

Transgenic *Bt*-Plants and the Future of Crop Protection (An Overview)

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Abstract

One of the best modern agricultural defenses against plant-eating insects is *Bacillus thuringiensis* (*Bt*), which either can be applied to the surface of the plant, to provide temporary protection, or can be genetically engineered into the plant to protect it against insects throughout its lifespan. Plants can be genetically engineered to produce their own *Bt* crystal protein (CP), which is toxic to the pest species of concern. As the insect feeds on the plant, it ingests the CP and suffers the same fate as if the leaf tissue was sprayed with *Bt*. The use of commercial crops expressing *Bt* toxins has increased in the recent years due to their advantages in plant protection and lower production costs, however, insects-developed resistance against plant defense mechanisms and the consequent effects of *Bt*-plants on non target species are hence considered disadvantages. This is still a controversial topic and the question is: Within the next few years, will *Bt*-plants provide hope for the future of crop protection?

Keywords: *Bt*-plants, transgenic, plants, crop, protection

Introduction

There are an estimated 67000 pest species that damage agricultural crops, of which approximately 9000 species are insects and mites (Ross and Lembi, 1985). Insect-pests are the major cause of crop losses (Kumar et al., 2008). An average of 15% of crops worldwide is currently lost to insects (Maxmen, 2013). Insects cause direct losses to the agricultural crops, in addition to the indirect losses due to impaired quality of the produce and their role as vectors of various plant pathogens (Kumar et al., 2006). In the past, humans have searched for crop plants that can survive and produce under different biotic and abiotic stresses. Ancient farmers searched for pest resistance genes in their

crops, sometimes by actions as simple as collecting seed from only the highest-yielding plants in their fields (COMESA, 2007).

Although the development of chemical insecticides during the last 40 years guaranteed a production increase in agriculture, contamination of the environment by pesticides is increasing due to their usage in crop protection (Oerke, 2006). Using of pesticides led to contamination of water and food sources, poisoning of non-target beneficial insects and development of insect populations resistant to the chemical insecticides (Kumar et al., 2008; Matsumura, 1975; Scheyer et al., 2005; Tanabe et al., 1983). The adverse effect of chemical insect-

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cides on the environment has increased the global public concern to seek alternative methods of insect control. One of the promising alternatives is the use of biological control methods, such as biopesticides and entomopathogenic microorganisms. Entomopathogens (i.e. bacteria, fungi, and viruses) are disease-causing organisms. They kill or debilitate their host and are relatively specific to certain insect groups. An important benefit of biological control methods is that they can replace, at least in part, some hazardous chemical pesticides (Kumar et al., 2008). In addition, biological control reduces expenses and health hazards associated with pesticide sprays (Kouser and Qaim, 2011).

Bt is short for *Bacillus thuringiensis*, a natural bacterium in the genus *Bacillus*. One of the best modern agricultural defenses against plant-eating insects is *Bacillus thuringiensis* (*Bt*), which either can be applied to the surface of plants to provide temporary protection or can be genetically engineered into the plant to protect against insects throughout the lifespan of the plant (James, 2006). The bacterial fermentation technology was led to optimization of better formulations and cheaper *Bt* products. New effective *Bt* species against insects from the orders Lepidoptera, Diptera and Coleoptera were isolated (Cannon, 1995). With the beginning of genetic engineering, genes for insect resistance can be moved into plants more quickly and deliberately (James, 2006).

Biotechnology affords opportunities to develop new tools to treat pest and disease problems. When a given pest or disease problem has no satisfactory cure or treatment, usually only a technological breakthrough can provide one, but the process whereby this happens can be undefined and unpredictable. *Bt* technology is one example of genetic engineering that may be used to develop insect resistant crops now and in the future (Miller, 2007). However, the widespread success of *Bt* has prompted two concerns; first, insects might someday become resistant to this important treatment (Addison, 2010; Bates et al., 2005; Tabashnik et al., 2009). Second, while primary pests are controlled through *Bt*, the lower use of chemical pesticides may entail the outbreak of secondary pests, especially mirids, mealybugs, and other sucking pest species, which are not controlled through *Bt* (Lu et al., 2010; Nagrare et al., 2009). Both factors could potentially lead to the increase

in using chemical pesticide again after a certain time of reduction (Krishna and Qaim, 2012).

In 1996, the commercialization of transgenic *Bt* maize (corn) hybrids to control corn rootworms generated more interest and excitement among corn growers who enthusiastically adopted the technology to control the most important corn pests in North America (Shelton et al., 2002). In due course, transgenic traits to control both corn borers and corn rootworms were “stacked” in elite corn hybrids with traits for herbicide tolerance, resulting in double, triple, and quad-stacked hybrids (Gray et al., 2009). Next to *Bt* maize, *Bt* cotton is currently the most widely grown *Bt* crop in North America (James, 2010). The largest *Bt* cotton areas are found in India and China, where the technology is mainly used to control the American bollworm *Helicoverpa armigera* and to a lesser extent, spotted bollworm *Earias vittella*, pink bollworm *Pectinophora gossypiella*, and related species (Qaim, 2009), with efforts at various stages in other countries to develop and release adapted *Bt*-cotton varieties, such as South Africa (Morse et al., 2004), Burkina Faso (Vitale et al., 2010), Egypt (Dahi, 2012) and Kenya (Midega et al., 2012).

Biological and Ecological Features of *Bt*

Bacillus thuringiensis (*Bt*) was first isolated about 112 years ago in Japan from silkworm larvae by Ishwata in 1901. Ten years later, in Germany, Berliner described the same pathogen from the flour moth *Ephestia kuehniella* (Berliner, 1915). For over 40 years, *Bt* has been applied to crops in spray form as an insecticide, containing a mixture of spores and the associated protein crystals. A unique feature of *Bt* is that the bacterium produces proteins in the form of crystalline structures, and these proteins have activity against some insect species (Riegler and Stauffer, 2003). *Bt* is naturally occurring, gram positive, spore forming soil bacterium (Figure 1), which differs from *Bacillus cereus* in terms of its insect pathogenicity (Meadows, 1993). The characteristic of the parasporal crystal, which is formed in the course of the sporulation, was described early in 50's (Hannay, 1953). Two years later correlated insecticidal activity with the parasporal crystal was reported (Hannay and Fitz-James, 1955). The first sub-

species of *Bt* toxic to dipteran (flies) species was found in 70's (Goldberg and Margalit, 1977) and the first discovery of strains toxic to species of coleopteran was in 80's (Krieg et al., 1986)

