DEFRA Research Contract EPG 1/5/156

Environmental Impact of *Bt* Exudates from Roots of Genetically Modified Plants

Final Report

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April 2002

Executive Summary

Within the specific terms of reference for this review, emphasis has been placed on the newly described pathway of entry of *Bacillus thuringiensis* (*Bt*) (Procaryotae: Firmicutes: Bacillaceae) toxin to soil via root exudates (Saxena *et al.*, 1999). In order to place this novel finding in context, the review has taken an ecological approach to evaluation of all aspects of *Bt* occurrence and biology spanning the crop and the putative host "environments". There have been enormous advances in the understanding of modes of action and genetic diversity of *Bt*, which is reflected in the literature database of 4500 pertinent references, the majority of which have been published during the past 5 years.

The review is divided into 5 principal Chapters, initially providing comprehensive background information on *Bt*, in nature and as a microbial insecticide, and then bringing together the various elements of interaction between the bacterial toxin and potential hosts in a framework previously used in reviewing the environmental risks from microbial insecticides (Evans, 1999).

Chapter 2: *Bacillus thuringiensis* and its toxin genes

Bt is a crystalliferous spore-forming bacterium, which typically contains both a spore and a crystal toxin within its cell wall. *Bt* has a long and safe history of use as a microbial insecticide and this has prompted research into the nature of the toxin moiety that is central to mortality of target organisms. Recognition that *Bt* has a diverse genetic structure within the range of toxin genes, referred to as Cry genes, has led to research into the interactions between crystal toxins and potential hosts. Although *Bt* was already the dominant microbial insecticide globally, the ability to identify and incorporate toxin genes into plants has accelerated interest in the agent and has expanded both research and uptake in pest management.

Bacillus thuringiensis *biology and mode of action* (Chapter 2.1)

Chapter sections 2.1.1 to 2.1.5 describe the history of *Bt* use, including conventional application, recognition of the cry genes responsible for toxicity and their classification, structure and roles in determination of host ranges.

Bt must be ingested before it can affect target organisms and detailed descriptions are provided of the host interactions at the gut level (Chapter 2.1.6). A three stage process is necessary to induce toxicity in potential hosts.

1. Initially gut pH conditions must be suitable to solubilise the protein protoxin. Among the insects, Lepidoptera and Diptera have gut pHs that are highly alkaline and solubilisation characteristics of Cry toxins active for these Orders reflect this. Coleoptera active Cry genes, however, are solubilised at pHs nearer to neutrality, again reflecting typical gut conditions in their normal hosts.
2. Proteolysis, the action of gut proteolytic enzymes, then results in dissolution of the toxin to remove amino acid sequences from the C- and N- terminal ends of the toxin, leading to activation of the toxin core.
3. Binding. The toxin binds to the midgut brush border epithelium and, by conformational changes and insertion into the epithelial border, develops a pore leading to cell lysis and death.

Incidence in Nature (Chapter 2.2)

Bt is present in most habitats, whether affected by human activity or not. The presence of the bacterium in stored product facilities, on aerial plant surfaces and, particularly, in soil indicates that

survival and growth of *Bt* is common in these environments. Despite the ubiquitous nature of the organism, its biology and ecology in relation to interaction with putative hosts is poorly understood. Presence in soil is linked closely to adsorption to soil aggregates, with particular linkage to presence of clay minerals and humic acids. This applies to all stages of *Bt*, including the new variant of truncated toxin that is associated with transgenic plants.

Chapter 3: Use of *Bt* as a pest management tool

Bt is the dominant microbial control agent, but still represents only about 2% of the global insecticide market in terms of conventional application of the organism.

Conventional application (Chapter 3.1)

Application of *Bt* against a range of pests is normally achieved through spray application of a formulated product containing spores, crystal toxins and various additives to aid adhesion and to protect against ultra violet light. Methods to optimise the application and retention of *Bt* in the field include improved activity of the spore/toxin combination, higher concentrations and use of sophisticated spray application and decision support tools. Despite these improvements in technology, a considerable proportion of the spore/toxin mixture misses the primary target and reaches the soil.

Effects of Bt on non-target organisms (Chapter 3.2)

One of the major perceived and demonstrated benefits of using *Bt* in pest management programmes is the lack of impact on non-target organisms relative to use of broad-spectrum chemical insecticides. A number of examples of interactions with non-target organisms are summarised, particularly in Table 3.2, and indicate that, on the basis of current knowledge, *Bt* effects on hosts are consistent with the known parameters leading to toxicity. However, there are complex interactions that indicate the need for greater research in this area.

Application as a component of transgenic plants (Chapter 3.3)

The successful incorporation of *Bt* toxin genes into a range of crop plants has revolutionised the application of *Bt* for pest management. Use of constitutive or tissue-specific promoters has enabled expression levels to be reached that result in high mortality of target organisms feeding on *Bt*-plants. As technology has improved, the quantity of toxin per g of plant tissue has increased. Expression levels in leaves, stem, roots and plant reproductive tissues vary both with the promoter employed and with the age of the plant. Currently, expression is achieved using truncated forms of the toxin genes so that it is not necessary to solubilise the entire toxin moiety or to undergo full activation through proteolytic cleavage. By contrast, Wild Type toxin requires pH specific and enzyme specific activity for activation in the host gut.

The scale of development and use of Bt crops (Chapter 3.4)

Areas of *Bt*-crops planted globally reached over 11 million ha in 2000. The range of crops and of the *Bt* transformation events is growing annually and is projected to continue to expand as more crops are registered. Apart from efficacy in pest control, there have also been benefits in reductions of chemical pesticide usage.

Within an overall evaluation of the ubiquitous presence of Wild Type (WT) *Bt* in soil (Chapter 3.5), the specific route of entry by exudation of *Bt* from the roots of plants is discussed in detail in

Chapter 3.6, using data from Stotzky and colleagues to evaluate the temporal and spatial influences of root exudates in the rhizosphere and bulk soil. Key findings include:

1. Truncated toxins retain activity in soil for at least 234 days (the longest period studied) but at a declining rate. Retention is linked closely to the proportion of clays, especially montmorillonite, in soil. Leaching of *Bt* vertically through soil is also dependent on clay content and varies from 75% to 16% of the original quantity added to soil columns.
2. Exudation of *Bt* has been demonstrated for corn and potato but not for cotton, canola (oilseed rape) and tobacco. Although the reasons for this were uncertain, it was postulated that it could be linked to proximity of the endoplasmic reticulum to the root cell membranes.
3. Crops grown in soil known to have *Bt* root exudates present from a previous crop do not take up the toxin through their roots.
4. Assessment of effects on earthworms and soil microfauna (nematodes, Protozoa and fungi) did not indicate any adverse impact of *Bt* exudates.

Root exudates in plants: rates and function (Chapter 3.7)

Root exudation and its influence in the rhizosphere are discussed in relation to the sphere of influence of *Bt*-plant exudates in the soil environment. Both the composition and the quantity of exudates change with plant age and with position along the root. Exudates are important in determining carbon and water flow in plants and there are complex interactions with factors such as soil pH and presence of soil microorganisms including mycorrhizae.

Chapter 4: Interaction effects

The process of encounter between potential hosts and expressed Bt - introduction (Chapter 4.1)

Assessment of impacts of various pathways of *Bt* entry into soil and their relative contributions depends on encounter between *Bt* and potential hosts. The ecological basis for assessing such interactions is considered in this section of the review. Conventional application introduces both spores and toxin crystals to the phylloplane and soil environments and the pathways for reaching the soil are discussed relative to introduction through transgenic plants. Pathways for encounter of hosts with *Bt* in transgenic plants include expression in various plant tissues, either constitutively or specifically within defined tissues and, ultimately, by incorporation into soil through plant debris and by root exudation.

Encounter between target and non-target organisms and wild type and truncated Bt (Chapter 4.2)

Encounter between organisms in the leaf and stem as well as the root zones of *Bt*-plants is linked both to the total presence of consumers and to their temporal availability. The range of organisms associated with corn, as a model system, is discussed in relation to their likelihood of ingesting *Bt*. Since the majority of constructs incorporated into transgenic plants are Lepidoptera-active, this order of insects is discussed in more detail.

Factors affecting the likelihood of expression and retention of lethal/sub-lethal dosages of Bt (Chapter 4.3)

Quantitative assessment of *Bt* through the life of the crop – a whole life budget for *Bt* – is considered in this Chapter. Data from many sources have been gathered to assess *Bt* expression in various tissues and at different stages of the crop, with emphasis on corn as a model system. Expression tends to increase to anthesis (pollen shed) and then decline, with much lower levels

during senescence. Exudation of *Bt* takes place through the life of the crop, but is greatest during the early growth stages. These data are extrapolated to quantities of *Bt* per ha and, thence, per plant in order to provide a basis for determining the amount per g of soil from exudates *vs* other routes, including *Bt*-plant debris and conventionally applied *Bt*. Depending on the degree of expression, there is a very wide range of concentration per g of plant tissue, although the majority of commercial transgenic plants have concentrations of < 1.0 µg/g of tissue. The exception is chloroplast expression of Cry2Aa2 in tobacco which reaches 2-3% of total soluble leaf protein ≡ up to 30,000 µg/g. Root exudation is calculated to produce at least 256 ng/g of soil and probably higher.

Conceptual framework for encounter between Bt toxin and putative hosts (Chapter 4.4)

The conceptual framework introduced by Evans (1999) to aid evaluation of risks from genetically modified bioinsecticides has been adapted to the particular risks from truncated *Bt* introduced to the soil by exudation or other means. Discussion is concerned with Sub-Model C that deals with *Bt*-host encounter determinants at both the host ingestion stage and, particularly, by impact determined within the midgut (type of Cry gene, gut conditions and specific gut receptors). Likelihood of encounter with the *Bt* load from transgenic plants commences in the phylloplane and is taken through to the rhizosphere and bulk soil.

In summary, estimated quantities of toxin from various pathways indicate that root exudation of *Bt* does not introduce as much truncated toxin to the soil as incorporation of plant debris (Table 4.4). Likelihood of encounter with the *Bt* pool in soil is, therefore, independent of the source of the toxin and relates directly to relative densities and distributions of potential hosts and *Bt* (Chapter 4.4.1.3). Rhizosphere soil is likely to retain more *Bt* than bulk soil, although this is tempered by higher microbial activity and degradation in this zone.

Encounter initially requires ingestion of *Bt* by potential hosts, but the final determinants of toxicity to target and non-target organisms are driven by interactions within the gut environment. The special case of truncated *Bt vs* full protoxin is the key to risk assessment for *Bt* from transgenic plants and is driven by the likelihood of Cry toxin binding to midgut receptors and, ultimately, to pore formation and toxicity (Chapter 4.4.2 to 4.4.4).

Conclusions and research needs (Chapter 5)

This Chapter identifies hazard and discusses risk associated with various methods of *Bt* application in relation to target and non-target organisms. The principal hazards relate to direct effects on non-target organisms and the prospect of dissemination and survival of transgenically delivered *Bt*. Irrespective of the route of delivery, risk is dependent on survival of toxin through a sequence of events, each of which contributes to overall susceptibility and host range. Ingestion and final proteolytic digestion of the truncated toxin to release the fully active toxin is an important initial stage but the final determinant is receptor binding. Data from current research suggests that receptor binding is not changed dramatically by truncation and that large shifts in host range have not been observed.

The role and fate of truncated toxins is one of the key aspects of risk assessment in comparing transgenic plants to conventional *Bt* applications. The mode of delivery of the toxin does not appear to alter the quantitative aspects and so research needs to address both exudation and entry to the soil from debris of transgenic plants. Research needs are addressed by posing a series of questions and, where appropriate, suggesting methods of approach or of presentation of data.

Key research needs (Chapter 5.3)

The main questions that remain to be answered relate to the relative fitness of truncated *Bt* toxins in the environment and in the midguts of potential hosts and, using this information, on how to identify vulnerable organisms in relation to risk profile. This approach recognises that it is impossible to assess impacts on all potential hosts in an ecosystem and, therefore, a more directed research effort is required, making use of the new molecular techniques to address priorities in apportioning research effort. Key questions requiring further research are:

1. **Do the truncated toxins differ in survival characteristics once they have entered the environment?**

2. **Does truncated *Bt* have a wider host range than the protoxin from which it is derived?**

3. **If gut conditions imply that changes in specificity can be expected from employment of truncated Bt, what are the implications for organisms in the field?**

These are inter-related topics but it is answers to question 2 that will provide a rational basis for determining impacts in the field in a quantitative way and on the basis of direct impacts on potential hosts accompanied by clear demonstration of receptor binding and toxicity events.

Ecological framework for rational risk assessment (Chapter 5.4)

The interactions that will determine whether *Bt* delivered to the environment pose an unacceptable threat to one or more non-target organisms take place at a number of trophic levels. However, the key effects are driven initially by encounter frequency between a defined *Bt* load and the array of organisms likely to consume the toxin. Such encounters only "count" if the gut environment is suitable for binding and pore formation, leading to mortality of the host. Ecological impact will, therefore, be driven both at the population level, requiring assessment of species diversity and abundance, and at the organ level within putative hosts, requiring broad risk categorisation of those groups that might be vulnerable to the truncated *Bt*. This poses a dilemma in determining which is the dominant factor in determining risk. In relation to return from effort expended, it would appear that accelerated effort into determining interactions at the gut level will provide the framework necessary for designing and interpreting field sampling programmes to assess both efficacy and unintentional impacts of transgenic crops.

Concluding summary (Chapter 5.5)

1. Despite a relative lack of detailed information on the interactions between putative hosts and truncated toxins, **the balance of probability indicates that truncation does not substantially change the host range of such toxins**.
2. In relation to the specific objective of assessing the relative risks from the root exudation pathway of entry of *Bt* to soil, data indicate that quantities of toxin introduced in this way are no greater than from other pathways. **Impacts of root exudation must, therefore, be considered as part of a total *Bt* load entering the soil as a consequence of planting transgenic *Bt* crops.**
3. There is a surprising lack of quantitative information on the **total load of *Bt* in soil beneath transgenic crops**, thus pointing to the need for more research in this area. Methods are available for accurate quantification and also for assessment of potential impacts, both at the gross population level and by direct study of the effects of reversible and irreversible binding to gut receptors.

4. At the same time, **indirect effects involved in tritrophic interactions** can also be considered, although these do not appear to be influenced directly by the Bt toxin and are more the result of change in abundance of prey or host items for natural enemies.
5. The planting of large areas of transgenic Bt-crops should be taken as an opportunity for quantitative assessment of impacts on agro-ecosystem function in comparison with isogenic lines of the same crops. Opportunities exist for fully replicated tests, taking advantage of the refuge strategies being adopted for management of potential resistance to transgenically delivered Bt. **Use of molecular and biological methods for assessing effects are essential in such studies, but the approaches adopted should be optimised in relation to investigations of the interactions in the gut environments of putative hosts**. Greater collaboration between research communities embracing studies of crop dynamics linked to soil flora and fauna carried out at both the organism and molecular levels are necessary to establish a framework for risk assessment.

