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Risk assessment of Bt crops on the non-target plant-associated insects and soil organisms

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Abstract

Transgenic plants containing *Bacillus thuringiensis* (Bt) genes are being cultivated worldwide to express toxic insecticidal proteins. However, the commercial utilisation of Bt crops greatly highlights biosafety issues worldwide. Therefore, assessing the risks caused by genetically modified crops prior to their commercial cultivation is a critical issue to be addressed. In agricultural biotechnology, the goal of safety assessment is not just to identify the safety of a genetically modified (GM) plant, rather to demonstrate its impact on the ecosystem. Various experimental studies have been made worldwide during the last 20 years to investigate the risks and fears associated with non-target organisms (NTOs). The NTOs include beneficial insects, natural pest controllers, rhizobacteria, growth promoting microbes, pollinators, soil dwellers, aquatic and terrestrial vertebrates, mammals and humans. To highlight all the possible risks associated with different GM events, information has been gathered from a total of 76 articles, regarding non-target plant and soil inhabiting organisms, and summarised in the form of the current review article. No significant harmful impact has been reported in any case study related to approved GM events, although critical risk assessments are still needed before commercialisation of these crops.

Keywords: biosafety; Bt crops; genetically modified plants; non-target insects; pest control; risk assessment; soil organisms

HISTORICAL BACKGROUND OF GENETICALLY MODIFIED CROPS

The evolution of genetically modified (GM) crops has come a long way for its adoption into modern agriculture.[1] In the late 1970s and early 1980s recombinant DNA technology tools were in constant use for crop improvement. In 1983, the first genetically modified tobacco plant (*Nicotiana tabacum*) was developed for antibiotic resistivity in China.[2] FlavrSavr tomato, with a prolonged shelf life, developed by Calgene, was the first GM crop to be planted commercially in 1994.[3] Monsanto's Roundup Ready soybean, approved in 1996, was another important step in GM crop development. A GM variety of soyabean was able to survive after being sprayed with the 'Roundup' herbicide containing glyphosate that is applied to kill weeds.[4] During recent decades, there has been a growing interest from the food crop industry to construct and produce GM crops with the primary goal to significantly increase the yield and avoid the use of pesticides. The best known example of transgene for insect resistance is the use of *Bacillus thuringiensis* (*Bt*) genes in cotton, corn and other crops.

The rapid adoption of transgenic crops during 1996 to 2015 reflects multiple benefits realised by large and small farmers both in industrial and developing countries. The global area of GM crop cultivation has dramatically increased as the technology has proved itself environmentally friendly as well as quiet promising in leading to significant socio-economic benefits. A recent study showed that from 1996 to 2012, a total global area of about 1.7 million hectares has been increased to 160.4 million hectares for GM agriculture. Four main crops with GM traits have been introduced in market: i.e soybeans, corn, canola and maize.[5] GM crops are gaining in popularity due to the success in the field; presently about 14 million farmers are deliberately growing GM crops in 25 countries[6] and 70% of the total cultivated area in China is used for planting transgenic cotton.[7] So far, GM plants with herbicide and insecticide traits have been introduced commercially while other traits are likely to be introduced in the near future.[8]

The transgenic plant technology can be useful to determine global food scarcity, especially in developing countries. Farmers could have higher crop yields within a shorter growing duration. In under-developed countries, about 800 million people, including 250 million children, are critically malnourished.[9] Similarly, with the depletion of natural resources and increasing demands for food worldwide, along with challenges by environmental changes, an increase in agricultural productivity is a prerequisite for food security in the long-term future.[10]

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Table 1. Common Bt plants with insect and disease resistant traits

Plant	Transgene	Resistant for	Reference
Cotton (*Gossypium*)	*cry1A* and *Cry2A*	Bollworm (*Helicoverpa armigera)*	14
Tobacco (*Nicotiana*)	*Bt and CPT1*	Bollworm (*Helicoverpa armigera*)	15
Indian potato (*Kufri Badshah*)	*Cry1Ab*	Potato tuber moth (*Phthorimaea opercullela* Z.)	16
Tomato (cv. *Money maker*)	*Bt* (*Cry 2Ab*)	American bollworm (*Helicoverpa armigera*) and potato tuber moth (*Phthorimaea operculella*)	17
Brinjal (*Solanum melongena* L.)	*cry1Fa1*	Brinjal fruit and shoot borer (*Leucinodes orbonalis*)	18
Sweet corn (*Zea mays* L.)	*Vip3A* (*Bt* gene)	Corn earworm (*Helicoverpa zea)*	19
Indica rice (*Oryza sativa*)	*Cry1Ab/Ac*	Lepidoptera, e.g. rice leaf folder (*Cnaphalocrocis medinalis*)	20
Elite Vietnamese rice	*Cry1Ab-1B* and hybrid *Bt* gene, *Cry1A/Cry1Ac*	Yellow stem borer (*Scirpophaga incertulas*)	21

