4.). Bacteria sometimes occurred among the destroyed cells of the pest.

The obtained results suggested that, if virus entry into insect cells is disturbed, then the adsorption of viruses is blocked. Some of the tested plant substances showed antifeedant properties. Using these extracts, it is necessary to avoid frightening away the larvae of the white cabbage butterfly to unsprayed plants, since the larvae have a high potential to migrate. The performed tests showed that, before recommending the use of active plant substances in plant protection, it is necessary to know how these extracts influence the pest.

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AUGU EKSTRAKTU UN GRANULOZES VĪRUSU LOMA AUGU KAITĒKĻU POPULĀCIJU REGULĒŠANĀ

Pētījumos analizētas iespējas lietot augu ekstraktus kopā ar *Pieris brassicae* granulozes vīrusu (PbGV) kāpostu balteņa *Pieris brassicae* L. populāciju regulēšanā. Samteņu, pelašķu un kliņģerīšu izvilkumi (bez granulozes vīrusa pievienošanas) iedarbojas kā antifidanti un trešajā dienā pēc apmiglošanas izraisa *P. brassicae* kāpuru nelielu mirstību — attiecīgi 11,5%, 4% un 4%. Tomātu lapu novārījums ir toksisks, tas izraisa 25% *P. brassicae* kāpuru mirstību jau trešajā dienā pēc miglošanas. Veikti citoloģiskie pētījumi, lai noskaidrotu augu ekstraktu iedarbības mehānismu uz *P. brassicae* kāpuriem un vīrusu infekcijas procesu. Lietojot minētos ekstraktus kopā ar granulozes vīrusu, kāpuru mirstība palielinās. Tomēr, analizējot vīrusslimības norisi, noskaidrots, ka izvilkumi negatīvi ietekmē vīrusu infekciju, jo mazina kāpuru barošanās aktivitāti un pagarina slimības attīstības periodu.

BIOLOGICAL CONTROL OF *MALACOSOMA NEUSTRIA* L. POPULATION WITH LATVIAN ISOLATE OF NUCLEAR POLYHEDROSIS VIRUS

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ABSTRACT

The Latvian isolate of *Malacosoma neustria* NPV was used as a source for production virus insecticide. In recent years novel virus insecticide formulations have been developed and tested using environmently - friendly matrix materials. Field trials with the new virus insecticide compositions indicated that high levels of mortality (89-96 %) of second and third instar larvae could be achieved 10 days after spraying. The persistence of NPV in the agroecosystem was measured. The results of bioassays demonstrated that the new virus insecticide formulations retained activity 3 weeks after spraying. Additives used: lysine KKL, polyglucine, a by-product of citric acid production and molasses of peat, increased persistence of polyhedra 7, 6, 4, and 3 times, respectively. The mean percentage of larval mortality in test variants was 2.5 to 3 times higher than that in the controls.

INTRODUCTION

Baculoviruses including nuclear polyhedrosis viruses (NPVs) are insect pathogenic viruses that cause diseases of insects and may control the population of their hosts. They are considered to be safe bioinsecticides and therefore have great potential in integrated pest control. They can substitute for chemical insecticides diminishing the amount of pests. NPVs in particular have been formulated and applied as biological insecticides against pest populations (Huber, 1986). The Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) is a highly active and selective agent for the control of European tent caterpillar *M. neustria* population (Zarins & Eglite, 1993). Mn NPV was used as a basis of 'VIRIN-KS' which is the virus preparation registered and produced in the Soviet Union.

In recent years we have been working on problems involved in the development of microbiological methods for plant protection. Initially we analyzed possibilities of using new biological plant protection methods both for ecological agriculture as well as for conventional agriculture. Biopreparations based on NPVs may be considered for the following reasons: i) high host - specificity, ii) no evidence of occurrence in non-arthropod hosts; no observations of harmful effects on the rest of the entomofauna including beneficial insects, or on other bioagents in the agroecosystem (Grôner, 1986), iii) spread and multiplication of these viable microbial preparations in pest populations leading to the possibility of control of pest populations several years after initial spraying (Bird, 1961), iv) local virus strains and isolates have high activity in the climatical conditions of Latvia. The nuclear polyhedrosis viruses described are inactivated by different environmental factors such as sunlight, summer temperature, humidity and rain. The inactivation rate may be slowed down by using various additives to the virus preparations. It has been shown that formulated viral preparations are

more or less easily washed out from foliage, depending on the formulation used (Mohamed et al., 1982).

The aim of our studies was to test new virus insecticide formulations developed in our laboratory with high potency and desirable physical characteristics and to compare the persistence of Mn NPV activity in the different formulations.

MATERIALS AND METHODS

The Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) strain KS-5 (Patent SU 1022350 A 01, 1983) isolated in the Institute of Biology, Latvia was used as the basis of the virus insecticide. The method of producing the virus formulation was developed in our laboratory (Patent SU 1314492 A1, 1987). Environmently-friendly matrix materials were used. The four additives used in the formulations were selected from 20 tested: polyglucine, molasses of peat (experimental product, produced in the Institute of Wood Chemistry, Latvia), lysine KKL (produced in the factory for producing lysine, Latvia), a by-product of citric acid production (produced in Institute of Microbiology, Latvia). Formulations containing bentonite were used as positive controls. All of the tested additives gave good wettability of dispersible dry formulations (Table 1), as well as promoting adhesion to the plants.

Table 1. Physical characteristics of virus preparations containing different additives

Additive in the virus preparation	Stability: concentration of virus suspension, polyhedra/ml		Dispersion, (sec)	Adhesion	Viscosity,
	before sedimentation	after 30 min of sedimentation			
Polyglucine	$2.8x10^7$	$2.7x10^6$	300-360	good	1.06
Molasses of peat	$6.9 x10^6$	$6.3x10^6$	15	satisfactory	1.03
Lysine KKL	$2.4x10^6$	$2.4x10^6$	30	satisfactory	1.01
By-product of citric acid production	$5.1x10^6$	$3.2x10^6$	40	satisfactory	1.25

Stability against u.v. light was measured after exposing working solutions for 20 and 60 min. to u.v. light. Apple leaves were sprayed with virus preparations using a hand sprayer. The virus concentration in the working solution was 10^7 polyhedra ml^{-1}. *M. neustria* larvae reared on natural food in special cages (0.5 x 0.35 x 0.35 m) were fed on sprayed leaves for one day. Experiments were repeated 4 times (30 larvae in each replica). The efficiency of virus preparation was expressed as the percentage mortality caused by the virus, ussing the method of Abbott (Abbott, 1925) and LT_{50} (Finney, 1971). Persistence of virus viability after storage of the virus formulations for two years was tested using bioassay, as described previously. Virus insecticide formulations were stored as pastes as well as dry powders. Dry formulations were developed by means of lyophilization.

The suitability of the additives used in the virus formulations was examined in field trials

from 1990 to 1995. Apple trees infested with second instar of *M. neustria* were sprayed with virus preparations (working solution $5x10^6$ polyhedra ml^{-1}, 50 to 70 litres/ha). Backpack air-blast sprayer (Yamar-10, Japan), nozzle type 1.6 mm, spray angle 50°, operating pressure 1.6 was used.

The persistence, distribution and accumulation of NPV in the agroecosystem was determined to optimise the successful biological control of the *M. neustria* population. An experiment was performed to study the influence of environmental factors on virus viability after its application. Apple trees were sprayed with virus preparations ($2x10^7$ polyhedra ml^{-1}). Virus was exposed on foliage. After 1, 8, 15, 22 days leaves were randomly collected for bioassays and 200 discs (10 mm diameter) were cut for DNA-DNA hybridization. Third instar larvae of *M. neustria* reared on artificial diets were used for biotest. Experiments were repeated 5 times (20 larvae in each replica). Virus sprayed leaves were homogenized and added to diet. Leaves sprayed with water were added to diet and used as a control.

RESULTS AND DISCUSSION

The new tested dry formulations produced by lyophilization showed very good efficiency after two years storage (Table 2). New additives protected the virus from u.v. light. Virus exposed to u.v. light for 60 min retained on 15 % of its efficiency compared to values 70-90 % for virus in preparations containing additives.

Table 2. Efficiency of virus preparation depending on additives used and conditions of storage

Additive in virus preparation	Concentration of additive in working solution, (%)	Form of storage	Efficiency after two years of storage, (%)
Polyglucine	0.4	dry powder	92.5
Molasses of peat	0.5	dry powder	95.0
Lysine KKL	0.5	dry powder	95.0
By-product of citric acid production	0.5	dry powder	78.0
		paste	89.3

Field trials with the new virus insecticide formulations indicated that high levels of mortality 89 to 96 % of second and third instar larvae could be achieved 10 days after spraying.

The results of biotest demonstrate that the new virus insecticide formulations secure the persistence of virus activity 22 days after spraying (Table 3).The weekly loss of polyhedra determined by specific DNA-DNA hybridization on apple leaves varied between 20 to 60% in variants with additives, in the control (virus in water suspension without additives) the loss was 80%. The use of the additives: lysine KKL, polyglucine, by-product of citric acid production and molasses of peat increased the persistence of the polyhedra 7, 6, 4, and 3 times, respectively. The mean percentage of larval mortality after 15 and 22 days was 2.5 to 3 times higher than that in the controls.

Table 3. Persistence of virus activity of preparation after its application

Additive in the virus preparation	Mortality corrected after Abbot, (%)	
	Time exposed on foliage, (days)	
	1	22
Polyglucine	98.0	73.4
Molasses of peat	80.0	51.8
Lysine KKL	88.1	66.0
By-product of citric acid production	86.6	50.0
Bentonite	84.4	58.0
Virus water solution	80.5	19.0

It may be concluded that the new virus insecticide formulations can be used to control populations of the European tent caterpillar. The additives used in the preparation of the virus insecticides strongly reduced the harmful effect of weather conditions on the persistence of viral activity.

ACKNOWLEDGMENTS

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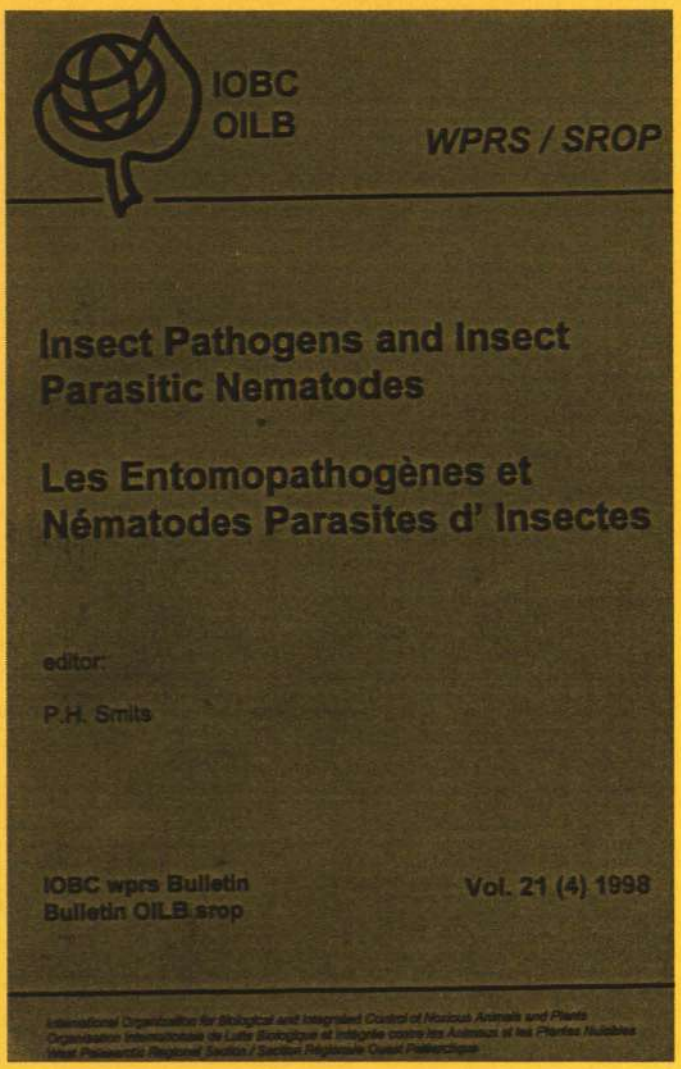

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Accumulation and migration of *Malacosoma neustria* nuclear polyhedrosis virus in soil

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Summary

Dispersal and accumulation of nuclear polyhedrosis virus (NPV) and persistence of their pathogenicity in the soil was investigated. The Latvian isolate of *Malacosoma neustria* NPV was used in model experiments. The amount of macro- and microelements in the loamy soil did not influence the pathogenicity of Mn NPV substantially. The pathogenicity and a reproduction ability of viruses did not differ in tested loamy soils within 1 month. Investigations showed that the virulent polyhedrae can leach through 25 cm layer of soil. Leaching efficiency of the polyhedrae through sand, peat and loamy soil was compared in model experiments. The smallest efficiency was established for loamy soil, because loam can adsorb the polyhedra. The leached polyhedrae maintained the ability to reproduce in larvae of *M. neustria* causing the mortality from 68 % to 100 %. Rainfalls influenced vertical transfer of the viruses and provided their dispersal in soil.

Introduction

In context of sustainable agriculture, it is necessary to maintain the environmental potential (beneficial insects, entomopathogenous fungi, bacteria, baculoviruses) in agrocenoses to diminish multiplying and spreading of plant pests. It is necessary to investigate the distribution and accumulation of NPVs and to secure their remaining in biocenoses for prolonged limitation of plant pests.

It is well known that NPVs cause epizootics of pest insects. Large quantities of virus are released into environment during epizootics. Between epizootics the virus might survive in the host population or to accumulate in the environment. The nuclear polyhedrosis viruses have different capabilities of surviving in abiotic environment and are inactivated by different environmental factors. Several studies shoved that soil is a natural reservoir for viruses and is highly favorable for survival of NPV (Jaques, 1969, Thompson et al., 1981). Nuclear polyhedrosis viruses persist overwinter in the soil (Tanada & Omi, 1974, Mohamed et al., 1982, Olofsson, 1987). Hukukura (1985) reported that the median infectious concentration of *Hyphantria cunea* NPV in the soil was 7.14 $x10^7$ polyhedra/g of soil. There are studies on a vertical distribution of viruses in soil (Jaques, 1969, Thomphson & Scott, 1979). Physicochemical properties of soil may affect pathogen survival. Jaques and Harcourt (1971) found that soil pH as low as 5.6 had no effect on the concentration of *T. ni* viruses, but Thomas reported that the *T. ni* NPV was inactivated rapidly in loamy sand with pH 4.83-7.7 (Thomas et al., 1972). However, the effect of soil on pathogen persistence is not well understood.

Rainfall may be suggested as an another factor affecting distribution and accumulation of viruses. Heavy rains may wash NPV into the soil and influenced spreading of viruses (Qgaard, 1988). However, the recirculation of insect viruses in soils is not well understood and needs further investigations.

The aim of our studies was to investigate accumulation and dispersal of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) in soil after rainfall to provide the necessary

background for developing an effective strategy of biological control for exploiting the pathogens, already present in the ecosystem.

Material and methods

The model experiment was performed to study the migration and accumulation of Mn NPV in soil after simulated rainfall. Mn NPV produced in the Laboratory of Experimental Entomology of Institute of Biology was used. Sand (pH -6.1), peat moss (pH- 5.5) and loamy soil (pH -6.1) was tested. Viruses were kept in 10% soil suspension to different time periods (week, month, year). Three loamy soil with different pH (6.05, 6.1 and 6.8) and content of humus (6.8%, 3.0% and 3.5%) were tested.

Application and virus migration test

Glass containers (10 x10 x 50 cm) were filled with soil (height of soil layer 25 cm). Mn NPV suspensions (100 ml) containing $2x10^{11}$ polyhedrae were applied in each container. To determine the influence of rainfall, we used artificially simulated rain, equal to June's average precipitation (69 mm) in Riga's district. Artificial rain was used to leach the polyhedra every 10 days - 3 times a month. Filtrates that leached through soil were collected, filtered through cheesecloth and taken for bioassay, electron microscopy and specific DNA - DNA hybridization.