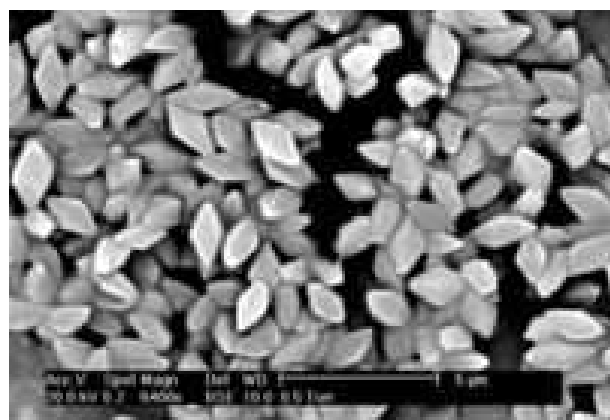


Figure 1: Spores and bipyramidal crystals of *Bacillus thuringiensis*

This diverse genus also includes more than 20 other *Bacillus* species and hundreds of different subspecies. Members of the genus *Bacillus* are generally considered soil bacteria, and *Bt* is common in terrestrial habitats including soil (Martin and Travers, 1989; Meadows, 1993), living and dead insects, insect feces, granaries, and on the surfaces of plants. *Bt* occurs in nature predominantly as spores that can disseminate widely throughout the environment. Experiments to isolate *Bt* from soil samples, which were treated before with spore suspensions, have shown that spores under natural conditions in the soil cannot germinate and also do not outlast for a long period of time (West et al., 1985). Other isolation attempts proved the fact that *Bt* is a ubiquitous bacterium and is being found less in the soil than on the plant parts, such as leaves and grain bran from mills (Meadows et al., 1992; Smith and Couche, 1991). A further source for *Bt* toxins could be larvae, which died of bacterial infestations, for example, in beehives (Muerrle and Neumann, 2004).

Bt can synthesize a set of insecticide working substances: *D*-Endotoxin, *A* and *B*-Exotoxins, Phospholipases, immunosuppressive substances and vegetative insecticide proteins (Krieg, 1986; Peferoen, 1997). *D*-Endotoxin is the most important protein, which developed as crystal elimination during the sporulation, and it has a specific

toxic effect against certain insect pests. In all spores-forming bacteria, there is an exchange that takes place between vegetative growth phase, with intensive metabolism, and the following dwell phase, as inactive spore formation. The vegetative cells do not need special requirements of the nutritive substrates and are therefore simply mass produced. During exhaustion of nutrients, the cell division is stopped and starts the sporulation forming sporangium. This contains the actual spore, surrounded by endo- and exosporium, as well as parasporal protein crystals. Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ -Endotoxins (*Cry* toxins) which are encoded by *Cry* genes. *Cry* toxins have specific activities against species of the orders Lepidoptera (moths and butterflies), Diptera (flies and mosquitoes) and Coleoptera (beetles) (Koziel et al., 1993; Van Rie, 2000). Thus, *B. thuringiensis* serves as an important reservoir of *Cry* toxins and *Cry* genes for the production of biological insecticides and insect-resistant genetically modified crops. After sporulation, the sporangium wall is dissolved, and the spore and the parasporal body are dismissed. Transgenic crops that contain *Cry* genes are widely adopted by farmers in many countries over the last 15 years (James, 2009). Several studies showed that *Bt* crops, which provide resistance to some Lepidopteran and Coleopteran insect species, have helped reduce chemical pesticide use and increase total yield (Carpenter, 2010; Huang et al., 2005; Krishna and Qaim, 2007; Morse et al., 2006; Qaim and De Janvry, 2005; Subramanian and Qaim, 2009; Wossink and Denaux, 2006) by 13-23% when insect infestation was severe (Mungai et al., 2005), but no significant differences were noticed in yield under low or moderate insect infestation (Ma and Subedi, 2005). Wang et al. (2010; 2012) found no significant differences on growth performance or total yield between *Bt*-transgenic rice and their non *Bt* counterparts.

Krieg (1986) classified the pathotypes of *Bt* species according to their effects on insect orders: pathotype A; effective against butterflies, pathotype B; effective against Diptera and pathotype C; effective against Coleoptera. Meanwhile, there are other Pathotypes which are also effective against other organisms like protozoan, mites and nematodes (Feitelson et al., 1992). Not all types of *B. thuringiensis* bacteria synthesize crystalline pro-

teins that are toxic to insects (Bernhard et al., 1997; Martin and Travers, 1989; Meadows et al., 1992). *Bt* toxins increased mortality, reduced growth rate, prolonged development time, and reduced adult body mass and size of the insect (Lang and Otto, 2010).

Structure of *Bt* D-Endotoxin

Bt D-Endotoxin is classified according to its pathogenicity and the crystalline form into three pathotypes (Schnepf and Whiteley, 1981). Crystalline proteins of the pathotype A have a bipyramidal form, proteins of the pathotype B are spheroid cuboids form and protein of the pathotype C is rhomboid. In consequence of the discovery of new toxin proteins and the inhomogeneous composition of the protein crystals, a new nomenclature was developed according to pathogenicity, sequence homology and molecule-size (kDa) (Höfte and Whiteley, 1989; Lereclus et al., 1993). A new systematic procedure was developed by Peferoen (1997) and Crickmore et al. (1998), which was based only on the homology of the amino acid sequence. Based on this, there were more than 100 well-known toxins, which were divided into 20 classes (Crickmore et al., 1998). The most studied D-Endotoxins belong to the classes *Cry1*, *Cry2*, *Cry3*, *Cry4* (crystal protein) and *Cyt* (cytase). Table 1 is briefly describes crystal proteins, their molecular weights and the target insects.

Table 1

Types of crystal proteins and the target insects:

D-Endotoxins Crystal protein	Molecular weight	Target insect (active against)
<i>Cry1</i>	130 kDa	Butterflies (and beetles)
<i>Cry2</i>	70 kDa	Butterflies (and Diptera)
<i>Cry3</i>	70 kDa	Beetles
<i>Cry4</i>	130 kDa	Diptera
<i>Cyt</i>	30 kDa	Nonspecific Cytotoxin

Produced as crystalline inclusions by the bacterium *Bacillus thuringiensis* (*Bt*), *Cry* toxins are the most widely used insecticidal trait in transgenic crops for insect control (James, 2009). Each crystal protein consists of two terminals; amino terminal and carboxyl terminal. During the activation of the

Cry protoxin, the carboxyl terminal is split by proteases of the insect. The amino terminal remains as active fragment of approximately 30 kDa (*Cry4*) - 60 kDa (*Cry1*), consisting of approximately 600 amino acids (*Cry1*). *Cry2* and *Cry3* toxins are hardly split (Gill et al., 1992), while the *Cyt* toxin must not be activated (Lereclus et al., 1993). By the means of the three-dimensional analysis of the active *Cry* fragment, three different domains were determined: Domain I; responsible for the pores formation in the epithelium of the midgut, domain II; interacts with the receptor (Peferoen, 1997) and domain III; responsible for the formation of a toxin oligomer (Pardo-Lopez et al., 2006) that leads to osmotic cell death (Zhuang et al., 2002).

Cry toxins target the insect midgut cells to compromise the gut epithelium barrier and facilitate the onset of septicemia (Raymond et al., 2010; Soberon et al., 2009). Although the specific mechanism resulting in enterocyte death is still controversial, commonly accepted steps in the intoxication process include solubilization of the crystal toxin and activation by the insect gut fluids. Activated toxins are attracted to the brush border membrane of the midgut cells through low affinity binding to glycosylphosphatidylinositol-anchored (GPI-) proteins (Arenas et al., 2010), such as aminopeptidase-N (APN) or membrane-bound alkaline phosphatase (mALP). This initial binding step facilitates subsequent binding of higher affinity to cadherin-like proteins (Bravo et al., 2004), which leads to further processing of the toxin, resulting in formation of toxin oligomers. Toxin oligomers display high binding affinity towards N-acetylgalactosamine (GalNAc) residues on GPI-anchored proteins (Pardo-Lopez et al., 2006), resulting in concentration of toxin oligomers on specific membrane regions called lipid rafts, where they insert into the membrane, forming a pore that leads to osmotic cell death (Zhuang et al., 2002). Alternatively, binding of toxin monomers to cadherin has been reported to activate intracellular signaling pathways that resulted in cell death by oncosis (Zhang et al., 2006).