1 Background and purpose of the review

The recent finding that the Cry1Ab toxin of a micro-organism, *Bacillus thuringiensis* (*Bt*), when expressed in transgenic corn (maize), *Zea mays*, is subsequently released into the rhizosphere in exudates from the roots has introduced a new risk pathway for a GM product (Saxena *et al.*, 1999). Current risk assessments pertinent to the parental *Bt* have concentrated on pathways for encounter between the organism and potential hosts when wild-type or GM *Bt* is released by "conventional" means such as spray applications, a typical process for a bioinsecticide (Evans, 1999). Exudation of the *Bt* toxin into the soil environment, and the clear demonstration that the toxin is bound to the clay components of soil, pose questions in relation to potential effects on soil dwelling organisms and on the persistence and spread of this source of the toxin (Saxena & Stotzky, 2000; Stotzky, 2000).

The purpose of this review is to place this new information in the context of the known pathways for *Bt* to enter soil and to determine whether root exudation of *Bt* from transgenic crops represents an enhanced environmental risk. At the same time gaps in knowledge will be identified to provide a basis both for research needs and also for provision of information of value in developing and implementing GM regulations in the UK and elsewhere.

An ecological approach has been adopted throughout, with particular emphasis on separating a putative hazard from the likelihood of that hazard being realised, i.e. risk. Although the review concentrates on *Bt* in the soil environment, the general role of *Bt* within pest management regimes will be assessed to provide a wider context for the exudate source of *Bt*.

2 *Bacillus thuringiensis* as a microbial insecticide

2.1 *Biology and mode of action of* Bacillus thuringiensis

Bacillus thuringiensis is a crystalliferous spore-forming bacterium. The bacterium typically contains within its cell wall a spore and, usually, a bi-pyramidal toxin crystal, although the shape of the crystal can be highly variable (Schnepf *et al.*, 1998). Modes of action and descriptions of Cry gene diversity and host-range implications of the crystal toxin genes are described below.

2.1.1 History of early use of *Bacillus thuringiensis*

Bacillus thuringiensis was first recognised as a mortality factor against insects by Berliner in 1915 (Berliner, 1915). Although work on the bacterium, particularly on methods for culture and characterisation of dosage requirements, took place after its discovery by Berliner, commercial development did not commence until the late 1930s when the product *Sporeine* was made available in France. However, it was only when the crystal toxin in the bacterium was recognised and its structure described, that increased commercial development took place (Hannay & Fitz-James, 1955; Angus, 1954). Later, the variant termed *kurstaki* was isolated from a meal moth in France and from pink bollworm in the USA (where it was termed the HD1 strain (Dulmage, 1970). New strains active against biting flies (Diptera; mosquitoes and blackflies in particular) and against plant feeding beetles (Coleoptera) were isolated, extending the range of activity and taking *Bt* into primary position as a bioinsecticide (Burges & Hussey, 1971; Burges, 1981; Becker & Margalit, 1993). Apart from its demonstrated efficacy and relatively narrow host range, one of the key advantages of *Bt* as a bioinsecticide was the ability to mass-produce the agent in fermenter culture (Bryant, 1994). This allowed both scale-up and also greater control over quality and the use of either or both of the spores and crystal toxin proteins that determine the efficacy of *Bt*.

Development of *Bt* as a microbial insecticide followed from increased efficiency in production and quality control so that formulations with high activity and improved spray characteristics could be developed (van Frankenhuyzen, 1993). Developments, particularly for forestry applications, were most significant during the 1970s and 1980s when attention was paid to matching formulation, spray atomizers and crop characteristics to the known biology and susceptibility of the target host organisms (Evans, 1997). Significantly, although cost reductions were bringing *Bt* to levels where they were regarded as suitable alternatives to chemical insecticides (van Frankenhuyzen, 1990), other constraints limited their penetration into major crop markets (Gelernter & Evans, 1999). Principal amongst these constraints was the high specificity of *Bt* that, in most crop systems, effectively meant that the agent was effective against one pest only. This was not a particular problem in forestry where there was normally only one target and the environmental benefits of such an approach added to the value of using *Bt*. However, in agricultural crops where a complex of pests were being managed by broad spectrum conventional pesticides, the higher cost, short persistence and high specificity of *Bt* were major barriers to uptake.

Nevertheless, discovery of the Diptera active *Bt israelensis* (*Bti*) in the late 1970s (for review see Keller & Langenbruch (1993)) resulted in major uptake and the development of several products for vector control, particularly of mosquitoes and blackflies. Similarly, discovery of the Coleoptera-active *tenebrionis* strain of *Bt* in the mid 1980s provided a valuable boost to use of *Bt* against some of the major beetle crop pests, particularly Colorado potato beetle, *Leptinotarsa decemlineata* (Becker & Margalit, 1993).

2.1.2 Historical overview of the genetic basis of *Bt* toxin crystal expression

Recognition that there were strains of *Bt* with differential activity spectra within and between insect orders led to a rapid expansion of research into the genetic basis of these differences. This, in turn, led to development of a much greater understanding of the modes of action of *Bt* through the interaction of the crystal toxin with the gut of potential target organisms. Amongst the critical milestones during the 1980s was the discovery that the genes coding for the toxin crystals were located on transmissible plasmids enabling exchange of genetic information between *Bt* strains (Gonzales *et al.*, 1981; Gonzales & Carlton, 1984). This opened the way to manipulation of genes, including transfer between *Bt* strains, thus enabling researchers to begin to make the link between genetic information and function, including toxicity and host range (Gonzales *et al.*, 1982). In parallel to this development, Schnepf & Whiteley (1981) succeeded in cloning the *Bt kurstaki* toxin gene in *Escherichia coli*, thus providing the means to clone a wide range of toxin genes from the increasing number of *Bt* strains being discovered in the environment (for review of this early development phase see Whiteley & Schnepf (1986)). Sequencing of the crystal protein genes soon followed and a number of authors published on the subject during 1985 (Adang *et al.*, 1985); (McLinden *et al.*, 1985) (Schnepf *et al.*, 1985); (Shibano *et al.*, 1985). Armed with techniques, such as hybridization to assess for known nucleotide sequences, the numbers of individual toxin genes being described rose rapidly and were designated Cry genes that were, by the late 1980s and early 1990s, grouped into those with known activity spectra against particular insect orders. Thus, CryI were classed as Lepidoptera active, CryII as Lepidoptera and Diptera, CryIII as Coleoptera, CryIV as Diptera, CryV as Coleoptera and Lepidoptera, while other genes with general toxicity were classed as Cyt genes (Lereclus *et al.*, 1993). Subsets of the Cry classification system were then recognised (e.g. CryIC with high activity against specific Lepidoptera compared with CryIE with limited activity (Visser *et al.*, 1990)). However, this distinction is now blurred and is recognised in the new classification described below.

2.1.3 Classification of *Bt* Cry genes

Studies of the natural incidence of *Bt* worldwide have revealed an increasing number of different crystal toxin genes (Cry genes). These have been classified on the basis of DNA profiles and also in relation to the modes of action of the genes (2.1.5). Recent comprehensive re-classification of the *Bt* Cry genes has been carried out by Crickmore *et al* (1998). Relationships between the Cry genes are now based on an alphanumeric system using Arabic numerals, upper and lower case letters. This

replaces the older classification based on the host ranges in which the toxin genes were first observed or tested. Thus the Cry genes are now recognised using four hierarchical levels based on sequence homology of the various proteins in each rank. Proteins with less than 45% sequence homology are separated in the primary rank, while further separation at the secondary and tertiary ranks is based on 78% and 95% homology respectively. De Maagd *et al* (2001) have recently provided a useful summary of the putative evolutionary relationships between the different Cry genes and listed 31 Cry genes and their phylogenetic interactions. Further additions to the list of Cry genes are administered through the database managed by Crickmore *et al* (2002), as indicated in Figure 2.1 and described more fully in Appendix 1 (Chapter 8). The diversity of genes and their virtually ubiquitous presence in a very wide range of habitats (see 2.2) suggests that potential exposure to *Bt* of organisms in many different taxa is high. This will have relevance to the overall risk of introducing *Bt* toxin by a range of different pathways, including transgenic plants and will be discussed in detail in Chapter 4.

2.1.4 Structure of the *Bt* toxin

In wild type (WT) *Bt*, the toxin crystal may constitute up to 25% of the weight of the bacterial cell. The crystal is proteinaceous and characteristic of the particular strain of *Bt*, with sizes ranging from 66 to 140 kDa encoded in plasmids (Schnepf *et al*, 1998). As described in Chapter 2.1.6 below, the intact toxin crystal from WT *Bt* must undergo solubilisation and proteolysis to strip off amino acid sequences at the N- and C-termini before they become active in the guts of hosts. In the case of transgenic plants, a truncated form of the toxin is employed that has most of the C-terminal end removed, thus requiring only minimal further proteolytic processing for activation. Schnepf *et al* (1998) provide a comprehensive review of the structure of *Bt* toxins and their genomic composition, including the presence of many transposable elements that are thought to be involved in horizontal dissemination of genetic information, such as *cry* genes within the *Bt* complex.

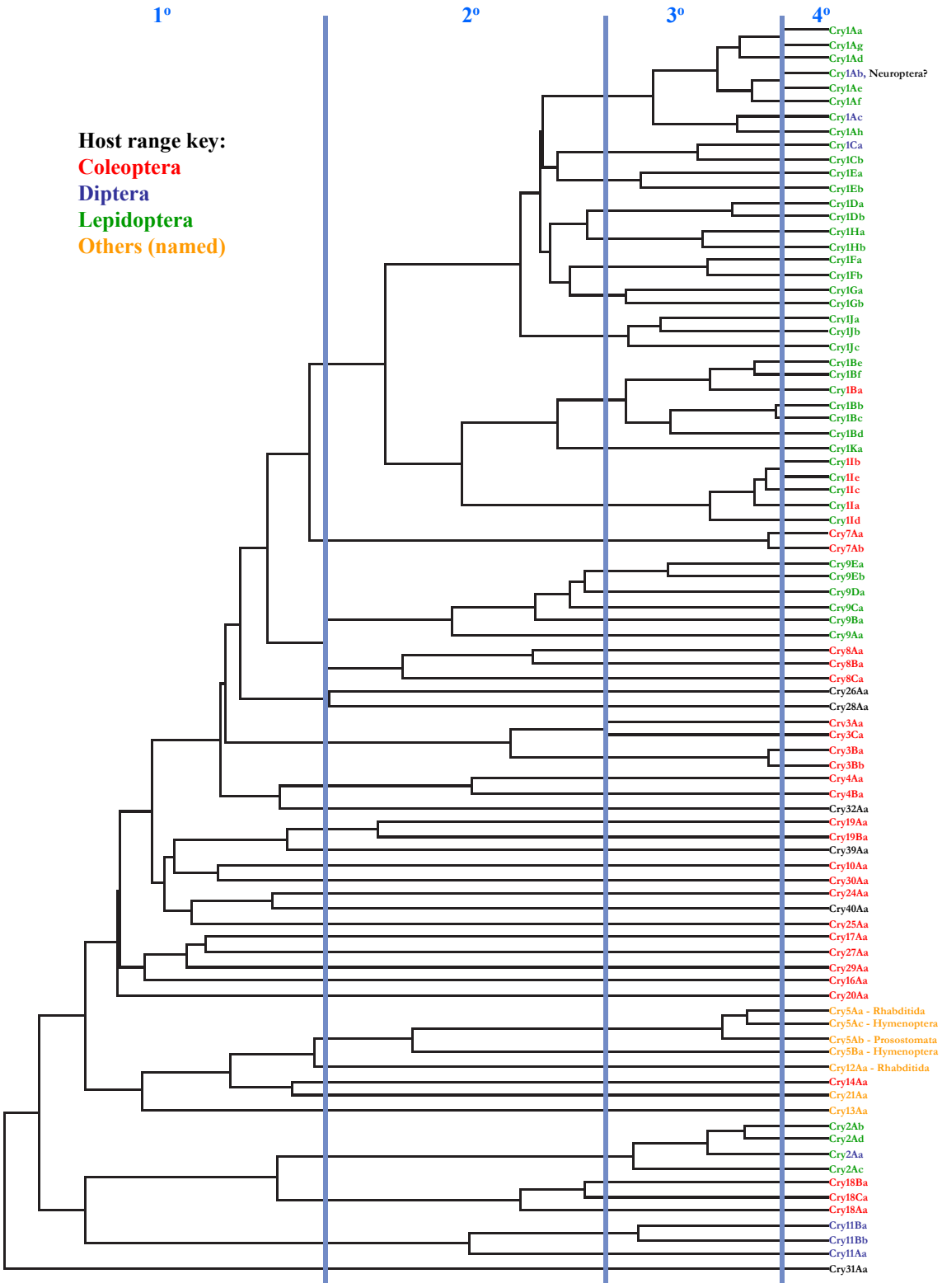
2.1.5 Cry genes and the host range of *Bt*

Recognition of the roles of Cry genes in toxicity was originally based on the recorded host ranges of the *Bt* variants that had been isolated, usually in association with a particular insect species. Recent re-organisation of the taxonomy of Cry genes has removed the emphasis on host range. Nevertheless, host range can be linked to the major Cry groupings and it useful to be able to explore this association in relation to environmental risks of both Wild Type (WT) and GM *Bt*. Information on host tests of Cry genes against a very wide range of organisms can be found in van Frankenhuyzen & Nystrom (1999) and detailed in the table indicating which Cry genes have been tested and the organisms involved provided in Appendix 2 (Chapter 8.2).

The evolutionary relationships between the different Cry genes is gradually being elucidated, based on sequence homology and Crickmore *et al* (2002) provide regular updates to the phylogenetic tree for *Bt*. The version of March 2002 is shown in Figure 2.1: . In this case, data on known host ranges, in terms of insect Orders or other taxonomic entities, have been indicated for each Cry gene.

The most significant conclusion from these data is that some Cry genes, notably Cry1 and Cry2 have activity patterns that cross taxonomic boundaries. This is particularly apparent for cross activity between Coleoptera, Lepidoptera and Diptera, although none of the genes in the table have demonstrated activity against all three orders of insect. However, recent research on a *Bt* isolate which, on cloning, was identical to *Bt* subsp, *thuringiensis* HD-2 and classified as a Cry1ba1 gene, indicated activity against the house fly (*Musca domestica*, Diptera), cottonwood leaf beetle (*Chrysomela scripta*, Coleoptera), and tobacco hornworm (*Manduca sexta*, Lepidoptera) in laboratory assay (Zhong *et al.*, 2000). In this study the gene was expressed in a *Bti* strain that did not normally produce crystals but which, after expression, produced irregular bipyramidal crystals on sporulation.

Figure 2.1: Domain tree for *Bacillus thuringiensis*, indicating the relationships between the Cry genes (from Crickmore *et al* (2002), accessed March 2002). Where known, the host range of each Cry gene is indicated (see host range key)



2.1.6 Host interactions at the gut level

Improved techniques for characterisation of Cry genes and a greater understanding of the processes of interaction between the toxin fragments and the guts of potential hosts offer prospects for prediction of the possible host ranges of both naturally occurring and manipulated *Bt* toxins. The process of activation of the crystal in the guts of putative hosts is being elucidated by a number of research groups and the findings are of considerable value in analysing environmental risks from widespread use of *Bt*. Table 2.1: summarises the main conditions that lead to activation of the *Bt* toxin and the factors that affect binding of the core toxin fragments to midgut cells.