Accumulation of polyhedrae in loamy soil (pH 6.05) was determined. Soil probes were taken through small holes in the container. 10 g of soil probe were add to 50 ml of sterilized water and agitated in a shaker for 2 hr. Each suspension was filtered through cheesecloth and tested.

Bioassay and DNA-DNA hybridization

Bioassays were used to test persistence of virus viability in soil and leaching efficiency. Third instar larvae of *M. neustria* reared on natural food in special cages (0.5 x 0.35 x 0.35 m) were fed for one day on apple-tree leaves sprayed with soil filtrates. In controls sterile water filtered through the soil was applied. Experiments were repeated 4 times, 30 larvae in each replica. The virus viability was expressed as the percentage of mortality caused by the virus and LT_{50} (Finney, 1971).

A specific- DNA-DNA hybridization assay (Sharipo, 1991, Kukan & Meyer, 1995) was used to detect virus in soil filtrates. We used ^{32}P labelled DNA probe capable to detect Mn NPV produced in the Institute of Microbiology and Virology and a modified method of DNA-DNA spot-hybridization (Sharipo, 1991) for the detection of Mn NPV in the environment. 500 µl of soil extracts were analyzed for each sample. The experiments were repeated 5 times, 100 µl in each replica. To lyse virions 100 µl of the soil extract were added to 100 µl of 2x SSPE Buffer and 0,4 N NaOH. Sterile water filtered through soils was used as a control. Extracts were vacuum-blotted onto nitrocellulose filters ("Hiju Kallur", Tallinn) and hybridized.

Results and discussion

The distribution and accumulation of NPVs in soil after artificial rainfall were determined to optimize biological controls strategies. Results of our investigations show that rainfall washed out the virus and do not differ from the conclusions of Qgaard et all. (1988). We

determined that polyhedra leached through 25 cm layer of tested soils, if they were sprayed and washed with water amount equal to average precipitations in June. The smallest leaching efficiency was established for loamy soil, because loam can adsorb the polyhedrae (Table 1). The bioassay showed that the larvae fed on the leaves sprayed with soil filtrates died after 6 - 17 days. The viruses leached through peat and sand had high activity and caused larval mortality 95 to 99% after first and second water spraying. Determined LT_{50} showed that the development of disease is faster after the first artificial rainfall, it means that after two weeks a part of viruses is inactivated or adsorbed in soil. Obtained results do not differ significantly from the conclusions of Hukuhara & Namura (1972) that physicochemical properties of soil affect the adsorption of viruses. Leaching efficiency could be decreased, if the additives were used in the virus preparations. Additives provided the persistence of Mn NPV activity in soil (Čudare unpublished).

Table 1. Leaching efficiency of *Malacosoma neustria* NPV leached through 25 cm soil layer determined by DNA-DNA hybridization and bioassay.

Soil	Number of artificial rainfall	Intensity of DNA-DNA hybridization	Larval mortality, %	$LT_{50} \pm sLT_{50}$ days
Loamy soil	I	+	33.6	*
	II	++	60.4	15.6±1.6
	III	+	18.9	*
Peat moss	I	+++	98.1	7.8±0.8
	II	++	98.4	11.7±1.0
	III	++	76.6	11.7±0.8
Sand	I	++	95.0	9.1±0.7
	II	++	98.3	10.7±0.5
	III	++	90.0	11.1±0.7

+ virus conc. more than 1000 polyhedra per 1 ml, ++ 10000 - 90000 polyhedra per 1 ml, +++ more than 100000 polyhedra per 1 ml, * larval mortality did not exceed 50%, in controls larval mortality 1.5 to 4% and no hybridization was observed.

Table 2. Persistence of *Malacosoma neustria* NPV in loamy soil after artificial rainfall (precipitation 3 x 23 mm).

Depth of soil layer, cm	Intensity of DNA-DNA hybridization	Larval mortality, %
0-5	+++	67.1
5-10	+++	73.0
10-15	++	12.1

++ virus conc. 10^5 - 10^6 polyhedra per 1 g, +++ more than 10^7 polyhedra per 1 g, in control no hybridization and larval mortality 1.8 % was observed.

Artificial rain spread viruses into loamy soil, DNA-DNA hybridization showed that viruses were accumulated in the soil layers (Table 2). The bioassay showed that after three applications of artificial rain the amount of viruses in tested soil is high, because third instar larvae of *M. neustria* get a sufficient dose of viruses by eating up 5-10 cm^2 of the leaves, which is sprayed with soil filtrates. Results showed that most of the polyhedrae were near the soil surface.

The viruses which were kept in soil suspension persisted activity within 1 year. Virus activity was significantly lower in the variant with humus content 6% than in the variants with humus content 3.0 % and 3.5 %. Probably the humus acids play the role in virus inactivation. This study shows that Mn NPV Latvian isolate accumulates and survives in the soil within 1 year and maintained the ability to reproduce in larvae of *M. neustria.*

The used modified method of DNA-DNA hybridization showed to have good possibilities for the evaluation of the presence of virus polyhedrae in soil filtrates.

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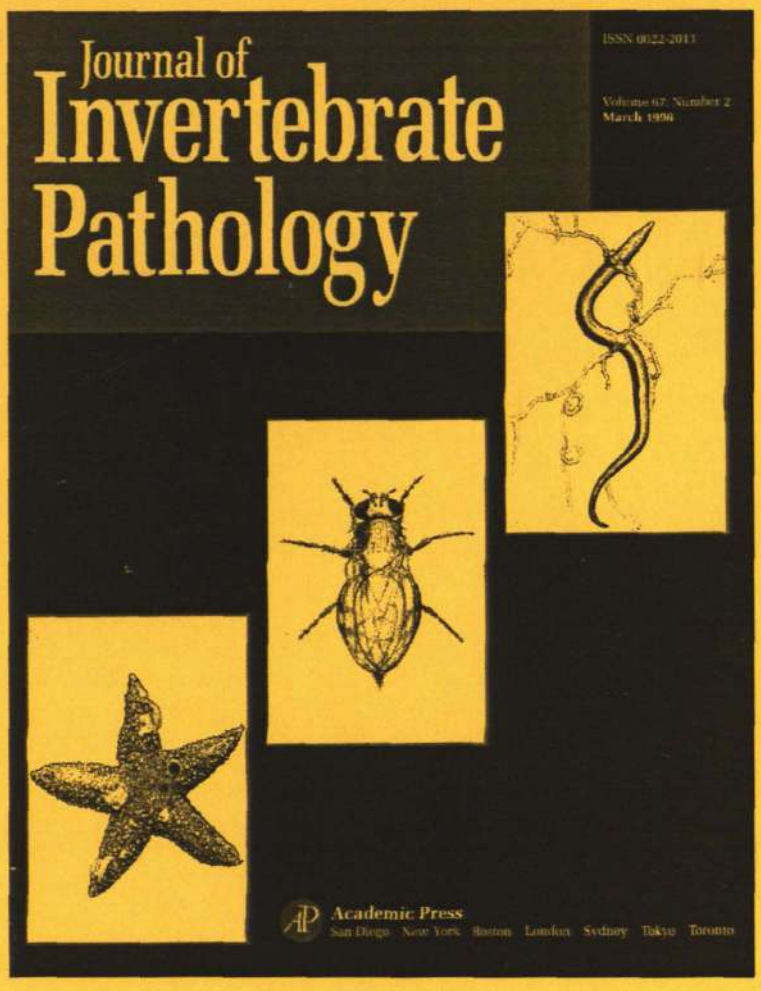

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NOTE

New Isolate of *Malacosoma neustria* Nuclear Polyhedrosis Virus in Latvia

Baculoviruses are large, enveloped, double-stranded DNA viruses that caused disease of insects. They are considered to be a safe biological insecticide and have a great potential in pest control. Baculoviruses rather rarely cause epizooty in pest populations under Latvian climatic conditions therefore nuclear polyhedrosis viruses (NPV) have been isolated from seven insect species (Zariņš and Eglīte, 1993), *Malacosoma neustria* NPV were isolated by the activation of latent infection in 1971 (Zariņš and Kalniņa, 1971).

The present aim was to search for new isolates of NPV in the European tent caterpillar *Malacosoma neustria* L. (*Lasiocampidae*) populations and to determine its biological and morphological characteristics. We isolated new Mn NPV from collected dead larvae, which had different biological activity and pathogenesis. Biological activity of new Mn NPV isolate was characterized by a bioassay using infection with disc method. The LD_{50} values were 20 ± 11, 55 ± 10, and 985 ± 19 polyhedral occlusion bodies (polyhedrae) per larvae for the first, second, and the third instar, respectively.

Cytological preparations made by fixing the larvae of *M. neustria* with characteristic symptoms of illness were investigated by light microscope (Amplival, Germany) and electron microscope (JEM-100C, JEOL, JAPAN).

Necrotic fat cells were visible under light microscope in body cavity of the infected larvae 5 days after infection. Cell-breakage was accompanied by disappearance of fat drops from fat cells as well as by condensation of chromatin. Tissues and cells in the latest stage of the disease contained a large amount of viral polyhedrae. It is well-known that NPV infection is polyorgano-

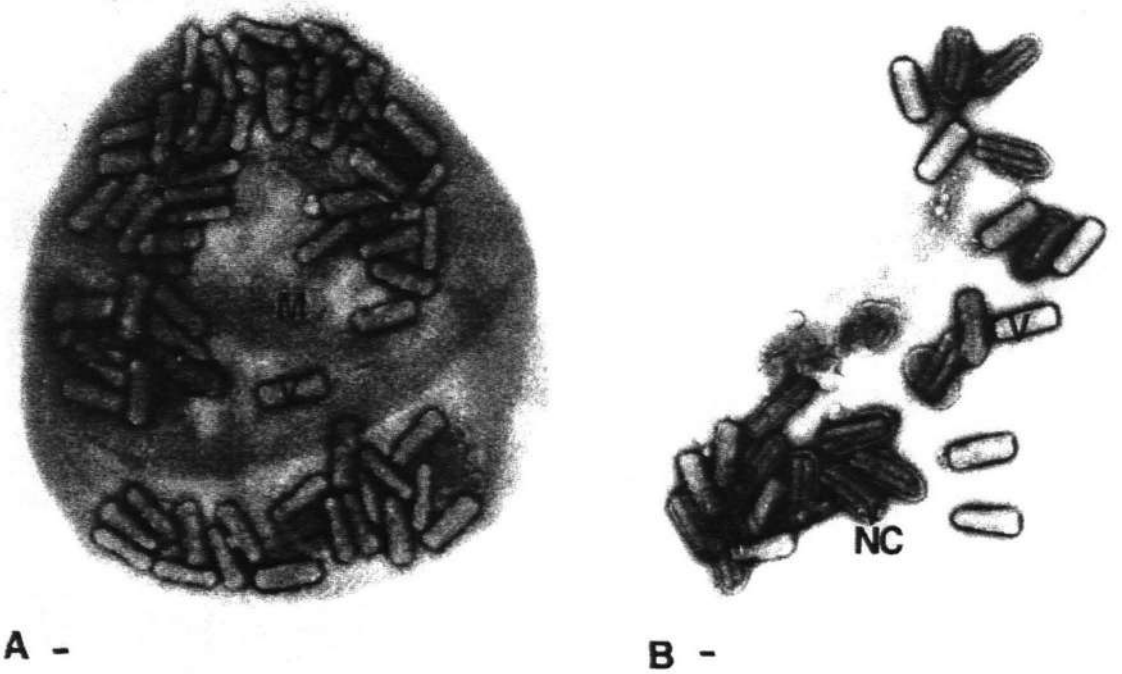

FIG. 1. Electron micrographs of *Malacosoma neustria* nuclear polyhedrosis virus. Polyhedra dissolved with 1% NaOH for 5 min. Preparations negatively stained with 2% phosphotungstic acid. Bars represent 0.1 μm. (A) Remains of dissolved inclusion bodie matrix (M) and virions (V). (B) Virions and nucleocapsids (NC).

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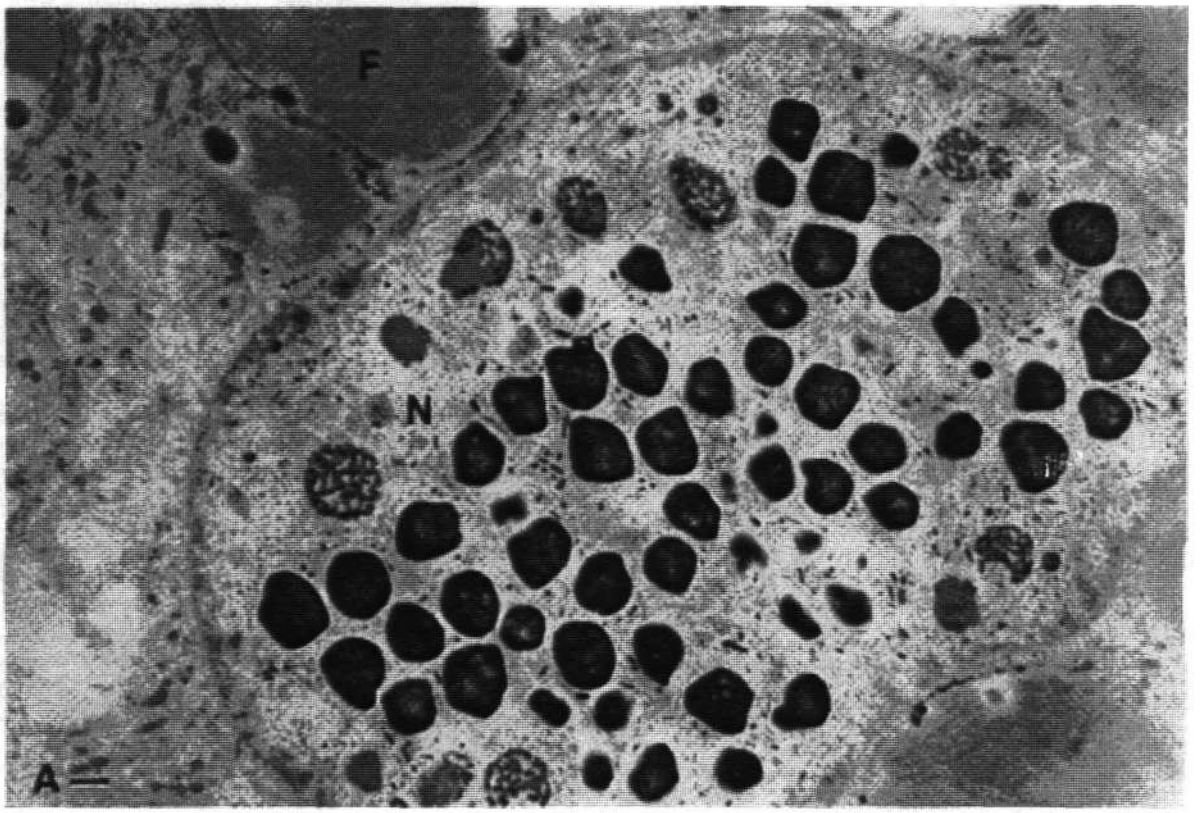
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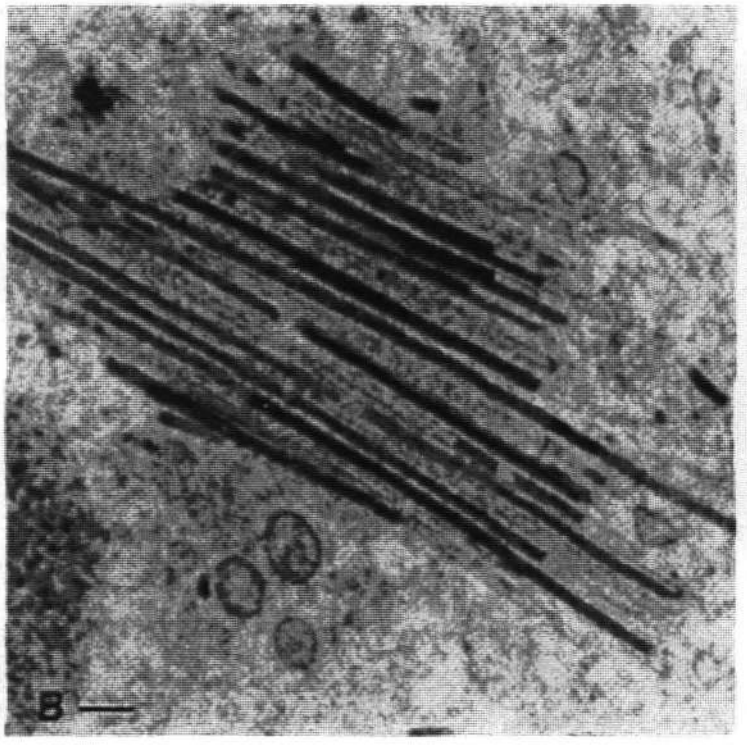
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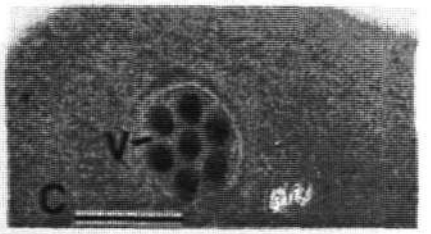
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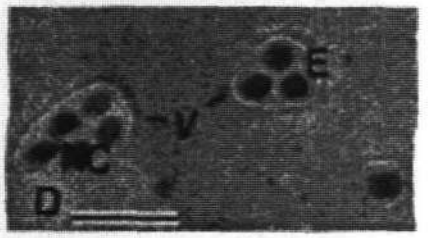
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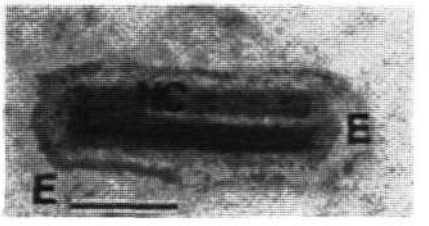
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tropic (Granados and Williams, 1986). Polyhedra of new isolate were observed in nuclei of midgut epithelial and fat cells, hypodermys, trachea, and muscular sheet. It is necessary to emphasize that new NPV isolate formed the polyhedra in the nuclei of gut epithelial cells that was not typical for isolate isolated in 1971. The dimension of polyhedra was 850 to 1400 nm. Polyhedra contained a large number of rod-shaped virions (Figs. 1A and 1B). Virions were 400× 100–200 nm; nucleocapsids were 360 × 80 nm. Based on the variable number of nucleocapsids per virion (Bilimoria, 1986), it was concluded that the type of new MnNPV Latvian isolate is multiple-nucleocapsid nuclear polyhedrosis virus, commonly as earlier investigated viruses isolated from several *Malacosoma* species *M. apicolata* MNPV and *M. distria* MNPV (Benz, 1963; Keddie and Erlandson, 1995). Electron microscopy shows that nucleocapsids, multiple virions, virogenic stroma, and developing polyhedrae were visible in the hypertrophied nuclei of fat cells at the 7th day after infection (Fig. 2A). Formation of nucleocapsids took place in nuclei of fat and gut epithelial cells (Fig. 2B). In cross-section of polyhedrae it was visible that multiple virions contain 1 to 11 nucleocapsids per virion (Figs. 2C and 2D). Virions contained enveloped, rod-shaped nucleocapsids (Fig. 2E).

