2

Baculovirus Biopesticides

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1. Introduction

Baculoviruses are a large group of double-stranded DNA viruses (almost 1000 species have been described); the majority have been isolated from a few insect orders: Lepidoptera, Diptera, Hymenoptera and Coleoptera. Viral genome ranges in size from 80 to 200 kb. Individual baculoviruses usually have a narrow host range limited to a few closely related species. The most widely studied baculovirus is the *Autographa californica* nuclepolyhedrovirus (AcMNPV).

Baculoviruses are arthropod viruses well known due to their potential as agents of biological control of pests in agriculture and forestry. They are also widely used as expression vectors in biotechnology. The family *Baculoviridae* contains diverse members and in the past the classification was based on virus morphology. It was divided into two genera: the *Nucleopolyhedrovirus* (NPVs) and the *Granulovirus* (GVs). Recently, this division was challenged (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, but the traditional division into two genera is still widely used.

Baculoviruses infect arthropods and they do not replicate in vertebrates, plants and microorganisms. Although they do not replicate, they may, under special conditions, enter animal cells. This unexpected property made them a valuable tool in the last few years for studies of transient expression of foreign genes under vertebrate promoters introduced into baculovirus genome (Boyce and Bucher, 1996; Kost et al., 2005).

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. Virions consist of one or more nucleocapsids embedded in a membranous envelope. Two morphologically distinct, but genetically identical, viral forms are produced at different times post-infection:

- Budded virus particles (BV) which serve for the transmission of the virus to other tissues of the caterpillar body.
- Occlusion bodies (OB) which 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) of Nucleopolyhedrovirus contain many occlusion-derived virions (ODV) surrounded by a matrix composed mainly of polyhedrin, a major structural protein (Braunagel et al., 2003). Polyhedrin is produced in large quantities (around 30% of total protein mass at the time of host death) but it is not needed for the transmission of the virus from cell to cell. Polyhedra are relatively stable and the protected virions in the favourable conditions can survive in the environment for more than twenty years. Under magnification of around 1000x, polyhedra resemble clear, irregular crystals of salt so they are big enough to be seen in a light microscope.

Baculoviruses have gained great attention in molecular biology laboratories because they are very versatile genetic engineering tools (for a 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 because high level of foreign gene expression is usually achieved compared to other eukaryotic expression systems. Baculovirus genome can accommodate large pieces (up to 50 kbp) of foreign DNA, so it is possible to express more than one foreign gene. Additionally, the 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.

Recombinant baculoviruses are usually constructed in two steps. 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 latter is another viral gene coding for a protein which is produced in large quantities late in the infection. It is the main component of the fibrillar structures which accumulate in the nucleus and in the cytoplasm of infected cells. 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* (Lepidoptera: Noctuidae) The most widely used line is Sf9 which grows well in suspension. BTI-Tn5B1-4 derived from *T. ni*, known as High Five cells, has been also largely used for viral growth (Granados *et al.*, 1994). Cell lines which can be used for the propagation of *Lymantria dispar* nucleopolyhedrovirus (LdMNPV), *Heliothis zea* nucleopolyhedrovirus (HzSNPV), *Bombyx mori* nucleopolyhedrovirus (BmSNPV), *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) and a few other baculoviruses are also currently available.

2. Baculovirus production technology

At present, commercial production of baculoviruses has been carried out only *in vivo*, either by applying the virus against the host insect in the field and collecting diseased or dead larvae, or by producing the target insect in the laboratory on an artificial diet. The latter is the most commonly used method for producing baculoviruses in many countries but both methods have been used successfully for commercial production of the *Anticarsia gemmatalis* baculovirus (AgMNPV) in Brazil (Moscardi, 1999, 2007). For some insects there are no available artificial diets and, therefore, commercial production of baculoviruses of these

insects is generally too difficult or impossible under laboratory conditions. In such cases, field production of baculovirus stocks may be sometimes a method of choice, also from financial point of view (Moscardi, 1999). This approach is, however, difficult when liquefaction of the insect body is very intense, as, for instance, in larvae infected by *Spodoptera* spp. baculoviruses. In this case, live larvae must be collected close to death when the body has not yet ruptured.

