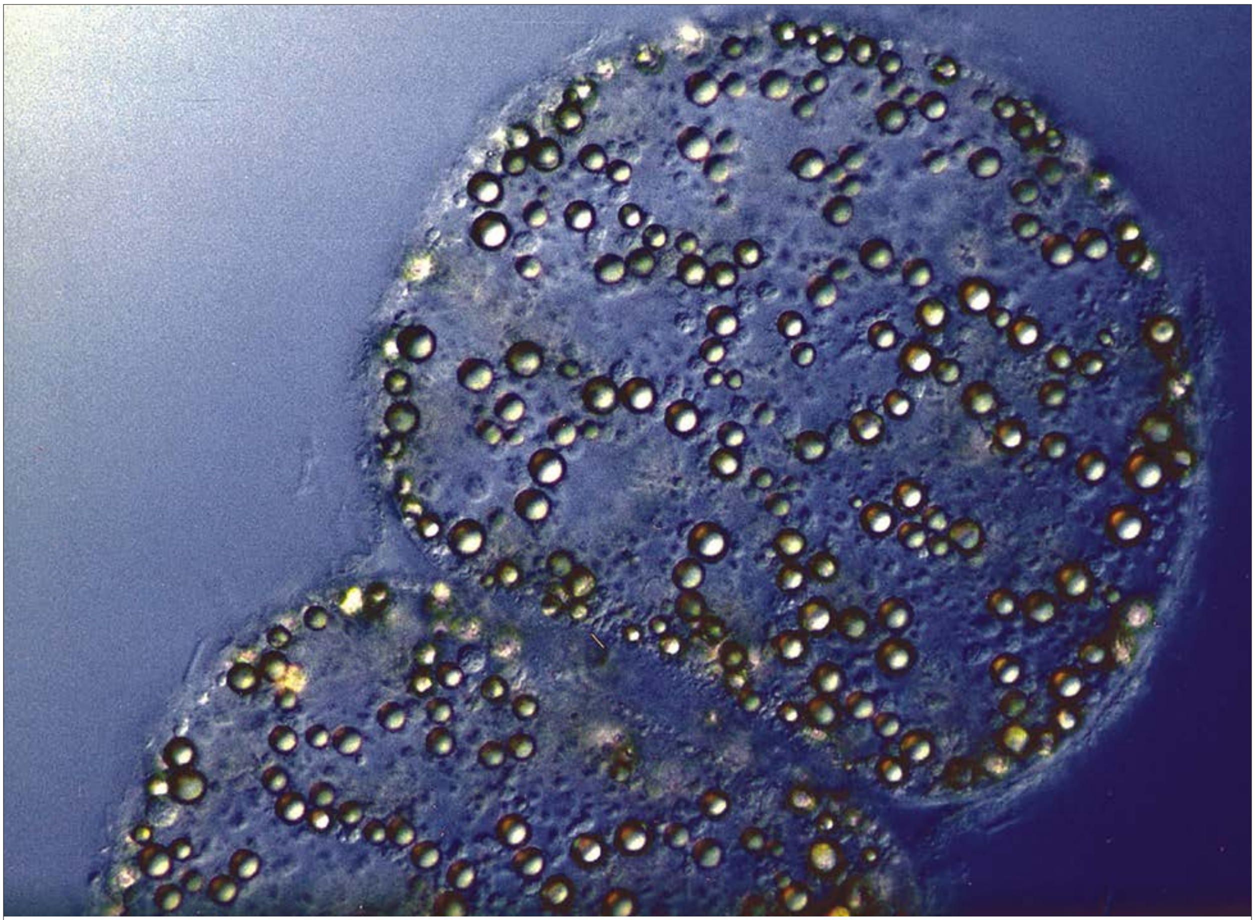

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Bacillus thuringiensis: An Environment Friendly Microbial Control Agent

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ABSTRACT

Bacillus thuringiensis (Bt) is an aerobic, gram positive, spore forming soil bacterium that produces different kinds of crystal inclusions during sporulation. These crystal 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 insects. 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. 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 describes about biology of Bt toxin, recent progress in the development of Bt technology, evolution of resistant insect populations against Bt and management strategy.

Key words: *Bacillus*, endotoxin, biopesticide, insecticidal properties, transgenic crop

INTRODUCTION

Presently the main problem in world is increased population and decreased arable land available for agriculture. In the past 40 years, the world population has increased by 90% while food production has increased by only 25%. In the worldwide, farmers will have to produce 39% more food grains because additional 1.5 billion people have to be fed by 2020 (Anonymous, 2000). The application of chemical insecticides in insect control programs, although very effective in most cases, have caused many environmental problems related to the appearance of insect resistance, emergence of secondary pests, environmental pollution and residues on the agriculture products and animals (Nester *et al.*, 2002). In many instances, biological insect management system give adequate levels of pest control and pose fewer hazards. Using microbial insecticides is such a system. With comparison to other commonly used insecticides, these biological agents are safe for both the pesticide user and consumers of treated crops. Among these bio-control agents, *Bacillus thuringiensis* based products have a major share i.e., up to 90% of the bio-insecticides used world over (Fernandez-Ruvalcaba *et al.*, 2010).

