Chapter 13

Microorganisms in Biological Pest Control — A Review (Bacterial Toxin Application and Effect of Environmental Factors)

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Additional information is available at the end of the chapter

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1. Introduction

In this section, the topic in biological control of pests considered. will take place. There has been an increased interest in biological control agents in last decade. More number of biocontrol agents was screened for their efficacy and environmental impact including mammalian safety. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. However, most of these agents were shown to be of little operational use, largely because of the difficulty in multiplying them in large quantities. Some species of organisms, those that have been introduced from elsewhere may be pest to other organisms as well. They are pests to the extend which efforts must have been made to control them both in terrestrial and aquatic/freshwater environments [1]. Prior to the advent of chemical pesticides, predators which are natural enemies of those specific pests, were an important subject in biological sciences with respect to agriculture and forest pest control.

Pesticides that include insecticides, herbicides, and fungicides are employed in modern agriculture to control pests and to increase crop yield. In both developed and developing countries, the use of chemical pesticides has increased dramatically during the last few decades. Control of pests with synthetic chemicals results in several problems. The residues ofthese synthetic insecticides cause toxic effects on wild life (e.g.,birds, beneficial insects like honeybees). These chemical insecticides also induce harmful chemical changes on non-target insects/pests on their predators, parasites, etc. They can also be harmful to humans and domestic animals. Other environmental concern is the contamination of ground water [2]

In addition, there have been several recent research on biological control of marine pests [3]. The introduction of marine pests to new habitats is as old as nautical experience. Atlantic shipworms were quite possibly the first for the applicaiton of some new predator, Mytilus gallprovincialis, and the western Atlantic populations of the European green crab have planted themselves so firmly as a neutralized part of the biota. Many other introductions, such as polychaetes, amphipods are cryptic and have been considered species with natural cosmopolitan distributions [4]

Agriculture and forests form an important resource to sustain global economical, environmental and social system. For this reason, the global challenge is to secure high and quality yields and to make agricultural produce environmentally compatible. Chemical means of plant protection occupy the leading place as regards their total volume of application in integrated pest management and diseases of plants. But pesticides cause toxicity to humans and warm-blooded animals.

Despite many years of effective control by conventional agrochemical insecticides, a number of factors are threatening the effectiveness and continued use of these agents. These include the development of insecticide resistance and use-cancellation or de-registration of some insecticides due to human health and environmental concerns. Therefore, an eco-friendly alternative is the need of the hour. Improvement in pest control strategies represents one method to generate higher quality and greater quantity of agricultural products. Therefore, there is a need to develop biopesticides which are effective, biodegradable and do not leave any harmful effect on environment [5].

2. Biological pesticides

The biopesticides are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals. For example, canola oil and baking soda have pesticidal applications and are considered biopesticides. Even at the end of 2001, there were approximately 195 registered biopesticide active ingredients and 780 products. Biopesticides are biochemical pesticides that are naturally occurring substances that control pests by nontoxic mechanisms. They are living organisms (natural enemies) or their products (phytochemicals, microbial products) or byproducts (semiochemicals) which can be used for the management of pests that are injurious to crop plants. Biopesticides have an important role in crop protection, although most commonly in combination with other tools including chemical pesticides as part of Bio-intensive Integrated Pest Management.

They are biological pesticides based on pathogenic microorganisms specific to a target pest offer an ecologically sound and effective solution to pest problems. They pose less threat to the environment and to human health. The most commonly used biopesticides are living organisms, which are pathogenic for the pest of interest. These include biofungicides (*Trichoderma*), bioherbicides (*Phytopthora*) and bioinsecticides (*Bacillus thuringiensis, B. sphaericus*). The potential benefits to agriculture and public health programmes through the use of biopesticides are considerable [6].

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The advantages of using biopesticides (in place of other chemical ones are based on these factors:

- Ecological benefit; inherently less harmful and less environmental load.
- Target specificity; designed to affect only one specific pest or, in somecases, a few target organisms,
- Environmental beneficiency; often effective in very small quantities and often decompose quickly, thereby resulting in lower exposures and largely avoiding the pollution problems.
- Suitability; when used as a component of an integrated pest management (IPM) programs, biopesticides can contribute greatly.

3. Microbial pesticides

They come from naturally occurring or genetically altered bacteria, fungi,algae, viruses or protozoans. Microbial control agents can be effective and used as alternatives to chemical insecticides. A microbial toxin can be defined as a biological toxin material derived from a microorganism, such as a bacterium or fungus. Pathogenic effect of those microorganisms on the target pests are so species specific. The effect by microbial entomopathogens occurs by invasion through the integument or gut of the insect, followed by multiplication of the pathogen resulting in the death of the host, e.g., insects. Studies have demonstrated that the pathogens produce insecticidal toxin important in pathogenesis. Most of the toxins produced by microbial pathogens which have been identified are peptides, but they vary greatly in terms of structure, toxicity and specificity. [7].

These microbial pesticides offer an alternative to chemical insecticides with increased target specificity and ecological safety so that they are used either uniqly or in combination with other pest management programmes. One definition for integrated pest management (IPM) which is most relevant to this practice comes from Flint and van den Bosch [1981]: "It is a ecologically based pest control strategy that relies heavily on natural mortality factors and seeks out control tactics that disrupt these factors as little as possible. Ideally, an integrated pest management program considers all available pest control actions, including no action, and evaluates the potential interaction among various control tactics, cultural practices, weather, other pests, and the crop to be protected"[8].

These microbials as biocontrol agents present u beneficiancy. They have efficiency and safety for humans and other nontarget organisms. They leave less or no residue in food. They are ecologically safe, so that other natural enemies are free of their threatening, leading to preservation of other natural enemies, and increased biodiversity in managed ecosystem. So, microbial agents are highly specific against target pests so they facilitate the survival of beneficial insects in treated crops. This may be the main reason that microbial insecticides are being developed as biological control agents during the last three decades.

Microorganism e.g., a bacterium, fungus, virus or protozoan as the active ingredient can control many different kinds of pests, although each separate active ingredient is relatively

specific for its target pest. For example, there are fungi that control certain weeds, and other fungi that kill specific insects. One bacterial species like *Bacillus thuringiensis* may be more effectiv on *Aedes aegypti* while one another *B. sphaericus* strain can be effective on a different types of mosquito like *Culex quinquefasciatus* [9].

3.1. Advantages of microbial insecticides

Individual products differ in important ways, but the following list of beneficial characteristics applies to microbial insecticides in general.

- The organisms used in microbial insecticides are essentially nontoxic and nonpathogenic to wildlife, humans, and other organisms not closely related to the target pest. The safety offered by microbial insecticides is their greatest strength.
- The toxic action of microbial insecticides is often specific to a single group or species of insects, and this specificity means that most microbial insecticides do not directly affect beneficial insects (including predators or parasites of pests) in treated areas.
- If necessary, most microbial insecticides can be used in conjunction with synthetic chemical insecticides because in most cases the microbial product is not deactivated or damaged by residues of conventional insecticides. (Follow label directions concerning any limitations.)
- Because their residues present no hazards to humans or other animals, microbial insecticides can be applied even when a crop is almost ready for harvest.
- In some cases, the pathogenic microorganisms can become established in a pest population or its habitat and provide control during subsequent pest generations or seasons.
- They also enhance the root and plant growth by way of encouraging the beneficial soil microflora. By this way they take a part in the increase of the crop yield.

3.2. Disadvantages of microbial insecticides

Naturally there are also the limitations which are listed below, but do not prevent the successful use of microbial insecticides. These factors just provide users to choose effective microbial products and take necessary steps to achieve successful results.

- Because a single microbial insecticide is toxic to only a specific species or group of insects, each application may control only a portion of the pests present in a field and garden. If other types of pests are present in the treated area, they will survive and may continue to cause damage. Conventional insecticides are subject to similar limitations because they too are not equally effective against all pests. This is because of selectivity indeed and this negative aspect is often more noticeable for both general predators, chemicals and microbials. On the other hand predators and chemicals may be danger for other beneficial insects in threatened area.
- Heat, desiccation (drying out), or exposure to ultraviolet radiation reduces the effectiveness of several types of microbial insecticides. Consequently, proper timing and application procedures are especially important for some products.

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- Special formulation and storage procedures are necessary for some microbial pesticides. Although these procedures may complicate the production and distribution of certain products, storage requirements do not seriously limit the handling of microbial insecticides that are widely available. (Store all pesticides, including microbial insecticides, according to label directions.)
- Because several microbial insecticides are pest-specific, the potential market for these products may be limited. Their development, registration, and production costs cannot be spread over a wide range of pest control sales. Consequently, some products are not widely available or are relatively expensive (several insect viruses, for example).

