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Microbial Biopesticides with a Focus on *Bacillus thuringiensis* and Baculoviruses

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1. INTRODUCTION

Agriculture has been facing the destructive activities of numerous pests since long, leading to radical decrease in yields. Chemical pesticides for plant protection occupy the leading place as regards their total volume of application in integrated pest management. But these chemical pesticides cause toxicity to humans and warm-blooded animals as well as pollute our environment.

Despite many years of effective control by conventional chemical insecticides, continued use of chemical pesticides and some other factors are threatening the effectiveness of these agents. This leads to development of pesticide resistance and de-registration of some insecticides due to human health and environmental concerns. Therefore, an alternative is needed which must be ecofriendly. Higher quality and greater quantity of agricultural products can be generated by improvement in pest control strategies. Therefore, there is a need to develop biopesticides which are effective, biodegradable and ecofriendly (Mazid et al., 2011).

Biopesticides are natural substances that control pests (insects). Biopesticides are living organisms (bacteria, virus, fungi, etc.) or their products (phytochemicals, microbial products) or byproducts (semiochemicals), which can be used for the management of pests that are injurious to crops. Biopesticides have an important role in crop protection. Biopesticides based on pathogenic microorganisms specific to a target pest offer an ecologically sound and effective solution to pest problems. They are not harmful to the environment and human beings. The most commonly used biopesticides are living organisms, which are pathogenic for the pest of interest. These include biofungicides (*Trichoderma*), bioherbicides (*Phytopthora*), bioinsecticides (*Bacillus thuringiensis*) and baculoviruses (Mazid et al., 2011). The biopesticides offer the following advantages:

Table 2.1: List of registered biopesticides under Insecticides Act, 1968 (Gupta and Dikshit, 2010)

S. No.	Name of the Biopesticide
1.	NPV of *Helicoverpa armigera*
2.	NPV of *Spodoptera litura*
3.	*Bacillus thuringiensis* var. *galleriae*
4.	*Bacillus thuringiensis* var. *kurstaki*
5.	*Bacillus thuringiensis* var. *israelensis*
6.	*Bacillus sphaericus*
7.	*Trichoderma harzianum*
8.	*Trichoderma viride*
9.	*Beauveria bassiana*
10.	*Pseudomonas fluorescens*
11.	Cymbopogan
12.	Neem based pesticides

4. BACTERIAL BIOPESTICIDES

Bacterial biopesticides are the most widely used and are cheaper than the other modes of pest bioregulation. Many species of bacteria can infect different varities of insects, but those bacteria belonging to the genus *Bacillus* are most widely used as pesticides. One of the *Bacillus* species, *Bacillus thuringiensis,* has developed many molecular mechanisms to produce insecticidal toxins; most of toxins are coded for by several Cry genes (Schnepf et al., 1998).

Since its discovery in 1901 as a microbial insecticide, *Bacillus thuringiensis* has been widely used to control insect pests important in forestry, agriculture, and medicine. Its principal characteristic is that during sporulation it synthesizes a crystalline inclusion containing proteins known as δ-endotoxins or Cry proteins, which have insecticidal properties. To date, more than one hundred *B. thuringiensis*-based bioinsecticides have been developed, which are mostly used against lepidopteran, coleopteran and dipteran larvae. In addition, the genes that code for the insecticidal crystal proteins have been successfully transferred into different crop plants (corn, brinjal, cotton, tomato, etc.), which has led to significant economic benefits (no need of spraying chemical pesticides and crop yield was increased). *B. thuringiensis* and Cry proteins are efficient, safe and sustainable alternatives to chemical pesticides for the control of insect pests because of their high specificity and their safety in the environment, (Roh et al., 2007; Kumar et al., 2008). The toxicity of the Cry proteins has traditionally been explained by the formation of transmembrane pores or ion channels that lead to efflux of ATP and finally osmotic cell lysis (Roh et al., 2007; Kumar et al., 2008).