GM CROPS WITH THE *BACILLUS THURINGIENSIS* (BT) GENE

Plants have been modified by genetic engineering to express insecticidal Bt proteins. These crystal Bt or the Cry proteins are derived from a natural soil spore-forming bacterium, *Bacillus thuringiensis* (Bt), and specifically affect the gut of target insects to kill or inactivate them.[11] Regarding the integrated pest management (IPM) system, plants genetically modified with Bt protein are considered to be the most effective bio-insecticides which have proved their importance in socio-economic benefits in developing countries.[12] Bt crops are grown globally over 35 million hectares in 13 different countries and are most important in biocontrol of stemborers and rootworms.[6] Plants such as tobacco, potato, rice, tomato, maize, eggplant and cotton have been successfully transformed with *Bt* genes (Table 1). Yet Bt cotton and Bt maize are considered to be the only insecticidal crops with respect to the market;[1] however, new varieties of transgenic plants with other insecticidal and fusion proteins are also expected to be introduced soon.[13]

RISKS ASSESSMENT OF BT CROPS

Bt crops are accepted as quite promising for increasing annual crop yield and eliminating the use of various insecticides but the global agriculture sector has plunged into a lively debate. Opponents emphasise that Bt crops with insect resistance traits could disturb the natural environment as well as human health. The question of environmental safety in the use of these plants, the chances of disturbing biodiversity, ingestion of *Cry* proteins in living beings along with Bt maize or corn, possible effects of Bt crops on non-target organisms (NTOs) and financial gain or loss in developing countries are the topics of dispute among populations.[22] From the last 20 years, risk assessment has been extensively studied for NTOs.[23] In this study, an effort has been made to gather the results from different experimental trials regarding risk assessments of Bt crops in NTOs and conclusions about risks in non-target plant associated micro- and macroorganisms can be assessed (Table 2).

EFFECT OF BT CROPS ON NON-TARGET INSECTS

Cry proteins attack the midgut of susceptible insects and cause wounds or lesions in the epithelial layer of the midgut, eventually producing septicaemia in the host insect along with multiplying enteric bacteria. One major aspect in risk assessment of Bt crops is the estimation of lethal effects of Cry proteins on beneficial or non-target arthropods.[24] Most of the experimental studies have been conducted by directly feeding the insects with Bt plant tissues, e.g. grains, seeds or pollens. The results from such trials favoured the use of Bt plants since no damaging effect to non-target insects were reported.[25] Varieties of Bt plants introduced commercially carry either *Cry1Ab* or *Cry3Bb1* toxic proteins to control *Lepidoptera* and *Coleoptera*, the most important pest groups which cause the major yield loss in maize.[26]

In practice, a small number of representative species should be selected for testing. The tested organism should be either ecologically active or considered as threatened, i.e. high exposure or at the risk of exposure to Bt proteins.[27] Negative effects of Bt corn were indicated by Losey and colleagues[28] when feeding trials of Bt pollens were carried out with the larvae of monarch butterfly, *Danaus plexippus*; however, the feeding strategy used in that study was considered inappropriate because high levels of Bt pollens were artificially used. Later experimental studies using an appropriate level of Bt supplemented feed indicated no lethal effects of this protein on non-target visiting insects or storage pests.[29] Muhammad *et al.*[29] also investigated the effects of transgenic basmati rice on various non-target insects, i.e. rice grasshopper (*Acridaexaltata*), green leaf hoppers (*Nephotillixcincticeps*), backed plant hoppers (*Sogatekkafurcifera*), paddy grasshopper (*Oxyahyla*), rice bug (*Leptocorisaacuta*), rice thrips (*Stenchaetothripsbiformis*), etc. and no false impression of Bt toxins has been observed. Since the *Cry* genes are specific in their attributes therefore the protein encoded by these genes has a narrow activity against particular target organisms and do not harm the other non-target insects or herbivores.[30] Combination of various related studies are summarised in this section.

Pollinator insects

About 70% of all crops undergo pollination by different insects or small animals and almost 1 million maize pollen grains are produced per day. The pollens from transgenic crops bear *Cry* protein toxins, therefore pollinators are supposed to be at high risk because of direct feeding with pollens. General experimental studies showed minimal or no harmful effect of Bt pollens on pollinators; however, the risks may be dependent upon the type of insect selected and experimental set-up.[31]

Honey bee (Apis mellifera L.)