PATHOGEN	PRODUCT NAME	HOST RANGE	USES AND COMMENTS
BACTERIA			
Bacillus thuringiensis var. *kurstaki* (*Bt*)	Bactur®, Bactospeine®, Bioworm®, Caterpillar Killer®, Dipel®, Futura®, Javelin®, SOK-Bt®, Thuricide®, Topside®, Tribactur®, Worthy Attack®	caterpillars (larvae of moths and butterflies)	Effective for foliage-feeding caterpillars (and Indian meal moth in stored grain). Deactivated rapidly in sunlight; apply in the evening or on overcast days and direct some spray to lower surfaces or leaves. Does not cycle extensively in the environment.
Bacillus thuringiensis var. israelensis (*Bt*)	Aquabee®, Bactimos®, Gnatrol®, LarvX®, Mosquito Attack®, Skeetal®, Teknar®, Vectobac®	larvae of *Aedes* and *Psorophora* mosquitoes, black flies, and fungus gnats	Effective against larvae only. Active only if ingested. *Culex* and *Anopheles* mosquitoes are not controlled at normal application rates.. Does not cycle extensively in the environment.
Bacillus thuringiensis var. *tenebrinos*	Foil® M-One® M-Track®, Novardo® Trident®	larvae of Colorado potato beetle, elm leaf beetle adults	Effective against Colorado potato beetle larvae and the elm leaf beetle. Like other *Bt*s, it must be ingested. It is subject to breakdown in ultraviolet light and does not cycle extensively in the environment.
Bacillus thuringiensis var. *aizawai*	Certan®	wax moth caterpillars	Used only for control of was moth infestations in honeybee hives.
Bacillus popilliae and *Bacillus lentimorbus*	Doom¨, Japidemic¨,® Milky Spore Disease, Grub Attack®	larvae (grubs) of Japanese beetle	The main Illinois lawn grub (the annual white grub, *Cyclocephala* sp.) Is NOT susceptible to milky spore disease.
Bacillus sphaericus	Vectolex CG®, Vectolex WDG®	larvae of *Culex, Psorophora,* and *Culiseta* mosquitos, larvae of some *Aedes* spp.	Active only if ingested, for use against *Culex, Psorophora,* and *Culiseta* species; also effective against *Aedes vexans.* Remains effective in stagnant or turbid water
FUNGI			
Beauveria bassiana	Botanigard®, Mycotrol®, Naturalis®	aphids, fungus gnats, mealy bugs, mites, thrips, whiteflies	Effective against several pests. High moisture requirements, lack of storage longevity, and competition with other soil microorganisms are problems that remain to be solved.

PATHOGEN	PRODUCT NAME	HOST RANGE	USES AND COMMENTS
Lagenidium giganteum	Laginex®	larvae of most pest mosquito species	Effective against larvae of most pest mosquito species; remains infective in the environment through dry periods. A main drawback is its inability to survive high summertime temperatures.
PROTOZOA			
Nosema locustae	NOLO Bait®, Grasshopper Attack®	European cornborer caterpillars, grasshoppers and mormon crickets	Useful for rangeland grasshopper control. Active only if ingested. Not recommended for use on a small scale, such as backyard gardens, because the disease is slow acting and grasshoppers are very mobile. Also effective against caterpillars.
VIRUSES			
Gypsy moth nuclear plyhedrosis (NPV)	Gypchek® virus	gypsy moth caterpillars	All of the viral insecticides used for control of forest pests are produced and used exclusively by the U.S. Forest Service.
Tussock moth NPV	TM Biocontrol-1®	tussock moth caterpillars	
Pine sawfly NPV	Neochek-S®	pine sawfly larvae	
Codling moth granulosis virus (GV)	(see comments)	codling moth caterpillars	Commercially produced and marketed briefly, but no longer registered or available. Future re-registration is possible. Subject to rapid breakdown in ultraviolet light.
ENTOMOGENOUS NEMATODES			
Steinernema feltiae (=*Neoaplectana carpocapsae*) *S. riobravis*, *S. carpocapsae* and other *Steinernema* species	Biosafe®, Ecomask®, Scanmask®, also sold generically (wholesale and retail), Vector®	larvae of a wide variety of soil-dwelling and boring insects	*Steinernema riobravis* is the main nematode species marketed retail in the U.S. Because of moisture requirements, it is effective primarily against insects in moist soils or inside plant tissues. Prolonged storage or extreme temperatures before use may kill or debilitate the nematodes.
Heterorhabditis heliothidis	currently available on a wholesale basis for large scale operations	larvae of a wide variety of soil-dwelling and boring insects	Not commonly available by retail in the U.S.; this species is used more extensively in Europe. Available by wholesale or special order for research or large-scale commercial uses.
PATHOGEN			
Steinernema scapterisci	Nematac®S	late nymph and adult stages of mole crickets	*S. scapterisci* is the main nematode species marketed to target the tawny and southern mole cricket. Best applied where irrigation is available. Irrigate after application.

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Table 1. Microbial Insecticides: A summary of products and their uses.

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3.2.1. Entomopathogenic fung

Entomopathogenic fungi are important natural regulators of insect populations and have potential as mycoinsecticide agents against diverse insect pests in agriculture. These fungi infect their hosts by penetrating through the cuticle, gaining access to the hemolymph, producing toxins, and grow by utilizing nutrients present in the haemocoel to avoid insect immune responses [10]. Entomopathogenic fungi may be applied in the form of conidia or mycelium which sporulates after application. The use of fungal entomopathogens as alternative to insecticide or combined application of insecticide with fungal entomopathogens could be very useful for insecticide resistant management [13].

The commercial mycoinsecticide 'Boverin' based on *B. bassiana* with reduced doses of trichlorophon have been used to suppress the second-generation outbreaks of *Cydia pomonella* L. Anderson *et al.* (1989) detected higher insect mortality when *B. bassiana* and sublethal concentrations of insecticides were applied to control Colorado potato beetle (*Leptinotarsa decemlineata*), attributing higher rates of synergism between two agents [14].

The use of the insect-pathogenic fungus *Metarhizium anisopliae* against adult *Aedes aegypti* and *Aedes albopictus* mosquitoes has also been reportedThe life span of fungus-contaminated mosquitoes of both species was significantly reduced compared to uninfected mosquitoes. The results indicated that both mosquito species are highly susceptible to infection with this entomopathogen [15].

3.2.2. Viral pesticides

There are more than 1600 different viruses which infect 1100 species of insects and mites. A special group of viruses, called baculovirus, to which about 100 insect species are susceptible, accounts for more than 10 percent of all insect pathogenic viruses. Baculoviruses are rod-shaped particles which contain DNA. Most viruses are enclosed in a protein coat to make up a virus inclusion body. Alkaline condition of insect's midgut dissolves the protein covering and the viral particles are released from the inclusion body. These particles fuse with the midgut epithelial cells, multiply rapidly and eventually kill the host. But, viral pesticides are more expensive than chemical agents. Furthermore, many baculoviruses are host specific. Therefore they cannot be used to control several different pests. The action of baculoviruses on insect larvae is too slow to satisfy farmers. These viral preparations are not stable under the ultraviolet rays of the sun. Efforts are being made to encapsulate baculoviruses with UV protectants to ensure a longer field-life.

First well-documented introduction of baculovirus into the environment which resulted in effective suppression of a pest occurred accidentally before the World War II. Along with a parasitoid imported to Canada to suppress spruce sawfly *Diprion hercyniae*, an NPV specific for spruce sawfly was introduced and since then no control measures have been required against this hymenopteran species. In the past, the application of baculoviruses for the protection of agricultural annual crops, fruit orchards and forests has not matched their potential. The number of registered pesticides based on baculovirus, though slowly, increases

steadily. At present, it exceeds fifty virus formulations, some of them being the same baculovirus preparations distributed under different trade names in different countries [16].

NPVs and GVs are used as pesticides but the group based on nucleopolyhedrosis viruses is much larger. The first viral insecticide Elcar™ was introduced by Sandoz Inc. in 1975Elcar™ was a preparation of *Heliothis zea* NPV which is relatively broad range baculovirus and infects many species belonging to genera *Helicoverpa* and *Heliothis*. HzSNPV provided control of not only cotton bollworm, but also of pests belonging to these genera attacking soybean, sorghum, maize, tomato and beans. In 1982 Sandoz decided to discontinue the production. The resistance to many chemical insecticides including pyrethroids revived the interest in HzSNPV and the same virus was registered under the name GemStar™. HzSNPV is a product of choice for biocontrol of *Helicoverpa armigera*]. Countries with large areas of such crops like cotton, pigeonpea, tomato, pepper and maize, e.g. India and China, introduced special programs for the reduction of this pest by biological means. In Central India, *H.armigera* in the past was usually removed by shaking pigeonpea [17].

The well-known success of employing baculovirus as a biopesticide is the case of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) used to control the velvetbeen caterpillar in soybean. In the early eighties this program was performed in Brazil. Since then, over 2,000,000 ha of soybean have been treated with the virus annually. Recently, after many new emerging pests in the soybean, this number dropped down. Although the use of this virus in Brazil is the most impressive example of viral bioregulation worldwide, the virus is still obtained by *in vivo* production mainly by infection of larvae in soybean farms. The demand for virus production has increased tremendously for protection of four million hectares of soybean annually. Because large scale *in vivo* production of baculoviruses encounters many difficulties the high demand for AgMNPV require studies dealing with inexpensive *in vitro* production of the virus. The use of AgMNPV in Brazil brought about many economical, ecological and social benefits. On the basis of this spectacular success of a baculovirus pesticide, it is needless to say that the advantages of biopesticides over chemical pesticides are numerous [18].