Bt Mode of Action

A unique feature of *B. thuringiensis* bacteria is the synthesis of crystalline proteins, which have

activity against some insect species. The main effect of *Bt* against insects is due to *D*-Endotoxin. *Bt* insecticides, whether in the form of spray or in *Bt*-genetically engineered plants, do not function on contact as most insecticides do. The insecticidal effect of *Bt* comes from the crystal proteins (CPs) produced during the bacterium's sporulation phase. These proteins are inactive in this phase. In order to be activated, the crystals must be loosened in an alkaline milieu (pH>9). The CPs can be converted into the active toxin by protease during digestion, which interacts with the epithelium of the midgut (Riegler and Stauffer, 2003).

Among the agricultural pests that are currently targeted with *Bt* insecticides are bollworms, stem borers, budworms, and leaf worms in cereal crops; the gypsy moth and spruce budworm in forests; and the cabbage looper and diamondback moth in vegetable crops (BCMAFF 2004; Olkowski et al., 2000; Weinzierl et al., 2000). Mosquitoes and black flies are also controlled with *Bt* sprays or by treating the aquatic breeding sites with *Bt* (Lacey and Merritt, 2004; Martin and Travers, 1989; Peairs, 2010).

The CPs must be ingested by the target organism to be effective. The process takes hours or even days; somewhat longer time than that is required for synthetic insecticides to kill insects. Active CP binds to specific receptors on the midgut, forming pores and leading to leakage of the midgut contents, paralysis and death of the insect. Recognition of these toxins is necessary by receptors, which locate on the microvilli of the midgut epithelium. After perforation of the epithelial membrane by the toxin, cations exchange increases between midgut contents and the epithelial cells. As a result of the increased osmotic pressure, the epithelial cells burst and the midgut contents move into the haemolymph causing blood poisoning and intestine paralysis that results in insect death after few hours to 2-7 days (Gill et al., 1992; Olkowski et al., 2000; Riegler and Stauffer, 2003; Wheeler et al., 2011). The pH of the insect gut (depending on how alkaline or acidic it is) is critical for dissolving the walls of the storage spore. Not all insects have the same acidity in their gut, and this is why some insects are susceptible to *Bt* poisoning (e.g., immature stages of certain moths (caterpillars), beetles (grubs), mosquitoes and black flies), while others are not (BCMAFF, 2004; Olkowski et al., 2000;

Wheeler et al., 2011). The good thing is that these reactions cannot take place in humans and other mammals (Wheeler et al., 2011).

***Bt* Applications**

1. *Bt* Preparations

For over 40 years, *Bt* has been applied to crops in spray form as an insecticide. *Bt* preparations are mostly suspensions that contain a mixture of spores and associated protein crystals. Unlike other pesticides that kill on contact, *Bt* must be eaten by insects to be effective. As an ingredient of commercial sprays, *Bt* is relatively expensive compared to chemical pesticides (Riegler and Stauffer, 2003). *Bt* preparations are highly specific with short persistence and thus have relatively high environmental compatibility. UV radiation breaks down *Bt* and rain washes it off the plants. Therefore, *Bt* must be applied exactly where and when the target insects are feeding and they must consume it quickly before it disappears (BCMAFF, 2004; Martin and Travers, 1989).

The use of conventional *Bt* preparations is limited against few insects. By the conjugation of different *Bt* strains, a combination of different plasmids with toxin genes in a specific strain can be made in order to expand the host spectrum of target insects (Cannon, 1995), for instance, most of the formulations found in retails indicate that they contain *B. thuringiensis* var. *kurstaki*. This strain is limited to controlling certain caterpillars. Other available strains of *Bt* are effective treatments against larval beetles and some flies, such as, incorporating *B. thuringiensis* var. *israelensis* in the soil to kill fungus gnats in greenhouse soil or in potted indoor plants. Other strains, such as *B. thuringiensis* var. *san diego* can be used to kill the Colorado potato beetle (Olkowski et al., 2000). *Bt* preparations degrade in short time after application, and this short duration of effect forces to repeated spraying (Behle et al., 1997).

Reliance on *Bt* in tree fruit production is also rapidly increasing, as the United States environmental protection agency (EPA) restricts post-bloom uses of higher-risk organophosphate (OP) and carbamate insecticides. Innovative growers are now perfecting insect integrated pest management (IPM) programs by combining pheromone-based mating disruption, *Bt* sprays, an application or two

of an insect growth regulator (IGR), and either spinosad or a lower-risk OP or carbamate when populations grow over thresholds (Benbrook and Suppan, 2000). Pheromone-based mating disruption *Bt* sprays are now used in strawberry pest management program in California (Devencenzi, 2013).

Rachel Carson promoted *Bt* as a natural insecticide in her book, *Silent Spring*, published in 1962. By the end of last century, the annual market extent of *Bt* preparations world-wide was estimated by \$60-120 million (Cannon, 1995). *Bt* products was constituting about 90-95% of the total biopesticides market (Feitelson et al., 1992), however, it shared only 0.5-2% of the entire plant protection market (Cannon, 1995; Mazier et al., 1997; Van Frankenhuyzen, 1993). By 1995, about 182 *Bt*-based products were registered by EPA; however, by 1999 the total sales of *Bt* formulations constituted less than 2% of the total sales of all insecticides (EPA, 2010). Agro magazine points out that the worldwide production of biopesticides in 2000 was close to \$160 million; of this, *Bt* biopesticides accounted for more than 90%. Although the worldwide pesticide market is in decline, under the future IPM concept, it is predicted that the biopesticides market will grow significantly. As of the end of 2001, about 195 different biopesticides effective ingredients and roughly 780 different products have already been marketed. According to Chuck Benbrook Consulting Company's forecast, after 2013, genetically modified crops and biological additives will increase significantly by 7.5 times and 5 times, respectively (Yuan, 2010). The global market for biopesticides was valued at \$1.3 billion in 2011 and is expected to reach \$3.2 billion by 2017, growing at a compound annual growth rate (CAGR) of 15.8% from 2012 to 2017. North America dominated the global biopesticides market, accounting for around 40% of the global biopesticides demand in 2011. Europe is expected to be the fastest growing market in the near future owing to the stringent regulation for pesticides and increasing demand from organic products (MAM, 2012).

