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Baculoviruses: A Safe Alternative in Pest Control?

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ABSTRACT

Broad spectrum chemical pest control agents have been widely regarded as ecologically unacceptable. Due to this opinion, there is the increased social pressure to reduce their use in the future and gradually replace them by biopesticides. Viruses of a few families are known to infect invertebrates, but only those belonging to the family *Baculoviridae* have been used for biocontrol of pests. They are safe to people and wildlife, their specificity is usually very narrow. Their application as bioinsecticides was limited until recently because of their slow killing action and technical difficulties for *in vitro* commercial production. Successful protection of over 2 million hectares of soybean in Brazil revived the hopes for using baculoviruses as effective biopesticides. Wider application of baculoviruses for pest control is very likely to be implemented in the future and two approaches for improvements of baculovirus killing properties can be foreseen. In countries where the use of genetically modified organisms (GMOs) is restricted, the improvements will be mainly at the level of *in vitro* production, diagnostics, and changes in biopesticide formulations. In countries that have fewer concerns towards GMOs, the killing activity of baculoviruses may be augmented by genetic modifications of the baculovirus genome. It is expected that baculoviruses improved by genetic modifications will compete successfully with other methods of pest control in many regions of our globe, especially in most densely populated countries of the world.

Keywords: biopesticides, genetic modifications, insect viruses, nucleopolyhedroviruses, safety and advantages
Abbreviations: AaIT, *Androctonus australis* toxin; **AcMNPV**, *Autographa californica multiple nucleopolyhedrovirus*; **AgMNPV**, *Anticarsia gemmatalis multiple nucleopolyhedrovirus*; **BmSNPV**, *Bombyx mori single nucleopolyhedrovirus*; **BV**, budded virus; **CpGV**, *Cydia pomonella granulovirus*; **DIPs**, defective interfering particles; **FP mutants**, Few Polyhedra mutants; **GMO**, genetically modified organism; **GV**, *Granulovirus*; **h p.i.**, hours post infection; **HzSNPV**, *Heliothis zea single nucleopolyhedrovirus*; **kbp**, thousand base pairs; **LdMNPV**, *Lymantria dispar multiple nucleopolyhedrovirus*; **LqhIT2**, *Leirus quinquestriatus hebraeus* toxin; **OB**, occlusion bodies; **ODV**, occlusion-derived virions; **MNPV**, multiple nucleocapsids; **NPV**, *Nucleopolyhedrovirus*; **PCR**, polymerase chain reaction; **SNPV**, single nucleocapsids

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INTRODUCTION

Apart from predators, insects have many less apparent pathogens; these include bacteria, fungi, nematodes and viruses. All of them may effectively suppress pests when applied artificially as microbial pesticides. A great advantage of biological control of this type lies in the fact that it can be potentially permanent. The natural enemies supplied from the outside will often establish themselves in the pest population and are likely to exert long-term protection against the target pest species.

At present bacterial microbial pesticides are the most widely used and cheaper than the other methods of pest bioregulation. Insects can be infected with many species of bacteria but the species belonging to the genus *Bacillus* are the most widely used pesticides. Of these *Bacillus thuringiensis* is the most successful. *B. thuringiensis* has developed many molecular mechanisms to produce pesticidal toxins, most of which are coded for by several *cry* genes (Schnepf *et al.* 1998). There are about 200 registered *B. thuringiensis* products in the USA and at the end of the last century worldwide sales amounted to about 2% of the total global insecticide market. Though the resistance to Cry proteins may develop after prolonged usage, safety considerations favour the future development of these toxins and their share in pesticide market steadily increases.

Although viruses of fifteen families are known to infect insects, those belonging to family *Baculoviridae* have almost exclusively been used as pesticides (Copping and Menn 2000; Lacey *et al.* 2001). Members of this family are regarded as safe to people and wildlife. Their specificity is usually very narrow, and often it is limited to only one species. They have been used in many countries around the world but their application as pesticides was not impressive

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in the past (sales amounted to a few millions of dollars annually). Wider use of baculoviruses as commercial insecticides was restricted because of their slow killing action and difficulties in *in vitro* large scale production. In the past, primary users used to fast-killing chemical insecticides regarded them as ineffective but this attitude changes with time and baculovirus protection becomes a method of choice for long-term protection of crops. The most successful project was carried out in Brazil where over 2.0 million hectares of soybean have been controlled by velvetbean caterpillar baculovirus (Moscardi 1999; Moscardi *et al.* 2002). Following the success of the Brazilian project many countries have begun to increase the area of fields and forests experimentally protected by baculovirus pesticides.

In this review we present programs in which baculovirus succeeded to control agricultural pests as well as the current limitations to large scale production of the virus. Furthermore, we examine the methods which are currently in use to make baculoviruses more efficient biopesticides and we critically analyse the future trends in the development of baculovirus technology.

BIOLOGICAL CHARACTERISTICS OF BACULOVIRUSES

More than 600 baculoviruses isolated from insect orders Lepidoptera, Hymenoptera and Diptera have been reported. Their genome is double-stranded DNA ranging from 80 to about 200 kbp in length. The family *Baculoviridae* contains diverse members and the classification is based mainly on virus morphology. Currently, it is divided into two genera (Fauquet *et al.* 2005): *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV). Recently, a new division has been proposed (Jehle *et al.* 2006) because the comparison of 29 fully sequenced baculoviral genomes indicated that virus phylogeny followed more closely the classification of the hosts than the virion morphological traits. According to this proposal, the classification of the family *Baculoviridae* should contain four genera: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs).