It is concluded that population of Mn NPV in Latvia is heterogenous. Isolates had different biological activity and pathogenesis.

The next step of investigations is a comparison of viral DNA isolated from new Mn NPV isolate.

Key Words: insecta; *Malacosoma neustria;* nuclear polyhedrosis virus; polyhedra formation.

Our investigations were supported by a grant of the Science Council of Latvia No. 93.252 and Danish Natural Sciences Council.

FIG. 2. Electron microscopy of *per os*-infected *Malacosoma neustria* larvae. The larvae were fixed in 2.5% glutaraldehyde in ph 7.2 phosphate buffer, postfixed in 1% OsO_4 in the same buffer, enclosed in epon, sliced. (A) Ultra-thin section through fat cell nucleus. Nucleus of fat cell is increased and filled with developing polyhedrae. Bar represents 1 μm. (B) Formation of nucleocapsids. Empty capsids and developing nucleocapsids are visible. (C, D) Cross-section through polyhedra and occluded virions of the *Mn* MNPV. Virions content variable number of nucleocapsids. (E) Section of multiple virions. Virions had two nucleocapsids enclosed by a trilaminar envelope. (B–E) Bars represent 0.1 μm. N, nucleus; P, polyhedrae; F, fragment of fat drops; NC, nucleocapsid; E, envelop of virion; V, virion.

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Biological control of the European tent caterpillar *Malacosoma neustria* L. (Lepidoptera, Lasiocampidae) population with different virus formulations

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Abstract

Jankevica L., Tenbergs G., Zariņš I. 1998. Biological control of the European tent caterpillar *Malacosoma neustria* L. (Lepidoptera, Lasiocampidae) population with different virus formulations. – Latv. Entomol., 36: 11-16.

The Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) was used as a source for the production of virus insecticide. In recent years novel virus insecticide formulations have been developed and tested using environmentally - friendly matrix materials. The persistence of NPV in the agroecosystems was measured and the adhesion of virus formulations to apple leaves was determined. Lysine KKL, polyglucine, a by-product of citric acid production and molasses of peat used as additives, increased the retaining of viral polyhedrae on apple leaves after artificial rainfall for 30%. The mean percentage of larval mortality caused by the virus formulations with new additives after exposing in artificially simulated rain was 0.5 to 3 times higher than that in the control.

Key words: *Malacosoma neustria*, nuclear polyhedrosis virus, biological control

Introduction

Baculoviruses including nuclear polyhedrosis viruses (NPVs) are considered to be safe bioinsecticides and therefore have great potential in integrated pest control. NPVs have been formulated and applied as biological insecticides against pest populations (Huber, 1986). Biopreparations based on NPVs may be considered for the following reasons: 1) high host-specificity, no evidence of occurrence in non-arthropod hosts and rest of benefical insects (Gröner, 1986); 2) distribution and multiplication of viruses in pest populations can control the pest population for several years after initial spraying (Bird, 1961, Young, Yearin 1982), 3) local virus strains and isolates have high activity in the climatic conditions of Latvia (Zariņš, Eglīte, 1993). The nuclear polyhedrosis viruses have different capabilities of surviving in abiotic environment. NPVs described are inactivated by different environmental factors such as sunlight, high summer temperature, humidity and rainfall. The survival capacity of NPV is influenced by chemical degradation in the water, soil or on plant surface. Rain is an important factor of the dispersal of viruses on trees, such as viruses of the tussock moths (Thompson, 1978) and sawflies (Cunningham, Entwistle, 1981). Thompson (1978) reported that in one case the percentage of contaminated current years foliage increased from 12% on the day before a light rain to 100% two days after the rain, and the percentage of virus-infected larvae increased from 23 to 99% during this period. Most studies of rainfall and virus transmission are concerned

1978) and sawflies (Cunningham, Entwistle, 1981). Thompson (1978) reported that in one case the percentage of contaminated current years foliage increased from 12% on the day before a light rain to 100% two days after the rain, and the percentage of virus-infected larvae increased from 23 to 99% during this period. Most studies of rainfall and virus transmission are concerned with artificial virus formulations. The inactivation rate of the viruses may be slowed down using various additives to the virus preparations. The formulated virus preparations are more or less easily washed out from foliage depending on the formulation used (Cunningham, Entwistle, 1981, Mohamed et all, 1982). The role of rainfall in naturally occurring epizootics has not been well documented. Because UV light is known to inactivate insect viruses (Ignoffo et all, 1977, Jacques, 1977) rainfall could do more than simply move virus and it can help to prolong the period of activity by bringing it to places protected from inactivation factors.

The aim of our studies was to test the new virus insecticide formulations developed in Laboratory of Experimental Entomology of Institute of Biology, University of Latvia, with good physical characteristics and use the preparations for biological control of the European tent caterpillar. We compared the persistence of polyhedra and Mn NPV activity in different formulations after artificial rainfall.

Material and methods

Mn NPV isolated in Latvia was used as the basis of a virus insecticide. Polyglucine, molasses of peat, lysine KKL, the by-product of citric acid production was used as additives in the formulations and tested. Formulations containing bentonite were used as positive control.

Suitability of the additives used in the virus formulations was examined in field trials from 1990 to 1995. The efficiency of the virus preparation was expressed as the percentage mortality caused by the virus using the method of Abbott (Abbott, 1925). The experiment was performed to study the influence of artificial rainfall on the persistence of viral polyhedra after application of virus formulation that contains different additives.

Virus application and artificial rainfall test

Apple-tree branches were sprayed with virus preparations ($2x10^7$ polyhedra/ml) using a hand sprayer, concentration of tested additives was 2%. When foliage had dried, simulated rain was applied to apple tree branches. Air-blast sprayer was used as water pump. The spray head was held of 2m distance from the branches. Rain was applied to branches for 10 and 30 minutes. By measuring a rain gauge under the branches we determined that these applications were be equivalent to 20 mm, 100 mm and 300 mm of naturally rainfall, respectively.

Bioassay and DNA-DNA hybridization

After simulated rain application branches were dried and leaves were randomly collected for bioassays and 200 discs (10 mm diameter) were cut out for DNA-DNA hybridization.

Persistence of virus viability after artificial rainfall was tested using a bioassay. Third instar larvae of *M. neustria* reared on natural food in special cages (0.5 x 0.35 x 0.35 m) were fed on sprayed and exposed to the simulated rain leaves for one day. Experiments were repeated 4 times, 30 larvae in each replica.

A specific- DNA-DNA hybridization assay (Ward et all, 1987, Kukan, Meyer, 1995) was used to detect virus on the foliage after the simulated rain. The amount of the persisted polyhedrae was determined. 200 leaf discs were washed and analyzed for each sample with DNA- DNA hybridization. The experiments were repeated 5 times, 40 discs in each replica. We used ^{32} P labeled DNA probe capable to detect MnNPV produced in the Institute of Microbiology and Virology (Sharipo, 1991) and a modified method of DNA-DNA hybridization (Vilnis et al., 1990, Sharipo, 1991) for the detection of MnNPV in the environment.

Results

Field trials with the new virus insecticide formulations indicated that high levels of mortality (89-96%) of second and third instar larvae could be achieved 10 days after the spraying. Table 1 shows the amount of Mn NPV polyhedrae on sprayed apple tree leaves after exposing them in artificial rain. Loss of the polyhedrae on apple-tree leaves after simulated rain was 70-93% in variants with additives and 99% in the control (virus-water suspension without additives).

Table 1

Amount of Mn NPV polyhedrae on apple-tree leaves after artificial rain containing different additives.

Additive in the virus preparation	Amount of polyhedrae on 1 cm^2 leaf surface depending on precipitation		
	0 mm	20 mm	100 mm
Polyglucine	20000	1250	620
Molasses of peat	19000	4400	225
Lysine KKL	20000	6200	620
By-product of citric acid production	18000	3200	200
Bentonite	12000	1250	175
Control - virus-water suspension	19000	300	20

The bioassay shows that the larvae fed on the leaves exposed to the artificially simulated rain died after 10-15 days. Efficiency of virus preparations containing different additives was 90 to 96% before and 76 to 84% after exposing the leaves to the artificial rain equal to 20 mm (Figure 1). After exposing the branches for 30 min to artificial rain equal to precipitation

Application of additives exert the influence of rainfall. The amount of polyhedra more than 200 polyhedrae on 1cm^2 leaf surface exceeds LD_{50} for 2nd instar *M. neustria* larvae and is enough to infect insects in population. The 3rd and 4th instar larvae with high feeding activity and LD_{50} of 1200 polyhedrae (Zariņš, Eglīte 1994), got a sufficient dose of viruses by eating up 5-10 cm^2 of the leaves which had been exposed to artificial rain.

Obtained results concur with the conclusion that the new virus insecticide formulations secure the persistence of virus activity 22 days after spraying and the lysine KKL, polyglucine increased the persistence of the polyhedrae and virus activity (Jankevica, Zarins, 1997).

Conclusions

The new virus insecticide formulations can be used to control populations of the European tent caterpillar. The new additives used in the preparation of the virus insecticides strongly reduce the harmful effect of weather conditions on the persistence of viral activity.

Tested additives of virus preparations retain virus persistence on plants after the rain.

The best efficiency was shown by the formulations with lysine KKL and polyglucine. The used method of DNA-DNA hybridization has been shown to have good possibilities for the evaluation of the amount of virus polyhedrae on leaf surface and shows no significant difference with the results obtained by the bioassay.

Acknowledgments

Our investigations were supported by the grant No 92.252 and 96.0113 from the Latvian Science Council. We acknowledge Dr. Sharipo for providing the DNA probe capable detect Mn NPV and technical assistance in DNA-DNA hybridization.

Kopsavilkums

Ābeļu vērpēja *Malacosoma neustria* kodolu poliedrozes vīrusa (MnKPV) Latvijas izolāts tika izmantots vīrusinsekticīda izveidošanai. Latvijas Universitātes Bioloģijas institūta Eksperimentālās Entomoloģijas laboratorijā tika veidotas un izvērtētas jaunas preparatīvās formas, izmantojot videi draudzīgas pildvielas. Darba mērķis bija noteikt pildvielu pieliptspēju, saglabāšanos ekosistēmā un to spēju pasargāt vīrusus no noskalošanās lietū. Lizīns KKL, poliglukīns, kūdras melase un citronskābes ražošanas blakusprodukts par 30% palielina MnKPV poliedru saglabāšanos uz ābeļu lapām. Izmantojot biotestu noskaidrots, ka vīrusa aktivitāte un izsauktā kāpuru mirstība, pielietojot jaunās pildvielas, pēc eksponēšanas lietū ir no 0,5 līdz 3 reizes lielāka kā kontrolē, kur pildvielas netika lietotas.

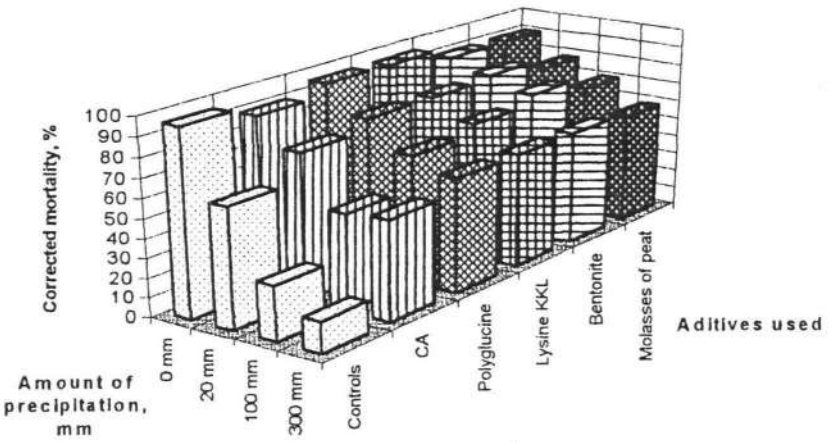

Figure 1. *Malacosoma neustria* larval mortality caused by different virus preparations after exposing to artificial rain. 300 mm the efficiency of the preparations containing additives was 52 to 60%, but in the control without additives - only 18%.

Discussion

The Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) is a active and selective agent for the control of the European tent caterpillar. All of the tested additives gave good wettability of dispersible dry formulations (Jankevica, Zarins, 1997) as well as promoted adhesion to the plants.

The persistence, distribution and accumulation of NPVs after artificial rainfall were determined to optimise successful biological control of the *M. neustria* population. Results obtained in the experiment performed to evaluate the influence of rainfall on the persistence of virus activity after the application of virus formulation does not differ significantly from the conclusions of Cunningham & Entwistle (Cunningham, Entwistle,1981) that additives reduce the influence of rainfall.

Used additives lysine KKL, the by-product of citric acid production, molasses of peat, polyglucine and Bentonite increased the persistence of the polyhedrae 20, 10, 13, 3 and 3 times, respectively in comparison with control. Results showed that directly after spraying the new tested additives increased adhesion of the virus polyhedrae to apple-tree leaves approximately 2 times in comparison with Bentonite. The used additives increased the efficiency and provided the persistence of activity of the virus preparations. The mortality of *M. neustria* larvae fed on the leaves exposed to artificial rain did not show significant differences among the preparations with additives lysine KKL, polyglucine, and molasses of peat. The effectivity of the preparations with the by-product of citric acid production is lower.