Bacillus thuringiensis (Bt) is an endospore former gram positive bacilli which is motile and facultative anaerobe. Bt can be isolated on simple media such as nutrient agar or Luria Bertani agar from a variety of environmental sources including soil, water, plant surfaces, grain dust, dead insects and insect feces (Federici, 1999). The spore germinates provided nutrients and environmental conditions are suitable and produces a vegetative cell that grows and reproduces by binary fission. The cells continue to multiply until one or more nutrients become insufficient for continued vegetative growth. Under this status, the bacterium sporulates producing a spore and parasporal body which is composed of one or more insecticidal proteins in the form of crystalline inclusions. This is the most distinguishing feature of *B. thuringiensis* from closely related *Bacillus* spp. (e.g. *B. cereus*, *B. anthracis*) (Bulla *et al.*, 1985). It is thought that *B. thuringiensis* is an insecticide producing variant of *B. cereus* (Gordon *et al.*, 1973). The plasmids coding for the insecticidal toxin of *B. thuringiensis* have been transferred into *B. cereus* to make it a crystal producing variant of *B. thuringiensis* (Gonzales *et al.*, 1982).

In the year of 1901 Japanese biologist Shigetane Ishiwatari discovered *Bacillus thuringiensis* (Ishiwata, 1901) as the cause of the sudden ("sotto") death disease of silkworms, larvae of the silkworm moth, *Bombyx mori*. After ten years of Ishiwata's discovery, the German bacteriologist Ernst Berliner (Berliner, 1915) unaware of Ishiwata's paper, described a similar bacterium as the cause of disease in larvae of the flour moth, *Ephestia kuehniella*. The species name "*thuringiensis*" is derived from Thuringia, the German state where the diseased flour moth larvae were found. Agronomists soon became interested in the entomopathogenic properties of Bt, because small amounts of preparations of this bacterium were sufficient to kill insect larvae rapidly. The first Bt based formulation was developed in France in 1938, under the name "Sporéine" but the first well-documented industrial procedure for producing a Bt based product dates from 1959, with the manufacture of "Bactospéine" under the first French patent for a biopesticide formulation. The commercial success was achieved in 1966 by the isolation of the economically important *B. thuringiensis* subsp. *kurstaki* HD-1 by Dulmage. Besides insecticidal activity, certain *B. thuringiensis* strains with activity against protozoa, mites and nematodes have also been reported (Marvier *et al.*, 2007).

According to Rowe and Margaritis (1987) and WHO (1999), nine different toxins are found in Bt strains namely α-exotoxin (phospholipase C), β-exotoxin (thermostable exotoxin), γ-exotoxin (toxic to sawflies), δ-endotoxin (protein parasporal crystal), louse factor exotoxin (active only against lice), mouse factor exotoxin (toxic to mice and *Lepidoptera*), water-soluble toxin, Vip3A (Bt vegetative insecticidal protein) and enterotoxin (produced by vegetative cells). Among these several toxins produced by Bt strains, δ-endotoxin have been more efficiently utilized for protection of a variety of crops from various insect pests.

CLASSIFICATION OF Bt

Previously Bt strains were classified into sub species based on morphological and biochemical characters (De Barjac and Franchon, 1990). Now a days, scientists use different methods for classification such as phage-typing (Ackermann *et al.*, 1995), esterase pattern of vegetative cells (Norris, 1971), crystal serology (Lynch and Baumann, 1985), plasmid pattern (Lereclus *et al.*, 1984), oligonucleotide probing (Prefontaine *et al.*, 1987), proteins profiling, use of monoclonal antibodies, H Flagellar serotyping (De Barjac and Franchon, 1990) and

PCR amplification based on sequences of known crystal protein genes (Porcar and Juarez-Perez, 2003). Between any one of these characterization methods, there is only a poor correlation. The insecticidal activity of a particular strain differs for several reasons such as the presence of multiple genes per strain, variable gene families in a given serotype, variation in expression levels of the genes present and solubility in the insect midgut (Porcar and Juarez-Perez, 2003; Du *et al*., 1994).

The Cry genes of *B. thuringiensis* have been reclassified several times as more individual genes and toxic proteins were identified. Hofte and Whiteley (1989) introduced the first systematic classification and nomenclature for toxin proteins on the basis of insecticidal activity. The major class is designated by Roman letter (I-IV). Subclasses of Cry proteins were later recognized based on their activity within the same group of insect itself e.g., CryIC with high activity against Lepidoptera compared with CryIE with limited activity (Visser *et al*., 1990). The Cry genes are characterized by different Arabic numerals which share <45% amino acid sequence homology and designated as primary ranks such as Cry1, Cry2, Cry3, etc. The Cry genes of the same primary ranks showing <78% amino acid homology are differentiated by secondary ranks using upper case letters such as Cry2A and Cry2B etc. The genes are assigned tertiary ranks whose products are different in amino acid sequence but are more than 95% amino acid sequence homology, designated by lowercase letters such as Cry2Aa, Cry2Ab, Cry2Ac etc. (Hofte and Whiteley, 1989). Now-a-day, Cry genes are classified into 70 classes and sub classes based on amino acid sequence similarity (Table 1).