Baculoviruses infect arthropods and they do not replicate in vertebrates, plants and microorganisms. However, though they do not replicate, they may, under special conditions, enter animal cells. After the entrance to mammalian cells they are rapidly inactivated which is a favourable biosafety profile. This unexpected property made them a valuable tool in the last few years for studies of expression of foreign genes under vertebrate promoters introduced into the baculovirus genome (Kost *et al.* 2005). Several aspects of the interactions of a baculovirus with mammalian cells still need to be clarified. It has been shown that baculovirus enters a mammalian cell through a low-pH-dependent endocytic pathway which is primarily clathrin mediated (van Loo *et al*. 2001; Long *et al*. 2006). Recently, Liang *et al*. (2005) found that gene product GP64 is a critical factor in the transduction of mammalian cells.

Individual baculoviruses usually have a narrow host range limited to a few closely related species. Baculoviruses produce a large number of occlusion bodies in infected cells (polyhedra and granules are shown in **Fig. 1**) which allow the virus to survive in the environment and to transmit the disease from one insect to another. Virions consist of one or more nucleocapsids embedded in a membranous envelope. The most widely studied baculovirus is the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). Early work on AcMNPV was directed towards the development of viral pesticides and construction of baculovirus-based expression vectors (reviewed by Wood and Granados 1991). The circular DNA genome of AcMNPV is surrounded by a small basic protein which neutralizes the negative charge of the DNA. This structure is protected by proteins forming a nucleocapsid. The genomic circular DNA is infectious in the naked form. Two morphologically

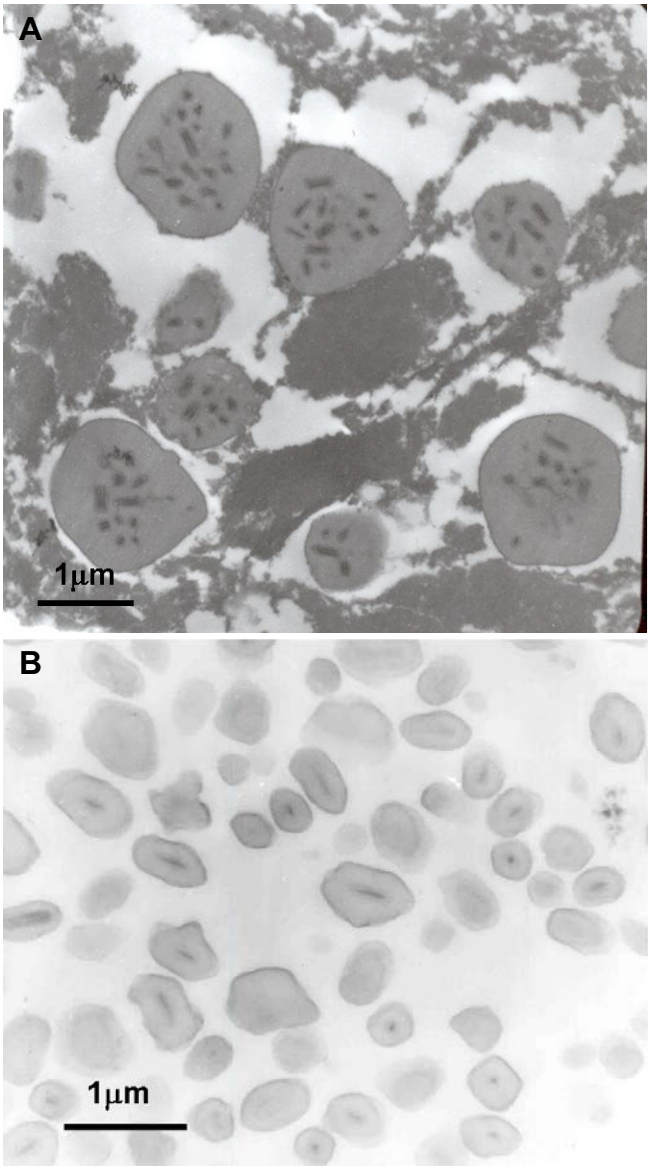

Fig. 1 Electron micrograph illustrating *Anticarsia gemmatalis* nucleopolyhedrovirus (A) and *Erinnyis ello* granulovirus (B) occlusion bodies. The large polyhedral occlusion body contains many virions (**A**). The cylindrical occlusion body contains one virion (**B**).

distinct, but genetically identical, viral forms are produced at different times post-infection. Budded virus particles (BV) serve for the transmission of the virus to other tissues of the caterpillar body. Occlusion bodies (OB) are responsible for the survival of the virus in the environment and the spread of the virus from insect to insect. The occlusion bodies (polyhedra) contain many occlusion-derived virions (ODV) surrounded by a matrix composed mainly of polyhedrin, a major structural protein (Braunagel *et al.* 2003). Polyhedra are relatively stable and the protected virions in the favourable conditions can survive in the environment for more than twenty years. They are big enough to be seen in a light microscope. Under magnification of around 1000x, polyhedra resemble clear, irregular crystals of salt.

