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Understanding the impact of *Bacillus thuringiensis* proteins on non-target organisms

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Abstract— *Bacillus thuringiensis* (*Bt*) is a spore-forming, gram-positive, aerobic, rod-shaped bacterium. During sporulation, *Bt* produces proteinaceous crystals called Cry proteins that are lethal to many insects' species, so are commonly used as biological pesticide. Transgenic *Bt* crops are genetically altered to express insecticidal toxins that cause fatality of a number of general agricultural pests. The insecticidal toxins formed by *Bt* crops possess narrow range of toxicity and therefore less non-target impacts as compared to conventional insecticides. A decrease in the amount and regularity of insecticide applications are financially advantageous. In numerous regions of the world, insecticide inputs have been significantly reduced because of *Bt*. The use of *Bt* crop technology might help in worldwide food security by escalating the amount and steadiness of crop yields. Though impact of *Bt* toxin on non-targeted organism is a serious issue yet no conclusion could still be drawn from several studies. This review summarizes the benefits of *Bt* crops including the impact on non-targeted organisms and *Bt* toxins having potential risks with respect to the environment.

Keywords— *Bacillus thuringiensis, Bt toxin, Cry proteins, environment, non-target organisms*

I. INTRODUCTION

Bacillus thuringiensis (*Bt*) is a gram-positive, aerobic, spore-forming, rod-shaped bacterium. An important trait of *Bt* is the production of proteinaceous crystals at the time of sporulation. These crystal proteins commonly called Cry proteins possess toxicity to many insects' species and thus are commonly used as biological pesticide. *Bt* belongs to *Bacillus cereus* group. However, it is differentiated from *B. cerus* by the presence of a plasmid that produces the crystal protein [1]. 2014). During sporulation, Cry protein encoding genes become active as they are controlled by RNA polymerase that gets synthesized with the formation of spores [1, 2, 3].

Bt was originally isolated in 1901 by a Japanese biologist Shigetane Ishiwatari. He investigated that the sotto disease (sudden-collapse disease), which killedhuge populations of silkworms(*Bombyx mori),* was caused by *Bacillus thuringiensis*. Initially, Ishiwatari named the bacterium *Bacillus sotto*. Later in 1911, Ernst Berliner isolated bacteria that killed a Mediterranean flour moth (*Ephestia kuehniella*) and rediscovered *Bt*. He renamed it *Bacillus thuringiensis* after Thuringia, the German town where the moth was found [4]. *Bt* is a soil dwelling, ubiquitous bacteria, and has been found in all kind of topography, including desert, beaches, and tundra habitats. Itis largely used in agriculture, especially organic farming [5].This article reviews impact of *Bt* toxins and *Bt* derived transgenic crops on the environment including non target organisms, their benefits and drawbacks. Rest of the paper is organized as follows, Section II describes the structure of *Bt* protein, Section III describes the mode of action of Bt endotoxins, Section IV contain the information about applications of *Bt* technology, section V explain thebenefits of *Bt* technology, Section VI describes potential risks of *Bt* crops, Section VII contain the impact of *Bt* toxins on non targeted organisms, and Section VIII concludes review with future directions.

II. Structure of *Bt* protein

Cry toxins and Cyt-toxins are two types of insecticidal proteins found during sporulation when crystalline bodies are produced. Cry toxins are so called as they are present in form of crystals. Mnemonic Cry was derived from parasporal crystal proteins of *Bacillusthuringiensis* which demonstrated noticeable toxic effects on target organismsor on other proteins which show homology to Cry protein. Cyt-toxins are named due to *in vitro* cytolytic activity possessed by them. Mnemonic Cyt referred to parasporal crystal proteins of *Bacillus thuringiensis* showing hemolytic activity, as well as to other protein having homology to Cyt protein. This kind of naming system has been accepted for the vegetatively produced *Bt*-toxins. These proteins have been précised the mnemonic Vip [6, 7].

