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«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»

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Review article

From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review

Vincent SANCHIS*

INRA, UMR 1319 Micalis, Domaine de Vilvert, 78352 Jouy-en-Josas, France

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Abstract – *Bacillus thuringiensis*, known as Bt, is a spore-forming bacterium that occurs naturally in soil and that produces highly specific insecticidal proteins called Cry proteins. These proteins are stomach poisons that specifically affect insects. Today, Bt preparations are considered as the most effective, specific and environmentally-friendly bioinsecticides; they have been used as biological pesticides in agriculture, forestry and in human health for the elimination of vectors of diseases for more than 60 years and their implementation far exceeds other microbial agents such as fungi, protozoa or viruses. This review on the use of this entomopathogenic bacterium in crop protection is not intended to be a compilation of the results of all the investigations made in this field. Instead, it is an attempt to provide an overview of the major trends and developments of Bt for the control of agricultural insect pests and to describe the main approaches that have been used to improve this natural bioinsecticide. *Bt*-based insecticides are considered safe for mammals and birds, and are safer for non-target insects than conventional insecticides; they have become the most widely used microbial insecticides. However, Bt products have several limitations, such as a narrow activity spectrum, instability in rain and sunlight, and inefficiency against pest feeding on internal tissues of the plants. The first step towards improving Bt has involved the isolation of new strains with higher and broader insecticidal activity against targeted insect pests and the cloning of *cry* genes encoding new insecticidal crystal proteins. A second strategy was to increase the persistence of its toxins in the field by encapsulation in recombinant asporogenic Bt strains or other heterologous recombinant microbial hosts; this protected the toxins against UV degradation and had the advantage that the transgenic microorganisms released into the environment were non-viable. Bt has also become a key source of genes for transgenic expression to provide pest resistance in plants and in so-called genetically modified plants. The engineering of plants to express Bt *cry* genes has been especially helpful against pests that attack parts of the plant that are usually not well protected by conventional insecticide application. The potential effects on human health and the environment of the large-scale use of these Bt crops are also in the scope of this review.

Bacillus thuringiensis **/ insecticidal bacteria / biological control / microbial pesticides / Cry proteins / transgenic Bt crops / insect pest control / resistance management / history**

Contents

* Corresponding author: vincent.sanchis@jouy.inra.fr

1. INTRODUCTION

It is now known that living organisms such as bacteria, viruses and fungi, or natural products derived from these organisms, can be used successfully as biological control agents, as principal or supplementary pest control measures, and represent a valuable arsenal for the control of pests. The study of the diseases of insects, now known to be caused by microorganisms, dates back to 2500 BC when humans, in China, began to raise useful insects and recorded diseases of the silkworm, *Bombyx mori* (L.) (Steinhaus, 1949, 1956). In the western world diseases of the honey bee, *Apis mellifera* (L.), were also recorded by the Greeks in 350 BC (Tanada and Kaya, 1993). Through the following centuries, insect pathology developed slowly until the seventeenth century when, in 1676, Antonie Philips van Leeuwenhoek, in 1676, using a single-lens microscope of his own design, observed microorganisms for the first time. In doing so he initiated the scientific field of microbiology. About one hundred years later, in 1805, Pierre-Hubert Nysten, a Belgian-French physiologist, studied silkworm diseases in southern France, and provided an early description of polyhedrosis, a virus affecting insects (Nysten, 1808). The name "bacterium", derived from the Greek βακτηριον meaning "small stick", was introduced in 1828, by Christian Gottfried Ehrenberg, a German zoologist and microscopist, who concentrated his studies on microscopic organisms and described thousands of new microscopic species, which until then had not been systematically studied. Soon after, Agostino Bassi, an Italian entomologist, was working on a silkworm disease, known in Italy as *mal del segno* or as *calcinaccio*, because of the white efflorescence and calcined appearance that developed after the worms had died. The disease caused heavy losses to the silkworm industry in Italy and in France, where it was called *la muscardine* (or muscardin). The research to find the cause of this disease took him 20 years. He finally published the results of his investigations in two papers entitled "On the Disease of the Sign, Calcification or Muscardine, a Disease that Afflicts Silkworms, Part I: theory and Part II: Practice" in 1835 and 1836, respectively (Bassi, 1835, 1836). Bassi's work clearly demonstrated, for the first time, that the silkworm disease was a living entity, was contagious and could be transmitted naturally by direct contact or infected food. The first part of his study clearly contained a theory proposing that some contagions of plants and animals had their source in the "germs" of plant or animal parasites, and that possibly certain diseases of man were caused by vegetable microorganisms. The causative agent was later shown to be a fungus, which multiplied in and on the body of the insect and was named *Beauveria bassiana* to honor his discovery. However, at that time, the theory that diseases were due to the growth of germs in the body still remained unacceptable to many biological and medical scientists. In 1837, Jean-Victor Audouin, a French entomologist employed by the French government to inquire into the diseases of the silkworm, and the insects that destroyed the vines, confirmed Bassi's discovery (Audoin, 1837). Audouin also reported that the transmission of the disease was not restricted to the silkworm. Soon after, Friedrich Gustav Jakob Henle, a German physician and pathologist, following the ideas of Agostino Bassi, developed the concepts of contagium vivum and contagium animatum, respectively; thus co-founding the theory of microorganisms as the cause of infective diseases. His essay "On Miasma and Contagia" (Henle, 1840) was an early and important argument for the germ theory of disease. These pioneering works certainly influenced the thinking of many other scientists. In 1850, Davaine along with Rayer isolated, from the blood of a diseased and dying sheep, a microbe which is now known as *Bacillus anthracis*, the causative agent of anthrax. In 1863, Davaine demonstrated that the bacterium could be directly transmitted from one animal to another. Later on, in 1865, the German microbiologist Robert Koch studied anthrax more closely. He invented methods to purify the bacterium from blood samples and grow pure cultures. The idea of using diseases to combat pest insects rapidly followed the recognition that pathogens of insects and animals were contagious, passing from diseased to healthy individuals, under both laboratory and natural conditions. Pasteur, who began studies on silkworm diseases in 1865, noted the presence of microbes in diseased silkworms and proposed that mortality was caused by infection (Pasteur, 1870). Pasteur was also the first to suggest that pebrine (a microsporidian disease of silkworms) could be applied for control of grape phylloxera, *Phylloxera vitifoliae* (Fitch), but he did not put in practice the idea. The works of Pasteur and of Koch on anthrax, from 1865 and onward, are considered as the first convincing discoveries on infectious diseases and the starting point of bacterial pathogenesis. While Pasteur was not the first to propose the germ theory (Bassi and Henle had suggested it earlier), he developed it and conducted experiments that clearly indicated its correctness and managed to convince the scientific community it was true; as such, he is often regarded as the father of germ theory together with Koch. He also remains in the history of sciences and medicine for the first applications of microbiological discoveries such as the vaccine against rabies. Koch, who was a student of Henle, became famous for isolating *Bacillus anthracis* in 1877 and for establishing the fundamental rules or guidelines to establish a standard for evidence of causation in an infectious disease. Today, Koch postulates for defining disease-causing microbes are still the gold standard to define microbial virulence. Ferdinand Julius Cohn, a German botanist and microbiologist, who studied bacteria, from 1870 onward, was the first to classify bacteria into groups based on shape, and the first to show, in 1876, that, under stressful environmental conditions, *Bacillus* species produce endospores that can stay dormant for extended periods. At about the same time, Elie Metchnikoff, a Russian associate of Pasteur, proposed the use of another fungus, *Metarhizium anisopliae*, to control the wheat cockchafer, *Anisoplia austriaca* (Hbst). He mass-produced spores of the fungus and brought about the first field trials in Russia (Metchnikoff, 1879). The success attained by Metchnikoff inspired one of his colleagues, Krassilstchik, to establish a small production plant in 1884 for the purpose of producing spores of the fungus on a large scale (Krassilstschik, 1888). He applied the fungus in the field for the control of the sugar-beet weevil, *Bothynoderes* (*Cleonus*) *punctiventris* (Germ.) (Tanada and Kaya, 1993). A few years latter, in 1911,