2. Biotechnology of *Bt* Products

By the means of new molecular biology methods, both the spectrum of use and the duration of effect of *Bt* products can be expanded (Cannon, 1995; Gelernter and Schwab, 1993). For example, by the

insertion of toxin crystals in other genetic systems, for example, in non sporulated bacterium *Pseudomonas fluorescens*, the persistence of the toxin was extended after application, since the toxin is strongly more protected against dismantling processes (Schnepf et al., 1998). Further toxin genes were inserted in bacteria, such as *Clavibacter xyli*, in order to ensure a systemic protection against stem borers, for example, in corn and sugar beet (Turner et al., 1991). Transferring *cry3Aa1* gene into root nodules forming bacteria, such as *Rhizobium leguminosarum* has protected leguminous roots against the larvae of *Sitona flavescens* (Skot et al., 1990; 1994). Another possibility is the transformation of insect baculovirus with *Bt* toxins, in order to increase their virulence against certain insect pests (Gelernter and Schwab, 1993; Vlak, 1995).

Reports on the emergence of insects resistance to *Bacillus thuringiensis* D-Endotoxins have raised doubts on the sustainability of *Bt* toxin-based pest management technologies (Manyangarirwa et al., 2006; Tabashnik et al., 2008), for instance, moth *Plutella xylostella* has developed field resistance to *Bt* toxin due to a reduction in toxin binding to gut receptors (Ferre and Van Rie, 2002; Kain et al., 2004; Shelton et al., 2002). Jurat-Fuentes et al. (2011) reported that reduced levels of midgut membrane-bound alkaline phosphatase (mALP) is a common feature in strains of *Cry*-resistant *Heliothis virescens*, *Helicoverpa armigera* and *Spodoptera frugiperda* when compared to the susceptible larvae. Insect resistance may also differ from one cultivar of the insect to the other, for example, there is evidence in the scientific literature that the “variant” western corn rootworm is more difficult to be killed with *Bt* proteins than the “normal” western corn rootworm (Siegfried et al., 2005). Since most of the insect-resistant transgenic plants were released for commercial cultivation and the target insect populations are consistently exposed to the single *Bt* *cry*-toxin protein, therefore, the possibility of insects evolving resistance to a single *Bt* toxin is high (Gunning et al., 2005; Zhao et al., 2005). This challenge has developed the innovation of gene pyramiding that entails the simultaneous expression of more than one toxin in a transgenic plant (Manyangarirwa et al., 2006; Shelton et al., 2002; Suresh and Malathi, 2013). Theoretical and practical evidence in insect population genetics suggest that gene pyramiding

cannot be sustained as a resistance management strategy per se. Pyramiding is useful as a strategy to broaden the range of insect pests controlled in each transgenic variety, and it still has to be deployed in tandem with *Bt* resistance management strategies, such as crop refugia, biological pest control, temporal and spatial crop rotations among others (Manyangarirwa et al., 2006). Pyramided transgenic chickpea plants with moderate expression levels of two *Cry* toxins showed high-level of resistance and protection against pod borer larvae of *Helicoverpa armigera* as compared to high level expression of a single *Cry* toxin (Mehrotra et al., 2011). The first two commercialized pyramided *Bt* corn technologies in the U.S. for managing lepidopteran pests include Genuity VT Triple Pro and Genuity Smart Stax. Both were first commercially planted during the 2010 crop season (Monsanto, 2012; EPA, 2010).

3. Transgenic *Bt*-Plants

In May 1995, transgenic *Cry3A* potatoes were certified for the first time in USA. One year later, both *Bt* cotton and *cry1Ab1* hybrid corn were also certified (EPA 2000; Mazier et al., 1997). These crops provide highly effective control of major insect pests, such as the European corn borer, southwestern corn borer, tobacco budworm, cotton bollworm, pink bollworm, and Colorado potato beetle and reduce reliance on conventional chemical pesticides. They have also provided notably higher yields in cotton and corn (Betz et al., 2000). In 1997, there were more than 3 million hectares cultivated with transgenic *Bt* cotton, corn and potatoes in the USA (EPA, 2000; Tabashnik et al., 1997) with a potential for *Bt* genetically modified crops to take up to 33% of the insecticide market by 2000 (Arozzi and Koziel, 1997). Plantings of *Bt*-maize (corn), expressing *cry* toxins harmful to Lepidoptera or Coleoptera (Koziel et al., 1993; Van Rie, 2000) grew from about 8 % of US corn acreage in 1997 to 26 % in 1999, then fell to 19 % in 2000 and 2001, before climbing to 29 % in 2003 and 67 % in 2012. Plantings of *Bt* cotton expanded more rapidly from 15 % of US cotton acreage in 1997 to 37 % in 2001 and 77 % in 2012 (USDA, 2012).

The story started with the surprising discovery of DNA interchangeability among different bacteria, animals and plants makes it possible to locate the gene that produces *Bt* proteins lethal to insects and

transfer this gene into crop plants. The plants modified in this way are called transgenic. So, plants can be genetically engineered to produce their own *Bt* crystal protein (CP) which is toxic to the pest species of concern. As the insect feeds on the plant, it ingests the CP and suffers the same fate as if it ingested leaf tissue sprayed with *Bt* (Roush and Shelton, 1998). Transgenic plants expressing insecticidal proteins from the bacterium, *Bacillus thuringiensis* (*Bt*), are revolutionizing agriculture. *Bt*, which had limited use as a foliar insecticide, has become a major insecticide because genes that produce *Bt* toxins have been engineered into major crops grown on 11.4 million ha worldwide in 2000 and expanded to 80 million ha in 2004. Constituting 19% of the worlds genetically modified (GM) crops; these crops (i.e. cotton, corn and potatoes) have shown positive economic benefits to the growers and reduced the use of other insecticides in a number of developed and developing countries (Bates et al., 2005; Cohen, 2005; Ferre and Van Rie, 2002; Ferry et al., 2004; Gao et al., 2010; James, 2006; Shelton et al., 2002; Wu and Guo, 2005). In United States, corn farmers have experienced significant changes since *Bt* corn was first introduced. Corn borer infestations have decreased considerably; new traits, such as corn rootworm and corn earworm resistance, have been engineered into *Bt* seeds, and many input costs have increased. Each of these changes has the potential to alter the farm-level costs and benefits of adopting and continuing to plant *Bt* corn (Fernandez-Cornejo and Wechsler, 2012). The global area of genetically modified crops is expected to increase significantly at 7.5 times after 2013 (Yuan, 2010).

The advantages of using transgenic *Bt* plants, compared to the foliar sprays of *Bt* or chemical pesticides, are: 1) Protection of the plant through the entire vegetation period with no need of repeated foliar sprays of *Bt*-biopesticides or other chemical pesticides (BCMAFF, 2004), 2) Systemic protection of plants against insects, for example, stem borers, or resistant insects, which already developed resistance against chemical insecticides (Mazier et al., 1997). *Bt* is less likely than chemical pesticides to cause field resistance in target insects due to its short biological half-life and its specificity (BCMAFF, 2004) and 3) Minimizing the residues of chemical pesticides in food and environment prevents the effects on non-target

organisms and benefits farm workers and others likely to come into contact with these pesticides (James, 2000; NAS, 2000) because *Bt* toxins attack sites are found only in target insects (Peairs, 2010).

