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Diversity of *Bt* toxins and their utility in pest management

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Abstract

Background The rising demand for food production along with the concerns regarding the injudicious use of chemicals in pest management has paved way for the alternatives that could promise sustainable pest management. *Bacillus thuringiensis* Berliner (*Bt*), a soil bacterium, is a potential biopesticide with its ability to produce crystal toxins that are insecticidal in nature.

Main body This article provides an insight into the diverse *Bt* toxins and their applications as biopesticides in pest management. The selective action of *Bt* towards target organism is based on its specific interactions with the insect gut receptors. The significance of *Bt* in the management of lepidopteran, coleopteran, hemipteran, dipteran and nematode pests of crops and livestock through its mode of action is extensively reviewed.

Conclusion Besides being a promising pest control option, the challenges faced through resistance development, variation in susceptibility across species and non-target effects of *Bt* are also discussed. Proactive approaches and multiple modes of action can mitigate this issue.

Keywords *Bacillus thuringiensis*, Lepidoptera, Coleoptera, Hemiptera, Diptera

Background

Bacillus thuringiensis (*Bt*) is an aerobic, gram-positive, spore-forming, soil bacterium that has now revolutionized pest management. *Bt* is acknowledged worldwide for its safety as a bioinsecticide. *Bt* occupies an intricate ecological profile. As per the recent assessments, *Bt* is seen in a wide range of habitats, even those lacking insects, thus underscoring the significance of various vectoring systems (Ruan et al. 2015). This broadens the boundaries set for *Bt* as an insecticidal toxin. The host specificity of *Bt* is favoured evolutionarily by changes in the population of certain insect species (Argôlo-Filho and Loguercio 2013). The lifecycle of *Bt* consists of four distinct life stages. The Phase I is the vegetative growth stage, Phase II is the progression to sporulation, Phase III is sporulation, and Phase IV is maturation of the spores and cell lysis (Berbert-Molina et al. 2008).

Delving into the historical background of *Bt*, it all began with the discovery of 'Sottokin', by Ishiwata from the diseased *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) larvae. It has been more than a century since he isolated *Bt* and identified that a toxin is responsible for death rather than septicemia. Ten years later, in 1911, Ernst Berliner isolated the bacterium from *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae) larvae in Thuringia province of Germany. Aoki and Chigasaki (1916) revealed that the toxicity originating from the sporulated cultures was due to an endotoxin protein. Mattes (1927) who re-isolated Berliner's isolate was successful in observing an additional body other than the spore in the sporangia. The parasporal crystal inclusion

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was hypothesized to be responsible for the insecticidal activity by Hannay (1953). This was proved by Angus and he inferred that only the alkali-treated ingested spores could lead to paralysis, septicemia and death (Angus 1954). The first commercial *Bt* formulation 'Sporeine' came to the forefront in 1938 as a consequence of severe infestation of European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) in France. In the 1950s, efforts of Steinhaus brought *Bt* to USA. The second *Bt* formulation 'Thuricide' was introduced in 1957 with the initiative of Steinhaus and R. A. Fisher (Heimpel and Angus 1960; Beegle and Yamamoto 1992). This was followed by the discovery of many potential novel isolates and their commercialization.

The lack of effectiveness of formulations and the internal feeding behaviour of the targeted pest species led to the idea of genetically modifying the plants. Genetic modification of *Nicotiana tabacum* (Linnaeus) (Solanales: Solanaceae) with truncated *cry* gene was done with *Agrobacterium tumefaciens* (Smith & Townsend) (Hyphomicrobiales: Rhizobiaceae) mediated gene transfer. The resistance exhibited by the plant to *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) paved the way for transgenic *Bt* plants (Vaeck et al. 1987). With all the efforts, in 1995, the United States Environmental Protection Agency (US EPA) sanctioned the commercial production of *Bt* crops. Cotton and corn were the predominant crops to be transformed (Abbas 2018). Since 1996, *Bt* cotton and corn have undergone substantial adoption in the USA, with 85% corn and 89% cotton acres planted with *Bt* engineered crops (US Department of Agriculture, Economic Research Service, 2023). The adoption rates seem to fluctuate depending upon the pest infestations. Currently, 15 *cry* genes and 3 *vip* genes for lepidopteran insect control, 5 *cry* genes for coleopteran insect control, 1 *cry* gene for hemipteran insect control and 1 *cry* gene for nematode control have been identified to have potential for genetic engineering in plants (International Service for the Acquisition of Agri-biotech Applications [ISAAA], 2024).

The *Bt* toxins and the toxin structure

Various strains of *Bt* possess diverse proteins that may exhibit specificity or the toxicity may be spread across two or more insect orders. Toxins that show order-specific toxicity and those that exhibit cross-order toxicity (van Frankenhuyzen 2017) are given in Table 1.

Owing to the continuous efforts made in the discovery of bacterial toxins for pest management, a variety of toxins have come to the forefront. The classification of *Bt* crystal proteins was initially based on insecticidal activities. However, limitations arose when proteins with sequence homology showed different insect specificity and the necessity for comprehensive bioassay data for classification. In 1998, a revised nomenclature based solely on amino acid similarity was introduced, which has remained robust. Recent advancements in genome sequencing and protein structure determination have highlighted the need for a classification system reflecting structural differences, suggesting a potential shift from

Table 1 Order specificity and cross-order activity of *Bt* toxins

S. No	Order	Protein
1	Lepidoptera	Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ad, Cry1Ae, Cry1Ba, Cry1Bb, Cry1Ca, Cry1Cb, Cry1Da, Cry1Db, Cry1Ea, Cry1Eb, Cry1Fa, Cry1Ia, Cry1Ie, Cry1Ja, Cry1Jb, Cry1Ka, Vip3Aa
2	Diptera	Cry4Aa, Cry4Ba, Cry11Aa, Cry11Ba, Cry11Bb; Cyt1Aa, Cyt1Ab
3	Coleoptera	Cry3Aa, Cry3Ba, Cry3Bb
4	Nematodes	Cry5Aa, Cry5Ab, Cry5Ba, Cry6Aa, Cry55Aa, Cry6Ba, Cry12Aa, Cry21Aa, Cry13Aa
6	Orthoptera	Cry7Ca
5	Lepidoptera + Diptera	Cry1Ca, Cry30Fa, Cry30Ga, Cry54Aa, Cry56Aa
6	Lepidoptera + Coleoptera	Cry1Ia, Cry8Da
7	Lepidoptera + Hemiptera	Cry1Ab
8	Diptera + Coleoptera	Cry10Aa
9	Diptera + Nematodes	Cry55Aa
10	Diptera + Hemiptera	Cry4Aa, Cry11Aa
11	Coleoptera + Hemiptera	Cry3Aa, Vip1A/Vip2A, Cry51Aa
12	Lepidoptera + Coleoptera + Diptera	Cry1Ba, Cyt1Ba
13	Lepidoptera + Diptera + Nematodes	Cry2Ab
14	Lepidoptera + Diptera + Hemiptera	Cry1Ac, Cry2Aa
15	Diptera + Coleoptera + Hemiptera	Cyt1Aa