3.2.3. Protozoa

Protozoan pathogens naturally infect a wide range of insect hosts. Although these pathogens can kill their insect hosts, many are more important for their chronic, debilitating effects. One important and common consequence of protozoan infection is a reduction in the number of offspring produced by infected insects. Although protozoan pathogens play a significant role in the natural limitation of insect populations, few appear to be suited for development as insecticides.

As an other example, the Microsporidia include species promising 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 indeed slow acting organisms, taking days or weeks to make harm 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

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commercially, but the technology is new and work is needed to perfect the use of these organisms [12]

3.2.4. Microscopic nematods

To be accurate, nematodes are not microbial agents. Instead, they are multicellular roundworms. Nematodes used in insecticidal products are, however, nearly microscopic in size, and they are used much like the truly microbial products discussed previously. 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., tetradomatids) or expensive (e.g. mermithids) to mass produce, have narrow host specificity against pests of minor economic importance, possess modest virulence (e.g., sphaeruliids) or are otherwise poorly suited to exploit for pest control purposes. The only insect-parasitic nematodes possessing an optimal balance of biological control attributes are entomopathogenic or insecticidal nematodes in the genera *Steinernema* and *Heterorhabditis*Nematodes used for insect control infect only insects or related arthropods; they are called entomogenous nematodes [19]

The entomogenous nematodes *Steinernema feltiae* (sometimes identified as *Neoaplectana carpocapsae*), *S. scapteriscae*, *S. riobravis*, *S. carpocapsae* and *Heterorhabditis heliothidis* are the species most commonly used in insecticidal preparations. Within each of these species, different strains exhibit differences in their abilities to infect and kill specific insects. In general, however, these nematodes infect a wide range of insects. On a worldwide basis, laboratory or field applications have been effective against over 400 pest species, including numerous beetles, fly larvae, and caterpillars.

The infectious stage of these nematodes is the third juvenile stage often referred to as the J3 stage or the "dauer" larvae. Nematodes in this stage survive without feeding in moist soil and similar habitats, sometimes for extended periods. *Steinernema* species infect host insects by entering through body openings--the mouth, anus, and spiracles (breathing pores). *Heterorhabditis* juveniles also enter host insects through body openings, and in some instances are also able to penetrate an insect's cuticle. If the environment is warm and moist, these nematodes complete their life cycle within the infected insect. Infective juveniles molt to form adults, and these adults produce a new generation withing the same host. As the offspring mature to the J3 stage, they are able to leave the dead insect and seek a new host [20]

Nosema locustae has been used to reduce grasshopper populations in rangeland areas, and adequate control has been achieved when treatments were applied to large areas while hoppers were still young. Although not all grasshoppers in the treated area are killed by *Nosema locustae,* infected hoppers consume less forage and infected females produce fewer viable eggs than do uninfected females. *Nosema locustae* persists on egg pods to provide varying degrees of infection the following season. The effectiveness and utilization of *Nosema locustae* for rangeland grasshopper control are likely to increase as research continues. This single-celled protozoan infects and kills over 90 species of grasshoppers, locusts, and some species of

crickets. Nosema Iocustae is non-toxic to humans, livestock, wild animals, birds, fish, and pets. Should be applied early in the season as over-wintering hoppers emerge. Unfortunately, small, one-pound packages of *Nosema locustae* preparations developed for sale to gardeners and homeowners offer much less utility or none. The mobility of grasshoppers, coupled with the fact that infected hoppers are not killed until a few weeks after they ingest the pathogen, means that application of baits containing *Nosema locustae* to individual lawns or gardens is unlikely to reduce grasshopper densities or damage substantially [21].

3.2.5. Bacterial biopesticides

Bacterial biopesticides are the most common and cheaper form of microbial pesticides. As an insecticide they are generally specific to individual species of moths and butterflies, as well as species of beetles, flies and mosquitoes. To be effective they must come into contact with the target pest, and may require ingestion to be effective. Bacteria in biological pesticides survive longer in the open than previously believed. Bacterial pathogens used for insect control are spore-forming, rod-shaped bacteria in the genus *Bacillus*. They occur commonly in soils, and most insecticidal strains have been isolated from soil samples. The *Bacillus* genus encompasses a large genetic biodiversity. *Bacilli* are present in an extremely large area of environments ranging from sea water to soil, and are even found in extreme environments like hot springs [22]. This bacterium could be one of the major sources of potential microbial biopesticides because it retains several valuable traits [23].

First of all, *Bacilli*, like *B. subtilis*, are well-studied organisms. Secondly, the US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *Bacillus subtilis* which is thus recognized non-pathogenic. This is of course essential with respect to its application as a biopesticide. Thirdly, *Bacilli* have the capacity to produce spores which are extremely resistant dormancy forms capable to withstand high temperatures, unfavorable pH, lack of nutrients or water, etc. [24]. They are produced by the bacteria when environmental conditions are unfavorable which probably helps these microorganisms to survive in the phytosphere. The phenomenon can also be exploited in industrial production as sporulation can be induced at the end of cultures. course essential regarding its application as a biopesticide [25].

Bacterial insecticides must be eaten to be effective; they are not contact poisons. Insecticidal products comprised of a single *Bacillus* species may be active against an entire order of insects, or they may be effective against only one or a few species.

3.2.6. Bacillus thuringiensis, BT

Bacillus thuringiensis (Bt) is an aerobic, gram positive, spore forming soil bacterium that shows unusual ability to produce endogenous different kinds of crystals protein inclusions during its sporulation. *B. thuringiensis* (commonly known as 'Bt') is an insecticidal bacterium, marketed worldwide for control of many important plant pests - mainly caterpillars of the Lepidoptera (butterflies and moths) but also mosquito larvae, and simuliid blackflies that vector river blindness in Africa. The commercial Bt products are powders containing a mixture of dried spores and toxin crystals. They are applied to leaves or other environments where the

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insect larvae feed. The toxin genes have also been genetically engineered into several crop plants. The method of use, mode of action, and host range of this biocontrol agent may differ within other Bacillus insecticidal species [10]

The *Bacillus* species, *Bacillus thuringiensis*, has developed many molecular mechanisms to produce pesticidal toxins; most of toxins are coded for by several *cry* genes. Since its discovery in 1901 as a microbial insecticide, *Bacillus thuringiensis* has been widely used to control insect pests important in agriculture, forestry and medicine. Its principal characteristic is the synthesis of a crystalline inclusion during sporulation, containing proteins known as endotoxins or Cry proteins, which have insecticidal properties [26]. The crystal protein inclusions are composed of one or more crystal (Cry) and cytolytic (Cyt) toxins which are also called δ-endotoxins or insecticidal crystal proteins. Some of these proteins are highly toxic to certain insects but they are harmless to most other organisms including vertebrates and beneficial insect. Since their insecticidal potential has been discovered, it has been produced commercially and accepted as a source of environment friendly biopesticide all over the world.

There are different strains of *B. thuringiensis*. Each strain of this bacterium produces a different mix of proteins, and specifically kills one or a few related species of insect larvae. While some Bt's control moth larvae found on plants, other Bt's are specific for larvae of flies and mosquitoes. The target insect species are determined by whether the particular Bt produces a protein that can bind to a larval gut receptor, thereby causing the insect larvae to starve.The most widely used strains of *B. thuringiensis* have started against three genera of mosquitos; *Culex*, *Culiseta* and *Aedes*[

Their study has shown that Bt spores can survive both on the ground and in animals. What's more, wind, rain and animals can carry them to neighbouring areas. In the splashing rain drops they can even "hop" from the ground up onto leaves - another means of transport. Bt bacteria are also known to be able to easily transfer their toxicity genes to other bacteria in the application area.

When the bacteria were sprayed on cabbage plants, and they were found to have killed all the cabbage white butterfly larvae. In addition, though, the field study revealed that the bacteria are able to survive for a considerable time. After spraying, by far the majority of the spores were found to be present in the upper two centimetres of the soil, *National Environmental Research Institute of Denmark.* "Their toxic effects disappeared after a few days, but half of the bacteria were still surviving as spores 120 days later, and one fifth were still alive after a year. They existed in a dormant state, however, and did not produce toxins, although the spores are able to germinate later and produce insecticide again," explain microbiologists Bjarne Munk Hansen and Jens Chr. Pedersen of the National Environmental Research Institute. Until now it has generally been believed that the majority of Bt bacteria disappear rapidly after they have been sprayed. "It was thought that when the toxic effect disappeared, the bacteria had also disappeared. What in fact happens, though, is that the bacteria convert to a dormant stage and become spores," continue the two scientists.

In the present era of transgenic technology, insecticidal toxins of *Bacillus thuringiensis* (Bt) assume considerable significance in the production of insect resistant crops such as cotton,

maize, potato, rice etc. This review also describes about biology of Bt toxin, recent progress in the development of Bt technology, evolution of resistant insect populations against Bt and management strategy.