X-ray crystallographic methods revealed the 3-dimensional crystal structure of the Cry-proteins *viz.*coleopteran specific Cry3Aa, Cry3Bb and Cry8Ea1 [8, 9, 10], dipteran specific Cry4Aa and Cry4Ba [11, 12], lepidopteran specific Cry1Aa [13] and lepidopteran/dipteran specific Cry2Aa [14]. Moreover, 3-dimensional structure of Cyt-proteins comprisedof stimulated Cyt2Ba and untreated Cyt2Aa [15, 16].

The general 3-D crystal structure of most of the Cry toxins and Cyt-toxins has been illustrated to be similar, each containing three domains [1, 17]. Domain I is made up of seven α-helices where the central helix-α5 is hydrophobic and is surrounded by six amphipathic helices. The helical domain I share structural resemblance with other pore forming bacterial toxins like diphtheria toxin, colicin A and cytolycin A [18]. Outer helices of domain I are amphipathic in nature and are longer than 30 Ǻ in length [19]. In domain II three antiparallel β-sheets piled up to form a β-prism with pseudo three-fold symmetry [8]. Out of three, two are made up of four strands in the form of a Greek key motif and are exposed to solvent [12]. The third sheet is stacked next to domain I and is also assembled in a Greek-key-like pattern with a petite alpha-helix and three strands [19]. Domain III has been revealed to comprise of two antiparallel β-sheets that look like a β-sandwich structure having jelly roll topology [12]. Both the sheets are made up of five strands, with the inner sheet packing against domain II and outer sheet towards the solvent. Two long loops stretch from one end of the domain and get in contact with domain I [13].

III. Mode of action of *Bt* endotoxins

Bt toxins get activated in the gut, therefore,for insect mortality to occur, they should be eaten by the insect. *Bt* endotoxin crystals dissolve in the alkaline ambiance of midgut of host insect [20].

The proteins are present as protoxins which get activated in the presence of specific protease. As a result of proteolysis, toxins bind to receptors present in the brush border membrane of midgut which open the pores that cause disruption in the solute movement through the gut epithelium and torrent of water, resulting in death of the host host [21]. Alkaline conditions, specific receptors and specific proteases are required for proper working of *Bt.*It is not detrimental to mammals as these conditions are not available as such [22]. Mode of action of *Bt* involves:

Firstly the insect consumes *Bt* crystals and spores. In alkaline medium of gut of insect, activation of *Bt* pro-toxin to activated *Bt* toxin occurs. Then *Bt* toxin bind to specific receptors present in the gut.These proteins then putrefy the wall of gut, permitting the gut bacteria and spores to penetrate the host body.The host insect dies due to starvation and proliferation of spores take place [23].

IV. Applications of *Bt* technology

Bt crops

Genetic engineering of certain crops has been done to integrate genes which are derived from other species and offer agronomic and nutritional advantages, like resistance to viruses, insect pests, or to ecologicalsettings like low water availability. Transgenic *Bt* crops are genetically altered to express insecticidal proteins that cause fatality toa number ofgeneral agricultural pests. The genes from *Bacillus thuringiensis*coding for such proteinsare introduced into the genome of the preferred crop plant. Crop plants that have been genetically altered to express *Bt* toxins include cotton, sweet corn, potato, eggplant, oilseed, rice, rape (canola), broccoli, tomato, chickpea, collards, spinach, tobacco, cauliflower and soybean [24].

Chitinase production

Chitinases are the chitin degrading enzymes that can be found in the exoskeletons of arthropods, cell walls of fungi, and the shells of nematodes and crustaceans [25]. Chitinases possess numerous uses in the sphere of medicinal services due to their immunomodulating and antibacterial impacts, as well as in horticulture for management of plant pathogens and creation of antifungal substances [26]. Chitinase enzymes were found to be present in *Bt*. After several decades of field use; studies have reported the biological activity of bacterial chitinase [27, 28, 29]. Chitinases act synergistically with Cry and therefore provide improved rates of mortality. These enzymes break the invertebrates' chitinous exoskeleton and allow bacteria to invade the tissues, thus leading to septicemia followed by death. Researchers have tried to accelerate the production of *Bt* biopesticides, as chitinases increase the *Bt* toxicity in biological tests. To ensure the increased production of *Bt* biopresticide, chitin was supplemented to the culture media to stimulate the secretion of chitinase by bacteria [24].