the amino acid-based classification towards a more structurally oriented approach. In total, sixteen classes have been added in the revised classification of pesticidal proteins. As a result, only those toxins that possess a three-domain structure is being included under the Cry protein family. Apart from toxins produced by *Bt*, the new classification has successfully addressed those pesticidal proteins from other bacteria (Crickmore et al. 2021). The binary toxins composed of two proteins are non-three-domain toxins whose action come to play when present together. The BinB toxin is responsible for receptor binding while BinA determines toxicity (Srisucharitpanit et al. 2014). *Bt* produces several underexploited toxins, including the sphaericolysins, alveolysins, β-exotoxins, enhancin-like proteins, and P19 and P20 helper proteins. These toxins have varying levels of toxic activity and mechanisms that are not fully understood, requiring further research to explore their potential applications (Palma et al. 2014).

Coming to the class of Cry toxins, though there are variations in amino acid sequences, the three domains of the three-domain Cry toxins are more or less conserved. Cry1 protoxins are approximately 130-140 kDa in size, whereas Cry2 and Cry3 protoxins are smaller, around 70–73 kDa, because they lack the extensive C-terminal protoxin domain. The Cry4 protoxins have a size of 130 kDa. The processing of these protoxins results in an active toxin core that is about 55–65 kDa in size. The Domain I decides the pore formation ability of the toxin. Domain II is a determinant of the specificity of the toxin and receptor as the length of the three antiparallel β sheets is highly variable. The structure forms a β prism. Domain III is the galactose binding domain composed of two antiparallel running β sheets forming a β sandwich involved in receptor binding and perforation. Apart from these three domains comprising the toxin core, additional four more domains have been discovered from Cry1Ac protoxin (Adang et al. 2014; Palma et al. 2014). All other pesticidal classes comprising of *Bt* toxins are given in Table 2.

Mode of action

Cry toxin

A clear knowledge about the putative receptors and the mechanism of Cry protein binding is available for lepidopteran insects. The differential susceptibility of insect species against various Cry toxins is associated with the changes in the midgut binding receptors. Insecticidal crystal protein binding has been viewed mostly in the anterior portion of midgut in Lepidoptera, whereas it is in the posterior part in the case of Coleoptera. The highly alkaline pH of lepidopteran gut plays an important role in toxin solubilization. The protoxins acted upon by the proteases are converted to active toxin by removal of amino acids from C-terminal. Cry3A protoxins specific to coleopterans lack cysteine groups from the C-terminal region. Earlier this was identified as the reason for toxicity under the acidic pH of coleopteran midgut. However, the identification of Cry7 toxicity in certain coleopterans points to the influence of additional factors in imparting toxicity. Serine proteases, such as chymotrypsin and trypsin, dominate in lepidopterans and dipterans, whereas cysteine and aspartic proteases dominate in coleopterans (Chougule et al. 2008; Domínguez-Arrizabalaga et al. 2020).

The mode of action is explained by three models:

The classical model: In this basic model, the toxin dissolves in the insect's alkaline midgut and undergoes proteolytic activation into toxic polypeptides. These fragments bind to receptors on midgut epithelial cells, creating pores in the membrane and disrupting function, which allows gut contents to leak through. This midgut damage, combined with spores spreading, germinating and multiplying in the haemolymph, leads to septicemia and the death of the larvae (Adang et al. 2014). This model fails to look deeply into the process.

The sequential binding model: The protoxin once activated by proteases, attach to the primary cadherin receptor from the domain II loops. This results in further cleavage of the α helix-1 at the N-terminal side, leading to the formation of a pre-pore oligomer (Rausell et al. 2004). Ultimately, this oligomer binds to the secondary receptors, the GPI-anchored proteins (APN or ALP) through the domain III epitope and exposed loops of domain II. The unification of Cry receptor and the toxin was reported to catalyse channel formation in the phospholipid membrane. The receptor facilitates pore formation by the oligomer paving way for midgut lysis and death (Vachon et al. 2012; Adang et al. 2014). A similar sequence of activities is noted to occur with the (Etx_Mtx2) like Cry toxins also (Szczesny et al. 2011). The mode of action of Cry toxin in insect gut is illustrated in Fig. 1.

The signalling pathway model: This model addresses the changes in cellular metabolism rather than the lytic effect of Cry toxins. The binding of Cry toxin with cadherin initiates several Mg^{2+}-dependent cell signalling cascades. This involves activation of the cell surface receptors, G proteins. Activation of G proteins stimulates adenylate cyclase, an enzyme responsible for producing the secondary messenger molecule, cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). Elevated cAMP levels further activate protein kinases A (PKAs), initiating a series

Table 2 Non-Cry *Bt* proteins and their structure

Pesticidal protein class	Description	*Bt* Toxins	Structure	References
App	α-helical pore-forming toxins	Cry6	Two-domain architecture with 7-α helices Size: 48.23 kDa	Huang et al. (2016)
Cyt	Non-specific cytolytic toxins	Cyt1, Cyt2 and Cyt3	Single domain comprising of a mixed β-sheet at the centre surrounded by two α-helices on either side	Li et al. (1996)
Gpp	Aegerolysin-related pore-forming toxins	Cry34Ab	Single domain of β sandwich conformation with 2 β-sheets. β-sheet I contains four β-strands while β-sheet II has five β-strands Size: 13.62 kDa	Kelker et al. (2014)
Mpp	Etx/Mtx2-related beta pore-forming toxins	Cry15Aa, Cry23Aa, Cry33Aa, Cry45Aa, Cry45Ba, Cry51Aa, Cry60Aa, Cry60Ba, Cry64Aa, Cry74Aa, Mtx2, Sip	Three domains Domain 1 is composed of a short N-terminal β-strand, an α/β structure, a β-hairpin and an α-helix. Domain 2 is a β-sandwich of two-stranded β-hairpin and a curled antiparallel five-stranded β-sheet. Domain 3 is a β-sandwich composed of antiparallel three-stranded and two-stranded β-sheets	Akiba et al. (2006)
Mtx	Mosquitocidal protein from *L. sphaericus*	Mtx1	Four ricin B-type domains curling around the catalytic domain (ADP-ribosyl transferase domain) in a hedgehog-like assembly Size: 100 kDa	Berry (2012)
Spp	Pore-forming toxin from *L. sphaericus*		Size: 53 kDa	Nishiwaki et al. (2007)
Tpp	β pore-forming proteins	Cry35Aa, Cry35Ab, Cry35Ac, Cry35Ba, Cry36Aa, Cry49Aa, Cry49Ab	Cry35Ab: Two domains, N-terminal domain is a β-trefoil fold, next domain contains six helices and three antiparallel β-sheets. A four antiparallel strand β-sheet sits below the N-terminal domain and another two β-strands form a β-sandwich Size: 44.07 kDa	Kelker et al. (2014)
Vip	Vegetative insecticidal proteins	Vip3	Vip3B: Five domains, N-terminal two α-helical domains connected by a twisted 70-residue contiguous helix, C-terminal with three discrete β-sheet domains Size: 360.29 kDa	Zheng et al. (2020)
Vpa	Active component of the Vpa/Vpb binary pesticidal protein	Vip2	Vip2A: Two domains structure. Both domains have a five-stranded mix β-sheet and a three-stranded antiparallel-sheet. Around the β-sandwich core, consecutive four α-helices and one additional α-helix present Size: 45 kDa	Syed et al. (2020)