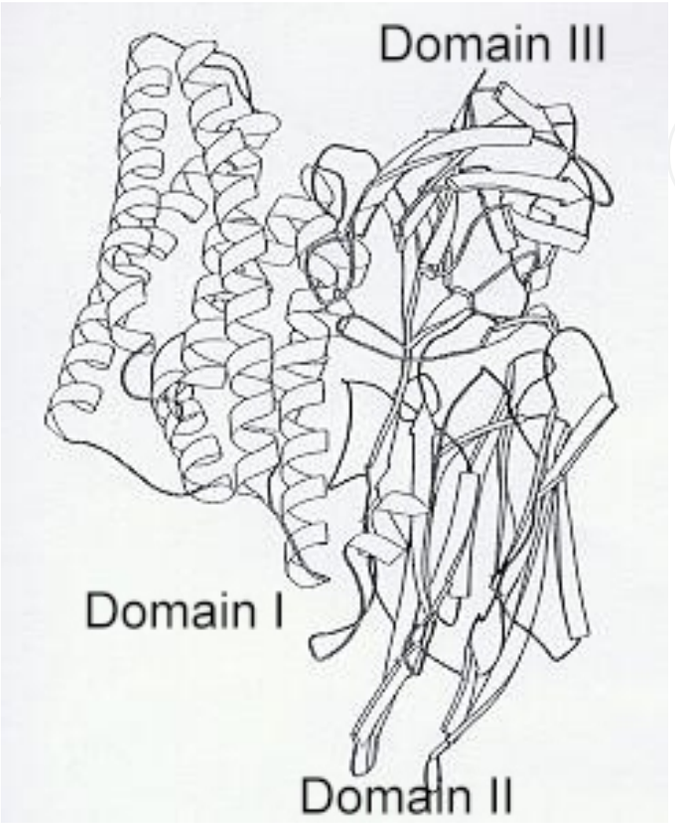

Figure 1. Different domains involved in the toxicity of *B.thuringiensis* toxin in the mid-gut of targeted insect. Source:Sharma et al., 2000. *Bt* bacteria are used by farmers, foresters and gardeners to destroy butterfly larvae, mosquito larvae and beetles. The Danish field study, which was undertaken in 1993 and 1994, is one of the first in the world where plants have been systematically sprayed with *Bacillus thuringiensis* bacteria, and where the research has been ecologically oriented. In contrast, the numerous field studies undertaken by the producers of biological pesticides have been oriented to commercial considerations.

Scientists Per Damgaard and Jørgen Eilenberg at the Royal Agricultural University in Denmark, have also observed examples of spores germinating in living but weakened flies. The flies were already suffering from a severe fungal infection of the lower abdomen, and it was exactly there that the spores germinated. They showed that, the bacterial spores germinate well in dead insects, as the two scientists confirmed by feeding spore and toxin-treated food to larvae of the large cabbage white butterfly.

Under good growth conditions a spore can produce up to a thousand million new spores in a single insect larva.

"There are no previous examples of the spores reproducing in living organisms, although they appear to be able to do so in dead flies. The advantage for the bacterium is that the spores can be spread when the fly moves around," continue Bjarne Munk Hansen and Jens Chr. Pedersen.

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Gene	Target pest	References
Cry 1A(b)	Striped stem borer and leaf folder	Fujimoto et al. (1993)
Cry 1A(b)	Yellow stem borer and striped stem borer	Wunn et al. (1996)
Cry 1A(b)	Yellow stem borer and striped stem borer	Ghareyazie et al. (1997)
Cry 1A(b)	Yellow stem borer	Datta et al. (2002)
Cry 1A(b)	Yellow stem borer	Alam et al. (1999)
Cry 1A(b)/ Cry 1A(c)	Leaffolder and yellow stem borer	Tu et al. (2000)
Cry 1A(b)/ Cry 1A(c)	Yellow stem borer	Ramesh et al. (2004)
Cry 1A(c)	Yellow stem borer	Nayak et al. (1997)
Cry 1A(c)	Yellow stem borer	Khanna and Raina (2002)
Cry 1A(c)	Striped stem borer	Liu et al. (2002)
Cry 2A	Leaffolder and yellow stem borer	Maqbool et al. (1998)
Cry 2A/ Cry 1A(c)	Leaffolder and yellow stem borer	Maqbool et al. (2001)
Cry 1Ie	Corn borer	Liu et al., 2004

Table 2. Successful examples to show *B. thuringiensis* genes (originated from *Bacillus thuringiensis*) integration for insect resistance in rice.

Insects can be infected with many species of bacteria but those belonging to the genus Bacillus, as alreadily mentioned, are most widely used as pesticides. *Bacillus thuringiensis* has developed many molecular mechanisms to produce called cry genes [28]. Since its discovery in 1901 over one hundred *B. thuringiensis*-based bioinsecticides have been developed, which are mostly used against lepidopteran, dipteran and coleopteran larvae[29]. In addition, the genes that code for the insecticidal crystal proteins have been successfully transferred into different crops plants by means of transgenic technology which has led to significant economic benefits. Because of their high specificity and their safety in the environment, *B. thuringiensis* and Cry protein toxins are efficient, safe and sustainable alternatives to chemical pesticides for the control of insect pests. The toxicity of the Cry proteins have traditionally been explained by the formation of transmembrane pores or ion channels that lead to osmotic cell lysis [30]. In addition to this, Cry toxin monomers also seem to promote cell death in insect cells through a mechanism involving an adenylyl cyclase/PKA signalling pathway. However, despite this entomopathogenic potential, controversy has arisen regarding the pathogenic lifestyle of *B. thuringiensis*. Recent reports claim that *B. thuringiensis* requires the co-operation of commensal bacteria within the insect gut to be fully pathogenic [31,32].

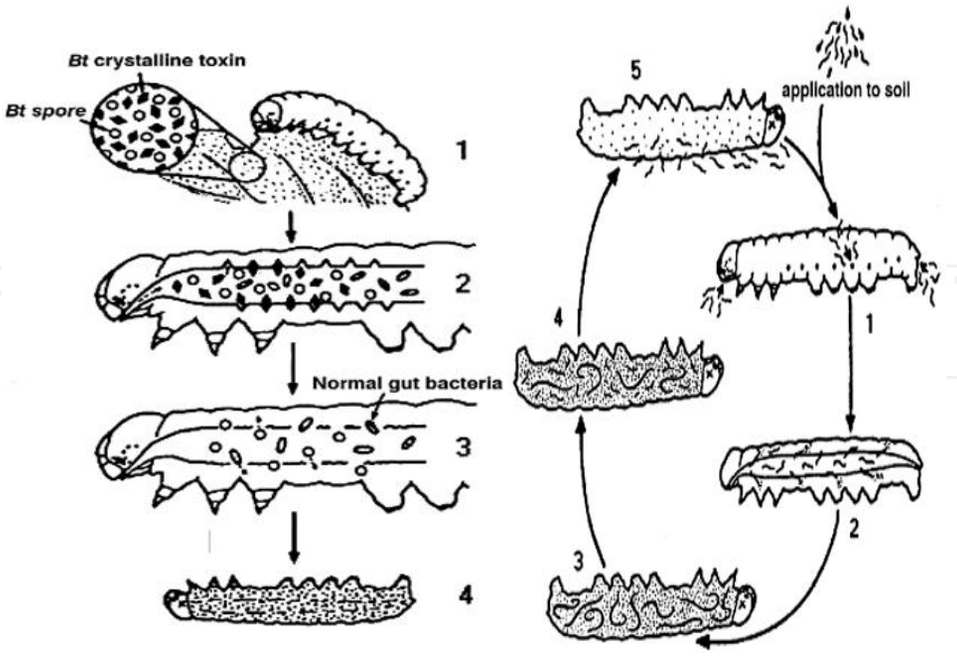

Figure 2. Life cycles of biopesticides, bacteria or nematods, to pest larvas. 1- enter the gut or respiratory system through body openings (mouth, anus, and breathing pores. 2- actively penetrate the gut wall, enter the body cavity, and release bacteria. 3- As it multiplies, host dies of septicemia, Adapted from Woodring,1988

The first developed *Bacillus thuringiensis* insecticidal agent is a mixture of *Bacillus thuringiensis* spores and its toxin. As a pesticide, (BT*)* accounts for over 90 percent of total share of today's bioinsecticide market and has been used as biopesticide for several decades. The discovery of the strain *B. thuringiensis* serovar *israelensis* made possible efficient microbiological control of Diptera Nematocera vectors of diseases, such as mosquitoes (Culicidae) and black flies [33]

In most countries of the world, products are available for control of caterpillars (var. *kurstaki*, *entomocidus*, *galleriae* and *aizawai*), mosquito and blackfly larvae (var. *israeliensis*) and beetle larvae (var. *tenebrionis*). Actively growing cells lack the crystalline inclusions and thus are not toxic to insects. The BT preparations remain stable without any disentegration over years even in the presence of UV sun rays. As the insect feeds on the foliage, the crystals too are eaten up. These are hydrolysed in the insect's midgut to produce an active endotoxin. The active toxin binds to receptor sites on gut epithelial cells and creates imbalance in the ionic make-up of the cell. This is seen by swelling and bursting of the cells due to osmotic shock. Subsequent symptoms are paralysis of the insect's mouthparts and gut. So obviously the feeding process is inhibited. [34,35]

Also, a relatively new mechanism of action of Cry toxins have been proposed which involves the activation of Mg2+-dependent signal cascade pathway that is triggered by the interaction of the monomeric 3-domain Cry toxin with the primary receptor, the cadherin protein BT-R1 [26]. The triggering of the Mg2+-dependent pathway has a knock-on effect and initiates a series of cytological events that include membrane blebbing, appearance of nuclear ghosts, and cell swelling followed by cell lysis. The Mg2+-dependent signal cascade pathway activation by Cry