Baculovirus production in insect cell cultures offers advantages over *in vivo* multiplication for being a controllable, sterile, highly pure product yield process. *In vitro* process of baculovirus production for agricultural pest control needs to be efficient, with competitive costs, leading to a final product which is highly pathogenic to the target pest. There is a strong limitation for *in vitro* production, however, since successive passages of the virus in cell culture result in genetic alterations leading to loss of virulence (Krell, 1996; Rhodes, 1996). In laboratory culture, production of occlusion derived virions (ODV) is not necessary for survival of the virus. The budded virus (BV) particle is the form used for cell-to-cell transmission in cell culture. The main protein of the BV particle is the GP64 (Blissard, 1996), essential for virus budding and responsible for entrance of the virus into the next host cell. Various culture conditions are known to influence infection of lepidopteran cells by baculoviruses and include temperature, pH, dissolved oxygen concentration, osmolality and nutrient composition of the culture medium. The investigation of factors associated with loss of genetic stability and the use of new strategies such as isolation of more stable variants, as well as the reduction of costs of cell culture medium components, are important requirements for process optimization of *in vitro* baculovirus production.

The requirements for productive insect cell lines (Jem et al., 1997) and for highly productive culture media (Chakraborty et al., 1999) are other challenges for *in vitro* production of baculovirus. Many cell lines are available for production purposes and are derived from various sources, thus exhibiting a wide variety of growth and production characteristics. Careful screening or formulation of media must be performed for a particular virus isolate-cell line combination, as different media can greatly affect polyhedra yields (Pedrini et al., 2006). Recently, a new strategy for *in vitro* production was proposed based on Many Polyhedra (MP) variants. These are clones selected using the plaque assay technique after several passages of the virus in cell culture. MPs maintain the wild type features such as formation of many polyhedra in the cell nucleus and Budded Virus high titer (Slavicek et al., 2001; Pedrini et al., 2005) which allow them, in principle, to compete with the population of Few Polyhedra mutants accumulated in cell culture.

3. Baculovirus pesticides in the past

Two strategies of pest management with baculovirus pesticides are usually employed (Fuxa, 2004):

- infested areas are sprayed with highly concentrated baculovirus to suppress the pest as quickly as possible,
- infested areas are sprayed with lower concentration of baculovirus and this results in establishment of the virus for more than one generation.

At present the number of registered pesticides based on baculovirus exceeds fifty formulations, some of them being the same baculovirus preparations distributed under different trade names in different countries. Both NPVs and GVs are used as pesticides but the former group is larger.

The first viral insecticide Elcar™ was introduced by Sandoz Inc. in 1975 (Ignoffo and Couch, 1981). Elcar™ was a preparation of *Heliothis zea* NPV which is relatively broad-range baculovirus and infects many species belonging to genera *Helicoverpa* and *Heliothis*. HzSNPV provided control of not only cotton bollworm, but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. In 1982 the production of this biopesticide was discontinued. The resistance to many chemical insecticides including pyrethroids revived the interest in HzSNPV and the same virus was registered under the name GemStar™. HzSNPV is a product of choice for biocontrol of *Helicoverpa armigera* (Mettenmeyer, 2002). Countries with large areas of such crops like cotton, pigeonpea, 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* in the past was usually removed by shaking pigeonpea plants until caterpillars fell from the plants onto cotton sheets. This technique is now used to obtain caterpillars which are fed on virus-infected seeds. Baculovirus preparations obtained in this way are used by farmers to prepare a bioinsecticide spray applied on pigeonpea fields. Another baculovirus, HaSNPV is almost identical to HzSNPV. It was registered in China as a pesticide in 1993 (Zhang et al., 1995). It has been used for large scale biopesticide production and has been extensively used on cotton fields (over 100 000 ha of cotton in the last decade). Broad spectrum biopesticide based on HaNPV is also used in India (Srinivasa et al., 2008).

The forests of temperate regions are very often attacked and defoliated by larvae of Lepidoptera (most common pest species are: *Lymantria dispar, Lymantria monacha, Orgiya pseudotsugata* and *Panolis flammea*) and some Hymenoptera species (mainly *Neodiprion sertifer and Diprion pini*). *L. dispar* MNPV formulations marketed under trade names: Gypchek, Disparivirus, Virin-ENSH, and *O. pseudotsugata* MNPV under trade names: TM BioControl-1 and Virtuss (Reardon et al., 1996) are sometimes used for forest protection. Forest ecosystems tend to be more stable than agricultural systems, allowing for natural or applied baculoviruses to remain in the environment for long periods of time increasing the chance of natural epizootics by these agents.