V. Benefits of *Bt* Technology

Decreased Risk as Compared to Conventional Insecticides

The insecticidal toxins produced by *Bt* crops have narrow range of toxicity and therefore less non-target impacts as compared to conventional insecticides. For example, numerous natural enemies reactedharmfully to foliar applications of wide-range pyrethroids in contrast to selective insecticides like *Bt* toxins, indoxacarb, and spinosad that were used to fight lepidopteran pests in sweet corn agroecosystems [30]. Lesser herbivore and predator abundance was found in non-transgenic control plots treated with insecticides as compared to unsprayed *Bt* fields. This effect was chiefly observed for predator populations in non-transgenic plots treated with pyrethroids like cyfluthrin, bifenthrin and lambda-cyhalothrin [31]. Likewise, more abundance of spiders was observed in *Bt* cotton, corn, and potato as compared to crops managed by conventional insecticides including systemic neonicotinoid seed treatments, foliar pyrethroid sprays, and organophosphate soil applications at planting [32].

Financial Savings

A decrease in the amount and regularity of insecticide applications are financially advantageous. In numerous regions of the world, insecticide inputs have been significantly reduced because of *Bt* cotton. It is apparent that *Bt*-based production systems are more sustainable with reference to pest management and also encompass the ability to improve agricultural diversity through reduced chemical inputs [33].

Worldwide Food Security

With the increasing human population there is need for augmented global food production and security. The use of *Bt* crop technology might help in this goal by escalating the amount and steadiness of crop yields, for example, corn yields were protected or increased by the management of European corn borer. In addition, stored *Bt* corn is protected against lepidopteran pests and mycotoxin which if introduced in food supply, pose a threat to the humans and livestock health [33].

VI. Potential Risks of *Bt* Crops

Presence in Human Food Supply

In digestive tract of vertebrates, *Bt* toxins hastily break down due to presence of acidic environment [34]. Transgenic corn (*Bt* corn) containing Cry9C proteins, commercially available under name StarLink™ were planted in the United States.Due to their importunate presence in vertebrate gut these were approved only for ethanol production and animal feed. StarLink and Taco Bell® taco shells which were meant for human consumption were confiscated from the market when Cry9C protein traces were found in cornmeal [35]. In spite of this, none of the allergenic reactions were reported due to presence of Cry9C in food products. Irrespective of lack of evidences about the risk associated with *Bt* food products, consumption by humans and commercial acceptance for some products like *Bt* potatoes has been persuaded in various countries [36].Thus, in spite of restricted outcomes on the human population, precautions need to be maintained to exclude the existence of unapproved genetically modified products inflowing the human food chain.

Pleiotropic Effects of Genetic Transformation

Incorporation of a *Bt* gene into a crop plant results in astounding and inadvertent pleiotropic consequence that alters the plant from its non-transgenic form [37]. Increased lignin content in transgenic plants was reported as pleiotropic effect in *Bt* corn. This trait resulted in decreased decomposition rates in the soil [38]. Nonetheless, further reports have challenged this inference and exhibited no dissimilarities in decomposition rate [39]. In corn, a superfluous pleiotropic consequence of Cry1F transformation in corn has been reported in corn leafhoppers *Dalbulusmaidis* (Hemiptera: Cicadellidae), which is a non *Bt* target organism, probably due to altered plant traits, like foliar pubescence, leaf vein characteristics and plant chemistry [40].