The consecutive steps of infection by AcMNPV are shown in **Fig. 2**. Caterpillars ingest polyhedra as contaminants of their food. The paracrystalline polyhedrin matrix is solubilized in the alkaline environment of the midgut caterpillars and the released virions enter midgut cells after fusion with microvilli. The virions are uncoated and nucleocapsids enter the nucleus where viral genes are expressed in a strictly controlled manner. Late AcMNPV genes are transcribed primarily between 6 and 24 hours post infection (h p.i.), whereas very late genes began to be transcribed around 18 h p.i. and the transcription continues until 72 h p.i (Lu

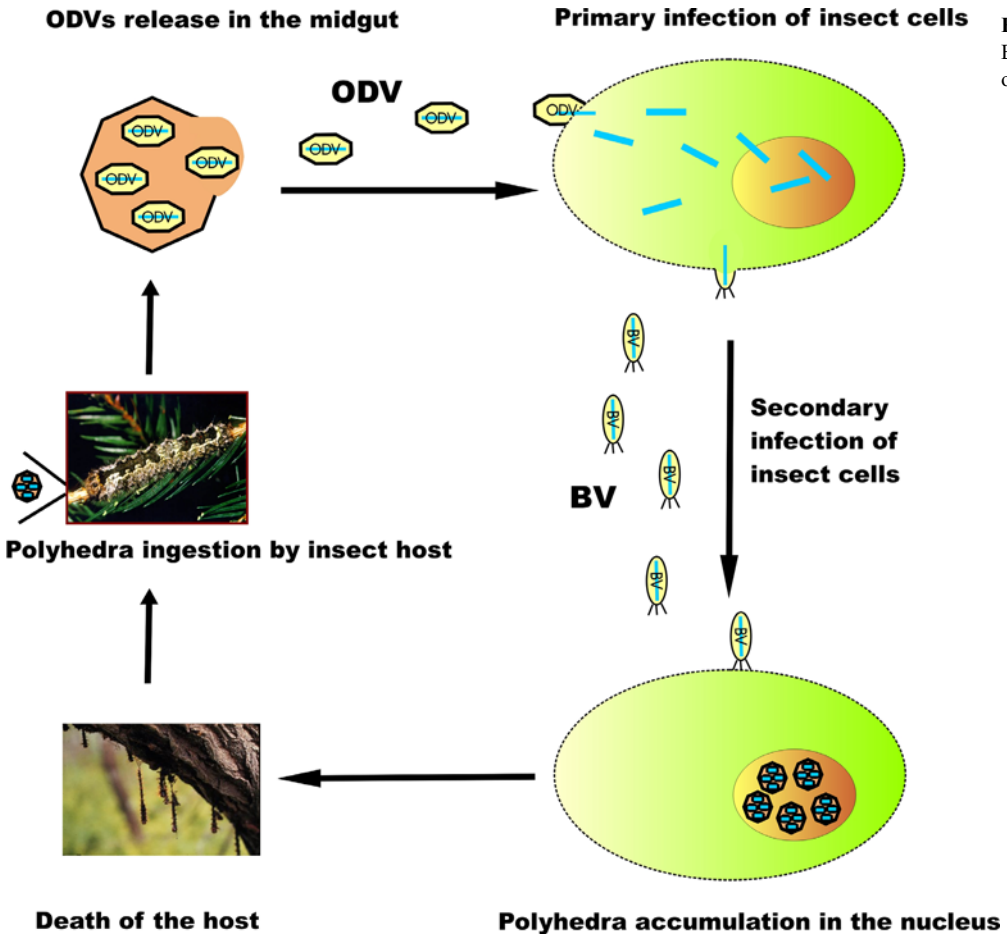

Fig. 2 Life cycle of a baculovirus. BV, budded form of the virus; ODV, occlusion derived form of the virus.

and Miller 1997). In the late phase nucleocapsid structural proteins are synthesized, including glycoprotein GP64 which plays an important role in the dissemination of the infection by budded virus inside the insect body. In the very late phase the production of infectious BVs is greatly reduced. Nucleocapsids become enveloped usually in groups of a few particles. This process appears to be an essential primary step in the process of occlusion of nucleocapsids by the very late protein – polyhedrin. The occlusion continues until eventually the nucleus becomes filled with occlusion bodies; usually more than 30 polyhedra per nucleus can be found. As a consequence, around 10^{10} polyhedra are produced per late instar larvae. This may account for over 30% of the dry weight of a larva (Miller *et al.* 1983). As occlusion proceeds, the fibrillar structures begin to accumulate in the nucleus and sometimes also in the cytoplasm. They are composed mostly of a very late protein P10. The function of these structures is not clear but they are probably involved in the controlled disintegration of the host cells (van Oers *et al.* 1994). In the terminal stages of infection, two viral enzymes, chitinase and cathepsin, act together to facilitate host cuticle breakdown (Hawtin *et al.* 1997). Finally the caterpillar liquefies. Polyhedra released from the cadaver can infect other caterpillars by horizontal transmission. This transmission is primarily through larvae ingesting occlusion bodies present on plants. The occlusion bodies can be further distributed by excrements of infected larvae and predators (Vasconcelos 1996). Vertical transmission may also play a role. It usually occurs through surface contamination of eggs or virus entering inside the egg (Fuxa *et al.* 2002).