Caterpillars of moths belonging to *Spodoptera* genus are of primary concern for agricultural industry in many countries of the world. Two commercial preparations based on *Spodoptera* NPV have been available. These are SPOD-X™ containing *Spodoptera 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 were controlled with *Spodoptera frugiperda* NPV in Brazil (Moscardi, 1999), but at present it has not been used due to technical problems in the virus production under laboratory conditions. Use of *Spodoptera litura* NPV has been tested on cabbage crops in India (Kumari et al., 2009). 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 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*.

Granulovirus CpGV is the active component of a number of biopesticides used for protection of apple and pear orchards against the coddling moth, *Cydia pomonella*. Some of the trade marks of GpGV-based products are following: Granusal™ in Germany, Carpovirusine™ in France, Madex™ and Granupom™ in Switzerland, Virin-CyAP in Russia. Annually up to 250 000 hectares of orchards have been protected with Madex™ in

different European countries (Vincent et al., 2007). Considering application of all trade names of the CpGV, this may be the most important worldwide viral insecticide currently applied in terms of treated area.

Another granulovirus, *Erinnyis ello* (cassava hornworm) granulovirus, was found to be very efficient for protection of cassava plantations (Bellotti, 1999). This GV has been used for spraying cassava crops in some South American countries. In Brazil a successful program for cassava pest control was carried out in the eighties based on recovering the virus that were multiplied in the field larval population. However, due to *Erinnyis ello* cyclical behaviour and the difficulty in the insect mass production in laboratory conditions, the program was discontinued.

Other important viruses that are currently employed to control insects include the tea tortricids *Adoxophyes honmai* and *Homona magnanima* granuloviruses (GV) in Japan. The area sprayed with GVs comprised 5,850 ha in Kagoshima in 1995, equivalent to 80 % of all the tea fields in the prefecture (Nishi and Nonaka 1996). The GVs of *H. magnanima* and *A. honmai* were registered in 2003, however, the use of GVs has recently declined. One reason for the reduction in use of GVs in Japanese tea fields is the changing pattern of occurrence of other pests. Mulberry scale, for example, has been increasing recently and chemical treatment is required to control this insect at the same time GVs are sprayed. However, the spray also kills *H. magnanima* and *A. honmai*. Furthermore, GVs have been applied in Kagoshima for more than ten years and the populations of *H. magnanima* and *A. honmai* have been reduced (Nakamura 2003). In China twelve baculoviruses have been authorized as commercial insecticides (Sun and Peng 2007), including *H. armigera* NPV (the most widely used virus in China for cotton, pepper and tobacco protection), *S. litura* NPV (vegetables), *S. exigua* NPV (vegetables), *Buzura suppressaria* NPV (tea), *Pieris rapae* GV and *Plutella xylostella* GV (vegetables). Use of baculoviruses in China is the greatest worldwide, regarding the number of viruses being registered for insect control. Sun and Peng (2007) also reported a Cypovirus (CPV) produced in China for control of *Dendrolimus punctatus*, an insect pest of pine forests.

The well-known success of employing baculovirus as a biopesticide is the case of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) used to control the velvetbeen caterpillar in soybean (Moscardi, 1999). This program was implemented in Brazil in the early eighties, and came up to over 2,000,000 ha of soybean treated annually with the virus. Recently this number dropped down, mainly due to new emerging pests in the soybean complex. The use of AgMNPV in Brazil brought about many economical, ecological and social benefits. At the soybean grower level, the financial savings from the use of the virus may reach up to ca. U$ 7/ha/season, including product cost and application cost. The annual savings at the grower level, in the total area sprayed with the virus was over US$ 11,000.000. Since the beginning of the program more than 17 million liters of chemical insecticides were not sprayed in the environment. The protection of soybean fields in Brazil has proven that baculoviral control agents can be effectively produced on a large scale and they may be an alternative to broad-spectrum chemical insecticides. On the basis of this spectacular success of a baculovirus pesticide, it is 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 agricultural 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 highly-developed countries which claim that environmental protection is one of their priorities in the development.

Genomic variability has been described for many wild type virus including *A. californica* MNPV, *S. frugiperda* MNPV, *S. litura* MNPV, *P. flammea* MNPV and *Mamestra configurata* NPV. Genotypic variants can be recognized by the presence of submolar fragments in the electrophoretic patterns of restriction endonuclease digestion products of a viral genome. Genotypic variation in baculovirus genomes can include point mutations, both small and large deletions and insertions (Krell, 1996). Though mutations can occur in any place of the genome, the presence of some hot spots was observed for certain genomic alterations such as insertions due to transposable elements or deletions in the hypervariable DA26 gene region (Kamita et al., 2003). 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. 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). These results indicated that the virus maintains considerable stability, even with the existence of some genetic changes shown in the DNA restriction profiles. It has been also observed that the virus retains its virulence to the host insect throughout the years of its application.

