BIOPESTICIDE PRODUCTION

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Summary

There are two aspects of economic problems caused by insects. One concerns the loss of production that results from damage to crops and to the health of human and domestic animals, the other concerns the cost of attempt to prevent or control such production losses. Mosquitoes and black flies are a constant threat to health and comfort, yet the chemical pesticides used to control them have created serious ecological problems. Population of resistant mosquitoes and black flies has evolved, beneficial insects and natural predators have been destroyed and environmental pollution has increased worldwide.

Therefore, scientists have sought new, environmentally safe technologies to combat mosquitoes and black flies.

Ecological problems created by chemical insect control methods and their relevance to human health are receiving serious attention everywhere. Various pathogens, including viruses, protozoa, fungi and nematodes can be used to regulate pest population. Biological control of pests and vectors has been studied to a limited extent for many years with several notable successes, of which microbiological control is one aspect.
The development of insecticide resistance in pest and vector population, the damage caused to non-target organisms and the realization of other environmental hazards of chemical insecticides have led to an increasing interest in biological, including microbiological control methods.

At present some strains of *Bacillus thuringiensis*, nuclear polyhedrosis virus, fungi and the nematode parasite of mosquitoes are commercially available. Biological control methods, especially those using an ecological approach, are arising interest in developing countries. It is important that microbial control of insect pests be further developed and that entomologists should be able to quantify and make contributions to the regulation of insect populations by naturally occurring pathogens. The emphasis given to each subject area reflects the efforts of individuals; hence the purpose of this publication is to provide a record as an instruction in microbial control of insects and biopesticide production.

1. Introduction

For the past five decades humans have almost been wholly dependent upon synthetic/organic insecticides. Agriculture has been revolutionized by the use of chemicals for crop protection, which started in the last 1800 with the introduction of arsenical insecticides and Bordeau mixtures as grape fungicide, and progressing to the very sophisticated compounds available now. Today, fewer people produce more food at less cost than ever before. The effect of synthetic chemicals on agriculture has been so dramatic that conventional agriculture now means using chemicals. Despite the immense benefits, they are used in increasing quantities designed to kill living organisms. However, the very properties that give these chemicals useful-long residual action and high toxicity for a wide spectrum of organisms, have given rise to serious environmental problems. Furthermore, the emergence and spread of increasing resistance in many vector species, concerns over environmental pollution, and the ever increasing cost of the new chemical insecticides, make it apparent that vector and pest control can no longer be safely based upon the use of chemicals alone.

Consequently, increasing attention has been directed toward natural enemies such as predators, parasites, and pathogens. Unfortunately, none of the predators or parasites can be mass produced and stored for long periods of time, since they all must be raised in vivo. It has become evident that there is an urgent need for a biological agent, possessing the desirable properties of a chemical pesticide making it highly toxic to the target organism, which can be mass produced on an industrial scale, has a long shelf life and can be safely transported.

In the mid seventies, WHO and other international organizations initiated studies into existing biological control agents and the development of new ones. Today, biological control is widely regarded as a desirable technique for controlling insects, due to its minimal environmental impact and its avoidance of problems of resistance in the vectors and agricultural pests.
2. Biological Control

Of the nearly one million known species of insects, about 15,000 species are considered pests and about 300 require some form of control. Fortunately, most insect pests have pathogenic microorganisms associated with them.

Entomopathogens have been suggested as controlling agents of insect pests for over a century, and belong to species of fungi, viruses, bacteria, and protozoa.

Insect pathology per se probably had its beginning in the nineteenth century under the stimulus of Bassi and Pasteur. A significant contribution to microbial control of insects was made by Mechnikoff in 1879 and Krassilnikow in 1888, who were the first to document that an entomopathogen, a muscordine fungus, *Metarrhizium anisopliae* could be mass produced and applied as a microbial insecticide to control the grain and the sugar beet pests. The control of insect pests with bacteria was probably first attempted by d'Herelle in 1914, approximately 35 years after Pasteur's description of silkworm diseases. Apparently the control was not consistent and therefore interest in bacterial pathogens was curtailed.

However, after a lag period of nearly 30 years, White and Dutky succeeded in 1940 in demonstrating a control of the Japanese beetle by distributing spores of the milky disease bacterium *Bacillus popilliae*. This success stimulated further investigations of bacteria and literature began appearing on the effectiveness of *Bacillus thuringiensis*. The issuing of eight patents between 1960 and 1963 for *B. thuringiensis* led to a revived interest in bacterial insecticides. The use of viruses to control insect pests was stimulated by the studies of Balch and Bird in 1944 and Steinhaus and Thompson in 1949, respectively. This initial interest is presently having a rebirth, as is evidenced by the recent registration of the first viral pesticide in the United States by the Environmental Protection Agency (EPA).

Of these, bacteria, viruses and some fungi, because of their known effectiveness and relative lack of toxicity or pathogenicity to nontarget animals and plants, have been developed into commercial products. Biological control is generally man's use of a specially chosen living organism to control a particular pest. This chosen organism might be a predator, parasite or infectious disease, which attack the harmful insects. Biological control methods can be used as part of an overall integrated pest management program to reduce the legal, environmental, and public safety hazards of chemicals. In addition, it may be a more economical alternative to some insecticides.

Some biological control measures can actually prevent economic damage to agricultural crops. Unlike most insecticides, biological controls are often very specific for a particular pest. There is less danger of impact on the environment and water quality and they offer a more environmentally friendly alternative to chemical insecticides. They could also be used where pests have developed resistance to conventional pesticides. Unfortunately, research and development of biological insecticides attracts very little financial support compared to that given toward the discovery of chemical pesticides.
It is becoming clear that more attention needs to be given to the selection of broad spectrum biopesticides and improvements in the production, formulation and application technologies. Efforts need to be made to optimize the impact of these agents by integrating them with other novel crop protection strategies. Successful use of biological control requires a greater understanding of the biology of both the pest and its enemies. In some cases, biological control may be more costly compared to the use of pesticides. Often the results of using biological control are not as dramatic or quick as it is with chemical pesticides. Most natural enemies attack only specific types of insects unlike broad-spectrum insecticides which may kill a wide range of insects.

Today biological control is regarded as a desirable technique for controlling insects, due to its minimal environmental impact and preventing the development of resistance in vectors. Specific bio toxin-producing strains of Bacillus thuringiensis var. israelensis or B.sphaericus have been used throughout the world to suppress or eliminate the larval stages of mosquitoes, particularly where malaria, filariasis or certain arboviruses are present. Bacillus thuringiensis var. israelensis is also effective against the larval stages of Simulium spp., vectors of river blindness in man (onchocerciasis) in tropical Africa, and the cause of severe ‘fly worry’ in domestic livestock in several regions of the world. Depending on the specific control programmed, chemical larvicides may precede or alternate with the use of Bacillus. Host treatment for onchocerciasis or filariasis may also be performed. Studies conducted to date have shown no significant effect of these bacteria and their toxins on vertebrates and only minimal effects on some non-target arthropods and crustaceans. Development of resistance is apparently less of a problem than with chemical pesticides. These and other potential problems are continuously being monitored and investigated.

Other potential tools which could be used in the future for area-wide biological control programme against insect vectors/pests of veterinary importance include species-specific sex pheromones. These are presently being used as attractants for trapping and monitoring of insects and for mating disruption. In addition, several parasites and pathogens of vector/pest species are under continuous investigation and are providing promising results. Hopefully, the not too distant future will witness the development of further biological control techniques of sufficient scope to free entire regions of pathogenic agents or vectors which cause or transmit significant diseases not only of domestic livestock and humans, but also of free-living wildlife. Virtually all pest populations are affected by natural enemies to some extent. In many cases, natural enemies are the primary regulating force of the pest populations. Natural controls include effects of natural enemies (predators, parasites, pathogens), other biotic (living) factors such as food availability and competition, and abiotic (non-living) factors such as weather and soil. In pest management, biological control usually refers to the action of parasites, predators or pathogens, on a pest population which reduces its numbers below a level causing economic injury. Herbivorous insects and pathogens that attack pest weeds are also considered bio-control agents. Biological control is a part of natural control and can apply to any type of organism, pest or not, and regardless of whether the bio-control agent occurs naturally, is introduced by humans, or manipulated in any way. Biological control differs from chemical, cultural, and mechanical controls in that it requires maintenance of some level of food supply (e.g., pest) in order for the bio-control agent to survive and flourish. Therefore, biological control alone is not a means
by which to obtain pest eradication. Biological control is defined as the action of natural enemies. It can be divided into 2 broad categories, natural biological control and applied biological control. Natural biological control occurs where native or co-evolved natural enemies reduce native arthropod populations, whereas applied biological control involves human intervention to enhance natural enemy activities. Applied biological control can be further separated into (a) classical biological control, where exotic natural enemies are introduced against an exotic or native pest, or (b) augmentative biological control, where human intervention occurs to enhance the effectiveness of the natural enemies already present in an area through manipulation of the environment.

Numerous species of plant-feeding insects have been evaluated for control of pest weeds. The greatest successes have been in rangelands, forests, and other natural habitats where other weed control approaches (e.g., herbicides, cultivation) are impractical or uneconomical. Some pathogens have also been looked at as weed biocontrol agents (e.g., plant rusts). The goal, while using the weed biocontrol agents, is generally to reduce the weed population and not to eradicate it. Importation of a biocontrol agent from the region of origin of the weed has been the most common approach. It is generally a long-term process which requires sustained efforts, but which can reap long-term benefits. A few of bacteria are highly effective at killing insects. The most important of these is Bacillus thuringiensis (Bt). It occurs naturally in insect-rich locations, including soil, plant surfaces and grain stores. It kills a range of insect orders and is the most widely used microbial biopesticide. It is also used in transgenic crops. There are over 40 Bt products available worldwide for control of caterpillars, beetles and blood-feeding flies such as mosquitoes. Together, these account for 1% of the world insecticide market.

As part of its life cycle, Bt produces protein crystals which have insecticidal properties. When ingested, the crystals paralyze the digestive tracts of insects, often killing them within 24-48 hours. Different Bt strains produce crystals with slightly different properties, and the crystals from each strain are specific for a small number of related insect species.

Over 1600 viruses have been recorded from more than a thousand species of insects. A family of viruses called baculoviruses is the most popular choice for microbial control as they are distinct from any type of virus recorded from vertebrates. They have been used regularly for pest control since the 1950s, particularly in forestry where they have been highly effective at controlling sawflies. Baculoviruses are very species, mostly caterpillars and sawflies, but also some species of beetle and flies. Baculoviruses infect their hosts through ingestion. Virus particles invade the cells of the gut before colonizing the rest of the body. Infection reduces mobility and feeding and insects are killed in five to eight days. Mass production of baculoviruses can be done only in insects, but this is economically viable for larger hosts such as caterpillars, and formulation and application are straightforward. At present, there are approximately 16 products available for use, or under development, mostly for control of caterpillar pests. Commercial products are available in Switzerland, Germany and Spain for the control of codling moth and the summer fruit tortrix. Products are also available in the USA for the control of tobacco bollworms on vegetables, ornamentals, tomatoes and cotton.
Over 750 species of fungi kill insects. Entomopathogenic fungi invade their hosts using spores that grow through the cuticle, and hence they are particularly suited for control of pests with piercing mouthparts, such as aphids and whiteflies, which are unlikely to acquire pathogens through feeding. Infection requires high humidity at the insect surface, but this can be overcome using oil-based formulations.

About 20 products are available worldwide for managing sap-feeding insects, beetles, caterpillars, flies and locusts. In the USA, and some countries in Europe, products based on the fungus Beauveria bassiana are becoming available for the control of a range of glasshouse pests.

Entomopathogenic nematode worms are just visible to the naked eye, being about 0.5 mm in length. Juvenile nematodes parasitize their hosts by directly penetrating the cuticle of through natural openings. They then introduce symbiotic bacteria, which multiply rapidly and cause death by septicaemia, often within 48 hours. The bacteria break down the insect body, which provides food for the nematodes. After the insect has died, the juvenile nematodes develop to adults and reproduce. A new generation of infective juveniles emerges 8-14 days after infection.

Unlike other entomopathogens, nematodes are exempt from registration and so have been popular choices for commercialization. Over 60 products are available in Europe. Nematodes require moist conditions to operate and have been marketed predominantly against soil pests, such as vine weevil and sciarid fly larvae. However they may also control foliar pests, for example Nemasys (Becker Underwood) which can be used to control western flower thrips. Like other natural enemies, nematodes are affected by environmental conditions.

Protozoan diseases of insects are ubiquitous and comprise an important regulatory role in insect populations. They are generally host specific and slow acting, most often producing chronic infections. The biologies of most entomopathogenic protozoa are complex. They develop only in living hosts and many species require an intermediate host. Species in the Microsporida are among the most commonly observed. Their main advantages are persistence and recycling in host populations and their debilitating effect on reproduction and overall fitness of target insects. As inundatively applied microbial control agents, only a few species have been moderately successful. The grasshopper pathogen Nosema locustae Canning is the only species that has been registered and commercially developed. The main disadvantages of the protozoa as inundatively applied microbial control agents are the requirement for in vivo production and low levels on immediate mortality.

3. Microbial Insecticides

Microbial insect control utilizes pathogenic microorganisms isolated from diseased insects during naturally occurring epidemics. Typically, such epidemics only occur when pest population densities are high and usually after appreciable damage have been done to crops. Over 400 species of fungi and more than 90 species of bacteria which infect insects have been described including Bacillus thuringiensis, varieties of which
are manufactured and sold throughout the world primarily for the control of caterpillar pests and more recently mosquitoes and black flies.

Among fungal pesticides, five have been introduced since 1979, and three in 1981. Many countries with centrally planned economies have been using fungal pesticides successfully for many years. So far, more than 40,000 species of *Bacillus thuringiensis* have been isolated and identified as belonging to 39 serotypes. These organisms are active against either *Lepidoptera*, or *Diptera* or *Coleoptera*.