Table 2 (continued)

Pesticidal protein class	Description	*Bt* Toxins	Structure	References
Vpb	Binding component of the Vpa/Vpb binary pesticidal protein	Vip1, Vip4	Vip4: Two domains—PA14 domain, bacterial Binary_ToxB domain Size: Vip4-108 kDa, Vip1-80 kDa	Syed et al. (2020)

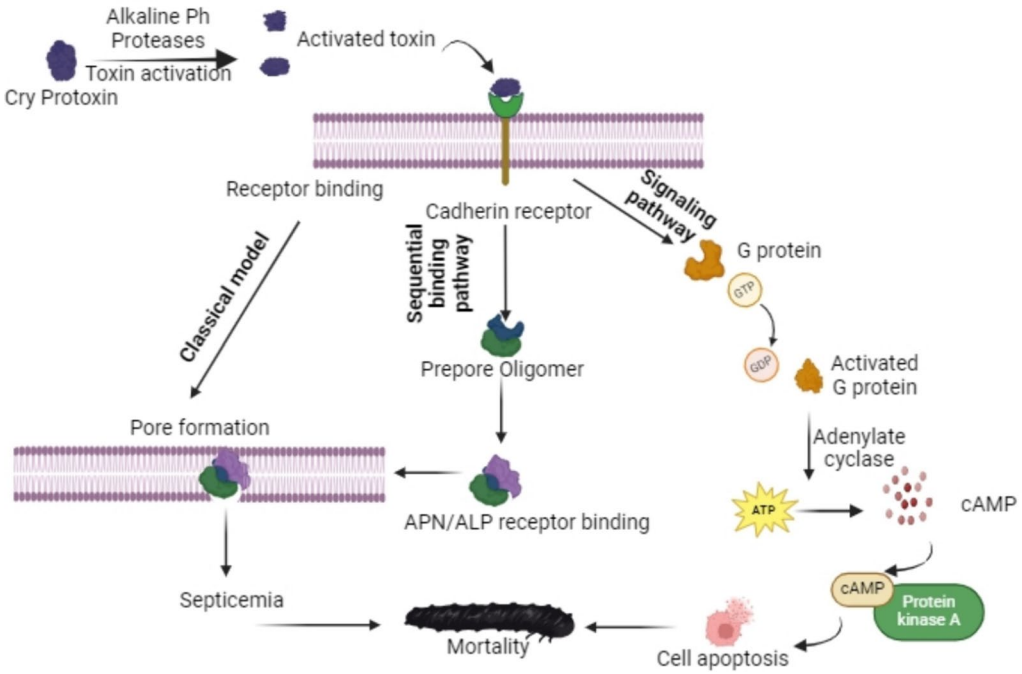

Fig. 1 Cry toxin mode of action

of downstream signalling pathways. These pathways ultimately result in the disruption of ion channels and cytoskeletons, as well as the acceleration of cell apoptosis (Vachon et al. 2012; Adang et al. 2014).

Vip toxin

Apart from the Cry toxins produced during sporulation, another set of toxins known as Vip (vegetative insecticidal protein) toxins are produced during the growth phase, beginning from the middle of the log phase and continues in sporulation phase.

In the case of Vip1/Vip2 binary toxins, the process commences with the ingestion of toxins followed by the action of trypsin-like proteases and oligomerization. This activated Vip1 toxin recognizes the receptors which is followed by membrane insertion and channel formation. Vip2 is hypothesized to enter into the cell by endocytosis or directly through the channels made by Vip1. The catalytic core of Vip2 transfers the ADP-ribose group from NAD to actin, thus disrupting the microfilament formation (Chakroun et al. 2016; Syed et al. 2020). Vip2 is cytotoxic to plants and that limits its application in transgenic plants. Figure 2 illustrates the mode of action of Vip1/Vip2 binary toxins in the insect body.

Coming to the Vip3 toxins, the mechanism of action is similar to Cry toxins. However, no binding sites are shared by Vip3A toxins with Cry toxins. Proteolytic activation of the toxin is not found to guarantee insecticidal effect. Once the toxin gets activated by the gut juice enzymes, the N terminus cleavage product (19–22 kDa) and the C terminus cleavage product (62–66 kDa) join to form a homotetramer (360 kDa). This is followed by receptor binding. Toxin binding to ribosomal S2 protein and *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)-fibroblast growth factor receptor-like protein (Sf-FGFR) was reported to cause changes such as DNA damage, disruption of the mitochondrial membrane and activation of caspases (caspase 3 or 9), which subsequently promotes apoptosis. A tenascin-like glycoprotein receptor identified in *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) was speculated to effect channel formation. The channels formed by Vip3 differed from that formed by Cry toxins in their conductance and ion specificity. Another protein, scavenger receptor class C-like protein (Sf-SR-C), was reported to influence Vip3A endocytosis (Chakrabarty et al. 2020; Syed et al. 2020). The detailed multistep process has not been completely clarified yet. As of now, ion channel formation, endocytosis activity and apoptosis activity are considered to be responsible for its toxicity. The mode of action of Vip3 protein is illustrated in Fig. 3.