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Virulence of *Malacosoma neustria* nucleopolyhedrovirus Latvian isolates

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Abstract

The virulence of Latvian isolates of *Malacosoma neustria* multiple nucleopolyhedrovirus (Mn NPV) was tested. The possibilities to enhance virus infection. We compared virulence and virus production of two *M. neustria* isolates. Studied Mn NPV isolates showed different biological activity.

Key words: *Malacosoma neustria*, nucleopolyhedrovirus, virulence, biological control

Introduction

In nature, nucleopolyhedroviruses (Baculoviridae), cause diseases of insects and regulate their host populations. Nucleopolyhedroviruses (NPVs), are considered to be safe biological insecticides and have a great potential in pest control. The European tent caterpillar *Malacosoma neustria* L. is widely represented in the apple-gardens in south and eastern part of Latvia (Ozols, 1963). Nuclear polyhedrosis viruses have been isolated from several *Malacosoma* species across North America and Europe (Stairs 1964, Schmidt et al., 1994, Keddie, Erlandson, 1995) and from *M. neustria* (Zariņš, Kalniņa 1971, Jankevica et al., 1998) here in Latvia. Mn NPVs have been used successfully against aple tree pest *M. neustria* (Magnoler, 1985, Zariņš, Eglīte, 1993). Mn NPV was used as a basis of 'VIRIN-KSh' that is the virus preparation registered and produced in Soviet Union. The method of producing the virus formulation was elaborated in Latvia (Zarinsh et all., 1987). It is well known that during storage virus virulence may be changed. Virulence and pathogeneity are important to studies of pathogen - host interactions and may be used in characterization of virus isolates and strains. Determination of time - dose - mortality relationships, along with morphological characteristics, is of importance in determining pathogen isolates which have the greatest potential for controlling target host. The improved bioassay system developed, allows rapid and accurate determination of median lethal dose (LD_{50}), median lethal time (LT_{50}), and should be an impetus for further, detailed studies on the biological properties of baculovirus strains (Evans, 1981). The voluminous literature of LD_{50} determined by log-dose probit experiments provides powerful laboratory evidence of the importance of pathogen population density. The basis of regression lines is that the response (i.e., percentage of infection or mortality) is directly dependent on dosage (i.e., pathogen population density) (Huber, Hughes, 1984). Researchers have demonstrated significant differences in the virulence, host range, and pathology of various strains and

isolates of baculoviruses (Gelertner, Frederici, 1986). Infectivity can be enhanced by factors produced by pathogens and by factors present in the environment.

Recently, Laboratory of Experimental Entomology of Institute of Biology, University of Latvia, either has been working to improve methods of biological control of *M. neustria* populations. The present aim was to search for new isolates of NPV in the European tent caterpillar *M. neustria* L. (Lasiocampidae) populations, to determine its virulence and to investigate the possibilities of enhancing virus infection. Quantitative and qualitative aspects of virus production in *M. neustria* larvae were studied.

Materials and Methods

Viruses

Two isolates of *Malacosoma neustria* nucleopolyhedrovirus (Mn NPV) were obtained from infected larvae with nuclear polyhedrosis symptoms. Infected larvae were collected from the well producing apple gardens located in district Saldus, and district Dobele.

Isolation and purification of NPV

Dead larvae were frozen with liquid nitrogen and homogenized in distilled water with 0,1 % sodium dodecyl sulphate (SDS). The homogenate was then filtered through cheese-cloth. The filtrate was centrifuged at 6,000 g for 40 min. Polyhedra were purified by centrifugation on 30-66 % (w/v) linear sucrose gradient. Inclusion bodies bands were collected and washed twice with distilled water. The virus pellet was resuspended in a small volume of H_2O and stored at -18° C.

Dosage- mortality assays

Leaf discs of apple leaves (1cm in diameter) were prepared using a cork borer, measured dose of virus were applied to leaf discs (Evans, 1981), then dried at room temperature. Using fine forceps, the dried discs were transferred, each in a well of the bioassay plate. Single third or fourth instar neonate larva was fed to each disc. After one day larvae were transferred to fresh, virus free foliage. The bioassay treatments were kept under constant conditions, 25 C°, 60-70% RH and 16 hour light, for 16 days. Mortality due to infection was recorded daily and up to 17 days. Experiments were repeated 5 times (20 larvae in each replica). Virus activity was expressed as the percentage mortality caused by the virus (Finney, 1971).

Evaluation of the yield of virus polyhedrae

Third instar *M. neustria* larvae fed on leaf discs with different virus doses were individually homogenised. The polyhedrae count per larva was determined by a haemocytometer.

Results

Biological activity of Mn NPV isolates were characterized by a bioassay. If third instar *M. neustria* larvae were infected, the rate of mortality reached 10%, 27%, 46%, 60%,69%,78%, 90%,100% for the tested doses of $5x10^1$, $1x10^2$, $5x10^2$, $1x10^3$, $5x10^4$ and $1x10^5$ polyhedrae/larva of MnNPV isolate (Saldus), respectively. LD_{50} values determined were 55 ± 10 and 985 ± 19 polyhedral occlusion bodies (polyhedrae) per larva for second and third instar, respectively. The rate of mortality caused by MnNPV isolate (Dobele) reached 10%, 18%, 37%, 40%, 48%, 64%, 82%,98% for the tested doses of $5x10^1$, $1x10^2$, $5x10^2$, $1x10^3$, $5x10^4$ and $1x10^5$ polyhedrae/larva, respectively. LD_{50} values were 80 ± 30 and 3280 ± 380 polyhedral occlusion bodies (polyhedrae) per larva for the second and the third instar, respectively. The relationship between time - dose and mortality for the virus isolates is shown in Figure 1 A,B.

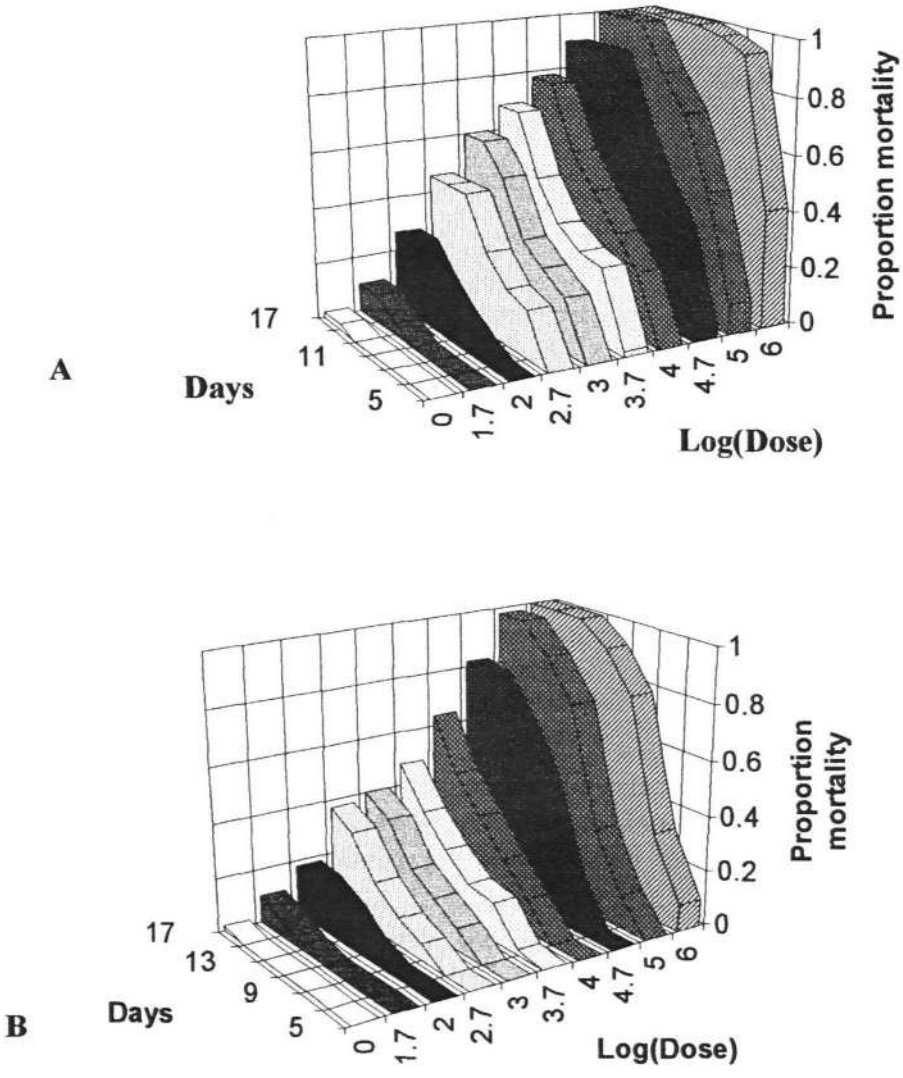

Figure 1. Relationship between cumulative proportion mortality, viral dose, and mortality time of *Malacosoma neustria* third instar larvae. Two isolates were used for bioasays. A-Mn NPV isolate (district Saldus), B - Mn NPV isolate (district Dobele).

The bioasays showed that incubation period of disease was 5 to 10 days after infection with different doses of Mn NPV.

Data in Table 1 show that the amount of NPV polyhedrae produced or virus yield per larva was $2,1x10^9$ to $9,82x10^9$ viruses/larva after feeding insects on leaves containing different virus doses. Estimated LT_{50} was 6.1 to 14.5 days.

Table 1

Effect of initial virus concentration on LT_{50} and obtained virus yield of Mn NPV in *Malacosoma neustria* 3rd instar larvae

Tested doses	Mortality corrected after Abbot, %	$LT_{50} \pm sLT_{50}$, days	Day of yield collection	Average yield/larva
$1x10^3$	62,8	$14.5 \pm 0,7$	8	$9.82x10^9$
$1x10^4$	78,6	11.9 ± 0.45	8	$3.84x10^9$
$1x10^5$	99	$8.4 \pm 0,26$	8	$2.87x10^9$
$1x10^6$	99	6.1 ± 0.17	8	$2.12x10^9$

Addition of plant lecitine (conc.1%) to low doses virus suspension increase larval mortality from 50% to 89%.

Discussion

Morphological characteristics of obtained isolates showed that the dimensions of isolated polyhedra were 850 to 1400 nm. Polyhedrae contained large number of rod-shaped multiple virions (Jankevica et al., 1998). The results of our experiments showed that tested isolates had different virulence. Isolate obtained in district Saldus had higher virus activity and shorter incubation period of disease. The increasing of virus doses caused decrease of the incubation period of disease and the time until insects death.

The comparison of biological properties of both isolates concurs with the DNA analysis (Jankevica and Jankevics, unpublished) that showed differences between isolates.

Results obtained in the experiment performed to evaluate the effect of initial virus concentration on obtained virus yield of Mn NPV in *Malacosoma neustria* 3rd instar larvae does not differ significantly from the conclusions of Rituma and Skujāne (1983), and Im et all. (1989), that to obtain maximum quantity of polyhedrae, it is purposeful to infest third or fourth instar larvae. In the case of using the MnNPV (Saldus) isolate and dose input of $1x10^3$ polyhedrae/larva, the highest yield per larva was obtained with the value of $9.8x10^9$ polyhedrae/larva which decreased to $3.8x10^9$, $2.8x10^9$ and $2.1x10^9$ polyhedra/larva for the tested concentrations of $1x10^4$, $1x10^5$ and $1x10^6$ polyhedra/larva, respectively. The virus yield was maximal on the 8th day postinoculation.

We conclude that the tested virus isolates showed high virulence and can be used as a source of virus insecticide to control populations of the European tent caterpillar.

Acknowledgments

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Kopsavilkums

Latvijas Universitātes Bioloģijas institūta Eksperimentālās Entomoloģijas laboratorijā no ievāktu slimu kāpuru materiāla, kas bija ievākts Saldus un Dobeles rajonos, tika izdalīti *Malacosoma neustria* kodolu poliedrozes vīrusa (MnKPV) izolāti. Tika salīdzināta divu izolātu virulence un produktivitāte. Noskaidrotas attiecības starp vīrusa devu, laiku un kāpuru kumulatīvās mirstības proporcionalitāti. Mn KPV (Saldus) izolāts uzrādīja augstāku virulenci.
Veiktie pētījumi ļāva secināt, ka analizētie MnKPV izolāti var tikt izmantoti izstrādājot jaunas vīrusinsekticīda formas.

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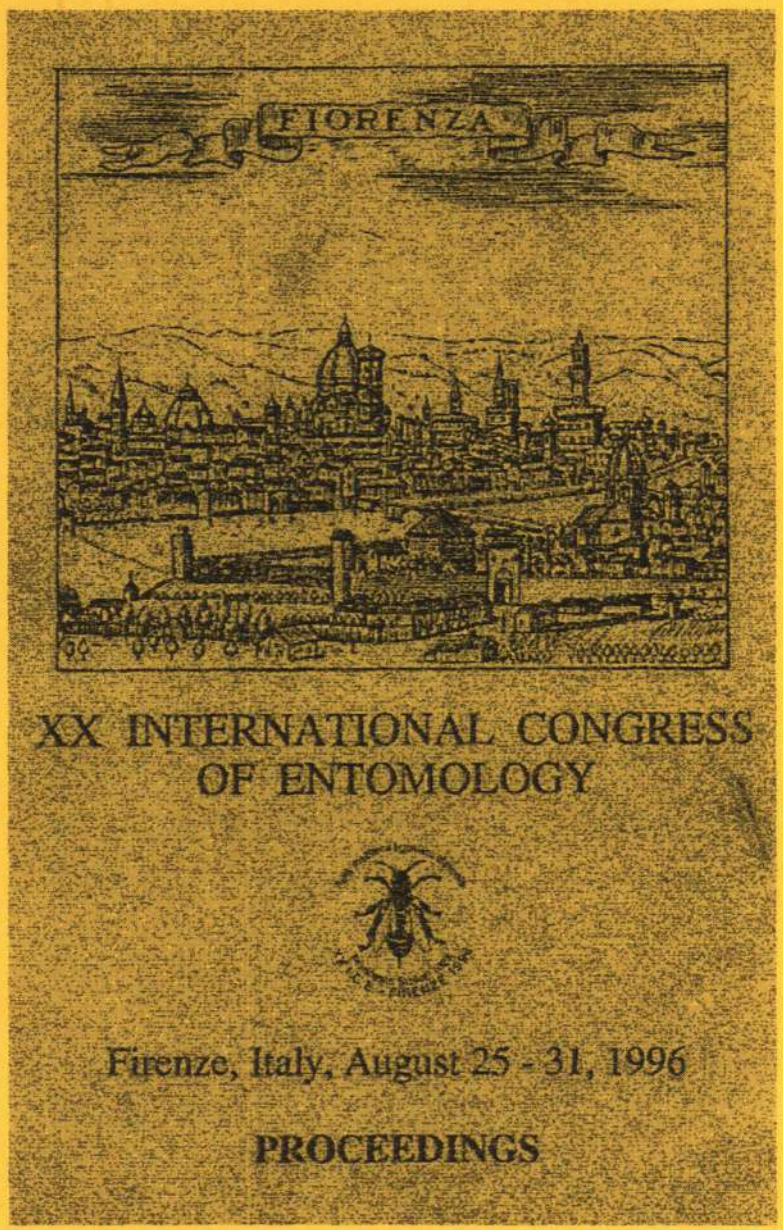

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FORMATION OF OCCLUSION BODIES OF NUCLEAR POLYHEDROSIS VIRUSES IN THE INFECTED CELLS OF *MALACOSOMA NEUSTRIA* L. (LEPIDOPTERA: LASIOCAMPIDAE).