Although, several methods were tried for classification, serotyping using H flagellar antigen, flagellin, remains the most widely used, simplest and practical method to classify Bt strains (De Barjac and Franchon, 1990). Today, the widely diverse *B. thuringiensis* strains are classified into 70 H serotypes (Table 2) (Reyes-Ramirez and Ibarra, 2005).

Table 1: Recent classification of Cry genes identified so far from *B. thuringiensis* (http://www/lifesci.sussex.ac.uk/home/neil_crickmore/Bt/toxins2.html)

S. No.	Class	Sub class	S. No.	Class	Sub class	S. No.	Class	Sub class	S. No.	Class	Sub class
1	Cry1	241	19	Cry19	2	37	Cry37	1	55	Cry55	2
2	Cry2	68	20	Cry20	3	38	Cry38	1	56	Cry56	2
3	Cry3	19	21	Cry21	3	39	Cry39	1	57	Cry57	1
4	Cry4	14	22	Cry22	6	40	Cry40	4	58	Cry58	1
5	Cry5	12	23	Cry23	1	41	Cry41	4	59	Cry59	1
6	Cry6	4	24	Cry24	3	42	Cry42	1	60	Cry60	6
7	Cry7	21	25	Cry25	1	43	Cry43	4	61	Cry61	3
8	Cry8	38	26	Cry26	1	44	Cry44	1	62	Cry62	1
9	Cry9	30	27	Cry27	1	45	Cry45	1	63	Cry63	1
10	Cry10	4	28	Cry28	2	46	Cry46	3	64	Cry64	1
11	Cry11	7	29	Cry29	1	47	Cry47	1	65	Cry65	2
12	Cry12	1	30	Cry30	11	48	Cry48	5	66	Cry66	2
13	Cry13	1	31	Cry31	10	49	Cry49	5	67	Cry67	2
14	Cry14	1	32	Cry32	7	50	Cry50	3	68	Cry68	1
15	Cry15	1	33	Cry33	1	51	Cry51	2	69	Cry69	2
16	Cry16	1	34	Cry34	11	52	Cry52	2	70	Cry70	3
17	Cry17	1	35	Cry35	11	53	Cry53	2		cyt1	12
18	Cry18	3	36	Cry36	1	54	Cry54	3		cyt2	24

Table 2: Classification of *Bacillus thuringiensis* strain according to the H serotype (Reyes-Ramirez and Ibarra, 2005)

Strain	Strain No.	Strain	Strain No.
Thuringiensis	1	Medellin	30
Finitimus	2	Toguchini	31
Alesti	3a, 3c	Cameron	32
Kurstaki	3a, 3b, 3c	Leesis	33
Sumiyoshiensis	3a, 3d	Konkukian	34
Fukuokaensis	3a, 3d, 3e	Seoulensis	35
Sotto	4a, 4b	Malaysiensis	36
Kenyae	4a, 4c	Andaluciensis	37
Galleriae	5a, 5b	Oswaldocruzi	38
Canadensis	5a, 5c	Brasiliensis	39
Entomocidus	6	Huazhongensis	40
Aizawai	7	Sooncheon	41
Morrisoni	8a, 8b	Jinghongiensis	42
Ostriniae	8a, 8c	Guiyangiensis	43
Nigeriensis	8b, 8d	Higo	44
Tolworthi	9	Roskildiensis	45
Darmastadiensis	10a, 10b	Chanapaisis	46
Londrina	10a, 10c	Wratislaviensis	47
Loumanoffi	11a. 11b	Balearica	48
Kyushuensis	11a, 11c	Muju	49
Thompsoni	12	Navarrensis	50
Pakistani	13	Xiaguangiensis	51
Israelensis	14	Kim	52
Dakota	15	Asturiensis	53
Indiana	16	Poloniensis	54
Tohokuensis	17	Palmanyolensis	55
Kumamotoensis	18a, 18b	Rongseni	56
Yosso	18a, 18c	Pirenaica	57
Tochigiensis	19	Argentinensis	58
Yunnanensis	20a, 20b	Iberica	59
Pondicheriensis	20a, 20c	Pingluonsis	60
Colmeri	21	Sylvestriensis	61
Shandogiensis	22	Zhaodongensis	62
Japonensis	23	Bolivia	63
Neoleonensis	24a, 24b	Azorensis	64
Novosibirsk	24a, 24c	Pulsiensis	65
Coreanensis	25	Graciosensis	66
Silo	26	Yazensis	67
Mexicanensis	27	Thailandensis	68
Monterrey	28a, 28b	Pahangi	69
Jegathesan	28a, 28c	Sinensis	70
Amagiensis	29		

TOXIN STRUCTURE

B. thuringiensis produce one or more crystalline inclusion (parasporal crystal) bodies during the sporulation and these can be seen under the phase contrast microscope. Several terminologies are used for the crystalline inclusions, for example, insecticidal crystal proteins (ICPs), Cry toxins or δ-endotoxin (Guerchicoff *et al.*, 2001).