Baculovirus has gained an immense attention in molecular biology laboratories because it is one of the most versatile genetic engineering tools (for a recent review see van Oers 2006). In fact, our current knowledge about the biology of AcMNPV is to a large extent a consequence of the developments of baculovirus-based expression vectors. Baculovirus system of expression of foreign genes has many advantages over other systems, namely:

- high level of foreign gene expression is usually achieved compared to other eukaryotic expression systems;
- it is possible to express more than one foreign gene;
- baculovirus genome can accommodate large pieces (around 20 kbp) of foreign DNA;
- insertion of specific signal sequences in front of a foreign gene leads very often to the export of the gene product outside of the infected cell;
- post-translationial modifications of eukaryot-derived proteins such as glycosylation, phosphorylation, etc. are efficient and are the same or very similar to those occurring in original organisms.

Recombinant baculoviruses are usually constructed in two steps (Luckow *et al.* 1993). Initially, a heterologous gene is introduced into a baculovirus transfer vector. It consists of a bacterial replicon of a multicopy plasmid, a selection marker gene, promoter and terminator regions along with flanking baculovirus sequences from a non-essential locus, and a multiple cloning site (or a single unique restriction site) downstream from a viral promoter. Most often the promoters and the flanking DNA originate from one of the late genes: polyhedrin or p10 gene. The baculovirus transfer vector containing foreign DNA and genomic viral DNA are then introduced into insect cells where they recombine yielding recombinant virus with an integrated heterologous gene. Polyhedrin and p10 promoters are very strong promoters, so high level of protein synthesis in insect cells is to be expected. For some purposes, weaker early promoters, such as basic protein promoter (p6.9), may be preferred.

Around 400 insect cell lines are known which potentially can be used for *in vitro* propagation of baculoviruses. Only a few of them support the growth of AcMNPV. These lines were obtained from two parental organisms: *Spodoptera frugiperda* and *Trichoplusia ni*. The most widely used

line is Sf9 which grows well in suspension (Summers and Smith 1987). BTI-Tn-5B1-4 derived from *T. ni*, known as High Five cells, has also been largely used for viral growth (Granados *et al.* 1994). Other cell lines such IPLB-LD-652Y (Goodwin *et al.* 1978), BCIRL-HZ-AM1 (McIntosh and Ignoffo 1981), BM-5 (Grace 1967), UFL-AG-286 (Sieburth and Maruniak 1988), can be used respectively for the propagation of *Lymantria dispar MNPV*, *Heliothis zea SNPV*, *Bombyx mori SNPV*, *Anticarsia gemmatalis MNPV*.

Recombinant baculoviruses can be used to infect caterpillars by ingestion of occlusion bodies but the formation of OBs requires a healthy polyhedrin gene, so in the past the foreign gene was usually introduced into the p10 locus for driving a high-level of foreign gene expression in baculovirus infected cells. Alternatively, recombinant occlusion-negative viruses can be packaged into polyhedra by cells infected with a second, occlusion-positive virus (e.g. wild type virus) (Wood *et al.* 1993). Another method is the duplication of a viral promoter. In this case none of the viral genes is lost. This allows for the expression under different promoters, e.g. basic protein gene promoter (p6.9). *Rachiplusia ou* MNPV (RoMNPV) expressing a gene coding for scorpion *Androctonus australis* toxin (AaIT) or *Leirus quinquestriatus hebraeus* toxin (LqhIT2) killed larvae of corn borer *Ostrinia nubilalis* most effectively when the gene was cloned behind p6.9 promoter (Harrison and Bonning 2000). A similar effect was found by Lu *et al.* (1996) for the production of toxin Tox34 of straw itch mite *Pyemotes tritici*, and by Harrison and Bonning (2001) for the recombinant AcMNPV-expressing protease, cathepsin L of the flesh fly (*Sarcophaga peregrine*). Recently, Tuan *et al.* (2005) showed that the early composite p-PCm promoter was better than the very late p10 for controlling insect pests when LqhIT2 scorpion depressant toxin gene was introduced into the AcMNPV genome. These facts are consistent with higher susceptibility of earlier instars of larvae to baculovirus infection. Other baculoviruses, including HzSN PV, *Helicoverpa armigera* SNPV, and RoMNPV are also employed for genetic modifications (Treacy *et al.* 2000; Sun and Wang *et al.* 2004) though AcMNPV, which infects many lepidopteran species, is by far most frequently used.