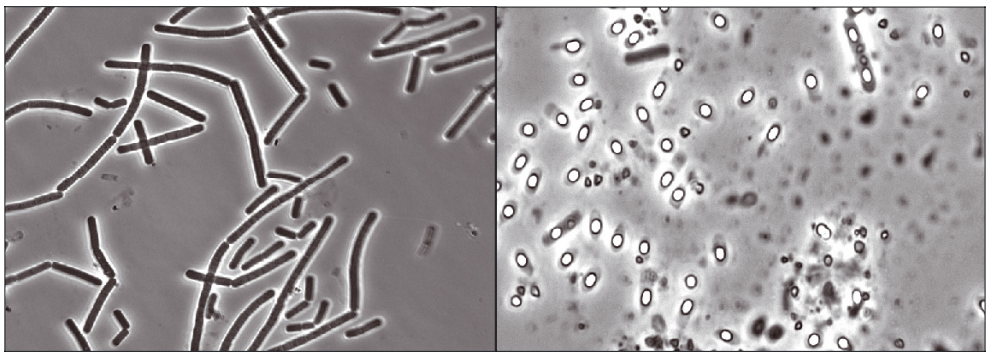

Figure 1. *Bacillus thuringiensis*: Phase photomicrographs of vegetative cells (left) and spores (right), 1000X. (Photographs, INRA).

Felix d'Herelle, a French-Canadian microbiologist, is credited with the first attempts to use bacteria for insect control. He observed epizootics in Mexican populations of the American grasshopper, *Schistocerca Americana* (Drury) (d'Herelle, 1911), and isolated a bacterium that he designated *Coccobacillus acridiorum* (d'Herelle, 1914). He then applied it in several Latin American countries with reportedly positive results in some places and not in others (d'Herelle, 1912). These first attempts and approaches were followed by many other scientists and have opened up the path for the successful use of microorganisms as biological control agents. Between 1920 and 1940 many insect pathogenic fungi, viruses and bacteria were tested under field conditions but only one, *Bacillus thuringiensis* (Bt), emerged as a good candidate for both efficient plant protection and large-scale production by industry. Other promising viruses or bacteria, such as *Bacillus popilliae*, had the disadvantage that they could only be grown within the host and were not easy to produce. Today, Bt sprays, which utilize naturally occurring Bt strains, account for approximately 75% of the global bioinsesticide market and comprise about four percent of the global insecticide spray market, estimated to be U.S. $8 billion per annum in 2005. This review article will start with the discovery of Bt in Japan at the beginning of the twentieth century. Then, I will describe the early work with Bt, in Europe, that showed that the bacterium had promise as a microbial control agent and the efforts directed at strain and *cry* gene isolation to improve Bt biopesticides. Between 1980 and the early 1990s the successful use of Bt sprays rapidly prompted the idea that plants could be protected by adding the genes that produce the Bt toxins to the plants. I will therefore provide a brief overview of the genetic modifications of the crystal protein genes that were necessary to obtain Bt crops expressing their protein at potentially commercially viable levels. The potential problems that could arise from the large-scale use of crops genetically engineered to produce Bt toxins will also be briefly discussed. Finally, future prospects for research to extend the potential and preserve the future of this biocontrol agent will be presented.

2. THE DISCOVERY OF Bt

Bacillus thuringiensis (Bt) is an aerobic Gram-positive endospore-forming bacterium, that was first isolated in 1901, from infected silkworms, *Bombyx mori* (L.), by the Japanese bacteriologist Shigetane Ishiwata (Ishiwata, 1901). From a taxonomic point of view Bt belongs to the family Bacillaceae (Fig. 1).

Ishiwata described his work with the bacterium and the pathology that followed the bacterium's ingestion by silkworm larvae in 1905 and he called the organism "Sottokin-Bacillus" which translates into "sudden death-Bacillus" but this name did not last (Ishiwata, 1905). Aoki and Chigasaki began working with the bacterium in 1911 and in a series of papers described the bacterium and the disease it caused when ingested by silkworm larvae (Aoki and Chigasaki, 1915; Aoki and Chigasaki, 1916). They noted that the bacterium was incapable of causing the disease unless old, sporulated cultures were fed to the insects. At approximately the same time, the bacterium was rediscovered by the German biologist Ernst Berliner, who isolated it, in 1911, from infected chrysalids of the Mediterranean flour moth, *Ephestia kuehniella* (Zell.), collected from a mill in the province of Thuringe (Berliner, 1911). He described the bacterium in 1915 and named it *Bacillus thuringiensis* Berliner, after the province where the infected moth was found (Berliner, 1915). Berliner reported the existence of a crystal within Bt, but the insecticidal activity of this crystal was not discovered until much later (Fig. 2).

This culture (Berliner strain of variety *thuringiensis*) was lost, but in 1927, Mattes reisolated the same organism from the same host as Berliner did and described the disease it caused in the flour moth (Mattes, 1927). Mattes' isolate was widely distributed and this strain (now known as "the German strain") is the representative strain for the type species of these crystal-forming bacteria. Bt was originally considered a risk for the silkworm rearing industry but agronomists and insect pathologists soon became interested in the entomopathogenic properties of Bt, because small amounts of preparations of this bacterium were sufficient to kill insect larvae, and Bt rapidly became the heart of microbial insect control. The first attempts to use Bt for insect control took place in the late 1920s against the gypsy moth, *Lymantria dispar* (L.), in the northeastern United States (Metalnikov and Chorine, 1929) and against the European corn borer, *Ostrinia nubilalis* (Hbn.), in Hungary (Husz, 1930) and in other eastern European countries (Metalnikov et al., 1930). In 1929, the Botanical Institute of the University of Zagreb, headed by

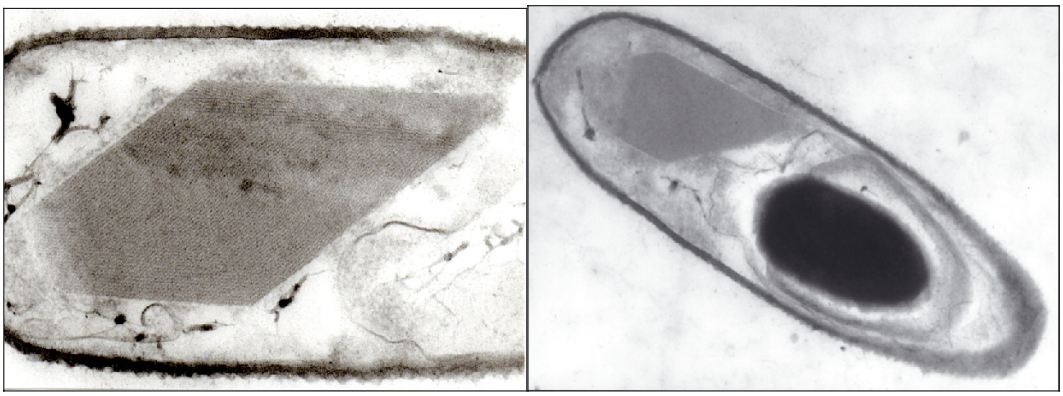

Figure 2. Transmission electron micrographs of longitudinal sections of *Bacillus thuringiensis* towards the end of sporulation, showing the spore (black ovoid structure) and the toxins with insecticidal properties (right), that accumulate to form a large bipyramidal crystal inclusion (left). (Photographs, Institut Pasteur and INRA).