### 3.1. *Bacillus Thuringiensis*

Maximizing the potential for successfully developing and deploying a biocontrol product begins with a carefully crafted microbial screening procedure, proceeds with developing mass production protocols that optimize product quantity and quality, and ends with devising a product formulation that preserves shelf-life, aids product delivery, and enhances bioactivity. Microbial selection procedures that require prospective biocontrol agents to possess both efficacy and amenability to production in liquid culture increase the likelihood of selecting agents with enhanced commercial development potential. Scale-up of biomass production procedures must optimize product quantity without compromise of product efficacy or amenability to stabilization and formulation. Formulation of *Bacillus* spp. for use against plant pathogens is an enormous topic in general terms but limited in published specifics regarding formulations used in commercially available products. Types of formulations include dry products such as wettable powders, dusts, and granules, and liquid products including cell suspensions in water, oils, and emulsions. Cells can also be microencapsulated. Considerations critical to designing successful formulations of microbial biomass are many fold and include preserving biomass viability during stabilization, drying, and rehydration; aiding biomass delivery, target coverage, and target adhesion; and enhancing biomass survival and efficacy after delivery to the target.

#### 3.1.1. General Overview

Over 90 species of naturally occurring, insect specific (Entomopathogenic) bacteria have been isolated from insects, plants and the soil, but only a few have been studied intensively. Much attention has been given to *Bacillus thuringiensis*, a species that has been developed as a commercial microbial insecticide since 1960 and is sold under various trade names. *Bacillus thuringiensis* (Bt) occurs naturally in the soil and on plants. Different varieties of this bacterium produce a crystal protein that is toxic to specific groups of insects.

The entomopathogenic microorganism *Bacillus thuringiensis* is one of the most promising biological control agents for pest and insect management since many strains are toxic specifically for *Lepidopteran* and strain *Bacillus thuringiensis* serotype H-14 is highly toxic to *Dipterans*. The first data on the existence and activity of the new strain *B. thuringiensis* H-14 appeared in 1977.
This bacterium was first recorded in 1901 as the cause of the damaging "Sotto" disease in silkworms in Japan. It was again isolated in 1927 by Maltes in Germany and given the name \textit{B. thuringiensis}.

\textit{Bacillus thuringiensis} (B.t.) is a gram positive bacterium characterized by a parasporal crystalline protein inclusion (Figure 1). The proteins are highly toxic to pests and specific in their activity over the past 40 years. The commercial use of \textit{B. thuringiensis} as a pesticide has been largely restricted to a narrow range of \textit{Lepidopteran} (Caterpillar) pests. In recent years, however, investigators have discovered B.t. pesticides with specificities over a much broader range of pests. The toxin genes have been isolated and sequenced, and recombinant DNA-based B.t. products produced and approved. Many of the newly discovered strains have activities that would extend the use of \textit{Bacillus thuringiensis} beyond traditional agricultural markets.

![Figure 1: Electron micrograph of \textit{Bacillus thuringiensis} parasporal body](image)

\textit{Bacillus thuringiensis} products account for 90-95 percent of the total biopesticide market which has grown from $24$ M in 1980 to $107$ M in 1989, and is forecast to expand at an annual rate of 11 percent. The availability of a large number of diverse B.t. toxins may also enable farmers to adopt usage strategies that minimize the risk of B.t. resistant pests.

The B.t. strains known are classified according to their H antigens into 27 groups and 7 subgroups and according to structure and molecular organization of the genes coding for the parasporal delta-endotoxins (Table 1 and 2). Only a few strains are used commercially as control agents. The main bacteria are different varieties of \textit{Bacillus thuringiensis}, \textit{Bacillus sphaericus} and \textit{Bacillus popilliae}. 

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<table>
<thead>
<tr>
<th>H-serotype</th>
<th>Serovar</th>
<th>Supposed Biovars or Pathovars</th>
<th>Abbreviation</th>
<th>First Mention and First Valid Description</th>
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<tr>
<td>1</td>
<td>Thuringiensis</td>
<td>THU</td>
<td></td>
<td>Berliner 1915; Heimpel and Angus 1958</td>
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<td>2</td>
<td>Finitimus</td>
<td>FIN</td>
<td></td>
<td>Heimpel and Angus 1958</td>
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<tr>
<td>3a</td>
<td>Alesi</td>
<td>ALE</td>
<td></td>
<td>Toumanoff and Vago 1951; Heimpel and Angus 1958</td>
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<td>3a,3b</td>
<td>Kurstaki</td>
<td>KUR</td>
<td></td>
<td>de Barjac and Lemille1970</td>
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<td>4a,4b</td>
<td>Sotto</td>
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<td></td>
<td>Ishiwata 1905; Heimpel and Angus 1958</td>
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<td>id</td>
<td>Id</td>
<td>Dendrolimus</td>
<td>DEN</td>
<td>Talalaev 1956; Bonnefoi and de Barjac 1963</td>
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<td>6</td>
<td>Entomocidus</td>
<td>ENT</td>
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<td>id</td>
<td>Id</td>
<td>Subtoxicus</td>
<td>SUB</td>
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<td>Aizawai</td>
<td>AIZ</td>
<td></td>
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<td>id</td>
<td>Id</td>
<td>Tenebrionis</td>
<td>TEN</td>
<td>Krieg, Huger, Langenbruch, and Schnetter 1983</td>
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<td>8a,8c</td>
<td>Ostrinia</td>
<td>OST</td>
<td></td>
<td>Gaixin, Ketian, Minghua, and Xingmin, 1975</td>
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<td>8b,8d</td>
<td>Nigeriensis</td>
<td>NIG</td>
<td></td>
<td>de Barjac et al. Unpub.data</td>
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<td>9</td>
<td>Tolworthi</td>
<td>TOL</td>
<td></td>
<td>Norris 1964; de Barjac and Bonnefoi 1968</td>
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<td>Darmstadiensis</td>
<td>DAR</td>
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<td>Krieg, de Barjac, and Bonnefoi 1968</td>
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<td>TOU</td>
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<td>KYU</td>
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<td>13</td>
<td>Pakistani</td>
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<td></td>
<td>de Barjac, Cosmao, Shail, and Viviani 1977</td>
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<td>14</td>
<td>Israelisens</td>
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<td>16</td>
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<td>17</td>
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<td></td>
<td>Ohba, Aizawa, and Shimizu 1981</td>
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<td>18</td>
<td>Kumamotoensis</td>
<td>KUM</td>
<td></td>
<td>Ohba, Ono, Aizawa, and</td>
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### Table 1: Strains classified according to their H-antigens

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<th>Delta Endotoxin gene</th>
<th>M.W. kDa.</th>
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<tr>
<td>CrylA (a)</td>
<td>130 a 133</td>
<td>L</td>
</tr>
<tr>
<td>CrylA (b)</td>
<td>138</td>
<td>L</td>
</tr>
<tr>
<td>CrylA (c)</td>
<td>135</td>
<td>L</td>
</tr>
<tr>
<td>CrylB</td>
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<tr>
<td>CrylC</td>
<td>71</td>
<td>L/D</td>
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<td>L</td>
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<td>C</td>
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<tr>
<td>CryllC</td>
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<td>C</td>
</tr>
<tr>
<td>CryllD</td>
<td>73</td>
<td>C</td>
</tr>
<tr>
<td>CrylVA, CrylVB</td>
<td>134 et 128</td>
<td>D</td>
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<td>CrylWC</td>
<td>78</td>
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<td>81</td>
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(1) L: lepidopteres; D: dipteres; C: coleopteres.


### Table 2: Molecular organisation of the genes coding δ - endotoxin
3.1.2. Mode of Action

Numerous moth and butterfly larvae and some beetle and fly larvae are susceptible to infection. Formulations of *Bt* variety *kurstaki* are available for the control of many caterpillar pests including imported cabbageworm, cabbage looper, hornworms, European corn borer, cutworms, some armyworms, diamond-back moth, spruce budworm, bagworms, tent caterpillars, gypsy moth caterpillars and other forest caterpillars, and Indianmeal moth larvae in stored grain. Less well controlled are corn earworms on corn, codling moth, peach tree and squash vine borers.

Formulations of *Bt* variety *tenebrionis* and variety *san diego* have been registered for the use against the Colorado potato beetle larvae and elm leaf beetle adults and larvae.

*Bt* variety *israelensis* is marketed for use against black flies and mosquitoes, fungus gnats. Unless used on a community-wide basis, it is probably more effective to eliminate standing water and control weeds at the edges of ponds. *Bt* variety *aizawai* is used to control wax moth larvae in bee hives and various caterpillars. It is important for the control of diamondback moth caterpillar which has developed resistance to *Bt* variety *kurstaki* in some areas.

The toxic crystal *Bt* protein in commercial formulations is only effective when eaten by insects with a specific (usually alkaline) gut pH and the specific gut membrane structures required to bind the toxin. Not only must the insect have the correct physiology and be at a susceptible stage of development, but the bacterium must be eaten in sufficient quantity. When ingested by a susceptible insect, the protein toxin damages the gut lining, leading to gut paralysis. The affected insects stop feeding and die from the combined effects of starvation and tissue damage. *Bt* spores do not usually spread to other insects or cause disease outbreaks on their own as occurs with many pathogens.

3.2. *Bacillus Thuringiensis* Var.*kurstaki*

In 1970, Dulmage isolated the HD-1 strain of *Bt* var. *kurstaki* and it became commercially available shortly thereafter. It is used today for the production of most *Bt* var *kurstaki* formulations used to control defoliating forest *Lepidoptera* in North America. The HD-1 strain is a serotype 3a 3b, and the crystal has a fairly broad spectrum of activity against a large number of *Lepidoptera*. Four companies produce various types of formulations (e.g., aqueous flowable suspension, nonaqueous emulsifiable suspension, oil flowable) of the HD-1 strain for use against gypsy moth. Each formulation contains inert ingredients which are unique and various additives (e.g., stickers) can be included to produce the final mix.

Loss of residual toxicity of *Bt* var *kurstaki* on foliage can result from degradation by sunlight, leaf temperatures, drying, washing off by rain, microbial degradation, and leaf chemistry. Solar radiation appears to be the key factor affecting the survival of *Bt* var *kurstaki* spores and crystals deposited on foliage. In a series of *Bt* var *kurstaki* bioassays, the half-life of its insecticidal activity for early stage gypsy moth larvae in the field has been estimated at 12-32 hours. In spite of this short half-life, a deposition
of 75 IU cm² from a 90 BIU ha⁻¹ application will give, on the average, insecticidal activity against early stage gypsy moth larvae of at least an LC₅₀ for 4 to 6 days.

Many safety tests have been performed with *Bt* var *kurstaki*. None of the vertebrates tested showed any abnormal reaction in terms of external symptoms or internal pathologies.

Nevertheless, vertebrate species that rely on lepidopterans as a food source (e.g., Virginia Big-eared bat, insectivorous birds) have the potential of being indirectly affected by the *Bt* var *kurstaki* suppression programs. For example, Rodenhouse and Holmes showed that a reduction in biomass of lepidopteran larvae following *Bt* var *kurstaki* application led to significantly fewer nesting attempts of certain birds.

Many lepidoptera that co-occur with a pest species are also susceptible to *Bt* var *kurstaki*. Of particular concern would be non-target impacts on lepidopterans that are important as pollinators, in the suppression of weedy plants, and other ecosystem functions. For example, James and coworkers showed in 1992 that *Bt* var *kurstaki* is toxic to late, but not early, instar larvae of the cinnabar moth (*Tyria jacobaeae*), which is an important species in the control of the noxious weed, tansy ragwort. Impacts on rare and endangered species are also of special concern.

*Bacillus thuringiensis* var *kurstaki* is a bacterium that offers natural biodegradable, safe control of pests, and can be sprayed onto plants, where it is eaten by leaf-eating insects, including loopers, hornworms, earworms, caterpillars, gypsy moths, oakworms, meal and flour moths, diamondback moths, fruitworms, leaf folders, and most species of leaf-feeding moth and butterfly larvae. The *Bt* var *kurstaki* disrupts the insects' digestive system and they starve. It is specific to certain pest insects, can be used with nematodes, and is completely environmentally safe. Crops can be harvested the day after any *Bt* variety is applied.

*Bacillus thuringiensis* var *kurstaki* formulations are specific to lepidopteran larvae which is preferable to previously used broader spectrum chemical insecticides.

Technical developments in genetic engineering and molecular biology are providing opportunities for the development of genetically manipulated strains of *Bt* var *kurstaki* and transfer of toxin-coding genes into other bacteria (cloning) or plant species (transgenic plants; see also – Transgenic Plants). In the near future, it may be possible to engineer more taxon specific strains of *Bt* var *kurstaki* that can be developed and commercially produced.

### 3.3. Bacillus Popilliae and Bacillus Lentimorbus

The bacteria grouped under the name *B. popilliae* cause milky diseases of beetles (coleoptera), especially of the beetle family *Scarabaeidae*. In practice, *B. popilliae* has been used intensively and almost exclusively for control of the Japanese beetle, *Popillia japonica*. 

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The beetle causes serious damage in North America, since the adult beetle feeds on a wide range of ornamental and crop plants, eating the tissues between the reins, and accumulates on ripening fruit, causing substantial damage. It is also a problem in the larval stage because the adult beetle lay their eggs in turf and the grubs destroy the grass roots.

Beetle larvae killed by *Bacillus popilliae* and *B. lentimorbus* may turn white, hence the name "milky disease".

By the 1930s, the infestation had become so extensive that a search for a control measure was undertaken which led to the discovery in nature of some diseased larvae. Milky spore bacteria were isolated. The term "milky disease" comes from the larva's pure white appearance when infected with *B. popilliae*. *B. popilliae* was the first insect pathogen to be registered in the U.S. as a microbial control agent.

*Bacillus popilliae* and *Bacillus lentimorbus* are related, naturally occurring bacteria that have been mass-produced for the control of Japanese beetle larvae in turf since the 1940s. Several commercial products are available. The bacteria, usually applied to the soil, cause "milky disease". Milky disease spores may reproduce within the beetle larvae and establish a resident population capable of causing mortality over several seasons if the soil is sufficiently warm and moist through the summer months. It may take several seasons for the disease to control the pest, and it is preferable to treat a broad area to reduce the impact of immigrating healthy beetles.