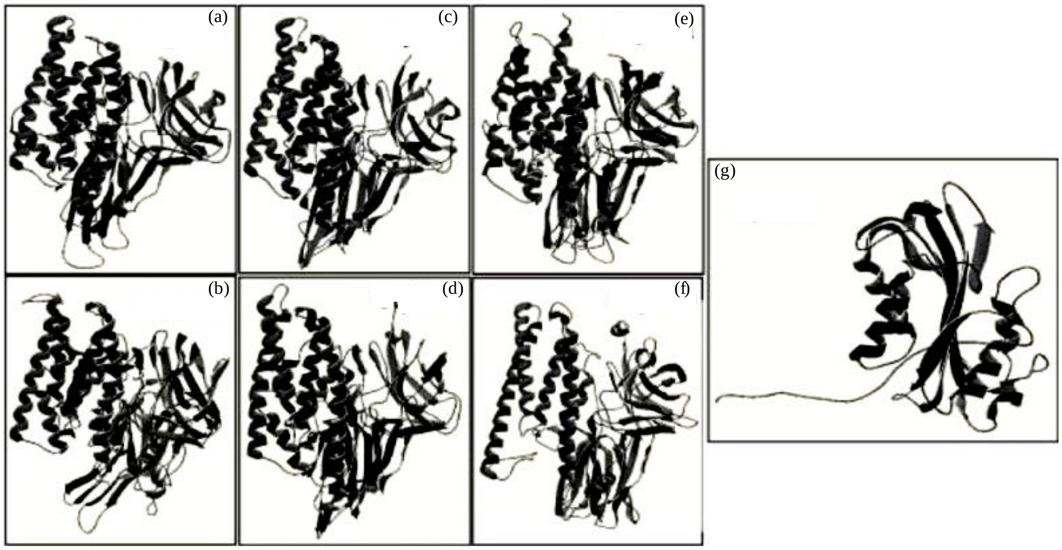

Fig. 1(a-g): A comparison of the 3D protein structures of (a) Cry1Aa, (b) Cry2Bb, (c) Cry3Aa, (d) Cry3Bb, (e) Cry4Aa, (f) Cry4Bb and (g) Cyt2A

Despite their sequence diversity, all Cry proteins share a similar overall tertiary structure, as exemplified by the six structures solved thus far by X-ray crystallography (Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa and Cry4Ba) (Fig. 1). The C terminal portion is rich in cysteine residues and involved in crystal formation. But it is not part of the mature toxin, as it is cleaved off in the insect gut. The N terminal portion is the toxin itself which is highly conserved and it comprises three domains (Kumar and Sharma, 1994). Domain I consists of seven hydrophobic alpha helices around a central core helix and involved in membrane insertion and pore formation. Domain II consists of three groups of anti-parallel beta-strands which are folded into loops and is responsible for the receptor recognition (De Maagd *et al.*, 2001). Domain III has a beta-sandwich structure with two twisted antiparallel **β**-sheets and may be responsible for the stability of d-endotoxins in the insect gut after activation. Several studies have suggested that domain III may also be involved in the specific binding of the toxin to its receptors (De Maagd *et al.*, 2003). A current model suggests that domains II and III initially bind to primary receptors (cadherins) that cleave the toxin inside domain I and induce oligomerization, that in turn promotes binding to high-affinity secondary receptors tethered to the membrane via C-terminal glycosylphosphatidylinositol anchors (Soberon *et al.*, 2009). The requirement for oligomerization has recently been confirmed through the isolation of dominant negative mutations of Cry1Ab (Rodriguez-Almazan *et al.*, 2009). An alternative model (Zhang *et al.*, 2006) suggests that initial binding triggers a Mg^{2+} dependent signalling cascade that causes G protein dependent cAMP accumulation and also the activation of protein kinase A. Phylogenetic analysis has established that the diversity of the Cry family evolved by the freelance evolution of the three domains and by swapping of domain III among toxins.

In contrast, cyt2A protein has a single domain in which two outer layers of α-helix wrap around a mixed **β**-sheet (Schnepf *et al.*, 1998). Unlike Cry proteins, cyt proteins do not recognize specific receptors on the epithelium and exhibit hemolytic activity (Crickmore *et al.*, 1998). When the sequences of crystal proteins are aligned, five conserved sequence blocks are common in the majority of them. Conserved block 1 is in the central helix of domain I, block 2 is at the domain I-II interface, block 3 is at the boundary between domains II and III, block 4 is in the central β-strand of domain III and block 5 is at the end of domain III (De Maagd *et al.*, 2001).