Table 2.1: The main interactions determining toxin activity in insect midguts

Gut conditions	Effect on crystal δ-endotoxin	Effect on truncated toxin
pH	• Initial solubilisation of the crystal is essential before any subsequent events leading to Bt toxicity. • For most Cry toxins, midgut pH must be strongly alkaline (pH >9.5) for dissolution of the crystal (Bradley *et al.*, 1995), some coleopteran-specific toxins function at a much lower pH (Koller *et al.*, 1992; Bauer, 1995). • Cry3a (Coleopteran active) toxin is dissoluted at acid and alkaline pH, but not at neutrality (Carroll *et al.*, 1997). • Rate and extent of crystal solubilisation influences toxicity levels in different hosts, and pH may influence the effectiveness and specificity of some toxins. (Bradley *et al.*, 1995). • Lepidopteran and dipteran midgut is highly alkaline, Coleopteran midgut neutral to acidic.	• Truncated genes do not require a specific mid-gut pH and solubilisation because the toxin is already processed to the solubilised core (Tapp & Stotzky, 1995; Koskella & Stotzky, 1997; Tapp *et al.*, 1994)
Gut enzymes	• Midgut enzymes are essential for the processing of the Cry protoxins to produce the active toxin core. • Midgut lumina of lepidopteran insect larvae have been shown to contain a variety of alkaline proteases. (Lightwood *et al.*, 2000). • δ-endotoxin proteolysis is a major determinant of toxicity (Lightwood *et al.*, 2000; Ogiwara *et al.*, 1992; Oppert *et al.*, 1994). • Cry1 crystals are solubilised to release protoxins (130-140kDa), which are subsequently processed to 55-66kDa proteins by gut enzymes. (Jurat-Fuentes & Adang, 2001) • Insufficient or excessive proteolysis can cause loss of toxicity e.g. the complete degradation of a toxin by proteases was the likely cause of loss of potency in *Spodoptera litura* (Inagaki *et al.*, 1992). • The ~130kDa Cry1 δ-endotoxins undergo extensive proteolysis at both their C- and N-terminal ends, producing a mature, toxic, and relatively protease resistant core of approx. 60kDa. (Lightwood *et al.*, 2000). • N-terminal site cleavage depends on the insect from which the proteases are derived (Lightwood *et al.*, 2000), (Ogiwara *et al.*, 1992). • CytA dissolves readily under alkaline conditions, but remains crystalline at neutral of slightly acidic pH (Federici	• Truncated toxins (e.g. of Cry1Ab in the Monsanto transgenic cotton line 81) are approximately 5kDa larger, but otherwise identical, to the active toxin molecules produced by proteinase digestion. (Malvar & Baum, 1994). • Limited further proteolysis is required to produce the fully active toxin.

	& Bauer, 1998). • The main digestive proteases of Lepidoptera and Diptera are serine proteases, and those of Coleoptera are mainly cysteine and aspartic proteases. (de Maagd *et al.*, 2001).	
Gut Structure	• Activated Cry toxins pass through the peritrophic membrane and bind reversibly to receptors on the brush border membrane of the midgut cells. Final, irreversible binding is linked to insertion of part of the toxin (Domain I) into the midgut membrane (Jurat-Fuentes & Adang, 2001). • Toxins interact with specific high affinity receptors and can recognise different or partially over-lapping sites. • Toxin binding to midgut brush border membrane vesicles (BBMV) is a key determinant of toxicity to insects (Luo *et al.*, 1999); (Van Rie *et al.*, 1990a) . • After a Cry1 toxin binds to the BBMV (Domains II and III of the 3D toxin structure), a large portion of the molecule (Domain I) inserts into the membrane, forming low-selective ion channels (Luo *et al.*, 1999; Miranda *et al.*, 2001). • Ion channels lead to osmotic swelling, cell lysis, and damage to the mid-gut haemocoel barrier, leading ultimately to death of the host. (Federici & Bauer, 1998). • Loss of binding sites is associated with acquired resistance (Luo *et al.*, 1999). Toxins may bind to the membrane but this is reversible, without insertion into the membrane (Luo *et al.*, 1999). • Multiple binding sites for cry toxins are present in many insects. (Luo *et al.*, 1999). • Cyt proteins also cause midgut lysis, although their primary affinity is thought to be for lipids in the microvillar membrane (Federici & Bauer, 1998; Thomas & Ellar, 1983; Knowles *et al.*, 1989).	• Specific receptors for the toxic (transgenic /truncated) proteins are required on the insect midgut epithelium. These receptors appear to be in higher numbers in susceptible larvae, although their absence from non-target organisms has not been established definitively (Tapp *et al.*, 1994).
Other factors	Feeding stimulants are known to greatly enhance *Bt* performance since most susceptible insects cease feeding after consumption of *Bt*-containing food (Bauer, 1995).	

2.1.6.1 Gut pH

Initial solubilisation of the WT crystal is dependent on gut pH. For the majority of Cry genes, gut conditions must be strongly alkaline in order to achieve dissolution, prior to proteolytic cleavage of the protoxin by gut enzymes (Hofmann *et al.*, 1988a). In the Lepidoptera and for many plant-feeding, as opposed to carnivorous, insect orders the midgut is alkaline and is an adaptation to the feeding strategy of the larval stage (Berenbaum, 1980). The gut conditions of Diptera larvae are also alkaline. However, some Coleoptera have mid-gut pH values that are slightly alkaline or slightly acidic and, significantly, this is reflected in the solubilisation characteristics of Coleoptera specific Cry3 genes that dissolute at these pHs but not at neutrality (Koller *et al.*, 1992). By contrast, Cry1 toxin crystals are only dissoluted at high pH and are poorly soluble in the more acid midguts of beetles such as *L. decemlineata* (Bradley *et al.*, 1995). However, pre-solubilisation of Cry1B gave rise to low toxicity in *L. decemlineata* larvae but this was not essential to induce activity against the cottonwood leaf beetle, *Chrysomela scripta*. Gut pH is, therefore, one of the factors that help to determine potential toxicity and, hence, the host range of *Bt* and is of greatest significance when hosts ingest intact toxin crystals. It has been postulated that

expression of a truncated (pre-solubilised) form of *Bt* in transgenic plants removes the need for the initial gut barrier of solubilisation and, therefore, may imply a higher risk of toxicity in both target and non-target organisms (Hilbeck, 2002; Stotzky, 2002b). Clearly, the demonstration that solubilisation of Cry1B enhances the toxicity, albeit at a very low level, against some Coleoptera implies that potential host range increases may be possible, although further barriers to gut penetration have to be overcome to ensure full toxicity (Bradley *et al.*, 1995).

Increased use of *Bt*, particularly through the transgenic plant delivery route, has led to concerns about the possible development of resistance to the toxin as a result of heavy selection pressure. A recent review by Ferré & Van Rie (2002) assessed and critically evaluated the separate and combined contributions of the elements in Table 2.1: in relation to the biochemical and genetic basis for observed resistance to *Bt*. No specific reference to pH was made, but some linkage to changed susceptibility to proteolytic digestion was considered to be significant in a few cases (see below). It appears, therefore, that gut pH affects *Bt* activity at a gross level in relation to effects on initial solubilisation of protoxin in intact crystals. To date, there appears to be no indication that exposure to pH concentrations present in insect guts has any adverse effect on activity of ingested truncated *Bt* derived from transgenic plants. However, the interaction of pH with other gut factors must be taken into account in assessing survival ability and toxicity of ingested *Bt*, either after solubilisation or by direct ingestion of truncated *Bt*. For example, Tran *et al* (2001) investigated the interactions of pH and ultimate pore forming ability of Cry1 toxins. Their results demonstrated that the pores formed by Cry1Ac are significantly smaller at pH 6.5 than under more alkaline conditions, whereas the pore-forming ability of Cry1C decreased rapidly above pH 8.5, which was consistent with the reduced activity of this Cry toxin against *M. sexta*. This moth is much more susceptible to toxicity from Cry1Aa, Cry1Ab, and Cry1Ac, all of which form larger pores under high pH conditions.

2.1.6.2 Proteolysis: gut enzyme effects

After solubilisation, *Bt* protoxins are cleaved by gut enzymes to form the active toxin core which is a protein of around 55-66kDa (Jurat-Fuentes & Adang, 2001). Table 2.1: includes some of the key findings in research on proteolysis of the *Bt* toxins. In relation to ultimate toxicity, proteolysis is both enzyme and time critical. If the toxin processing is not completed optimally, which may take place if there is rapid movement of food through the insect gut, or if the toxin is exposed to enzymes for an extended period, ultimate activity may be compromised (Lightwood *et al.*, 2000). Taking Cry1Ac as an example, proteolysis of the 130 kDa toxin takes place at both the C- (removal of 500-600 amino acid residues) and N- (removal of 27-29 residues) termini to leave a core of around 60 kDa that is relatively resistant to further protease degradation (Schnepf & Whiteley, 1985). The enzymes responsible for Cry1 activation in Lepidoptera and Diptera include alkaline proteases having trypsin and chymotrypsin activity, mainly as serine proteases (Christeller *et al.*, 1992; de Maagd *et al.*, 2001). Studies on Colorado potato beetle gut enzymes have demonstrated that they have chymotrypsin-like activity (Novillo *et al.*, 1997) as well as cysteine and aspartic proteases (Terra & Ferreira, 1994) and produce a core toxin fragment of around 55 kDa during activation of Cry3 toxins.

As indicated in Chapter 2.1.6.1, there is an interaction between gut enzymes and gut pH and also with the rate and position along the gut of digestive activity. This, in turn, is linked to the food consumed by the insect such that, in the guts of Lepidoptera, there are gradients of pH both tangentially and longitudinally (Skibbe *et al.*, 1996). In particular, in studies of the midgut of *Spodoptera litura*, the authors found that there was a remarkably steep gradient of pH from the centre of the gut lumen (pH 7) to the periphery, adjacent to the mid gut epithelium (pH 11). Woods & Kingsolver (1999), in developing a model for the transformation of plant material into insect tissue via midgut absorption indicated that amino acid gradients were steep in the opposite direction to pH, thus posing a barrier to solute movement. Both factors reflect the rate of movement of food along the gut so that, when the gut is full, there is slower movement and relatively little passage and mixing of food and of digestive enzymes along the epithelium surface itself. While the Woods & Kingsolver (1999) study was concerned with protein digestion and uptake, it also implied that the rate of breakdown of *Bt* toxins, either directly from the crystals or as the already truncated form associated with transgenic plant material, will be subject to the same dynamic constraints. A factor that adds to the complexity of understanding the dynamics of gut dissolution and activation of *Bt* toxins is the fact that, when initial toxicity commences, affected hosts will normally stop feeding quite rapidly (Burges, 1981). However, low dosages or relatively high resistance to the applied *Bt* toxin can result in recovery of the larva and re-commencement of feeding which can only lead to mortality if there is sufficient toxin remaining to bind to healthy midgut cells (van Frankenhuyzen *et al.*, 2000). In these circumstances, the total quantity of toxin within the gut lumen and, particularly, the ability of that toxin to survive the pH and digestive enzyme combinations will be critical in determining possible further toxicity to the host.

A further physical barrier to movement of solids and digested material from the gut lumen to the surfaces of the gut cells is the peritrophic membrane (PM) that is present in the majority of insects. The PM is a chitinous sheath that lines the midgut and is thought to provide protection against physical abrasion of the midgut epithelium and to selectively influence transfer of digested material and pathogens from the gut lumen to its periphery (Richards & Richards, 1977). The protective function of the PM in relation to potential impact of *Bt* was assessed in larvae of Douglas fir tussock moth, *Orgyia pseudotsugata* (Adang & Spence, 1983). Molecules and particulates up 800 nm could pass through the PM but microorganisms the size of *Bt* (spores and toxin crystals that had not been solubilised) could not penetrate the intact PM. However, pores in the PM did allow smaller microorganisms, such as nucleocapsids of baculoviruses, to pass through. Solubilised *Bt* toxin had no effect on PM permeability, although the proteinase ficin increased permeability.

Confirmation that the PM could act to reduce the potency of ingested *Bt* was provided in studies of Cry1C on larvae of *Spodoptera littoralis* that are normally only slightly susceptible to the toxin (Regev *et al.*, 1996). When larvae were fed an *E. coli* produced recombinant endochitinase (coded by the bacterium *Serratia marcescens*) alone, perforation of the PM was noted. A combination of bacterial endochitinase and truncated CryIC fed to larvae produced an approximately 7-fold decrease in the dosage required to induce a measurable toxic effect. This was attributed to perforation of the PM by the action of the endochitinase, which allowed the *Bt* toxin to pass through and directly attach to the midgut cells. Chitinase as a contributory or

synergistic factor in insect pest management is now being considered as an additional gene for inclusion in transgenic plants (Kramer & Muthukrishnan, 1997). For example, tobacco expressing the chitinase gene constitutively has been shown to enhance the effects of *Bt* applied to the foliage for control of tobacco hornworm *Manduca sexta* (Ding *et al.*, 1998). Recognition of the potential role of chitinases in enhancing the activity of *Bt* has led to screening for strains of the bacterium that naturally express chitinase, with the result that strains with enhanced activity against various hosts have now been recognised (Sampson & Gooday, 1998; Wiwat *et al.*, 2000; Guttmann & Ellar, 2000; Thamthiankul *et al.*, 2001). Not surprisingly, recombinant gene technology is now being employed to attempt to combine chitinase and Cry genes into novel carrier systems. For example, natural phylloplane bacteria, such as *Pseudomonas fluorescens*, have been engineered to express Cry1Ac7 and chitinase from *Serratia marcescens* in an attempt to control sugarcane borer *Eldana saccharina* (Downing *et al.*, 2000).

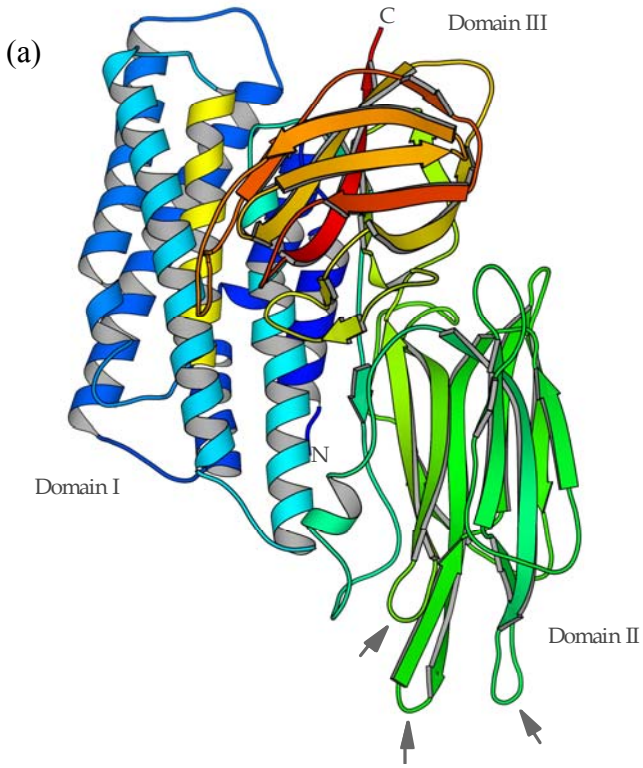
Presence of the PM and, particularly, its structure and ability to act as a protective barrier within the midgut is a further, but increasingly recognised, factor in determining potential activity of *Bt*. The PM role in the guts of both target and non-target organisms exposed to truncated *Bt* from transgenic plants has not been elucidated directly, but may have an important influence in relative susceptibility to this source of the bacterial toxin.