*B. popilliae* is a Gram-negative spore-forming rod of 1.3 to 2.5 0.5 to 0.8 micrometers. A fastidious organism, *B. popilliae* can only be cultured on rich media containing yeast extract, casein hydrolysate, or an equivalent amino acid source, and sugars. Several amino acids are known to be required for growth, as well as the vitamins thiamine and barbituric acid. Trehalose, the sugar found in insect hemolymph, is a favored carbon source, although glucose can also be used.

Japanese beetle is the exclusive host of the strain of *B. popilliae* which is sold commercially. However, other *B. popilliae* strains (and *B. lentimorbus*, which is considered a strain of *B. popilliae* by some experts) have other scarab hosts and are specific to different beetles in the family *Scarabaeidae*, which includes the Japanese beetle and the chafer, important Pasture pests, but also the beneficial dung beetles.

Spores which reside in the soil and have been ingested by beetle larvae germinate in the larva's gut within 2 days and the vegetative cells proliferate, attaining maximum numbers within 3 to 5 days. By this time, some of the cells have penetrated the gut wall and have begun to grow in the hemolymph, where large numbers of cells develop after 5 to 10 days.

High quality milky spore powder, containing the bacterium *Bacillus popilliae*, inoculates an area of turf and can control Japanese beetle grubs (*Popillia japonica*) for decades. The milky spore bacteria infect and then multiply within the grub host. When the larva dies, the disease is spread to surrounding areas of the lawn or garden. The spores produced in a host are almost invulnerable to climatic conditions and can spend years in
the soil waiting to infect other grubs. For best results, the application should be done when the soil is warm.

### 3.4. Bacillus Thuringiensis H-14

*Bacillus thuringiensis* serotype H-14 (Figure 2 and 3) is highly toxic to dipterans. The first data on the existence and activity of the new strain appeared in 1977.

![Figure 2: Electron micrograph of *Bacillus thuringiensis* H-14 parasporal body](image)

![Figure 3: Electron micrograph of *Bacillus thuringiensis* H-14 parasporal production](image)
*Bt* var *israelensis* is highly effective against mosquito larvae and black fly larvae and comes in two forms: granular (Aquabac) and solid dunks. Both can be used wherever mosquitoes breed: the periphery of ponds, lakes, flooded orchards, ditches, pastures, sewage or animal waste lagoons, roof gutters, run-off areas, and so on.

The action of the delta-endotoxin of *Bt*. H-14 is rapid. A stop of feeding can be observed within minutes following ingestion of crystals. The first histopathological changes can be shown within the microvilli which increase in size and loose their internal structure. Vacuoles appear within the gut epithelial cells. The size of the cells increases until they burst. The breakdown in the control of permeability leads to a free exchange between hemolymph and gut contents which are lethal for the insect larvae. The mode of action of all the different delta-endotoxins of *B. thuringiensis* seems to be based on the same principle. On the other hand several factors are responsible for the unique specificity of the delta endotoxin. The crystals have to be soluble in the gut juice. the gut juice proteases have to generate the right active polypeptide. Finally the appropriate receptors have to be present in the cell membranes of the gut tissue.

Public policy matters and environmental considerations dictate that proof of safety to nontarget organisms (NTO) and fish and wildlife is to be documented before any pest-control agent can be employed in actual pest control programmes. Microbial control agents are not exempted from this mandate.

A comprehensive review of the safety of *B. thuringiensis* varieties to NTO has been rendered by Lacey and Mulla in 1990.

The desired effectiveness of *Bacillus thuringiensis* H-14 on the reduction of larvae is dependent on a number of factors such as the type of insecticide, the age of larvae, formulation type, water quality of larvae habitat, temperature, physical conditions and culture media used in the production process, inhibiting and accelerating factors of larvae food intake, depth of larvae nests, amount of food available to larvae, concentration of larvicide and its mode of application.

Various studies have shown that *B. thuringiensis* H-14 is far more effective against *Culex* and *Aedes* than *Anopheles* mosquitoes. However, even for the different strains of the same type, the level of sensitivity to this bacterium is different. Of course, this situation may be due to the particular feeding place of *Anopheles* mosquitoes, i.e., the surface of water. As the larvicide formulation sinks into the water, the time available for the mosquitoes to ingest the former is reduced. In almost every experiment carried out, *Culex* species have shown relatively greater sensitivity to powder formulations and Bacillus tablets, as compared to *Anopheles*. But all three mosquito types have shown similar sensitivities to the floating formulations, which is possibly due to the longer stability of this formulation on the water surface.

The formulation type can have an important role in the effectiveness of a microbial larvicide, and indeed, the formulation of a microbial larvicide is a key stage of its production. Since the protein crystals of the delta-endotoxin are not soluble in water, formulating this bacterial material in a manner which renders it floating at the larval ingestion level is of paramount significance.
The granular *Bt var israeliensis* should be broadcast at 2.5 lbs acre\(^{-1}\), every 7-14 days, depending on the organic properties of the water: black, murky water needs more than clear water. 10-20 lbs acre\(^{-1}\) every 7-14 days may be needed to control 3rd & 4th instar larvae in highly organic waters. *Bt var Israeli* does not harm people, pets, fish, or plants.

### 3.5. Bacillus Sphaericus

The early history of *B. sphaericus* is marked by the isolation of key strains, which may have not been as active as the present candidates, but did play an important role during the development of the *B. sphaericus* series of cultures (Figure 4; Table 3).

![Figure 4: Electron micrograph of Bacillus sphaericus parasporal production](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Historical significant of key strain</th>
<th>Phage group, larvicidal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (USA)</td>
<td>First reported active isolate</td>
<td>Phage group I, low larvicidal activity</td>
</tr>
<tr>
<td>SS 11-1 (India)</td>
<td>First active isolate fermentation and population stability problem</td>
<td>Phage group or moderate larvicidal activity</td>
</tr>
<tr>
<td>1593 (Indonesia)</td>
<td>One of key field candidates</td>
<td>Phage group 3 high larvicidal activity</td>
</tr>
<tr>
<td>2362 (Nigeria)</td>
<td>The prime field candidate</td>
<td></td>
</tr>
<tr>
<td>Lysenko isolates Guyana, Hungary</td>
<td>Active strain isolated from nonmosquito terrestrial sources</td>
<td></td>
</tr>
<tr>
<td>2297 (Sri Lanka)</td>
<td>Crystal first noted in this strain</td>
<td>Phage group 4 moderate larvicidal activity</td>
</tr>
</tbody>
</table>

Table 3: *Bacillus sphaericus* strains
Unlike *Bacillus thuringiensis* subsp. *israeiliensis*, which is the result of only one documented isolation, the history of *B. sphaericus* is the history of the isolation of a whole series (Table 3). Of the 30 to 50 strains of *B. sphaericus* presently available, unlike *B.t.* subsp. *israeliensis*, *B. sphaericus* is limited to effects against mosquito larvae. Even among the mosquito larvae, certain genera (e.g. *Aedes*) are less susceptible to *B. sphaericus* toxin. The prime advantage of the *B. sphaericus* group of strains lies in their ability to perform for a longer period of time in the environment.

The classification of *B. sphaericus* strain by H-serotypes is again interesting because usually the toxic strains of the same serotype are given a comparable pathogenic power, but it has become a need, or a must, for their identification and remains as a first approach to anticipate their pathogenic potentialities.

4. Production of Bacillus Thuringiensis and Bacillus Sphaericus

4.1. Culture Maintenance and Preservation

There are over a thousand strains of *B. thuringiensis* active against agriculturally important insects. Similarly there are several hundred isolates of *B. sphaericus*, 25 percent of which are larvicidal to mosquito larvae.

The most important single need for the production of microbial insecticides is a supply of reproducible, reliable, authentic cultures of the microorganism. The principle bacteria used in the control of insects (*B.t.* and *B.s.*) are relatively easy to maintain (see also—*Microbial Cell Culture*).

4.1.1. Liquids

Coconut milk (waste product), crude sugar, e.g., jaggery, whey (waste product), molasses, corn steep liquid, inorganic nitrogen and (NH$_4$)$_2$SO$_4$;

4.1.2. Materials of Plant Origin

Legumes and other seeds, chick peas, peanuts, lime beans, cowpeas, soya beans, bambara beans, kidney beans, cotton seed meal, peanut cake, soy peptone, cotton seed, hydrolysate, horse beans, lentils; cereals, corn, guinea corn millets, wet mash from breweries, wheat flour, wheat bran carbohydrate, dextrin, maltose, sucrose, glucose Plant extracts, potato tubers, sweet potato roots, minced citrus peels, ground seed of dates, carrots; Tubers, cassava, yams, sweet potatoes; Yeast powder, fodder yeast

4.1.3. Materials of Animal (nonmammalian) Origin

Fishmeal

4.1.4. Materials of Mammalian Origin

Blood, chicken slaughterhouse residue
4.1.5. Minerals

Minerals are essential in the nutrition of organisms. Five metallic ions are considered to be particularly important in the growth and sporulation of bacilli: Mg$^{++}$, Mn$^{++}$, Fe$^{++}$, Zn$^{++}$, and Ca$^{++}$. These are all normally present in the carbon and nitrogen sources used in fermentations and there may be no need to include these ions in the fermentation media.

In many media 0.3 gl$^{-1}$ MgSO$_4$.7H$_2$O; 0.02 gl$^{-1}$ MnSO$_4$.H$_2$O; FeSO$_4$.7H$_2$O; ZnSO$_4$.7H$_2$O; and 1.0 gl$^{-1}$ CaCO$_3$ are added. Adding them to a medium will not damage a fermentation, even if there are already sufficient levels of these minerals present. They are also high enough to correct any deficiencies.

4.2. Fermentation

The fermentation of the different isolates of B.t., regardless of subspecies, have some general characteristics in common. They all use sugar (usually glucose, molasses, or starch), producing acid during the fermentation (see also – Cell Thermodynamics and Energy Metabolism). In general, they have similar requirements for proteins or protein hydrolysates, can use NH$_4^+$ salts, and respond similarly to minerals. However, the individual isolates are unique entities, and a particular medium that may support good growth or toxin production by one isolate may be less satisfactory for another. Different isolates of B.t. may produce toxins with different spectra of activities.

After sterilization, the pH of the fermentor is pH 6.8-7.2. Immediately after inoculation, the pH falls steadily as the glucose is utilized, reaching a pH of about 5.8-6.0 after 10-12 hours. At this point, the pH starts to rise at the same rate that it fell, reaching pH 7.5 after 25 hours. The rise in pH slows gradually, reaching a pH of 8.0 after about 30 hours. The pH may continue to rise, reaching pH 8.8 after 50-60 hours. With some cultures, the initial drop in pH may only reach pH 6.4-6.6. In such fermentations, there may be little or no increase in pH as the fermentation continues, reaching a pH of 8.0 at about 30 hours. The pH may continue rising, reaching a pH of 8.8 after 50-60 hours.

The pH in B.s. fermentations– in contrast to B.t. fermentations– moves continuously upward throughout the growth and sporulation of the bacteria. Since the bacteria do not use sugars as a source of carbon, acids are not formed. Rather, ammonia accumulates in the broth, probably due to deamination of amino acids. The final pH may range from 8.0 to 9.0 depending upon the protein content of the medium. It is possible to control the pH by the addition of acid, and this may enhance toxin production by some strains but not by others.

The "log-phase" of any bacterial fermentation is that period during which the organism is vigorously growing and rapidly dividing (see also Microbial Cell Culture and Cell Thermodynamics and Energy Metabolism). This first phase lasts 16-18 hours. Sporulation is complete 20-24 hours after inoculation, although the cells have not yet lysed. Lysis is complete by 35-40 hours.
4.3. Recovery Process

Large quantities of the bacteria are readily recovered by spray drying. A flow-sheet for this procedure is given in Table 4. A continuous-flow type centrifuge is used to separate the \textit{B. thuringiensis} from the beer.

![Flow-sheet for spray drying recovery process](image)

Table 4: The spray drying recovery process for the spore-crystal complex of \textit{B. thuringiensis} and \textit{B. sphaericus}

### 4.4. Formulation and Storage

An enormous number of amendments have been utilized in experimental and commercial formulations of Bacillus spp. and other biocontrol agents. In turn, these amendments can be grouped into any number of amendment types. Generally, amendments can be grouped as either carriers (fillers, extenders) or amendments that improve the chemical, physical, or nutritional properties of the formulated biomass. A selection of amendment types along with a limited number of examples of each type are shown in table 5 below.

<table>
<thead>
<tr>
<th>Amendment type</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid carriers</td>
<td>Vegetable oils</td>
</tr>
</tbody>
</table>
Table 5: Types of amendments and example materials for formulating Bacillus biomass

<table>
<thead>
<tr>
<th>Mineral carriers</th>
<th>Kaolinite clay, diatomaceous earth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic carriers</td>
<td>Grain flours</td>
</tr>
<tr>
<td>Stabilizers</td>
<td>Lactose, sodium benzoate</td>
</tr>
<tr>
<td>Nutrients</td>
<td>Molasses, peptone</td>
</tr>
<tr>
<td>Binders</td>
<td>Gum Arabic, carboxymethylcellulose</td>
</tr>
<tr>
<td>Desiccants</td>
<td>Silica gel, anhydrous salts</td>
</tr>
<tr>
<td>Thickeners</td>
<td>Xanthan gum</td>
</tr>
<tr>
<td>Surfactants</td>
<td>Tween 80</td>
</tr>
<tr>
<td>Dispersants</td>
<td>Microcrystalline cellulose</td>
</tr>
<tr>
<td>UV protectants</td>
<td>Oxybenzone</td>
</tr>
<tr>
<td>Sunscreens</td>
<td>Blankophor BBH</td>
</tr>
<tr>
<td>Optical brighteners</td>
<td>Lignin (PC 1307)</td>
</tr>
<tr>
<td>Light blockers</td>
<td></td>
</tr>
<tr>
<td>Stickers</td>
<td>Pregelatinized corn flour</td>
</tr>
</tbody>
</table>

Formulation plays a significant role in determining the final efficacy of a Bacillus-based product, as do the processes of discovery, production, and stabilization of the biomass of the biocontrol agent. If a Bacillus strain reaches the stage of formulation development via poorly conceived or understood discovery, production, or stabilization protocols, the biocontrol and therefore commercial potential of this agent will be compromised. Effective formulations of biomass of Bacillus spp. for biocontrol of plant diseases are currently in the marketplace but this in no way indicates that improvements in formulation technology are not needed. The formulations of commercial products containing B.thuringiensis have undergone a wide range of transitions over the years including aqueous suspensions, wettable powders, oil flowables, insect pellet baits, dry flowable-fluidized bed agglomerates, and dry flowable low pressure extrusion granules. With continued research similar improvements in formulations can be anticipated for plant disease biocontrol products.