4.1 *Bacillus thuringiensis*

Bacillus thuringiensis is capable of synthesizing during sporulation a protein crystal known as δ-endotoxins and it has insecticidal activity. This crystalline inclusion may contribute about 25 percent of the dry weight of the bacterium. Bt bacterium was first isolated in 1901, by the Japanese bacteriologist S. Ishiwata from infected silk worms, *Bombyx mori* (Ishiwata 1901). It was subsequently rediscovered by the German biologist Berliner in 1911, who isolated it from infected chrysalids of the Mediterranean flour moth, *Ephestia kuehniella*, collected from a mill in the province of Thuringe (Berliner, 1915). He called this bacterium *Bacillus thuringiensis*. Agronomists soon became interested in the insecticidal properties of Bt, because small amounts of preparations of this bacterium were sufficient to kill many insect larvae rapidly. The first formulation based on Bt was developed under the name "Sporéine" in France in 1938, but the first well-documented industrial procedure for producing a Bt-based product dates from 1959, with the manufacture of "Bactospéine". It was the first French patent for a biopesticide formulation. Commercial formulations of Bt composed of spore/crystal preparations obtained by culturing the bacterium in fermentors; the preparations are purified, dried and used in a granulated or wettable powder formulation for use as a spray. δ-endotoxins are highly diverse, resulting in a generally restricted activity spectrum for each individual toxin, and are innocuous to plants, animals and almost all non-target insects (ladybirds, bees and other auxiliary biological control agents) (Marvier et al., 2007). The industrial-scale production of Bt is now well controlled. It is relatively simple and competitive in terms of cost, and this obviously contributes to its success.

4.1.1 Processing of Parasporal Crystal Protein in Insect Midgut after Ingestion

The insecticidal activity (toxin) of *B. thuringiensis* subsp. *kurstaki* (first discovered in 1911) and other strains is contained within a very large structure called a parasporal crystal, which is synthesized during bacterial sporulation. Although no significant role on behalf of the bacterium has been attributed to the parasporal crystal structure, by synthesizing the crystal, the bacterium is "providing for its future" in that a dead insect provides sufficient nutrients to allow germination of the dormant spore. The parasporal crystal contains approximately 20 to 30 percent of the dry weight of a endospore culture and usually composed of mainly protein (~95%) and a small amount of carbohydrate (~5%). About 150 different parasporal crystal proteins (Cry proteins) are known. The parasporal crystal is an aggregate of individual crystal protein that can generally be dissociated by mild alkali treatment into individual subunits (Aronson and Shai, 2001). The individual subunits can be further dissociated *in vitro* by treatment with β-mercaptoethanol, which reduces disulfide linkages (Fig. 2.1).

The parasporal crystal does not usually contain the active form of the insecticide. Rather, once the crystal has been solubilized, the protein that is released is generally a protoxin, a precursor of the active toxin. The protoxin of many of the Cry toxins that are directed against lepidoptera have a molecular mass of approximately 130 kilodaltons (kDa) (Fig. 2.1). When a parasporal crystal is ingested by a target insect, it is processed by

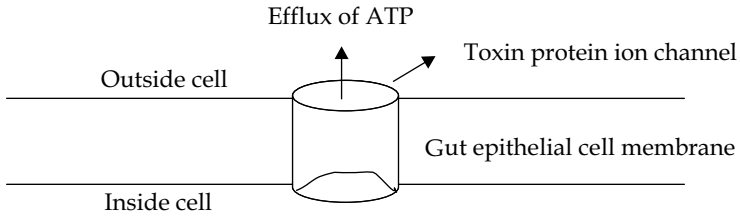

Fig. 2.2 Insertion of the Bt toxin into the membrane of an insect midgut epithelial cell. The Bt toxin creates an ion channel between the cell cytoplasm and the exterior environment.

Activated Bt toxins bind to specific proteins (cadherins) which are present on the surfaces of the microvilli of the insect midgut epithelial cells. Binding of active toxin monomers to cadherins, which are transmembrane glycoproteins containing 12 cadherin repeating domains and one membrane-proximal extracellular domain (Fig. 2.3), facilitates the development of a multimeric form of the toxin monomers and formation of a pore in the membrane. Loss of cadherin protein or mutation in cadherin genes is generally associated with resistance of insects to *B. thuringiensis* (Glick et al., 2010).