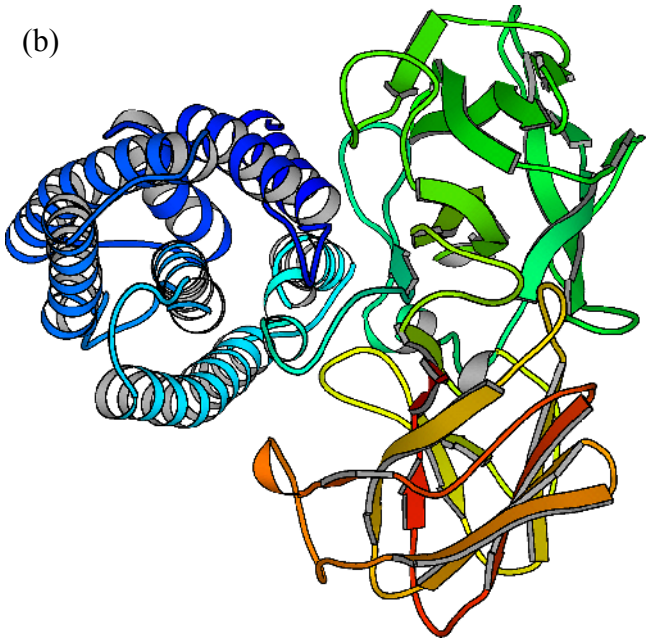
2.1.6.3 Receptor binding: the final determinant of toxicity

Activated toxins, in fragments of 55-60 kDa, depending on the nature of the Cry gene, must encounter the brush border membranes of midgut epithelial cells in order for the toxin to bind to and possibly form pores in the cells (Schnepf *et al.*, 1998; de Maagd *et al.*, 2001). As research progresses, using a range of techniques including cloning and mutagenesis to alter toxin structures at the molecular level, it is clear that the key to determining toxicity and specificity is the ability of the activated Cry toxin to bind to brush border membranes and, ultimately, to form a pore through which ion exchange takes place freely, leading to cell lysis and death (Knowles, 1994). Although the precise mechanism by which pore formation takes place is not yet elucidated fully, there is general consensus that binding and subsequent pore formation is linked to the three domain structure of the activated toxin (Hofmann *et al.*, 1988a; Hofmann *et al.*, 1988b; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990a; Schnepf *et al.*, 1998).

Elucidation and visualization of the three dimensional structure has aided the understanding of the final processing of the toxin and its conformation to initiate binding at the midgut cell membrane. An example of the three dimensional structure is provided in Figure 2.2 where both side and top views are provided.

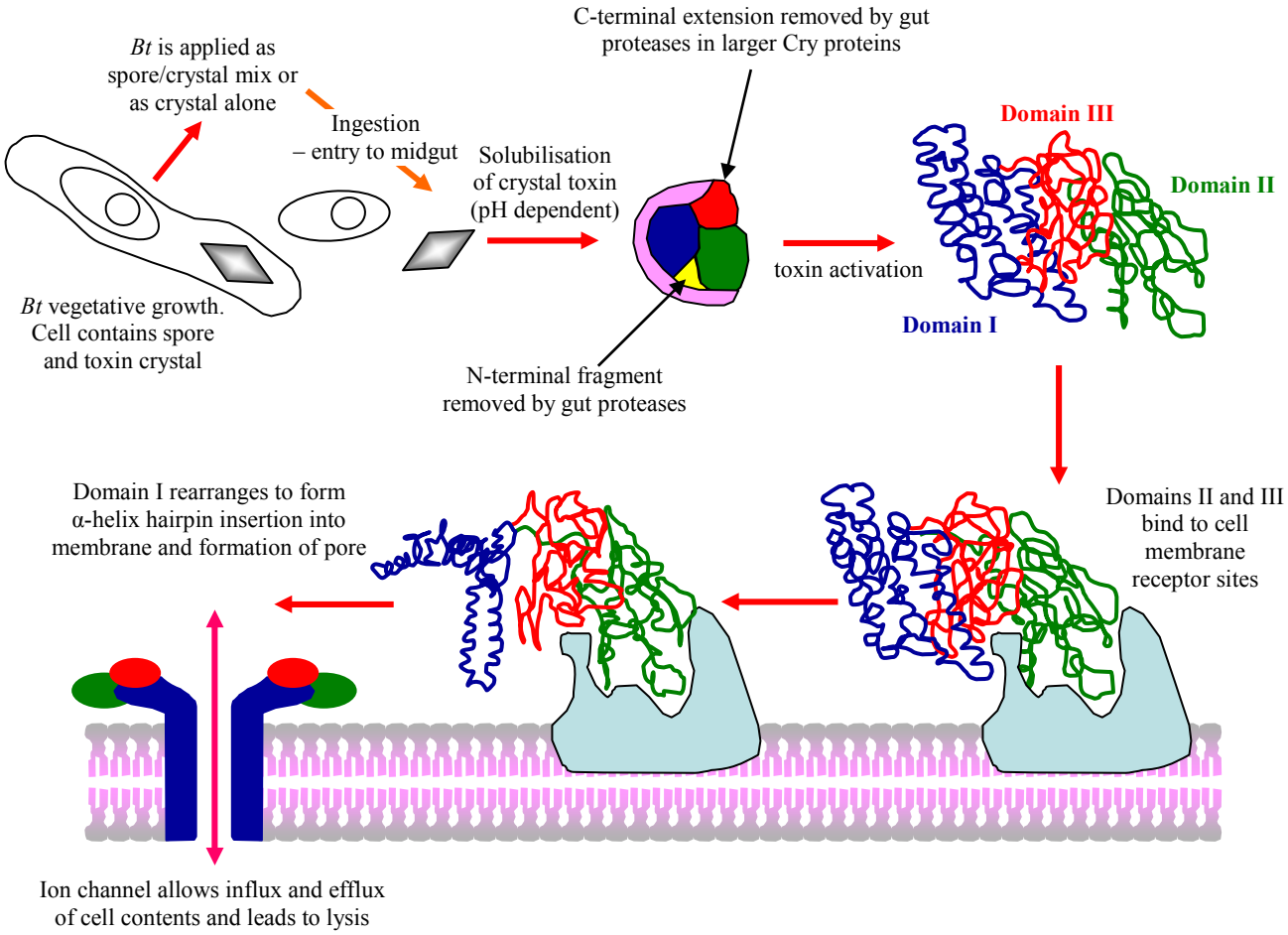
Figure 2.2 : (a) Side and (b) top views of the three dimensional structure of the Cry3Aa toxin, showing the three Domains and C- and N-terminal ends. Figures courtesy of Professor D. J. Ellar, Cambridge University.





In the Cry3Aa example in Figure 2.2 , the three domains are, from the N- to the C-terminus, a seven helix bundle (Domain I), a triple antiparallel beta sheet domain (Domain II) and a beta-sheet sandwich (Domain III). The top view emphasises the seven alpha helix domain I structure which resembles the pore forming domain of Colicin A and is now regarded as central to the insertion of the toxin through the cell membrane (D. J. Ellar personal communication). The elements of the binding and insertion processes have been illustrated conceptually by de Maagd *et al* (2001) and are reproduced in Figure 2.3:

Figure 2.3: The process of solubilisation, proteolysis and receptor binding of Domains II and III, followed by insertion and pore formation of Domain I of WT *Bt* toxin. Adapted from de Maagd *et al* (2001). Colours match those of Figure 2.2 .



The truncated and activated toxin produced after digestion by gut enzymes has a three domain structure that binds to the cell membrane of epithelial cells. There has been considerable effort to determine the nature of the receptor sites (reviewed by Schnepf *et al* (1998)) leading to proposals that aminopeptidaseN (APN), which is ubiquitous in gut epithelial membranes, might be universally important in receptor binding (Knight *et al.*, 1994). Separate actions of domains II and III in binding to *Lymantria dispar* APN have been shown to alter specificity in mutagenesis studies of Cry1Ac (Jenkins

et al., 2000). Presence of N-acetylgalactosamine resulted in loss of domain III contact and complete loss of APN binding. Detailed mutagenesis studies have indicated that binding to APN is not necessarily linked to toxicity and have cast doubt on the role of APN as an universal binding site (Burton *et al.*, 1999; Jenkins *et al.*, 1999; Lee *et al.*, 2000; Lee *et al.*, 2001). Other binding sites, such as a cadhedrin-like receptor (Nagamatsu *et al.*, 1999) and BTR-270 or a anionic brush border membrane glycoconjugate (Valaitis *et al.*, 2001), have been shown to be involved in binding and may be more likely to act *in vivo.* Overall, binding is initiated by Domains II and III that, in combination, are partial determinants of the specificity of a particular Cry gene (Aronson & Shai, 2001).

It appears from the majority of studies that domain II is the important determinant of receptor binding and, in many cases, is the dominant factor in action of *Bt*. For example, studies of the actions of Cry1Ab, Cry1Ac, Cry1Ja, Cry1Fa and Cry1Aa in *Heliothis virescens* have revealed the importance of domain II in irreversible receptor binding in these Cry genes (Jurat-Fuentes & Adang, 2001). The five Cry toxins investigated in this study all shared sequence homology in domain II, but did not all recognise the same proteins in binding to brush border membrane vesicles (the three Cry1A toxins, Cry1Fa and Cry1Ja recognised aminopeptidase binding sites, while Cry1Ab and Cry1Ac also recognised a 130 kDa molecule). It was concluded that shared binding properties contributed to cross resistance to *Bt* toxins in tobacco budworm *Heliothis virescens*. Strategies to overcome resistance could, therefore, include selection of toxins with low homology to Cry1A toxins in domain II. For example, Cry2Aa2 has low homology in the domain II loop and could be a useful candidate for incorporation into transgenic crops. This has already been tested with transgenic tobacco in which chloroplast expression of the full protoxin of Cry2Aa2 (the protoxin is naturally truncated to 65 kDa) led to very high levels of expression and 100% mortality of *H. virescens*, cotton bollworm *Helicoverpa zea* and beet armyworm *Spodoptera exigua*, all of which were resistant, to some extent, to Cry1A (Kota *et al.*, 1999).

Development of an improved understanding of binding kinetics has resulted from attempts to elucidate and overcome resistance to *Bt* in both field and, particularly, laboratory selected insect species. This has been well reviewed by Ferré & Van Rie (2002) who used the example of resistance in *Plutella xylostella* to propose a model for Cry binding sites, based on work by a number of authors. They proposed that at least four Cry binding sites are present in the midgut cells of *P. xylostella*:

Site 1: Cry1Aa
Site 2: Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, Cry1Ja
Site 3: Cry1B
Site 4: Cry1C

Resistance in moth colonies was correlated to alterations of binding sites in two ways:

Type I alteration: Site 2 – no binding of Cry1Ab
Type II alteration: Site 2 – no binding of Cry1Ab and Cry1Ac, plus Cry1Aa but this is masked because the toxin still binds to Site 1

Although direct evidence was lacking, the fact that cross-resistance to cry1Fa and Cry1Ja was apparent suggested that alteration of Site 2 also affects these toxins.

Initial binding processes involving domains II and III are, therefore, complex and incompletely understood and, in themselves, do not guarantee that insertion of domain I and subsequent pore formation takes place. Binding only becomes irreversible when domain I activity commences. Various models for Domain I membrane insertion have been proposed, with general agreement that alpha-helices 4 and 5, form a hydrophobic helical hairpin, which produces a membrane pore, eventually leading to cell leakage and eventual lysis (Schnepf *et al.*, 1998; Li *et al.*, 2001; Aronson & Shai, 2001). Aronson & Shai (2001) assessed current data on insertion events for domain I and summarised the combined receptor binding and insertion events in relation to an APN receptor. Initial reversible binding of domain III is followed by secondary binding of domain II, which brings domain I, particularly the α7 helix, close to the membrane. Domain I swings out bringing the region between α2 and α3 into contact with the membrane. All helices, except α1, then interact with the membrane and the hydrophobic α4-α5 region is inserted with α5 forming the sides and α4 the lining of the ion channel.

Once the pore is formed then ion channel activity commences, functioning as a cation channel in alkaline conditions corresponding to the large K^+ gradient in the midgut (Schwartz *et al.*, 1997; Guihard *et al.*, 2000). After cell lysis and leakage takes place, gut pH tends to fall and the pore may then function as an anion channel, leading to further cellular disruption (Schwartz *et al.*, 1997).

2.2 Incidence of Bacillus thuringiensis *in nature*

Although *Bt* was originally associated only with certain substrates and with affected host insects, the development of methods for effective extraction of the bacterium from a wide range of environments has resulted in an enormous proliferation of new isolates from virtually all locations where searches have been carried out (Meadows, 1993; Bernhard *et al.*, 1997). In relative terms, the most common locations for isolation of new *Bt* strains have been in grain storage (Burges & Hurst, 1977). This is not surprising in view of the close proximity between the often very high populations of susceptible pest species, especially in the Lepidoptera and Coleoptera, and the protected reservoir of *Bt* that is likely to be present in the grain storage environment. Of more ecological significance is the finding that *Bt* is present in virtually all natural environments, whether they are affected by human activity or not (Martin & Travers, 1989). Despite the ubiquitous presence of various forms of *Bt*, there is surprisingly little known about the ecology of the organism in nature. The fact that most of the isolations apply to the spore stage, which can be multiplied by growing on selective media, implies that populations of bacterial spores are maintained by a combination of pathogenicity against susceptible hosts and saprophytic growth when suitable abiotic conditions prevail.

It has been postulated that *Bt* has evolved a strategy to maximise the probability of the spore finding suitable conditions for germination and reproduction (Ellar, 1990). The metabolic load during vegetative growth placed on *Bt* in producing the crystal toxin, which can constitute 35% of the cell dry weight, is offset by the combined effects of gut paralysis followed by penetration and lysis of the gut cells. The former retains the

spore in the gut lumen while the latter generates a suitable environment for spore germination and reproduction. In this context it is significant that the majority of *Bt* isolates have the ability to produce toxin crystals and, consequently, retain the ability to cause rapid mortality on ingestion, solubilisation and activation of the toxin in the guts of potential hosts. The spore, therefore, represents a long-term survival mechanism that is selectively activated when the correct combination of gut conditions and presence of toxin crystals is achieved. However, as is demonstrated by the ability to isolate new strains of *Bt* by germinating spores on nutrient-rich media, the correct conditions outside a potential host can trigger germination (Chilcott & Wigley, 1993; Johnson & Bishop, 1996). The biology and ecology of *Bt* in the environment, therefore, remains unclear but, in terms of potential effects on hosts, whether they are regarded as pest or beneficial species, retention of either or both of the spores and toxin crystals in the feeding arena is the critical factor. The fact that most environments have one or several *Bt* isolates already present implies that exposure of potential hosts is commonplace and that expression of the toxin may be under-recorded in nature.