The efficacy of unformulated primary powders and whole fermentation beers of several isolates of B. sphaericus under a variety of field conditions is well documented. However, formulation of the primary powders and fermentation beers will make them more suitable for use and more effective under a variety of environmental conditions. The formulation requirements for B. thuringiensis and B. sphaericus are essentially the same as for B. thuringiensis H-14.

Although B. sphaericus is not being produced commercially at present, experimental formulations have been made in granular, flowable concentrate and sustained release forms similar to those available for B. thuringiensis H-14 have been made.

In addition to the impact of environmental parameters on the effectiveness of the larvicide, consideration must be given to the impact of the formulation components on the environment. These components should be innocuous to non-target organisms, both plant and animal.
Toxicity ratings should be provided for each batch of formulated material. A standardized bioassay protocol suitable for use in standardizing *B. sphaericus*, using 48h old larvae of *C. quinquefasciatus* as the assay insect, should be known. The Institut Pasteur has prepared a proposed international standard of *B. sphaericus* (Rb-80, isolate 1593) for use in this assay.

*B. thuringiensis* and *B. sphaericus* toxins remain extremely stable under optimal storage conditions of neutral pH and 4°C, and fairly stable at room temperature. Toxin that is exposed to high pH (10.8) will become denatured immediately.

### 4.5. Bioassay Protocol for *Bacillus Thuringiensis* and *Bacillus Sphaericus* Preparations

#### 4.5.1. Standard Bacterial Preparation

The standard powder IPS 82 and "SPH-88" can be obtained from the Pasteur Institute for handling and storing.

#### 4.5.2. Assay Species

Use early 4th instar larvae of the Culex pipiens complex (*C. quinquefasciatus*). In assay with Anopheles sp. (e.g. *An. stephensi*), which are less susceptible than Culex, third instar larvae should be used. *Ae. aegypti* should not be used because this species is relatively unsusceptible to the toxin of *B. sphaericus*.

#### 4.5.3. Preparation and Reading of the Bioassay

For the bioassay of preparations of unknown activity, time can be saved by first making a range-finding assay with widely spaced (e.g. 10 fold) concentrations. The SPH-88 standard powder is prepared for the assay as follows: A 50 mg L$^{-1}$ stock solution of the SPH-88 powder is prepared by thorough blending (sonication, homogenization) in chlorine-free water. To each plastic cup containing 150 ml of water, add up to 25 larvae. A total of 50-100 larvae should be used at each dilution. Add 120l, 90l, 60l, 30l, 24l, and 15l of the bacterial stock solution to the cups to obtain final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg L$^{-1}$ respectively of SPH-88. Fifty to 100 larvae in cups containing only water are held as controls. A small amount of food (e.g. ground mouse biscuit or a few drops of 10 percent w/v autoclaved bakers yeast) is added to each cup. The assay should be incubated at 25°-27°C.

After an exposure of 48 hours, count the number of live larvae.

#### 4.5.4. Calculation of Potency in International Units (IU)

The relative potency of the test preparation (see Table 6) with respect to the standard is given by the equation:

$$\text{Potency (IU/mg)} = \frac{\text{LC}_{50} \text{ Standard}}{\text{LC}_{50} \text{ test preparation}} \times \text{IU/mg standard}$$
4.6. Bioassay Protocol for Bacillus thuringiensis H-14 Preparations

4.6.1. Standard Bacterial Preparation

Use the material "IPS-82" as the standard, which is obtainable from the Pasteur Institute. It should be stored and handled according to the suppliers’ recommendations. These should include the necessity of storage at 2-5°C, equilibration at room temperature before opening the plastic box to avoid condensation and, after the taking of a sample, resealing the container and returning it to 2-5°C in darkness.

4.6.2. Assay Species

Use early 4th instar larvae of the Bora-Bora strain of Aedes aegypti. Eggs to start breeding colonies can be obtained from the Pasteur Institute, or one of several other centres.

In order to obtain larvae at an equal developmental stage, induce eggs to hatch by a stimulant such as the addition of 100 mg ascorbic acid L⁻¹ of water. This deoxygenates the eater and all the eggs hatch very quickly. Purchase a large stock of suitable mosquito food to avoid variation due to the use of different batches of food in the course of time. Store this food under dry, cool (5°C) conditions to prevent infestation by storage pests or infection by moulds. Feed larvae with standardized optimal quantities of food, using a standardized breeding routine at a constant temperature selected from a range between 22 and 28°C. Do not vary this temperature for different experiments. Harvest the larvae on filter-paper, with a strainer or with a pipette, at a pre-selected age when all have just moulted into the fourth instar. If Ae.aegypti is unacceptable in a country for safety reasons, use Culex quinquefasciatus or C. pipiens.

4.6.3. Calculation of Potency in International Units (IU)

The convention of using the term "International Unit" can easily lead to confusion. There is necessarily no correlation between the IUs of one standard and those of another. For example, as seen in Table 6, HD-1-S-1980 has been assigned 16.000 IU mg⁻¹ whereas IPS-78 is 1.000 IU mg⁻¹. These two materials affect different insects. HD-1-S-1980 has very little activity against mosquitoes whereas IPS-78 is very active against them. The assay insect and the standard used in the bioassay to interpret potencies expressed in international units must be known.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Name of standard</th>
<th>Serotype</th>
<th>Potency- IU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.t.</td>
<td>HD-1-S-1971</td>
<td>H-3a,3b</td>
<td>18.000</td>
</tr>
<tr>
<td>B.t.</td>
<td>HD-1-S-1980⁺</td>
<td>H-3a,3b</td>
<td>16.000</td>
</tr>
<tr>
<td>B.t.</td>
<td>IPS-78</td>
<td>H-14</td>
<td>1.000</td>
</tr>
<tr>
<td>B.t.</td>
<td>IPS-82⁺</td>
<td>H-14</td>
<td>15.000</td>
</tr>
<tr>
<td>B.s.</td>
<td>RB-80</td>
<td>H-5a,5b</td>
<td>1.000</td>
</tr>
</tbody>
</table>

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Table 6: Determination of potency [IU mg⁻¹] of *B. thuringiensis* products

It would be perfectly valid to use these ratios to express activity. For example, if the LC₅₀ of a test sample is one-half that of the standard, one can say that the test sample is twice as active as the standard, or, that the ratio of its activity to that of the standard' is 2.0, according to the following formula:

\[
\text{Ratio of activity, sample to standard} = \frac{\text{LC}_{50} \text{ standard}}{\text{LC}_{50} \text{ Sample}}
\]

In practice, this is difficult. One must deal with decimal numbers, many of them less than 1.0, and one must define the standard each time. This can be avoided by assigning units of potency to the standard and then expressing the activity of the test sample in similar units. This has been done in the case of *B. thuringiensis* and *B. sphaericus* formulations, and, by agreement, no matter which standard is defined, one expresses these units as "International Units" or "IUs". The formula thus becomes:

\[
\text{Potency of Sample, (IU/mg)} = \frac{\text{LC}_{50} \text{ Standard}}{\text{LC}_{50} \text{ Sample}} \times \text{Potency of standard}
\]

Several standard formulations have been used in the assay of bacterial larvicides. The names of these standards and the potencies assigned to them are given in Table 6.

4.7. Safety and Quality Control

4.7.1. Chemical Contamination

Care is required to ensure that the final products do not contain unwanted chemical contaminants. Occasionally however, an applicator will use a dirty tank or a barrek, thus contaminating the Bacillus formulation in situ. There is no good method of preventing or detecting this.

4.7.2. Microbial Contamination

Any breakdown in the fermentation process can lead to contamination. The formulation applied to a field contains, in addition to the *B. thuringiensis* toxin, viable spores of the bacilli which, in theory, could spread and multiply in the environment. These bacilli do persist over a few months, but they have never been known to spread out from the treatment area or to have harmed anything in the environment. However, while research over twenty years has demonstrated that these spores of *B. thuringiensis* are harmless,
there maybe other contaminating microorganisms. Microbial contaminants in a formulation are much harder to detect than chemical contaminants. Therefore, since living cells are being applied in any Bacillus treated plots and fields, it is prudent to examine all commercial batches to be sure that they are free from harmful contaminants. To ensure this, four types of tests are used.

**In vitro tests**: The fermentation beers, the development of the vegetative stages, maturing cells with spores, and the final product should be carefully examined and monitored using a microscope (1000 magnification). If a microscope with phase contrast is available, good differentiation of spores and crystals can be seen. Otherwise, several stains are available that will differentiate spores and crystals. (Spores of *B. thuringiensis* are oval; spores of *B. sphaericus* are round or spherical).

**In vivo tests**: In order to make sure that a formulation does not contain bacteria other than *B. thuringiensis* or *B. sphaericus*, six 20g mice are subjected to a single subcutaneous injection of $10^6$ spores of the test bacilli, after which they are observed for 7-21 days. If a mouse dies, lesions develop, or any signs of sickness are seen, the batch should be rejected until further studies have been done to see if the death or disease reflected a bad formulation, or was due to an experimental error.

### 4.8. Packaging and Distribution

As a dry powder, *B. thuringiensis* is quite stable. If it is protected from water and not subject to extremes of heat, it can be stored indefinitely. Thus packaging water-dispersible powders, granular formulations and dusts, is not difficult; the only requirement is that they should be packed in water-proof drums or bags.

Flowable formulations of *B. thuringiensis* are more difficult to prepare and stabilize. They tend to be quite sensitive to heat and must be protected in storage. They have a tendency to settle and form sludge at the bottom of the container. If the user does not adequately agitate the container, the active material may be left at the bottom of the drum. Shelf-life of the flowables is much shorter than that of dry materials. Thus, care must be taken both in the distribution and in storage of flowable materials. Flowables, being liquids, are packaged in plastic bottles or drums.

### 5. Entomopathogenic Viruses

#### 5.1. General Overview

Insect-specific viruses can be highly effective natural controls of several caterpillar pests. Different strains of naturally occurring nuclear polyhedrosis virus (NPV) and granulosis virus are present at low levels in many insect populations. Epizootics can occasionally devastate populations of some pests, especially when insect numbers are high.

Insect viruses need to be eaten by an insect to cause infection but may also spread from insect to insect during mating or egg laying. In some cases, for example while searching
for suitable hosts for egg laying, beneficial insects such as parasitoids may physically spread a virus through the pest population.

No threat to humans or wildlife is posed by insect viruses. Virus diseases of caterpillar pests may cause indirect mortality of some beneficial larval parasitoids if the host insects die before the parasitoids have completed development. Predators and adult parasitoids are not directly affected. Viruses can over winter in the environment or in over wintering insects to re-establish infection in subsequent seasons.

The successful commercialization of insect-pathogenic viruses has been limited. Thus far, NPV strains have only been mass produced in living insects, a costly procedure. Viral insecticide development is further hindered by the fact that the viruses are specific to one species or genus, ensuring a relatively small market.

Most crops and habitats are affected by caterpillar pests. Naturally occurring viruses may affect many caterpillar pests. NPV affects alfalfa looper, corn earworm, imported cabbageworm, cabbage looper, cotton bollworm, cotton leafworm, tobacco budworm, armyworms, European corn borer, almond moth, spruce budworm, Douglas fir tussock moth, pine sawfly and gypsy moth. Preparations of granulosis virus have been isolated from several caterpillar species, including imported cabbageworm, cabbage looper, armyworm, fall webworm, and mosquitoes, among many others.

Viruses invade an insect's body via the gut. They replicate in many tissues and can disrupt components of an insect's physiology, interfering with feeding, egg laying, and movement. Different viruses cause different symptoms. NPV infected larvae may initially turn white and granular or very dark. Some may climb to the top of the crop canopy, stop feeding, become limp, and hang from the upper leaves or stems, hence the common name "caterpillar wilt" or "tree top" disease. Victims of a granulosis virus may turn milky white and stop feeding. In both cases, the body contents of the dead larvae are liquefied and the cuticle ruptures easily to release infectious viral particles. Death from a virus infection usually occurs within three to eight days.

A naturally occurring viral epizootic can seriously deplete a pest population. Transmission of the virus through the population may take days or weeks but, if conditions are suitable, the entire population may eventually collapse. In some instances, the combination of naturally occurring viruses and other natural enemies will maintain pest populations at acceptable levels. For example, a virus has been recorded as destroying up to 28 percent of imported cabbageworm populations in cole crops, with up to 55 percent of the remaining population parasitized by several parasitoids. Cabbage looper populations experienced up to 40 percent infection by virus in the same studies.

Mass reared viruses have been successfully applied in limited areas as microbial insecticides against pests. Infected caterpillars have been mashed into a water solution and applied to pest populations as a form of microbial insecticide. Viruses are adversely affected by ultraviolet radiation and are best applied in the late afternoon. Pest abundance and the general fitness of the pest population will affect its susceptibility to virus attack, and the effectiveness of different strains of the same virus can vary considerably. Viruses can be stored frozen for many years. Research to reduce the
killing time of viruses is ongoing and includes the use of genetic engineering to improve the performance of viral insecticides.