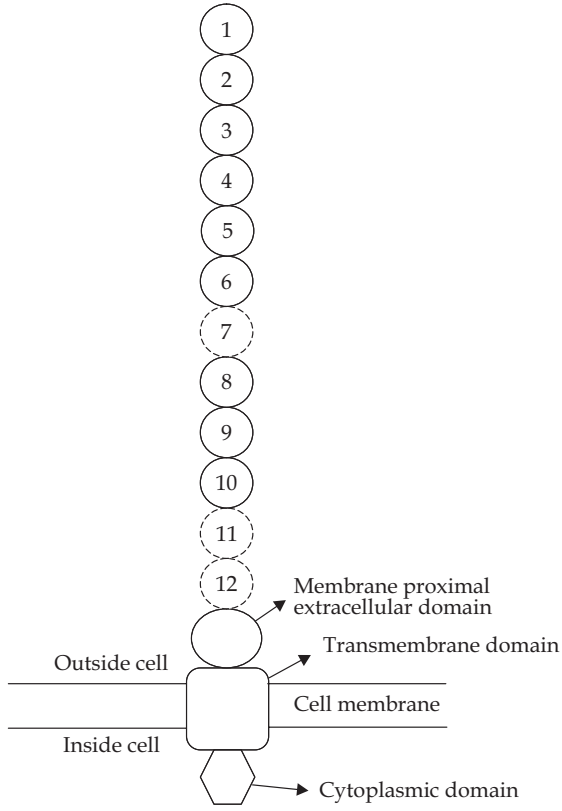

Fig. 2.3 Schematic representation of a cadherin protein molecule embedded in an insect midgut epithelial membrane. Twelve cadherin domains are labeled. Domains 7, 11, and 12, which are highlighted as dashed circles, have been implicated as binding sites for Cry proteins.

4.1.3 Properties of the Insecticidal Toxin Protein from Different Strains of *B. thuringiensis*

This bacterium comprises a large number of strains and subspecies, each of which produces a different crystal toxin that can kill specific insects only. There are more than 150 different subspecies of *B. thuringiensis* (Table 2.2).

Table 2.2: Some important properties of the insecticidal toxins from different strains of *B. thuringiensis* (Lereclus et al., 1993)

B. thuringiensis strain or subspecies	Protoxin size (kDa)	Target insects	Serotype
Berliner	130–140	Lepidoptera	1
kurstaki KTO, HD-1	130–140	Lepidoptera	3
entomocidus 6.01	130–140	Lepidoptera	6
aizawai 7.29	130–140	Lepidoptera	7
aizawai IC 1	135	Lepidoptera, Diptera	7
kurstaki HD-1	71	Lepidoptera, Diptera	3
tenebrionis (*san diego*)	66-73	Coleoptera	8
morrisoni PG14	125-145	Diptera	8
israelensis	68	Diptera	14

For example, *B. thuringiensis* subsp. *israelensis* kills diptera, such as blackflies and mosquitoes. *B. thuringiensis* subsp. *tenebrionis* (also known as *B. thuringiensis* subsp. *san diego*) kills coleoptera (beetles), such as the boll weevil and potato beetle. *B. thuringiensis* subsp. *kurstaki* is toxic to lepidopteran larvae, including those of butterflies, moths and skippers; cabbage worms; and spruce budworms. In addition, some subspecies of *B. thuringiensis* produce insecticidal toxins that are effective against orthoptera (grasshoppers, crickets, and locusts), hymenoptera (sawflies, wasps, bees, and ants) and mallophaga (lice).

4.1.4 Classification of *B. thuringiensis* Toxin Protein

The insecticidal toxins from the *B. thuringiensis* strains were previously grouped into four major classes, i.e., CryI, CryII, CryIII, and CryIV, based on the insecticidal activity of the toxin. These proteins were further organized into subclasses (A, B, C, etc.) and subgroups (a, b, c, etc.). In the past few years, as increasing numbers of *B. thuringiensis* strains were isolated and their genes were characterized, it became clear that the original classification was unable to accommodate many of the newly discovered *B. thuringiensis* toxin genes. Therefore, a new system of *B. thuringiensis* gene classification was introduced.

In the current classification scheme (established in 1998), *B. thuringiensis* insecticidal (Cry) proteins are assigned designations based on their degree of evolutionary divergence and this parameter was estimated by certain mathematical algorithms. In this scheme, a phylogenetic tree was constructed based on the amino acid sequences of *B. thuringiensis*

4.1.5 Limitations of Bt Toxin

The mode of action of *B. thuringiensis* toxins imposes certain constraints on its application. To kill an insect pest, *B. thuringiensis* parasporal crystals must be ingested. Contact of the bacterium or the insecticidal toxin with the surface of the target organism has no effect on it. The requirement that the insecticide be ingested, in part, limits the susceptibility of nontarget insects and other animals to the insecticide. *B. thuringiensis* is generally applied by spraying, so it is usually formulated with insect attractants to increase the probability that the target insect will ingest the toxin. However, insects that bore into plants or attack plant roots are less likely to ingest a *B. thuringiensis* toxin that has been sprayed on a host plant, so other strategies have been devised to control such pests. One approach is to create transgenic plants that carry and express a *B. thuringiensis* toxin gene so that they are protected from infestation throughout the growing season.