Cyt toxins

Ability of Cyt toxin to bind with lipid membrane makes them receptor-independent toxins (Chougule et al. 2013). Two pathways were suggested to explain the

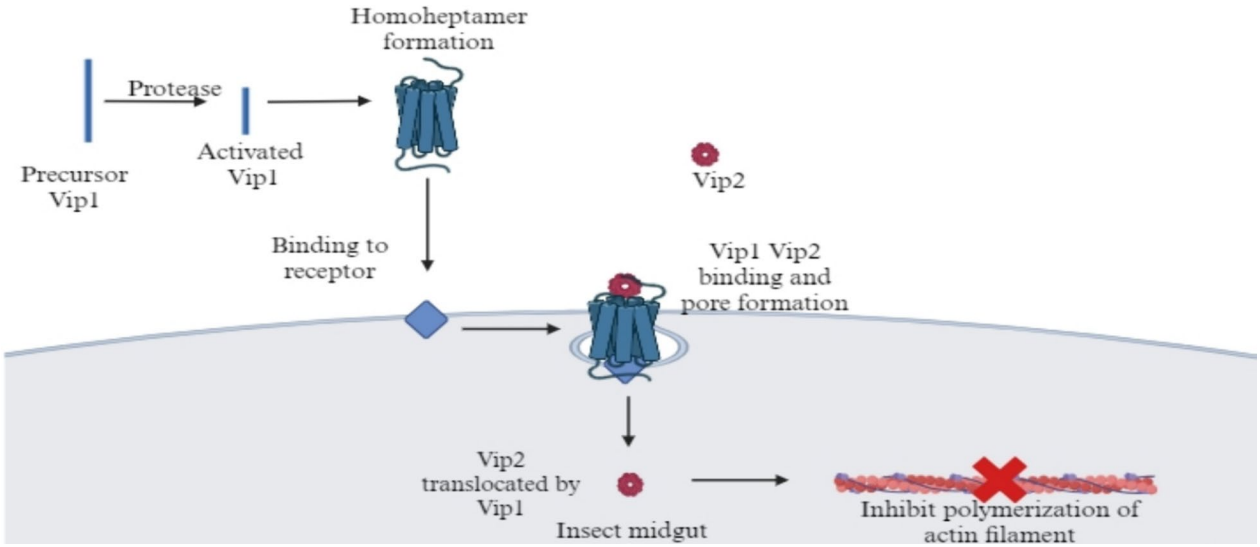

Fig. 2 Vip1/Vip2 mode of action (apoptotic pathway)

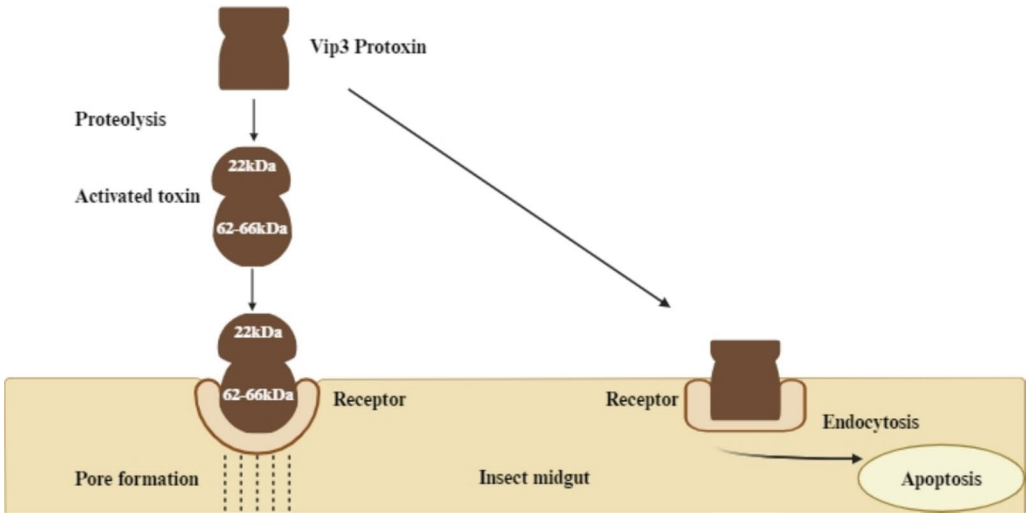

Fig. 3 Vip3 mode of action

mode of action of Cyt toxin: pore formation model and detergent action model. In the pore formation model, they just behave like the Cry toxins. Cyt toxins undergo enzymatic processing at the N and C terminals forming a protease-resistant product that imparts toxicity (Al-yahyaee and Ellar 1995). In the detergent action model, the cytolytic activity of Cyt toxins is explained by its specific binding to lipids that disturb the phospholipid layer of the membrane. Membrane permeabilization studies with Cyt1A concluded that rather than small precisely defined protein channels, they cause an overall disturbance over the membrane with a detergent-like effect (Butko et al. 1996; Manceva et al. 2005).

Applications in IPM

Bt-based products are being adopted widely in integrated pest management (IPM) strategies which reflects the growing recognition of its efficacy, environmental safety and sustainability. The utility of *Bt* in targeting various insect orders is discussed herein.

Lepidoptera

The order Lepidoptera is very popular when coming to pest management using *Bt*. It is the order with greatest number of *Bt*-related GM events until now. The strains of *Bt* such as HD 73; *Bt aizawai*-HD 68, HD 137; *Bt dendrolimus*-HD 37; *Bt darmstadiensis*-HD 146, etc., and several other strains from various geographical locations were tested against lepidopterans. Those virulent strains were made into commercial formulations such as Dipel, Halt and Xentari (Pinheiro and Valicente 2021; Vimala Devi et al. 2020). Rather than field pests, storage pests can also be managed using *Bt*. The toxins can be used for surface treatment during storage or engineered into the crop itself (Oppert et al. 2010; Malaikozhundan and Vinodhini 2018).

Lepidopteran-resistant transgenic crops

The idea of transgenic *Bt* plants took shape when the *Bt* spray formulations failed in exercising pest control. The reasons for failure with respect to the formulations are their short period of persistence and quick environmental degradation. Along with this, the tunnelling pests and root feeders escape from the toxin interaction (Sanahuja et al. 2011). Initial efforts to transform plants with the full-length toxin led to reduced insecticidal toxicity and phytotoxicity. The reason was found as due to the AT nucleotide-rich nature of toxin genes. Later this was rectified by sequence addition (Castagnola and Jurat-Fuentes 2012).

Currently, a number of crops exist in the market carrying *Bt* toxin genes. Those GM crops approved for cultivation by ISAAA is mentioned in Table 3.

Coleoptera

The story of *Bt* used against coleopterans began with the discovery of *Bt tenebrionis* in 1982 from *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) at Darmstadt (Krieg et al. 1983). The coleopteran-specific Cry toxin studies are mostly limited to the Cry3 protein family. Meanwhile, *Bt* var *sandiego* was identified to be toxic to boll weevil and Colorado potato beetle. Later both were identified to be the same (de Barjac and Frachon 1990). Now, many strains that carry Cry3 protein crystals during sporulation have become familiar such as *Bt* subsp. *tolworthi*, *Bt* subsp. *kurstaki* , etc. The activity of Cry3Aa, Cry3Ba, Cry3Bb and Cry3Ca is observed mostly against the coleopteran families Tenebrionidae, Curculionidae, Scarabaeidae, Chrysomelidae (Domínguez-Arrizabalaga et al. 2020). After its initial usage as spray formulations, successful efforts were made towards *cry3* expressing potato plants resistant to the most destructive pest, Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Adang et al. 1993). Transgenic maize carrying *cry3Bb1* for the control of *Diabrotica* sp. also came to the forefront.