5.2. Baculoviruses (Baculoviridae)

Scientific advancements built on an understanding of what nature already provides are leading to environmentally friendly crop-pest control by either biological agents or specifically designed synthetic anti-insect compounds. Baculoviruses are rod-shaped DNA viruses, many of which begin their life cycle reproducing inside cells. In the nuclei of caterpillar cells infected with baculoviruses, viral progeny multiply and are incorporated into protective polyhedron-shaped protein structures called occlusion bodies. Infected caterpillars die and contaminate the leaf surfaces with the occlusion bodies. Then, healthy caterpillars ingest the occlusion bodies and release the virus when feeding on contaminated leaves, thus continuing the life cycle of infection and replication.

“Each year, natural baculovirus epidemics nearly wipe out populations of some caterpillar pests, such as corn earworm, cotton bollworm, tobacco budworm, and cabbag’s nemesis—the diamondback. With an eye to worldwide marketing, several companies are developing technology to mass-produce baculoviruses in cultures of insect cells rather than in whole insects. Such technology is expected to save on labor costs and to keep bioinsecticides free of microbial contaminants. Besides serving as growth media for baculoviruses, insect cell cultures—particularly those from embryonic and nerve tissue—may someday be used to screen natural or synthetic chemical compounds for their potential as environmentally friendly anti-insect compounds. For example, the cultures might help to determine whether an insect growth regulator effectively disrupts development of pest insects without harming other insects. Cell cultures may also help scientists learn why certain chemicals paralyze or kill a particular pest insect or cause it to stop eating.

Recently, entomologist developed recombinant baculoviruses containing colored fluorescent protein markers. These markers may help researchers determine more quickly if the recombinants possess desired traits, such as the ability to kill pests with low for baculovirus production. The researchers have established eight insect cell lines from embryos and ovaries from members of the Helicoverpa and Heliothis species. One of the cell lines produced 10 times more of a baculovirus known as AcMNPV than did the other lines. This virus infects a wide range of caterpillar pests, infection levels were up to 2,000 times greater than rates of infection in caterpillars exposed to either AcMNPV or to another baculovirus. Baculoviruses don’t persist well in the environment, mainly because they are inactivated by ultraviolet-B (UV-B) rays of sunlight, which probably cause DNA damage. If they could be made a little more persistent, they might become much more practical alternatives to conventional chemical insecticides.

Baculoviruses are pathogens that attack insects and other arthropods. Like some human viruses, they are usually extremely small (less than a thousandth of a millimeter across), and are composed primarily of double-stranded DNA that codes for genes needed for virus establishment and reproduction. Because this genetic material is easily destroyed
by exposure to sunlight or by conditions in the host's gut, an infective baculovirus particle (virion) is protected by a protein coat called a polyhedron (plural polyhedra: see Figures 5A and B). Most insect baculoviruses must be eaten by the host to produce an infection, which is typically fatal to the insect.

The majority of baculoviruses used as biological control agents are in the genus *Nucleopolyhedrovirus*, so "baculovirs" or "virus" will hereafter refer to nucleopolyhedroviruses. These viruses are excellent candidates for species-specific, narrow spectrum insecticidal applications. They have been shown to have no negative impacts on plants, mammals, birds, fish, or even on nontarget insects. This is especially desirable when beneficial insects are being conserved to aid in an overall EPM program, or when an ecologically sensitive area is being treated.
On the other hand, the high specificity of baculoviruses is also cited as a weakness for agricultural uses, since growers may want one product to use against a variety of pests. Currently, researchers are attempting to use genetic engineering techniques to expand virus host ranges to the desired pest species.

5.2.1. Life Cycle

Viruses are unable to reproduce without a host since they are obligate parasites. Baculoviruses are no exception. The cells of the host's body are taken over by the genetic message carried within each virion, and forced to produce more virus particles until the cell, and ultimately the insect, dies. Most baculoviruses cause the host insect to die in a way that will maximize the chance that other insects will come in contact with the virus and become infected in turn.

As seen in the animation on the right, infection by baculovirus begins when an insect eats virus particles on a plant-perhaps from a sprayed treatment. The infected insect dies and "melts" or falls apart on foliage, releasing more virus. This additional infective material can infect more insects, continuing the cycle.

5.2.2. Relative Effectiveness

It is widely acknowledged that baculoviruses can be as effective as chemical pesticides in controlling specific insect pests. However, the expense of treating a hectare of land with a baculovirus product invariably costs more than an equally efficacious chemical treatment. This difference in price is due primarily to the labor intensive nature of baculovirus production. Some viruses can be produced in vitro (within cell cultures in the laboratory, not requiring whole, living insects). These are less expensive than those that can only be produced in vivo, that is, inside of living insects. The cost of rearing live hosts adds greatly to the final cost of the product. It is to be hoped that insect cell culture systems currently being developed for other uses may ultimately make viral pesticides more cost-effective.

5.2.3. Appearance

Insects killed by baculoviruses have a characteristic shiny-oily appearance, and are often seen hanging limply from vegetation. They are extremely fragile to the touch, rupturing to release fluid filled with infective virus particles. This tendency to remain attached to foliage and then rupture is an important aspect of the virus life-cycle. As discussed above, infection of other insects will only occur if they eat foliage that has been contaminated by virus-killed larvae.

It is interesting to note that most baculoviruses, unlike many other viruses, can be seen with a light microscope. The polyhedra of many viruses look like clear, irregular crystals of salt or sand when viewed at 400x or 1000x magnification. The fluid inside a dead insect is composed largely of virus polyhedra, of which many billions are produced inside of one cadaver.
5.2.4. Habitat

Baculoviruses can be found wherever insects exist. Since rain and wind readily carry baculoviruses from place to place, it is likely that every piece of land and body of water contains some virus particles. It is widely accepted by researchers that most products currently on the shelves is "contaminated" by baculovirus particles. In fact, the presence of baculovirus particles along with the results of tests performed in conjunction with registration may be considered both indirect and direct evidence for the safety of these agents.

Like most viruses, baculoviruses tend to be species or genus specific, although there are some exceptions to this rule, notably the *Autographa californica nuclear polyhedrosis virus*. Much of the genetic work currently being done to improve baculovirus-based pesticides is concentrated in the area of the virus genome controlling its host range.

5.2.5. Current use of Baculoviruses as Insecticides

Use of biological control agents, most notably baculoviruses, as alternatives to chemical pesticides is among the most promising approaches to reaching these goals (Table 7). Hundreds of baculoviruses have been described. They all infect invertebrates and about 90 percent infect lepidopterous insects, many of which are major agricultural pests.

<table>
<thead>
<tr>
<th>Commodity</th>
<th>Insect pest</th>
<th>Virus Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple, pear, walnut and plum</td>
<td>Coding moth</td>
<td>Coding moth granulosis virus</td>
</tr>
<tr>
<td>Cabbage, tomatoes, cotton, (and see pests in next column)</td>
<td>Cabbage moth, American bollworm, diamondback moth, patato tuber moth, and</td>
<td>Cabbage army worm nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Cotton, corn, tomatoes</td>
<td>Spodoptera littoralis</td>
<td>Spodoptera littoralis nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Cotton and vegetables</td>
<td>Tobacco budworm, Helicoverpa zea, and Cotton bollworm Heliothis virescens</td>
<td>Helicoverpa zea nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Vegetable crops, greenhouse flowers</td>
<td>Beet armyworm (Spodoptera exigua)</td>
<td>Spodoptera exigua nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Vegetables</td>
<td>Celery looper (Anagraphe falcifera)</td>
<td>Anagraphe falcifera nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Alfalfa and other crops</td>
<td>Alfala looper (Autographa californica)</td>
<td>Autographa californica nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Forest Habitat, Lumber</td>
<td>Douglas fir tussock moth (Orgyia psuedotsugata)</td>
<td>Orgyia psuedotsugata nuclear polyhedrosis virus</td>
</tr>
<tr>
<td>Forest Habitat, Lumber</td>
<td>Gypsy moth (Lymantria dispar)</td>
<td>Lymantria dispers nuclear polyhedrosis virus</td>
</tr>
</tbody>
</table>

Table 7: Current use of Baculoviruses as biological insecticides
In many instances, baculoviruses appear to play an integral role in the natural regulation of insect populations. Extensive health safety and environmental testing with baculoviruses has been conducted over the past 25 years. This testing has indicated a total lack of environmental and health concern with the use of baculovirus pesticides.

Based on their safety and potential to replace chemical pesticides, five baculoviruses have been registered as pesticides: Helocoverpa zea nuclear polyhedrosis virus (HzSNPV) in 1975, Orgyia pseudotsugata (Ot) MNPV in 1976, Lymantria dispar (Ld) MNPV in 1993. However, the only privately produced and commercially available viral pesticide comes from the U.S.A. and is the SeMNPV (Spod-X™).

Genetic engineering offers a potential solution to this shortcoming. A foreign pesticidal gene can be inserted into the viral genome and expression of the pesticidal gene product during replication will allow the virus to kill insects faster or cause quick cessation of feeding. Foreign genes which have been inserted into baculoviruses for this purpose include the Buthus eupeus insect toxin-1, the Manduca sexta diuretic hormone, the Bacillus thuringiensis ssp. kurstaki HD-73 delta-endotoxin, the Heliothis virescens juvenile hormone esterase, the Pyenotes tritici Tsp-I toxin, Androctonus australis neurotoxin, Dol m V gene and T-urf 13 genes. The most efficacious gene inserts have been the neurotoxins and the T-urf 13 gene which is responsible for cytoplasmic male sterility of maize. Several major pesticide companies are currently involved in the commercial development of these and other genetically enhanced viral pesticides.

6. Entomopathogenic Fungi

The global consensus to reduce inputs of chemical pesticide which are perceived as being hazardous by some consumers has provided opportunities for the development of novel, benign, sustainable crop protection strategies. A great many chemical pesticides have been or are being phased out (e.g. organochlorine insecticides, methyl bromide) either because of potential human health risks, environmental pollution, effects on non-target organisms or the development of pest resistance.

There are a number of biological agents commercially available for use in crop protection, most notably products based upon Bacillus thuringiensis, but it was clear from the presentations that considerable progress has been made in the development of fungal BCAs in the last decade. Several agents are currently being used in niche markets and many others are at various stages of commercialization.

Many entomopathogenic fungi, especially those in the Entomophthorales, are responsible for epizootics that often successfully regulate pest insect populations. Although inoculation of insect populations with entomopathogenic fungi has provided classical biological control of some pests, most notably against the gypsy moth, the most common method of employing fungi for insect control is through inundatory means. Most species of entomophthoralean fungi are relatively difficult to produce and their primary conidia are short lived, making timing of inundatory applications difficult or impossible. Development of effective methods for production of resting spores and competent mycelia of entomophthoralean species will ultimately increase the utility of these fungi.
Species in the Hyphomycetes demonstrate activity against a broad range of insect pests and are the main contenders for commercial production and use against homopterous pests. Several species offer good potential for production on inexpensive artificial media and have good shelf lives. Entomopathogenic Hyphomycetes have been investigated for use against a broad range of insect pests, including whiteflies, aphids, thrips, termites, grasshoppers and locusts, beetles, and others.

The prospects of genetic engineering for improvement of entomopathogenic fungi have steadily increased within the past decade, but still lag somewhat behind those of the recombinant technology developed for B.thuringiensis and baculoviruses. Developments in the molecular biology of entomopathogenic fungi will provide the tools for elucidating the mechanisms of pathogenesis.

One major criticism of fungal BCAs is that they act slowly and, therefore, give limited protection to crops. Clearly, more aggressive strains of fungal BCAs can be sought i.e. those which work more quickly and require a lower inoculum. Factors that determine pathogen virulence (virulence determinants) should be identified and used in strain selection and quality control. Fortunately, some progress has been made in this area with enzymes and metabolites having been identified which is important virulence or antagonistic determinants.

All fungi perform well in the laboratory but most are less effective in the field. Fungal BCAs must be “primed” for field conditions, i.e. strains must tolerate a wide range of climatic (fluctuating temperatures, humidities, UV light), seraphic (soil types) and biotic (antagonists) factors.

Many fungi produce biologically active secondary metabolites, some of which are very toxic and this is a major concern with all fungal BCAs as their presence would represent a health risk. Very little is known about many of these compounds and the following areas need urgent investigation:

- Screening for bioactive compounds from fungal BCAs. Techniques must be developed for the identification of toxin producers and the selection of strains that are good crop protection agents but not toxin producers. The methodologies and tools developed would help detect toxins in foodstuffs and the environment (target and non-target hosts, plant, soil, water).
- Determining the role of bioactive compounds. Are they pathogenicity determinants? Do they help in the survival of the BCA? Are they waste products?
- The mode of action of bioactive compounds. Are they i) a risk to living systems and ii) do they have any commercial value as pharmaceuticals, agrochemicals or research tools?
- Safety is a major concern for all crop protection products and more studies are needed to evaluate the risks involved in the use of fungal BCAs. The focus should be on:
  - allergic properties
  - risks of toxic metabolites
  - genetic recombination and displacement of natural strains
  - effect on biodiversity (i.e. impact on non-target organisms)
These data would be useful for registration purposes and could reduce development costs considerably.

Fungi often become attenuated (lose virulence or antagonistic characteristics) when maintained on artificial media. Cultural conditions must be identified which retain virulence without increasing production costs. At present little progress has been made in this area partly because the underlying mechanisms for attenuation have not been elucidated.

Entomopathogenic fungi are widely distributed throughout the fungal kingdom, although the majority occurs in the *Deuteromycotina* and *Zygomycotina* families. Some insect-pathogenic fungi have restricted host ranges, for example, *Aschersonia aleyrodis* infects only scale insects and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific, for example, *Metarbizium anisopliae* and *Beauveria bassiana*. The potential of fungal pathogens for the control of insect pests has been recognized since the latter part of the 19th century when *M.anisopliae* was tested against the wheat cockchafer *Anisoplia austriaca* and sugar beet curculionid *Cleonus punctuventris*. Over the past century there have been many attempts to exploit *Verticillium lecanii*, *A.aleyrodis*, *B.bassiana*, *Nomuraea rileyi*, *M.anisopliae* and some species of *Entomophthorales* for pest control. At present, fungi are being used for pest control on a moderate scale in China.