2.2.1 Stored product facilities

Retention of populations of *Bt* in stored product facilities is related both to the low exposure of the spores and toxin crystals to ultra violet light (a known factor leading to rapid degradation of *Bt*) and to the high "turnover" of the bacterium as a result of ready availability of susceptible hosts (Meadows *et al.*, 1992). Presence of cadavers containing spores and crystals and potential adherence to stored product substrates provide further mechanisms for retention of high *Bt* presence in a viable state (Burges & Hurst, 1977). In a similar niche, *Bt* has been isolated from sericulture farms where, in these circumstances, it may pose a threat to the silkworms (Kim *et al.*, 1998). Although the hosts being reared in sericulture were limited, there was a surprising variety of activities among the bacteria isolated. The rate of discovery of isolates was high (33% in spring and 25% in the autumn) and included Cry 1, 3a, 3b, 4a, 4c, 6, 7 and 12. No ecological explanation was provided for the seasonal variability in the rates of discovery of the various Cry genes. Although strains active against Diptera only were uncommon (7% in spring and 3% in autumn of the strains isolated) there was a high incidence of Cry genes with dual activity to both Lepidoptera and Diptera (19% in spring and 62% in the autumn). The most common activity spectrum was to Lepidoptera (70% in spring and15% in the autumn). Such studies indicate that even in situations where a high level of microbiological hygiene is maintained, *Bt* can be introduced and maintained in the environment. PCR analysis indicated that CrylAa and CrylC genes are frequently found in the spring and CrylAa in the autumn (Kim *et al.*, 1998).

Isolation of *Bt* strains from tobacco storage facilities at a number of locations worldwide has also been recorded, both in the tobacco leaves and in the tobacco beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae) (Kaelin *et al.*, 1994). In this case, 78 strains of *Bt* were recognised using morphological and serological techniques and most were Coleoptera active. Three *Bt* strains isolated from these studies have since been assessed for potential use as microbial control agents against *L. serricorne* and have shown considerable promise, although highest activity was obtained only when both spores and crystals were employed (Kaelin *et al.*, 1999).

There has been relatively little work on the ecological role of *Bt* in the stored product environment (Burges & Hurst, 1977). Observations of the rate of infection in host insects exposed to intrinsic levels of *Bt* do, however, reveal that, given the appropriate set of conditions, epizootic levels of mortality arising from toxin activity can be achieved (Subramanyam & Cutkomp, 1985). In relation to the Encounter Model discussed in Chapter 4.4, there is a very high encounter frequency between pest populations and the large pool of inoculum that is frequently present in the stored product environment. This represents an extreme example of exposure to *Bt* and would provide a useful model system for studying the limits of impact of WT *Bt* in relation to overall risk assessment from this organism. Indeed, the high levels of exposure of the main stored product pest, *Plodia interpunctella* (Indian meal moth) have prompted studies of potential development of resistance to *Bt* in this moth. Laboratory studies indicated that selection over 15 generations resulted in a 100 fold decrease in activity and was the first demonstration of resistance to the bacterium (McGaughey, 1985). This has led to intensive studies on potential for resistance development in the moth in commercial stored product facilities. Although laboratory cultures showing various levels of resistance to *Bt* can be selected after a relatively low number of generations (McGaughey & Beeman, 1988), there is little evidence that resistance has developed in populations in commercial stored product facilities. As indicated by Van Rie *et al* (1990b), resistance to one Cry gene (Cry1Ab – 800 fold less susceptible) does not necessarily imply resistance to other genes (Cry1Ca – 4 fold more susceptible). The multiplicity of Cry genes found naturally occurring in stored product facilities may, therefore, explain the apparent lack of resistance development in field populations of *P. interpunctella*. It also indicates that, even when populations of susceptible hosts are exposed to high levels of the *Bt* toxin, levels of mortality are rarely high enough to give rise to catastrophic population decreases. This will come as no surprise to those using *Bt* for practical pest management where great care in optimising dose levels, placement and timing is necessary to achieve acceptable control (1998).

2.2.2 Aerial plant surfaces

In many respects, it is surprising that *Bt* has been isolated so frequently from the aerial parts of plants, particularly the leaves (the phylloplane). It is known that when *Bt* is applied in conventional spray applications, the retention time on foliage is relatively short as a result of attrition from UV radiation and other factors such as rainfall and physical abrasion (Ignoffo, 1992). Isolation of viable spores and toxin crystals from the leaves of plants in areas where there has been no history of *Bt* application indicates that mechanisms for persistence in this apparently hostile environment must exist (Smith & Couche, 1991). Several reasons have been offered for this level of persistence, including retention within *Bt*-killed cadavers (Prasertphon *et al.*, 1973; Brownbridge, 1991; Strongman *et al.*, 1997), adherence to the undersides of leaves and on the more protected parts of plants, such as bark crevices (Meadows, 1993), and gradual washing out onto the leaves themselves (Smith & Couche, 1991). It seems unlikely that spores would find conditions suitable for germination and hence production of toxin crystals on the leaf surfaces themselves. However, there are numerous niches on the upper parts of plants that may occasionally provide nutrient conditions suitable for germination and, in this respect, *Bt* was considered to be an

epiphyte, rather than a pathogen by Smith & Couche (1991). They postulated that survival and reproduction over prolonged periods, on both broadleaves (by wind transfer and rain splash onto deciduous cycles of leaf availability) and conifer needles, was the result of factors such as exudation of nutrients from the leaves that enabled *Bt* to reproduce and, inadvertently, provide protection to the plant from leaf feeding pests.

It is also interesting to note that the activity spectra of *Bt* isolated from the leaves of plants are not linked closely to the ranges of host invertebrates likely to be present on those plants. For example, the ubiquitous presence of *Bt* on plant leaves was demonstrated in Japan (Mizuki *et al.*, 1999). The authors isolated a total of 120 *Bt* strains from the phylloplanes of 35 species of trees and shrubs in a relatively small area near Tokyo. Analysis revealed that there were at least 17 serotypes and 25 out of the 120 isolates produced bipyramidal parasporal toxin crystals while the remainder produced spherical or irregular-shaped crystals. The most interesting finding was that some of the isolates were active against mosquitoes (Diptera) and others against diamondback moth, *Plutella xylostella*, neither of which was likely to be present on the leaves. This finding provides further support for the view that *Bt* might have a saprophytic mode of action for longer term survival in habitats where availability of insect hosts is severely limited, e.g. mosquito active toxins on plant surfaces despite lack of available hosts.

Presence of *Bt* on grass foliage which has not received any direct application of the bacterium has also been demonstrated (Damgaard *et al.*, 1998). In this case, the close proximity to soil might explain the presence of the 32 isolates, most of which were Diptera-active and could have been linked to the presence of *Tipula oleracea* larvae in the soil environment below the plants. However, there was also high activity against *Aedes aegypti*, confirmed by the presence of a number of serotypes of *Bt* on the leaves.

2.2.3 Soil

There is a long history of recording of *Bt* in soil (Addison, 1993), although it is only the advent of improved extraction methodology that has resulted in the current proliferation of isolates from this environment. In view of the purpose of this review, this section deals in more detail with the factors within soil that determine persistence and activity patterns of *Bt* in that environment.

2.2.3.1 Factors in the soil environment that determine long-term survival of viable bacterial spores

A comprehensive review by Hattori & Hattori (1976) provides a useful framework for description of the interactions that result in retention and dynamic fluctuation of bacteria, such as *Bt*, in soil. They described the major features of soils that affected the ability of that medium to retain and allow survival and/or reproduction of many bacterial species and other soil microbiota.

Bacteria are retained by soil aggregates of various sizes and it is generally believed that the majority of bacterial cells in soil are attached to the surfaces of soil particles and rarely occur in soil solution. It appears that, although various bacteria may distribute randomly on the outsides of soil particles, bacteria in capillary pores inside larger particles (aggregates) may be able to metabolise substances more actively and sometimes succeed in multiplication.

Pores in soil are important in determining the bacterial/soil aggregate interface and can be divided into 2 groups according to their water retention capacities:

a) Non-capillary/large pores that do not hold water tightly,

b) Capillary/small pores that hold water tightly, and which retain water after most free drainage is completed.

Hattori & Hattori studied microbial composition and variation in the cores and outer components of soil aggregates using a washing-sonication technique. They found that the ratio of gram negative bacteria to total bacteria, under field conditions, was usually ≤10% in the outer part; but was frequently > 30% in the cores of aggregates. In their studies, most bacterial cells in the outer surface of an aggregate were in the spore, rather than vegetative form. In addition, halotolerant and nitrifying bacteria were also more abundant in the core. Differences in bacterial compositions between the core and outer part were thought to be related to water and salt contents of each microhabitat. In terms of the potential for bacterial growth in the soil habitat, they indicated that water and nutrient supply generally limit bacterial numbers, but this is additionally dependent on whether or not the bacterial cells are surface adsorbed or adsorbed in the capillary pores and are, therefore, effectively trapped. They found that when bacteria grow in soil aggregates, cells may multiply first on internal surface of pores of both the core and surface layers and may become detached if they saturate the adsorption capacity of the surface area of the aggregate. Those on the surface of aggregates may also be more vulnerable to attack by Protozoa and other soil microbiota or to be eluted by percolating liquid. In general, soil microorganisms are too large to penetrate a stable aggregate, and some bacterial cells may be trapped inside when an aggregate is formed. These cells would find conditions for growth unsatisfactory and, if they did grow, cell growth would split the aggregate. This concept is supported by:

- The existence of a great variety of bacteria, most of which probably dwell as single or several species mixed cultures in different pores and thus survive without microbial antagonism
- The persistence of microbial compounds in the aggregate
- Some bacteria in the core may not be trapped rigidly in closed pores, and may become locomotive if water is supplied.
- Only bacterial cells on the outside surface of the aggregate may be washed out by liquid if they are not in an adsorbed state.

A further critical factor is the rate of oxygen diffusion into soil aggregates which may limit the potential for bacterial germination and, certainly, growth. The rate of oxygen diffusion into soil aggregates is determined by:

1) Size of specific surface area through which oxygen enters the structure.
2) Difference in concentration between the surface and the core.
3) Geometry of the pathways for oxygen diffusion.
4) Diffusion coefficient of the medium.

Soil is, therefore, a complex environment that can be extremely hostile to bacterial spores and to the toxic protein core of *Bt*. The complexity of the interactions, particularly competition for nutrients and the high activity of Protozoa in scavenging for available protein sources (Couteaux & Darbyshire, 1998; Foissner, 1999), suggests that *Bt* would not survive well in soils. However, as indicated below, this is not the case and the interactions of the bacterium and its toxin with soil are part of a complex microcosm where each organism has evolved mechanisms to survive the hostile conditions.

2.2.3.2 The presence of *Bacillus thuringiensis* in soils

Methods such as acetate selection (Travers *et al.*, 1987) have enabled more precise extraction and quantification of bacterial isolates from soils and there have in recent years been many studies in which large numbers of new isolations have been made around the world. Consequently, *Bt* is now considered to be a relatively common soil bacterium with a world wide distribution (Bernhard *et al.*, 1997).

Martin and Travers (1989) isolated *Bt* from 70.4% of the 1115 soils they investigated in 30 countries. They found that *Bt* was represented in savannah, desert, agricultural and forest soils as well as arctic tundra, urban environments, beaches, deserts and steppes. Regionally, *Bt* was most abundant in Asian, Central and South African and European soils, whereas the lowest recoveries were from New Zealand and USA. However, even at the lowest recovery rates, over 50% of soil samples contained viable *Bt*. The natural occurrence of *Bt* strains in Canada is not well documented, but it has been reported that *Bt* was found in 85% of pre-spray samples taken from organic horizons in coniferous forests in Quebec during control operations against spruce budworm, *Choristoneura fumiferana* (Cardinal & Marotte, 1987). However, it was suspected that contamination from previous spray activities may have affected these levels considerably.

Manonmani *et al*., (1991) successfully isolated several serotypes of *Bt* from root surfaces of hydrophytes, and postulated that the bacterium may have been utilising root exudates. They also found that *Bt* was more readily isolated in the rhizospheres of aquatic plants than in sick/dead mosquito larvae. More recent studies confirmed that new *Bt* isolates with high activities against mosquitoes were present in soils in Bangladesh (Manonmani & Balaraman, 2001).

In accordance with the general findings of Hattori & Hattori (1976; 1993), the rate of degradation of bacteria is soil is a dynamic balance between retention and percolation/microbial degradation. In particular, the rate of *Bt* loss appears to be much greater in natural soils than in sterile soils, thus confirming the important role of competition and predation/antagonism in determining *Bt* survival. Koskella & Stotzky (1997) showed that toxins from *Bt kurstaki* and *Bt tenebrionis* were rapidly adsorbed to clays and were resistant to the action of a mixture of bacteria. The antagonistic

bacterial culture contained *Proteus vulgaris*, *Enterobacter aerogenes* and a mixture containing mainly *Agrobacterium* spp. and was obtained from several soils. In addition, the culture from soil contained fungi such as *Streptomyces griseus* (a producer of pronase E) and *Streptomyces hygroscopicus* (a producer of natural proteases). Toxins (Cry1 and Cry3) were exposed to the bacterial/fungal mixture either free or bound on montmorillonite or kaolinite clays. After incubation the insecticidal activity of the remaining toxins was tested against *Manduca sexta* (for Cry1). Utilisation of the free toxins by the microbial antagonists was high but when the toxins were bound on montmorillonite or kaolinite, they were protected from microbial degradation. In the case of Cry1, bioassays confirmed that the activity of bound toxins was retained whereas there was a significant decline when free toxins were exposed to the various combinations of microbes.

Retention of *Bt* in soil for long periods, despite potential exposure to microbial antagonists, has been demonstrated in a number of studies (West *et al.*, 1984a; Dupont & Boisvert, 1986; Addison, 1993; Sundaram, 1996; Yara *et al.*, 1997; Crecchio & Stotzky, 1998; Stotzky, 2000). In a follow-up study in relation to aerial application of *Bt* to control gypsy moth, *Lymantria dispar* in the USA, densities of spores on leaves and in soil were monitored over a five year period (Smith & Barry, 1998). Soil data indicated that *Bt* spores persisted for up to 2 years after application but there was no indication of germination and multiplication. Significantly, *Bt* was isolated from leaves 12 months after applications in both sprayed and unsprayed areas, providing supporting evidence that the bacterium is capable of surviving successfully in the phylloplane, even if it does not cause significant mortality to potential host invertebrates.

It is not always clear from the literature whether the decline in *Bt* is due to loss of spores, vegetative cells or crystals or some combination of these stages, each of which contributes to *Bt* growth and survival. Pruett *et al* (1980) compared the relative survival and potency of *Bt* spores and crystals in natural soil and concluded that toxin crystals degraded more rapidly than spores, although initial decline was slow, with no loss after 21 days. By contrast a similar study by West *et al* (1984b) indicated rapid losses of activity within 7 days (50% loss). The role of microbial antagonism was also confirmed by West *et al* (1984a) when they demonstrated that *Bt* spores retained viability over a three year period in sterilized soil. Detailed studies of persistence of *Bt* spores and crystals using strains modified with antibiotic-resistant marker genes applied as a spray to potato and corn indicated that spores reaching the soil declined to about 10% of their starting level in three weeks (Martin & Reichelderfer, 1989). In addition, the nature of the soil, particularly the amount and type of clay minerals, has a very significant influence on the likelihood of adsorption and protection from the majority of microbial antagonists (Addison, 1993). Humic acids have also been shown to be important in the rapid and persistent binding of *Bt* toxins to agricultural and forest soils (Crecchio & Stotzky, 1998). Addition of *Bt* subsp *kurstaki* to humic acids from forest and cultivated soils resulted in rapid 75-85% adsorption of the toxins to the acids, followed by continuing adsorption as more toxin was added, until a plateau was reached, signifying saturation of the adsorption capacity of the humic acid. The proportion of toxin adsorbed varied with total acidity and concentrations of phenols but, in all cases, the toxin-acid complexes retained the ability of the toxin to kill tobacco hornworm, *M. sexta*, in bioassays. This study also confirmed that free toxin not bound to the humic acids was rapidly utilized by microbial antagonists in the soil.