A limiting feature of the action of the *B. thuringiensis* toxin is that it can kill a susceptible insect only during a specific developmental stage. Therefore, the toxin must be applied when the pest population is at a particular stage in its life cycle (generally, the larval stage). The other major impediment to more widespread application of *B. thuringiensis* subsp. *kurstaki* is that it costs from 1.5 to 3 times as much as chemical insecticides. Some subspecies of *B. thuringiensis* that have been approved for use in the field and have rapidly gained widespread. These are *B. thuringiensis* subspecies *aizawai, kurstaki, israelensis, tenebrionis* (Glick et al., 2010).

4.1.5.1 *Bacillus thuringiensis subsp. kurstaki*

It was first discovered in 1901, although its commercial potential was ignored until 1951. Within recent decades, however, *B. thuringiensis* subsp. *kurstaki* has become the major means of controlling the spruce budworm in Canada. In 1979, approximately 1 percent of the forest area in Canada that was treated with an insecticide to combat the spruce budworm (about 2 million hectares, or 8000 square miles) was sprayed with *B. thuringiensis* subsp. *kurstaki*. The remainder of the treated forests were sprayed with chemical insecticides. By 1986, the use of *B. thuringiensis* subsp. *kurstaki* had increased dramatically. It was used to treat approximately 74 percent of the forests sprayed in that year for spruce budworm.

In other countries, *B. thuringiensis* subsp. *kurstaki* has been used against tent caterpillars, gypsy moths, cabbage worms, cabbage loopers, and tobacco hornworms. For the biological control (biocontrol) of insect pests, *B. thuringiensis* subsp. *kurstaki* is typically applied by spraying approximately 1.3×10^8 to 2.6×10^8 spores per square foot (1 square foot is equivalent to 0.093 m^2) of the target area. Administration of the spores is timed to coincide with the peak of the larval population of the target organism, because the parasporal crystals, being sensitive to sunlight, are short-lived in the environment. Under simulated conditions, sunlight degrades over 60 percent of the tryptophan residues of the parasporal crystal within a 24-hour period, thereby rendering the protein inactive. Depending on the amount of sunlight present, parasporal crystals may persist in the environment for as little as a day or as long as a month. The lack of persistence of the insecticidal protoxin in the natural environment means that natural selection of resistant insects is highly unlikely (Glick et al., 2010).

5. VIRUSES AS BIOPESTICIDES

Biopesticides mean the use of bacteria, fungi, protozoa, viruses, and nematodes for the biological control of insect pest, diseases, nematodes and weeds in agriculture, veterinary, medicinal, horticultural and forestry ecosystems. At present, the global market for microbial pesticides is more than US $ 125 million per annum which is still less than 1 percent of the total global market for chemical pesticide crop protection of $ 20–25 billion. The market is dominated by *Bacillus thuringiensis* (80%) followed by nematodes (13.3%) and others (6.67%). Nearly Rs. 14, 926 million have been spent during last decade by government of India for biocontrol programme in different crops. In India, some major states, adapting IPM strategy are Madhya Pradesh, Maharashtra, Uttar Pradesh, Andhra Pradesh and Karnataka. It is, therefore, clear that proper encouragement is needed for biological suppression of crop pests. Insect viruses are obligate, intracellular and pathogenic parasites. The viruses belonging to 11 families which are pathogenic to insects. Baculoviruses are associated with the orders of Hymenoptera, Lepidoptera, Coleoptera, Diptera, Trichoptera, Crustacea, Neuroptera, and mites. The virions are rod-shaped, 40–70 × 250–400 nm, comprising an envelope around a protein capsid containing DNA-protein core. The core (DNA) and protein capsid is known as the nucleo-capsid (Steinhaus, 1949).