Russia and Brazil, and several European and North American companies are selling small amounts of mycoinsecticides based on isolates of *V.lecanii*, *M.anisopliae* and *Beauveria* species. Fungal conidia require a high relative humidity to germinate and infect insects. However, recent work with locusts using oil-based formulations of *Metarbizium flavoviride* suggests that good control can be achieved even at very low environmental humidities. This all suggests that the potential of insect-pathogenic fungi for pest control may yet be realized. Understanding the mechanisms of fungal pathogenesis in insects will help in the production of more-efficient mycoinsecticides, either by identifying fungal virulence determinants that need to be selected or by identifying genes that could be upregulated or otherwise manipulated to enhance virulence. This article reviews the latest developments on our understanding of fungal pathogenesis in insects, focusing on *M.anisopliae*, and also highlights comparable studies of plant-pathogenic fungi.

### 6.1. Formation of an Infection Structure

Entomopathogenic fungi invade their hosts by direct penetration of the host exoskeleton or cuticle. This has two layers, the outer epicuticle and the procuticle. The epicuticle is a very complex thin structure that lacks chitin but contains phenol-stabilized protein and is covered in a waxy layer containing fatty acids, lipids and sterols. The procuticle forms the majority of the cuticle and contains chitin fibrils embedded in a protein matrix, together with lipids and quinones. Protein may account for up to 70 percent of the cuticle. In many areas of the cuticle, the chitin is laid down helically, giving rise to a laminate structure. As occurs in many plant-pathogenic fungi, conidia germinate on the host surface and often differentiate to form an appressorium. An infection hypha
penetrates down through the host cuticle and eventually emerges into the haemocoel of
the insect.

6.2. Penetration of the Cuticle

As with plant pathogens, entry into the host involves both enzymic degradation and
mechanical pressure. A range of extracellular enzymes that can degrade the major
components of insect cuticle is produced when *M. anisopliae* is grown in vitro with the
cuticle as the sole carbon and nitrogen source. The complex refractory nature of insect
cuticle suggests that penetration would require the synergistic action of several different
enzymes.

The production of cuticle-degrading enzymes by *M. anisopliae* during appressorium
formation has been investigated both in vitro and in vivo. The transparent wings of
some insect species provide an ideal form of cuticle for combined biochemical and
histo-chemical analysis. Among the first enzymes produced on excised blowfly wings
are endoproteases and aminopeptidase, which are produced at the same time as the
formation of appressoria. N-acetylglucosaminidase is produced slowly compared with
proteolytic enzymes, and chitinase and lipase activities are not detected. In situ
histochemical localization confirms that high levels of proteolytic enzymes are secreted
by appressoria.

6.3. Production of Toxins

Growth of fungi in the haemolymph of insects may be as yeast-like blastospores, hyphal
bodies or protoplasts, rather than in the form of a mycelium. This switch in growth
form, which also occurs in some human (for example, *Candida albicans*) and plant-
pathogenic (for example, *F. oxysporum*) fungi, may aid dispersion and colonization of
the haemocoel, optimize nutrient acquisition by increasing surface area and dissipate
the efforts of the host cellular immune system. Many fungi do not penetrate internal organs
before the death of the host, and may kill the host by consuming nutrients in the
haemolymph. When fungal pathogens kill their hosts after a limited period of sparse
vegetative growth, toxins may cause death. A high-molecular-mass insecticidal protein
toxin (>10 kDa) has been extracted from the haemolymph of the army worm
*Spodoptera exigua* infected with *B. bassiana*. Compounds toxic to or otherwise
biologically active against insects have also been extracted from the culture filtrates or
mycelia of several entomopathogenic fungi. Some of these compounds have been
identified in mycosed insects, but their role in disease development is not always clear.

Destruxins (DTXs), a group of cyclic depsipeptides (peptides containing ester linkages)
produced by *M. anisopliae*, have received most attention. Their general structure is
based on a core pentapeptide cyclized by the incorporation of a hydroxy acid; 19 closely
related variants have been described, 17 from *M. anisopliae*, most of which differ in the
number of methylated amino acids and/or in the type of hydroxy acid. DTXs are
insecticidal by injection and, in some cases, when ingested by mouth toxicity is most
acute among lepidopteran larvae and adult Diptera.
DTXs have diverse effects on various insect tissues. They depolarize lepidopteran muscle membrane by directly or indirectly activating Ca\(^{2+}\) channels. This leads to tetanic then flaccid paralysis, which is reversible at low doses, implicating detoxification processes. Interestingly, DTX B has been shown recently to be a specific, dose-dependent and reversible inhibitor of vacuolar-type ATPase, which maintains acidic homeostasis in membrane-bound organelles in eukaryotic cells. Acidification of intracellular compartments, a pivotal event in many aspects of cell physiology, was also found to be blocked by DTX B. Inhibition of vacuolar-type ATPases by DTX could account for most, if not all, of the effects of the toxin.

Some insect species, including many pests, are particularly susceptible to infection by naturally occurring, insect-pathogenic fungi. These fungi are very specific to insects, often to particular species, and do not infect animals or plants. Fungal growth is favored by moist conditions but fungi also have resistant stages that maintain infection potential under dry conditions. Fungi have considerable epizootic potential and can spread quickly through an insect population and cause its collapse. Because fungi penetrate the insect body, they can infect sucking insects such as aphids and whiteflies that are not susceptible to bacteria and viruses.

Several fungal species have potential as microbial insecticides and, in some countries, are commercially available in formulations that can be applied using conventional spray equipment.

Most crops including soybeans, greenhouse crops, vegetables, cotton, citrus, and ornamentals, and also interior plantscapes and forests are sprayed with such formulations. An aquatic fungus infects mosquito larvae of some genera.

Most insect pests are susceptible to fungal pathogens. Some fungi, such as the Entomophthora and related species, are fairly specific with regard to the groups of insects affected; others, such as Beauveria, have a wider host range.

### 6.4. Mode of Action

Fungi invade insects by penetrating their cuticle or "skin". Once inside the insect, the fungus rapidly multiplies throughout the body. Death is caused by tissue destruction and, occasionally, by toxins produced by the fungus. The fungus frequently emerges from the insect's body to produce spores that, when spread by wind, rain, or contact with other insects, can spread infection.

Whiteflies, aphids, grasshoppers, termites, Colorado potato beetle, Mexican bean beetle, Japanese beetle, boll weevil, cereal leaf beetle, bark beetles, lygus bugs, chinch bug, fire ants, European corn borer, codling moth, and Douglas fir tussock moth can be infected.

### 6.5. Lagenidium Giganteum

*Lagenidium giganteum* is a watermold that parasitizes the larval stage of mosquitoes (Figure 6). This microbial parasite belongs to a group of organisms that, although they look like fungi and have "fungal lifestyle", nonetheless are related to diatoms and brown
algae. The infective stage is a motile spore that goes on a search-and-destory mission. The basis of its host specificity is selective recognition and attachment to its mosquito hosts. If a spore encounters, for instance, a water beetle, a dragon fly larva or a rice plant, it recognizes that a susceptible host has not been encountered. It will back off from that surface and swim on looking for a suitable host.

![Figure 6: Light micrograph of L. giganteum.](image)

*L. giganteum* is most easily recognized after it has matured, and ovoid, septate cells have formed. Infected larvae are recognized by a characteristic grey-white to almost completely white appearance. (Note that infection by some other parasites, e.g. microsporidians, will also result in pale white larvae). In the absence of competing bacteria or protozoa, infected larvae will be completely filled with cells that under a microscope will appear translucent. These cells are often most easily seen either in the larval head capsule or the anal papilla (the breathing apparatus at the tail end of the larva).

Although *L. giganteum* is not an obligate parasite, and can grow vegetatively (for example, on rotting vegetation or dead insects) in the absence of its hosts, it grows much faster and is easier to isolate from mosquito larvae. It can be found in freshwater habitats supporting mosquito populations. It will become dormant at temperatures below 16°C or above 32°C. Moderate levels of salinity or organic load prevent it from sporulating, which is necessary for mosquito infection.

The parasite will infect and kill most species of mosquito breeding in fresh water, from temperatures of 16-32°C. It will also infect the closely related dipteran *Chaoborus astictopus*, the Clear Lake gnat, and at very high concentrations, some species of daphnids.
The infection of a larval host is initiated by motile biflagellate zoospores that selectively recognize chemical signals on the epicuticle (outer exoskeleton) of mosquitoes. After attaching (A) the zoospores inject themselves into the larva, and ramify throughout the body of the host (B). Depending upon the temperature and zoospore density, the larva dies of starvation within 1-4 days. At that time each individual cell can form an exit tube and release 10-50 asexual spores, which in turn seek out a new host (C). Alternately, two cells can fuse (D), ultimately resulting in the formation of a thick-walled dormant oospore (E). This sexual stage of *L. giganteum* can remain viable in a dehydrated state for at least 7 years. It is this spore that is responsible for multi-year recycling of the parasite even though a habitat may be dry for months or years before reflooding and colonization by mosquito larvae. Under appropriate environmental conditions, oospores will germinate, resulting in the production of infective biflagellate zoospores similar to those produced during asexual reproduction.

Operational levels of mosquito control have been obtained by ground or aerial application of *L.giganteum* at rates ranging from ca. 0.9 $10^{10}$ to 5 $10^{10}$ CFU's (colony forming units) per hectare. The application rate depends upon the susceptibility and developmental rate of the target species, and habitat characteristics (temperature, organic load and salinity). For instance, control of floodwater *Aedes* species in early fall, in which there is synchronous hatch of large numbers of eggs in relatively cold water, would require treatment at the higher rates. At the other extreme, for 3 to 4 month control of very susceptible mosquito species such as *Culex tarsalis* breeding in rice fields at very low densities, the lower rates can be used.

Zoospores do not have a cell wall, so are much too fragile to be applied directly in a breeding habitat. The sexual stage of *L. giganteum* has many advantages in an operational control program including multi-year stability, resistance to desiccation and abrasion, and its inherent slow-release characteristics. Unfortunately, fermentation yields of oospores remain 2 order of magnitude below that of the less stable mycelial (asexual, presporangial) stage. Research is continuing on improving oospore yields, which would be much more useful in large scale operational mosquito control programs.

*L.giganteum* is a facultative parasite, and has been grown in large fermentation tanks using inexpensive culture media.

This parasite is registered with the Environmental Protection Agency. It is currently the only commercially available fungus biological control agent for mosquitoes. Besides being host specific, *L. giganteum* has the ability to recycle for weeks, months, or even years in a given breeding habitat after a single application.

6.6. Verticillium Lecanii

*Verticillium lecanii*, or the white halo fungus, is a fungal species which belongs to the class Deuteromycetes, and the order Moniliales. The *V.lecanii* species contains a complex of several fungal strains, which differ little in appearance but rather in their host range (Figure 7)
Verticillium lecanii is a commonly occurring fungus that can, among others, affect arthropods. The fungus has been observed on several kinds of insects, but particularly on aphids, scale insects and on whitefly. It has also been found as a saprophyte, which is an organism living or dead, organic material. Verticillium lecanii also occurs as a hyperparasite on rusts and other plant pathogens. The fungus can easily be isolated from soil.

Verticillium lecanii has a white to pale yellow, cottony appearance. Colonies on malt extract agar at 20°C attain a diameter of 18-22 mm in 10 days. Under the microscope the mycelium is visible as shiny, white threads (hyphae). On the phialides, conidia (asexual spores) are formed in terminal heads of slime.

The conidia are cylindrical to ellipsoidal with symmetrically rounded ends, usually one-celled. 2.3-10 1.0-2.6 m. Chlamydomspores are absent.

The additives consist of spreaders, stickers and dispersants of natural origin. Moreover, the formulation stimulates the spores to germinate and infect and protects the spores against rapid desiccation during periods of low humidity. It contains dried spores of Verticillium lecanii which are formulated into a product with the aid of additives.

The mode of action of V.lecanii is based on a direct contact between fungal spores and insects. After applying, the spores land on the target insects and germinate. Mobile insects, such as thrips, can also pick up the spores while moving within the crop.
V. lecanii, under the right environmental conditions, kills the insects after 7-10 days. After spraying, the spores germinate and grow producing hyphae that penetrate into the body cavity where they proliferate destroying the tissues. The fungus then grows through the insect cuticle and under high humidity conditions spores are produced on the outside of the insect body which may spread the infection to other insects.

Verticillium lecanii is not able to spread quickly and effectively within a pest population since the spores do not become airborne. They are covered with slime, by which the spread of the infection can only take place by passing insects or by splattering water droplets. With their slimy surface the spores may adhere to passing insects. Hosts will be infected, whereas non-hosts will act as vectors.

Controls and checks throughout the different stages of manufacturing and formulating guarantee the biological activity and purity of each production batch is routinely screened for microbiological purity, spore viability and infectivity to whitefly.

Verticillium lecanii has been used successfully against greenhouse whitefly Trialeurodes vaporariorum, tobacco whitefly Bemisia tabaci, western flower thrips Frankliniella occidentalis, and onion thrips Thrips tabaci.

7. Biopesticide Production

7.1. Use of New Genetic-Engineering Technology

Biological control is the most important alternative to chemical pesticides in protecting crops from pests, pathogens, and weeds. Major breakthroughs in molecular biology and biotechnology since the early 1980s indicate that quick improvement in the competitive ability of biological control methods is possible, and that biopesticides can play a major role in crop protection in the future. It has become possible to improve some of the critical properties that earlier hampered the usefulness of many biocontrol agents. Valuable genes from completely unrelated organisms can now be utilized for biological control purposes.

Biological control using recombinant DNA (genetic engineering) technology can be achieved in several different ways: control agents may be improved; crop plants can be engineered to carry better resistance genes; or organisms associated with the plant may be modified to provide protection. All these approaches have successfully been used in several different ways experimentally (see also – Methods in Gene Engineering).