Similarly, Sundaram (1996) studied the adsorption of solubilised (in carbonate buffer at pH12) *Bt* subsp. *kurstaki* toxin to sterilized sandy or clay loam forest soils. Adsorption of the toxin increased gradually to peak at 2 hours in sandy loam (30.1% adsorption of the applied toxin) and 4 hours in clay loam (47.4% adsorption). Desorption of the bound *Bt* was assessed by repeated washing of the *Bt*-soil mixtures with buffer at pH 10.2 over six desorption cycles. There was a linear decline in desorption over the course of the first five cycles, after which there was no further loss so that 54.5% and 67.4% of the *Bt* toxin remained in the sandy and clay loams respectively. These short adsorption times and high retention during the desorption cycles support the conclusions of Stotzky and co-workers that *Bt* toxin is rapidly and strongly bound to soil structures, especially clay micelles (see Stotzky (2002b) for a comprehensive review of a wide range of experimental studies on the *Bt*-soil interaction).

Soil and the aquatic environment are obviously closely associated and the environmental fate of *Bt israelensis* (*Bti*) (Cry 4A, 4B, 11) and other Diptera-active Cry genes has also been studied. As with all the data on persistence, it is difficult to distinguish between *Bt* spores/crystals still present in the cadavers of hosts that have been killed by the bacterium and those that have been liberated and are free-standing. The original isolation of the Diptera-active *Bti* was from a pond in Israel where one of the few epizootics of *Bt* in external environments was inadvertently recorded (Margalit & Dean, 1985). Subsequently, a number of Diptera-active isolates of *Bt* have been discovered and there has been considerable interest in their potential environmental persistence, both from the pest management and from the environmental impact points of view. Isolations from a number of aquatic or semi-aquatic habitats indicate that survival of Diptera-active *Bt* isolates is good. For example, active isolates of *Bt* (*israelensis* and other pathotypes) have been found in brackish sediments in mangrove swamps in Japan (Maeda *et al.*, 2001), in running and still fresh water in Japan (Ichimatsu *et al.*, 2000), in soils in Bangladesh (Hossain *et al.*, 1997), Korea (Kim *et al.*, 1999) and Sweden (Ankarloo *et al.*, 2000) and in sericulture in Japan (Ohba, 1997). Specific studies of persistence in relation to applied *Bti* have indicated survival of spores for at least 9 months after application, especially in tyres and other containers (Siegel *et al.*, 2001). Naturally occurring *Bti* was also found in local soils and in the same aquatic environments as the applied microbial insecticide, further confirming the ubiquitous presence of a wide variety of *Bt* isolates.

3 Use as a pest management tool

Pest management using a range of microbial agents offers prospects for high selectivity and relatively limited persistence, thus giving an environmental profile that is seen as much more benign than chemical insecticides. Both conventional microbial agents (Fuxa, 1995) and genetically modified bioinsecticides (Evans, 1999) have been reviewed in relation to their ecological interactions with target and non-target organisms. The importance of the ecological context of the use of *Bt* and other microbial agents cannot be over-stressed, particularly in differentiating between laboratory host range and ecological host range (Evans, 1999). The ecological link is central to the discussions below and in the remainder of this review.

3.1 Conventional application

Bt has become the most widely used microbial insecticide and its implementation far exceeds other microbial agents such as fungi, Protozoa or viruses (Lacey *et al.*, 2001). As indicated in Chapter 2, expansion in the use of *Bt* coincided with the development of methods of large-scale fermentation and formulation (1997; Burges, 1998). Further impetus has been provided by the search for, and successful isolation of, new strains of *Bt* with increased activities against a wide range of potential hosts.

Irrespective of the target host or of the Cry toxin(s) employed in the applied agent, the methods of conventional application of *Bt* are essentially the same. Formulation of *Bt* is based on either the purified crystals alone (isolated from the rest of the bacterial cell and spore) or in combination with the spore. In either case, the product is separated from the cellular debris remaining from growth of the bacterium in suspension culture (Bryant, 1994; Watkinson, 1994). Most commercial products based on *Bt* employ a combination of spores and crystals and there is some evidence that efficacy of the two component in mixtures is greater than use of the toxin alone (Miyasono *et al.*, 1994; Tang *et al.*, 1996). Indeed, the effects of combining both crystals and spores can be synergistic and it has been demonstrated that addition of spores of *Bt* subsp. *kurstaki* to purified Cry1Ab and Cry1C crystal proteins gave 35-50 fold and 25-44 fold increases in toxicity to normal and *Bt*-resistant *Plodia interpunctella* respectively (Johnson & McGaughey, 1996). The effects were demonstrated both as increased overall mortality and as more rapid mortality in normal larvae but there was no effect on lethal time in resistant larvae.

3.1.1 The scale of use of conventional *Bt*

World markets for *Bt* up to 1995 amounted to around $90 million annually although, in the context of total insecticide use, this represented only about 2% of the market (Schnepf *et al.*, 1998). A more recent estimate places the market for conventional use at around $100 m in the vegetable, fruit, cotton, soybean, corn, forestry and vector control markets (Gelernter & Lomer, 2000). The market share, relative to conventional insecticides, has not changed. Nevertheless, there is an impressive array of *Bt* products registered for use in the USA, which represents the main market for this agent. Table 3.1 lists the number of products based on the various subspecies of

Bt that are recognised internationally and includes more novel approaches to overcoming some of the drawbacks of conventional application of *Bt*.

Constraints to wider use of conventionally applied *Bt* are discussed further by Gelernter & Evans (Gelernter & Evans, 1999) and Gelernter & Lomer (2000). Gelernter & Lomer make the significant point that if the methodology of application and the product is good enough, as was the case for use of conventional *Bt* against diamondback moth *Plutella xylostella*, the product can actually be over-used, leading to the development of serious levels of resistance to the bacterium. In this case, the initial success of *Bt* was as much due to its intrinsic efficacy as to the need to find alternatives to the widespread resistance to a spectrum of chemical insecticides that had essentially removed those active ingredients from available use against *P. xylostella*. From an initially slow penetration of the market in the early 1980s, *Bt* application against *P. xylostella* grew exponentially to reach a staggering 50 applications per year against this pest on cruciferous crops in Hawaii and to the first record of resistance development in the field (Tabashnik *et al.*, 1990). Later analysis revealed that the resistance from use of the commercial product Dipel (classified as *Btk* and containing Cry1Ab) had also developed to a range of other Cry genes, including Cry1Aa, Cry1Ac, Cry1Fa and Cry1Ja (Tabashnik *et al.*, 1996). Further records of resistance to *Bt* in this moth have now been recorded in Japan (greenhouses only), Malaysia, the Philippines, Honduras, Costa Rica, Guatemala and the USA (Florida, South Carolina) (Perez & Shelton, 1997; Ferré & Van Rie, 2002).

Table 3.1: *Bt*-crops and products available commercially or under development. Some products, such as StarLink corn have been withdrawn by the registering company. General information on conventional *Bt* spray formulations is also included

Cry gene	Event	Crop	Brand name	Target Pest	Company	Other Information
Cry1Ab	176	Corn	Knockout/ Naturegard	European corn borer	Syngenta Seeds, Inc.	Gene from *B. thuringiensis* subsp. *kurstaki Btk,* expressed in foliage and pollen.
Cry1Ab	Bt11	Corn	Yieldgard	Lepidopteran corn pests	Syngenta Seeds, Inc.	Insect-resistant and herbicide tolerant. *Btk* gene expressed constitutively, and phosphinothricin N-acetyltransferase (PAT) encoding gene from *S. viridochromogenes.*
Cry1Ab	Bt11	Corn	Yieldgard, Attribute	European corn borer or corn earworm	Novartis	Corn line HE89 transformed with a plasmid containing a truncated (and altered to improve its GC ratio) Cry1Ab gene, pat and ampr genes. The ampr gene was not found in the plant. Cry1Ab gene constitutively expressed.
Cry1Ab	MON80100	Corn		European corn borer	Monsanto company	ECB-resistant maize, full length gene from *Btk* expressed.
Cry1Ab	MON802	Corn		Lepidopteran corn pests	Monsanto company	Insect-resistant and glyphosate tolerant maize, truncated Cry1Ab gene from *Bt*, and the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene from *A. tumefaciens.*
Cry1Ab	MON809	Corn		European corn borer	Pioneer Hi-Bred International Inc.	Resistant to ECB, and glyphosate resistant by introduction of EPSPS from *A. tumefaciens.*
Cry1Ab	MON810	Corn	Yieldgard	European corn borer	Monsanto company	ECB-resistant maize, using a truncated form of the gene from *Btk* expressed constitutively. Initial year of registration 1996.
Cry1Ac	31807/ 31808	Cotton		Lepidopteran cotton pests	Calgene Inc.	Insect and herbicide tolerant. Genes from *Bt*, and a nitrilase encoding gene (against bromoxynil) from *Klebsiella pneumoniae*.

Cry gene	Event	Crop	Brand name	Target Pest	Company	Other Information
Cry1Ac	Mon531/757/ 1076	Cotton	Bollgard	Cotton bollworm, tobacco budworm, pink bollworm	Monsanto company	Insect resistant, with gene from *Btk* HD-73. Registered for use 1995, first commercialisation in 1996, registration expired 01/01/01
Cry1Ac	5345	Tomato		Lepidopteran pests	Monsanto company	Gene from *B. thuringiensis* subsp. *kurstaki Btk*.
Cry1Ac	DBT418	Corn	BtXtra	European corn borer	Dekalb Genetics Corporation.	Insect and glufosinate ammonium tolerant. Gene from *Btk* (expressed constitutively), and PAT gene from *Streptomyces hygroscopicus*.
Cry1Fa2	TC1507	Corn		Lepidopteran pests, specifically ECB	Mycogen; Pioneer	Insect and glufosinate ammonium tolerant. Genes from *Bt(aizawai*) and PAT from *Streptomyces viridochromogenes* (a common soil bacterium).
Cry3A	ATBT04-6,04-27,04-30,04-31,04-36; SPBT02-5,02-7	Potato		Colorado potato beetle (CPB)	Monsanto company	CPB resistant, inserted gene for Cry3A from *Bt* (*tenebrionis* - *Btt*).
Cry3A	BT6, BT10, BT12, BT16, BT17, BT18, BT23	Potato		Colorado potato beetle (CPB)	Monsanto company	CPB resistant, inserted gene for Cry3A from *Btt*
Cry3A	Russet Burbank (RB) RBMT15-101, SEMT15-02, SEMT15-15	Potato		Colorado potato beetle (CPB)	Monsanto company	CPB resistant, inserted gene for Cry3A from *Btt*, also potato virus Y (PVY) resistant by introducing the coat protein encoding gene from PVY.
Cry3A	RBMT21-129, RBMT21-350, RBMT22-082	Potato		Colorado potato beetle (CPB)	Monsanto company	CPB resistant, gene from *Btt*, also potato leafroll virus (PLRV) resistant, encoding the replicase gene from PLRV.
Cry9C	CBH-351	Corn	Starlink	European corn borer and other lepidopteran pests.	Aventis CropScience	Resistant to ECB, modified and truncated gene from *Bt* (*tolworthi*) expressed constitutively, also glufosinate ammonium tolerant, encodes the PAT gene from *S. hygroscopicus*. Used for livestock feed only.

Conventional *Bt* application.			
***Bt* source**	**Conventional Application**	**Target Pests**	**Other information**
Btk, Bti, Bta, Bt sandiego, Btt	*Bt* biopesticides: spray of whole spores, toxins and crystals as produced by the *Bacillus thuringiensis* bacterium. Most are a mixture of Cry genes.	Coleoptera (*Btt and sandiego*), Diptera (*Bti*) and Lepidoptera (*Btk*, *Bta*)	*Btk* biopesticide registered for forestry use on trees for control of caterpillars and for rangeland use for control of caterpillars. *Bt* initially registered in 1961 as a bacterial insecticide to US EPA (Dipel - *Btk*), registration reissued 1986. Dipel 2X contains~50 million spores of *Bt* per (g). *Bt* subsp. *kurstaki* (*k*), *israelensis* (*i*), *aizawai* (*a*), *sandiego* and *tenebrionis* (*t*) are all subspecies produced as commercial formulations.
Btk, Bt san diego, Cry1Ac, Cry1C	Endotoxin bearing plasmid transferred to *Pseudomonas fluorescens* cells in which the crystal toxin forms around 20% of total cell protein. The *Pseudomonas* cells are subsequently killed providing a protective cell wall "capsule" around the *Bt* crystal. The technology has been developed commercially as the "Cellcap" system.	Lepidoptera (*Btk* and Cry1 genes) Coleoptera (*Bt san diego*)	(Gaertner *et al.*, 1993)

By contrast to the above, extreme, example of over-use of *Bt*, application in forestry situations has a long and effective track record and there is no apparent development of resistance despite many years of use, particularly in North America and in eastern Europe (Evans, 1997). In many respects, the forestry market is ideal for microbial agents such as *Bt*; there is normally only one pest species to be managed at a time, they are usually univoltine, requiring a single application per year, and may not reach damaging levels in the following year. The economic threshold is relatively high so that tolerance of superficial damage is acceptable and the environmental premium for a "green" product is high. Van Frankenhuyzen (1993) analysed the operational use of *Bt* against one of the most serious and regular pests of conifers in eastern Canada, spruce budworm *Choristoneura fumiferana*, over a 10 year period from 1980. Although the area treated fluctuated from 0.8 m to 3.0 m ha, the proportion of that area treated with *Bt* increased dramatically from a low of 2% to reach 63%. A similar story of increasing use of *Bt* in forestry operations in eastern Europe was summarised by Evans (1997).

Perhaps the most concentrated use of *Bt* with a specific aim of eradication of a pest population, rather than suppression management, was the programme to eliminate the exotic white-marked tussock moth, *Orgyia thyellina*, in Auckland, New Zealand (Thorogood *et al.*, 1996; Aer'aqua Medicine Ltd & (formerly Jenner Consultants Ltd): 2001). During two consecutive larval feeding periods of the moth, which has three generations per year, *Btk* was applied aerially to the affected suburbs of Auckland such that a total of 9 applications by DC6 fixed wing aircraft (130,000 litres of diluted Foray 48B – Abbott Laboratories) and 23 applications by helicopter (28,000 litres) and a number of ground based applications were carried out at approximately weekly or fortnightly intervals. There was extensive monitoring of both environmental impacts and also of human health, including assessment of penetration of the *Bt* spray into domestic and business premises. The operation was successful in eradicating the moth and there were no significant health or environmental problems, although detailed studies of fate in soil were not carried out.

