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Review Article

Impact of Insecticidal Cry Proteins on Soil Biota: A Metareview

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Abstract

Genetic engineering offers the opportunity to develop plants with a variety of new traits that may be useful in pest control, provide resistance to extreme weather conditions as well as aid in bioremediation. One of the most popular genetically modified traits is Insect Resistance (IR). Larvicidal Cry protein producing genes of Bacillus thuringiensis are inserted in the plant body to make it resist the pest attack. But fate of these proteins in the plant biomass, soil and entire agroecosystem is highly contentious, as such genetic modification in plants requires detailed and deep understanding of the plant mechanisms. There is great complexity in this area of research as current understanding of the interactions of Bt crops with soil biota is lacking. This paper is in direction to assimilate and correlate the existing secondary data on the impact of Cry proteins on the soil biota. Aim is to bring together variegated research at the same table and analyse it through the impartial eye of a scientist to draw a honest and fair conclusion. Thus, a comprehensive analysis of published literature reporting the fate and effect of Cry protein on soil ecosystem was assessed. A meta review approach was adopted with three-dimensional study of effects of cry protein on soil microorganisms, soil dwelling invertebrates and other non-target organisms. The review highlighted the contrasting and statistically insignificant findings in many studies. Further, it calls for rigorous chronic studies on complicated system of plant-microbe interface, to develop a better and improved understanding of ecological compatibility of Bt crops and their impacts on soil biota.

Keywords: *Bt Crops, Cry Protein, Soil microorganisms, Insect resistance, Soil biota, Non-target organisms.*

Introduction

Bacillus thuringiensis (Bt) an aerobic, gram-positive, spore forming soil bacterium produces insecticidal crystal proteins. These spores and protein crystals have been in use as microbial insecticides since the early 1960s, using different strains of the bacterium to produce various combinations of Cry proteins, which are considered to be selective and target insects of a specific order. This bacterium has been extensively used as a source of genes for the expression of pest resistance in transgenic plants. Truncated forms of the genes that code for these toxins have been genetically engineered into plants that express the active toxins rather than the inactive protoxins. When modified cry genes are inserted into a plant species like Corn, Cotton and Potato, the plant expresses active larvicidal proteins in its tissues, making the plant toxic to various insect pests (Icoz *et al.*, 2008) these cry proteins are synthesized and secreted throughout the plant body including pollens and root exudates all through its growth cycle. As a high gut pH and specific proteases are not essential to activate these proteins, they could have potential impact on beneficial nontarget insects, as well as organisms at higher and lower trophic levels (Icoz and Stotzky, 2008). Hence, the largescale release of Bt crops remains a concern due to their presumed potential ecological and environmental risks. It has been claimed that the impact of these proteins and their persistence in soil is essentially governed by

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the amount added; rate of consumption and inactivation by insect larvae; rate of degradation by microorganisms; and rate of abiotic inactivation (Han *et al*., 2013). If production exceeds consumption, inactivation, and degradation, the toxins could accumulate to concentrations that may enhance the control of target pests or constitute a hazard to nontarget organisms, such as the soil microbiota, beneficial insects and other animal classes (Romies *et al* 2013). This persistence is also increased when the toxins are bound on surface-active particles in the environment and, thereby, rendered more resistant to biodegradation while retaining toxic activity. This could also result in the selection and enrichment of toxin-resistant target insects offsetting a severe agro-ecological mayhem. In this study, an extensive review of literature examines the reported and well documented impact of Cry proteins on soil biota including soil microorganisms, invertebrates and other non target species in the following sections.

Materials and Methods

The impact of cry protein from Bt plants was researched and reviewed on the soil biota. The detailed meta-review was synthesized by assessing the impact of insecticidal proteins on the following three aspects:

1. Effect on soil microorganisms
2. Effect on soil dwelling invertebrates
3. Effect on other non-target organisms

The published research on these aspects was studied and analyzed. The highlights of the various research findings were assessed for statistical significance and tabulated summaries were generated for better comprehension. The results were drawn into a three-dimensional Meta review for increased lucidity in understanding the risk Cry proteins pose to agro-ecosystem.