Vale Vouk, and the entomologist Boidar Hergula, also used different bacteria, isolated from diseased larvae, in field trials near Zagreb. They demonstrated that plants treated with a bacterial spray withstood the attack of the European corn borer much better than the non-treated plants. By far the best results were achieved by *Bacillus thuringiensis* (Vouk, 1930; Hergula, 1930). Soon after, in 1938, the first commercial *B. thuringiensis* product, "Sporéine", was produced in France by Laboratoire Libec (Entwistle et al., 1993). Further progress on the development of this product was halted with the discovery in 1939, by Swiss chemist Paul Muller, of the insecticidal properties of DDT and by the outbreak of World War II. With World War II came a surge in chemical advances and German scientists synthesized the organophosphorous insecticide parathion, which further reduced interest in the use of insect pathogens. After World War II, steady growth in pesticide use began with the introduction of broad-spectrum insecticides, such as DDT, with activity against both insect pests of agriculture and human health. Throughout the 1950s and 1960s, these types of chemicals became major pest-control agents that helped farmers solve many formerly unsolvable problems.

3. FIRST GENERATION OF Bt PREPARATIONS

In 1950, Jacobs tested the French product Sporéine against *A. kuehniella* (Zell.) in a series of excellent experiments (Jacobs, 1950) and again attracted attention to the potential of Bt. In 1951, Toumanoff and Vago reported the isolation of a bacterium closely resembling Bt and *Bacillus sotto* from silkworms dying of "flacherie" (Toumanoff and Vago, 1951). Since the bacterium also resembled *Bacillus cereus*, these investigators named it *Bacillus cereus* var. *alesti* (after the region of Ales, in France). In a second paper they compared *B. sotto*, *B. thuringiensis* and *B. cereus* var. *alesti* as to their cultural characteristics and came to the conclusion that these three bacteria were all varieties of *B. cereus* (Toumanoff, 1952). The resurgence of interest in Bt has actually been attributed to Edward Steinhaus, who began tests with bacteria, including *B. thuringiensis* (Steinhaus, 1951). Although field trials yielded inconsistent results, his conclusions were optimistic and he reported that infected alfalfa caterpillars *Colias eurytheme* (Bdv.) ceased feeding within a few hours after ingestion of spores. In 1956, Steinhaus convinced the president of the Pacific Yeast Products company of Wasco in California to produce Bt, and the product Thuricide® was soon available. In the United States, Thuricide became available for testing in 1958. Bt-based products were also made on a large scale in the late 1950s in several other countries including the USSR, Germany and France. In France, 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. Bactospéine was produced and distributed by Biochem Products S.A., a French subsidiary of the Belgian Solvay group (Fig. 3). Originally it was the fruit of research carried out in France by agronomists of the French National Institute for Agricultural Research (INRA), microbiologists from the Pasteur Institute in Paris and researchers at the Roger Bellon pharmaceutical laboratory.

These products were joined by Entobacterin-3 and Dendrobacilline produced in the USSR. In 1964, "Biospor" became the first Bt preparation to be licensed as a pesticide in Germany. However, despite these advances, Bt remained only a minor component of pest management, because highly efficient synthetic pesticides were always readily available. In 1962, Edouard Kurstak isolated another variety of Bt, from a larva of *A. kuehniella* (Zell.), from a flourmill in Bures-sur-Yvette in France, and named it *kurstaki.* In 1970, Dulmage isolated another more potent strain of this variety from diseased mass-reared pink bollworm, *Pectinophora gossypiella* (Saund.), larvae and designated it the HD-1 strain (Dulmage, 1970). Kurstak's and Dulmage's isolates were serotyped by De Barjac and Lemille and designated the variety *kurstaki* (de Barjac and Lemille, 1970). HD-1, which proved 2 to 200 times more toxic against key agricultural pests, and became the basis for products that were competitive with chemical insecticides in performance and cost. This strain became commercially available through Abbott Laboratories as Dipel® in

Figure 3. Various formulations of Bactospéine®, a Bt-based biological insecticide commercialized by Biochem Products. (Photograph, Biochem Products SA).

the early 1970s. Since this strain was active and more potent than previous strains against numerous lepidopteran species, it was used (and still is) for production of many formulations of Bt. One of the key advantages of Bt as a bioinsecticide, as compared with insect viruses, was its ability to be mass-produced in fermenters. Expansion in the use of Bt, in the 1970s and 1980s, was largely due to the development of methods of large-scale fermentation, and increased efficiency in production and quality control, so that formulations with high activity could be developed (Van Frankenhuyzen, 2000). Bt products commercially available for use were generally applied as liquid base formulations, water-dispersible granules, or wettable powders. Formulations were based on ease of application, end-user preferences, and improved spray characteristics, using the application equipment already available. Nevertheless, Bt products were still essentially restricted to niche markets, particularly for forestry applications, that were favored by environmental pressures and in which the application of conventional chemical agents was largely restricted. During the 1970s and 1980s, its use against the spruce budworm, *Choristoneura fumiferana* (Clem.), and the gypsy moth, *Lymantria dispar* (L.), in North American forests accounted for 60% or more of world sales (van Frankenhuyzen, 2000). Commercial interest in Bt grew rapidly when scientists and environmentalists became aware that the chemicals were harming the environment. This represented a significant opportunity for Bt-based products as a replacement for conventional insecticides. After forestry, organic farming became a major market for *B*t because, due to stringent regulation, synthetic chemicals were also phased out. With these food crops, Bt had the advantage that it could be used up to the day of harvest and had no entry restrictions. A further important impetus for the adoption of biopesticides containing Bt, apart from its demonstrated insecticidal efficacy, was when many popular synthetic insecticides became ineffective due to insect resistance. In 1979, the United Nations Environmental Program declared pesticide resistance one of the world's most serious environmental problems. Subsequently, governments and private industries started to fund research to search for new strains of Bt with increased activities against a wide range of potential hosts.