ACTION MECHANISM OF DELTA ENDOTOXINS

Cry protein: The crystal proteins of *B. thuringiensis* show host specificity. For this reason, each type of Cry protein can be toxic to one or more specific insect species. The specificity of these insecticidal crystal proteins (ICPs) derives from their mode of action (Gill *et al.*, 1992). In order for the δ-endotoxin to elicit its insecticidal affect, it has to be ingested. Following ingestion, δ-endotoxin is activated by the gut proteases and takes place under the alkaline conditions (pH >9.5) of the insect midgut which for most Lepidopterans (Hofman *et al.*, 1988a). The degree in which protein solubilization occur may be attributed to differences in the degree of toxicity among Cry proteins. The major proteases implicated in protein solubilization within the Lepidopteran insect midgut are either trypsin-like (Lecadet and Dedonder, 1964) or chymotrypsin like (Johnston *et al.*, 1995). The active form of δ-endotoxin then binds to specific receptors on the cell lining (Hofman *et al.*, 1988b) and interaction with the receptors results in the incorporation of the activated toxin components into the membrane (Carroll and Ellar, 1993). The hydrophobic surfaces then face the exterior of the bundle and this initiates the penetration of the cell membrane and the formation of pores (Siqueira *et al.*, 2006). This produces selective ion channels by oligomerization of toxin monomers. The loss of osmotic pressure regulation induces paralysis of the gut, halting the insects feeding activity and inevitably leading to the death of the insect (Aronson *et al.*, 1999). After this the spores may germinate in the gut of the insect leading to propagation (Yang and Wang, 1998). The δ-endotoxins are highly insoluble in normal digestive conditions. Moreover, mammals, including human, do not have δ-endotoxin receptors in their guts and all the δ-endotoxins tested so far (except Cry9) are unstable when heated and degrade within 20 sec by the mammalian digestive enzymes (EPA, 1998). Therefore, the toxins are not harmful to mammals, birds, fishes or to most of the beneficial insects.

Vip protein: The mode of action of Vip protein is similar to that of the δ-endotoxins. Vip3A is processed in the lepidopteran gut and that proteolysis of Vip3A alone was not considered sufficient for insect specificity (Shotkoshi *et al.*, 2003). Further processing was necessary for its bioactivity. The biotinylated Vip3A-G toxin predominantly binds to a low abundance 80 kDa and 100 kDa bands and generates a pattern that clearly differs from that of Cry1Ab. The Vip3A-G pores have the capability to destroy the transmembrane potential which suggests that pore formation may play a vital role in bioactivity. Competition binding assays demonstrated that Vip3A did not inhibit the binding of either Cry1Ac or Cry2Ab2 and vice versa (Lee *et al.*, 2006).

Cyt proteins: Cyt proteins are typically only occur in mosquitocidal strains of Bt and received little study in comparison to Cry proteins. As far as is known, Cyt proteins do not require a protein receptor and react directly with the non-glycosylated lipid portion of the microvillar membrane. Within the membrane, they appear to aggregate and then lipid faults form that cause an osmotic imbalance and cell lysis occur (Butko, 2003).

FACTORS RESPONSIBLE FOR GROWTH AND TOXIN PRODUCTION

The growth of Bt may be described by three phases: Vegetative growth, transition phase and sporulation phase. As carbon source, Bt generally uses sugars usually maltose, ribose, glucose, fructose, molasses, dextrin, starch, wheat flour and inulin (El-Bendary, 2006). With respect to the nitrogen sources appropriate for Bt production, the overwhelming majority of literatures revealed the inability of most of Bt varieties to utilize inorganic nitrogen source as a sole nitrogen source within the growth medium. Instead, a minimum of one amino acid notably glutamate, aspartate, valine, leucine, serine or threonine needs to be added in order to allow growth of the organism in a minimal medium. But cysteine and cystine amino acids showed clear inhibitory effect on growth, sporulation and toxin formation by Bt (El-Bendary, 2006). Icgen *et al.* (2002) found that peptone was the most effective organic nitrogen source supporting sporulation and toxin production by Bt. Potassium ion is crucial for toxin production by Bt (El-Bendary and Magda, 1999). An effective synthesis of Cry4Ba by Bti HD500 required high concentrations of inorganic phosphate (50 to 100 mM K_2HPO_4) (Ozkan *et al.*, 2003). Metal ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} are essential for production of the highest sporulation and δ-endotoxin formation by Bt. Mn^{2+} was the most critical element for the biosynthesis of Cry4Ba and Cry11Aa by Bti HD500 at 10^{-6} M concentration (Ozkan *et al.*, 2003). The growth of Bt occurs in the pH range of 5.5-8.5. The usual initial pH is 6.8-7.2; decreasing to 5.8 as acetate is released, then rising to 7.5-8 as it is consumed (El-Bendary, 2006). The normal temperature for growth and toxin production of Bt is 30°C. Aeration is very important for Bt fermentation. At low aeration levels, the organism was unable to survive or sporulate. The most submerged fermentation of Bt is done using aeration rates approximately one air volume/volume of medium/minute (Rowe, 1990).