BACULOVIRUS PESTICIDES IN THE PAST – SUCCESSES AND FAILURES

First reports to use baculoviruses for the protection of forests date back to the 19th century but there are no reliable data on the efficacy of these attempts. The first successful introduction of baculovirus into the environment which resulted in effective suppression of a pest occurred accidentally before World War II. A parasitoid was imported to Canada to suppress spruce sawfly *Diprion hercyniae*. Along with a parasitoid, an NPV specific for this hymenopteran species was introduced and since then no control measures have been required against *D. hercyniae*. This case of introduction followed by establishment of an alien baculovirus, which becomes a permanent part of an ecosystem, is rather an exception. Usually other strategies are more common in pest management (Fuxa 2004):

- infested areas are sprayed with a highly concentrated baculovirus to suppress the pest as quickly as possible (short-term insecticide approach),
- a lower concentration of baculovirus results in the establishment of a pest population for more than one generation (seasonal colonization approach) (Fuxa 2004).

In the past, the application of baculoviruses for the protection of agricultural annual crops, fruit orchards and forests has not matched their potential. Though relatively slowly, the number of registered pesticides based on baculovirus increases steadily. At present, it exceeds fifty formulations, some of them being the same baculovirus preparations distributed under different trade names in different countries (e.g. there are at least five commercial products consisting of *Anticarsia gemmatalis multiple nucleopolyhedrovirus* and *Cydia pomonella granulovirus*). Both NPVs and GVs are used as pesticides but the former group is much larger.

The first viral insecticide Elcar™ was introduced by Sandoz Inc. in 1975 (Ignoffo and Couch 1981) but in 1982 Sandoz decided to discontinue the production probably, because more profits were obtained from new chemical pesticides. Elcar™ was a preparation of *Heliothis zea* NPV which infects many species belonging to genera *Helicoverpa* and *Heliothis*. HzSNPV provided control, of not only cotton bollworm (*Heliothis zea*), but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. The resistance to many chemical insecticides including pyrethroids revived the interest in HzSNPV and the same virus was registered under the name GemStar™ (marketed by ThermoTrilogy Company). HzSNPV is a product of choice for biocontrol of *Helicoverpa armigera* (Mettenmeyer 2002). Countries with large areas of such crops like cotton, pigeon pea, tomato, pepper and maize, e.g. India and China, introduced special programs for the reduction of this pest by biological means. In Central India, *H. armigera* was traditionally removed by shaking pigeon pea plants (high protein food in vegetarian India) until caterpillars fell from the plants onto blankets. Nowadays, this technique is used to obtain caterpillars which are fed on virus-infected seeds. The caterpillar carcasses are then used by farmers to prepare a bioinsecticide spray applied on pigeon pea fields. Another baculovirus, HaSNPV is almost identical to HzSN PV and has similar host range. It was registered in China as a pesticide in 1993 (Zhang *et al.* 1995), adopted for large scale production and has been extensively used on cotton fields (over 100,000 ha of cotton in the past ten years).

European and North American forests are often attacked and defoliated by Lymantridae (most common pest species are: *Lymantria dispar*, *Lymantria monacha* and *Orgiya pseudotsugata*), Noctuidae (mainly *Panolis flammea*) and some Hymenoptera species (mainly *Neodiprion sertifer* and *Diprion pini*). Most widely distributed *L. dispar* MNPV formulations are marketed under trade names: Gypchek, Disparivirus, Virin-ENSH, and *O. pseudotsugata* MNPV under trade names: TM BioControl-1 and Virtuss (Reardon *et al.* 1996; Arif 1997). Ecological concern is a stimulus for forest managers to increase the area protected with biopesticides which at present is still marginal.

Spodoptera is another genus which is of primary concern for the agricultural industry in many countries of the world. At least two commercial products based on *Spodoptera* NPV are available in the USA and Europe: SPOD-X™ containing *Spodopera exigua* NPV to control insects on vegetable crops and Spodopterin™ containing *Spodoptera littolaris* NPV which is used to protect cotton, corn and tomatoes. About 20,000 hectares of maize annually are controlled with *Spodoptera frugiperda* NPV in Brazil (Moscardi 1999). Many other species belonging to the *Noctuidae* family are economically important pests of sugarcane, legume, rice and others. *Autographa californica* and *Anagrapha falcifera* NPVs were registered in the USA and were field-tested at a limited scale. These two NPVs have a relatively broad host spectrum and potentially can be used on a variety of crops infested with pests belonging to a number of genera, including *Spodoptera* and *Helicoverpa*. Even codling moth, *C. pomonella*, a common pest of orchards in temperate zones is susceptible to these two broad-range baculoviruses (Lacey *et al.* 2002). The susceptibility of codling moth is, however, much greater to the granulovirus CpGV, and this baculovirus is the active component of a number of biopesticides used for protection of orchards. Some of the trade marks of GpGV-based products are: Granusal™ in Germany, Carpovirusine™ in France, Madex™ and Granupom™ in Switzerland, Virin-CyAP in Russia. *C. pomonella* is a worldwide pest, so the use of granulovirus-based products gradually increases. It is prognosed that annually up to 100,000 hectares of orchards will be protected with CpGV. Another granulovirus, *Erinnyis ello* (cassava

hornworm) *granulovirus*, was found to be very efficient for protection of cassava plantations (Schmitt 1985; Bellotti 1999). Virus-based products are now commercially available in some South American countries.