These viruses are specific and highly virulent to their hosts. They are restricted in their virulency to the class insecta; they are often genus or species specific. About 60 percent of the total known insect viruses (1200) belong to the family Baculoviridae and it is estimated that such viruses can be used against nearly 30 percent of all the major pests of food and fibre crops.

Nucleopolyhedrosis virus (NPV) is known for high epizootic levels and is naturally occurring, self-perpetuating, safe to natural enemies due to host specificity and eco-friendly. Since, NPV is an obligate parasite, it multiplies only in living insect larvae. So, mass production of NPV is a tough job and requires skilled workmen (Mclntosh et al., 1987).

Nuclear polyhedrosis viruses recorded in India include *Helicoverpa armigera, S. exigua, S. litura, Agrotis ipsilon, A. segetum, Amsacta moorei, Anadividia peponis, Trichoplusiani, Thysanoplusia orichalcea, Plutella xylostella, Corcyra cephalonica, Adisura atkinsoni, Phthorimaea operculella* and *Mythimna separata* (Erayya et al., 2013).

5.1 Baculoviruses

Baculoviruses are one of the largest and most diverse groups of double-stranded DNA viruses (almost 1000 species have been described) pathogenic only for insects mostly of the orders Lepidoptera (butterflies, moths), Hymenoptera (sawflies) Coleoptera (beetles) and Diptera. The size of viral genome ranges from 80 to 200 kb. Individual baculovirus usually have a narrow host range restricted to a few closely related species. *Autographa californica* nucleopolyhedrovirus (*Ac*NPV) is the most widely studied baculovirus. Baculoviruses are arthropod viruses and they are well known due to their potential as agents of biological control of pests in agriculture, forestry and horticulture. They are also widely used as expression vectors in biotechnology. The family Baculoviridae contains

The occlusion bodies (polyhedra) of nucleopolyhedrovirus contain many occlusion-derived virions (ODV) surrounded by a matrix of polyhedrin protein, a major structural protein (Braunagel et al., 2003). Polyhedrin is produced in large quantities (nearly 30% of total protein mass at the time of host death), but it is not needed for the transmission of the virus from infected cell to healthy cell. Polyhedra are relatively stable and the protected virions can survive in the environment for more than twenty years under favourable conditions. Polyhedra resemble clear, irregular crystals of salt under 1000× magnification so they are big enough to be seen in a light microscope.

In nature, baculoviruses are found embedded within proteinaceous matrix known as 'polyhedra' on plant foliage, plant debris and soil. Insect larvae acquire infection when they feed on plant foliage, plant debris. Baculoviruses primarily infect insect larvae and it is uninfectious to the adult insects. The insect larvae, while feeding on the plant foliage, plant debries accidentally feed upon the polyhedral. Upon ingestion by an insect, the polyhedra move to the midgut, where the alkaline environment facilitates the dissolution of the polyhedrin protein coat, releasing infectious nucleocapsids. The nucleocapsids are taken up by the insect midgut cells and then migrate through the cytoplasm to the nucleus, where the nucleocapsid is removed. After viral replication, which takes place in the nucleus, and nucleocapsid assembly, some nucleocapsids are released by budding through the plasma membranes of infected midgut epithelial cells into the circulatory system of the insect or get embedded in the polyhedral late in the infection process. Consequently, the infection spreads to other cells throughout the insect (Figs. 2.6 and 2.7). It usually takes about 10 rounds of viral replication, or about 5 to 9 days, for the insect to die. At that stage, about 25 percent of the dry weight of the insect consists of polyhedron/occlusion bodies. Masses of these polyhedral are released in the soil environment upon death (after tissue liquefaction and then rupturing of midgut epithelial cells) of the infected larvae. From here, they are again ready to be ingested and infect their healthy caterpillar hosts (Huh and Weaver, 1990).