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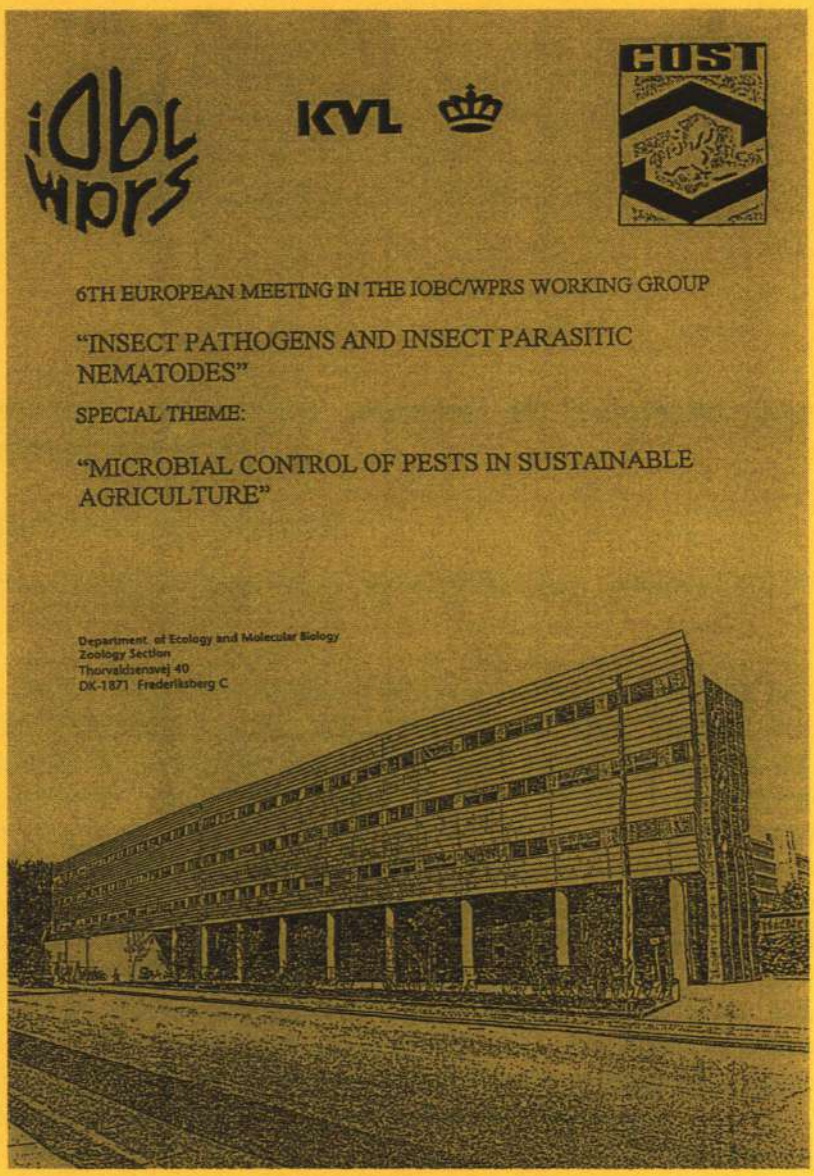
Baculoviruses rather rarely cause epizootics in pest populations under Latvian climatical conditions. Nuclear polyhedrosis viruses have been isolated from 7 insect species. *Malacosoma neustria* L. has been used as a model insect in the cytological researches.

Infectional process of *M. neustria* caused by the Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis viruses (Mn NPV) was investigated. The LD50 values were 75 and 985 polyhedral occlusion bodies (polyhedrae) per larvae of the second and third instars, respectively. Tissue and cells in the latest stage of the disease after per os infection content a large amount of viral polyhedrae. Electron microscopy shows that at the 6th day after infection polyhedrae, multiple virions and nucleocapsids are visible in the nucleus of gut epithelial and fat cells. The dimension of polyhedra is 900-5000 nm. Rod-shaped nucleocapsids are 220 - 360 nm in length and 30 - 60 nm in width. Polyhedrae of the MnNPV Latvian isolate content multiple virions, which content 2-6 nucleocapsids each. The development process of the membrane of virions and polyhedrae has been investigated. In particular cases polyhedrae have been observed with necrotical changes.

Obtained results of the natural way of polyhedrae formation will give the possibilities to estimate the influence of substances used in virus preparation, on this process. The influence of inhibitors and stimulators will also be determined.



Čudare Z., Jankevica L. 1997. Accumulation and vertical migration of* Malacosoma *neustria nuclear polyhedrosis virus in soil. In: 6th EU meeting in the IOBC/WPRS working group, Microbial control of pests in sustainable agriculture. KVL, Copenhagen, Danmark, 10-15 August. Abstr.

Accumulation and vertical migration of *Malacosoma neustria* nuclear polyhedrosis virus in soil.

Zigrida Čudare, Līga Jankevica

Institute of Biology, University of Latvia, Miera iela 3, Salaspils LV-2169 LATVIA

In context of sustainable agriculture, it is necessary to maintain the environmental potential (beneficial insects, entomopathogenous fungi, bacteria, baculoviruses) in agrocenoses that diminishes the multiplying and spreading of plant pests. The use of entomophagous and entomopathogenic microorganisms in plant protection provides the possibilities to diminish or substitute the spraying of chemical insecticides. Using new effective biological plant protection methods, the pollution of the environment is gradually diminished. The use of the baculoviruses in plant protection is one of the preconditions for producing competitive agricultural production using scientifically well founded system of forecasts and recommendations.

The aim of the studies was to investigate accumulation and dispersal of *Malacosoma neustria* nuclear polyhedrosis virus (Mn NPV) in soil to provide the necessary background for developing an effective strategy of biological control for exploiting the pathogens, already present in the ecosystem.

Nuclear polyhedrosis viruses cause the disease of *Malacosoma neustria* larvae in warm springs in Latvia. The Latvian isolate of Mn NPV isolated in Laboratory of Experimental Entomology was tested. Investigations of Mn NPV dispersal, accumulation and persistence of their pathogenicity in the soil showed that the virulent polyhedrae can leach through 25 cm layer of soil. Leaching of the polyhedrae through sand, peat and loamy soil was compared in model experiments. Less polyhedrae leach through loamy soil, because loam can absorb them. The leached polyhedrae maintained their ability to reproduce in larvae of *M. neustria* causing the mortality 68 % to 100 %. The amount of macro,- and microelements in the loamy soil, did not influence the pathogenicity of Mn NPV essentially. Rainfalls influenced vertical transfer of the viruses and provide their dispersal in soil. The pathogenicity and the reproduction ability of viruses does not differ in different soil types for 1 month.

XXIV
Nordic Congress of Entomology

Tartu, Estonia, 8–11 August 1997

Excursion to South-Estonia on 7 August 1997
Excursion to the island of Saaremaa on 12–16 August 1997

PROGRAM & ABSTRACTS

LIST OF MEMBERS

University of Tartu
Institute of Zoology and Hydrobiology
&
Estonian Naturalists' Society

Jankevica L., Tenbergs G., Čudare Z. 1997. Occurrence of pathogens and parasites in the populations of Malacosoma neustria L. (Lasiocampidae) in Latvia. In: XXIV Nordic Congress of Entomology. Program& Abstracts, Tartu, Estonia, 8-11 August, 21

Occurrence of Pathogens and Parasites in the Populations of *Malacosoma neustria* L. (Lasiocampidae) in Latvia

Jankevica L., Tenbergs G., Čudare Z.
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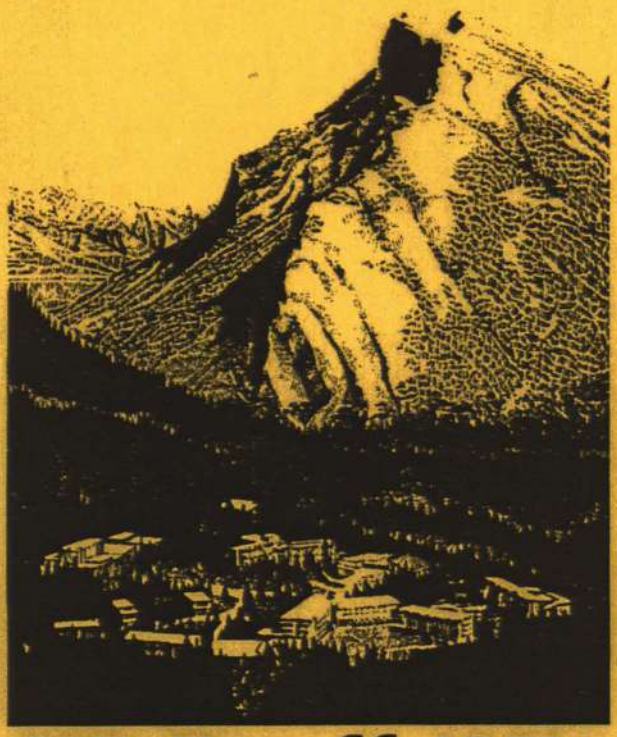
Miera street 3, Salaspils LV-2169, LATVIA

The use of the enthomopathgenic microorganisms and parasites in plant protection is one of the preconditions for producing competitive agricultural production using scientifically well founded system of forecasts and recommendations. It is necessary to investigate the occurrence of entomopathogens and parasites to secure their remaining in biocenoses for prolonged limiting of plant pests with the help of this environmental potential. The European tent caterpillar (*Malacosoma neustria* L.) is widely represented in the biocenosis of apple-garden and is limited by wide range of natural enemies.

The aim of studies was to observe *M. neustria* populations, to search for pathogens and parasites and to increase knowledge concerning their occurrence and environmental effects. Randomly collected 2nd instar larvae were reared on natural food in special cages (0.5 x 0.35 x 0.35 m) under laboratory conditions to determine the presence of pathogens and parasites. In Jelgava's district a population of *M. neustria* was observed in a well producing apple-garden where no insecticides had been used. We determined that 27 % of *M. neustria* collected there were infested by the parasitic flies (*Tachinidae*). Approximately 10 % of the larvae were infected by *Malacosoma neustria* nuclear polyhedrosis viruses (*Baculoviridae*) and 6 % by microsporidia (*Microsporidia*). Mixed infections were found in 9 % of dead larvae.

We did not detect any parasites and pathogens in the observed population isolated naturally on the island in the restricted Lake Engure in Talsi district.



SIP Banff '97

Society for Invertebrate Pathology

Program and Abstracts

30th Annual Meeting

The Banff Centre
Banff, Alberta, Canada
24-29 August, 1997

sip

Jankevica L, Čudare Z., Jankevics E. 1997. Determination of** Malacosoma neustria **nuclear polyhedrosis virus in the ecosystem and in pest populations after virus application. In: Programm and Abstracts, 30th Annual SIP Meeting. Banff, Alberta, Canada 24-29 August. (A3) 33

Tuesday, STUDENT POSTER A3

Determination of *Malacosoma neustria* nuclear polyhedrosis virus in the ecosystem and in pest populations after virus application

L. Jankevica[1], Z. Čudare[1], and E. Jankevics[2]

[1]Dept. of Experimental Entomology, Institute of Biology, University of Latvia, Miera str. 3, Salaspils, LV 2169, Latvia; [2]LU Biomedical Research and Study Center, Ratsupites str. 1, Riga, LV 1067, Latvia.

Malacosoma neustria nuclear polyhedrosis virus (Mn NPV) is a highly active and selective agent for the control of the European tent caterpillar *Malacosoma neustria* (Lasiocampidae) that is widely represented in apple-gardens in Latvia. It is important to estimate the possibilities of baculoviruses to accumulate in the environment and subsequently to initiate epizootics. To improve the biological control of a *M. neustria* population, we have estimated the persistence, distribution and accumulation of NPV in biocenosis and in the *M. neustria* population.

The aim of our studies was to estimate dispersal and persistence of Mn NPV after aple-trees treating with virus insecticide formulations developed in our laboratory. Previously we used specific- DNA-DNA hybridization assay and a bioassay to detect MnNPV on the foliage and in soil. The weekly loss of polyhedra determined by the specific DNA-DNA hybridization on apple-tree leaves varied from 20 to 60% in variants with additives; in the control (virus in water suspension without additives) the loss was 80%. We have detected that the polyhedra can leach through at least 20 cm layer of garden soil, if the amount of precipitations exceeds 100 mm.

Present study demonstrates the use of Polymerase chain reaction (PCR) for identification of nuclear polyhedrosis virus in several developmental stages of insects (egss, larvae, imago and coccons). Primers based on polyhedrin gene were designed and synthesized. The set of primers were used to amplified 505 bp of the polyhedrin gene. The PCR procedure will be optimized, to use it for identification and characterization of individual virus strains. New improved methods for virus identification will be used to describe the natural occurrence of NPV in pest populations and the homology of virus population.

Monday, 11:00 SYMPOSIUM

Molecular typing of fungi: An industrial perspective

Stefan T. Jaronski

Mycotech Corporation, Butte MT

Industry's needs for molecular typing technology are several fold. First of all, a molecular "fingerprint" of a registered fungus is needed to supply to regulatory agencies, eg., the U.S. Environmental Protection Agency. Such agencies generally recognize the uniqueness of deuteromycete fungal strains, and thus need a tool to definitively identify a registered active ingredient and differentiate it from other similar products. Similarly, a company with a registered fungal product needs to be able to protect its investment by being able to conclusively differentiate its active ingredient from other, competing products.

Manufacturing facilities concerned with more than one strain of the same fungal species need a diagnostic quality assurance tool to differentiate strains. This tool needs to be rapid, i.e., take less than 48-72 hours, and be able to consistently discriminate among strains of the same fungal species.

During field trials it is often necessary to be able to differentiate between a released fungal strain and the background population. Again, good, consistent intraspecific differentiation is required, but with perhaps not as short a response time as in Quality Assurance. In this situation a large sample burden is often the case, eg., a large number of colonies from soil dilution platings from replicate samples from a number of treatments.

Thursday, POSTER B41

Persistence of *Beauveria bassiana* conidia on lower and upper leaf surfaces of cantaloupe (*Cucumis melo* L.) and cotton (*Gossypium hirsutum* L.)

Stefan T. Jaronski, J. C. Lord, and J. Rosinska

Mycotech Corporation, Butte MT

Conidia of *Beauveria bassiana* (Mycotech Strain GHA) were applied to individual upper and lower leaf surfaces of cantaloupe and cotton plants grown under normal agronomic practice in Brawley CA (cantaloupe) and Phoenix AZ (cantaloupe and cotton) in May-July 1994, and June 1995. Treated leaves were collected at intervals, the spores eluted into water and conidial viabilities determined by plating the suspensions on Sabouraud dextrose agar. Corresponding solar irradiance data was derived from nearby meteorological stations.

In cantaloupe having a thin canopy the half-life of conidia on lower leaf surfaces ranged from 4.5 to 9 days; on the upper surfaces conidia rapidly lost viability for a half-life of 1 day. In cantaloupe having a dense canopy, and thus considerable lateral shading, conidia on lower leaf surfaces suffered little loss in viability during the observation period of 10 days; a half-life could not be calculated. On cotton leaves (in somewhat exposed upper third of the canopy) conidial persistence was much shorter, with a half-life of 2 days on the lower and 1 day on the upper leaf surfaces.

Thursday, POSTER B42

Field evaluation of the effects of *Beauveria bassiana* Strain GHA on the hymenopterous whitefly parasite, *Eretmocerus* sp. in commercial melons

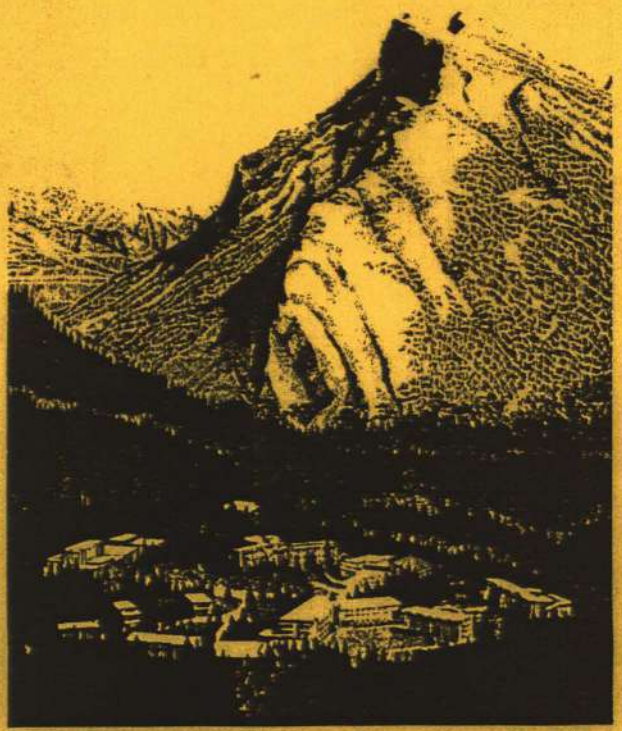
Stefan T. Jaronski[1], J. C. Lord[1], G. Simmons[2], and K. Hoelmer[2]

[1]Mycotech Corporation, Butte MT; [2]USDA/APHIS, Brawley CA

Artificially augmented field populations of *Eretmocerus sp.* (Padappai India strain, APHIS, Mission, Texas, Biological Control Laboratory (MBCL) Culture #M92019) were exposed to Mycotrol WP, which contains the fungus *Beauveria bassiana* Mycotech Strain GHA. The test was conducted in two commercial cantaloupe fields in Imperial County, California, during the Spring of 1995, and involved several weeks of wasp releases onto 0.75 acre plots, followed by three applications of Mycotrol WP, at 1 lb per acre, or carrier alone onto replicated subplots, at five day intervals. No comparisons with chemical insecticide treatments were made.