DEVELOPMENT OF Bt BIOPESTICIDES

In the late 1930s, the insecticidal Bt products were first commercialized in France. For over 80 years, Bt has been one of the most consistent and vital biopesticides for use on crops as an insecticidal spray, which contains a combination of spores and the insecticidal crystals. 182 Bt based products were registered by the U.S. Environmental Protection Agency (EPA) by 1995. But in 1999 Bt formulations constituted less than two percent of the entire sales of all insecticides and represented around 80% of all biopesticides sold. Bt sprays constituted $100 million in annual sales, however with the appearance of transgenic plants engineered with the insecticidal cry gene, sales have decreased to $40 million. The OECD (Organization for Economic Cooperation and Development) predicts that the biopesticide might grow to 20% of the world's pesticide market by 2020. Bt sprays are used sporadically and usually over tiny areas. The sprayable Bt formulations have penetrated cotton, fruit and vegetable, aquatic and other insecticide markets (Roh *et al.*, 2007).

In the 1960s, strain improvement led to the replacement of many of the early products with new Bt strains that were more potent than their predecessors and finally resulted in the production of commercial products. Bt strain NT0423 (named "Tobaggi" and produced from Dongbu Hannong Chemicals) is one of the registered Bt biopesticides in Korea. This strain had at least five known crystal protein genes, Cry1Aa, Cry1Ab, Cry1C, Cry1D and Cry2A and one new gene, Cry1Af1 (GenBank Accession No. U82003). It has dual toxicity against lepidopteran larvae-like *Plutella xylostella, Spodoptera exigua* and *Hyphantria cunea* and dipteran larvae-like *Culex pipiens* and *Musca domestica*. The developmental procedure for the Bt NT0423 product might be a typical example of Bt sequential research for Bt biopesticides. The Bt biopesticide market is mostly dominated by Abbott Laboratories (Chicago, IL) (since the acquisition in 1995 of Novo-Nordisk's biopesticide business) and Novartis (created through the merger in 1996 of Ciba and Sandoz), together accounting for >70% of global production (Roh *et al.*, 2007; Sanahuja *et al.*, 2011).

EXPRESSION OF Bt GENES IN CROPS

The most significant advancement in the use of Bt as a bio-control agent in recent years has been the expression of toxin in agricultural crops. Since 1996, crops expressing a Bt toxin have been grown commercially and insect resistant varieties are now the second-most widely employed genetically modified crops after herbicide-tolerant ones. The obvious advantage to these crops is that there is no need to control certain pests with foliar sprays as the toxin is constitutively expressed in the plant that they target. The use of Bt crops has increased dramatically in recent years with 22.4 million Ha grown worldwide in 2004 (James, 2004). Many crops, such as cotton, maize, potato, tomato, rice, eggplant and crucifer vegetables, have been genetically transformed with genes derived from soil bacteria Bt coding for proteins that are highly active against many important pests.