The most spectacular success of employing baculovirus as a biopesticide is the case of *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) used to control the velvetbean caterpillar in soybean (Moscardi 1999; Moscardi *et al.* 2002). This program was implemented in Brazil in the early eighties, and currently over 2,000,000 ha of soybean are treated annually with the virus. Although the use of this virus in Brazil is the most impressive example of bioregulation with viral pesticide worldwide, the virus is still obtained by *in vivo* production mainly by infection of larvae in soybean farms. The details of the production, implementation and evolution of the use of AgMNPV in Brazil were presented by Moscardi *et al.* (2002). The demand for virus production has increased tremendously reaching currently the need for protection of four million hectares of soybean annually. Recently, a "Biofabric" for large-scale *in vivo* production of baculoviruses has been installed in South Brazil.Larvae are reared in artificial diet in controlled laboratory conditions. Basically, daily 3% of the larvae are selected to become pupae in order to maintain the insect colony. The rest of the larvae (97%) is used for virus production, where approximately 350 larvae are kept in plastic box containing 420 g of diet inoculated with the virus. After 7 days the larvae are collected, processed and used for the formulation of the bioinsecticide (Moscardi and Santos 2005). This high demand for AgMNPV calls also for the studies aiming at the sustained inexpensive *in vitro* production of the virus. *In vitro* production requires initially the scale up of the cells from small volumes (skakers) to large volumes (bioreactors). The virus is obtained directly from the insect haemolymph or from supernatant (BVs) of previously infected cells. Scale-up technology varies according to the cell line and the culture conditions and different laboratories establish their own protocols and methodologies. However, a major problem in production of baculovirus in cell culture is the generation of less virulent mutants (Krell 1996).

In the beginning of the 1980's, a pilot program for AgMNPV use in soybean fields was initiated by a Unit of the Brazilian Agricultural Research Corporation (Embrapa) located in Londrina, State of Paraná. The implementation of the program began in 1982 when ca. 2000 ha of soybean was treated with AgMNPV. Initially frozen killed larvae were distributed for treatment of demonstration plots and virus production in the field. This provided inoculum for treatment of other areas in the same season or for subsequent seasons. In the early to mid 1990's, AgMNPV use approached 1,000,000 ha. An important step of the program was the development of a wettable powder formulation of the virus in 1986. The transfer of technology from Embrapa to private companies started in 1990. Field production of the virus became a profitable business and large quantities of virus-killed larvae were produced at a low cost. However, since 1999 field production of the virus has not been sufficient to fulfill the rapidly growing demand for this bioinsecticide. Therefore, much effort has been devoted to the elaboration of methods for the commercial production of AgMNPV in the laboratory. In November 2004, the Research Station of the Farmers' Cooperatives for the State of Paraná (COODETEC) inaugurated a "Biofabric" for large-scale production of the virus (COOPER VIRUS PM). This laboratory is increasing its virus production with the aim to inoculate 600,000 larvae/day, which may be sufficient for spraying the virus over 1,3-1,5 million of hectares of soybean per year. Recently, a "Pilot Plant" was built at Embrapa Soja, Londrina, for improvement of the laboratory process and also for training people in virus production. This laboratory will have the capacity to inoculate 25,000-30,000 larvae per day (Moscardi and Santos 2005).

The use of AgMNPV in Brazil brought about many economical, ecological and social benefits. The current annual savings at the grower level, in the total area sprayed with the virus is over U$ 11,000.000. Since the beginning of the program more than 17 million liters of chemical insecticides were not sprayed in the environment, resulting in considerable environmental benefits (Moscardi *et al*. 2002).

The occurrence of genomic alterations in wild type isolates of baculovirus populations is a well-documented phenomenon. Genomic variability has been described for many wild type virus including *Autographa californica* MNPV, *Spodoptera frugiperda* MNPV, *Spodoptera litura* MNPV, *Panolis flammea* MNPV and *Mamestra configurata* NPV. Genotypic variants are easily recognized by the presence of submolar fragments in the electrophoretic patterns of restriction endonuclease digestion products. Plaque purification of wild type isolates confirmed that they contain a mixture of these variants. AgMNPV genomic variability has been also carefully studied because the selection pressure due to the application of AgMNPV in the field during subsequent years could lead to alterations in virus stability. The method of choice was the technique of restriction endonuclease analysis (REN). It was used to monitor the genetic stability of this virus, by comparing the DNA profiles of eleven different seasonal isolates. Viral DNA were initially purified from diseased larvae collected during several crop seasons and compared to AgMNPV-79, a wild-type virus that was used originally and subsequently in this program (Souza *et al*. 2001). In general, when a change was introduced in the viral population, such as a new cleavage site, it persisted in subsequent years. The most important conclusion inferred from the analysis of the virulence of these isolates by bioassays was the fact of retaining the pathogenicity of the virus throughout the years of its application. These results indicated that the virus maintains considerable stability, even with the existence of some genetic changes shown in the DNA restriction profiles.

BACULOVIRUS PESTICIDES - FUTURE PROSPECTS

The Brazilian success story described in the preceding section proved that the baculovirus protection at large scale is possible and can be done at relatively low cost. It is very likely that, after long-lasting lag period, the need to develop environment-friendly pesticides and the awareness of the benefits that baculovirus protection offers, will result in the reevaluation of the prospects for this type of biological protection. We expect that the development of baculovirus pesticides will depend on the attitude of societies towards genetically modified organisms (GMOs). In countries, where use of GMOs is restricted, only naturally occurring baculoviruses will be used for protection of crops. The improvements in the application of the biopesticide in this case will be at the level of diagnostics of infection, development of the *in vitro* cultures and changes in the formulations of the biopesticide. In countries with less stringent attitude towards GMOs, the improvements will aim at the acceleration of the killing activity of baculoviruses. This action can be achieved by introduction of exogenous genes into baculovirus genome.