Results and Discussion

Any change to the quality of crop residues and rhizosphere inputs could modify the dynamics of the composition and activity of organisms in soil. Insect-resistant Bt crops have the potential to change the microbial dynamics, biodiversity, and essential ecosystem functions in soil, as they produce insecticidal Cry proteins through all parts of the plant all through its life cycle. Soil micro and macro-organisms are exposed to these proteins via root exudations or during the decomposition of Bt-plant in the soil (Stotzky *et al*., 2009). Moreover, biodegradation of Cry protein in soil is highly contested as many reported studies claimed that it cannot accumulate in soils for a long, as its degradation in soil takes place quickly during initial stages of its entry in the soil and a little quantity can persist. (Zurbrugg *et al*., 2010).

The fate of these insecticidal proteins in soil has been highly conflicted by researchers due to methodological limitations in many of the studies conducted so far. In one of the experiments reported, 14C-labeled Cry1Ac protein was used to study its mineralization in soil (Accinelli *et al*., 2008). Low mineralization of around 6% of the radio-labeled protein was observed in autoclaved soil, indicating that microbial processes play a major role in the dissipation of the Cry1Ac in soil. The results of this study suggested that there may be limited risk of the bioaccumulation of Cry1Ac in soil due to the eventual release of this insecticidal toxin by Bt crops (Accinelli *et al*., 2008). Multiple studies have been conducted to evaluate the impact of Bt crops on soil organisms, with varied results. Thus, requiring an in-depth review of literature on the fate and impact of cry proteins as analyzed here.

Effect on soil microorganisms

Soil ecosystem is a highly dynamic area with multifarious changes at different crop growth stages (Wei *et al*., 2012). Microorganisms are the dominant organisms, both in terms of biomass and activity, in soil, and they are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens, decomposition of agricultural chemicals, and improvement of soil structure (Li *et al*., 2013). The close interaction between crop cultivation and microbe-mediated soil processes inadvertently leads to contact of soil organisms with Cry proteins released from Bt crops.

Initial studies on the impact of Cry proteins on soil microbiota reported significant changes in the mycorrhizal growth (Table I). Similar observations were reported in the bacterial community of rhizospheric soil planted with Bt crops (Helassa *et al*., 2011). Contrastingly, Hu *et al*. (2013) did not find any impact on the useful population of rhizospheric bacteria even after cultivating transgenic Bt Cotton for several years. No considerable effect of Bt Maize cultivation was observed on bacterial and fungal groups involved in transforming soil organic matter (Becker *et al*., 2014). Castaldini *et al*., (2005) reported difference in bacterial population, soil respiration, colonization and establishment of mycorrhizal in fields of Bt Maize and non-Bt Maize, however the risk was not well known.

Table I Effect of Cry proteins on soil micro-organisms

S.No.	**Research paper**	**Organism**	**Expt Variable**	**Effect**
1.	Turrini *et al*., 2004	Arbuscular mycorrhizal fungus	Rhizosphere soil of Bt and non-Bt Maize	Significantly lower presymbiotic hyphal growth of mycorrhizae in soil with Bt-Maize than with non-Bt Maize
2.	Gupta and Watson, 2004	Composition of soil microbiota	Rhizosphere soils of Bt Cotton versus herbicide-tolerant (Roundup Ready) Cotton	Significantly different microflora in soil with Bt Cotton residues than in soil with herbicide-tolerant Cotton (by microscopic observations)
3.	Devare *et al*., 2004	Microbial activity and bacterial community structure	Soil with Bt and non-Bt Maize	No effects on microbial activity or bacterial community structure
4.	Blackwood and Buyer, 2004	Rhizosphere bacterial community	Soil with Bt and non-Bt Maize	No significant effects on bacterial and fungal PLFA profiles, Small significant effect of Cry proteins on community structure observed by CLPP
5.	Xue *et al*., 2005	Culturable bacteria and fungi	Soil with Bt and non-Bt Maize & Soil with Bt and non-Bt potato	Lower ratio of gram-positive to gram-negative bacteria in soil with Bt Maize than in soil with non-Bt Maize, and no differences in the ratio of fungi to bacteria; Higher ratio of gram-positive to gram-negative in soil with Bt potato than non-Bt potato, and no differences in the ratio of fungi to bacteria
6.	Icoz and Stotzky, 2007	Microbial populations and activity of enzymes	Soil with Bt and non-Bt Maize	No consistent significant effects on numbers of culturable bacteria, gram-negative bacteria, chitin- and cellulose utilizing bacteria, nitrifyers, denitrifiers, protozoa, and fungi by

				plate and MPN counts; no consistent significant effects on activities of some enzymes
7.	Devare *et al*., 2007	Microbial biomass	Soil with Bt and non-Bt Maize	No adverse effects of Bt Maize on microbial biomass
8.	Zhou *et al*., 2011	Microbial populations and soil enzymes	Soil with Bt and non-Bt Maize	Reduced mycelium length of AMF