4. DISCOVERY OF NEW Bt STRAINS AND EXPANSION OF THE INSECTICIDAL SPECTRA OF Bt

Until the 1970s it was generally accepted that lepidopteran insects (moths and butterflies) were the only targets of Bt. In 1976, Goldberg and Margalit reported that a new Bt subspecies found in the Negev Desert, called israelensis (or *bti*), killed mosquito and black fly larvae; both are from the order Diptera. This was the first documented case of a Bt strain killing an insect other than a caterpillar (Goldberg and Margalit, 1977). The Dipteran-active Bt subsp. *israelensis* was used extensively for vector control, particularly of black flies and mosquitoes, providing both medical and environmental benefits. An example of an outstanding success in cooperation between industry and a governmental organization to achieve those benefits was the Onchocerciasis Control Program of the World Health Organization (WHO) during the 1980s and 1990s in western Africa, wherein Bt subsp. *israelensis* applications comprised up to 50% of all insecticide applications. In 1983, a second new subspecies of Bt, subsp. *morrissoni* var. *tenebrionis*, was isolated (Krieg et al., 1983). This isolate, discovered in Germany, had excellent activity against the larvae of certain coleopteran species, and enhanced commercial development of this organism has a bioinsecticide. More recently, Bt crystal proteins were screened for activity against

the free-living larval stages of nematode pests that infect animals and plants and some of them were identified with significant activity in inhibiting larval development, thus demonstrating that the phylum Nematoda was also a target of Bt crystal proteins (Wei et al., 2003). To date, several thousand natural strains have been isolated from various geographical areas and from different sources, including grain dust, soil, insects and plants (Martin and Travers, 1989; Smith and Couche, 1991). These isolates have been classified into about 60 serotypes based on biochemical properties and flagellar antigens or H-antigens (de Barjac and Frachon, 1990), producing well over 300 crystal proteins that are active against several orders of insects (>500 species) and some other invertebrates and recently, leukemic cells (Ohba et al., 2009). However, this classification does not reflect the pathotype of the bacteria, which is essentially defined by the *cry* genes that make up the crystalline inclusion (Lereclus et al., 1993). It is also important to note that most Bt strains produce more than one type of crystal protein that can act in combination.

5. MODE OF ACTION OF THE Bt PESTICIDAL CRY TOXINS

In 1956, Tom Angus demonstrated that the crystalline protein inclusions formed in the course of sporulation, and that subsequently became known as Cry proteins, were responsible for the insecticidal action of Bt (Angus, 1954). Recognition that there were strains of Bt with differential activity spectra within and between insect orders also led to a rapid expansion of research into the genetic basis of these differences (Dulmage, 1981). This, in turn, led to development of an understanding of the modes of action of Bt through the interaction of the Cry toxins with the gut of the target organisms. The mechanism of toxicity has been reviewed in detail by Knowles (Knowles, 1994). Briefly, the Cry proteins are stomach poisons that cause lysis of the epithelial cells. Following ingestion, the crystals first dissolve in the intestinal tract. Crystal solubilization is dependent on gut pH. For the majority of the crystal inclusions produced by Bt, gut conditions must be strongly alkaline in order to achieve dissolution (Hofmann et al., 1988). Gut pH is, therefore, one of the factors that help to determine potential toxicity and, hence, the host range of Bt, when hosts ingest intact toxin crystals. After solubilization, the inactive protoxin molecules must be activated through proteolytic cleavage by the insect midgut proteases (Lecadet and Dedonder, 1967) to generate mature toxins consisting of the amino-terminal part of the protoxin (Choma et al., 1991). In the case of Cry1A protoxins, the cleavage is performed by the chymotrypsin-like or trypsin-like proteases (Johnston et al., 1995). The initial molecular weight of the protein decreases from about 130–140 kDa to 55–65 kDa. Following their solubilization and activation, the Cry toxins could pass through the peritrophic membrane, a chitinous sheath, thought to provide protection against physical abrasion of the midgut epithelium (Richards and Richards, 1977). Activated Cry proteins then bind to specific receptors on the apical brush border of the midgut microvilliae in susceptible insects (Hofmann et al., 1988; Van Rie et al., 1990) (Fig. 4).

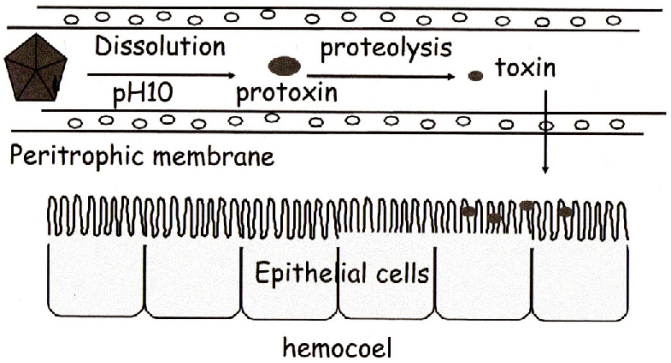

Figure 4. Schematic representation of the mode of action of the insecticidal crystal proteins of *Bacillus thuringiensis* in the intestine of lepidopteran insects.

Following binding, the toxin rapidly and irreversibly inserts into the cell membrane. Insertion results in the formation of pores which leads to epithelial cell lysis as a result of selective cation permeability (English and Slatin, 1990). The specific receptors of some of the Cry proteins have been identified and shown to be membrane aminopeptidases (Knight et al., 1994) or proteins of the cadherin family (Vadlamudi et al., 1995). Currently, 38 different aminopeptidases have been reported for 12 different lepidopterans (Pigott and Ellar, 2007). At the physiological level, the lysis of the epithelial cells leads to paralysis of the insect's digestive system and it quickly stops eating. Alone, this effect of the Cry toxins can cause the death of the susceptible insects one to three days after the ingestion of the crystals. However, generally the insects also ingest Bt spores along with the crystals. The result is that, when Bt sprays are used, a septicemia, due to the germination of the spores and the development of the bacteria, is almost always associated with the toxemia, and this may optimize the toxic effect of the Cry toxins.

6. CLONING AND CLASSIFICATION OF Bt INSECTICIDAL *CRY* GENES

By the early 1980s, Gonzalez revealed that the genes coding for crystal proteins were localized on transmissible plasmids, using a plasmid curing technique (Gonzales et al., 1981). Soon after, Schnepf and Whiteley first cloned and characterized the genes coding for crystal proteins that had toxicity to larvae of the tobacco hornworm, *Manduca sexta* (Lin.), from plasmid DNA of Bt subsp. *kurstaki* HD-1 (Schnepf and Whiteley, 1981; Schnepf et al., 1985). The cloning of the first *cry* gene in 1981 was quickly followed by the cloning and DNA sequence determination of many other *cry* genes. In 1989, Hofte and Whiteley proposed a systematic nomenclature and classified the crystal proteins into major groups according to their insecticidal and molecular relationship (Cry I, Cry II, Cry III, Cry IV and Cry V, etc.) (Hofte and Whiteley, 1989). The availability of cloned *cry* genes also permitted a better analysis and

definition of the spectrum of insecticidal activity of each of the gene products. As new strains were discovered containing new *cry* genes, a need for a new nomenclature arose and the Cry proteins have been further classified on the basis of amino acid identity into about 300 Cry sub-groups, to date (Crickmore et al., 1998). According to this new nomenclature Roman numerals were exchanged with the Arabic numerals and the Cry proteins were named exclusively on the basis of their evolutionary divergence. Additionally, underneath the capital letters small letters were placed, indicating minor amino acid differences like the capital letters denote for the major differences. Thus the Cry genes are now recognized using four hierarchical levels based on sequence homology of the various proteins in each rank. The Cry proteins with less than 45% sequence identity are separated in the primary rank, while further separation at the secondary and tertiary ranks is based on less than 78% and 95% identity, respectively. As a result of this classification, today there are as many as 60 major Cry protein classes (the Cry60Ba was added on February 2010). A current list of *cry* genes can be found on the Internet at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/holo2.html. Each individual Cry protein generally has a restricted spectrum of activity, limited to the larval stages of a small number of species. However, it has not been possible to establish a correlation between the degree of identity of Cry proteins and their spectrum of activity. The Cry1Aa and Cry1Ac proteins are 84% identical, but only Cry1Aa is toxic to *Bombyx mori* (L.). Conversely, Cry3Aa and Cry7Aa, which are only 33% identical, are both active against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Other Cry toxins are not active against insects at all, but are active against other invertebrates. For example, the Cry5 and Cry6 protein classes are active against nematodes.