4. Prognoses for future use of baculovirus pesticides

Large-scale application of AgMNPV in Brazil has proven that the baculovirus protection can be done at relatively low cost. It is very likely that the growing awareness of the benefits of the environment-friendly pesticides will result in the reevaluation of the prospects for biological protection with baculovirus preparations. However, until today, baculovirus insecticides have not met their full potential to control pest insects worldwide. In his review, Moscardi (1999) previewed the following: the expansion of baculovirus use, in the following five years, i.e., up to 2004, would depend on new developments in the areas of recombinant baculoviruses and in the *in vitro* commercial production of these agents. The development of recombinant baculovirus was efficiently completed by researchers in several countries, but the *in vitro* commercial technology still lags behind today, due to technical problems. Future development of baculovirus pesticides will probably depend on the attitude towards genetically modified organisms. In countries where use of genetically modified organisms is restricted, only naturally occurring baculoviruses will be used for protection of crops. In this case the improvements 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 which favour the introduction of genetically modified organisms, the improvements will be achieved by introduction of exogenous genes into baculovirus genome, thus greatly enhancing the killing activity of bioinsecticide formulations.

Reliable assays for the progress of infection with baculovirus are necessary because the major problem in using biopesticide for crop protection is their slow action and lack of morphological changes in larvae in first stages of baculovirus propagation. Lack of such assays may incline agricultural services to use subsequent chemical means of protection which, from the ecological point of view, may be redundant. Fast and sensitive methods in

diagnostics based on baculovirus genome detection will probably play a predominant role in future. They are relatively simple analytical methods giving precise information about occurrence and spread of the virus. Using specific primers, not only target larvae, but also vectors for baculovirus transfer - invertebrate and bird predators can be quickly analysed. For strictly quantitative assays, real-time PCR is a method of choice. The equipment required – light cyclers, are relatively expensive, but their prices decrease very quickly.

The *in vitro* production is still a strong requirement on a commercial perspective of baculoviruses use as insecticides. However the accumulation of genotypic variations by serial passage in cell culture prevents its large scale production. One of the most important effects of the viral passage is the change from the parental, many polyhedra per cell (MP) phenotype, to the few polyhedra per cell (FP) phenotype. The major problem of the passage effect is the reduced occlusion and loss of virulence of the occluded virus (Krell, 1996). Frequent mutations have been identified within a specific region in the Few Polyhedra mutants (FP) that contains the 25k *fp* locus (Harrison and Summers, 1995; Lua et al, 2002). This gene encodes a 25KDa protein that is essential for virion occlusion and polyhedron formation. Another type of mutants generated during serial passage of baculovirus is the formation of Defective Interfering Particles (DIs). These mutants have lost the ability to be replicated in the host cell without the aid of a helper virus and large sizes of their genome are usually deleted (Pijlman et al., 2001). These particles replicate faster because they are smaller, and inhibit the replication of a standard virus. The challenge to make *in vitro* commercial production of baculoviruses a viable initiative depends on development of new techniques to sustain MP production through passages in cell cultures from small flasks to large scale commercial fermentors.

The stability of baculoviruses is influenced by temperature, pH, humidity, presence of additives but ultraviolet light is probably the most detrimental factor to viral survival. Under field conditions little activity is left when the virus is not shaded by plant canopy, therefore much effort has been devoted to the development of UV protectants (Shapiro and Dougherty, 1994; Zou and Young, 1994, Morales et al., 2001). The best results were obtained for stilbene fluorescent brighteners which are marketed under many trade names (e.g. Phorwite AR, Blankophor and others). Future developments in the formulations of brighteners may lead to the reduction of cost of baculovirus production. Inactivation of baculoviruses may be also caused by plant metabolites such as peroxidases which generate free radicals (Hoover et al., 1998). The inactivation can be reduced by addition of free radical scavengers such as mannitol or enzyme superoxide dismutase to baculovirus preparations (Zhou et al., 2004).