Prospects for improvements in formulations, diagnostics and *in vitro* propagation of baculovirus for crop protection

The major problem in using baculoviruses for crop protection is their slow action and lack of morphological changes in larvae in first stages of baculovirus propagation. Reliable assays for the progress of infection may prevent respective agricultural services from using chemical means of protection which, from the ecological point of view, may be redundant. Therefore, it is crucial in pest management with baculovirus to have a sensitive method to assess the effectiveness of infection. Fast and sensitive methods in diagnostics can be roughly divided into immunological methods

based on protein composition and content, and genome detection methods usually based on PCR techniques. The latter group of techniques will probably play a predominant role in future. They are relatively simple analytical methods giving wealth of information about occurrence and spread of the virus. Using specific primers, not only target larvae, but also vectors for baculovirus transfer – predating invertebrates and birds can be quickly analysed. Most of analytical laboratories associated with pest management are able to buy this equipment and train technicians to perform routine analysis. For strictly quantitative assays, real-time PCR is a method of choice. The equipment required – light cyclers are relatively expensive now, but their prices decrease very quickly and it is very likely that they will soon become routine equipment in pest management laboratories.

The relative genetic stability of baculoviruses *in vivo* may not be exactly the same as in the continuous passages in cell cultures. The most common mutations that occur in cell culture are DIPs (defective interfering particles) and Few Polyhedra mutants (reviewed by Krell 1996). The virus which has gone through a long period of multiple infection cycles gradually losses the ability to infect insect cells due to formation of these particles. The DIPs require the presence of an intact wild type virus as helper and they replicate faster because they are smaller. Insect cells should be infected at low multiplicity of infection to minimize the probability of a DIP entering a cell along with an intact virion. The generation of FP mutants is due to changes in the *25 k FP* viral gene, like transposones or point mutations. The search for more stable viral isolates is one strategy to try to overcome the generation of FP mutants during *in vitro* production. A stable isolate of *Lymantria dispar* MNPV that exhibits enhanced polyhedra productions in cell culture was described by Slavicek and collaborators (1996, 2001).

The studies of the effects of *Anticarsia gemmatalis* MNPV due to the serial passages in cell culture as well as the analysis of susceptibility of different lepidopteran cell lines to AgMNPV are currently being carried out (Castro *et al.* 2002, 2006). Recently, studies to optimize the AgMN PV production in spinner bottles and in bioreactors using Sf9 cells had been started (Rodas *et al.* 2005). The use of other baculoviruses requires similar studies if they have to be produced at large scale in *in vitro* conditions. In the coming years the research directed towards producing baculovirus biopesticides in cell culture will have to be greatly intensified to simplify commercial production of baculoviruses and to reduce the cost of large-scale production, so that they will become competitive with chemical pesticides. Recently, a two step method for producing commercial quantities of baculovirus based on production of infectious virus in caterpillar larvae and then using the resultant infectious virus as an inoculum for a limited number of serial passages in cell culture was patented by Steven Reid and Linda Lua from The University of Queensland, Australia (WO/2005/045014).

Viral survival can be influenced by temperature, pH or the presence of additives. The most detrimental effect on virus activity is attributed to UV light. Under field conditions little activity is left when the virus is not shaded by plant canopy. Much effort has been devoted to the development of UV protectants (Shapiro and Dougherty 1994; Zou and Young 1994). Many formulations have been tried but the most promising are at present stilbene fluorescent brighteners which are marketed under many trade names (e.g. Phorwite AR, Blankophor or Tinopal LPW). Future developments in the field of brighteners may lead to the reduction of cost of baculovirus production. Inactivation of baculoviruses may be also caused by plant metabolites. One of such possibilities is inactivation by plant peroxidases which generate free radicals (Hoover *et al.* 1998; Sun *et al.* 2004). The inactivation can be reduced by the addition of free radical scavengers such as mannitol or the enzyme superoxide dismutase to baculovirus preparations (Zhou *et al.* 2004).

Prospects for improvements by genetic modification of baculoviruses

The killing action by baculoviruses may be accelerated by interference with host physiology and/or introduction of an insect-specific toxin (Bonning and Hammock 1996; Inceoglu *et al.* 2001). The experiments aiming to change host physiology by genetic engineering means were done by introducing genes coding for some insect hormones or hormone-modifying enzymes into baculovirus genome, or by deletion of the baculovirus-encoded ecdysteroid glucosyltransferase (*egt*) gene. The most exploited case of the former approach is the regulation of the level of juvenile hormone which controls the onset of metamorphosis. Juvenile hormone is regulated by juvenile hormone esterase which overexpressed decreases the concentration of the hormone and this is a signal for a caterpillar to stop feeding and pupate. This approach encountered many difficulties in practice but it is being pursued in some laboratories (Hammock *et al.* 1990; Inceoglu *et al.* 2001). The latter approach – the deletion of the baculovirus-encoded *egt* gene - was used first by O'Reilly and Miller (1991). The product of the *egt* gene interacts with larval moulting and indirectly increases the time of feeding of infected caterpillars. The infection with *egt*-deleted virus resulted in 30% faster killing of larvae. Additional advantage of this approach is the fact that the *egt* gene is not essential for viral replication and can be replaced with an insecticidal exogenous gene, thus enhancing the insecticidal activity of the recombinant virus (Arif 1997; Sun and Wang *et al.* 2004).