Expression of *Bt* gene (*cry1A*) in tobacco and tomato provided the first example of genetically engineered plants for insect resistance (Barton et al., 1987; Vaeck et al., 1987), which showed a significant tolerance against Lepidopteran larvae (Ely, 1993). Subsequently, several *Bt* genes have been expressed in transgenic plants, including tobacco, potato, tomato, cotton, brinjal, rice, and so on (Kumar et al., 2008). In 1995, EPA registered the first *Bt* potato-incorporated protectants (*cry3A*) for use in the United States. Since then, EPA has registered 11 *Bt* plant-incorporated protectants, although 5 of these registrations are no longer active and the rest are different cultivars of corn, cotton and potato (EPA, 2002; 2011). By the cultivation of transgenic *Bt* cotton in America, the application of insecticides reduced from 5-12 to 0-3 times, while in Australia about half of the insecticides amount was saved (Carpenter et al., 2002; EPA, 2000; Roush, 1997). The largest *Bt* cotton areas are found in India and China, where the technology is mainly used to control the American bollworm (*Helicoverpa armigera*) and to a lesser extent, spotted bollworm *Earias vittles*, pink bollworm *Pectinophora gossypiella*, and related species (Qaim, 2009). In both countries, the cotton sector is heavily dominated by smallholder farmers with land areas of less than 5 ha, who benefit from *Bt* technology adoption in terms of higher incomes and lower occupational health hazards associated with pesticide sprays (Hossain et al., 2004; Huang et al., 2002; Kouser and Qaim, 2011; Qaim et al., 2009). *Bt* cotton contributes to poverty reduction and broader rural development in these countries (Ali and Abdulai, 2010; Subramanian and Qaim, 2010). A unique panel survey of *Bt* cotton farmers conducted in India between 2002 and 2008 shows that the *Bt* pesticides reducing effect has been sustainable. Total pesticides use has decreased significantly over time and *Bt* has also reduced pesticides applications by non-*Bt* farmers. Increase in total yield ranged usually from 30 to 60% and the reduction in number of insecticide sprays averaged around 50% causing 50 to 110% increase in profits from *Bt* cotton, equivalent to a range of \$76 to \$250 per hectare (Choudhary and Gaur, 2010). *Bt* cotton biotechnology includes Bollgard I

technology, containing the *Cry1Ac* gene that was officially commercialized in India in 2002 and Bollgard II technology, containing stacked *Cry1Ac* and *Cry2Ab* genes that was also approved in 2006 (Krishna and Qaim, 2012). By 2010, over six million Indian farmers had adopted *Bt* cotton on 9.4 million hectares – almost 90% of the country's total cotton area (James, 2010). India is currently the biggest producer of *Bt* cotton in the world since 2012 (Krishna and Qaim, 2012).

Outgoing from these transformation models, over 50 plants were successfully modified with the toxin genes, *cry1Aa1*, *cry1Ab1*, *cry1Ac1*, *cry1Ca1* and *cry3Aa1* (Kumar et al., 2008). Transgenic crops are grown over large areas in the America (James, 2011; USDA, 2012). For 2012, US planted area of wheat is estimated at 56.0 million acres, soybean at 76.1 million acres, corn at 96.4 million acres and cotton 12.6 million acres (NASS, 2012). In Alabama alone, between 300,000 and 400,000 acres of *Bt* cotton has been grown annually since 1996. Syngenta Seeds Inc. reported development of transgenic cotton plants expressing *vip3Aa* gene across the US cotton-belt during 2000-2002. The transgenic cotton plants were reported to provide excellent protection against Lepidopteran insects throughout the season and resulted in significantly higher yields (Artim, 2003), however, in crops such as cotton that are plagued by several pests with varying degrees of susceptibility to *Bt*, there is a concern that the toxins will not be strong enough to kill all pests, because *Bt* toxins are highly specific against insects without affecting predators and other insects (Christou, 2005). The result would be reduced *Bt* efficiency and increased risk of pests developing *Bt* resistance (Tabashnik and Carrier, 2010). Additionally, reliance on a single (or similar) *Bt* protein(s) for insect control increases the probability of *Bt* resistance development in target pest populations (Addison, 2010; Bates et al., 2005; Tabashnik et al., 2009). Moreover, while primary pests are controlled through *Bt*, the lower use of chemical pesticides may entail the outbreak of secondary pests, especially mirids, mealybugs, and other sucking pest species, which are not controlled through *Bt* (Lu et al., 2010; Nagrare et al., 2009). Both factors could potentially lead to chemical pesticide use increasing again after a certain time of reduction. The probability of this happening may be higher in the small farm sector of develop-

ing countries, where implementation of *Bt* refuge strategies and careful monitoring are more difficult (Wang et al., 2008). However, beyond such undesirable effects, there are also possible positive spill-overs: widespread use of *Bt* technology may suppress bollworm infestation levels regionally, such that non-*Bt* adopters may also be able to reduce their pesticide applications (Carrière et al., 2003; Hutchinson et al., 2010; Wu et al., 2008). Also, the insertion of toxin genes directly into the chloroplasts DNA, which is similar to the bacterial DNA, resulted in further chloroplasts is present in large number (up to 5000) per cell. By specific transformation of the chloroplast DNA, the toxin part in the soluble protein content in the leaf can be increased up to 3-5%, for instance, tobacco chloroplasts contain 5000-10,000 of their genome per cell (McBride et al. 1995) with no native untransformed chloroplast genome without the toxin gene present. This has established the homoplasmic nature of transformed plants with massive number of toxin gene per cell, explaining the high level of tolerance to a specific insect in transgenic plants with no effect on plant growth rate, physiological processes or productivity (Daniell, 1999; 2000). In addition, chloroplast genetic engineering offers a number of other advantages as a plant-based expression system includes multi-genes engineering in a single transformation event, lack of gene silencing and position effects due to site specific transgenic integration, minimal, or lack of pleiotropic effects due to subcellular compartmentalization of toxic transgene products, and transgene containment via maternal inheritance (Daniell et al., 2002; Maliga, 2004). Chloroplast transformation has been achieved in a much wider range of dicot plant species, such as soybean, cotton, potato, tomato, lettuce, sugar beet, eggplant, citrus etc. (Verma and Daniell, 2007). Chloroplast transformation of monocots, including the most important food crops, such as rice, maize, wheat and sorghum has not been successful yet (Clarke et al., 2011). The levels of recombinant protein accumulation achieved by chloroplast transformation vary enormously ranging from less than <1% to more than 70% (Oey et al., 2009; Ruhlman et al., 2010).