7. IMPROVEMENT OF Bt STRAINS AND PRODUCTS

The ability to identify and clone Bt *cry* genes and the characterization of the specific activities of individual Cry proteins, as well as the availability of recombinant DNA technology, led to the development of new strategies for improving the exploitation of Bt or increasing its entomopathogenic potential. The first step towards improving Bt strains naturally involved the isolation of new strains with new or higher insecticidal activity against targeted insect pests. Amongst the critical milestones was the discovery that the genes coding for the toxin crystals were located on transmissible plasmids enabling exchange of genetic information between Bt strains (Gonzales et al., 1982). This opened up the way to manipulation of genes, including transfer between Bt strains. Conjugation was used to develop strains with optimized activity against a given insect pest or strains with a broadened toxicity spectrum (Sanchis, 2000). For example, conjugation has been used by Ecogen, a small biotechnology firm, to construct Bt strains carrying new combinations of *cry* genes; Ecogen constructed strain EG2348, the active ingredient of the Condor® bioinsecticide product, that contained a combination of *cry* genes encoding crystal proteins particularly active against specific lepidopteran pests of soybean crops. Another strain, EG2424, the active ingredient of Foil®, contained two plasmids, one carrying a *cry* gene whose product is active against the European corn borer, *Ostrinia nubilalis* (Hbn.), and a second encoding a crystal protein with activity against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say.), a coleopteran pest of potatoes (Baum, 1998). A second strategy for improving the exploitation of Bt or increasing its entomopathogenic potential involved diversifying or improving the way the pesticidal Cry toxins were delivered, by using recombinant DNA technology. An important delivery system was the encapsulation of the *cry* genes in a bacterium other than Bt: the non-pathogenic *Pseudomonas fluorescens*. This approach has been used by another biotechnology firm, Mycogen, to produce two commercial products: MVP® for controlling lepidopterans and M-TRAK® for controlling coleopterans. The bacteria are killed by means of a physical chemical process after fermentation and the toxins remained enclosed in the cell wall of the dead microorganisms as crystalline inclusions. This process significantly increased the efficacy of the Cry proteins, increasing their persistence in the environment by protecting them against degradation and inactivation by UV irradiation (Gaertner et al., 1993). Using a different approach, Crop Genetics International (CGI) transferred a *cry* gene into *Clavibacter xyli* var. *cynodontis*, an endophytic bacterium, that colonizes the vascular system of various plants including maize. The gene introduced into this bacterium encoded a protein toxic to the larvae of the European corn borer (Lampel et al., 1994).

8. DEVELOPMENT OF TRANSGENIC Bt CROPS

Until the 1980s, Bt was rather ineffective against certain pests, because the sprays could not reach the cryptic insects inside the stalk or near the root (Fig. 5).

At that time, several scientists successively demonstrated that plants can be genetically engineered and this rapidly led to the development of transgenic Bt plants (Fig. 6). The Belgian company Plant Genetic Systems was the first company to develop a genetically engineered plant with insect tolerance by expressing a *cry* gene from Bt in tobacco, but this first attempt did not lead to sufficient expression of the insecticidal proteins (Vaeck et al., 1987).

The same year, other groups of scientists spliced Bt genes into tomato and cotton plants. The transformation technique used was based on transfer of the Ti plasmid from *Agrobacterium tumefaciens*. This method was used to introduce foreign DNA into plants other than tobacco but its application was restricted to a limited number of plant species, most of them dicotyledons. The development of new methods of transformation, such as electroporation, or particle bombardment, made it possible to transfer DNA into most plants, including monocotyledons such as maize (Koziel et al., 1993). Initial attempts at transformation employed a full Cry1A toxin gene, using the cauliflower mosaic virus (CaMV) 35S promoter, but resulted in very low levels of expression of the Cry proteins, and consequently poor protection against insect

Figure 5. Example of damage of European corn borer, *Ostrinia nubilalis* (Hbn.), on corn. The young caterpillars burrow into the apical bud and then penetrate into the interior of the stem, creating a network of holes in the soft tissue. Due to the way it attacks plants, this insect is particularly difficult to control with standard insecticide treatments. (Photograph, INRA).

damage (Barton et al., 1987). Protection was then improved when only the toxic N-terminal part of the protein was expressed in plants (Fischhoff et al., 1987), but again the protein level was still relatively low. It was believed that high AT-content of *cry* genes led to aberrations in mRNA processing and translation (Ely, 1993). To try to enhance the expression of *cry* genes in plants, DNA sequence modifications were performed, for removal of mRNA processing and polyadenylation signal sites, without changing the encoded amino acid sequence of the toxin. Depending on the range of modifications, this site-directed mutagenesis enabled increase in the toxin protein production in tobacco from a non-detectable level to 0.02% of soluble protein (Perlak et al., 1991) or the minimal insecticidal level. However, these levels of expression were still not sufficient for reliable control of pests in transgenic crops produced in the field. Soon after, the total DNA resynthesis of *cry* genes became technically feasible and enabled obtaining adequate levels of expression to stabilize the control of target pests in field conditions. Achieved levels of expression usually varied between 0.2–1% of soluble protein. Much more effective plants, that used synthetic genes designed to be more compatible with plant expression, were introduced a few years later (Koziel et al., 1993) and even higher levels of expression were achieved by transforming tobacco chloroplasts (McBride et al., 1995). In 1995, the US Environmental Protection Agency (EPA) approved the first registration of Bt potato, corn and cotton products with Bt toxin genes expressing their protein at potentially commercially viable levels (Fig. 7). Bt potatoes, expressing the Cry3A coleopteran active toxin, were first commercialized in 1995–1996 by Monsanto in the United States under the NewLeaf® trademark for control of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). In 1995, two transgenic corn hybrids containing a Cry1Ab gene active against *Ostrinia nubilalis* (Hbn.) were also registered by Syngenta Seeds and Mycogen seeds, under the names of KnockOut® and NatureGard®, respectively; both hybrids contained Event 176, but sales of these varieties were rapidly discontinued.