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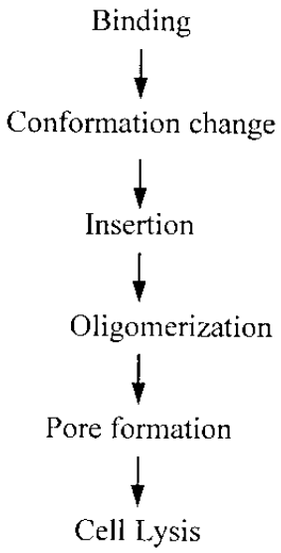

Figure 3. Characterization of the steps require for formation of pores in cell membranes

toxins have been shown to be analogous to similar effect imposed by other pore forming toxins on their host cells when they are applied at subnanomolar concentration [37, 38]

Though the two mechanisms of action seem to differ, with series of downstream events following on from toxin binding to receptors on target cell membranes, there is a degree of commonality in that initially the crystals have to be solubilised in vivo or in vitro, and activated by proteases before and/or after binding to receptors such as cadherin [39, 40]

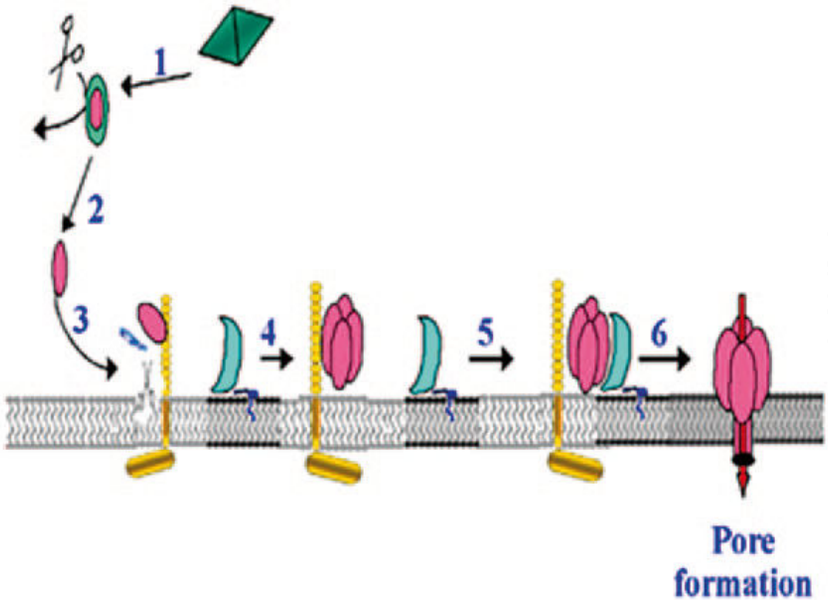

Figure 4. Model of the mode of action of Cry1A toxins. *1* Crystal toxin solubilisation, *2* Initial cleavage by gut proteases, *3* Toxin monomer binding to receptors and second cleavage bymembrane bound protease, *4* Membrane insertion-competent oligomer formation, *5* Binding of oligomeric toxin to receptors, *6* Lytic pore formation

4. Plant-Incorporated-Protectants (PIPs)

One approach, to reduce destruction of crops by phytophagous arthropod pests, is to genetically modify plants to express genes encoding insecticidal toxins. The adoption of genetically modified (GM) crops has increased dramatically in the last 11 years. Genetically modified (GM) plants possess a gene or genes that have been transferred from a different species.

The production of transgenic plants that express insecticidal δ-endotoxins derived from the soil bacterium *Bacillus thuringiensis* (*Bt* plants) were first commercialized in the US in 1996. The expression of these toxins confers protection against insect crop destruction. The lethality of *Bt* endotoxins is highly dependent upon the alkaline environment of the insect gut, a feature that assures these toxins are not active in vertebrates, especially in humans [41]. These proteins have been commercially produced, targeting the major pests of cotton, tobacco, tomato, potato, corn, maize and rice, notably allowing greater coverage by reaching locations on plants which are inaccessible to foliar sprays. There are numerous strains of *Bt*, each with different Cry proteins, and more than 60 Cry proteins have been identified. Most *Bt* maize hybrids express the Cry1Ab protein, and a few express the Cry1Ac or the Cry9C protein, all of which are targeted against the European corn borer (*Ostrinia nubilalis* Hubner) (Lepidoptera), a major pest of maize in North America and Europe. Some recent maize hybrids express the Cry3Bb1 protein, which is targeted against the corn rootworm complex [*Diabrotica spp.* Coleoptera], also a major pest of maize, especially in North America. Cotton expressing the Cry1Ac protein is targeted against the cotton bollworm [*Helicoverpa zea- Lepidoptera*] [42].

Crop target	Gene	Target pest	References
Corn	*Cry 1A(b)*	European corn borer	Koziel et al. (1993)
Soybean	*Cry 1A(c)*	Bollworm and Bud worm	Stewart (1996)
Tobacco	*Cry 2aa2*	Cotton bollworm	De Cosa et al. (2001)
Sugar cane	*Cry 1A(b)*	Stem borer	Arencibia et al. (1997)
Potato	*Cry 5*	*B. thuringiensis* Potato tuber moth	Douches et al. (1998)
Alfalfa	*Cry 1C*	Leaf worm	Strizhov et al. (1996)
Tomato	B. thuringiensis (k)	Tobacco hornworm, tomato pink worm and tomato fruit worm	Dellannay et al. (1989)
Brassica	*Cry 1A(c)*	Pod borer	Stewart (1996)
Cotton	*Cry 1A(b)/(c)*	Lepidoptera	Stewart(2001), Chitkowski et al. (2003)
	Cry 2A	Pink Bollworm	Tabashnik et al. (2002)

Table 3. Development of some other *B. thuringiensis* transgenic crops for insect resistance [35]

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5. *Bacillus thuringiensis* applications in agriculture

Bacillus thuringiensis and its products have been formulated into various forms for application as biological control agents. Such formulations could be solid (powdery or granulated) or liquid. Presently there are over 400 of *Bt* based formulations that has been registered in the market and most of them contain insecticidal proteins and viable spores though the spores are inactivated in some products. Formulated *Bt* products are applied directly in the form of sprays). An alternative, and highly successful, method for delivering the toxins to the target insect has been to express the toxin-encoding genes in transgenic plants [43,44]

5.1. *Bacillus sphaericus*, BS

Entomopathogenic bacteria, namely *Bacillus thuringiensis* Bt, have been known from the early 1900's but the control of dipteran species has been establ,shed only since the discovery of *B. thuringiensis* serovar *israelensis* Bti in 1977 and a highly toxic strain of *B. sphaericus* B.s. strain 1593 in [45].

Bacillus sphaericus is the another aerobic bacterium in Bacillus genus that has been used in the biological control of the insects. *Bacillus sphaericus* Bs, like *Bacillus thrungiensis* Bti is a naturally occurring soil bacterium with mosquitoe larvicidal properties from the genus *Bacillus*. It has become an alternative agent for microbial control of mosquitoes since the isolation of highly larvicidal strains of this bacteria. *Psorophora*, and some members of the genus *Aedes*. *Ae. aegypti* and *Ae. albopictus* are insensitive to *B. sphaericus*

The first reported *B. sphaericus* strain (BS) active against mosquito larvae was isolated from Moribund area in Argentina to mosquito larvae of *Culiseta* incidens in 1965 [46]. Strain 2362, isolated from *Simulium* in Nigeria, is not toxic to black flies, but it is regarded as the most promising isolate for field use against mosquitoes. Pasteurization of the soils make the medium selective for *B. sphaericus.* The efficacy of strain 2362 against field populations of mosquitoes from the genera *Culex* has been demonstrated. Since the sixties, when a strain of BS was discovered to have larvicidal activity against mosquito species, a large number of other mosquitocidal Bs strains have been described. The larvicidal activity of this first isolate was so low that its use in mosquito control would not have been considered indeed. But only after isolation in Endonesia from dead mosquito larvae of strain 1593 which exhibited a much higher mosquitocidal activity against *Culex quinquefasciatus* was potential of *Bacillus sphaericus* as a biological control agent for some species of mosquitoes, and used as insecticide in the field as part of vector control programmes [47].

It has terminally located sphearical spores. One of the phenotypic characthers examined was patogenicity of some of them to mosquito larvae. A pro-toxin produced during sporulation as in the case of BT, causes fatal cellular alterations when ingested by larvae of some dipteran species. This bacterium has been used to control *Culex* and *Anopheles* populations in various countries replacing chemical larvicides with certain advantages. They include reduction in cost and selectivity to the target populations. The toxic activity of the *Bacillus sphaericus* strains increased at the time of sporulation, it is logical to look for parasporal inclusions in this

bacterium. Since filter sterilized culture supernatants had been shown to be nontoxic, all of the toxin must be retained on or within the cells themselves. In the cells fractioned in the process of sporulation, the cell walls gave more toxic characthere than the cytoplasmic part. On the other hand, the mature spores isolated from the cells were more toxic than the cell wall fraction, thus it appeared that some toxin may be located in several parts of the cell but that the spore contains the highest concentration of the toxin [48,49,50].