Apart from Cry3 toxins, Cry7, Cry8 and binary toxins have demonstrated its insecticidal activity. Cry7Aa and the binary toxin Cry23Aa/Cry37Aa were found effective against *L. decemlineata* and *Cylas* sp. after in vitro solubilization (Ekobu et al. 2010; Domínguez-Arrizabalaga et al. 2019). The lack of toxicity in Coleoptera is due to the acidic pH that hinders the proper unfolding of the protein. Using of binary toxins proved a significant rise in toxicity as observed for Cry23Aa and Cry37Aa protein combination, when applied on beans (*Phaseolus vulgaris* (Linnaeus) (Fabales: Fabaceae)) for the management of storage pest, *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae) (Rodriguez-Gonzalez et al. 2020). The same response was recorded in the case of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Popilia japonica* (Newman) (Coleoptera: Scarabaeidae). Another pair of toxins dependent on each other for toxicity is Cry34 and Cry35. But Cry34 toxin alone is also able to effect action (Oppert et al. 2010). Cry8 toxins had demonstrated its effect against scarab beetles. Cry8Da and Cry8Db showed activity against larva and adults, whereas Cry8C worked against larva of Japanese beetle (Yamaguchi et al. 2008). Two novel toxins, Cry8Sa1 protein and Cry8Ib-like protein, were reported to be effective in controlling the sugarcane whitegrub, *Holotrichia serrata* (Fabricius) (Coleoptera: Scarabaeidae) (Naveenarani et al. 2022; Srikanth et al. 2024). A novel gene, *cry8Ka5* displayed its potential for genetic transformation of cotton against the major pest, cotton boll weevil, *Anthonomus grandis* (Boheman) (Coleoptera: Curculionidae) (Oliveira et al. 2011).

Even though Cyt proteins are primarily toxic to dipterans, Cyt1Aa showed the same effect against Cottonwood leaf beetle *Chrysomela scripta* (Fabricius) (Coleoptera: Chrysomelidae) and Cyt2Ca was toxic to *Diabrotica* sp., *L. decemlineata, Diaprepes abbreviates* (Linnaeus) (Coleoptera: Curculionidae). Transgenic citrus rootstock expressing Cyt2Ca1 was proved to be protective against *D. abbreviatus* (Mahmoud et al. 2017). Cyt toxins can be used to subdue resistance to Cry3A (Federici and Bauer 1998; Weathersbee III et al. 2006).

Besides the δ-endotoxins produced during sporulation, Vip and Sip proteins were also reported against coleopteran pests. The insecticidal activity of Sip1A protein has been reported initially against Colorado potato beetle (*L. decemlineata*), Southern corn rootworm (*Diabrotica undecimpunctata howardi* (Barber) (Coleoptera: Chrysomelidae), Western corn rootworm (*D. virgifera virgifera*), *Colaphellus bowringi* (Baly) (Coleoptera: Chrysomelidae) (Donovan et al. 2006; Sha et al. 2018). The binary toxins, Vip1/Vip2 effecting corn

Table 3 The lepidopteran-resistant GM plants approved for commercial cultivation (ISAAA, 2024)

***Bt* protein introduced**	**Targeted pests**	**References**
Cotton		
Cry1Ab	*Spodoptera exigua, S. frugiperda, Helicoverpa armigera*	Adamczyk Jr and Mahaffey (2008), Khan et al. (2013), Khan et al. (2011)
Cry1Ac	*H. armigera, Pectinophora gossypiella, Helicoverpa zea, S. exigua, S. frugiperda, S. litura, Pseudoplusia includens*	Adamczyk Jr and Gore (2004), Tindall et al. (2009), Sivasupramaniam et al. (2014)
Cry1F	*S. frugiperda, S. litura, H. zea, P. includens, S. exigua*	Tindall et al. (2009), Adamczyk Jr and Gore (2004), Siebert et al. (2008a, 2008b)
Cry2Ab2	*P. gossypiella, S. frugiperda, S. exigua, S. litura*	Sivasupramaniam et al. (2014)
Vip3A	*H. zea, P. gossypiella, H. armigera, S. exigua, S. frugiperda*	Adamczyk Jr and Mahaffey (2008), An et al. (2010), Chen et al. (2017), Yang et al. (2022), Tabashnik et al. (2022)
Cry1Ac + Cry2Ab	*H. armigera, Sylepta derogata*	Héma et al. (2009)
Cry1Ab + Vip3A	*S. exigua, S. frugiperda, H. zea*	Adamczyk Jr and Mahaffey (2008)
Cry1Ac + Cry1F	*S. frugiperda, S. exigua, Chloridea virescens, H. zea*	Adamczyk Jr and Gore (2004), Siebert et al. (2008b)
Cry1Ac + Cry1F + Vip3A	*H. armigera, H. zea*	Marques et al. (2023)
Maize		
Cry1Ab	*H. zea, S. frugiperda, Plodia interpunctella*	Buntin (2008)
Cry1F	*S. frugiperda, H. zea, Elasmopalpus lignosellus, A. ipsilon*	Buntin (2008), Siebert et al. (2008a), Marques et al. (2019)
Cry9C	*O. nubilalis, Diatraea grandiosella, S. frugiperda*	Reed and Halliday (2001), Bokonon-Ganta et al. (2003)
Vip3Aa20	*A. ipsilon, H. zea*	Marques et al. (2019), Niu et al. (2021)
Cry1A.105 + Cry2Ab2	*E. lignosellus*	Marques et al. (2019)
Cry1Ab + Vip3Aa20	*H. zea, O. nubilalis, S. frugiperda*	Burkness et al. (2010), Lin et al. (2022), Eghrari et al. (2022)
Cry1A.105 + Cry1F + Cry2Ab2	*E. lignosellus, A. ipsilon*	Marques et al. (2019)
Brinjal		
Cry1Ac	*Leucinodes orbonalis*	Hautea et al. (2016), Prodhan et al. (2019)
Rice		
Cry1Ab	*Chilo suppressalis, Scirpophaga incertulas, Cnaphalocrosis medinalis, Herpitogramma licarisais, Sesamia inferens, Naranga anescens, Mycalesis gotama, Parnara guttata*	Shu et al. (2000)
Cry1Ab + Cry1Ac	*Tryporyza incertulas, C. medinalis*	Wang et al. (2010)
Tomato		
Cry1Ac	*Tuta absoluta*	Jalapathi et al. (2020)
Sugarcane		
Cry1Ac	*Diatraea saccharalis, Telchin licus*	Gao et al. (2016), Sakuno et al. (2024)
Populus		
Cry1Ac	*Hyphantria cunea*	Liu et al. (2016)
Soybean		
Cry1Ac	*Anticarsia gemmatalis, C. virescens, Helicoverpa sp., Chrysodeixis includens, S. litura*	Yu et al. (2013), Horikoshi et al. (2021)
Cry1Ac + Cry1F	*Spodoptera eridania, Spodoptera cosmioides, Spodoptera albula, E. lignosellus, A. ipsilon, H. armigera*	Marques et al. (2017), Machado et al. (2020)
Cowpea		
Cry1Ab	*Maruca vitrata*	Addae et al. (2020)