The honey bee is the most efficient and important pollinator among all the pollinators. Nearly 80% of the total pollination is carried out by honey bees in cultivated and natural ecosystems.[32] Experimental trials have been conducted to evaluate the lethal

Table 2. Physiological effects of Bt toxin on major non-target organisms

Experimental organism	Cited by	Effect on physiological parameters: Development	Reproduction rate	Survival/ mortality	Ecological behaviour	Body mass/ size
Paddy grasshopper (*Oxyahyla*)	Muhammad *et al.*[29]	Harmless	Harmless	Harmless	Harmless	Harmless
Honey bee (*Apismellifera* L.)	Han *et al.*[35] Hofs *et al.*, 2008[36]	Harmless	Harmless	Harmless	Harmless	Harmless
Ladybird (*Adaliabipunctata*)	Schmidt *et al.*[37]	Disturbance in larval development	Harmless	High mortality was observed in tested larvae	Harmless	Reduced larval weight
	Alvarez-Alfageme *et al.*[25]	Harmless	Harmless	Harmless	Harmless	Harmless
Monarch butterfly (*Danausplexippus* L.)	Sears *et al.*[42]	Harmless	Harmless	Harmless	Harmless	Harmless
	Perry *et al.*[45]	Reduced larval development	Harmless	Harmless	Alteration in larval behaviour	Reduced wing size/body mass
Aphid (*Aphidoidea*)	Ramirez-Romero *et al.*, 2008[33,49]	Harmless	Harmless	Harmless	Harmless	Harmless
Soil microbes	X. Li *et al.*, 2011[46]; Lijun and Zhijie 2004[59]	Slight reduction in phosphatase activity	Harmless	Harmless	Harmless	Harmless
Earthworm (*Lumbricina*)	Zeilinger *et al.*[73]	Harmless	Harmless	Harmless	Harmless	Harmless
Nematode (*C. elegans*)	Hoss *et al.*, 2011[75,76]	Harmless	Reduced reproduction rate	Harmless	Harmless	Harmless

effects of Bt toxin on honey bees but only a few have assessed the risks on physiology and behaviour.[33] Babendreier *et al.*[34] studied the effect of Bt toxin on honey bee by feeding it directly with Bt mixed pollens and reported no noticeable adverse effect of Bt toxin on its survival or other life activities. He observed a very small amount of Bt toxin in hypo-pharyngeal glands of young bees after continuously feeding them with Bt protein for 10 days but found negative results on development of their hypopharyngeal glands. Various other experimental trials also rendered no effect of Bt pollens to the survival, developmental stages, learning behaviour, diversity and abundance of honey bees.[35]

Ladybird (Adalia bipunctata)

The two-spotted ladybug is a carnivorous pollinator and also considered as an important biological control agent. Schmidt and co-workers performed an eco-toxicity test on *Adalia bipunctata* by direct feeding of immature larvae with *Cry1Ab* toxin to evaluate the effects of Bt transgenic products. High mortality was observed in tested larvae as compared to control group.[37] These results might be due to poor study designs or low nutritional quality,[38] because later subsequent studies revealed no lethal or harmful effects of *Cry1Ab* and *Cry3Bb1* to larvae. Further studies also confirmed that Bt protein had no adverse effect on the weight, development and differentiation of *A. bipunctata* larvae.[25]

Green lacewing (Chrysoperla carnea)

This is a common and predominant pollen-consumer in maize fields. In successive experimental trials, their adults were fed on Bt pollens (supplemented with *Cry3Bb1* or *Cry1Ab*) from transgenic maize and rice for ≥28 days. No difference of the fitness parameters like survival, pre-oviposition period, fertility and dry weight was observed between Bt and non-Bt treatments.[39]

Flower bug (Orius insidiosus)

These natural predators are found in cotton and maize fields. A study by Lumbierres *et al.*[40] indicated reduced nymph development of *Orius insidiosus* when fed with Bt-containing spider mites, but later experimental trials revealed that ingestion of Bt proteins either through plant leaves or via food was not lethal for these beneficial predatory insects.[41]

Phytophilous insects

Butterfly (Rhopalocera spp.)