In 1996, two new Bt corn varieties, both expressing the Cry1Ab1 toxin, were commercially released by Northrup King and Monsanto, under the names of Agrisure™ CB (Event Bt11) and YieldGard® (Event MON 810), respectively. The first transgenic cotton varieties, expressing a modified *cry1Ac* gene derived from the Bt subsp. *kurstaki* strain HD73, were also released by Monsanto in 1995, under the trademarks Bollgard® and Ingard® (Events 531, 757 and 1076). In 1998, the US EPA also approved an insect-resistant tomato line (Event 5345) expressing the Bt insecticidal Cry1Ac protein. In 2001, a new corn variety, expressing the Cry1F toxin, was developed jointly by Pioneer Hi-Bred and Dow AgroSciences, and commercialized under the name "Herculex™ I (Event TC 1507). This product specifically targeted the black cutworm, *Agrotis ipsilon* (Hufn.), the fall armyworm, *Spodoptera frugiperda* (Smith) and the European corn borer, *Ostrinia nubilalis* (Hbn.), three major lepidopteran pests of corn. In 2002, a new type of Bt cotton, Bollgard® II (Event 15985), that expressed two Bt toxins, Cry1AC and Cry2Ab, both active against various lepidopteran pests of cotton, was also released by Monsanto. A year later, in 2003, Monsanto released YieldGard® Rootworm (Event MON 863), a new variety of transgenic corn resistant to the western corn rootworm, *Diabrotica virgifera virgifera* (Lec.). It was developed using a synthetic variant of the wild-type coleopteran-active *cry3Bb1* gene from Bt subsp. *kumamotoensis*. A second, new Bt corn transgenic variety, YieldGard® Plus (Event MON810 + Event MON863), also developed by Monsanto, was also commercialized in the USA in 2003; YieldGard® Plus contained both Cry1Ab1 and Cry3Bb1 toxins, designed to control two different types of insects (lepidopteran and coleopteran). More recently, in 2005, Dow AgroSciences and Pioneer Hi-Bred constructed transgenic corn, Herculex®RW (Event DAS-59122-7), expressing binary toxins from Bt designated as Cry34Ab1/Cry35Ab1, active against various other coleopteran insect pests of the Chrysomelidae family. These toxins, also produced as crystalline inclusions in Bt, have been assigned a Cry designation, although they have little homology to the other members of the Cry toxin family. The Cry34A and Cry35A are 14-kDa and 44-kDa proteins, respectively, that function as binary toxins showing good activity on the western corn rootworm, *Diabrotica virgifera virgifera* (Lec.) (Ellis et al., 2002) (Tab. I).

In addition, various isolates of Bt have also been shown to produce other types of insecticidal toxins. One such toxin class is the Vegetative insecticidal protein (Vip) 3A that was discovered in 1996 by Estruch and collaborators (Estruch et al., 1996) and which has broad toxicity against lepidopteran species. Genetically engineered products expressing Vip3A are also being evaluated in cotton and maize plants. The most

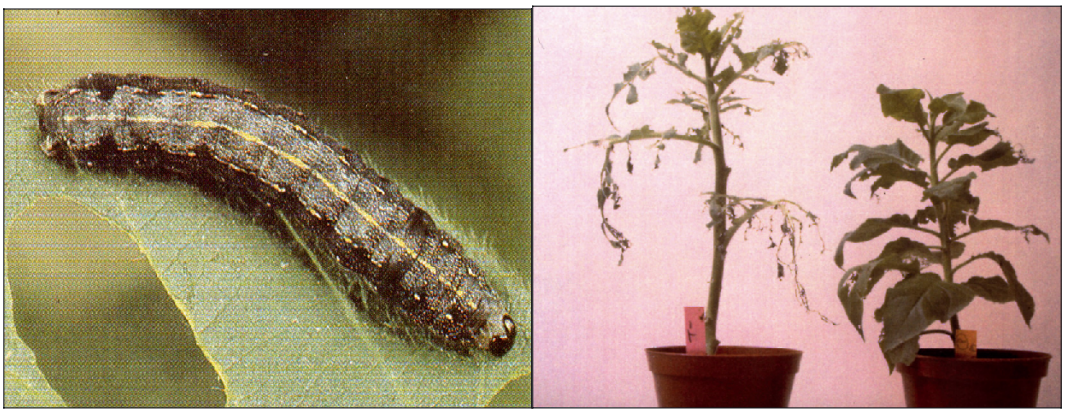

Figure 6. African cotton leafworm, *Spodoptera littoralis* (Bdv.), caterpillar feeding on cotton leaves (left) and transgenic tobacco transformed with the *cry1C* gene (right). Forty *Spodoptera littoralis* (Bdv.) second instar larvae were placed on the leaves of an untransformed control plant (left) and a tobacco plant transformed with the *cry1C* gene (right). The photograph shows the damage after 72 h. (Photograph, INRA).

Figure 7. Effects of a natural infestation of European corn borer, *Ostrinia nubilalis* (Hbn.), on two versions of the same variety of corn: a non-modified (left) and a transgenic version expressing a *cry* gene (right). A difference of 9% in yield in favor of the transgenic version was obtained in this trial. (Photograph, courtesy of Cayley, AgrEvo).

obvious advantage of Bt transgenic crops was that parts of the plant that cannot be reached by foliar sprays, such as the inside of the stalk, were also protected against internal invasion of the pest. Several other benefits can be cited: the insecticide does not need to be reapplied several times as the toxin is produced for the entire season and the effects of weathering on the insecticide are lessened. The use of these Bt insect-resistant crops, since 1996, has led to a significant reduction in pesticide use and cost savings for growers and the confidence in the benefits of Bt crops has rapidly increased throughout the world, except in Europe. Indeed, between 2008 and 2009, thirteen years after the introduction of the first transgenic Bt crops, the global hectarage planted with Bt insect-resistant crops still grew by 9.5% worldwide whilst the cultivation of Bt crops in the European Union fell 12% to 94 750 h; this is less than the approximately 115 000 h of commercial Bt cotton planted in 2009 in Burkina Faso. In 2009, worldwide, transgenic insect-resistant Bt crops covered 21.7 million hectares (or 15% of the area under genetically modified organisms (GMOs)), whereas crops with a combination of transgenic traits (Bt insect resistance and herbicide tolerance) occupied a larger area, 28.7 million hectares (or 21% of the global biotech crop area) (James, 2010). Rice varieties have also been transformed with genes encoding various Bt Cry proteins and have been shown to be resistant to one or more lepidopteran pests of rice. Field trials of Bt rice were conducted in China in 1998, in India in 2001 and in Pakistan in 2003. In China, a series of transgenic Bt rice lines transformed with modified *cry1A*, *cry1Ab* or *cry1Ac* genes were approved for biosafety assessment and large-scale trials (Huang et al., 2007); biosafety certificates were approved on 27 November

2009, clearing the way for crop registration. Other Bt crops under development worldwide are canola/rapeseed, tobacco, tomato, apples, soybeans, broccoli and peanuts.