High variation in microbial population and diversity index in soil varied with different of hybrids, different depths in the soils of Bt and non Bt Cotton, and the amount of root exudates and distinct inherited properties of the plants (Velmourougane and Sahu, 2013). Under Ecogen project of UK to study interaction of Bt Maize with soil processes and ecosystem reported no direct effects of Cry1Ab protein on laboratory test organisms (Nicholas *et al*., 2007). But in the glasshouse experiments total nematode and protozoan numbers increased in field soil under Bt Maize relative to conventional Maize, whilst microbial community structure and activity were unaffected. Therefore, the study concluded that any negative effects of Bt Maize at field scale were indirect and no greater than the impacts of crop type, tillage and pesticide use (Nicholas *et al*., 2007).

A survey of 217 stream sites in Indiana USA, was done to determine the extent of Maize detritus and presence of Cry1Ab protein in the stream network (Tank *et al*., 2010). The study reported Cry1Ab protein in stream-channel Maize at 13% of sites and in the water column at 23% of sites. Interestingly, the Maize detritus and associated Cry1Ab proteins, were found to be widely distributed and persistent in the headwater streams of a Corn Belt landscape in Indiana. In another study using a tiered approach for exposure assessment, worst-case exposures of Bt Maize were estimated using standardized models, and factors mitigating exposure were described (Carstens *et al*., 2012). An Indian research study reported a significant reduction in count of actinobacteria (17%), bacterial (14%) and the activities of acid phosphatase (27%), phytase (18%), nitrogenase (23%) and dehydrogenase (12%) in the Bt Cotton fields versus non-Bt Cotton fields (Singh *et al*., 2013b). The amount of lignin because of Bt cultivation can also modify the intensity and role of decomposers in the soil (Xue *et al*., 2011). It was reported that intercropping of Bt crops with non Bt crops can facilitate in avoiding any harmful effect of Bt toxin on microbes (Singh *et al*., 2013b), although high density of microbial community was also observed in soil cultivated with transgenic Cotton (Kapur *et al*., 2010).

Effect on soil dwelling invertebrates

Although, soil-borne communities are dominated by microorganisms, which account for more than 80% of the total biomass in soil, soil invertebrates are also an essential species in the soil ecosystem (Koch *et al*., 2015). The microbial communities are part of complex food webs, together with numerous and varied soil-dwelling invertebrates like earthworms, collembolans, mites, woodlice and nematodes. The effect of Cry proteins on soil dwelling invertebrates has been studied via in-situ field experiments as well as laboratory experiments discussed here (Table II).

Stotzky and Saxena reported no effect on common earthworms (Lumbricus terrestris) in addition to nematodes, protozoa, and bacteria after 40 days in soil that had been planted to or amended with plant material from Bt Corn. Growth was used as one endpoint for the assessment of the effects on the earthworm. However, in this experiment, growth was probably not an appropriate endpoint because the individuals used were already large, with fully developed clitella, and less likely to exhibit sensitive changes in growth (Stotzky and Saxena, 2009). It is further stressed that exposure of juveniles would provide a more appropriate growth endpoint. In similar lab experiments on Eisenia fetida fed on Bt and non-Bt Maize leaves reported no deleterious effects on the survival and reproduction in the worm (Barruiso *et al*., 2012). But another study on collembola and mites reported significant effect on feeding pattern and preference when fed with Bt and non-Bt Maize biomass (Singh *et al*., 2013a).