Abbott Laboratories has recently formulated a commercial product (Vectolex) of *B. sphaericus* 2362. Generally, *B. sphaericus* strains with high larvicidal activity have been isolated from dead insects and as well as from other sources. However, five isolates of *B. sphaericus* from soil samples in Israel have been reported to belong to phage group three and were found to be as toxic as strain 2362 to *Culex* sp. Larvae. During a screening for entomopathogenic bacteria in soil samples carried out at Cenargen / Embrapa in Brazil, several *B. sphaericus* isolates were obtained [51].

5.2. Systematics of the *Bacillus sphaericus* Neide

According to one the old view *Bacillus sphaericus* is a heterogeneous species of bacteria that contains strains belonging to at least five different DNA homology groups that are sufficiently phenotypically similar without a need to establish each as a new distinct species of which homology group IIA differ from the other genospecies in that it contains strains that are pathogenic for mosquitoe larvae. According to a recent study based on phylogenetic analysis of the 16S rRNA DNA sequences from 58 strains identified as *B. sphaericus*, which were also confirmed by whole-cell fatty acid profiles and other phenotypic determinations, *B. sphaericus*-like strains segregated into seven distinct clusters in a phylogenetic tree and is a genetically and phenotypically a highly heterogeneous taxon. Among these, one cluster represented *B. sphaericus* and another *B. fusiformis*. A third cluster containing all of the pathogenic strains was closely related to or was possibly part of the *B. fusiformis* group. The remaining four groups were distinct and represented unnamed taxa that are more closely related to *B. sphaericus* and *B. fusiformis* than to the psychrophilic, round-spored species, *B. globisporus* and *B. psychrophilus*. The pathogenic strains are members of a distinct group and not of the species *B. sphaericus* sensu stricto. The apparent variability of mosquitoe pathogenicity among *B. sphaericus* strains can partially be explained by this heterogeneity [52].

5.3. Mode of living of *Bacillus* spp. and *Bacillus sphaericus* Neide

The growth of *Bacillus sphaericus* is in four stages depending on the presence of food and water in their environment. These are; (1) lag phase, where active microbial growth is to be commenced, (2) log phase, where there are active bacterial growth and the number of bacteria increases logarithmically, hence the name suggests, and (3) stationary phase, where growth of bacteria ceases due to food limitation and/or some other factors, and (4) death phase, where death of bacteria starts if they are not sporulating. If the bacterium is of a sporulating type like the genus *Bacillus*, then at the late stationary stages sporulation starts and we usually do not speak of death phases for the bacteria in the genus *Bacillus*. *B. sphaericus* starts producing endospores at the last stage of its growth cycle [51,52]

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5.4. Endospore formation of *Bacillus sphaericus* Neide

Endospore formation is a trait found in several microorganisms, which can provide positive benefits to agriculture and varying affects in humans as well. Species of *Bacillus*, *Clostridium*, *Sporosarcina*, and *Heliobacteria* produce typical endospores. Endospores are formed when a vegetative cell discontinues protein synthesis for proteins needed for normal cell function and instead activates genes specific for sporulation. Endospores are the product of aging cells in environments low in nutients. All of the cell's materials remain inside the protoplast, or core of the endospore, but metabolism is dormant. The endospore is refractile, dehydrated, and surrounded by numerous thick layers of peptidoglycan. The coat, a keratin-like protein, contains diplicolinic acid (DPA) and a high calcium content. These help make the endospore highly heat resistant, boiling, radiation, pressure, dessication and chemical treatment. Endospores also contain large amounts of small acid-soluble spore proteins (SASPs). The function of SASPs is to bind to DNA as a form of protection and to serve as a carbon energy source for when the endospore germinates to form a new vegetative cell [52]

5.5. Growth Cycle of *B. sphaericus* and production extra-cellular enzymes

During their growth cycle, strains of *Bacillus* grow as undifferentiated rod-shaped vegetative cells. Usually when the carbon source (or some other required nutrient) becomes limited, the cell enters a sporulation sequence during which a resistant resting-stage endospore is formed. The process of cellular differentiation leading to spore formation can be divided into three successive phases. Sporulation in *B. subtilis* has been well studied and it includes four stages. The first (stages 0 to II) is the stage of differentiation, and during this there is a single cell type, which eventually contains two completed chromosomes. The phase is completed with the division of this cell into two cells that differ markedly in size, but otherwise appear to be rather similar. In the second phase (stages II to III) the differentiation becomes fixed; the two cells have their own genomes, which presumably functions differently, and by stage III the two cell types differ dramatically. The developing spore at stage III exhibits none of the properties that characterize the mature spore, and the development of these properties takes place in the next phase (stages III to VII). Production of entomopathogenic toxin is a biochemical changes that accompany the morphological changes during sporulation. When fresh medium (or a pulse of a carbon source) is made available, the spore will germinate and produce a vegetative rod-shaped cell [53,54]

Because *B. subtilis*, like other Gram-positive eubacteria, lacks an outer membrane, many of these proteins are directly secreted into the growth medium. In most cases, these secreted proteins are enzymes involved in the hydrolysis of natural polymers such as proteases, lipases, carbohydrases, DNases and RNases. Such degradative enzymes are usually synthesised as part of an adaptive response indeed to changes in the environment. So that the cell to optimally can benefit from available resources.

5.6. Pathogenicity and properties of toxins of *B. Sphaericus*

The bulk of toxicity in Bs comes from the second toxin which is produced at the time of sporulation and it accumulates in the sporangium as a parasporal body, parasporal crystal packed with bacterial spores, in much the same way as that found in *B. thuringiensis*. The crystal

of *B. sphaericus* 2362, which becomes visible in the cytoplasm at about stage III of sporulation contains two polypeptides, a 51 kDa protein,P51 and a 42-kDa protein, P42, which together form the binary toxin. The binary toxin consists of two distinct proteins of 51.4 and 41.9 kDa. Both proteins are cleaved by endogenous proteases to form active toxins of 43 and 39 kDa protein subunits that are believed to associate as a hetero-dimer named the binary toxin Bin, in a 1:1 ratio, which form the active hetero-dimer complex [42]. These protein toxins have been sequenced and were found to be unrelated to each other or to the *B. thuringiensis* toxins. The *bin* genes of several highly toxic strains have also been completely sequenced, and their amino-acid sequences have been found to be highly conserved [54].

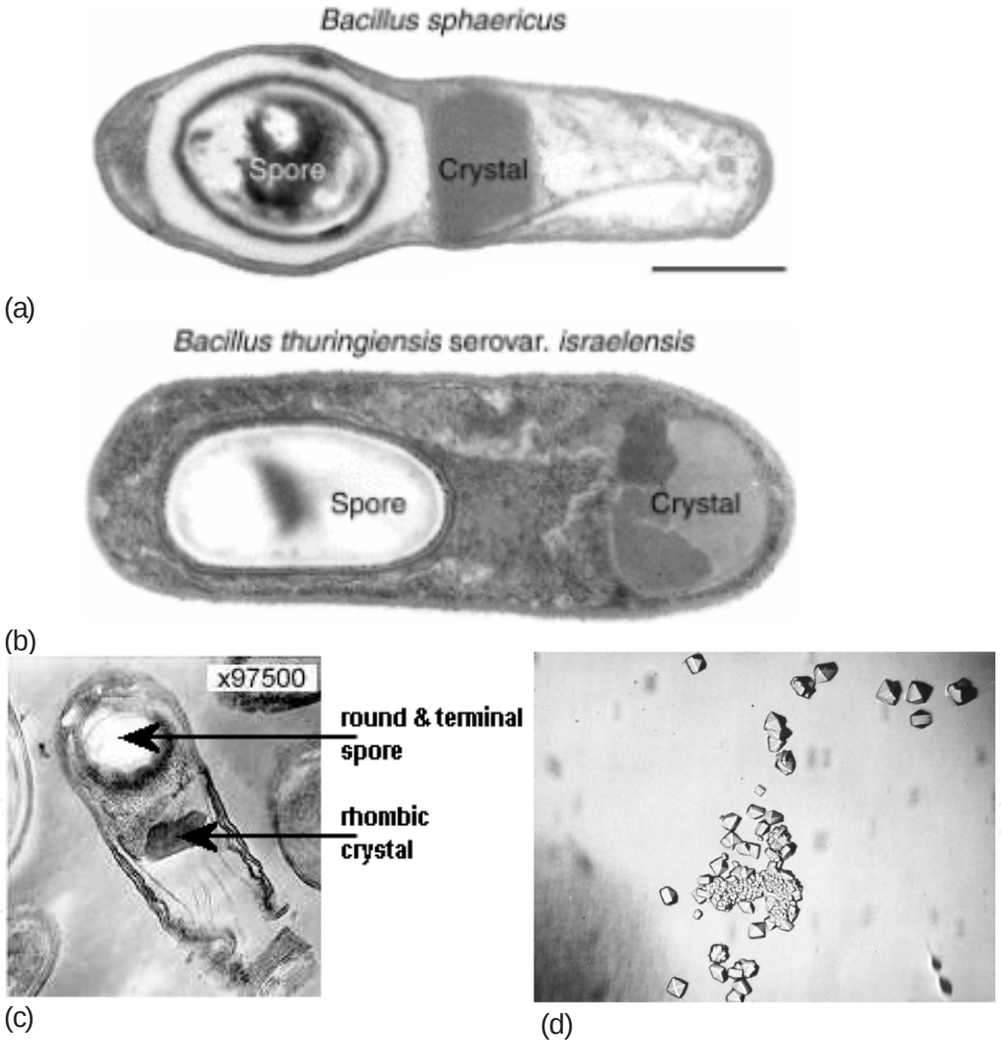

Figure 5. Sporangia of *Bacillus sphaericus* (**a**) and *Bacillus thuringiensis* serovar. *israelensis* (**b**) before the end of the sporulation process as seen by transmission electron microscopy. The parasporal inclusions (crystals), which contain the entomopathogenic toxins, are visible close to the spore. Scale BAR = 0.5 µm (from Regis et al., 2001). A closer view of *B. sphaericus* crystal showing its rhombic shape from the **a** (top) and crystals of the 51 kDa protein of *B. sphaericus* grown using the seeding method. The largest crystals are 0.2 mm in length and have well defined facets consistent with the tetragonal space group. Note that the crystals grow preferentially along the streaking path, which is almost vertical [55]