VII. Impact of *Bt Toxins* on Non Targeted Organisms

Extensive studies have been conducted past 20 years on the harmful effects of *Bt* toxins on non- targeted organisms and various aspects of environment. Contrasting evidence of effects on non-targets, ranging from unobservable effects of intake of *Bt* transgenic crops [41, 42, 43, 44], to number of harmful effects (*viz.* delay in development, reduction in weight gain, changes in behavior or increased mortality) on beneficial organisms like pollinators [45], non-target arthropods [46,47,48], parasitoids [49] and predators [50, 51] are present.

Pollinators and butterflies

In terrestrial ecosystem, pollinators occupy an important place. Honey bees being the most profuse and prevailing pollinators globally, were used as indicators for the Pest Management Regulatory Agency of *Bt* crops [52]. Feeding trials with plant pollen containing *Bt* toxins have been carried out on honey bees extensively with no lethal effect on their prolonged existence, nourishment, behavior, dismutase activity, growth of hypopharyngeal glands and bacterial communities present in their intestines [53, 54, 55, 56]. In contrast to these studies Han et al. [57] reported that honey bees reared on pollens of cotton plants expressing Cry1Ac and CpTI toxins exhibited a disturbing feeding behavior. Duan et al. [58] reported no adverse effect on the survival and development of *Oriusinsidiosus* (Heteroptera: Anthocoridae) nymphs, when the nymphs were continuously fed on bee pollen diet with a hazard exposure dose of the Cry3Bb1 protein for about 14 days. Niu et al. [59] conducted a study on *A. suturalis* and *H. luteolus* to estimate the toxicity of *Bt* cotton varieties expressing Cry1Ac, Cry2Ab, Cry1Ac toxins. No considerable inflation was observed in the mortality of either species after nurturing them on *Bt* cotton plants for about 7 days. General studies exhibited negligible or no lethal effect of *Bt* toxins on pollinators, nevertheless the risks associated may be depend on the type of insect and experimental system [60, 61].

Microorganisms and macroorganisms
The possible impact of *Bt* crops on micro and macroorganisms depend on the biological activity and persistence of *Bt* proteins. *Bt* toxins have the characteristic to bind to clay particles and humus in soils, and become defiant to biodegradation, but retain larvicidal activity [62].

Table 1: Important studies on effect of *Bt* proteins on non target organisms (2015-2019).

Non Target Organism: *Category*	Non Target Organism: *Name*	Cry protein	Host plant	Effect	Reference
Fungi	arbuscular mycorrhizal fungi	*Cry1Ab*	maize	No negative effect	[83]
Plant	*Brassica juncea*	-	Rapeseed	Protected plant growth, increased biomass and seed production	[84]
Insects	*Diabrotica virgifera virgifera*	*Cry3Bb1 Cry34/35Ab1*	corn	reduced survival	[85]
	Daphnia magna	*Cry1Ab*	maize	negative fitness effect	[86]
	Propylea japonica	*Cry1C or Cry2A*	rice	negligible risk	[87]
	Apis cerana cerana	*cry1Ah*	corn	no risk for the survival and development	[88]
	Spodoptera litura	*Cry 1 Ac*	cotton	larval mortality and increased the stages and duration of development	[89]
	Diabrotica virgifera virgifera	*Cry34/35Ab1*	corn	No effect	[90]
	Aphis gossypii, Propylea japonica	*Cry1Ah and Cry2Ab*	-	No effect	[91]
	Apis mellifera	*Cry1Ie*	-	no risk to survival, pollen consumption, or learning capabilities	[92]
	Apis mellifera	*Cry1Ac/EPSPS or Cry1Ac/2Ab*	cotton	minimal risk for negative effects	[93]
	Macrocentrus cingulum	*Cry1Ac*	maize	No negative effect	[94]
	Helicoverpa armigera.	*Cry1Ac*	-	no effect on resistance	[95]
	Nilaparvata lugens, *Pardosa pseudoannulata*	*Cry1Ac, Cry2Aa and Cry1Ca*	rice	negligible effect	[96]
	Adelphocoris suturalis, Haptoncus luteolus	*Cry1Ac/Cry2Ab Cry1Ac/EPSPS*	cotton	No effect	[97]
	Folsomia candida	*Cry1C and Cry2A*	rice	Nontoxic and No negative effect	[98]
Mammals	rats	*Cry1AC*	cotton	No lethal effects	[81]
	Wuzhishan Pigs	*Cry1Ab*	rice	no unintended adverse effects	[99]
	rabbits	*Cry1Ac, Cry2A*	Cotton seed	Adverse affect on rabbit's haematological profile.	[100]