This is one of the most important organisms among the Phytophilous, or sap-sucking, insects. Since Bt products could affect different non-target species in different ways, butterfly species are also needed to be tested in terms of risk assessment. A 2-year study on the Monarch butterfly (*Danaus plexippus* L.) suggested that Bt corn pollens are harmless to their population.[42] Another field study suggested non-lethal effects of Bt corn on the swallowtail butterfly population and confirmed that wide use of Bt corn did not affect swallowtails living near Bt cornfields.[43] However, the feeding trials with Monarch butterfly by another group of scientists showed that Bt proteins somehow negatively affect the development, survival, body weight, wing size and larval behaviour of this natural pollinator.[44,45] Similarly, the larvae of Peackock butterfly (*Inachisio* spp. L.) when subjected to direct feeding with Bt maize pollen, a reduced larval body weight was observed and it was assumed that reduction in larval weight is directly proportional to the concentration of Bt protein in maize pollen.[47] Bt pollens are observed to somehow adversely affect the living characteristics of butterfly species but no lethal effect has been claimed by any researcher. However, more critical experimental studies are needed to evaluate the ecological effects of Bt plants on butterfly populations.

Aphid (Aphidoidea spp.)

Aphids are common plant feeding insects and also occupy an important position in the food chain as a prey for many other insects. Therefore aphid is considered a significant tool in Bt plant risk assessment on non-target phytophilous insects.[48] Aphids, when exposed to Bt-maize and quantified experimentally for the presence of *Cry1-Ab* protein, show only traces of toxin, or no toxin was detected. No negative effects on developmental stages, survival or other biological parameters were observed in tested aphid species, i.e. *Rhopalosiphum maidis* F., *R. padi* L. and *Sitobion avenae*.[49] Since Cry proteins do not translocate into the phloem, no toxin could be transferred to the aphids while sucking or feeding the phloem. However, the presence of traces of Bt proteins in some aphids could be due to intracellular sap uptake during piercing the plant tissue.[50]

EFFECT OF BT CROPS ON SOIL ORGANISMS

Experimental studies revealed the degradation of *Cry* proteins in soil after their release from root exudates or plant decay, no long-term persistence or accumulation of these toxins has been proved in soil, in any study.[29] Since the Bt toxin can adhere to soil particles just after release into the soil, soil organisms are considered to be at greater risk of contact with these toxins. Experiments have been performed for this purpose and results revealed negligible or no harmful effect of Bt toxins on soil ecology.[51]

Soil microorganisms

During the risk assessment of Bt crops, their effects on soil microorganisms should be considered because of their significant roles in in nitrogen fixation, salt tolerance, nutrient solubilisation, plant pathogen control by toxin production and plant hormone production to promote plant growth.[52] Experimental trials have been carried out for this purpose for the last few years and results revealed negligible or no harmful effect of Bt toxins on soil ecology.[53]

Plant growth promoting rhizobacteria

The term ‘rhizobacteria’ was first presented in 1978 by Kloepper and Schroth after their collective work on radish colonising soil bacteria.[54] The concept of plant growth promoting rhizobacteria (PGPR) is confined to the specific bacteria that can accomplish at least two of the three basic criteria, i.e. strong colonisation, plant growth stimulation and biocontrol.[55] Soil bacteria such as *Azorhizobium*, *Allorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, etc. are included in endophytes which promote plant growth by invading the root cell walls for nodule formation.[56] Free living PGPR include *Azospirillum*, *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Enterobacter* and *Flavobacterium* which fix atmospheric nitrogen for plants and also increase the organic content of soil by biodegradation.[57]

Very few risk assessment studies have been made on PGPR strains regarding Bt crops. In a study, reduced efficiency of soil bacteria in a medium supplemented with Bt-maize residue was reported. However, the experimental set-up used there was considered to be unsatisfactory, as the direct addition of Bt-maize straw greatly increased CO_2 concentration of substrate and eventually resulted in high mortality rates of soil bacteria.[58] Another evaluation made by Lijun and Zhijie showed the decreased phosphatase activity of rizhospheric bacteria in Bt soil as compared to bacteria in non-Bt soil.[59] But later studies suggest that the cultivation of transgenic Bt plants has minor or no effects on rhizobacteria.[60] A 4-year experimental study by Barriuso *et al.* on risk assessment of rhizobacteria in Bt-maize fields concluded no change in the structure and ecology of Bt-maize rhizobacterial communities when compared to those in the non-Bt maize fields.[61] Successive comparative studies of Bt crop soil with control soil showed that neither the soil microbial biomass nor the microbial enzymes are adversely affected by incorporation of *Cry* proteins into the rhizosphere[62] but it is suggested that microbial colonies are also co-affected by other biotic and abiotic environmental factors of rhizosphere.[63] Bt toxins do not directly cause negative effects on rhizobacteria, but parallel factors, such as plant growth stage, duration of plant straw decomposition, plant hybrid, variety, fibre decay, soil aeration and soil organic content might have stronger effects on the microorganisms than the presence of the *Cry* protein.[64]