Baculovirus genome modifications by introduction of exogenous toxin genes were widely exploited in many research laboratories. Most of the reported research was focused on arthropod toxin genes isolated from scorpions, mites or spiders (Bonning and Hammock 1996; Inceoglu *et al.* 2001). The most potent insect-specific toxin gene used for construction of baculovirus recombinants was probably the gene coding for AaIT toxin originating from the scorpion *Androctonus australis*. The speed of kill of this baculovirus recombinant was increased by about 40% and the feeding damage was reduced by about 60% (Inceoglu *et al.* 2001). Toxin genes isolated from other scorpions, e.g. *Leiurus quinquestriatus hebraeus* (Froy *et al.* 2000), straw itch mite *Pyemotes tritici* (Lu *et al.* 1996) or spiders (Hughes *et al.* 1997), introduced into baculovirus genomes have also been intensively studied as potential biopesticides. Arthropod toxins described here usually attack insect sodium channels, which means, that they produce final effect similar to the chemical pesticides of the pyrethroid group. The specific target within sodium channels is different, so, when combined, they may have synergistic effect (McCutchen *et al.* 1997).

Chang *et al.* (2003) constructed a baculovirus that produced occlusion bodies incorporating *Bacillus thuringiensis* toxin by making a fusion protein consisting of polyhedrin and Bt toxin. The pathogenicity of the recombinant was greatly increased compared to wild-type virus. This experiment proved that a potential biopesticide, which combines the advantages of the virus and the bacterial toxin, is feasible.

The line of research presented above proved to be highly successful but the reluctant attitude of many countries to genetically engineered products was a reason for a slow pace of consecutive steps necessary for the transfer of a potential biopesticide from a laboratory bench to industry. Though rarely supported by sound evidence, the attempts to perform field trials, even at a small scale, promoted web and mass media attacks in Europe. It is a general belief in the scientific world that baculoviruses pose no hazard to other animals than their hosts. This belief is based on a number of studies. Recombinant baculoviruses expressing AaIT scorpion toxin gene were not pathogenic to bees and all vertebrate species that were used in this study (Sun *et al.*

2004). Natural enemies of larvae such as parasitoids and predators were not adversely affected by preying upon larvae infected with recombinant viruses (Boughton *et al.* 2003). The theoretical possibility of the cloned gene to jump from recombinant baculovirus to the any other organism has not been observed (Inceoglu *et al.* 2001). The question arises – what is the reason for the fact that after almost 20 years since first experiments with recombinant baculoviruses, we do not have in use excellent biopesticides of this kind? On the contrary, preliminary field trials of genetically modified baculoviruses raised massive public protests which put on hold further trials for a long time. In our opinion the slow progress in application of genetically modified baculoviruses as pesticides is at least in part due to scientists themselves who did not take into account the perception of their experiments by general public. Baculovirus genomes were often modified by introduction of genes coding for "exotic" toxins from the African scorpion or Australian spider (Stewart *et al*. 1991; Cory *et al*. 1994; Kamita *et al*. 2005). These very elegant and exciting scientific experiments were strongly criticized by non-specialists with high impact on man-in-the-street awareness of ecological threats, e.g. by members of "green" movements in rich Western democratic countries. So, though genetic modification of baculoviruses is probably a right path to go if we want to introduce effective baculoviral pesticides, the choice of toxins used for this purpose should be reexamined and modification of baculoviruses with more "natural" toxins, e.g. parasitoid wasp toxic polypeptides, should be explored much more thoroughly.

CONCLUDING REMARKS

Our aim was to show that after many years of stagnation, baculoviral pesticides have again come into the light and to indicate which are the most likely ways to make their role more significant. The protection of soybean fields in Brazil has proved that baculoviral control agents can be effective on a large scale and they may be an alternative to broad-spectrum chemical insecticides. Needless to say, that the advantages of biopesticides over chemical pesticides are numerous. Safety for humans and non-target organisms, preservation of biodiversity in the environment, reduction of toxic residues in end-products are just the examples of potential benefits. However, the cost of biopesticide production has been usually higher than the cost of conventional pesticides. So, paradoxically, countries where the cost of human labour is low are more open towards the use of baculoviral pesticides than rich Western countries that claim that environmental protection is one of their priorities in the development. Hopefully, more rational approach will be adopted towards microbial pesticides in the near future and short-term profits from chemical pesticides will not determine the fate of biopesticides. The more rational approach is also needed in the social perception of dangers associated with genetically modified baculoviruses and here, we hope, scientists will also have a share in convincing the public on risks and benefits of genetically modified biological pesticides.

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