At one site, differences in the number of *Eretmocerus* in untreated, carrier-treated and *Beauveria*-treated plots were not statistically significant on each of two sample dates. The percent parasitism was numerically higher in *Beauveria*-treated plots than in control plots, while peak whitefly population reduction by the fungus was 71-72%.

In the second site numerical differences among treatments in both parasite numbers and percent parasitism were not statistically significant. Whitefly control from the fungus applications was only 43-44%. A low, variable percentage of parasitized nymphs were overtly infected by the fungus. In summary, even though parasites can be infected by the fungus, large percentages of parasites survived field use of *B. bassiana* in cantaloupe and the overall levels of parasitism were unaffected, which may have been at least partly due to an increase in the parasite to host ratio.



SIP Banff '97

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Program and Abstracts

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Tenbergs G., Jankevica L., Zarins I. 1997. Persistence of Neodiprion sertifer nuclear polyhedrosis virus after the application of virus preparations. In: Programm and Abstracts, 30th Annual SIP Meeting. Banff, Alberta, Canada 24-29 August. (A6) 62-63

Monday, 5:15 STUDENT

The novel toxicity of *Bacillus thuringiensis* against some harmful species belonging to Nemathelminthes, Protozoa and Platodes

Ming Sun[1], Zhaohui Xu[1], Baoan Yao[2], and Ziniu Yu[1]

[1]Dept. of Microbial Science and Technology, [2]Faculty of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei 430070, P. R. China

Some strains of *Bacillus thuringiensis* with toxicity to *Dirofilaria immitis* microfilariae (Nemathelminthes), *Plasmodium berghei* (Protozoa) or *Schistosoma japanicum* (Platodes) were isolated from Chinese soils. Strain YBT-032 shows toxicity to filaria.

The toxin is a kind of water soluble, dialyzable and unstable low molecular weight metabolite. The mortality may up to 100% within 24 hr *in vitro*. While the δ-endotoxins of strain YBT-007, YBT-021 and YBT-032 played the leading roles in controlling plasmodia. Mice carrying plasmodia can survive one week longer or so than control when treated with solubilized crystal proteins through intravenous injection. Blood smears also showed that the ability of plasmodia infecting erythrocytes was decreased with postponing infection four or five days compared with control.

Strain YBT-008, YBT-032 and YBT-037 were toxic to *Schistosoma*. The moralities of cercariae or adults of *Schistosoma* were up to more than 90% within 24 hr if they were treated in vitro with solubilized crystal proteins. Tests *in vivo* with mice also displayed that these toxins postponed the symptoms and prolonged the life expectancy of mice.

The further study is being undertaken.

Tuesday, POSTER A45

Production of fruiting body using cultures of entomopathogenic fungal species

J. M. Sung, H. K. Lee, Y. O. Kim, S. H. Kim, Y. S. Choi, and G. H. Sung

Dept. of Agricultural Biology, Mycological Lab., Kangwon National University, Chuncheon 200-701, Korea

One hundred and six *Cordyceps* cultures including five cultures of *Paecilomyces tenuipes* were used for production of artificial fruiting body. In the test of artificial fruiting body formation, no fruiting bodies were induced on media containing PDA and ground silkworm pupae with liquid nitrogen. And the best fruiting body formation was showed on unpolished rice media containing three and half fold water to rice grain and long polished rice containing two and half fold water to rice grain. Optimum temperature in inducing artificial fruiting body was at 20°C. Twenty seven isolates were selected as good cultures for production of artificial fruiting body. Maturation of fruiting bodies incubated on rice grain media was completed for about 50 to 65 days.

Friday, 9:15

Modes of inheritance of resistance to *Bacillus thuringiensis* toxins in diamondback moth

Bruce E. Tabashnik[1], Yong-Biao Liu[1], Luke Masson[2], David G. Heckel[3], Thomas Malvar[4], and Juan Ferré[5]

[1]Dept. of Entomology, University of Arizona, Tucson, AZ 85721, USA; [2]Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2; [3]Dept. of Biological Sciences, Clemson University, Clemson, SC 29634, USA; [4]Ecogen Inc., Langhorne, Pennsylvania 19047, USA; [5]Dept. de Genetica, Universitat de Valencia, 46100 Burjassot, Valencia, Spain

Efforts to delay evolution of resistance by pests with two or more toxins from *Bacillus thuringiensis* (Bt) assume that independent mutations are required to counter each toxin. Two other key assumptions are that resistance is recessive and resistance alleles are rare in susceptible populations. We tested these assumptions by conducting single-pair crosses in conjunction with bioassays of diamondback moth (*Plutella xylostella*), the first insect with documented resistance to Bt in open field populations. An autosomal recessive gene conferred extremely high resistance to four Bt toxins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F). A surprisingly high proportion (21%) of the individuals from a susceptible laboratory-reared strain were heterozygous for the multiple-toxin resistance gene. The gene or genes controlling resistance to Cry1C differed from and were not linked to the multiple-toxin resistance gene. The dominance of resistance to Cry1C depended on concentration; inheritance was increasingly dominant as concentration decreased. The results show that the mode of inheritance of resistance to Bt toxins in diamondback moth depended on the toxin. This variation affects the choice of strategies for combating resistance.

Thursday, 4:15

Spray dried formulations of the *Anagrapha falcifera* NPV: shelf life, solar stability and rainfastness

Patricia Tamez-Guerra[1,2], M. R. McGuire[1], B. S. Shasha[1,3], and R. L. Pingel[1,3]

[1]Bioactive Agents Research Unit, USDA- ARS, Peoria, IL., USA. 61604; [2]Dpto. De Microbiología e Inmunlogía, Fac. De Ciencias Biológicas, UANL. A.P. 414. Sn. Nicolás de los Garza, N. L. México. 66450; [3]Dept. Biology, Bradley University, Peoria, IL., USA. 61603

The *Anagrapha falcifera* nucleopolyhedrovirus (AfNPV) has shown potential for use as a bioinsecticide. Environmental conditions such as sunlight and rainfall can affect viral persistence, however, viral activity can be maintained with improved formulations. We produced 15 formulations of AfNPV by a spray drying technique and determined the effect 1) of the spray drying process on virus viability, 2) of different storage temperatures during a period of four months on viability, and 3) of persistence of viral activity after simulated rain or sunlight. The majority of ingredients tested included types of pregelatinized corn flours and kraft lignin. Droplet bioassays with *Trichoplusia ni* neonates were used to determine effect of spray drying and shelf life on activity of AfNPV. Cotton leaf bioassays were used to determine effect of artificial rainfall (5 cm in one hour) and solar stability (8 hours exposure in a Suntest CPS) of formulations. Differences occurred among the formulations after spray drying. Virus spray dried only with lignin did not survive the process well whereas formulations containing pregelatinized flours survived the best. Shelf life studies indicated that formulations containing nixtamalized corn flour, sugar, and oil survived well (90% OAR) for four months. Formulations containing both flour and lignin resisted solar degradation (65% OAR) and rainfall (75% OAR) better than other formulations. We believe these preliminary results demonstrate that natural products can enhance the stability, activity and performance of viral biopesticides.

Tuesday, STUDENT POSTER A6

Persistence of *Neodiprion sertifer* nuclear polyhedrosis virus after the application of virus preparations

G. Tenbergs, L. Jankevica, and I. Zarins

Dept. of Experimental Entomology, Institute of Biology, University of Latvia, Miera str. 3, Salaspils, LV 2169, Latvia

Accumulation of *Neodiprion sertifer* nuclear polyhedrosis virus (Ns NPV) and persistence of its activity in the environment after spraying was estimated depending on virus insecticide formulations. Ns NPV Latvian isolate was used as a source of the virus preparation, and was applied to control the populations of the European pine sawfly *Neodiprion sertifer* (Geoffr.) in Latvia. Novel preparations were successfully used in pest control in years 1992 -1995 in various

regions of Latvia. Field trials in Talsi' and Liepaja's districts with the new virus insecticide formulations' indicated that high levels of mortality - 83 to 92 % of second and third instar larvae could be achieved 10 days after spraying.
Persistence of virus activity on pine needles depending on the used formulations of the virus preparation and ecological conditions has been studied. An optimized method of DNA-DNA hybridization has been used for the evaluation of the amount of virus polyhedrae in the soil and on plant surface. Relation between the persistence of virus activity and time after spraying has been clarified. Positive correlation between the amount of polyhedrae on pine needles and rainfall intensity has been found. In variants of the virus preparation with additives and the amount of precipitation 22 mm per week, the number of polyhedrae diminished 10-30 times, but in controls without additives -- 120 times. We have determined polyhedrae in soil probes taken from 0 - 5 cm and 5-15 cm layers under virus sprayed trees with 95% mortality of sawflies observed.

Tuesday, Virus WORKSHOP

Baculovirus taxonomy: problems, solutions, and limitations

David A. Theilmann

Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada Summerland, B.C. V0H 1Z0 Canada

The recent taxonomy statement released by the ICTV committee has been an attempt to provide consistency to the classification and nomenclature of viruses in general. This has resulted in several changes in the taxonomy statement of the family Baculoviridae. This includes a streamlining of the information defining the characteristics that must be met for a virus to be classified as a family member. The Baculoviridae now consists of the genus Polyhedrovirus, and the genus Granulovirus. In addition, the taxonomy statement has removed the non-occluded baculoviruses as a separate genus. Future problems for classification of the Baculoviridae include defining what constitutes the "same" virus (species concept) and nomenclature based on the name of the virus host species.

Tuesday, 5:30

Expression of a cytolysin gene from *B. thuringiensis* variety *medellin* in an acristalliferous *B. thuringiensis* and in *B. sphaericus* toxic strains

Isabelle Thiery and Armelle Delécluse

Institut Pasteur, Bactéries Entomopathogènes, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France.
Fax : 33 1 40 61 31 83, Email: ithiery@pasteur.fr

Bacillus thuringiensis variety *medellin* is highly toxic to mosquito larvae and belongs to class 2 of the mosquitocidal toxins, after *Bti*. The only *Btmedellin* protein related to *Bti* toxins is the 30kDa polypeptide. The gene encoding this cytolysin, called Cyt1Ab1, has been isolated and its genetic environment studied. The sequence of the Cyt1Ab1 protein is 86% identical to that of the Cyt1Aa1 protein. The *cyt1Ab1* gene is flanked upstream by a *p21* gene, in the same orientation, encoding a 21,370-Da protein that shows 84% similarity to the P20 protein from *Bti*, and downstream, on the opposite strand, by a sequence showing 85% identity to the IS*240*A insertion sequence. Hemolytic and mosquitocidal activities of Cyt1Ab1 protein expressed in a crystal-negative *B. thuringiensis* has been compared to that of Cyt1Aa1. Purified Cyt1Ab1 crystals are as hemolytic as those of the Cyt1Aa1 protein. Cyt1Ab1 protein is 4 times less toxic than Cyt1Aa1 protein and 150 times less than the *Btmedellin* native crystals on *Culex pipiens* larvae. Introduction of the *cyt1Ab1* gene in *Bacillus sphaericus* has been performed in order to enhance the larvicidal activity of *B. sphaericus* and to avoid risk of larval resistance to the binary toxin by addition of other toxins. *B.sphaericus* strains 2362, 2297, Iab 59, Iab872 and Iab881 have been transformed by electroporation with a shuttle vector pMK3 containing the *cyt1Ab1* gene and its environment. Larvicidal activity of the different recombinant *B. sphaericus* strains expressing both cytolysin and binary toxin will be discussed.

Thursday, 10:05 SYMPOSIUM

Application of ecology to the practical use of mycoinsecticides

Matthew Thomas[1] and Simon Wood[2]

[1]Leverhulme Unit for Population Biology and Biological Control, NERC Centre for Population Biology, Imperial College, Silwood Park, Ascot, Berks SL5 7PY, UK; [2]The Mathematical Institute, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9SS, UK

Diseases can have dramatic effects on populations of insects in both natural and agricultural settings. However, despite their importance, the dynamics of insect-pathogen interactions remain relatively poorly studied. In particular, studies in which theoretical approaches are linked or supported with quantitative empirical data are rare. Thus, whereas biological control based on predators and parasitoids has available to it a large and established body of relevant theory, biological control using pathogens has only a limited theoretical basis. At the same time, because of environmental concerns and increasing insecticide resistance, the opportunities for using conventional chemicals for insect pest control are becoming more and more restricted and the interest in the potential of insect pathogens for biological control, particularly as biorational pesticides, has been stimulated by recent developments in molecular biology and biotechnology and is growing rapidly. Thus, efforts to improve understanding of host-pathogen dynamics and ecology are now both necessary and relevant.
This paper describes how ecological approaches are being applied in an on-going biological control programme which is developing a fungal-based biopesticide product for control of locusts and grasshoppers (the LUBILOSA programme). The paper does not attempt to address insect-pathogen interactions from a broad theoretical perspective but instead, focuses on how basic ecological techniques are being used to help interpret and predict the impact of spray applications and, in more general terms, how this is contributing to a greater appreciation of both the similarities and the differences between chemical pesticides and their biological counterparts.

Thursday, 5:00

Unlocking the catch - effects of expressing an active Cry1 toxin in *E. coli*

Candy Tickner, Claudia Walczak, and Neil Crickmore

School of Biological Sciences, University of Sussex, UK.

In their 1995 paper (Mol. Gen. Genet. 247:482-487) Martens *et al* observed that when a truncated Cry1Ab toxin lacking the first 28 amino acids was expressed in *E. coli* growth of the host cell was severely restricted. The authors postulated that this effect was due to the toxin forming pores in the cytoplasmic membrane. They speculated that the 28 N-terminal amino acids might be acting as a "safety catch" preventing the toxin from inserting into inappropriate membranes until it had been proteolytically removed in the gut of the target insect.
We have demonstrated a similar phenomenon when expressing a Cry1C fragment (amino acids 28-627) in *E. coli*. When induced it has a dramatic effect on cell growth which can be quantified. Data will be presented on the effect of various site-directed mutations on the activity of this fragment which will then be discussed in relation to the Umbrella and Penknife models of toxin action.

THE VIth EUROPEAN CONGRESS OF ENTOMOLOGY
České Budějovice, Czech Republic
August 23-29, 1998

BOOK OF ABSTRACTS

(Proceedings of the VIth European Congress of Entomology)

THE INSTITUTE OF ENTOMOLOGY
ACADEMY OF SCIENCES OF THE CZECH REPUBLIC
in collaboration with
THE UNIVERSITY OF SOUTH BOHEMIA AND THE CZECH ENTOMOLOGICAL SOCIETY

Jankevica L., Tenbergs G. , Jankevics E.1998. Occurrence of nuclear polyhedrosis viruses in the populations of *Malacosoma neustria L.* ***(Lasiocampidae). In: Proceedings of the VI th European Congress of Entomology. Ceske Budejovice, August 23-29, 608-609.***

Abstract of a communication presented at the VI^th European Congress of Entomology, České Budějovice, August 23-29, 1998

Section 7

OCCURRENCE OF NUCLEAR POLYHEDROSIS VIRUSES IN THE POPULATIONS OF *MALACOSOMA NEUSTRIA* L. (LEPIDOPTERA, LASIOCAMPIDAE)

JANKEVICA L.*, TENBERGS G.* and JANKEVICS E.**

*Department of Experimental Entomology, Institute of Biology, University of Latvia, Miera str. 3, Salaspils, LV 2169, Latvia,

** LU Biomedical Research and Study Center, Ratsupites str.1, Riga, LV 1067, Latvia

In nature, nuclear polyhedrosis viruses (Baculoviridae), which are frequently associated with outbreak or declining populations of Lepidoptera, cause diseases of insects and can control the population size of their hosts. Baculoviruses, including nuclear polyhedrosis viruses (NPV), are considered to be safe biological insecticides and have a great potential in pest control. Polyhedrae protect the virus from inactivation by environmental factors. Survival may be achieved by persistence of the polyhedrae in the soil, litter, on leaf surface e.t. or within the pest populations. Generally natural epizooties caused by NPVs have been observed in most areas were host populations reach high density, however epizooties caused by baculoviruses are rather rare in pest populations under Latvian climatic conditions. NPVs have been isolated from just 11 insect species in Latvia. It is necessary to investigate the occurrence of baculoviruses and to provide their remaining in biocenoses for prolonged period to limit the amount of plant pests with the help of this environmental potential. There are a little information on persistence and transmission of viruses in pest populations with low density.