Advantages and Disadvantages of *Bt* Plants

There are several advantages of expressing *Bt* toxins in transgenic *Bt* crops;

- 1) The resistance is inherited in a stable and Mendelian fashion (Gould, 1988).
- 2) The level of toxin expression can be very high, thus delivering sufficient dosage to the pest, which is considered necessary to delay the evolution of resistance (Bates et al., 2005).
- 3) Because the toxin expression is contained within the plant system (in microgram quantities), the potential for exposure to farm workers and nontarget organisms is extremely low, and hence only those insects that feed on the crop can be controlled effectively (Betz et al., 2000), in addition, the toxin is highly specific against insects without affecting predators and other beneficial insects (Christou, 2005; Kumar et al., 2008) because *Bt* toxins attack sites are found only in target insects (Peairs, 2010)
- 4) The toxin expression can be modulated by using tissue-specific promoters that are found in insect's midgut, so *Cry* proteins are non-toxic to humans and pose no significant concern for allergies. Food derived from *Bt*-protected crops, which have been fully approved by regulatory agencies, have been shown to be substantially equivalent to the food derived from conventional crops. Also, the *Cry* proteins are rapidly degraded when crop residue is incorporated into the soil or plant products that were fed to animals or human. Thus the environmental impact of these crops is negligible (Betz et al., 2000; Wheeler et al., 2011).
- 5) *Bt*-corn produces *Cry1Ab* protein throughout the plant, which virtually eliminates corn borer-induced tissue damage in corn products making fungal spores less able to germinate and produce fumonisins (fungal toxins) (Betz et al., 2000). Fumonisin cause death and morbidity in horses (Norred, 1993) and have been linked in epidemiological studies to high rates of esophageal and liver cancer in African farmers (Marasas et al., 1988).
- 6) Reduced applications of broad spectrum insecticides (Carpenter et al., 2002; Edge et al.,

2001; Krishna and Qaim, 2012; Romeis et al., 2006), which save money and time (Morse et al., 2004) and reduce health risks to farmers and consumers (Kumar et al., 2008), in addition to indirect positive impact on preserving beneficial insects populations and supplemental insect control by natural enemies (Betz et al., 2000; Head et al., 2001).

Perceived disadvantages of *Bt* transgenic crops may be:

- 1) Exchange of genetic material between the transgenic crop and related plant species leading to the development of so called “Super weed” (Kumar, 2002).
- 2) Increase in toxin levels in the soil may affect soil microflora (Kumar, 2002; Wu et al., 2004), however, this requires more research because Wang et al. (2006) reported no *Cry1Ab* protein in the rhizosphere soil of field-grown *Bt* transgenic rice.
- 3) Potential impact on nontarget species and the environment (Kumar, 2002). Although the transgenic *Bt* crop plants provide high levels of protection against certain insect pests, they may have consequent effects on natural enemies specializing on such pests (Ahl-Goy et al., 1995). A better understanding of the interaction between transgenic plants, pests and parasitoids is important to limit disruption of biological control and to provide background knowledge essential for applying measures for the conservation of parasitoid populations. It is also essential for investigation into the potential role of parasitoids in delaying the build-up of *Bt*-resistant pest populations (Schuler et al., 2003; Stewart et al., 2004). For example, by feeding larvae of the European corn borer *Ostrinia nubilalis* on transgenic *Bt* corn and submitting it to the natural predator *Chrysoperla carnea*, doubled the mortality rate of this predator (Hilbeck et al., 1998). Feeding the wasp *Meteorus laeviventris* on *Bt* treated larvae *Ostrinia nubilalis* increased the mortality and reduced the life span of the wasp (Hafez et al., 1997). In USA, the cultivation of transgenic *Bt* cotton has successfully reduced infestation by both *Pectinophora gossypiella* and *Heliothis virescens*, whereas *Helicoverpa zea* caused substantial damage to the crop (Roush, 1997). In other experiments with

transgenic *Cry1Ab* cotton in Australia the infestation rates by *Helicoverpa armigera* were reduced, however other pests, such as soft bugs and Thrips increased, which made the application of synthetic chemicals necessary (Fitt et al., 1994; Hardee and Bryan, 1997). Repeated cultivation of transgenic *Bt*-plants may increase *Bt* toxins in the soil and cause changes in the soil microbiology, for example research on *Bt* corn, which includes *Cry1Ab* toxin, revealed high mortality rate of the soil collembola, *Foliosomia candida* (Riegler and Stauffer, 2003).

However, recent research findings negated the effect of *Bt*-plants on non target species. Ramirez-Romero et al. (2008) found that the *Bt* maize expressing *Cry1Ab* toxin does not affect the development of the non-target phytophagous aphid, *Sitobion avenae* on young maize and that no presence of the toxin is detected in this aphid species. They suggested that there is no direct or mediated risk effect at the third trophic level (parasitoids and predators) associated with the aphid, *Sitobion avenae* on *Bt*-maize. Li et al., (2011b) stated that *Bt* cotton has no direct positive or negative effect on a non-target pest *Apolygus lucorum* in northern China and the observed outbreaks in the insect numbers is due to the decrease in the pesticide application. It was also reported that *Bt* maize plants had no sub-chronic adverse effects on non-target Coleopteran insect; *Tenebrio molitor* (Kim et al., 2012a) or *Rhopalosiphum padi* aphids (Kim et al., 2012b); however, they stated that *Cry* toxins can be transferred to higher predatory insects if they consume the aphids feeding on *Bt* maize. There would be a potential negative impact of *Bt* maize expressing *Cry3* in the food web in a scenario where *R. padi* feeding on *Bt* maize expressing *Cry3* is consumed by a typical Coleopteran predator, such as the lady beetle, as *Cry3* toxin is selectively toxic to Coleopteran insects. Thus, more detailed studies on the potential impacts of the *Bt* toxin on the food web are required.

- 4) Evolution of new and more virulent biotypes of the pests because insects are capable of developing high levels of resistance to one or more *Cry* proteins (Kumar et al., 2008). As

such, the development of resistance in target pests to *Bt*-plants is considered the main risk for long-term success of this technology (Farinós et al., 2011). So far, field evolved resistance to *Bt* maize has been documented in two species, the African stem borer, *Busseola fusca* (Lepidoptera: Noctuidae) (Kruger et al., 2009; Van Rensburg, 2007) and the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Matten et al., 2008; Storer et al., 2010). In addition, laboratory selection assays have shown that laboratory populations of *Ostrinia nubilalis* (Lepidoptera: Crambidae) can develop low to moderate levels of resistance under intense selection pressure from *Bt* toxins (Crespo et al., 2009).

- 5) Reliance on one *Cry* protein may increase the probability of *Bt* resistance development in target pest (Addison, 2010; Tabashnik et al., 2009), for example, pyramided *Bt* corn hybrids were very effective, compared to single gene *Bt* corn hybrids, against sugar cane borer, *Diatraea saccharalis* (Ghimire et al., 2011; Wangila et al., 2012), the dominant corn borer species in many areas of US gulf coast region (Huang et al., 2012).
- 6) Fluctuations in toxin concentration occurs due to various factors such as leaf age (Le et al., 2007; Wei et al., 2005), growth conditions (Le et al., 2007; Sachs et al., 1998), nutrient availability (Coviella et al., 2002), CO₂ level (Chen et al., 2005; Coviella et al., 2002; Wu G. et al., 2007) and elevated CO₂; temperature and tropospheric ozone (O₃) could hasten *Bt* resistance in target insect (Himanen et al., 2009).
- 7) The toxin gene can be expressed in the chloroplast genome and thus the possibility of gene transfer via pollen (Daniell, 2000) is highly variable (Szekacs et al., 2010). It was reported that pollen from *Bt* corn is highly toxic to Monarch butterflies in the laboratory (losey et al., 1999), but follow up field studies showed that under real-life conditions Monarch butterfly caterpillars rarely come in contact with pollen from corn (losey et al., 1999; Sears et al., 2001). A similar conclusion was reached for the pale grass blue butterfly, *Pseudozizeeria maha* in Japan (Wolt et al., 2005). Recently, Holst et al. (2013) reported a potential environmental risk of the field