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The two major protein subunits, the 42 and 51 kDa, are both required for full activity and maximum toxicity if they are present in equimolar amounts, suggesting a 'binary toxin' mode of action. Studies on the mode of action of Bin toxin suggested that BinB is responsible for the initial binding to the surface of midgut epithelial cells and that BinA confers toxicity. It was also reported that the BinA compound alone can confer toxicity at high doses These two protein subunits are homologous, with 25% identity and four conserved regions between their sequences. P51 is the primary component of binding to the *Culex* midgut epithelium, while P42 binds efficiently only in the presence of P51 but is responsible for the larvicidal action Upon ingestion by larvae, these proteins are processed to 43 and 39 kDa, respectively. Studies on the mode of action of Bin toxin suggested that BinB is responsible for the initial binding to the surface of midgut epithelial cells and that BinA confers toxicity Neither subunit of the binary toxin is not toxic by itself both BinB and BinA in order to achieve full toxicity and a BinA–BinB or BinA–BinB–receptor complex formation in strains of 1593 and LP1-G of *B. sphaericus* has been proposed to cause larval. In confirmation that both subunits are required for full toxicity in vitro binding studies were performed to show that the N-terminal region of BinB interacts with the larval gut receptor, whereas the C-terminal region interacts with the N-terminal region of BinA, leaving the C-terminal end of BinA to facilitate internalization of the toxin complex. Binding of the binary toxin to midgut epithelium causes swelling of mitochondrial and endoplasmic reticula and enlargement of vacuoles, followed by lyses of epithelial cells, midgut perforation, and the death of larvae. How the Bin toxin causes the death of larvae is not clearly established. There is evidence that a single class of receptor is expressed on the surface of microvilli in the gastric caeca and posterior midgut of susceptible *C. pipiens* and *A. gambiae* and binding of toxin to midgut cells of susceptible mosquitoe larvae is a key step after the initial solubilization and activation of the toxin. A recent report has shown that this binding is specific, and mediated by a receptor with a unique binding site, present at the surface of epithelial cells from *Culex* and *Anopheles*. It was found that BinB alone is involved in receptor binding in *C. pipiens,* whereas both BinA and BinB seem to be involved in receptor binding in *A. Gambiae* [55]

6. Mode of action of the crystal toxin

The mode of action of *Bacillus sphaericus* crystal toxin has only been studied in mosquito larvae. A number of studies have established that the action of the crystal toxin on susceptible larvae involves th efollowing series of steps: (i) ingestion of the crystal- spore- cell complex; (ii) solubilization on th e midgut by the alkaline pH; (iii) processing of the 51- and 42-kDa proteins to 43 and 39-kDa proteins respectively, (iv) binding of toxin proteins to cells of the gastric cecum and posterior midgut; and (v) exertion of a toxic effect by means of a unknown mechanism [56].

6.1. Binding to a specific receptor in the brush- border membrane fractions

After ingestion of the spore-crystal complex by mosquito larvae, the protein crystal matrix quickly dissolves in the lumen of the anterior stomach through the combined action of midgut

proteinases and the high pH *Bacillus sphaericus* crystals release the toxin in all species such as *A. aegypti*. Indeed, some studies reported that the differences in susceptibility to Bacillus sphaericus between mosquito species do not result from differences in solubilization and/or activation of the crystal toxin. Physiological effects in midgut start as soon as 15 min. after ingestion of spor crystal complex. Midgut damages may be the same after ingestıon of spore crystals but the semptoms of intoxication produced differ in mosquito species. Large vacuoles or lysosomes appear in *Culex pipiens* midgut cells, whereas large areas of low electron density appear in Anopheles stephensi midgut cells. A generally occuring sypmtom is mitocondrial swelling, described for *C. pipiens* var. *pipiens* and *Aedes stephensi*, as well as for *A. Aegyptin* when intoxicated with a very high dose of spore crystals. The midgut cells are the cells most severely damaged by the toxin, also reported late damage in neural tissue and in skeletal muscel. Ultrastructural effects have been reported in cultured cells, of which swelling of mitochodrial cristae and endoplasmic reticula, followed by enlargement of vacuoles and condensation of mitochondrial matrix [55,56].

6.2. The effectiveness of the toxin

Differences in susceptibility between mosquito species seem to differences at the cellular level.The binding of the crystal proteins, P42 and P51 depend on each other. In addition, the internalization of toxin only seems to occur when both components are present. The hyphothesis that a single receptor is involved in the toxin binding was confirmed by in vitro binding assays using radio-labeled activated crystal toxin and midgut brush-border membrane fractions isolated from susceptible mosquito larva It is assumed that the P42 component is the toxic moiety and the P51 is the binding component, the *B. sphaericus* crystal toxin is more likely to be similar to an A/B toxin than to a binary toxin. The nature of the receptor is still unknown [56].

6.3. Mtx1: Vegetative growth mosquitocidal toxin single protein of 100 kDa

B. sphaericus is also known for the ability of some strains to produce another mosquitocidal toxin. The gene encoding one such toxin, Mtx1 (formerly known as Mtx, Mosquitocidal toxin, Mosquitocidal toxin), was first isolated from the low.

Toxicity *B. sphaericus* strain SSII-1 and has been shown to be widely distributed amongst many, but not all, high and low toxicity isolates of this species [59]. It was later shown that there are other Mtx toxins which are then started to be called Mtx1, Mtx2 and Mtx3 the genes for these toxins have been partially characterized and shown to have an extremely high level of similarity [46]. Unlike the binary toxins Mtx1 is produced as a 100 kDa protein that is processed by trypsin-like proteinases in the mosquitoe gut to yield a product with ADP-ribosyl transferase activity, 27 kDa and a putative receptor binding domain, 70 kDa [56].

In initial experiments, activity of the protoxin was demonstrated against larvae of the mosquitoe, *Culex quinquefasciatus*, and protoxins of *Escherichia coli* cells were shown to be active against *Aedes aegypti* but not the predatory mosquitoe *Toxorhynchites splendens*. In contrast to the Bin toxin, Mtx1 was also found to be highly potent against larvae of *Ae. Aegypti*, on the other hand, Bin toxin shows low or zero toxicity against this insect. This high-level toxicity to

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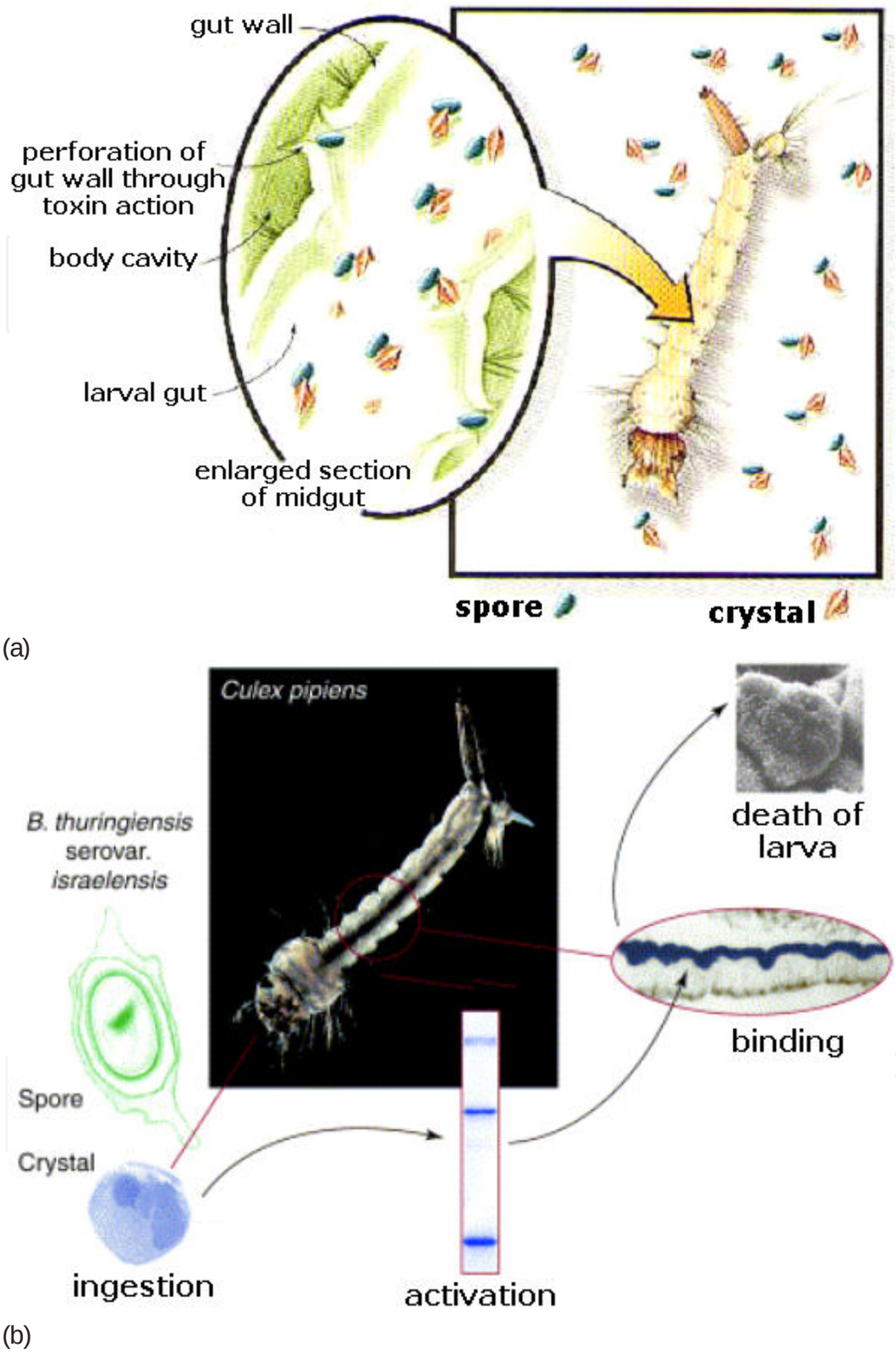