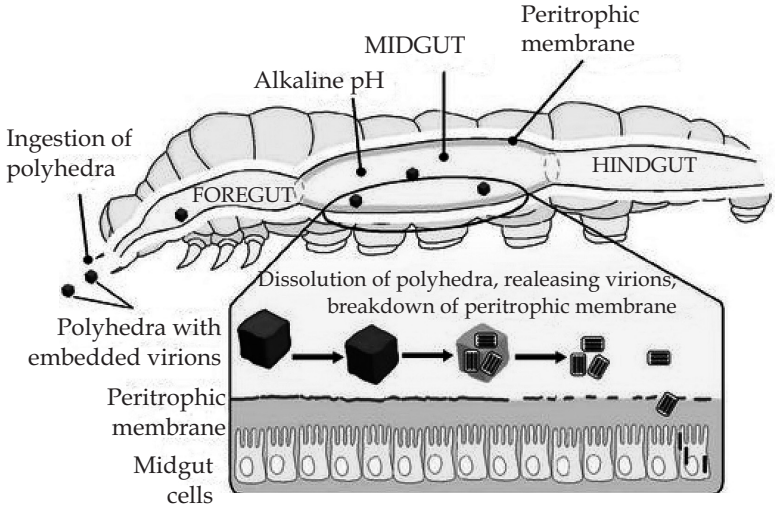

Fig. 2.6 Infection of an insect caterpillar larva by a nucleopolyhedrovirus

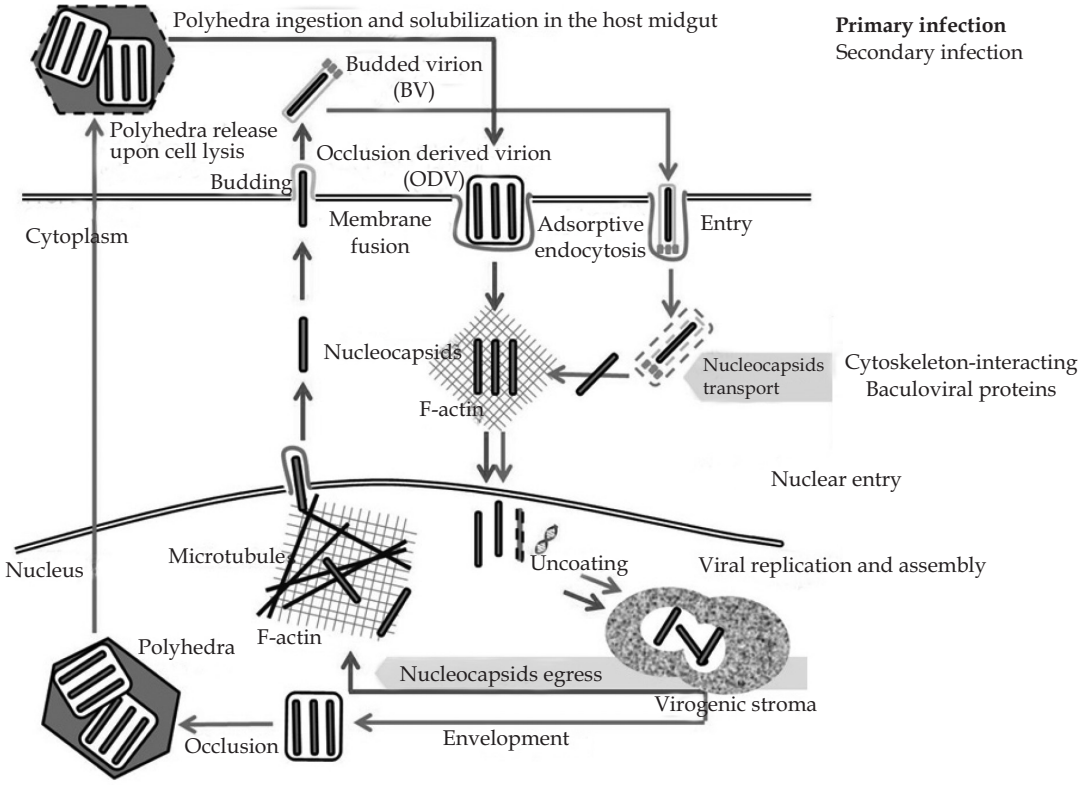

Fig. 2.7 Diagram representing different stages of life cycle of a typical baculovirus and formation of polyhedral, occlusion-derived virion (ODV) and budded virion (BV) (Monteiro et al., 2012).