9. ECOLOGICAL RISKS ASSOCIATED WITH THE USE OF TRANSGENIC Bt CROPS

A first concern associated with transgenic Bt crops is that Cry toxins are produced in an active form in plants, whereas in bacteria they are produced as inactive protoxin molecules that must be dissolved and activated in the insect's gut to become toxic. The use of a truncated toxin in transgenic plants removes some of the steps that contribute to host specificity (pH, proteolysis) and could result in extension of the host range to non-target organisms. Very little is known about the effects of activated Bt toxins on non-target insect species and too little is known to exclude the possibility that the toxicity and host range of the transgenic organism is not significantly altered or broadened by genetic manipulation (Hilbeck et al., 2002; Stotzky et al., 2000). Another ecological risk associated with the use of transgenic plants is that of the dissemination of the Bt toxin genes into cultivated varieties of the same crop or related wild species that are interfertile to some extent with the transgenic variety. It is unclear whether such gene flow is likely to be frequent and whether it could have adverse effects in a given agricultural or ecological context. The probability of gene transfer, particularly via the pollen, is not negligible, especially for transgenic characters that are advantageous, such as those conferring greater tolerance to insect pests (Thuriaux, 1996). One report has shown such an escape of a Bt toxin gene from oilseed rape (Steward et al., 1997). Transgenes could be biologically contained by insertion into the chloroplast genome which is, in most crops, maternally inherited. Chloroplast transformation has only been achieved in tobacco (McBride et al., 1995), but the transfer of this technique to other agronomically important plant species could result in the development of new varieties producing very large amounts of insecticidal toxins in which the absence of gene flow via pollen can be guaranteed. However, this approach is only useful for leaf-eating insects and cannot be used to control stem and fruit borers or root- and tuber-damaging subterranean insects. Another concern is that transgene spread could occur, not only through pollen, but also through seed dispersal (Van Raamsdonk and Schouten, 1997) and this has become an issue of open public debate, especially in Europe. Furthermore, transgenic plants producing Bt toxins may persist in the soil for a long period of time, and the Cry toxins produced by the transgenic plants may be broken down less quickly than those sprayed during standard treatments, thereby increasing the selection pressure and the risk of selecting resistant insects more quickly (Addison, 1993). In addition, these toxins may accumulate in the soil in an active form and this may affect soil invertebrates not normally in contact with Bt toxins. Finally, the remote possibility of horizontal gene transfer to other bacterial organisms must also be considered given the greater persistence of the DNA in the environment (Lorenz and Wackernagel, 1996). Assessment of these risks requires both rigorous and independent scientific examination.

10. INSECT RESISTANCE TO TRANSGENIC Bt CROPS

Given the wide use of Bt technology in crops and organic farming, and although researchers initially believed that insects would not develop resistance to biological insecticides, development of Cry toxin resistance among target populations was rapidly considered a serious threat to the long-term use of Bt products. By killing pest insects, Bt crops create selection pressure on pests to evolve resistance. In 1985, the first evidence of resistance developing against Bt was published. Low levels of resistance were found in the Indianmeal moth, *Plodia interpunctella* (Hbn.), in storage bins of Bt-treated grain, and laboratory studies indicated that selection over 15 generations resulted in a hundredfold decrease in activity (McGaughey, 1985). Recognition of the potential of the Bt resistance problem became greater when populations of the diamondback month, *Plutella xylostella* (L.), developed resistance outside the laboratory, following Bt-based treatments. The first resistant lines of this lepidopteran were detected in two populations sampled on watercress in Hawaii (Liu and Tabashnik, 1997). One of these populations had been subjected to 15 treatments with Bt-based biopesticides over the course of 18 months and the other had been subjected to between 50 and 400 such treatments between 1982 and 1989. Resistance in *P. xylostella* (L.) was also detected after intensive use of Bt sprays in several other countries, including Japan, China, the Philippines and Thailand (Liu and Tabashnik, 1997). After that, attempts were made to select in the laboratory for resistance in populations of different insect species, and insect strains resistant to one or several Bt toxins have since been selected in about 10 insect species (Tabashnik, 1994; Tabashnik et al., 2005), while none of them had yet developed resistance in the field. These laboratory studies confirmed that insects exhibit a remarkable ability to develop resistance and showed that the potential to develop resistance to Bt is real. The multiplicity of Cry genes found naturally occurring in Bt strains used as sprays may explain, in part, the apparent lack of resistance development in field populations. More recently, Tabashnik and collaborators analyzed the results of studies from Australia, China, Spain and the United States monitoring the resistance to Bt crops in field populations of six major insect pests: *Helicoverpa armigera* (Hbn), *Helicoverpa zea* (Boddie), *Heliothis virescens* (F.), *Ostrinia nubilalis* (Hbn), *Pectinophora gossypiella* (Saund.) and *Sesamia nonagrioides* (Lef); field-evolved resistance was not detected in *H. armigera* (Hbn), *H. virescens* (F.), *O. nubilali* (Hbn), *P. gossypiella* (Saund.) or *S. nonagrioides* (Lef). However, for *H. zea* (Boddie), field samplings done from 1992 to 1993, and from 2002 and onward, indicated that some populations of *H. zea* (Boddie), sampled during 2003 and 2004, in Arkansas and in Mississippi, clearly showed resistance ratios for Cry1Ac >50. Similarly, data from field populations, sampled in 2005 and 2006 in Arkansas, also demonstrated *H. zea* (Boddie) yielding resistance ratios for Cry1Ac

Table I. Examples of genetically engineered Bt crops approved for sale.

Crops	Target insects	Genes	Event	Trade name	Company
Potato	Colorado potato beetle	*cry3A*	various	New Leaf	Monsanto
Cotton	Bollworms and Budworms	*cry1Ac + cry2Ab*	15985	Bollgard II	Monsanto
		cry1Ac + cry1F	281-24-236 + 3006-21-23	WideStrike	Dow Agrosciences and Pioneer Hi-Bred
Corn	European Corn Borer	*cry1Ab*	MON810	YieldGard	Monsanto
		cry1Ab	Bt11	Agrisure CB	Syngenta
Corn	Western Bean Cutworm, European Corn Borer, Black Cutworm , Fall Armyworm	*cry1F*	TC1507	Herculex I	Dow Agrosciences and Pioneer Hi-Bred
Corn	Western Corn Rootworm	*cry3Bb1*	MON863	YieldGard Corn Rootworm	Monsanto
Corn	Western Corn Rootworm Northern Corn Rootworm Mexican Corn Rootworm	*cry34/35Ab1*	DAS-59122-7	Herculex RW	Dow Agrosciences and Pioneer Hi-Bred
Corn	European Corn Borer Corn Rootworm	*cry1Ab + cry3Bb1*	MON810 + MON863	YieldGard Plus	Monsanto
Corn	Western Bean Cutworm, European Corn Borer, Black Cutworm Fall Armyworm Western Corn Rootworm Northern Corn Rootworm Mexican Corn Rootworm	*cry1F + cry34/35Ab1*	TC1507 + DAS-59122-7	Herculex Xtra	Dow Agrosciences and Pioneer Hi-Bred

>100. By contrast, other studies showed no decrease in susceptibility to Cry1Ac in *H. zea* (Boddie) populations from North Carolina. After a detailed analysis of refuge abundance, during each of three generations when *H. zea* (Boddie) fed on cotton, meticulously estimated by other authors, in Arkansas, in Mississippi and in North Carolina, Tabashnik came to the conclusion that higher refuge sizes in North Carolina most probably explained the delayed resistance in *H. zea* (Boddie) observed in this state (Tabashnik et al., 2008). Although field-evolved resistance to Cry1Ac occurred in some Arkansas and Mississippi populations only 7–8 years after commercialization of Bt cotton, to date, *H. zea* (Boddie), *P. interpunctella* (Hbn.) and *P. xylostella* (L.) are the only insect species in which resistance has been found to develop outside of the laboratory. In fact, the sustained efficacy of the first generation of Bt crops for more than a decade against nearly all targeted pest populations is quite remarkable and exceeded the expectations of many entomologists working on population genetics (Bourguet, 2004). There are several possible reasons for the general lack of emergence of resistance to Bt plants in target pest populations during the first 12 years of Bt plant cultivation. The first is that resistance management programs have been implemented and that the principal areas in which Bt cotton and Bt maize crops have been planted on a large scale over the last few years – the US and Canada, in particular – have been managed properly. The second reason could be that the alleles conferring such resistance are present in the insect populations at such a low frequency that, despite possible increases over the last decade, these alleles remain too rare for detection in the field. A third possible reason is that the cost of resistance is sufficiently high for there to be a selection against these alleles in the absence of the toxin (Sanchis and Bourguet, 2008).