Table II Effect of Cry proteins on soil dwelling invertebrates

S.No.	Research paper	Organism studied	Expt. Variable	Effect
Earthworm				
1.	Saxena and Stotzky, 2001b	Lumbricus Terrestris	Soil amended with biomass of Bt and non-Bt Maize; soil planted to Bt and non- Bt Maize	No significant differences in mortality and weight
2.	Zwahlen *et al*., 2003b	Lumbricus Terrestris	Fed residues of Bt and non-Bt Maize	No differences in mortality and weight
3.	Lang *et al*., 2006	Lumbricidae Community	Cultivation of Bt and non-Bt Maize;	No effect of Cry protein on numbers in soil planted with Bt and non-Bt Maize
4.	Clark and Coats, 2006	Eisenia fetida	Fed leaves of Bt and non-Bt Maize	No deleterious effects on survival and Reproduction
Collembola & Mites				
5.	Al-Deeb *et al*., 2003	Natural Populations	Cultivation of Bt and non-Bt Maize	No deleterious effects on numbers of collembolans and mites in soil
6.	Griffiths *et al*., 2006	Natural Populations	Cultivation of Bt and non-Bt Maize in pots	Lower collembolan abundance and higher mite populations under Bt Maize
7.	Bakonyi *et al*., 2006	Heteromurus nitidus, Sinella coeca	Fed dried leaves of Bt and non-Bt Maize	Species specific effects in distributions and Sinella coeca feeding preference
8.	Heckmann *et al*., 2006	Protaphorura Armata	Fed purified protein or biomass of Bt and non-Bt Maize	No effect of purified or Bt Maize-Cry1Ab protein on growth and reproduction
Nematodes				
9.	Manachini and Lozzia, 2003	Caenorhabditis elegans	Cultivation of Bt and non-Bt Maize	Lower abundance in soils with Bt Maize than with non-Bt Maize
10.	Griffiths *et al*., 2005	Natural populations	Field Cultivation of Bt and non-Bt Maize	Significantly lower abundance in soils with Bt Maize than with non-Bt Maize
11.	Griffiths *et al*.,	Acrobeloides	Cultivation of Bt	Significantly higher

	2006	sp. Pratylenchus sp.	and non-Bt Maize in pots	populations under Bt Maize than non-Bt Maize
12.	Lang *et al*., 2006	Caenorhabditis elegans	Cultivation of Bt and non-Bt Maize	Negative effect of Cry1Ab protein on growth, number of eggs, and reproduction

A review of the impact of Bt plants on arthropod natural enemies in laboratory experiments showed that Cry toxins and proteinase inhibitors often have non-neutral effects on natural enemies (Lovei *et al*., 2009). The input of senesced Corn tissue expressing the Bt gene may impact stream-inhabiting invertebrates that process plant debris, especially trichopteran species related to the target group of lepidopteran pests as reported by Jensen *et al*. It was highlighted that such adverse effects to aquatic non-target shredders involve complex interactions arising from plant genetics and environment that cannot be ascribed to the presence of Cry1Ab proteins (Jensen *et al*., 2010).

Icoz and Stotzky (2008) from New York University discussed and reviewed data on the effects of Cry proteins on below-ground organisms. They studied the fate of these proteins in soil as well as the techniques and indicators that are available to study these aspects. They reported no significant difference in earthworm population while lower abundance of nematodes was observed in the soils cultivated with Bt crops. Another toxicological study, used Eisenia fetida and the springtail Folsomia candida under artificial conditions using Cry protein isolated from Escherichia coli reported marginal to no adverse effect on test species(Xiao *et al*, 2005). Although studies with purified protein help to provide insight into potential effects, their ecological relevancy is probably low. Similarly, a study fed four Cry proteins to two springtail species, F. candida and Xenylla grisea, incorporated into a diet of baker's yeast. No adverse effects were observed, but it is unclear if the protein was consumed by the test organisms (Shu *et al*., 2011). It is important to note that the route of exposure in lab studies is ecologically unrealistic, as any possible effect on collembolans would likely occur during their participation in the breakdown of plant residues. It is important to note, that Collembola often do not even feed on the plant material, but rather would consume fungi growing on the decaying plant material. While, another study used transgenic plants expressing Cry1Ac reported no adverse effects on F. candida and Ocypus nitens were observed. This study was a more realistic reflection of ecological effects because it used Bt or non-Bt plant material and soil rather than purified protein mixed with yeast (De Castro *et al*., 2012).

Effect on other non-target organisms

In light of the environmental fate of the Cry proteins, non-target organisms are likely to be exposed to the protein in decomposing plant material that is incorporated into the soil matrix. There is potential for long-term exposure due to season long expression of the proteins and the persistence of active protein in the soil. Potentially, the protein could also reach the soil through shedding of leaf material and pollen, in addition to the post-harvest input of plant residue incorporation. Effect of Bt crops on population dynamics and community structure of non-target soil animals was reviewed by researchers at Chinese Academy of Sciences. The pathway of the Cry protein , presence of residual Cry protein and its degradation in soil was also assessed, which paved way for further experimentation studying the complex soil-organism interface. Initially, no harmful effect on soil macro-organisms was reported under laboratory and field experiments when exposed to Cry proteins (Bai *et al*., 2010). While harmful effect of Bt Maize was observed on development and eggs hatchability of snails (Kramarz *et al*. 2009), the hazard was not recognized due to limited information and requirement of further testing. Instead, the crop itself, environmental settings and use of pesticide were reported to impact diversity and uniformity of species as recommended by field observations (Birch *et al*., 2007).