Table 2.3: Some insect pests that are currently controlled with baculoviruses (Glick et al., 2010)

Pest	Common name	Crop
Anticarsia gemmatalis	Velvetbean caterpillar	Soybean
Chrysomela scripta	Cottonwood leaf beetle	Trees
Chrysomela scripta	Codling moth	Apple, walnut
Heliothis sp.	Cotton bollworm	Cotton, sorghum
Lymantria dispar	Gypsy moth	Deciduous trees
Mamestra brassicae	Cabbage moth	Vegetables
Neodiprion sertifer	European pine sawfly	Pine
Oryctes rhinoceros	Rhinoceros beetle	Coconut
Spodoptera littoralis	Egyptian cotton leaf worm	Cotton
Spodoptera exigua	Beet armyworm	Vegetables, flowers
Trichoplusia ni	Cabbage looper	Brassicas

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, essential for virus budding and necessary for entrance of the virus into the next host cell is the GP64 (Blissard 1996). Infection of lepidopteran cells by baculovirus is influenced by various culture conditions including dissolved oxygen concentration, pH, temperature, osmolarity and nutrient composition of the culture medium. For process optimization of *in vitro* baculovirus production, main requirements are 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 in the costs of components of cell culture medium.

Some other challenges for *in vitro* production of baculoviruses are the requirements of productive insect cell lines (Jem et al., 1997) and highly productive culture media (Chakraborty et al., 1999). At present, many cell lines are available for production purposes and are derived from various sources, thus exhibiting a wide variety of growth and production characteristics. Screening or formulation of media must be performed carefully for a particular virus isolate cell line combination, as different media can greatly affect yields of polyhedra (Pedrini et al., 2006). Recently, a new strategy for *in vitro* baculovirus production was proposed based on many polyhedra (MP) variants. These are clones selected after several passages of the virus in cell culture using the plaque assay technique. MPs maintain the wild-type features such as formation of many polyhedra in the infected cell nucleus and high titer of budded virus (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.

5.1.3 Advantages of Baculovirus Based Biopesticides

A positive feature of using baculoviruses as biocontrol agents is that they generally have limited host ranges and do not affect nontarget organisms. However, this means that any particular baculovirus can be used to control only a limited number of insect pests. Since baculoviruses coevolved with their insect hosts over thousands of years, they are well adapted to avoid the insect's defence mechanisms, and resistance to these viruses develops only rarely, and much less frequently than resistance to *B. thuringiensis* (Glick et al., 2010).

Control of the European spruce sawfly *(Gilipinia hercyniae)* population in eastern Canada is the best example of insect control by a baculovirus. Population of European sawfly were reduced to below economic threshold levels by 1943 and remain under control today. Ironically, the reason why some baculoviruses are not used commercially is related to the effectiveness of the virus. If a virus is effective for preventing proliferation of a particular insect species, the virus has to be applied only once every year or so, making it difficult for the industry to justify the high registration costs. Farmers and growers prefer to use a single insecticidal agent that can control many different insect pests rather than a number of different insecticides, so if baculoviruses are to be used more extensively, their limited host range needs to be expanded (Glick et al., 2010).

6. CONCLUSION

Due to "chemical pesticide" problems in India, there is an urgent need to promote environment-friendly 'biopesticides'. Moreover, recent government policies also favour biopesticides. Application of biopesticides has thus become an integral component of nutrient management system. Apart from these, they play a vital role in increasing the agricultural production.

Demand for biopesticides is increasing steadily in all parts of the world. Efficacy of biopesticides can be equal to or better than conventional products, especially for crops like vegetables, fruits, flowers and nuts, when they are used in integrated pest management systems. By combining safety and performance, biopesticides perform with high efficiency while providing the flexibility of minimum application restrictions, resistance management potential, superior residue and human and environmental safety benefits. As a natural agent, they pose little danger to the environment as compared to the dangerous, persistent and broad-spectrum chemical pesticides.

It is expected that in future they will play more important role in agriculture and forestry. Biopesticides clearly have a potential to play a significant role in development of future integrated pest management strategies. Hopefully, in the near future more rational approach will be gradually adopted towards biopesticides and fate of biopesticides will not be determined by short-term profits from chemical pesticides (Usta, 2013).

Baculoviruses have been confirmed to be safe in terms of their effect on beneficial insects and vertebrates. NPV is one of the important biopesticides, because it has less residual toxicity, is ecofriendly, has self-perpetuating nature and is compatible with many chemical pesticides. Hence, NPV can be implemented as one of major component in integrated pest management programme. But, scope is always there to develop quality control guidelines and methodologies, systematic registration policies, to identify effective stains and to develop UV (ultraviolet) resistant strains. In addition, the guidelines and training for implementation of biocontrol agents should be made available by competent agencies (Erayya et al., 2013).

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