3.1.2 Stability of sprayed applications of *Bt*

Studies of spore survival and, particularly, retention of activity of the toxin crystals of *Bt* have indicated that, when the agent is applied as a conventional spray, loss of activity can be very high. In fully open conditions where spores/crystals are exposed to UV light, losses of spores are generally rapid initially but may stabilise over a longer term. For example, comparison of *Bt* stability in agricultural crops indicated that the half life of the initial deposit was 1-2 days, which was five times less than the equivalent half life in forest applications (Fuxa, 1989). It also appears that the toxin is degraded more rapidly than the spores or vegetative stages of *Bt* and any formulation to improve stability has to account particularly for the toxin itself (Burges, 1998).

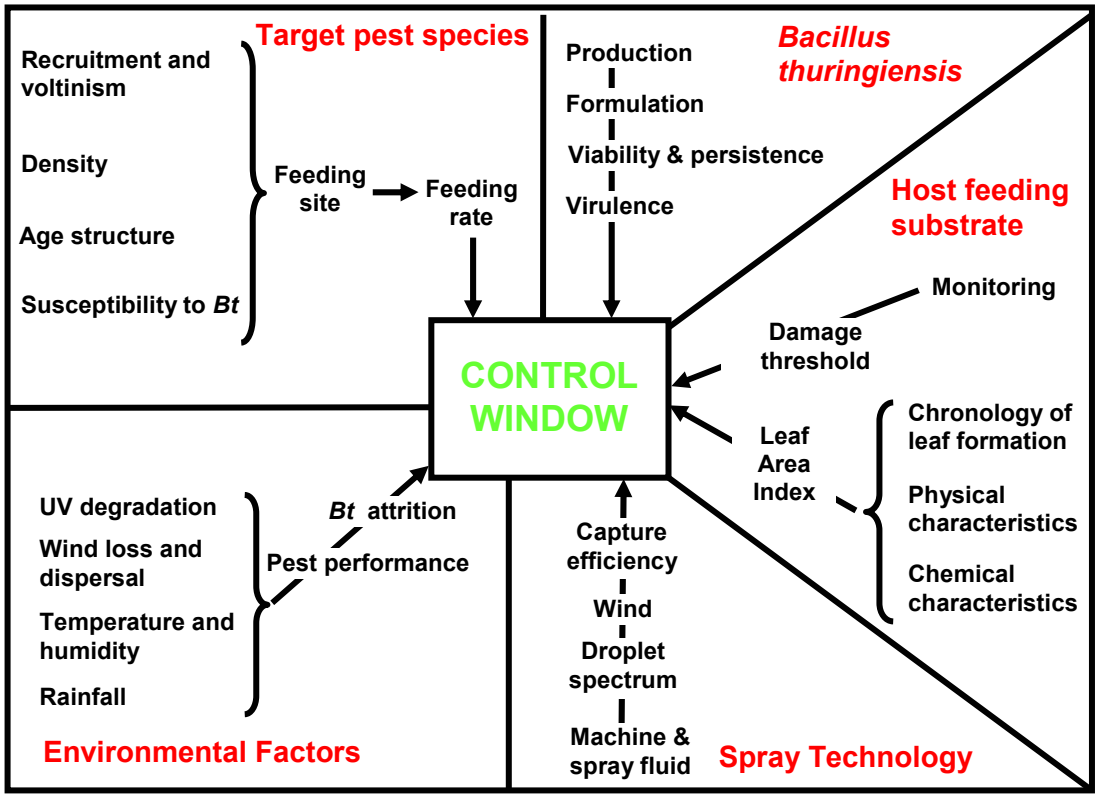
3.1.3 Application technology to deliver the dose to the target

Recognition of the key attributes of delivering the correct *Bt* dose to the right place at the right time (i.e. to a susceptible stage of the pest before it has caused unacceptable damage) is essential in developing a suitable pest management regime based on *Bt*.

There have been major advances both in the formulations of *Bt* available commercially and in the physics of spray generation, making use of more sophisticated atomisers and technology such as GPS and electronic track guidance to guide the tracks of sprayers (1998). Improved delivery of the microbial agents results in a greater proportion of the spray reaching the precise target area where the pest stages are feeding. At the same time as improving the performance of the agent in delivering effective control, such an approach also minimises the quantity of *Bt* that lands on non-target areas such as surrounding vegetation and, particularly, the soil. The value of ultra low volume (ULV) controlled droplet application (CDA) is now well established and has been particularly well developed in the forestry sector (Picot & Kristmanson, 1997).

Delivery of the droplets to the right place must be combined with knowledge of the dosage required to kill an acceptable proportion of the target host population and also the rate of attrition of the applied *Bt* during a defined period of feeding by the target stages. This has been assessed in detail by Evans (1998) who developed the *Control Window* concept to take account of measurable and, particularly, controllable parameters of a spray application programme involving microbial agents. The *Control Window* is reproduced and adapted for *Bt* in Figure 3.1. Many of the parameters within the *Control Window* can be established under controlled laboratory conditions and will enable practitioners to have a much higher level of confidence in establishing an initial field dosage for a given microbial insecticide. It is also important to take account of interaction effects, such as the phenology of the host plant if this affects synchrony between the target host and the expanding leaf substrate. As with many other microbial control agents and, to a lesser extent, with chemical pesticides, the susceptibility of larval stages of potential target organisms tends to decline with increasing age and size of larvae. It is often the case that targeting of the younger larval stages is essential for effective control, both to minimise the dose requirements and to kill the pests before they can cause unacceptable damage.

Figure 3.1: The *Control Window* concept to aid more accurate and effective application of *Bacillus thuringiensis* in conventional spray application programmes. Adapted from Evans (1998)



Knowledge of the inter-relationship of the target insect stage with the availability of its feeding substrate is, therefore, important, especially if the physical and chemical composition of the substrate changes to the disadvantage of the applied *Bt*

This is well illustrated in the case of spruce budworm, *Choristoneura fumiferana*, on balsam fir (*Abies balsamea*) and on black spruce (*Picea mariana*) (Cadogan & Scharbach, 1993). Aerial applications of *Bt* were less effective when they were timed to correspond with flushing of the balsam fir (early flushing) but not for black spruce (a late flushing species). Both larval mortality and damage reduction were significantly improved by timing the spray for the flushing of black spruce but the authors noted the difficulty of timing in mixed forests where it may not be possible to optimise application for a particular tree species.

The nature of the leaves themselves, particularly the presence of secondary plant compounds such as tannins, may also impact on the efficacy of applied *Bt*. In bioassays of larval stages of *Spodoptera exigua* fed on foliage of white oak (*Quercus alba*) or sweetgum (*Liquidamber tyraciflua*) there was greater mortality on the oak foliage than on the sweetgum foliage, even though dosages received by the larvae were the same in both cases (Farrar *et al.*, 1996). The authors attributed this to the higher levels of secondary plant compounds in sweetgum that presumably affected the solubilisation and/or activation of the toxin. A similar result was described in studies of two *Apium graveolens* var. *rapaceum* cultivars that were intrinsically different in their ability to support larvae of *S. exigua* and *Trichoplusia ni* (Meade & Hare, 1994).

Efficacy of applied *Bt* was greatest on the plants that were least suitable for normal larval development, suggesting a link with host plant secondary compounds.

By contrast, some leaves contain compounds that bind to the *Bt* toxin and render it less vulnerable to gut solubilisation and activation and, hence, results in lower toxicity to test larvae. Applications of Thuricide, a commercial formulation of *Btk*, were made against gypsy moth, *Lymantria dispar*, on leaves of red oak, *Quercus rubra*, chestnut oak, *Quercus prinus* and quaking aspen, *Populus tremuloides* (Appel & Schultz, 1994). In relative terms, efficacy of similar quantities of *Bt* was least on red oak, followed by chestnut oak and then quaking aspen. This was linked to the quantities of total phenolics and gallotannins giving rise, in a quantitative way, to binding of the *Bt* proteins. Both tree species differences and also the temporal dynamics of tannins and other secondary compounds in the leaves must be taken into account. On oak, tannins tend to increase rather rapidly after leaf flush in the spring so that, even without the interaction with agents such as *Bt*, the leaves tend to become less suitable for larval development over time. Such is the case for winter moth, *Operophtera brumata*, that must have close synchrony between egg hatch and bud burst of oak in order to maximise early instar survival before tannin concentrations increase to damaging levels (Hunter, 1992; Buse *et al.*, 1998). Data of this nature need to be taken into account not only in designing spray operations but also in assessing environmental risk in an ecological context.

Concepts like the *Control Window* have been developed to aid timing and dosage requirements against major insect pests. Spruce budworm has been better studied than most species and models for improving efficacy were presented by Regnier & Cooke (1998) and Cooke & Regnier (1999). Parameters such as daily max-min air temperatures, spray deposition, initial budworm density and stage-specific survivor rates of the larvae were input to the models and used to simulate applications with single or double treatments using two different rates of Foray (48B and 76B). The proportion of larvae ingesting a lethal dose of *Bt* within the control window period of 48 hours predicted total mortality well but, overall, the model was most sensitive to the quality of population density sampling. In a further study to improve aerial application of *Bt* against spruce budworm, rates of feeding and movement of 2^{nd} to 4^{th} instar larvae over the target foliage were found to be good predictors in a model called Pesticide Drop Simulator which also took account of the formulation characteristics of the applied *Bt* (Hall *et al.*, 1995).

3.2 Effects of Bt *on non-target organisms*

One of the major perceived and demonstrated benefits of using *Bt* in pest management programmes is the lack of impact on non-target organisms relative to use of broad-spectrum chemical insecticides (Milner, 1994; Lacey *et al.*, 2001). The relatively selective nature of the agent and the increased understanding of the mode of action and possible effects on non-target hosts tend to confirm that effects across insect orders or even families are small. Indeed, the difference between laboratory-demonstrated host ranges and ecological host ranges must also be taken into account in relation to encounter frequency between potential hosts and lethal dosages of *Bt* (see Chapter 4.4). An understanding of the ecological role of *Bt* in the environment, however, is scant and is only now receiving attention because of the increasing use of this microbial agent in genetically modified plants (Fuxa, 1989). Nevertheless,

information on non-target effects gathered during the course of conventional spray programmes has provided some valuable data on significance of potential environmental impacts of *Bt*.

As might be predicted from the modes of action of *Bt* Cry genes, some potentially susceptible organisms that possess the correct combination of gut conditions and suitable binding sites in the midgut cells, could succumb to the toxin if they are in the same spray zone as the target pests. This was the case for three species of tree-feeding swallowtail butterflies in the same zone as other hosts such as spruce budworm that are frequently subjects of spray applications of *Bt* (Johnson *et al.*, 1995). Experimental application of equivalent to 40 BIU/ha to potted tulip trees followed by monitoring for toxicity to early instar *Papilio glaucus* demonstrated some mortality of the early instar larvae for up to 30 days after application. Some of the most intensive studies of possible non-target effects of conventionally applied *Bt* have been carried out in connection with suppression of gypsy moth populations in the USA. Evaluation of effects on native, non-target arthropods was carried out in eastern West Virginia by Sample *et al* (1996). They collected adult and larval arthropods using foliage samples and light-trapping at 24 20 ha plots, including sprayed and unsprayed treatment with and without noticeable defoliation from gypsy moth. Abundance and species richness of larval and adult non-target Lepidoptera decreased at all plots but was greater in the *Btk*-treated plots. The authors pointed out that although the short-term (<1 year) impacts on non-target Lepidoptera were negative, they were more than compensated for by long-term reduction of gypsy moth (an introduced exotic species), which led to increases in native species of Lepidoptera. Richness and abundance of some larval and adult Lepidoptera declined at defoliation plots without *Bt* treatments.

The increasing use of Diptera-active *Bt* isolates, specifically Cry2A, has been studied in relation to possible effects on non-target organisms in treatment zones (1997). Tests of purified Cry2A (known to be Lepidoptera and Diptera-active) were carried out against one isopod (Crustacea, Isopoda) and 35 insect species representing the orders Coleoptera, Collembola, Diptera, Hemiptera, Homoptera, Hymenoptera, Isoptera, Lepidoptera, Neuroptera, and Orthoptera. Only Lepidoptera and Diptera (Culicidae) were sensitive to the protein. Among the Lepidoptera, the most susceptible species, *Anticarsia gemmatalis* was >12500-fold more sensitive than the cutworm *Agrotis ipsilon* and the fall armyworm *Spodoptera frugiperda*. However, sub-lethal concentrations of Cry2a reduced larval growth of all Lepidoptera species tested. Among the Culicidae, the most susceptible species, *Anopheles quadrimaculatus* was >500-fold more sensitive than *Culex pipiens*. Other Diptera (*Musca* and *Drosophila*) and non-Lepidoptera species were not affected by the toxin. Although these results provide useful data on the relative susceptibilities and, particularly, the lack of activity against Orders other than Lepidoptera and Diptera to Cry2A, they must be interpreted with caution in relation to the ecological likelihood of effects in the field.

More studies combining laboratory sensitivity with actual field impacts should be carried out in order to evaluate fully the non-target effects of *Bt*. Interaction effects, in which impacts on non-targets are evaluated both directly (+/- mortality) and indirectly (e.g. reduction of competition, reduced availability of prey/hosts for specialist natural enemies) are fundamental to the understanding of ecological effects of *Bt*, whether it is applied conventionally, as discussed in this section, or through GM routes, as discussed in Chapter 3.3. Interactions with natural enemies are particularly important

and must be considered in the context of short and long-term ecological effects. Some laboratory studies have indicated very little or nil negative effects of *Bt* on natural enemies. For example *Bt tenebrionis* (Coleoptera-active) was tested against Australian plague soldier beetle *Chauliognathus lugubris*, a predator of the Tasmanian eucalyptus leaf beetle *Chrysophtharta bimaculata* a pest of eucalyptus (Beveridge & Elek, 1999). No negative effects were noted and the authors considered that *Btt* could be included in an Integrated Pest Management regime against *C. bimaculata* with the express aim of conserving natural enemies, while still applying a biocide. There are numerous other studies of the interactions between conventionally applied *Bt* and natural enemies (often considered under the general title of tritrophic interactions) and some of these are summarised in Table 3.2.

Table 3.2: Effects on natural enemies of *Bt* applications, applied alone or in comparison with or in combination with chemical insecticides.