Interestingly in a field study on the headwater agricultural streams in the Midwestern United States indicated that Bt Corn pollen was being eaten by caddis-flies, which are close genetic relatives of the targeted Bt pests (Rosi-Marshall *et al*., 2007). The follow-up laboratory studies suggested that consumption of Bt Corn byproducts reduced the growth rate of shredding caddis-flies by half. In addition, 43% of the insects died when fed with high concentrations of Bt Corn pollen compared to 18% mortality in controls (Rosi-Marshall *et al*., 2007). In the peer-reviewed literature, reduced emergence and development of the parasitoid wasp (Zele chlorophthalmus) reared on Bt-fed Spodoptera littoralis was reported. Performance of the diamondback moth parasitoid (Cotesia plutellae) was investigated in Bt-resistant and -susceptible host diamondback moths (Plutella xylostella) that were fed Bt Rape. The parasitoid was unable to develop normally on susceptible P. xylostella that had consumed Bt Rape.

It is a known fact that Cry protein targets lepidopteran insects too. Initial laboratory studies indicated that there was a hazard to the monarch butterfly (Danaus plexippus) larvae that consumed pollen containing high levels of Cry protein (Losey *et al*., 1999). In 2001, a series of studies were published that examined pollen deposition, spatial and temporal overlap of pollen and monarchs, and the sensitivity of larvae to the Bt Corn pollen (Dively *et al*., 2004). Because of concern about potential sublethal effects on monarch larvae due to consumption of Bt pollen, this risk assessment was further updated to incorporate data on the long-term exposure to Bt pollen. The study concluded that there would be only 0.6% additional mortality due to low level of exposure of larvae to Bt pollen.

There has been an increasing concern on the potential toxicity of Bt plant anthers to monarch larvae. Anderson *et al*. (2004) examined the effect of feeding on milkweed leaves contaminated with Bt plant anthers and found adverse effects at moderate to high densities of 15 anthers/leaf. However, in a field survey, the peak level of anther deposition was found to be around 3-5 anthers per leaf which was replicated in a caged field exposure with monarch larvae and showed no adverse effects. The potential toxicity to another nontarget butterfly, the black swallowtail was examined in field studies reported sublethal toxicity.

Conclusion

The introduction and proliferation of transgenic crops in agroecosystems present a unique challenge in determining or predicting the environmental fate and effects of Cry proteins incorporated in these crops. The widespread adoption of Bt crops represents a shift in how insect control is conducted and should be investigated with the ecology of the agroecosystem in mind. Literature review highlighted that environmental fate of Cry protein is still not well documented and described. Degradation of Cry protein in decomposing plant material after harvest reported great variability in the observed results, even within the same study. It is important to note that, the process of degradation of plant incorporated Cry protein was found to be different from the degradation of purified protein, involving macrofaunal and microbial decomposition of plant material before Cry proteins interact directly with soil. For such reasons, the presence and persistence of the plant-produced Cry proteins is currently more uncertain in comparison to many conventional insecticides.

The lack of adequate analytical techniques currently available for analysis of environmental concentrations of Cry proteins has led to a shortage of reliable, accurate data for the estimation of environmental exposure concentrations. In addition, the examination of ecological impacts of Cry toxins to nontarget organisms is still in need of ecological studies that relate estimated Cry toxin concentrations in the environment to the responses of representative elements of the biota. This lack of a quantitative chemical or biochemical assay hinders the development of accurate estimates of exposure of organisms to the protein in the agroecosystem. The lack of standardized bioassays for the transgenic crop system, the lack of taxonomic diversity tested, and the short duration and artificial conditions of many of the toxicity tests previously conducted represent additional areas that can be improved to further our understanding of the effect of Cry proteins on the agroecosystems.

It is also worth mentioning, that many of the studies that have been conducted were for regulatory purposes and have not been peer-reviewed or published. For several groups of nontarget organisms, the existing results are still somewhat conflicting, unclear, or incomplete. The meta review also highlighted the fact that chronic effects of Cry proteins on soil ecosystem have not been well documented in the published literature. Thus, there is an urgent need to develop and implement accurate and specific analytical methods for soil micro and macro-organism studies and their intricate biochemical relations with soil components, to understand the environmental fate of plant produced toxins.

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