rootworms (*D. virgifera, Diabrotica longicornis* (Say) (Coleoptera: Chrysomelidae), *D. undecimpunctata*) and Scarabaeids (*Holotrichia oblita* (Falderman) (Coleoptera: Scarabaeidae), *Holotrichia parallela* (Motschulsky) (Coleoptera: Scarabaeidae), *Anomala corpulenta* (Motschulsky) (Coleoptera: Scarabaeidae)) have been reported (Bi et al. 2015). Vip1Aa/Vip2Aa, Vip1Aa/Vip1Ab, Vip1Ba/Vip2Ba and Vip1Bb/Vip1Ba toxin combinations have shown its potential against *D. virgifera virgifera* (Chakroun et al. 2016; Domínguez-Arrizabalaga et al. 2020).

Coleopteran-resistant transgenic crops

Formulations successful for the control of various coleopteran pests are being devised and used (Eski et al. 2017; Kim et al. 2015). Other than that *Bt* crops provide an extended control as the entire plant expresses the gene and encounter the pest, which is a limitation of formulations. Potato and maize plants expressing *Bt* genes are being approved and used in many countries. Table 4 shows the GM events approved by ISAAA.

Hemiptera

The lower toxicity of *Bt* to hemipterans compared to other pest orders can be attributed to the fact that *Bt* has not evolved for infecting hemipterans. This is inferred from the ecology of the bacteria and the piercing and sucking feeding behaviour of hemipterans (Schnepf et al. 1998). *Bt* toxicity in hemipterans is accounted in *Bt* transgenic plants rather than spray formulations due to their sap sucking behaviour. The observed toxic effect may be seen as a consequence of the similarity of glycoproteins present in hemipterans and other insect orders (Porcar et al. 2009). With the advent of transgenic cotton, the number of insecticidal sprays reduced significantly. This resulted in a notable surge in the mirid pest population such as *Lygus lucorum* (Meyer-Dür) (Hemiptera: Miridae), *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae) and *Adelphocoris* spp. (Lu et al. 2010; Li et al. 2010, 2011a). Plant bug *Lygus* sp., a major sap feeder, is an economic pest of cotton in the USA. The proteins Cry15, Cry23, Cry33, Cry45 and Cry46 were expressed in cotton plants that caused mortality and mass reduction in *Lygus hesperus* (Knight) (Hemiptera: Miridae) (Baum et al. 2012). A non-preference strategy for feeding and oviposition was shown against *Bt* plants when cotton thrips and tarnished plant bugs (*Lygus lineolaris* (Palisot) (Hemiptera: Miridae) were subjected to choice tests between *Bt* Cry51Aa2 plants and non-*Bt* plants (Graham et al. 2019).

Coming to homopterans, the solubilized Cry1Aa, Cry1Ab, Cry1C, Cry1F, Cry2A, Cry3A and Cry4D caused significant mortality of the potato aphid, *Macrosiphum euphorbiae* (Thomas) (Hemiptera: Aphididae). A strong mortality was observed for Cry2A toxin (Walters and English 1995). The bioassays could infer that unlike the other orders, reluctance to feed after toxin ingestion was not observed. A low to moderate toxicity was observed in *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) when administered with solubilized forms of Cry3A, Cry4Aa, Cry11Aa and Cyt1Aa (Porcar et al. 2009). The Cry1Ac toxin could undergo complete processing following the action of gut proteases and the receptor binding of activated toxin was reported to be glycan (GalNAc) mediated (Li et al. 2011b). A homopteran-specific Cry protein having 40% sequence similarity to Cry41Aa1 and Cry41Ab1 parasporins was found to be toxic to green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae). Homopteran-specific toxins, Cry64Ba/Cry64Ca, displayed a high level of activity against the rice plant hoppers *Laodelphax striatellus* (Fallén) (Hemiptera: Delphacidae) and *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae) (Liu et al. 2018). Vip1 and Vip2 proteins were seen to have a notable effect against cotton aphid (*Aphis gossypii* (Glover) (Hemiptera: Aphididae). The Vip1Ae/Vip2Ae binary toxins also exhibited toxicity (Sattar and Maiti 2011).

As these fluid feeders excrete out significant amounts of their diet rapidly, the retention time of toxin in the midgut will be less. This is the reason for the low toxicity in hemipterans compared to lepidopterans and coleopterans (Walters and English 1995). Studies indicate the presence of enzymes, aminopeptidase and α-glucosidase in the posterior midgut and cysteine protease in the anterior midgut. The modified perimicrovillar membrane is involved in reversible binding for enhancement of amino acid absorption, prevention of excretion of cathepsin-L-like cysteine proteinase, maintenance of osmolarity, etc. The acidic pH of the gut is yet another factor that can affect toxin solubility and thereby reduce toxicity (Cristofoletti et al. 2003). Transgenic crops having insecticidal action against hemipterans have been developed, though it was not much pronounced as in Lepidoptera and Coleoptera. Transgenic cotton with a modified Mpp51Aa2 toxin showcased potential toxicity against hemipterans and thrips. This is the only approved GM event used in hemipteran pest management (Asiimwe et al. 2023).

Table 4 The coleopteran-resistant GM plants approved for commercial cultivation (ISAAA, 2024)

Crop	*Bt* protein introduced	Targeted pests	References
Potato	Cry3A	*L. decemlineata*	Salehian et al. (2021)
Maize	Cry34Ab1/Cry35Ab1 + Cry3Bb1	*Diabrotica barberi*	Ludwick et al. (2017)
	Cry3Bb1	*D. barberi*	Siegfried et al. (2005)
	mCry3A	*D. barberi*	Oyediran et al. (2016)
	DvSnf7 + Cry3Bb1	*D. undecimpunctata*	Levine et al. (2015)

Diptera

Bacillus thuringiensis subsp. *israelensis, B. sphaericus* and *B. thuringiensis* subsp. *jegathesan* are the major bacterial species and subspecies associated with specificity towards dipterans. *Bt israelensis* produces parasporal inclusions containing Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba (Federici et al. 1990; Pérez et al. 2007). *Lysinibacillus sphaericus,* formerly known as *B. sphaericus* produces binary toxins, Tpp1/Tpp2 (formerly known as BinA/BinB) and Cry48/Tpp49 (Tpp49 formerly known as Cry49) along with Mtx1, Mpp2 (formerly known as Mtx2), Mpp3 (formerly known as Mtx3), Mpp4 (formerly known as Mtx4) and Sphaericolysin (Berry 2012). *Bt jegathesan* is reported to produce eight protoxins, namely Cry11Ba, Cry19Aa, Cry24Aa, Cry25Aa, Cry30Ca, Cry60Aa, Cry60Ba and Cyt2Bb (Sun et al. 2013). The presence of novel toxins makes this strain to be potent over *Bt israelensis.* Apart from the above-mentioned species, several other subspecies such as *Bt canadensis*, *Bt thompsoni* and *Bt malaysiensis* were identified to carry the mosquitocidal toxins Cry4A, Cry4B, Cry11A and Cyt1A (Ragni et al. 1996). *Bt kyushuensis, Bt tenebrionis, Bt medellin* and *Bt darmstadiensis* producing a different cytolytic toxin from *Bt israelensis* have been investigated. This suggests the variability observed among Cyt toxins (Knowles et al. 1992; Guerchicoff et al. 1997; Juárez-Pérez et al. 2002).