Mycorrhizae

Mycorrhizae are an obligate symbiont of more than 80% plants which can grow in any soil ecosystem and considered to be sensitive for the genetic changes within the host plant. Arbuscular mycorrhizal fungi (AMF) are crucial for the growth of higher plants, disease tolerance and nutrition. Previous risk assessments of Bt crops on AMF have been discrepant because most studies were conducted under heterogeneous experimental conditions. As some of the experimental results revealed the negative interference of Bt toxins to the symbiotic association of AMF.[65] Similarly, reduction in AMF colonisation in multiple Bt maize lines has been reported when compared with control lines which showed unexpected harmful effects of Bt crop cultivation on non-target soil fungi.[66] However, further related studies using molecular fingerprinting and nucleic acid based pyrosequencing methodologies indicated no risk for AMF in Bt cultivation.[67] We can say that AMF risk assessment needs more critical experimentation than previous trials to accurately evaluate the possible impacts of Cry proteins.

Soil protozoa

Soil protozoa are important agents for soil mineralisation which fed upon bacteria or fungi. Unfortunately, very few studies have been made to assess the risks or benefits associated with these microscopic organisms regarding GM events. Lack of an experimental set-up under laboratory conditions is one of the major problems in their study. However, results from some novel trials showed non-significant effects of Bt toxin on the overall community structure of soil protozoans.[68]

Soil macroorganisms

The effects of Bt crops on soil macroorganisms such as mites, collembola, earthworms, and snails, have been studied and no adverse effects of Bt toxin was reported in laboratory or field experiments. According to the collected data, only one experimental approach reported the harmful effect of Bt maize on growth and egg hatchability of snails.[69] However, the risk was not well demonstrated due to the lack of additional tests. Furthermore, field investigations proposed that crop management protocols, environmental conditions (e.g. rainfall during growing season) and use of pesticides are also factors which impact greatly on the diversity and occurrence of these species, rather than the transgenic crop itself .[70]

Earthworms (Lumbricina spp.)

Earthworms are the most active macroorganisms in soil which alleviate water and nutrient transport through soil layers and are termed as natural ploughs.[71] Since the earthworm ingests Bt toxins along with bulk soil particles, researchers assumed that Bt toxin could directly impact upon earthworm activity, or other factors like plant associated organisms can also be the indirect source that affects the earthworm.[72] But keeping in view the results from different experimental studies in this regard, it has been evident that Bt proteins neither harm the adult or juvenile earthworm in soil nor affect its characteristics like reproduction, growth rate, abundance, biomass and mortality rate.[73]

Nematodes (e.g. Caenorhabditis elegans)

Millions of nematodes, commonly known as round worms, are found in the top soil layer. Nematode species play a significant role in soil nutrient cycling and they are also a worthy indicator of soil pollution.[74] The effects of Bt crops are also assessed in free living nematodes and it is estimated that *C. elegans* was not affected significantly by *Cry* proteins, their abundance and diversity were essentially the same between different Bt maize cultivars.[75] Contrarily, later studies revealed that *Cry* proteins somehow negatively affect the reproduction rate of *C. elegans* due to the presence of similar receptors of nematicidal *Cry* proteins.[76] Meanwhile, additional studies showed the ability of *C. elegans* to defend itself against the *Cry6Aa2* toxin through up-regulation of defence initiating genes as well as behavioural responses, i.e. reduced oral uptake and physical avoidance.[77] More experimental trials are needed to analyse the ecological effect of Bt toxins in soil nematodes.

CONCLUSIONS

Primarily, GM foods are playing a promising role in desirable quality, broadened shelf-life and long-term food security. Therefore not only users but also farmers are also getting benefits from the technology. However, preservation of the environment is an important aspect to be considered when introducing GM crops like Bt varieties into the field. Since Bt crops have a narrow spectrum of activity they therefore do not adversely affect the non-target organisms.[38] The comparative results of different experimental trials regarding risk assessments of Bt crops in NTOs favour their use and show no lethal impact of Bt protein to biodiversity. The risk of horizontal gene transfer to other plants is also not reported in any study.[78] The present review article can be helpful in successful deployment of GM crops. Similarly, risk assessment of every new GM event will be helpful to demonstrate the fate of the biotechnology in the future.

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