The European tent caterpillar *Malacosoma neustria* L. is widely represented in the biocenosis of apple - gardens in Latvia. Nuclear polyhedrosis viruses have been isolated from several other *Malacosoma* species across North America and Europa (Stairs 1964) and from *M. neustria* (ZARIŅŠ, Kalniņa, 1971), here in Latvia.

The aim of our studies was to observe *M. neustria* populations, to search for nuclear polyhedrosis viruses and to increase knowledge concerning their occurrence and environmental effects.

These studies were based on microscopic examination of larval smears to ascertain the presence of virus polyhedral inclusion bodies. This method is too labour intensive. One alternative to this method is to use DNA hybridization techniques (KEATING et al.1991) or the sensitive technique of DNA amplification by polymerase chain reaction (PCR) to detect viral DNA. We have elaborated a sensitive PCR - based method usable for detecting the presence of viral DNA in the extracts of M neustria larvae. Sequence analysis of the polyhedrin gene of Malacosoma neustria nuclear polyhedrosis virus (Mn NPV) Latvian isolate was done and two sets of primers (20 - mers) based on polyhedrin gene were designed and synthesized. We used nested PCR to increase the sensitivity of determination.

M. neustria populations from two study sites were observed and analysed in 1997. Density of observed populations was low. Virus insecticides are not used in selected study plots. We determined the presence of NPV in M. neustria populations in a well producing apple-garden in Jelgava's district and in a population isolated naturally on the island in the restricted Lake Engure in Talsi district. 2^nd^ and 4^th^ instar larvae collected randomly from natural habitats were analysed. Each individual larva was homogenized. For the extraction of baculovirus and host genomic DNA from individual insect, we used the method described by SAVILLE et al. (1997).

Nested PCR analysis showed the presence of Mn NPV in approximately 21 % of the 2^nd^ and 4^th^ instar larvae collected in the plots in Jelgava's district. Results obtained by microscopy showed

that 10 % of 2[nd] instar larvae were infected with Mn NPV. Comparison of the results obtained by both methods showed that the new method was most sensitive.

We did not detect presence of NPV in the 2[nd] and 4[th] instar larvae collected in population isolated naturally on the island in the restricted Lake Engure.

The method for the detection of polyhedrin - specific DNA sequences using PCR has been proved to have good possibilities for the determination of presence of viruses in pest populations.

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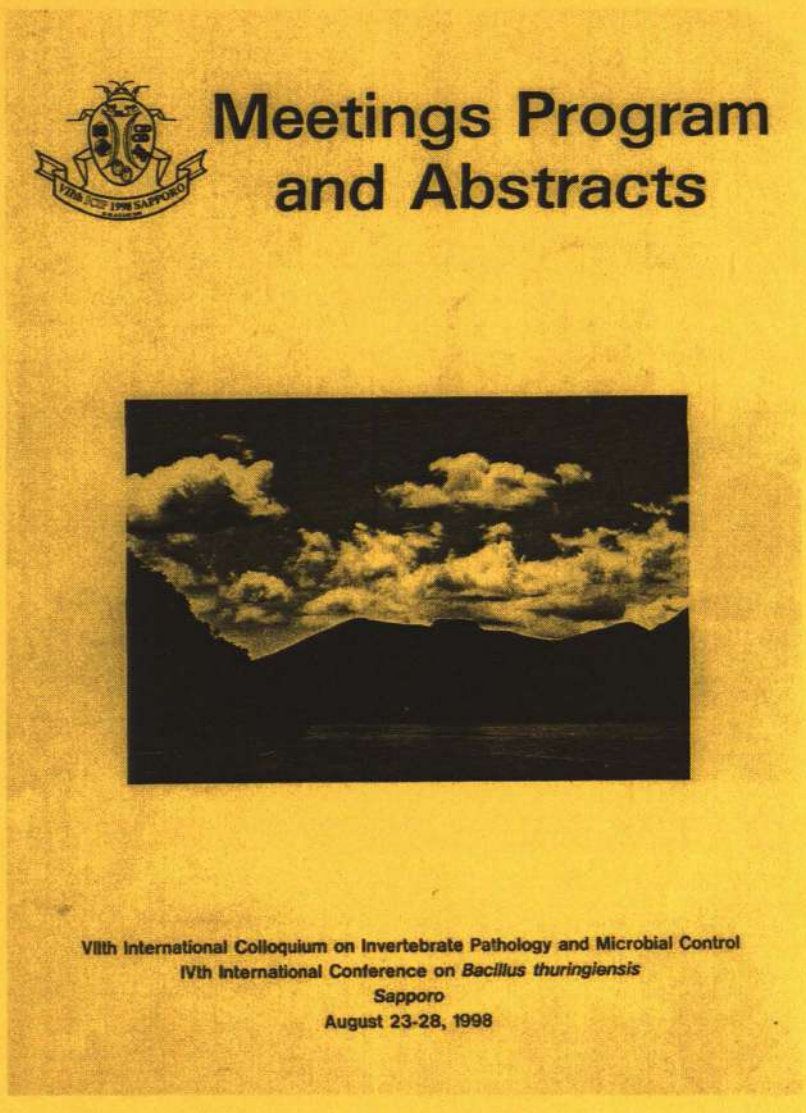

Jankevica L., Cudare Z., Jankevics E. 1998. Use of PCR in detection of *Malacosoma neustria* ***nuclear polyhedrosis virus in pest populations after virus application. In: VIIth International Colloquium on Invertebrate Pathology and Microbial Control, Sapporo August 23-28, 62***

*Bm*CPV dsRNA genome in infected insect cells.

Thursday, POSTERS-Viruses V21-S

Use of PCR in detection of *Malacosoma neustria* nuclear polyhedrosis virus in pest populations after virus application.

L. JANKEVICA[1], Z. Cudare[1] and E. Jankevics[2].

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A Latvian isolate of *Malacosoma neustria* nuclear polyhedrosis virus (*Mn*NPV) is a highly active and selective agent for the control of the European tent caterpillar *Malacosoma neustria* (*Lasiocampidae*) populations. We have estimated the persistence, distribution and accumulation of nuclear polyhedrosis virus (NPV) in the pest populations after treating them with a virus preparation developed and produced in Laboratory of Experimental Entomology. New sensitive and specific methods of virus diagnostics, which can be used for risk assessment of released baculoviruses, are highly required.

Our purpose was the elaboration of a highly sensitive method for detecting *Mn*NPV in the extracts of *M. neustria* larvae and the use of new improved method for detecting *Mn*NPV in pest populations after virus application.

We used a sensitive technique of DNA amplification by polymerase chain reaction (PCR) usable for detecting DNA, and developed a PCR-based method that can detect the presence of polyhedrin-specific *Mn*NPV DNA, in the extracts of *M. neustria* larvae. Therefore sequence analysis of a fragment (1200 bp) of polyhedrin gene of *Mn* NPV Latvian isolate was done. To increase the selectivity of developed method two sets of primers (20-mers) based on polyhedrin gene were designed and synthesized and nested PCR was used. The first set of primers amplified 945 bp, and the second set - 668 bp of the polyhedrine gene. No polyhedrin-specific sequences were detected in DNA of granulosis viruses that was used as negative controls. We determine *Mn*NPV in all instar *per os* infected *M. neustria* larvae. The developed and optimized method showed the presence of *Mn*NPV in second and third instar larvae just a day after treatment (field exsperiment) with the virus preparation. Presence of *Mn*NPV was determined also in the second generation of treated *M. neustria*.

The method for the detection of polyhedrin-specific DNA sequences using PCR has been proved to have good possibilities for the determination of the presence of viruses in pest populations and can be a useful tool for the risk assessment of the releasing of baculoviruses in the environment.

Friday, POSTERS-Viruses V22-S

Monoclonal antibodies to a penaeid rod-shaped DNA virus (PRDV) of *Penaeus japonicus*.

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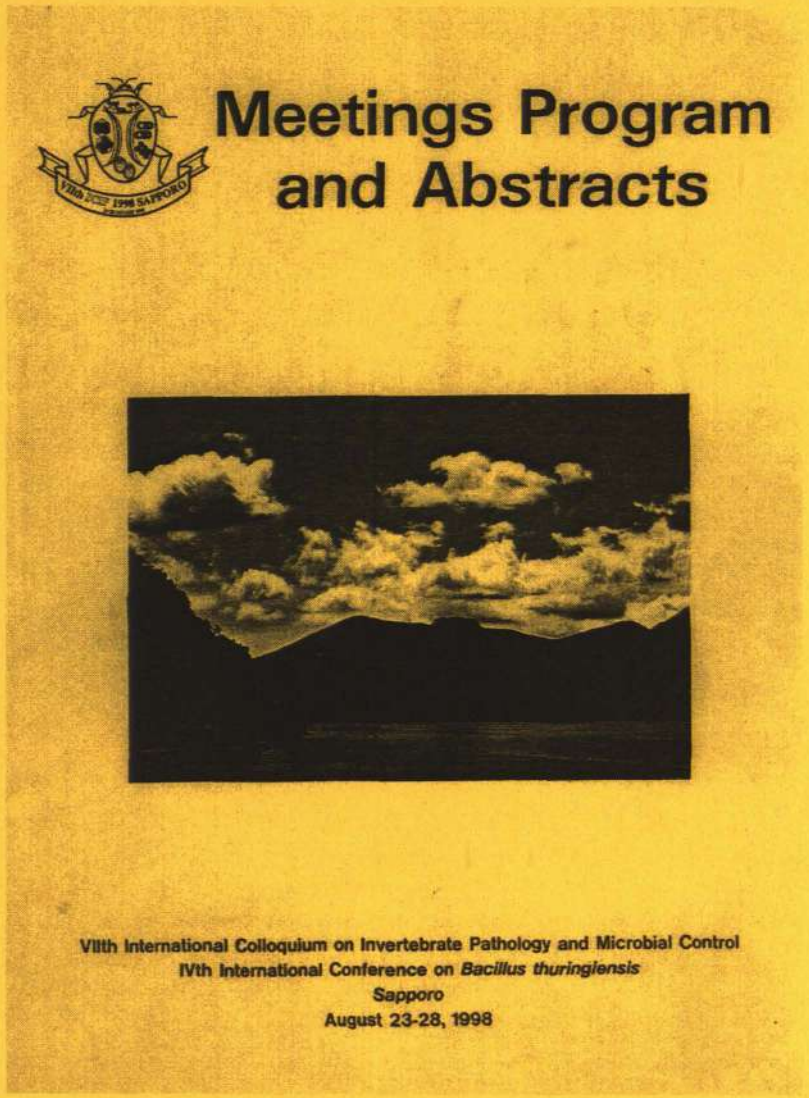
[1] Institute of Applied Biochemistry, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-0004, Japan; [2]Department Microbiology , University of Hawaii at Manoa, 207 Snyder Hall/2538 The Mall, Honolulu, Hawaii 96822 USA

In 1993, a great amount of kuruma shrimp *Penaeid japonicus* in western Japan was found motalized seriously due to the virus infection, which resulted in severe economic loss to shrimp farmers. The causative virus named penaeid rod-shaped DNA virus (PRDV) caused penaeid acute viremia (PAV), and the virus



Tenbergs G., Jankevica L., Zarins I. 1998. Persistence of** Neodiprion sertifer **nuclear polyhedrosis virus depending on used virusinsecticide formulations. In: VIIth International Colloquium on Invertebrate Pathology and Microbial Control, Sapporo August 23-28, 57

In dual infection experiments in BM-N cells with *Ac*NPV and *Bm*NPV, it was found that: 1) when the two viruses were inoculated simultaneously, multiplication of *Bm*NPV overcame that of *Ac*NPV, as judged by the accumulations of viral genomic DNA and structural polypeptides in the infected cells; 2) this overcome of *Bm*NPV multiplication occurred even when *Bm*NPV was superinfected at 2 hr after the *Ac*NPV infection; and 3) when the superinfection with *Bm*NPV was delayed to 4 hr after *Ac*NPV infection, multiplication of *Ac*NPV overcame that of *Bm*NPV. These results indicate that *Ac*NPV is less active than *Bm*NPV to establish productive infection in BM-N cells, but interferes with *Bm*NPVwhen it was added to BM-N cells minimum 4 hr before *Bm*NPV infection.

Thursday, POSTERS-Viruses V4-S

Complete nucleotide sequence and genome structure of the densovirus from smoky-brown cockroach, *Periplaneta fuliginosa* (*Pf*DNV).

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Densovirus (DNV) is an invertebrate parvovirus with the ability of autonomous replication and is widespread pathogen of insects. In 1994, we characterized a small spherical virus newly isolated from the smoky-brown cockroach *Periplaneta fuliginosa* (CSSV), and suggested that it would belong to DNV on the basis of some physicochemical properties of the virus particles.

We here determined the complete nucleotide sequence of the virus DNA. The sequence was 5457 nucleotides long and contained inverted terminal repeats (ITRs) of 202nt at both ends. The end of 118 bases in ITRs can fold into a typical hairpin structure. This terminal structure of the virus DNA is similar to those of other parvoviruses such as adeno-associate viruses, *Bombyx mori* densovirus (*Bm*DNV) and *Junonia coenia* densovirus (*Jc*DNV), suggesting a common mechanism of DNA replication for these parvoviruses.

A computer-assisted sequence analysis suggested that *Pf*DNV resembled *Jc*DNV in genome organization. In one strand, there are 3 ORFs (ORF 1, 2, and 3) which will encode the capsid proteins of *Pf*DNV. The polypeptide encoded in ORF2showed the most significant sequence similarities to the coat proteins of parvoviruses, especially to the VP1 of parvoviruses. In addition, our observation implies the possibility of post-transcriptional and/or post-translational modification in the production of the structural proteins. The complementary also contained 3 ORFs (ORFα,β,γ). The predicted polypeptides encoded in these ORFs showed restricted sequence homologies to the nonstructural proteins of *Jc*DNV and other parvoviruses. Above all, ORFγencoded the polypeptide that showed significant sequence similarity to the parvovirus NS-1. These results indicated that *Pf*DNV would be assigned to the subfamily *Densovirinae*, genus *Densovirus*.

Thursday, POSTERS-Viruses V5-S

Improvement of the *Bm*NPV ie1 promoter.

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Expression vector is important tool for biological analysis and mass production of proteins. Nowadays, the baculovirus vector system is widely used for foreign gene expression in insect cells. The effectiveness of the protein production using the virus vector system is arisen from its high infectivity and the use of the strong promoter that is highly regulated during virus replication. However, the virus replication may, in some cases, cause disadvantages for the functional analyses of the gene products expressed in the cells and/or for mass production of proteins. On the other hand, the expression system using a plasmid vector is usually not so efficient to express foreign genes compared with the virus vector system. One of the critical steps to develop the plasmid-derived foreign gene expression system is to improve the activity of the promoter that is independent of the virus replication. The immediate early gene, ie1, promoter of NPV is a promoter that acts in the host cells efficiently without any viral product. We improved the ie1 promoter of *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) in gene expression activity by manipulating the flanking region of the ie1 promoter sequence. A tandem repeated ie1 promoter sequence was one of the effective constructs to improve the promoter activity. Expression level of a reporter gene from the improved ie1 promoter was 4 to 6-fold higher than that from the original one. Primer extension analysis revealed that the improvement of the promoter activity arose from the increase both of the transcripts from the correct transcription initiation site and the transcription initiation site. Furthermore, the promoter activity was strongly influenced by the sequence just downstream of the transcription initiation site.