Figure 6. Action mechanism of VectoLexBC, a Biological Larvicide of *Bacillus sphaericus* (from Abbott Laboratories, 2003) **a.** top. The mode of action of crystal toxins from an entomopathogenic bacteria *Bacillus thuringiensis* serovar. *israelensis*, in this case **b.** below

Ae. aegypti gives Mtx1 great potential for use, by over-expression, in the strain improvement of *B. sphaericus*. It was proposed that the enhanced toxicity against *Ae. aegypti* would widen the utility of resulting strains whilst the distinct mechanism of action of Mtx1 compared to the Bin toxin would be expected to delay the development of resistance in target *Culex* populations. So far, thus Mtx toxin(s) of *B. sphaericus* may further enhances toxicity of this species both to mosquitoe and non-mosquitoe dipteran species [51].

7. Comparison of The Two Important Insect pathogens of *Bacillius*

Though *B. sphaericus* has a narrower range of host species than the main mosquitoe control agent *B.t.* subsp. *israelensis*, it is able to persist in the environment for a longer time than *B.t.* subsp. *israelensis*, especially in waters polluted with organic materials. However, populations of *Culex* mosquitoes resistant to the binary toxin of *B. sphaericus* have been selected under laboratory conditions. Field resistance, as a consequence of vector control programs based on *B. sphaericus* application, has also been reported in some countries, such as France and Brazil. In spite of massive field usage of *B.t.* subsp. *israelensis* in mosquitoe, chironomid midge, and black fly control, no resistance has been detected in field populations of these dipterans. This event has been explained by the presence of a set of toxic proteins of a different nature that interact synergistically, increasing larvicidal activity of *B.t.* subsp. *israelensis* and suppressing development of resistance [52].

The development of a larvicide for use in public health programmes demands selectivity. It should be active against the target species without affecting humans and other non-target populations. The development of a biological larvicide is a process similar to that of the chemical insecticides in that it aims to identify the ideal concentration and form of administration in the field. Formulation is the process used to convert a technical slurry or powder containing the active ingredient produced by the bacterium into a useful and use larvicide compatible with existing application systems. It should also ensure biological stability of the active ingredient and must have an adequate shelf life. It should be easily produced and administered, conveniently stored, and economic [51,52].

8. Resistance of insects to mosquitoe toxin

It was recently found that the decomposition of organic matter present in aquatic bodies by bacteria lead to the evolution of certain volatile compounds, which attract and/or stimulate gravid female mosquitoes to lay eggs. This finding is a clear indication that bacteria are in great association with mosquito species.

The risk of emergence of resistance should be considered when designing application strategies. *Bacillus sphaericus*, has been shown to recycle in the field conditions and exert larvicidal activity for a long period. Field resistance has been only reported for Bs, while for Bti, it seems more difficult for mosquitoes to develop resistance even under intensive laboratory selection,

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which may be due to the multiple toxin complex of this bacterium. One mechanism of resistance is the reduced binding of the toxin to the midgut receptor sites. The mechanism of resistance to *B. sphaericus* crystal toxin has been studied extensively in only two *C. pipiens* populations. Bioassays indicated that the resistance level was increased as the treatment increased, and the best way to produce bacterial strains that simultaneaously express different toxins binding to different receptors

One mechanism of resistance is the reduced binding of the toxin to the midgut receptor sites. As genes for production of insecticidal compounds are added to crop plants, developpers devise methods of preventing or managing insecticide resıstance in target pests. The mechanism of resistance to *B. sphaericus* crystal toxin has been studied extensively in only two *C. pipiens* populations. Bioassays indicated that the resistance level was increased as the treatment increased, and the best way to produce bacterial strains that simultaneaously express different toxins binding to different receptors.

Despite the reports of the resistance, the future of *B. sphaericus* in the control of mosquito larvae is promising. Indeed, resistance in the field seems to decline very quickly when treatments are suspended. The best way to prevent resistance has been seemed to produce bacterial strains that simultaneaously express different toxins binding to different receptors. On the other hand, there still exist some resistance or there are some other factors effecting the toxin in the application medium.

Development of mosquitoe larval resistance against the toxin of commercial microbial larvicide *B. sphaericus* has been first studied in cultured mosquito cells and later in *Culex quinquefasciatus* by many authors. It was recently shown that pattern of resistance evolution in mosquitoes depended on continuous selection pressure, and the stronger the selection pressure, the more quickly resistance developed. However repeated exposure of an insect population to *B. thuringiensis* induces the emergence of resistant pests. The number of toxin genes, together with the qualitative and quantitative differences among them and the properties of the resulting toxin, affect the quality of the developed strains [58].

Depending on the formulation and environmental conditions, *B. sphaericus* is generally effective from 1-4 weeks after application. The persistence of toxicity against *Culex quinquefasciatus* larvae, during a considerable period of time, the ability to recyle under certain environmental conditions have been studied.

On the other hand, there still exist some resistance or there are some other factors effecting the toxin in the application medium. The effects of aquatic bacterial proteases have been determined only in one study yet. In that study, about 500 bacterial isolates have been obtained from different aquatic mosquito habitats in Turkiye, and then the B. s. larvacidal toxin proteins have been exposed to these extracellular proteases of these bacterial isolates to establish the preliminary screening of the possible effect of these proteases on the B.s. binary toxin proteins. In this study, it was found that, there are also the effects of the environmental microorganisms specifically bacteria due to their extracellular proteases released in the area naturally, so that the B.s. toxin effectiveness in controlling the mosquitoes, especially *Culex* spp., can be affected by this factor [59,60]. The decrease or variability in the efficiency of the B.s. toxin may be not

only due to the genetic capability of the insect organism to develope resistance against the microbial protein, but also due to the environmental microbiological character.

9. Conclusion

The increasing of biological control due to both ecological beneficiancies including the human health as part of world ecology, has been renewed.

The demand for bio-pesticides is rising steadily in all parts of the world. When used in Integrated Pest Management systems, biopesticides' efficacy can be equal to or better than conventional products, especially for crops like fruits, vegetables, nuts and flowers. By combining performance and safety, biopesticides perform efficaciously while providing the flexibility of minimum application restrictions, superior residue and resistance management potential, and human and environmental safety benefits.

In the study in which the sensitivity of the Bs crystal binary toxin to extracellular proteaese of the aquatic microorganisms were detected, it was shown that there are also the effects of the environmental microorganisms due to their extracellular proteases released in the toxin application area naturally. So that, the Bs toxin effectiveness in the controlling the mosquitoes, especially *Culex* spp.can be affected by this factor. The resistance against the microbial entamopathogens by the target organisms, has been usually thought to be genetic capability of the insects, specifically mosquito species. In this study it is found that, the decrease or variability in the efficency of the Bs toxin may be not only due to the genetic capability of the insect organism to develope resistance against the microbial protein, but also may as well be due to the environmental microbiological character [61].

In the future other studies can be done as well to detect the type and charactheristics of the effective proteases released into the Bs toxin application areas, so that the preventive manıpulatıons of the Bs toxin protein or some other genetic derivations of the toxin protein may well b eestablished, so that the specific proteaeses would not be able to effct the toxin, while the toxin still can kill the mosquito spp. It is very likely that in future their role will be more significant in agriculture and forestry. Biopesticides clearly have a potential role to play in development of future integrated pest management strategies Hopefully, more rational approach will be gradually adopted towards biopesticides in the near future and short-term profits from chemical pesticides will not determine the fate of biopesticides [62].

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