Generally, no deadly effects of Bt proteins on macroorganisms like collembola and mites have been reported [63, 64, 65]. In a study, *Bt* maize straw brought about alteration in bacterial community of *Eisenia fetida* casts [66]. A number of studies on the effects of *Bt*crops on soil microorganisms have failed to find any significant effects in laboratory experiments, in microcosm, and under field conditions. To date, the direct effects of *Bt* protein on soil microorganisms is unclear [67, 68, 69, 70, 71, 72].

Aquatic organisms

Even though water organisms are not directly exposed to *Bt* proteins present in crop plants but indirectly *Bt* toxins can effect aquatic organisms, exposed due to run-off transfer of *Bt* residues bound to soil and crop material. Quite a few studies were done to assess the effects of *Bt* vegetation on water organisms [73, 74, 75, 76]. Order Trichoptera have aquatic larvae. Numerous Cry toxins like Cry1Ab, Cry1Ac, Cry1A.105, Cry1F, Cry2Ab2, and Vip3A aim the Lepidopterans and consequently, can also target the phylogenetically near Trichoptera. Also a few studies on crustaceans pointed out the possible risks of *Bt* toxins to Daphnids [77]. Different effects on *Daphnia* fed with Cry1Ab-containing maize were, decreased fecundity, decreased total number of eggs, less individuals attaining maturity and less number of juveniles per stage [73, 77]. However, Holderbaum et al. [73] showed no effect of Cry1Ab maize on cumulative fecundity and age at maturation. In *Hyallelaaszteca* no effect on mortality and growth was observed when fed with Cry1Ab-containing maize but showed increased mortality when fed with Cry1Ac-containing cotton [74, 78]. Since literature provides mixed response of *Bt* in aquatic organisms, therefore additional research is needed for the evaluation of effects of *Bt* crops on aquatic organisms.

Birds and mammals

Experiments were carried out to study the effect of *Bt* transgenic crops fed to livestock, poultry and other animals. The parameters studied were hematology, immune response, growth rate, weight gain, food intake, feed efficiency. No effect of *Bt* maize was observed on calves and pigs in some studies. However, certain other studies report higher level of severe inflammation in stomach, higher uterus weight in pigs, lower proportion of T helper and T cytotoxic cells within lymphocytes and higher spleen weight in broilers when fed with *Bt* maize [79]. Sajjad et al. [80] studied the effect of *Bt* cotton on mice and concluded no harmful effect on model animal. Also, no fatal consequence of transgenic *Bt* crystals on the continued existence of rats and earthworm was observed [81]. Thus, several researchers have reported the toxic effects of *Bt* proteins on animals while others showed no harmful effects [82]. It is the task of researchers to evaluate of effects of *Bt* crops on animals with further studies.

VIII. Conclusions

The chance of occurrence of *Bt* toxins in the environment and its effect on non target organisms is complicated and inconsistent. It totally depends upon crop type, transgenic incidents, geographic conditions, and other factors. The impact of *Bt* toxins on the environment is extensively debatable. Various approaches have been proposed for future research regarding environmental management of *Bt* crops and their incorporation into integrated pest management and resistance management systems. Regardless of the concerns linked with *Bt* crops, substantial reductions in chemical usage is observed and this approach is environmental friendly as compared to other pest restraint approaches, especially those methods in which broad-spectrum insecticides are used.

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