Expression of Bt gene in tobacco and tomato provided the first example of genetically engineered plants for insect resistance (Barton *et al.*, 1987; Vaeck *et al.*, 1987). Subsequently, several Bt genes have been expressed in transgenic plants, including tobacco, potato, tomato, cotton, brinjal, rice, etc. Delannay *et al.* (1989) for the first time reported field performance of transgenic tomato plants expressing δ-endotoxin gene. Though Cry1Ab protein was effective against tobacco hornworm, higher level of gene expression was needed for the control of tomato fruit worm (*Helicoverpa* sp). Results of field trials of Bt transgenic tobacco (Hoffmann *et al.*, 1992) and cotton (Wilson *et al.*, 1992) expressing truncated δ-endotoxin genes were encouraging. Since then, there have been several reports of field-tested δ-endotoxin expressing transgenic crops. Bt potatoes were first commercially produced in the USA in 1995 but issues with consumer acceptance led to their retraction from the market after 5 years (Grafius and Douches, 2008). In contrast, Bt cotton was first commercially produced in 1996 in Australia, Mexico and the USA and its adoption and use has spread to six additional countries. So far, Bt maize and Bt cotton are the only insect-resistant GE crops for commercial planting (James, 2010a). Bt genes (Cry1Ac, Cry1Ab, Cry2Ab, and Cry1F) of cotton were commercialized in 11 countries in 2009 and the total planting area reached 15 million hectares, which comprised approximately half of all the cotton grown in the world in 2009 (Naranjo, 2011). The total area where Bt cotton was planted globally in 2010 was 19.6 million hectares, up by 4.6 million hectares in 2009 (James, 2010b). China and India are the two major cotton-growing countries. In India, Bt cotton was first planted in 2002 by 54,000 farmers on 50,000 ha (James, 2003). In 2009, 8.4 million hectares of hybrid Bt cotton were planted in India, which made India displace China as the largest Bt cotton-growing country. To delay the development of pest resistance, Bt cotton varieties containing two different Cry proteins (Bollgard II and Wide Strike) have been gradually adopted by some countries in recent years. Since 2004, growers in Australia have been exclusively using Bollgard II (expressing Cry1Ac and Cry2Ab) instead of Bollgard I (expressing Cry1Ac) (Naranjo *et al.*, 2008). Bt cotton varieties with two Cry proteins is becoming common and most Bt cotton is also genetically engineered to be herbicide tolerant (Naranjo, 2011). Maize transformed with Bt genes (Cry1Ab, Cry1F, Cry3Bb1, Vip3A, Cry34Ab1/Cry35Ab, Cry2Ab) was commercially planted in 16 countries worldwide in 2009 and the total planting area reached 35.3 million hectares. In 2010, Bt maize was grown on 39 million hectares, an increase of 3.0 million hectares or a year-over-year growth rate of 10% (James, 2010b). After the USA, Brazil is the second largest Bt maize-growing country, with 5 million hectares in 2009 (Marshall, 2010). In South Africa, both Bt cotton (more than 85% of the country's crop) and Bt maize are grown. This is the only country to date where white Bt maize, 0.9 M ha representing 67% of the country's total production, was planted for food (James, 2007).

There are a number of Bt crops under development and evaluation including broccoli, cabbage, apples, soybeans, pulses, peanuts, cauliflower and eggplant (Shelton *et al*., 2008). Bt potatoes are likely to be re-introduced, probably in Asia, Africa and Eastern Europe, in the future (Grafius and Douches, 2008) and Bt rice is being evaluated in several countries (Cohen *et al*., 2008). By the end of 2009), China also approved Bt rice and GM phytase maize for commercial cultivation (James, 2009). The perceived disadvantages of Bt transgenic crops may be: (1) potential impact on non target species, (2) increase in toxin levels in the soil may affect soil microflora, (3) exchange of genetic material between the transgenic crop and related plant species leading to the development of so called "Super weed" and (4) evolution of new and more virulent biotypes of the pests (Kumar, 2002).

DEVELOPMENT AND MANAGEMENT OF RESISTANCE

There is increasing concern by scientists, agriculturalists and environmentalists regarding the potential of insect developing resistance to Bt owing to the widespread use as an insecticide and in transgenic plants. The major concern to the continued success of Bt crops is the evolution of resistance by pests (Tabashnik *et al*., 2003). The first well documented instance of resistance occurring against Btk in the field was presented by Tabashnik *et al.* (1990). But earlier reports had recommended the possibility of *Btk* resistance occurs in the Philippines, in populations of *Plutella xylostella* (Kirsch and Schmutterer, 1988). Even statistically significant resistance to Bti has been reported in mosquitoes *Culex quinquefasciatus* Say and *Aedes aegypti* Linnaeus (Georghiou *et al*., 1983; Goldman *et al*., 1986). The strain of Colorado potato beetle *Leptinotarsa decemlineata* say has been selected for resistance to Bt subsp. *tenebrionis* which is active against Coleoptera (Miller *et al*., 1990).

In Laboratory conditions, resistance to Bt toxins has been found in many insects, like Indian meal moth, *Plodia interpunctella* (McGaughey, 1985), tobacco budworm, *Heliothis virescens* (Gould *et al*., 1992), diamondback moth, *Plutella xylostella* (Tabashnik, 1994), beet armyworm, *Spodoptera exigua* (Moar *et al*., 1995), European corn borer, *Ostrinia nubilalis* (Huang *et al*., 1999a; Siqueira *et al*., 2004), pink bollworm, *Pectinophora gossypiella* (Tabashnik *et al*., 2004) and *Helicoverpa armigera* (Xu *et al*., 2005). In field conditions, resistance to formulated Bt microbial insecticide sprays have developed by three lepidopteran insect pests which include *P. interpunctella* (McGaughey, 1985), *P. xylostella* (Tabashnik, 1994) and *Trichoplusia ni* (Janmaat and Myers, 2003). More importantly, field resistance to commercial Bt crops that lead to field control failures or reduced efficacy are documented in three cases. The primary case is the resistance of fall armyworm, *Spodoptera frugiperda,* to Cry1F corn in Puerto Rico (USEPA, 2007) the second case is the resistance of an African stem borer, *Busseola fusca*, to Cry1Ab corn (e.g., YieldGard® corn) in South Africa (van Rensburg, 2007) and the third case is the resistance of *P. gossypiella* to Cry1Ac cotton in India (Dhurua and Gujar, 2011).