cultivation of insect-resistant (*Bt*-toxin expressing) transgenic maize due to the consumption of *Bt*-containing pollen by herbivorous larvae of *Inachis io* butterflies (Lepidoptera) in European farmland. In a more detailed analysis, they found that in northern Germany, where *Inachis io* is mostly univoltine, the cultivation of *Bt* maize would pose a negligible risk, because pollen shedding is predicted later than larval feeding, but in southern Germany, where *Inachis io* is bivoltine, the second generation of larvae coincides with the peak of maize pollen deposition and consequently is at risk. They concluded that the population-wide effect of *Bt* maize on insect species will depend on: the species-specific susceptibility to *Bt* toxins; the actual exposure to the toxin, and the ecology of the species. This conclusion confirmed previous report by Peterson et al. (2006) who modeled the phenology of both maize pollen shedding and Karner blue butterflies, *Lycaeides melissa samuelis* larval feeding, and combined this with a GIS-based analysis of the co-occurrence of *Bt* maize fields and larval habitats, concluding that in most places and for most years, maize pollen shedding would occur after the majority of the butterfly larva population had finished eating.

Bt-Resistant Pest Population

The potential of pest to develop resistance against the defense mechanisms of crops is documented and is not unique to genetically engineered plants only. More than 500 insects and mites already have acquired resistance to a number of insecticides (McGaughey and Whalon, 1992) and similar resistance to *Bt* toxins were developed in several major pests, including the tobacco budworm, Colorado potato beetle, Indian mealy moth (Tabashnik et al., 2003), maize stalk borer (Van Rensburg, 2007), cotton bollworm (Tabashnik et al., 2008) and the fall armyworm (Storer et al., 2010). The diamondback moth (Marois et al. 1991; McGaughey et al., 1998) and fall armyworm is already known to have evolved a resistance to *Bt* in spray form (Blanco et al., 2010), consequently loss of the effectiveness of *Bt* preparations. This would mean an enormous damage particularly for the organic agriculture, where these preparations are

only certified as efficient Bio-pesticides (Madigan and Martinko, 2005).

Due to constant exposure to the toxin, an evolutionary pressure is created for resistant pests and the expression of the *Bt* gene can vary. For instance, if the temperature is not ideal, this stress can lower the toxin production and make the plant more susceptible to insects attack. More importantly, reduced late-season expression of toxin has been documented, possibly resulting from DNA methylation of the promoter (Dong and Li, 2007; Müller-Cohn et al., 1996). *Bt*-toxin resistance evolution in herbivore insects has been raised as a severe threat for the continuing success of *Bt*-transgenic crops (Tabashnik et al., 2008).

There is also another risk that, for example, transgenic maize will crossbreed with wild grass variants, and that the *Bt*-gene will end up in a natural environment, retaining its toxicity. An event like this would have ecological implications (Wu et al., 2004), as well as increasing the risk of *Bt* resistance arising in the general herbivore population (Kumar, 2002). The frequency of resistance alleles in *Helicoverpa zea* to *Bt Cry1Ac* cotton have been reported to increase in field populations, but resistance management tactics, such as refuge requirements (Frisvold and Reeves, 2008) and concurrent expression of several toxins in the same plant (Ibargutxi et al., 2008) have been successful in delaying the onset of resistance (Tabashnik et al., 2008), especially that insect-specific CPs cannot be changed during a growing season (Habuřtová et al., 2012; Webber 2006).

Resistance Management

There are many methods for delaying the appearance of resistant strains of the insect to *Bt* crops. One of the key factors for successful resistance management is the timely implementation of monitoring programs to detect early changes of susceptibility in the field populations in relation to the original susceptibility of the population (Farinós et al., 2004), jointly with the implementation of resistance management strategies to prevent or delay the emergence of resistant populations. Different strategies have been proposed to delay insect resistance to *Bt* crops, but the “high dose/refuge strategy” (HDR) is the most widely used, and it is mandatory in most countries where

Bt crops are grown (Farinós et al., 2011). This strategy combines the use of *Bt* crops that express high concentrations of *Cry* toxins and the planting of refuges of non-*Bt* crops near *Bt* crops. The aim is to encourage a large population of pests so that any genes for resistance are greatly diluted, and thus reduction of resistance heritability. This technique is based on the assumption that resistance genes will be recessive (Bravo and Soberón, 2008; Blanco et al., 2010; Carrière et al., 2010; Liu and Tabashnik, 1997; Storer et al., 2010; Tabashnik, 1994; Tabashnik et al., 2004). Gao et al. (2010) stated that the resistant alleles will be diluted by susceptible moths produced from surrounding areas, which may be a major factor contributing to maintenance of susceptibility in this species to *Cry1Ac* after a decade of large-scale planting of *Bt* cotton. Li et al. (2011a) found that based on the relative average development rates (RADR) of *H. armigera* larvae in F1 generation, no substantial increase in *Cry1Ac* tolerance was found over the 3-years period. It appears so far to be a successful method of delaying widespread resistance to *Bt* toxins (Carrière and Tabashnik, 2001; Frisvold and Reeves, 2008; McGaughey et al., 1998; Tabashnik et al., 2003). Increasing *Bt* expression levels (high dose/structured refuge strategy), which has been adopted for planting the first generation *Bt* corn that expresses a single *Bt* protein (e.g. Yield Gard *Bt* corn), is based on the assumptions that resistance in the target species should be recessive so that a high percentage of resistant heterozygotes can be killed by “high dose” expressed *Bt* corn (Andow and Hutchison, 1998; EPA, 2001); however, some recent findings reported that single gene *Bt* corn did not express a high dose of *Bt* protein, as desired for the high dose/refuge strategy (Ghimire et al., 2011; Wu X. et al., 2007).

Alternately, creating a mosaic genetically modified (GM) crop expressing many different *Bt* toxins would have a greater chance of eliminating the entire pest population and thus eliminating resistance alleles (Atkinson, 2006; Ibargutxi et al., 2008). Expressing multiple toxins, that is, gene pyramiding, which is a strategy employed to develop transgenic plants that express that multiple *Bt* proteins is more effective than one protein in targeting the same group of insect pests (Manyangarirwa et al., 2006; Monsanto, 2012; Shelton et al., 2002; Suresh and Malathi, 2013; Wangila et al., 2012). Because *Cry1Ab* and *Cry1Ac* are very

similar in their structure and function, resistance to one *CryIAb* protein would most likely impart resistance to another *CryIAc* protein as has already been observed with the tobacco budworm. Nowhere is this more of a concern than with cotton bollworm/corn earworm that usually feeds on corn during spring and early summer, then migrates to cotton to complete several more generations during summer and early fall (Wearing and Hokkanen, 1995). Clearly, different *Bt* proteins are needed to decrease the development of resistance (Atkinson 2006; Ibargutxi et al., 2008).), for example pyramided *Bt* corn hybrids were very effective, compared to single gene *Bt* corn hybrids, against sugar cane borer, *Diatraea saccharalis* (Ghimire et al., 2011; Wangila et al., 2012), the dominant corn borer species in many area of US gulf coast region (Huang et al., 2012).