Thursday, POSTERS-Viruses V6-S

Persistence of *Neodiprion sertifer* nuclear polyhedrosis virus activity depending on used virus insecticide formulations.

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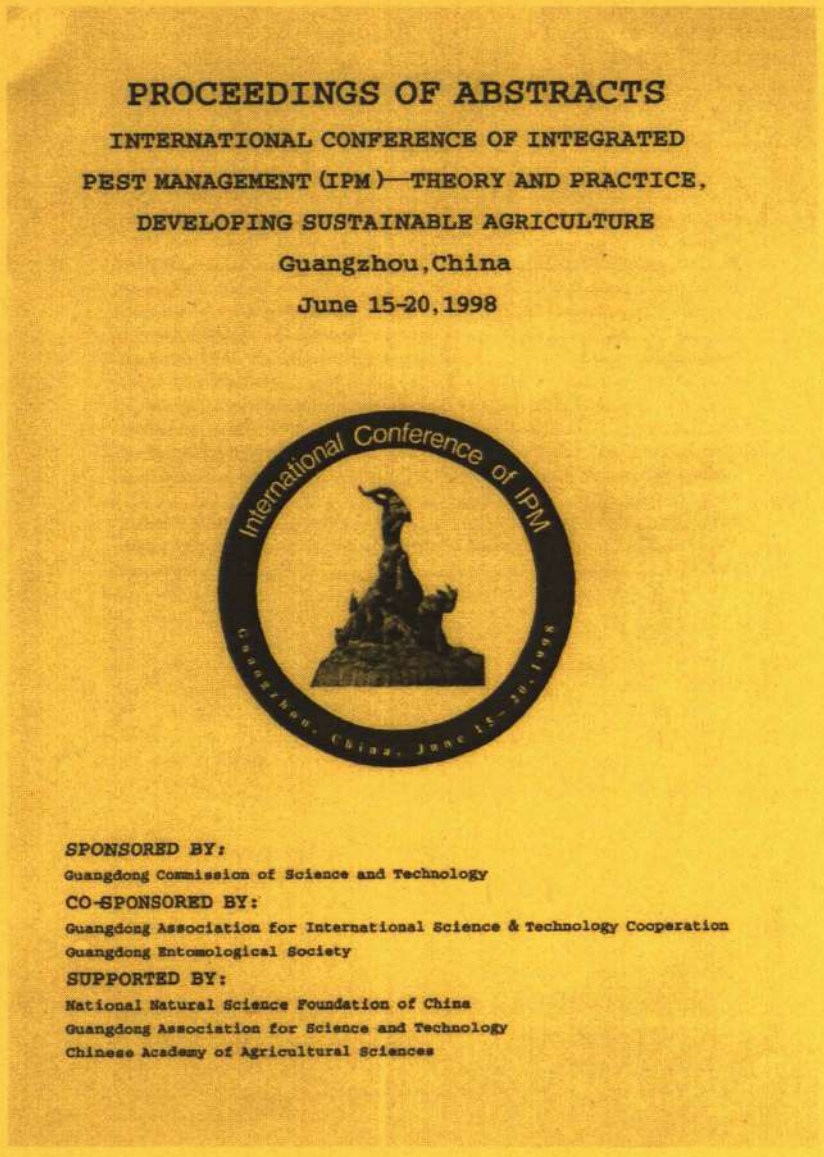
Neodiprion sertifer nuclear polyhedrosis virus (*Ns*NPV) is a highly active and selective agent for the control of the European pine sawfly *Neodiprion sertifer* (Geoffr.) - a serious pest in pine stands in Latvia. *Ns*NPV Latvian isolate was used as a source of the virus preparation applied to control the populations of the European pine sawfly in Latvia. The aim of our studies was to test new virus formulations produced in the Laboratory of Experimental Entomology, that secure the persistence of virus activity. New virus insecticide formulations using environmentally safe matrix materials have been developed and tested. Tested additives had desirable physical characteristics (dispersibility, UVstability, wettability, stickability). Persistence of virus activity after 2 -4 year storage of virus preparation was tested. After two year storage, the decreasing of viral activity was not essential in the variants where novel dried formulations were used. Field tests of the new virus insecticide formulations indicated high mortality levels of *N. sertifer* larvae (84 -95%) 15 days after spraying ($5x10^{10}$ PIB/ha). Persistence of virus activity in the environment after spraying was estimated depending on virusinsecticide formulations and ecological conditions. We estimated the persistence of *Ns*NPV on pine needles. A biotest showed, that virus activity in the variants with novel additives had decreased for 15- 20% after 8 days and for 25- 36% after 15 days (in control without additives 75% and 86%, respectively). Possibilities for using and improving the methods of DNA-DNA hybridization and PCR have been described to determine and evaluate the amount of *Ns*NPV polyhedrae on plant surface.



PROCEEDINGS OF ABSTRACTS

INTERNATIONAL CONFERENCE OF INTEGRATED PEST MANAGEMENT (IPM)—THEORY AND PRACTICE, DEVELOPING SUSTAINABLE AGRICULTURE

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Zariņš I., Jankevica L. 1998. Baculoviruses isolated from horticultural pests and their potential as pest control agents in Latvia. In: International Conference of Integrated Pest Management (IPM) - theory and practice, developing sustainable agriculture, Guangzhou, China, June 15-20, 260

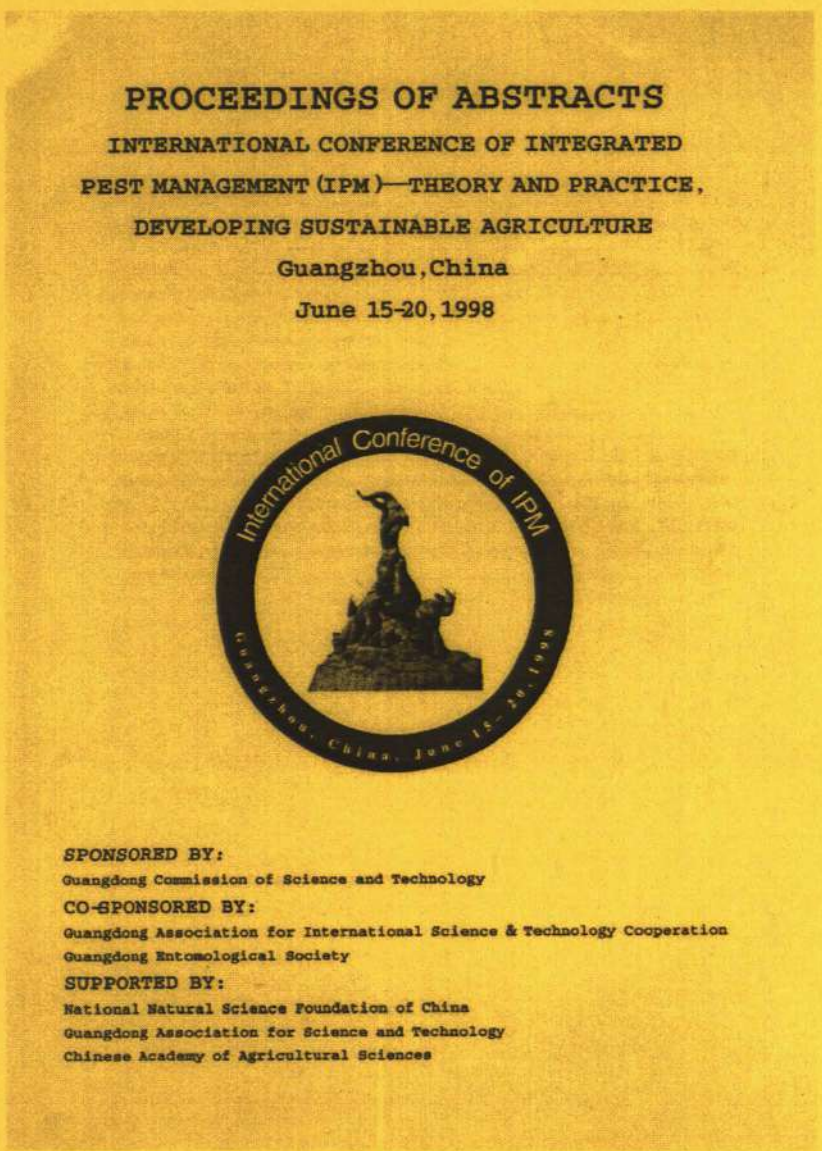
BACULOVIRUSES ISOLATED FROM HORTICULTURAL PESTS AND THEIR *POTENTIAL AS PEST CONTROL AGENTS IN LATVIA*

ZARINS, I. JANKEVICA, L.

Department of Experimental Entomology, Institute of Biology, University of Latvia, Miera str. 3, Salaspils, LV 2169, Latvia

Outbreaks of widespread pest species cause important loses in fruit-growing and horticulture in Latvia. Therefore the aim of our studies was to elaborate new environmentally safe and effective methods of biological plant protection using baculoviruses. Baculoviruses were isolated from the most dangerous pest species and their activity were determined. Experimental strains with high virulence were developed by selection and used as a basis of virus preparations. Nuclear polyhedrosis viruses (NPV) were isolated from the cabbage pest, *Mamestra trassicae* L.; the vegetable pest, *Agrotis segetum* Schiff; fruit-tree pests, *Orgyia antiqua* L., *Malacosoma neustria* L. and *Operophthera brumata* L. Granulosis viruses were isolated from the fruit-tree pests, *Cydia pomonella*, *Hyponomeuta padella* L.; and cabbage pests, *Pieris brassicae* L. and *P. rapae* L. Experimental strains had 20-25% higher efficiency than natural isolates. The novel virus preparations had high efficiency (70-100%) in the climatical conditions of Latvia. NPV isolated from *Leptinotarsa decemlineata* Say had low infectivity (20-30%). We suggest it may be concluded that the novel virus preparations can be used to control pest populations.

Key words: Biological plant protection, nuclear polyhedrosis viruses, baculoviruses



Zariņš I., Jankevica L. 1998. Efficiency of** Pieris brassicae **granuloses viruses isolated in China under environmental conditions of Baltic region. In: International Conference of Integrated Pest Management (IPM) - theory and practice, developing sustainable agriculture, Guangzhou, China, June 15-20, 255

EFFICIENCY OF *PIERIS RAPAE* GRANULOSES VIRUSES ISOLATED IN CHINA UNDER ENVIRONMENTAL CONDITIONS OF BALTIC REGION

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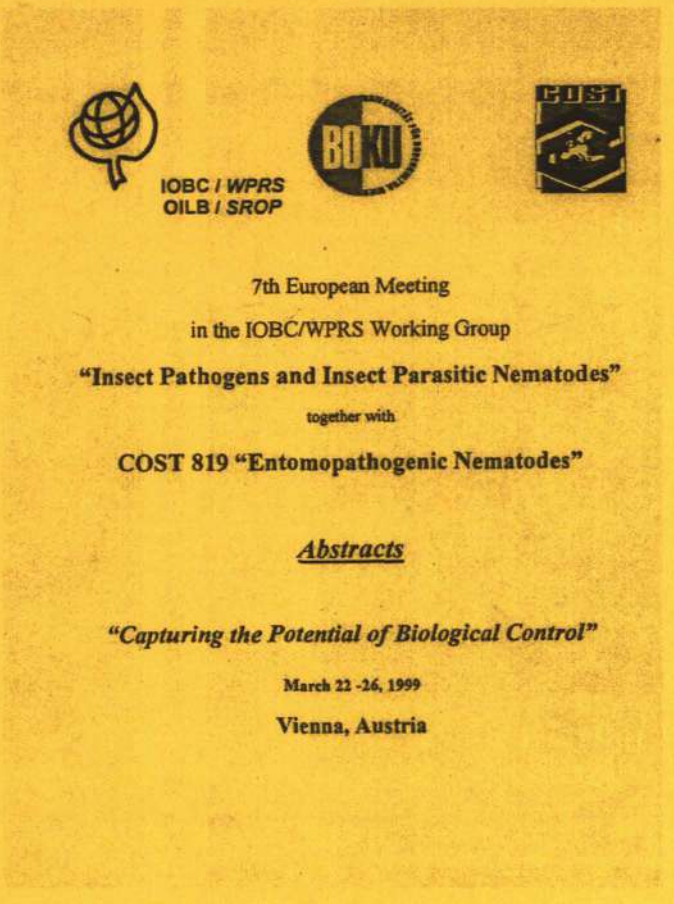
The cabbage white butterfly *Pieris brassicae* L. and cabbage butterfly *P. rapae* L. are widespread dangerous pests of cruciferous plants in Northwest region of Europe and in the People's Republic of China. Granulosis viruses (CV) can be used for successful control of these pests. We have tested the efficiency of virus preparation based on *Pieris rapae* granulosis virus (PrGV) that was produced in the Institute of Virology, University of Wuhan of People's Republic of China under environmental conditions of Latvia. During three years the activity of the virus preparation was tested in field tests in Latvia. Cabbages infested with first and second instar *P. brassicae* larvae were sprayed with virus reparations. The concentration of viruses in working suspensions was 1×10^7 capsules/ml, 150 to 200 l/ha. During the field tests, the average daily temperature was 20-23°C and relative air humidity was 65-75%. Water was used as a control. The mortality of the larvae of *P. brassicae* caused by the preparation produced in China was 50.5% - 68.0%. Incubation period of the infection was 7-10 days, the disease lasted for 6-12 days. The level of larval mortality did not exceed 10% in control.

Key words: *Pieris brassicae, P. rapae,* granulosis viruses



Jankevica L. 1999. Persistence and accumulation of** Malacosoma neustria **nuclear polyhedrosis virus in the ecosystem and in pest population after virus application. In: 7th European Meeting in the IOBC/WPRS Working Group: Capturing the Potential of Biological Control, Vienna, Austria, March 22-26.

Persistence and accumulation of *Malacosoma neustria* nuclear polyhedrosis virus in the ecosystem and in pest populations after virus application

Līga JANKEVICA

Department of Experimental Entomology, Institute of Biology, University of Latvia, Miera street 3, Salaspils, LV 2169, Latvia

Malacosoma neustria nucleopolyhedrovirus (Mn NPV) is a potential agent for the control of the European tent caterpillar *Malacosoma neustria* (*Lasiocampidae).* The Latvian isolates of *Mn* NPV was used as a source of virus insecticide. To improve the successful biological control of a *M. neustria* population, we have estimated the persistence, distribution and accumulation of NPV in biocenosis and in the *M. neustria* population after application of elaborated virus formulations.

The aim of our studies was to estimate dispersal and persistence of Mn NPV after apple-trees treating with virus insecticide formulations developed in our laboratory.

Persistence of virus activity on apple-tree leaves depending on the used formulations of virus preparation and ecological conditions has been studied. Different virus insecticide formulations have been developed and tested. Environmentally safe matrix materials were used. Tested additions had good stickerability to the plants, wettability of dispersible dry formulations, stability against solar radiation. Field trials with new virus insecticide compositions indicated that high levels of mortality 80-96 % of second- and third instar larvae could be achieved 10 days after spraying. An optimized method of DNA-DNA hybridization has been used for the evaluation of the amount of virus polyhedrae on plant surface. Relation between the persistence of virus activity and time after spraying has been clarified. Correlation between the amount of polyhedrae on apple-tree leaves and rainfall intensity has been found. The results of specific- DNA-DNA hybridization and bioassay demonstrate that new virus insecticide formulations secure the persistence of virus 3 weeks after spraying. Weekly loss of polyhedra on apple-tree leafs was 20-60% in variants with additions, in control (virus water suspension without additions) 80%. Bioassay showed that the mean percent of larva's mortality in test variants was 2,5- 3 times higher than in control.

In conclusion the use of novel virus insecticide formulations against the European tent caterpillar can give adequate control for the pest. Owing to virus insecticide's additions the influence of weather conditions on activity of virus was reduced.