11. CONCLUSION

Increasing global agricultural production and preserving the environment are major challenges facing our society in the twenty-first century. Currently, insect pests destroy about 30% of pre-harvest and 10% of post-harvest yields, and will continue to be a major cause of damage to the world's commercially important agricultural food and fiber crops. As the world's population increases, the need to keep insects from destroying food crops will certainly become more urgent in the future and the use of pesticides will remain critical in meeting the demand for foodstuffs. Farmers started to spray Bt as a pesticide as early as in the 1930s, but for many years, it remained a minor component of pest management, because highly efficient synthetic pesticides became readily available after World War II. As the application of synthetic pesticides resulted in environmental damage and pest resistance, use of Bt products increased in the 1960s and 1970s and finally Bt emerged as a highly valuable alternative in pest control. In the 1980s, some of the limitations of the Bt microbial preparations, such as field stability and lack of capacity to reach cryptic pests, were overcome by the development of better conventional products and with the expression of the Bt toxins in transgenic

plants. Although concerns have been raised about transgenic Bt crops, this technology is generally, ecologically and environmentally, less destructive than the use of chemical insecticides. Bt corn and Bt cotton have permitted consistent reductions in insecticide use, thereby reducing the environmental impact associated with pesticide use and greenhouse gas emissions. Farm workers have also greatly benefited from using transgenic Bt crops in place of much more hazardous insecticides. Furthermore, Bt crops have indirectly contributed to food safety as it has been shown that Bt corn grain was generally less contaminated than conventional corn grain by fungal toxins produced by the genus *Fusarium*. Indeed, these fungal pathogens enter the plant through wounds caused by boring insects and Bt corn is better protected against damage from corn borers. Although using Bt in the form of transgenic crops is now very common (50 million hectares of Bt crops were planted worldwide in 2009) and has ensured a place for Bt as a highly commercially viable product, the more traditional spray form of Bt is still widely used (Liu and Tabashnik, 1997) by organic farmers. It is one of their most valuable biocontrol tools and the loss of its use would be an extremely unfortunate occurrence. Moreover, the use of transgenic plants will be of little value if the important insect pests become rapidly resistant to Bt. Therefore, the possible development of resistance to Bt toxins in insect pests has become a critical issue. Past experience in the domain of development of resistance to chemical pesticides in insect pests tells us that it has taken 10–15 years for key cotton insects to develop resistance to each new type of insecticide applied to control them. There is also evidence for development of resistance where Bt has been over-used in conventional application systems, and therefore, there is no reason why this should not also become true when Bt crops are used. Resistance of a pest to any insecticide is a serious concern and, in the USA, Bt was recognized, from the beginning, as a valuable insecticide whose continued efficacy required government action to protect against the evolution of resistant insects. As a result, the EPA established programs to preserve the efficacy of Bt toxins through the close monitoring of pest populations and the use of refuges for susceptible insect populations. The resistance management program that has been most frequently implemented in several countries for commercialized Bt crops is the "high dose refuge strategy" (or HDR). It involves growing plots of Bt crops producing large amounts of toxin (with a goal of more than 99.9% lethality) alongside non-Bt crop plots (referred to as refuge zones), in which the larvae of target insects are not exposed to the toxin. These conventional plants serve to sustain susceptible alleles within the insect population and to provide susceptible insects that may mate with resistant insects (Sanchis and Bourguet, 2008). The resistant management plans imposed by the EPA on Bt cotton and Bt corn have, for the most part, been effective. However, the recent discovery that the frequency of resistance alleles to the Cry1Ac toxin in transgenic Bt cotton has increased substantially in some field populations of the Corn Earworm, *Helicoverpa zea* (Boddie), stands as a reminder that Bt resistance must be closely monitored and managed (Tabashnik et al., 2008). Even if the HDR strategy appears quite efficient in controlling the evolution of resistance, the best management strategy would certainly be to reduce the toxin exposure of all insects to minimize selection pressure as much as possible. One solution, as in some cases Bt is just as effective as transgenic plants when properly applied in a spray form, would be to use Bt only as foliar sprays, when the benefits of using transgenic Bt crops are not significant enough to justify their use. Rotation of Bt crops with non-transgenic plants would also slow down the development of resistance, particularly if resistance is not stable in the insect population. Another solution may be to express the Bt toxins in plants only when needed by the use of tissue-specific promoters (time-specific expression). Resistance management strategies using time-specific expression must be thoroughly tested and validated in the field. However, tissue-specific expression would not be a viable option for some pests which target nearly the entire plant, such as the European corn borer. New Bt crops that express multiple Cry proteins (that recognize different receptors) are also currently being developed (gene stacking strategy). The search for new insecticidal proteins, in Bt or other microorganisms, may also provide new weapons for the fight against insect damage. The screening of Bt strains for the production of insecticidal proteins at various physiological stages (other than sporulation, when Cry proteins are produced) has already shown that Bt produces various other insecticidal proteins during vegetative growth. One such protein, Vip3A, is highly toxic to lepidopteran pests (Estruch et al., 1996) and the corresponding genes have been cloned. Genetically engineered products expressing Vip3A are currently being evaluated in cotton and maize plants. As yet, no strategy has proved broadly applicable to all crop species, and a combination of approaches may prove most effective for engineering the next generation of insect-resistant crops. Ultimately, the quest to improve bioinsecticides may lead to the introduction of foreign insecticidal genes into *B. thuringiensis* and/or to the development of new genetically engineered crops that express multiple insecticidal proteins of various origins in addition to the Cry toxins. Genes encoding proteins with potential insecticidal properties such as protease inhibitors, lectins and chitinases have been isolated from various sources (plants, insects and microorganisms). The use of these genes may delay the appearance of resistance or synergy may be found with Bt Cry toxins. Some of these genes have already been expressed in various plants (e.g; tobacco, potato, cotton) and in some cases this has reduced the damage caused by insect pests (Hilder et al., 1987; Shade et al., 1994; Down et al., 1996). However, these proteins are effective insecticides only if produced in large amounts in the plant. The long-term value of the expression of these genes in plants, in association with the Bt *cry* and *vip* genes, is being assessed. Future research should also strongly focus on the gaps in our knowledge, especially regarding the characteristics of insect resistance that we do not yet fully understand (e.g. selection intensity, size of refuges or presence of other host plants, pest mortality due to natural enemies, population dynamics, mating behavior, gene flow). A better understanding of the biology of the pests, the possible mechanisms of resistance and the frequency of resistant alleles in the insect populations are clearly required to devise optimum and correct resistance management strategies. More recently, the use of Bt biotechnology in food crops such

as tomato, potato and rice has also raised some concerns about their safety for consumption by humans, despite the good toxicology data already existing for Bt (when used as sprays Bt is the only insecticide for which there are no mandated residue limits on foods). The generally negative image of genetically modified foods relies heavily on perceived risks, and has led, in many European countries, to the regulatory "precautionary principle" which restricts their cultivation or commercialization, irrespective of their apparent economic or environmental benefits. Implementing programs aimed at communicating to the general public the risks and benefits of using Bt crops could be useful for achieving a better understanding and public acceptance of some of these transgenic crops, but this goal may remain quite difficult in several countries. Finally, the current and recent interest by European consumers in organic and non-genetically engineered foods has also raised the problem of the contamination of organic products with genetically engineered materials.

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