The activity of baculoviruses against their natural hosts may be enhanced by introduction of insect-specific toxins or by interference with insect physiology (Bonning and Hammock, 1996; Inceoglu *et al*., 2001). Baculovirus genome modifications by introduction of exogenous toxin genes were extensively studied in many laboratories. Most of the research was devoted to the studies of arthropod toxin genes isolated from the scorpion or spiders (Bonning and Hammock, 1996; Inceoglu et al., 2007). The most potent insect-specific toxin gene used for construction of baculovirus recombinants was the gene coding for a toxin from scorpion *Androctonus australis*. The feeding damage caused by larvae infected with this modified baculovirus was reduced by about 60% in comparison to a wild type baculovirus (Inceoglu et al., 2001). Toxin genes isolated from other scorpions, e.g. *Leiurus quinquestriatus hebraeus* (Froy et al., 2000), straw itch mite *Pyemotes tritici* (Burden et al., 2000), ants

(Szolajska et al., 2004) or spiders (Hughes et al., 1997) have been intensively studied as potential enhancers of baculovirus activity. Arthropod toxins usually attack insect sodium channels producing final effect similar to the chemical insecticides of the pyrethroid group. However, the specific target in sodium channels is different, so there is a potential possibility to produce synergistic effect by biopesticide/chemical pesticide application (McCutchen et al., 1997).

Baculovirus recombinants that produced occlusion bodies incorporating *Bacillus thuringiensis* toxin were constructed by making a fusion protein consisting of polyhedrin and Bt toxin (Chang et al., 2003). The pathogenicity of the recombinant was remarkably increased compared to wild-type virus. These studies proved that it is possible to construct a biopesticide which combines the advantages of the virus and the bacterial toxin.

The changes to host physiology 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 former approach was employed by cloning juvenile hormone esterase gene into baculovirus genome which overexpressed decreases the concentration of the juvenile hormone which is a signal for a caterpillar to stop feeding and pupate. This line of research is being pursued in some laboratories (Hammock et al., 1990; Inceoglu et al., 2001). 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 *egt*-deletion from baculovirus genome resulted in 30% faster killing of caterpillars. Another advantage of this genomic modification is the fact that the *egt* gene is not essential for viral replication and can be replaced with an exogenous gene; the product of which may enhance the insecticidal activity of the recombinant virus (Sun et al., 2004).

In the future, genetically modified baculoviruses will contribute to the expansion of baculovirus use worldwide, as these GMOs are considered safe through extensive research conducted over many years. The scientific data indicate that baculoviruses pose no hazard to other animals than their hosts and this was documented by a number of studies from different laboratories. Recombinant baculoviruses were not pathogenic to bees and all vertebrate species (Sun et al., 2004) as well as to the natural enemies of larvae such as parasitoids and predators (Boughton et al., 2003). However, in spite of this sound evidence, preliminary field trials of genetically modified baculoviruses raised massive public protests which put on hold further trials for a long time. The slow progress in application of genetically modified baculoviruses as pesticides may be in part due to the choice of toxin genes used for modifications of the baculovirus genome which were isolated from highly dangerous invertebrates. Taking into account the origin of these social conflicts, the choice of toxin genes used for genome modifications should be restricted to genes coding for ecologically natural insect toxins, e.g. the genes coding for toxic polypeptides of parasitoid wasps occurring in regions infested by a particular pest. The more rational approach is also needed in the social perception of dangers associated with genetically modified baculoviruses by educating the public on risks and benefits of recombinant baculovirus pesticides.

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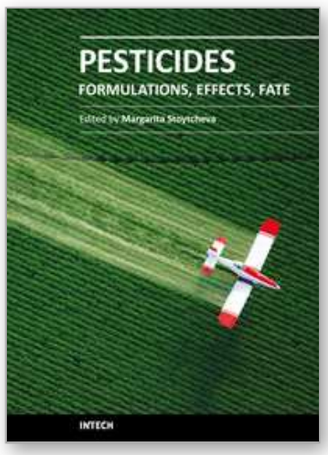

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This book provides an overview on a large variety of pesticide-related topics, organized in three sections. The first part is dedicated to the "safer" pesticides derived from natural materials, the design and the optimization of pesticides formulations, and the techniques for pesticides application. The second part is intended to demonstrate the agricultural products, environmental and biota pesticides contamination and the impacts of the pesticides presence on the ecosystems. The third part presents current investigations of the naturally occurring pesticides degradation phenomena, the environmental effects of the break down products, and different approaches to pesticides residues treatment. Written by leading experts in their respective areas, the book is highly recommended to the professionals, interested in pesticides issues.

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