Coming to the Cry toxins, as seen in lepidopterans, the Cry11A toxin was found to bind with a cadherin receptor in *Aedes aegypti* (Linnaeus) (Diptera: Culicidae), enabling oligomerization which subsequently will bind to secondary receptor ALP (Fernandez et al. 2006). Cry11B, Cry4A and Cry4B competing with Cry11A for the toxin binding site clarify the reason for cross-resistance observed to varying extend among these toxins (Buzdin et al. 2002; Chen et al. 2009). The synergistic action of cadherin (AgCad1) on Cry4Ba toxin was demonstrated in *Anopheles gambiae* (Giles) (Diptera: Culicidae) (Hua et al. 2008). This may be due to the oligomerization of toxin after contact with cadherin protein. Cry4Aa protein toxic to larva of *Aedes* sp. and *Anopheles* sp. and Cry4Ab protein toxic to larva of *Culex* sp., *Aedes* sp. and *Anopheles* sp., structurally and functionally resembles the lepidopteran toxic Cry1A (Boonserm et al. 2005, 2006).

Rather than individual toxicity, the proteins exhibit high lethal effect when present together. The reason behind synergistic action of Cyt1A on Cry11A toxin revealed the role of Cyt1A in the formation of pre-pore oligomer. The role of Cyt1A toxin as a receptor of Cry11A was reported to be responsible for the synergistic action of Cyt1A toxins on Cry11A. Thus, *Bt israelensis* can be viewed as a bacterium capable of manufacturing toxin and its binding receptor (Pérez et al. 2007). *Bt israelensis* possess 3 Cyt toxins: Cyt1Aa, Cyt2Ba and Cyt1Ca (Cohen et al. 2008). Even then strains resistant to *Bt israelensis* are reported to occur. The β-exotoxins in *Bt* also have a profound effect on dipteran life stages as understood from the studies on houseflies. In house flies, treatment with toxin led to delayed larval development, arrested moulting, prevented pupation and adults exhibited teratological effects. *Bt israelensis* toxicity is utilized against agricultural and veterinary important pests *Tabanus triceps* (Fabricius) (Diptera: Tabanidae), *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (Mexican fruit fly), *Bradysia coprophila* (Lintner) (Diptera: Sciaridae) (Fungus gnats), *Rivellia angulata* (Diptera: Platystomatidae) and Chironomid midges (Margalith and Ben-Dov 2000).

Nematodes

The initial studies on nematicidal *Bt* toxins were focussing on animal parasitic nematodes (Burrows and De Waele 1997). Strains of *Bt israelensis* and *Bt kurstaki* tested against the ruminant nematode *Trichostrongylus colubriformis* (Giles) (Rhabditida: Trichostrongylidae) had an ovicidal effect. No effect was seen in the third stage larva and adult helminths (Bottjer et al. 1985). β-exotoxins effecting vertebrates and invertebrates have a profound effect on nematodes also. In a study conducted with *Bt* β-exotoxin, Thuringiensin, on *Caenorhabditis elegans* (Maupas) (Rhabditida: Rhabditidae) and *Meloidogyne incognita* (Kofoid and White) (Tylenchida: Heteroderidae), 100% mortality was observed against the former and significant effect was seen against the latter also. But only a very high quantity of the active toxin could produce an appreciable effect on nematode population (Devidas and Rehberger 1992). Cry5 and Cry6 proteins are the most studied with respect to nematicidal property. Cry5B, Cry14A, Cry21A and Cry6A exhibited toxicity against four phylogenetically diverse nematode species (Wei et al. 2003). Cry5B, identified as a nematicidal toxin, prevented blood feeding nematode *Ancylostoma ceylanicum* (Rhabditida: Ancylostomatidae). Exposure resulted in decreased egg production by the nematodes (Cappello et al. 2006).

Compared to insects, the toxin specificity to intestinal receptors is less in nematodes, as observed in the free-living nematode *C. elegans.* Optical microscopy revealed the toxin action to occur in two phases within 24 h. The dissolution of crystals occurred at a slower pace. During the initial 12 h of toxin ingestion, there is a progressive breakdown of the four cells at the anterior intestinal ring, posterior to pharynx. The second phase witnesses the degradation of the remaining intestine which is believed to be because of decomposition of deceased nematode (Borgonie et al. 1995). On comparing this mode of

action to that in insects, differences and similarities are observed. An increasing level of toxicity is seen from younger stage to adult stage, lacking a specific stage for toxicity in the case of nematodes. In contrast, insecticidal toxins are specific to larval stage. Another notable difference is the slower activity in nematodes compared to the rapid toxicity observed in lepidopterans and dipterans. Similar to insects, toxins display species specificity in nematodes also. Once germinated in the gut, they colonize the entire nematode body in 24 h.

Field trials with a potent *Bt* strain demonstrated reduction in galls due to *M. incognita* and reduction of *Rotylenchulus reniformis* (Linford and Oliveira) (Tylenchida: Hoplolaimidae) population in tomato and pepper plants. It was then applied onto the seed coats of strawberry, which demonstrated control over *Pratyenchus penetrans* (Cobb) (Tylenchida: Pratylenchidae) and the pathogen *Rhizoctonia fragariae* (Husain and McKeen) (Cantharellales: Ceratobasidiaceae) (Zuckerman et al. 1993). Cry1Ea11 protein showed significant nematicidal activity against the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) (Aphelenchida: Parasitaphelenchidae), marking the first instance of Cry1 proteins being effective against plant-parasitic nematodes (Huang et al. 2018). Till now, only one *Bt*-transformed nematode-resistant plant is approved for cultivation. GMB151 soybean combines an herbicide-resistant gene as well as a nematode toxic gene, *cry14Ab-1.b.* The toxin targets cyst nematodes infesting soybean (Organisms et al. 2021).