Another method is expressing the protein only in tissues highly sensitive to damage (tissue specific expression) (Fearing et al., 1997; McGaughey and Whalon, 1992). By means of spatial and temporal regulation of toxin expression, DNA- technology is offering promising solutions to minimize the residues of *Bt* toxins in soil and delaying build-up resistant strains of insect pests. For example, by expressing insecticidal proteins in chloroplasts, toxins can be put in the part of a plant where they are most likely to be consumed. The toxin gene can be expressed in the chloroplast genome and thus the possibility of gene transfer via pollen (Daniell, 2000), in which toxin concentration is highly variable (Szekacs et al., 2010). Most caterpillars feed on green tissues that are rich in chloroplasts; therefore, they consume the highest level of insecticidal toxins if the toxins are placed in chloroplasts. Related studies indicated that high levels of expression of *Ccry2Aa2* in transgenic tobacco did not affect growth rates, photosynthesis, chlorophyll content, flowering, or seed forming under laboratory conditions (EPA, 1995; Fearing et al., 1997). Gómez-Barbero et al. (2008) reported a significant yield advantage of *Bt* maize over conventional maize. Incorporating these genes in the chloroplast offers several advantages, including the ability to place foreign genes at a specific location in the plant cell and to increase the levels of toxic proteins in the plants. Furthermore, because chloroplast genes are inherited through the mother (ovary) instead of the father (pollen), the risk for out-crossing (foreign genes escaping to other

species) to other plants, such as weeds, is reduced (Mikkelsen et al., 1996). Gene transfer between cultivated plants and wild species is well-known. By out-crossing or introgression, the inserted genes of transgenic plants can be transferred over pollen to the same species of cultivated plants and wild species. The out-crossing rate is however different depending upon plant type and geographical location and must be regarded (Pascher and Gollmann, 1997). There are many examples such as strawberries, carrot, corn, sorghum, sunflowers and sugar beet where out-crossing is conceivable into other species (Gray and Raybould 1998; Kling 1996). Stewart et al. (1997) enumerates nine species of *Brassica napus*, to which an out-crossing is possible within short time.

Horizontal gene transfer simply means spreading of genes between very different species in non sexual ways. Moreover, it takes place via transduction, transformation, conjugation, as they arise frequently with bacteria, as well as via the activity from transposing, retroviral and other infectious agents. Thus, it differs from the vertical gene transfer of vegetative and sexual form inclusive species hybridizing and introgression. Like that some sequence homologue between bacteria and plants are well-known, which suggest a gene transfer in the course of the evolution. A possible gene transfer of *Bt* toxin genes on related *Bacillus* sp. is also conceivable (Schlüter and Potrykus, 1996). During the risk estimation of transgenic plants, a possible horizontal gene transfer should be considered primarily by plants to soil bacteria and mushrooms (Lorenz and Wackernagel, 1994). A restriction of the out-crossing can take place via transformation of the chloroplast. The propagation of the transgenes over pollen is thus prevented, since plastids are left purely maternal (Daniell et al., 1998; Schlüter and Potrykus, 1996). McBride et al. (1995) reported that there is a high level of expression of *Cry2Aa2* in tobacco chloroplasts compared to *Bt* genes inserted into plant nuclei. This is likely because DNA can be expressed better in chloroplasts than in nuclei, and there are many chloroplasts within a plant cell, but only one nucleus. DNA placed in the chloroplasts will be copied 5,000 to 10,000 times in a cell, while typically there are only one to four copies of the gene per cell when it is contained within the nucleus. Also, plant cells can express smaller genes (*Cry2Aa2*) better than larger (*CcryIAc*) genes

(Daniell et al., 1998; Feitelson et al., 1992; Fitt et al., 1994).

Conclusion

Bacillus thuringiensis (*Bt*) is insect-pathogen bacteria, its impact is mainly due to the synthesis of *D*-Endotoxin. This diverse genus also includes more than 20 other *Bacillus* species and hundreds of different subspecies. Members of the genus *Bacillus* are generally considered soil bacteria, and *Bt* is common in terrestrial habitats including soil, living and dead insects, insect feces, granaries, and on the surfaces of plants. When a susceptible host, eats the crystal, part of it binds to specific gut-receptors, penetrates, and collapses the cells lining its gut, causing death.

For decades, produced *Bt* preparations consisting of spores and toxins are registered as Bio-pesticides. Advantages of the *Bt* preparations are high specificity, short persistence and thus a relatively high environmental compatibility. UV radiation breaks down *Bt* and rain washes it from the plants. Therefore, *Bt* must be applied exactly where and when the target insects are feeding and they must consume it quickly before it disappears. The use of conventional *Bt* preparations is limited against few insect pests. By the conjugation of different *Bt* strains, different plasmids can be combined with toxin genes in a single strain, in order to expand the host spectrum of targeted insects. *Bt* preparations degrade in a short time after application and this short duration of effect forces repeated spraying. *Bt* in spray form is environment friendly and considered as certified efficient Bio-pesticide for organic agriculture.

Plants can be genetically engineered to produce their own *Bt* crystal protein, which is toxic to the pest species of concern. As the insect feeds on the plant, it ingests the crystal protein and suffers the same fate as if it ingested leaf tissue sprayed with *Bt* (Roush and Shelton, 1998). The use of commercial crops expressing *Bt* toxins has increased in recent years due to their advantages over crops that require traditional chemical insecticides.

Some advantages to the use of transgenic *Bt* plants compared with foliar sprays of *Bt* are as follows: protection of the plant through the entire vegetation period; minimizing the residues and the side effects of pesticides in the environment, and systemic protection of plants against insects, for example, stem borers, or resistant insects, which already developed resistance against chemical insecticides. On the other hand, due to the constant exposure to the toxin an evolutionary pressure is created for resistant pests, and hence the expression of the *Bt* gene can vary. The potential of pests to develop resistance against the defense mechanisms of crops is well-known, and is not unique to genetically engineered plants. Because more than 500 insects and mites already have acquired resistance to a number of insecticides, there is concern that similar resistance to *Bt* toxins could develop. In addition, although the transgenic *Bt* crop plants provide high levels of protection against certain insect pests, their consequent effects on non target species is still controversial, in spite of recent findings that proof that there are little or no effect on non target species.

Within the next few years, crops that have been genetically engineered for *Bt* resistance could dramatically lower production costs and provide farmers with new insect control options. The success of their commercialization depends on several factors, including the regulatory climate, patent issues, and the ability of scientists to deal with targeted insects that develop resistance to lethal proteins. Before a *Bt* plant is released, and especially before it is authorized for commercial cultivation, tests have to be carried out to check that this plant will not be associated with any harmful impacts on non-target organisms. In addition, they have to reach a result within a reasonable amount of time, as well as take into account complex ecological relationships.

Hence, it can be concluded that using of transgenic *Bt*-plants is so far promising for the future of crop protection, however, some cautions, related to the unknown future effects on human health and other organisms, should be taken into account and more research conducted to proof that.

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