Product development has been very active in the area of incorporating resistance genes—mainly from Bt—directly into plants. Successes include potato, tomato, tobacco, and cotton. General root colorizing bacteria of plants have also been engineered to produce insecticidal toxins, which protect against pests such as the corn rootworm (see also – Crop Protection through Pest-resistant Genes). Another bacterium living in the vascular tissues of corn has also been modified to give protection against the corn borer. None of these modified plants or associated organisms is available commercially yet. Similar approaches are used for the biological control of plant pathogens and weeds, but research has been most active in the area of insect control.
In the wake of the enthusiasm about the new possibilities, some serious doubts have arisen. How safe are these organisms for actual use? How do they affect the environment or humans?

Therefore, a very critical approach is necessary toward the use of genetic-engineering technologies in agriculture.

In principle, genetic engineering can be used for biological pest control in two ways: one is improving the properties of the biological control agents, and the other is engineering crop plants to be resistant to pests.

### 7.2. Engineering Biological Control Agents

The genetic improvement of biological agents is a relatively new concept. For this, a great deal must be known about the biology, ecology, and behavior of the organism. This is a very crucial step.

### 7.3. Engineering Crop Plants

The first published reports of successful engineering of crop plants to produce insecticidal or antifeedant proteins appeared in 1987. The crop plants were tobacco and tomato, producing the delta endotoxin of *Bacillus thuringiensis* to make them resistant against caterpillars. To date, transgenic crop plants have been produced of at least 27 different species, including potato, cabbage, sugarbeet, rice, soybeans, corn, rapeseed, sunflower, walnut, and poplar. Within only two years of the first reports, at least 53 field trials in seven countries were conducted, involving eight plant species.

Instead of being inserted directly into the crop plant genome, the protective insecticidal genes can be engineered into associated organisms. Two bacteria have been successfully tested for this purpose. *Pseudomonas fluorescens*, which colonizes the root systems of crops, has been engineered to express *Bacillus thuringiensis* (Bt) toxins, and thus provide continuous protection against such pests as corn rootworm.

The genes for all the major proteins that account for the insecticidal properties of *Bt* have been cloned and sequenced. Now we have nucleotide sequences for more than 20 *Bt* genes that encode proteins active against lepidopterans, eight genes encoding proteins active against dipterans, and two genes encoding proteins active against coleopterans.

To increase the environmental stability and effectiveness of the various *Bt* toxins in the field, genes encoding proteins active against beetles and caterpillars have also been cloned into the rhizobacterium *Pseudomonas fluorescens*. After fermentation, the bacteria are killed and the cell walls hardened chemically. The endotoxins are thereby microencapsulated, resulting in insecticides with greatly enhanced residual activity. Large-scale field trials with this product have been performed, and the product obtained full registration in 1991.
Similar strategies have been employed to develop mosquitocidal species of algae (see also – Genetic Engineering of Algal Species). Even more significantly, several major crop species, including cotton, tobacco, and soybeans, have been transformed with *Bt* genes, becoming resistant to attack by caterpillars and beetles.

Through genetic-engineering techniques, the *Autographa californica multinucleocapsid nucleopolyhedrosis virus* (AcMNPV) has been engineered to kill insects more quickly by expressing either enzymes or toxins soon after host invasion. Of particular interest is the possibility of making viruses produces insect neurohormones, which can cause rapid physiological disruptions in minutely defined target hosts. This strategy is in its early stages of development, but there is little doubt that within the very near future we will have viruses with extended or specifically designed host ranges, capable of killing insects within 24 to 48 hours. These genetically engineered viruses should have an advantage for use against hosts that are not easily controlled by *Bt*.

Very little is known about the genetics of entomopathogenic fungi. The first transformation system for an entomopathogenic fungus was developed using *Metarhizium anisopliae* protoplasts mixed with a fungicide-resistant plasmid. A benomyl-resistant strain of *M. anisopliae* has thus been obtained. Fungal enzymes involved in the penetration of the insect cuticle have now been identified. Knowledge of these genes and gene products will eventually lead to the possibility of genetic alteration of fungal pathogens that possess those genes. Transformation systems for some fungi exist already and may soon be applied to the entomopathogenic species (see also – Genetic Engineering of Fungal Cells).

Parasexual recombination not only facilitates genetic analysis in asexually reproducing fungi, but also provides an important tool in strain improvement of bioprotectant fungi.

Entomopathogenic fungi, which are facultative parasites, are subjected to many environmental factors, and the host insect can exert a selective pressure by favoring one or a few genotypes. Pathogenicity tests showed that some strains are selective hosts for virulence.

The distinct genetic homogeneity of this population could be the sign of an evolutionary history with particular adaptation of pathogenicity towards this host insect. Nuclear markers will allow a reexamination of host specificity and characteristics of populations in terms of evolutionary history, and maybe co-evolution.

The first applications are likely to be the utilization of various *Bacillus thuringiensis* toxins, of some insect baculoviruses, and of some antagonists for plant-disease control. Other applications appear remote for the time being.

The formulation distinguishes pathogens from chemical pesticides, where the method of exposure is less critical and can be reliant on indirect means such as translocation leading to systemic action of activity via the vapor phase. This requirement for direct contact places unique and strong demands on the formulation and application methods for microbial pesticides. In fact, the operational concept of delivery is much more useful than that of application. The microbial active ingredient must be placed or brought into
contact with the target, delivered to it, as it were. The principle commercially relevant genera of entomopathogenic fungi, *Metarhizium* and *Beauveria*, are contact pesticides. The infective fungal conidia penetrate the insect cuticle to initiate the infection process, leading to insect death.

Nonetheless, most efforts to employ them as insecticide active ingredients have been based on the use of a particular isolate against a particular insect pest, usually in isolates found associated with the pest in some natural infection.

8. Entomopathogenic Protozoa and Microsporida

Protozoans are one-called life forms. Some species are responsible for serious human diseases, such as malaria, vectored by mosquitoes. However, there are about 1200 species, out of 15,000 described, specific to and causing diseases in insects.

One group, the *Microsporidia*, contains many species that have promise for biological control. Microsporidian infections in insects are thought to be common and responsible for naturally occurring low to moderate insect mortality. But these are relatively slow acting organisms, taking days or weeks to debilitate their host. Frequently they reduce host reproduction or feeding rather than killing the pest outright. *Microsporidia* often infect a wide range of insects. Some microsporidia are being investigated as microbial insecticides, and at least one is available commercially, but the technology is new and work is needed to perfect the use of these organisms.

Most microsporidia must be eaten to infect an insect, but there may also be some natural transmission within a pest population, for example by predators and parasitoids. The pathogen enters the insect body via the gut wall, spreads to various tissues and organs, and multiplies, sometimes causing tissue breakdown and septicemia.

Infected insects may be sluggish and smaller than normal, sometimes with reduced feeding and reproduction, and difficulty molting. Death may follow if the level of infection is high. One advantage of this type of infection is that the weakened insects are more likely to be susceptible to adverse weather and other mortality factors.

*Nosema pyrausta* (*Perezia pyraustae*) is a microsporidium that infects several insect species, including European corn borer, for which it can be an important natural control. This disease was widespread in the midwestern United States during the 1950s and 60s, causing considerable natural mortality, but its commercial use is still in the developmental phase. Infection can spread from diseased to healthy larvae via contaminated grass, and by migration of infected larvae between plants.

*Nosema locustae* is the only commercially available species of microsporidium, marketed under several labels for the control of grasshoppers and crickets. It is applied with insect-attractant bait. Because of its slow mode of action, this product is better suited to long-term management of rangeland pests than to the more intensive demands of commercial crop or even home garden production. Other *Nosema* species have been shown to infect spider mites and webworms, but have yet to be developed sufficiently for commercial use.
Vairimorpha necatrix is another microsporidium with commercial potential. It has a wide host range among caterpillar pests, including corn earworm and European corn borer, various armyworms, fall webworm, and cabbage looper. It can be more virulent than other species and infected insects may die within six days of infection.

9. Entomopathogenic Nematodes

A plethora of nematode species in more than 30 families is associated with insects and other invertebrates. The major focus of research and development has been on nematode species in 7 families, Mermithidae, Tetraonematidae, Allantonematidae, Phaenopsisylenchidae, Sphaerulariidae, Steinernematidae, and Heterorhabditidae, because of their potential as biological control agents of insects.

The entomopathogenic activity of steinernematid and heterorhabditid species has been documented against a broad range of insect pests in a variety of habitats. These nematodes are especially efficacious against insects in soil and cryptic habitats. They have been used inundatively in a number of high-value cropping systems. For example, the citrus root weevil, D. abbreviatus, in citrus; the black vine weevil, Otiorhynchus sulcatus, in nurseries and cranberries; the black cutworm, Agrotis ipsilon, and mole crickets, Scapteriscus spp., in turfgrass and the peach borer moth, Carpocapsa niponensis Walsing-ham, in apples have been successfully controlled. The entomopathogenic steinernematid and heterorhabditid nematode species possess many attributes of parasitoids and pathogens. They are analogous to parasitoids because they have chemoreceptors and can actively search for their hosts. They are similar to pathogens because of their association with mutualistic bacteria in the genera Xenorhabdus, for steinernematids, and Photorhabdus, for heterorhabditids. The nematode/bacterial complex is highly virulent, killing its host within 48 h through the action of the mutualistic bacteria. These nematodes can be cultured in vitro, have a high reproductive potential, and have a numerical, but no functional, response. Steinernematids and heterorhabditids have been used successfully against a number of soil-inhabiting insect pests. However, this realm of insect nematology is a very young discipline with major contributions being made since the mid-1980s.

Genetic improvements in entomopathogenic nematodes may expand their potential as biocontrol agents by increasing search capacity, virulence, and resistance to environmental extremes, among other attributes. Recently, using molecular techniques, have inserted a heat-shock protein into H. bacteriophora, resulting in transgenic nematodes that were 18 times better than the wild type at surviving high-temperature stress. Field release of the transgenic and wild-type nematodes showed no differences in their abilities to persist.

Significant advances have been made with these entomopathogenic nematodes, but the high costs associated with production and formulation in comparison to those costs of chemical pesticides and other biologicals (i.e., B.thuringiensis) will restrict their use to high-value niche markets.

Nematodes are simple roundworms. Colorless, unsegmented, and lacking appendages, nematodes may be free-living, predaceous, or parasitic. Many of the parasitic species
cause important diseases of plants, animals, and humans. Other species are beneficial in attacking insect pests, mostly sterilizing or otherwise debilitating their hosts. A very few cause insect death but these species tend to be difficult (e.g., tetratomatids) or expensive (e.g. mermithids) to mass produce, have narrow host specificity against pests of minor economic importance, possess modest virulence (e.g., sphaerulids) or are otherwise poorly suited to exploit for pest control purposes. The only insect-parasitic nematodes possessing an optimal balance of biological nematodes can be found in the genera Steinernema and Heterorhabditis. These multi-cellular metazoans occupy a biocontrol middle ground between microbial pathogens and predators/parasitoids, and are invariably lumped with pathogens, presumably because of their symbiotic relationship with bacteria.

Entomopathogenic nematodes are extraordinarily lethal to many important soil insect pests, yet are safe for plants and animals. This high degree of safety means that unlike chemicals, or even Bacillus thuringiensis, nematode applications do not require other safety equipment; and re-entry time, residues, groundwater contamination, chemical trespass, and pollinators are not issues. Most biologicals require days or weeks to kill, yet nematodes, working with their symbiotic bacteria, kill insects in 24-48 hr. Dozens of different insect pests are susceptible to infection, yet no adverse effects have been shown against nontargets in field studies. Nematode production is easily accomplished for some species using standard fermentation in tanks up to 150,000 liters. Nematodes do not require specialized application equipment including pressurized, mist, electrostatic, fan, and aerial sprayers. Application through irrigation systems has improved grower acceptance. Insecticidal nematodes are virtually without competition from other biological agents for control of soil-inhabiting and plant-boring insects.

More than forty countries are working to develop nematodes as biological insecticides. Nematodes are sold in the U.S., Europe, Japan, and China for control of insect pests in high-value horticulture, agriculture, home and garden niche markets.

Steinernematidae and Heterorhabditidae have similar life histories. The none-feeding developmentally arrested infective juvenile seeks out insect hosts and initiates infections. When a host has been located, the nematodes penetrate into the insect body cavity, usually via natural body openings (mouth, anus, spiracles) or areas of thin cuticle. Once in the body cavity, a symbiotic bacterium (Xenorhabdus for steinernematids, Photorhabdus for heterorhabditids) is released from the nematode gut, which multiplies rapidly and causes rapid insect death. The nematodes feed upon the bacteria and liquefying host, and mature into adults. Steinernematid infective juveniles may become males or females, whereas heterorhabditids develop into self-fertilizing hermaphrodites although subsequent generations within a host produce males and females as well. The life cycle is completed in a few days, and hundreds of thousands of new infective juveniles emerge in search of fresh hosts.

Thus, entomopathogenic nematodes are a nematode-bacterium complex. The nematode may appear as little more than a biological syringe for its bacterial partner, yet the relationship between these organisms of one of classical mutualism. Nematode growth and reproduction depends upon conditions established in the host cadaver by the bacterium. The bacterium further contributes anti-immune proteins to assist the
nematode in overcoming host defenses, and anti-microbials that suppress colonization of the cadaver by competing secondary invaders. Conversely, the bacterium lacks invasive powers and is dependent upon the nematode to locate and penetrate suitable hosts.

Growers will not adopt biological agents that do not provide efficacy comparable with standard chemical insecticides. Technological advances in nematode production formulation, quality control, application timing and delivery, and particularly in selecting optimal target habitats and target pests, have narrowed the efficacy gap between chemical and nematode agents. Nematodes have consequently demonstrated efficacy in an number of agricultural and horticultural market segments.

Like other biological control agents, nematodes are constrained by being living organisms that require specific conditions to be effective. Thus, desiccation or ultraviolet light rapidly inactivates insecticidal nematodes; chemical insecticides are less constrained. Similarly, nematodes are effective within a narrower temperature range than chemicals, and are more impacted by suboptimal soil type, thatch depth, and irrigation frequency.