During the past 20 years, Bt resistance mechanism has been discussed as one of the hottest topic in the agricultural science. Several mechanisms of insect resistance to Bt toxins have been proposed (Gill *et al*., 1992). As numerous steps are involved in the full process of Bt's mode of action, the resistance mechanisms are complicated. There are many ways of stopping the process and resisting the toxin are possible. With considerations of about the importance of the reversible binding step for toxicity, it is recommended that the most frequent and best characterized resistance mechanism involves a disturbed interaction between the ICPs and their specific receptors in the insect midgut. The reduced affinities or loss of specific ICP binding due to a change in the receptor molecule have been observed in a Dipel resistant *P. xylostella* strain (Ferre *et al*., 1991), *Plodia interpunctella* larvae (Lepidoptera: Pyralidae) (Van Rie *et al*., 1990) and other insects (Gonzalez-Cabrera *et al*., 2003). However, lower numbers of midgut receptor molecules have also been reported in resistant *P. xylostella* and *H. virescens* strains (Jurat-Fuentes and Adang, 2004). Moreover, altered receptor glycosylation patterns due to the loss of a β-1,3-galactosyltransferase in *C. elegans* have been related with acquired Cry5B resistance after selection (Griffiths *et al*., 2001). Resistance to *B. thuringiensis* ICPs that cannot be explained by altered receptor binding has also been described (Jurat-Fuentes *et al*., 2003). Changes within the gut physiology and protease arsenal have been demonstrated to hinder normal protoxin solubilization and activation (Huang *et al*., 1999b). Additionally, activated ICPs can be detoxified by proteolytic degradation (Bah *et al*., 2004). Based mostly on the observation that endogeneous phospholipase can release the APN receptor molecules from the midgut membrane by cleavage of the GPI anchor, it has been hypothesized that an analogous process *in vivo* could also lead to *B. thuringiensis* resistance (Lu and Adang, 1996). On the other hand, premature termination of translation could result in loss of the GPI-anchor and secretion of the ICP receptor molecules in the midgut lumen. Finally, increased regeneration of damaged midgut epithelium in resistant strains has been proposed as a possible resistance mechanism explaining the similar initial histophathological injury observed in a susceptible and Cry1Ac resistant *H. virescens* strain (Forcada *et al*., 1999). Some factors such as pH, enzymes, peritrophic membrane, enzyme detoxification and antimicrobial characteristics of gastric juice of insect gut make insects resistant to the toxin (Davidson, 1992).

The resistance management programs typically use three basic approaches to delay resistance. First approach based on reducing exposure to toxins and/or enable for mating between resistant insects and an oversized population of susceptible insects, to retain susceptible traits continuing in the gene pool. The strategies include tissue-specific and time-specific expression of toxins, mosaics, rotations, mixtures refuges and occasional release of susceptible males into the field. Second approach focuses on combining pest control techniques and relies on the belief that an insect is more likely to develop resistance to just one type of control than more than one type of control simultaneously (Sharma *et al*., 2004). Strategies in this category include high doses, gene stacking, combinations of low toxin dose and natural enemies and combinations of toxins with completely different modes of action. The final approach is very different in nature from those listed above. This strategy employs “trap plants” to lure pests away from productive crops. But among all approaches, the “high dose-refuge strategy” has gained the most attention and is currently imposed by the US Environmental Protection Agency when growing *B. thuringiensis* crops (USEPA, 2001). This strategy involves to plant “high dose” Bt plants that can kill = 95% heterozygotes for Bt resistance. The strategy also requires Bt crop growers to plant a specified proportion of their crop to a non-Bt variety of the crop to serve as a refuge for hosting susceptible insects. Bt susceptible insects ought to emerge from refuge areas and mate with the rare potentially resistant homozygous individuals that might emerge from the Bt crop so that most offspring will be heterozygous and thus be killed by the “high dose” Bt plants. Therefore, resistance allele frequencies in field populations should remain low for long period of time. The 15 years of success of transgenic Bt crops in managing four major corn and cotton pests, *O. nubilalis, Diatraea grandiosella*, *H. virescens* and *P. gossypiella* in North America without any signs of resistance is believed to be resulted from a successful implementation of the “high-dose/refuge” IRM strategy.

CONCLUSION

When organic pesticides are increasingly proving ineffective against many of the insect-pests, the Bt transgenic technology should be utilized to its full potential by its strategic deployment. The durability of insect resistance in transgenic crops can only be ensured if IPM practices are followed (Ranjekar *et al.*, 2003). The use of these safer and biodegradable biological control agents also has a number of ecological advantages. These benefits include increased crop yields, reduced costs for pesticides, less fungal contamination and reduced labor. The magnitude of each benefit varies by geography and crop. Further investigations are needed to identify and create novel Bt strains and toxins with more potent and specific efficiency and to generate transgenic plant that suppress agricultural pests and reduce opportunities for the evolution of resistant strains.

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