Thysanoptera

Potential of *Bt* in thrips management has been dealt very recently. The thrips in cotton ecosystem witnessed a hike in population with the advent of *Bt* cotton as seen in the case of hemipterans. Novel isolates with enhanced effectiveness are being discovered (Maurastoni et al. 2023). Transgenic cotton varieties with an extended action spectrum developed by incorporating Cry51Aa repelled the female thrips from ovipositing and reduced the larval and adult feeding. However, the susceptibility was noticed to be prominent in *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) compared to *Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae) (Huseth et al. 2020). 'Thryvon', a transgenic cotton variety expressing Cry51Aa developed against sucking pest, could effectively control the thrips population in field (Whitfield et al. 2022). Overall sucking pest control using *Bt* is an emerging area and needs further research.

Acari

Tyrophagus putrescentiae (Schrank) (Sarcoptiformes: Acaridae), a mould mite under storage conditions evaluated against *Bt israelensis* and *Bt tenebrionis* showed significant effect on growth and biology (Ahmed et al. 2016). Many studies on livestock mites and ticks concluded that *Bt* has potential in exercising control. Cry3A toxin was reported to have effect against mites and ticks (Dunstand-Guzmán et al. 2015; Erban et al. 2009). Varroa mite, a major pest in the apiary, was seen to be controlled by *Bt* application without effecting any of the bee castes (Alquisira-Ramírez et al. 2014). A non-preference strategy was seen towards *Bt* maize when carmine spider mite, *Tetranychus cinnabarinus* (Boisduval) (Trombidiformes: Tetranychidae), was subjected to *Bt* and non-*Bt* maize (Prager et al. 2014). The crude pellets of *Bt kurstaki* could significantly reduce the population of spider mite, *Eutetranychus orientalis* (Klein) (Trombidiformes: Tetranychidae) under laboratory conditions (Veloorvalappil Narayanan et al. 2018). However, still there is no clear idea regarding the mode of action of *Bt* in these organisms. Further intensive studies are needed in this area to manage these emerging crop pests.

Challenges and solutions

Bt formulations face significant challenges in the field, impacting their effectiveness and sustainability. Their widespread use is hindered by production and formulation costs, with harvesting and formulation efficiency being critical for marketability and effectiveness. Formulation instability and the degradation of pesticidal proteins due to ultraviolet radiation reduce the activity of *Bt* products. Additionally, varying environmental conditions lead to inconsistent efficacy, complicating their application. Furthermore, *Bt* formulations have a limited shelf life and maintaining optimal concentrations in the field poses difficulties (Devi et al. 2019). A major concern is the potential for resistance development in target pests, primarily caused by changes in target receptors. The extensive use of specific Cry proteins in transgenic crops like cotton and corn has accelerated resistance in pests, prompting regulatory bodies like the Environmental Protection Agency for Integrated Resistance Management plans (Storer et al. 2012). While *Bt* is generally safe for non-target organisms, there are ecological impacts that need careful assessment. Adverse effects on predators and parasitoids can occur indirectly through preys that have ingested *Bt* toxins (Yu et al. 2011; Mandal et al. 2020). Although honeybees are largely unaffected, some pollinators like butterflies have shown detrimental effects from *Bt*-contaminated pollen. Transgene flow occurring when *Bt* genes are unintentionally transferred to wild relatives, non-*Bt* plants or other organisms is another concern (Pretty 2001).

To address the challenges associated with *Bt*, several strategies have been developed to enhance its effectiveness and delay resistance development. Initially a high

dose/refuge strategy was implemented, relying on the rare occurrence of resistance alleles in heterozygous condition. This approach involves planting non-*Bt* crops (refuges) alongside *Bt* crops to maintain a population of susceptible pest that can dilute the resistance genes through mating. However, improper implementation, complex behaviour of pests and the chance for transfer of *Bt* genes to the non-*Bt* refuge crop over time are certain drawbacks (Storer et al. 2012). Manipulating the suitability of refuge host plants to be lower can enhance fitness costs for *Bt*-resistant pests, thereby improving the effectiveness of the refuge strategy in delaying resistance development (Carrière and Tabashnik 2024). Gene pyramiding is a strategy which involves combining multiple *Bt* proteins with different modes of action in a single plant, making it harder for pests to develop resistance. Examples include Bollgard II (Cry1Ac + Cry2Ab), Widestrike (Cry1Ac + Cry1F) and VipCot (Cry1Ab + Vip3A) (Storer et al. 2012). Additionally, proper screening of the genetic variability of target pests and DNA-based resistance allele detection in heterozygotes are essential for timely interventions (Morin et al. 2003). These strategies, informed by pest population dynamics and ecology, aim to sustain the efficacy of *Bt* crops and manage resistance effectively. Advances in *Bt* formulations, such as microencapsulations and micro-granules, aim to overcome environmental challenges, and future alternatives like fermented wastewater and wastewater sludge formulations promise for cost-effective and environmentally friendly solutions (Devi et al. 2019). Protein engineering is another area of interest that aims at broadening the toxicity spectrum of a particular toxin or designing a new version of a toxin against any group of insects. Hybrid proteins formed by the modification of already existing toxins have the potential to delay resistance development (Torres-Quintero et al. 2018; Hou et al. 2019).

Conclusion

Bt stands out as a naturally occurring bacterium that has gained widespread attention for its remarkable ability to produce protein toxins. The toxins, lethal to specific insect groups upon ingestion, have garnered significant attention as biopesticides owing to their selectivity and minimal impact on non-target organisms, including humans and beneficial insects. The current status of the effective *Bt* toxins, their utilization and their application as *Bt* transgenic lines specific to lepidopterans, coleopterans, hemipterans, dipterans, thrips, mites and nematodes has been discussed. The utilization of *Bt* toxins in agriculture has primarily targeted pests from lepidopteran and coleopteran orders, showcasing notable effectiveness. While the effectiveness of *Bt* toxins in Lepidoptera, Coleoptera and Diptera is well established, further research is warranted to extend its efficacy to other insect orders. Understanding the toxicity variation among different insect orders is crucial, with the gut conditions playing a pivotal role in mediating this variation.

In conclusion, advancing our understanding of molecular aspects of *Bt*, toxicity variations across insect orders and strategies for managing resistance are paramount for maximizing its potential as a safe and effective tool in agricultural pest management practices. Comprehensive studies elucidating the pest population dynamics, diverse mode of action of various *Bt* toxins, effect on natural enemies and the development of resistance management strategies are imperative. Continued interdisciplinary research efforts are essential for addressing the evolving challenges in pest control while ensuring environmental sustainability and food security.

Abbreviations

Bt	*Bacillus thuringiensis*
Vip	Vegetative insecticidal protein
Sip	Secreted insecticidal protein
ISAAA	International service for the acquisition of agri-biotech applications
BBMV	Brush border membrane vesicles
LC_{50}	Lethal concentration 50
GM	Genetically modified
ALP	Alkaline phosphatase
APN	Aminopeptidase N
IPM	Integrated pest management

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