Because the symbiotic bacterium kills insects too quickly, there is no intimate host-parasite relationship as is characteristic for other insect-parasitic nematodes. Consequently, entomopathogenic nematodes are lethal to an extraordinarily broad range of insect pests in the laboratory. Field host range is considerably more restricted, with some species being quite narrow in host specificity.

10. Biological Control of Aflatoxin Contamination of Crops

Aflatoxin contamination of crops compromises the safety of food and feed supplies and causes significant economic losses each year. Of the many research approaches being studied to reduce and, ultimately, eliminate aflatoxin contamination, biological control is one of the more promising, particularly for the near-term. Numerous organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, and nontoxigenic strains of the causal organisms, Aspergillus flavus and A.parasiticus. Most of the field successes to date have been achieved by applying certain nontoxigenic strains of A.flavus and A.parasiticus to soil of susceptible crops, such as peanuts, cotton, and corn. The applied strains occupy the same niche as the naturally occurring toxigenic strains and competitively exclude them when crops are susceptible to infection. Various formulations have been used to apply the nontoxigenic strains to soil, but the most effective methods have been to combine the desired strain with a carrier/substrate, such as a small grain. This was done either by minimally growing the desired strain on sterilized grain or by coating the surface of the grain with conidia of the strain. After application to the field and uptake of moisture, the fungus completely colonizes the grain, and abundant sporulation provides inoculum levels sufficient to achieve a competitive advantage for the nontoxigenic strain. In several years of field studies, particularly with peanuts and cotton, significant reductions in aflatoxin contamination in the range of 70-90% have been achieved consistently. Two separate products have recently received EPA registration as biopesticides to control aflatoxin contamination in cotton (AF36) and peanuts (afla-guard).
11. Integrated Pest Management

The history of integrated pest management (IPM) traces its first real beginning to the 1960s, where a number of factors came together to initiate a search for better control than simple reliance on prophylactic pesticide use. These factors include well known litany of pesticide misuse problems (resistance and non-target effects), rapid development of technologies enabling more sophisticated approaches, rapid advances in communication and computing, with the allied new sciences research, systems analysis, and modeling.

With the advent of genetic engineering, the increased interest of the agriculture "biorationale" products, and the challenge of new and more stringent regulation of agricultural products, a probable adoption of integrated pest management as a national goal for agriculture hopes to position itself as a provider of unbiased IPM knowledge and technology, expanding our membership base to include more grower associations, food processes and biotechnical firms, government agencies, and other agricultural groups with affordable and safe food production.

12. Market

Biological products currently represent just one per cent of the world market, and 80 per cent of that is taken by one product: Bt. Even though this is the case, some commentators have estimated that biological control products could replace at least 20 per cent of chemicals, a market valued at US$ 7 billion. For this to be possible, the financial, regulatory and technical support would need to be forthcoming to develop and expand the industry.

Biocontrol manufactures are not well equipped to develop markets. Most are small enterprises with small turnovers, and just one or two products aimed at niche markets. They do not have the resources to mount international distribution, sales and extension programmers for their products. There is also very little general understanding of the capability and benefits of biocontrol to drive demand by farmers or consumers. Essentially while the knowledge and capability exists, it remains immobilized.

Control of pest insects with chemical pesticides has generated several problems including insecticide resistance, outbreaks of secondary pests normally held in check by natural enemies, safety risks for humans and domestic animals, contamination of ground water, decrease in biodiversity, and other environmental concerns. These problems and sustainability of programs based predominantly on conventional insecticides have stimulated increased interest in integrated pest management. Sustainable agriculture in the 21st century will rely increasingly on alternative interventions for pest management that are environmentally friendly and reduce the amount of human contact with chemical pesticides.

Another environmental issue is that pesticide residue in crops or animal meat can cause importing countries to ban the produce. Also, any pesticide spraying disbars land from qualifying as organic and prevents farmers from selling in this more lucrative market (see Figures 8-10).
Effective microbial control agents that can fill the void of phased out chemicals exist, but their further development and implementation will require the following advances: improvements in the pathogens, their production, and formulation; better understanding of how they will fit into integrated systems and their interaction with the environment.
and other IPM components; greater appreciation for their full advantages (efficacy, safety, selectivity, etc.), not simply their comparison with chemical pesticides; and acceptance by growers and the general public.

There is no doubt that microbial biopesticides can play a key role in controlling pests and make a significant contribution to the reduction of chemical inputs. The use of microbial biopesticides has potential to expand greatly in the future as products are improved and better methods for using them in Integrated Pest Management systems are developed.

**Table 8: The Global Market is rapidly expanding**

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Source: International Biocontrol Manufacturer’s Association, 2004

13. Conclusion

No plant lives in isolation. It is part of a food chain involving many other organisms, including insects, fungi, bacteria, and other plants and viruses. Some feed on the plant, some on the organisms that feed on the plant and so on. The plant powers this system by taking nutrients from the soil and converting solar energy by photosynthesis. In an agricultural system, where the human goal is to maximize the food value of the plant for human or animal consumption, any organisms in the system that impede this objective are called pests or diseases. The most common way to reduce their impact on food production has been to use chemical sprays (insecticides and fungicides) that destroy the pests but not the food plants.

Biological control uses a different approach to pest management, focusing on natural enemies of plant pests and diseases to manage their populations. The strategies rely on detailed knowledge of the ecology, the life cycles, and the food chains in each system,
developing highly target-specific control strategies that leave the non-target plants, insects, or other animals unharmed.

Not all pests are exotic. Many are indigenous; they have found rich niches in agricultural crops where they cause problems. They may have wild host plants in the same area, but the density of planting of crops makes these so much more attractive for feeding or egg laying. Indigenous natural enemies usually exist, but are not capable of keeping these particular pests below tolerable levels. In many ways these pests present much more difficult challenges for biological control.

Researchers employ a host of strategies; often looking for means to strengthen local, natural enemies or to produce them en masse as biopesticides. Fungi, insect viruses, competing but harmless strains of the same pest are being tried, often with great success.

Chemicals are mainly used as herbicides, fungicides and insecticides. These chemicals characteristically have good storage, relatively wide spectrum of activity, fast speed of kill, relatively short persistence, so need frequent applications, and a potential for environmental harm and toxicological concerns.

In contrast, biological control agents tend to have relatively poor storage, high target specificity, slow speed of kill, potentially long persistence through secondary cycling and consequently lower frequency of application. They are environmentally friendly, and are a low hazard for humans and livestock. Biological control agents include pathogens (bacteria, fungi, viruses) and entomopathogenic nematodes usually formulated as biopesticides, and insect predators and parasitoids (small wasps that parasitize insects). Biocontrol can be applied through introductions, augmentative releases inundatively as biopesticides, or through conserving existing field populations. There has been a tendency however, to develop and used biocontrol agents and particularly biopesticides, just as if they have the same properties as chemicals.

The effectiveness of a biocontrol agent depends on two factors: its capacity to kill and to reproduce on pests (compounding its killing action); in ecological terms, its functional and numerical responses. Currently, biopesticides based on viruses and fungi that have the potential for persistence and the compounding benefits of numerical responses, have been developed using the traditional chemical pesticide model involving a quick kill, low persistence and frequent application. In this way, all the shortcomings of these biopesticides relative to chemicals emerge, and few of the benefits. Hence, there are many opportunities to exploit the ecological benefits of biopesticides that have yet been little explored.

Biologicals cannot be successfully used against all pests. Biocontrol agents can be used effectively against a whole range of high threshold pests including aphids, whiteflies, stemborers, leaf miners, locusts and grasshoppers. Hence, there are specific opportunities for use of biological control agents, but as a group they do not provide a panacea, as alternatives to chemical pesticides.
There is considerable pressure on growers to reduce or eliminate the use of pesticides in crop production systems because of concerns about the effects of pesticide residues on human health and on the environment. Entomopathogens are microorganisms that cause disease in arthropods, particularly insects and mites. There is considerable interest in using these microbes as biological control agents of pests, as alternatives to chemical pesticide. Entomopathogens are naturally widespread in the environment and include bacteria, fungi, viruses, nematodes and protozoa. Most are host specific, and some cause natural epidemics in insect populations.

Some entomopathogens can be mass-produced and applied against pests in a way that is similar to a pesticide, using sprays, dusts and drenches. Entomopathogens used this way are called microbial biopessticides. Their advantages are that they leave no toxic residues and create little or no environmental pollution. They are also compatible with many chemical pesticides, parasitoids and predators.

Over a hundred microbial products are available worldwide for use in horticulture, agriculture and forestry. They are being used increasingly in America, Japan and Europe. However, like other natural enemies, they tend to be more expensive than chemicals and they can be affected by environmental conditions. In addition, although they are an attractive option, the development and implementation of commercially viable systems often takes years of research and development.

Phytophagous insect pests account for billions of dollars of losses in agricultural and forest production each year. Synthetic chemical insecticides have limited these losses, but are becoming more costly and more restricted in availability. Because of the increasing development and registration costs, the number of new synthetic pesticides brought to market each year has steadily declined over the past 10 years. At the same time, insect populations have developed resistance to many products, resulting in the need to increase application doses or to abandon pesticides. In response to the documented and potential health/environmental risks associated with synthetic chemical pesticides, in 1988 the U.S. Congress mandated that pesticides registered prior to 1984 must undergo a re-registration process. Because of the costs of satisfying current registration requirements, registration of many pesticides for use in small markets has been abandoned. Subsequently, particularly with medium to small market crops, the need for effective insect control is increasing, but the availability of acceptable chemicals is decreasing.

The need to develop "safer pesticides" has become a priority of both the current administration and the Environmental Protection Agency (EPA). The current EPA policy is to facilitate the testing and registration of pesticides which have "reduced risks".

Consequently, increasing attention has been directed toward natural enemies such as predators, parasites, and pathogens. Unfortunately, none of the predators or parasites can be mass-produced and stored for long periods of time. They all must be raised in vivo. It became evident that there was an urgent need for a biological agent that possessed the desirable properties of a chemical pesticide, which is highly toxic to the target organism, able to be mass-produced on an industrial scale, have a long shelf.
Guidelines for the evaluation of the infectivity of Entomopathogens to nontarget organisms have been formulated by the Environmental Protection Agency (EPA).

A new more active strain of *B.t.* was produced which has increased the performance and acceptance of commercial products and broadened its use against other insect pests. Development of entomopathogens or their by products into microbial insecticides is a reality. Safe, effective entomopathogens formulated as used by growers. The technical successes reported herein undoubtedly will continue to be translated into commercial successes and will in turn stimulate additional research on use of entomopathogens as safe, selective insecticides.

**Acknowledgments**

I am indebted to Dr. M. Mazaheri and Dr. M. Azin for their aid in manuscript preparation.

**Glossary**

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACMNPV:</td>
<td>Antographa califonia multinucleocapside nuclear polyhedrosis virus.</td>
</tr>
<tr>
<td>B.t. :</td>
<td><em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>B.s. :</td>
<td><em>Bacillus sphaericus</em></td>
</tr>
<tr>
<td>C:</td>
<td>Coleoptera</td>
</tr>
<tr>
<td>Cry:</td>
<td>Delta endotoxin gene</td>
</tr>
<tr>
<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>D:</td>
<td>Diptera</td>
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<tr>
<td>DTXs:</td>
<td>Destruxins</td>
</tr>
<tr>
<td>EPA:</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>H:</td>
<td>Flagelle Antigen of <em>Bacillus thuringiensis</em></td>
</tr>
<tr>
<td>HD-1-S-1971:</td>
<td>Standard powder of <em>Bacillus thuringiensis</em> H-3a3b</td>
</tr>
<tr>
<td>HD-1-S-1980:</td>
<td>Standard powder of <em>Bacillus thuringiensis</em> H-3a3b</td>
</tr>
<tr>
<td>I.P.M. :</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>I.U. :</td>
<td>International unit</td>
</tr>
<tr>
<td>I.T.U. :</td>
<td>International Toxic Unit</td>
</tr>
<tr>
<td>IPS-78:</td>
<td>Standard powder of <em>Bacillus thuringiensis</em> H-14</td>
</tr>
<tr>
<td>IPS-82:</td>
<td>Standard powder of <em>Bacillus thuringiensis</em> H-14</td>
</tr>
<tr>
<td>L:</td>
<td>Lepidopteran</td>
</tr>
<tr>
<td>LD 50:</td>
<td>Lethal dose 50% mortality of larvae.</td>
</tr>
<tr>
<td>R.B.-80:</td>
<td>Standard powder of <em>Bacillus sphaericus</em></td>
</tr>
<tr>
<td>RNA:</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SPH-88:</td>
<td>Standard powder of <em>Bacillus sphaericus</em></td>
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Lacey L.A. and Davidson E.W.) C.R.C. Press, Baton. [This article presents the safety of both organisms when applied in the aquatic environment]


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**Biographical Sketch**

**Dr. Nasrine Moazami** began her research career as a self-described 'Medical Microbiologist Biotechnologist' in 1976 after receiving her PhD from the University of Laval in Canada. Dr. N. Moazami is the pioneer of biotechnology in Iran. She has 16 years experience in biopesticide research and production, work she started in 1980. She opened up vast ever-expanding possibilities of agriculture, industry, medicine and public health for solving problems through the introduction of biotechnology in Iran.

Dr. Nasrine Moazami is also the founder of the Persian Type Culture Collection (PTCC), which is mainly a collection of microorganisms of industrial importance. This Culture Collection is an affiliated member of WFCC [World Federation of Culture Collections] and MIRCEN International Network since 1985. She established the first marine biotechnology center in Qeshm Island in the Persian Gulf in the south of Iran.

On July 27, 1995, Dr. Moazami was presented with the Prestigious French Award, the 'Chevalier dans l'ordre des palmes Academiques', a citation given for outstanding professional research.

Dr. Moazami is also the recipient of the February 9, 1989 International Kharasmi Science Festival first prize for the research on and production of biological pesticides.

On November 2, 1996, the President of Iran presented her the National Governmental Award for Research.

At present she is head of the Institute of Advanced Technology of the Iranian Research Organization for Science and Technology as well as Director of the Tehran MIRCEN.