Natural enemy group/species	Agents applied	Observed effects	Reference
Spiders: Salticidae, Linyphyiidae, Clubionidae, Philodromidae, Theridiidae in an apple orchard.	Dipel – *Btk* MVP – *Pseudomonas* encapsulated *Bt* Summer oil Diflubenzuron – insect growth regulator (IGR) Phosmet – organophosphate Carbaryl – carbamate Esfenvalerate – pyrethroid Permethrin - pyrethroid	Most generally harmless to the spiders +/- *Bt*. Pyrethroids harmful.	(Bajwa & Aliniazee, 2001)
Parasitoids of Nantucket pine tip moth, *Rhyacionia frustrana*. *Lixophaga mediocris* – Hymenoptera: Tachinidae *Haltichella rhyacioniae* – Hymenoptera: Chalcidae	Orthene - acephate Foray – *Btk* Mimic – tebufenozide, an IGR	*Bt* and tebufenozide harmless. Parasitism of the moth lower in acephate treated plots, with greater impact on *H. rhyacioniae*	(Wirth *et al.*, 2000)
Generalist predators: spiders (Araneae), big-eyed bugs (*Geocorus* spp.), damsel bugs (*Nabis* spp.) and minute pirate bugs (*Orius* spp.).	Transgenic potato expressing Cry3Aa Weekly spray of *Btt* containing Cry3Aa Bi-weekly spray of permethrin Early- and mid-season sprays of systemic insecticides – phorate and disulfoton	Neither the plant-expressed nor the sprayed *Bt* had any effect on natural enemies. Permethrin significantly reduced predators, accompanied by rise in green peach aphid, *Myzus persicae*. Systemic insecticides had no effect on natural enemies	(Reed *et al.*, 2001)

Natural enemy group/species	Agents applied	Observed effects	Reference
General predators: Coccinellidae, Neuroptera, Araneae and predatory Heteroptera in sprays against *Helicoverpa* spp. on cotton.	Neem – azadirachtin Talstar – bifenthrin Methomyl Thiodicarb Endosulfan *Btk*	Chemicals and biorationals used alone or in combination. No effects on natural enemies of neem or *Bt*. Chemical insecticides very destructive.	(Ma *et al.*, 2000)
Spined soldier bug, *Podisus maculiventris* (Heteroptera: Pentatomidae)	Teflubenzuron – IGR Methomyl – carbamate Deltamethrin – pyrethroid Bactospeine - *Btk*	Tested in laboratory against fourth instar and adult female bugs. *Bt* had no effect and deltamethrin relatively safe to 4th instars compared with adults. Methomyl and teflubenzuron were very toxic to 4th instars.	(Mohaghegh *et al.*, 2000)
Cotesia plutellae (Hymenoptera: Braconidae), parasitoid of *Plutella xylostella*	*Btk* as a spray and as toxin-affected *P. xylostella* larvae	No direct or indirect effects on mortality (male and female) or oviposition behaviour of adult parasitoids.	(Chilcutt & Tabashnik, 1999)
Predatory Heteroptera and general biodiversity in plots treated against Colorado potato beetle, *Leptinotarsa decemlineata*.	5 weekly applications of: The insect pathogenic fungus *Beauveria bassiana* GM modified *Bt* – Raven® (Cry1Ac, Cry3A, Cry3Bb) with dual Lepidoptera and Coleoptera activity Temik – Aldicarb	Biodiversity and, especially, predatory Heteroptera decreased with aldicarb. *Bt* had nil effects on predatory Heteroptera and non-targets *B. bassiana* similar to *Bt* plots	(Lacey *et al.*, 1999)
Stinkbug, *Perillus bioculatus* (Heteroptera: Pentatomidae) in management of *L. decemlineata*.	*Bt tenebrionis* applied to potato crop	Laboratory studies showed extended development in *Btt* affected larvae and greater impact of predation. Confirmed in the field – synergistic interaction between *Btt* and the predator.	(Cloutier & Jean, 1998)

Some of the studies in Table 3.2 distinguish between the potential direct effects of *Bt* on natural enemies and also the indirect effects through possible avoidance of hosts by parasitoids and removal of prey items, especially for more specialist natural enemies. The time from ingestion of *Bt* by host larvae may have a significant effect on whether or not parasitoid larvae complete development in the affected host. In the case of *Cotesia marginiventris* (Hymenoptera: Braconidae) parasitizing tobacco budworm, *Heliothis virescens*, larvae affected by *Bt* or the insecticide thiocarb, there were significant effects of timing of exposure to the agents (Atwood *et al.*, 1997). Although there was a significant increase in mortality when *Bt* and *C. marginiventris* were used in combination, the numbers of the parasitoid emerging decreased if parasitism took place soon after *Bt* was fed to the larvae. In the case of thiocarb application, there was a significant negative effect on parasitoid emergence, especially if application was

delayed. An interesting variation on this type of interaction was noted by Chilcutt & Tabashnik (1997b) when they evaluated the effects of *Bt* and the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) on susceptible and resistant *P. xylostella*. The impact of *Bt* declined with the level of known resistance in the selected *P. xylostella* colonies but parasitism was the same in all cases. The authors found that delaying treatment with *Bt* from 0 to 4 days after parasitism in resistant moths did not affect overall *P. xylostella* mortality but it significantly increased parasitoid survival. This was thought to be due to a reduction in competition for the host, thus allowing better parasitoid development and eventual population increase (Chilcutt & Tabashnik, 1997a). Such an effect could have a longer term positive impact in managing the problems of resistance to *Bt* in populations of this moth in the field.

These examples illustrate the complexity of assessing impacts and potential effects on the environment from use of *Bt*, whether it is applied conventionally or by novel means.

3.3 Application as a component of transgenic plants

Incorporation and successful expression of the *Bt* toxin gene in plants is a relatively new phenomenon and was reported first for tobacco plants in 1987 (Barton *et al.*, 1987; Vaeck *et al.*, 1987). General reviews of the early progress in developing and initial marketing of transgenic crops expressing *Bt* were provided by Estruch *et al* (1997) and Peferoen (1997). The essential technology is based on the introduction of novel genes into the cells of the host plant using a range of physical (biolistics) or vector-delivered insertion techniques (Jenkins, 1999). The soil bacterium *Agrobacterium tumefaciens*, that infects dicotyledonous plants leading to crown gall disease, has been used extensively as a vector for delivery of foreign DNA. It contains large Ti plasmids that are responsible for transfer of DNA designated the T-DNA region, which is flanked by border sequences that determine precisely the DNA sequences that are transferred to the plant. The T-DNA segment can be engineered to contain marker or functional genes and it is this sequence that undergoes cell insertion and incorporation into the host genome. A further set of virulence genes (V-DNA) are expressed at acidic pH and particularly when phenolic inducers are present, as would be case if the plant is damaged (Hansen & Wright, 1999).

As a general requirement, the vector system for delivery of the toxin gene is constructed to maximise the likelihood of integration into the plant genome. The Ti plasmid used in transformation of plants is normally disarmed so that, although it infects the plant, it does not result in formation of tumours (the crown gall) and, instead, inserts the *Bt* gene into the plant genome. Expression requires promoter and terminator sequences flanking the *Bt* gene; cauliflower mosaic virus (CaMV35S) is a constitutive promoter whereas others, such as phosphoenolpyruvate carboxylase (PEPC) (green tissue), pith tissue and maize pollen specific promoter are tissue specific in their modes of action (Koziel *et al.*, 1993; Datta *et al.*, 1998).

Initial attempts at transformation with *Bt* employed the full Cry1A toxin gene, using the CaMV35S promoter but resulted in very low levels of expression attributable to low efficiency in forming full size mRNA (Van Aarssen *et al.*, 1995). Early strategies to improve expression of *Bt* were based on truncation of the full toxin gene to

synthesize the N-terminal core toxin portion of the genome, a method that gave some enhancement to reach about 0.02% of total leaf soluble protein. The key to greater success in transformation was conversion of the AT-rich (60-70%) amino-acid sequences that are typically found in bacteria to the GC-rich (40-50%) sequences found in higher plants (de Maagd *et al.*, 1999). The AT-rich regions of the *Bt* gene were found to have various sites contributing to the poor performance; these included polyadenylation termination sites, hidden mRNA splicing sites and areas (motifs) of mRNA instability. Removal of some of the polyadenylation (7 out of 18) and mRNA instability (7 out of 13 ATTTA sequences) sites increased expression 10-fold relative to unmodified Cry1Ab and Cry1Ac genes (Perlak *et al.*, 1991). Further manipulation to remove the remaining polyadenylation and mRNA sites and to modify 356 out of the 615 codons increased expression to a level that was 100 times higher than achieved with the full toxin gene. As indicated by van Aarssen *et al* (1995), it is the presence of cryptic introns in the unmodified toxin gene that causes mRNA splicing, leading to interference with full transcription within the plant cells. In these cases the *Bt* introns act like plant introns. Efforts have been made to remove these introns in a more specific manner in a number of Cry genes, including Cry3A which had initially proved difficult to express at levels sufficient to result in mortality of Colorado potato beetle (Perlak *et al.*, 1993). As knowledge of the interaction between the codon usages of both the *Bt* and of the plants themselves has increased, more sophisticated manipulation of the Cry genes have been made (Koziel *et al.*, 1996; Estruch *et al.*, 1997).

Promoters other than the ubiquitous CAMV35S have increasingly been used to improve and to allow more selective expression. Thus, wound-inducible promoters (Vaeck *et al.*, 1987), chemically induced promoters (Williams *et al.*, 1992) and tissue specific promoters (Koziel *et al.*, 1993; Koziel *et al.*, 1996; Stoger *et al.*, 1999) have all been employed, with a range of expression enhancements compared with a constitutive promoter.

3.4 The scale of development and use of Bt crops

From the initial successes in transformation of tobacco plants (Ely, 1993), there has been remarkable progress in both the range of plants that have been successfully engineered to express *Bt* and in the numbers of crops that have been registered for commercial use world-wide. A recent review by Shelton *et al* (2002) includes an assessment of the areas of *Bt*-crops grown commercially, reaching a total of 11.4 million ha world-wide during 2000. A forward look estimated that world markets for transgenic crops as a whole could be 8$ billion in 2005 and $25 billion by 2010. The scale of growth of the transgenic plant market as a whole (dominated by herbicide tolerance) is indicated by the rise from the numbers of countries growing them commercially from 1 in 1992 to 13 in 1999, accompanied by global increases in areas planted from 1.7 m ha in 1996 to 44.2 m ha in 2000 (98% of which was accounted for by USA, Canada and Argentina) (C. James quoted in Shelton *et al* (2002)). Herbicide tolerance has been the most common trait engineered into crop plants (74%), while *Bt*-corn constituted up to 18%, of which 3% was combined *Bt*-herbicide tolerance. Table 3.1 in Chapter 3.3, listed some of the *Bt* crops registered currently. Some of the transformations, such as event 176, are now grown over relatively small areas (<2% of total corn grown in the USA and declining (Sears *et al.*, 2001). Mon810 is the dominant product and accounts for >85% of the *Bt*-corn planted globally.

One of the often-quoted attributes of planting crops expressing *Bt* is the expected reduction in use of chemical insecticides to control those pests that are susceptible to the expressed bacterial toxins. Among the list of corn pests regarded as the most damaging (European corn borer (ECB), *Ostrinia nubilalis*; Asiatic corn borer (ACB), *O. funicalis*; southwestern corn borer (SWCB), *Diatraea grandiosella*; corn earworm (CEW), *Helicoverpa zea*; fall armyworm (FAW), *Spodoptera frugiperda* and black cutworm (BCW), *Agrotis ipsilon*), ECB is the most damaging internationally. In North America, the moth is estimated to cost in excess of $1 billion annually from direct losses and the cost of control measures (Shelton *et al.*, 2002). The extensive uptake and planting of *Bt*-corn in the USA has prompted more detailed appraisals of the effects on insecticide use. It is interesting to note that most growers do not use insecticides to manage ECB, despite the losses caused by the moth. Data from field use indicate that from 1995, prior to commercial planting of *Bt*-corn, to 1999 the use of five insecticides recommended for ECB had declined by 1.5% (≡ 400,000 ha) (Carpenter & Gianessi, 2001). The number of users reducing the quantity of insecticide used directly as a result of switching to *Bt*-corn rose from around 13% to 26% during a three year survey of corn producers from six US States (Hellmich *et al.*, 2000). In relation to the premium paid by farmers for the *Bt*-corn seed, it has been estimated that it is only when ECB populations are high that the full economic benefits of the new technology are apparent; this was estimated to have been the case in 10 out of 13 years between 1986 and 1998 (Carpenter & Gianessi, 2001).

The comprehensive review by Shelton *et al* (2002) provides further cost-benefit assessments of the value of growing other transgenic *Bt*-crops, including cotton (generally positive economic and environmental benefits, through reduced pesticide usage) and potato (small area and limited reduction in pesticides, but still a significant positive benefit). Assessments of the influences of *Bt*-crops on the environment, including non-target organisms and other crops were also included in the review. In relation to the main purpose of the current review, Shelton *et al* conclude that "although the data to date do not indicate striking problems with Bt proteins in the soil, they point out the difficulty in working in a complex soil system. Studies often focus on single organisms under specific environmental conditions and over an often short period of time. Under these conditions the power to test for differences is relatively low, and longer-term and more complex studies are needed to ensure the integrity of important soil organisms". The complexity of the soil environment and the need to focus on the total risk, rather than a potential hazard is paramount in assessing the impacts of *Bt* entering the soil. This will be discussed in detail in Chapters 4 and 5.

3.4.1 Expression within the host plant

Levels of expression that provide the prospect of 100% insect mortality for the principal pests attacking a particular crop has been one of the main aims of research, particularly in truncation of the *Bt* genes and selection of the promoters being employed to carry out the insertion into the engineered plants. Apart from the constitutive promoters and whole plant expression achieved with CaMV35S, there are examples of more tissue-specific expression that are linked to the feeding habits of particular target pests.

3.4.1.1 Leaves

Expression in this zone targets pests that preferentially consume leaves, either or both as larvae and adults. Even though the leaf is regarded as the target zone for expression, the end result may be achieved by constitutive expression, provided that sufficient toxin is expressed to cause acceptable mortality in the target host.

Initial integration of toxin genes concentrated on Cry1 and Cry 3 and the first commercially available crops, registered in 1995 and 1996, were based on these two genes. Cry3A was incorporated into potato to protect against Colorado potato beetle, while Cry1Ab and Cry1Ac were engineered into corn and cotton, respectively, to protect against lepidopteran pests. By this stage in technology, the levels of expression were sufficient to provide protection comparable with conventional methods of managing pests on the selected crops (Estruch *et al.*, 1997; Briggs & Koziel, 1998). More recently, other Cry genes have been incorporated and expressed in the leaves of major crop plants. Cry9Aa2 has a 129 kDa protein active against Lepidoptera and has been incorporated into tobacco for control of the larvae of potato tuber moth, *Phthorimaea operculella* (Gleave *et al.*, 1998). The full toxin was truncated at the N-terminal end to remove motifs that prevented full transcription and stability in the plants, along with alteration of the codons to match more closely the codons of dicotyledenous plants. Various permutations of the mutagenesis were carried out, with progressive removal of nucleotides at the 5' end prior to incorporation into tobacco. Mortality of *P. operculella* was greatest when the 5'-terminal 693 nucleotides were modified compared with nil mortality with the wild type Cry9Aa2 gene. This study further confirmed the positive gains arising from improved stability of transcripts after specific modification of the toxin gene.

There has been considerable interest in the prospects of overcoming insect resistance to *Bt* that has developed in diamondback moth. Use of specific *Bt* genes and their expression precisely at the points of feeding are proving to be potentially valuable in re-establishing the bacterium as a viable pest management tool against this important pest. Transformation of cabbage plants (*Brassica oleracea* var *capitata*) with a synthetic Cry1Ab3 gene using a wound-inducible promoter (soybean vspB) proved to be effective against *P. xylostella* (Jin *et al.*, 2000). Tests with the full Cry11a3 gene did not indicate any toxicity towards the moth.

As a staple crop in many countries of the world, there is enormous interest in protecting rice, *Oryza sativa* against various pests and, hence, there have been a number of papers on incorporation of *Bt* into rice plants. The main target pests in rice are striped stem borer, *Chilo suppressalis* and yellow stem borer, *Scirpophaga incertulas* and attempts have been made to introduce a range of Cry genes to protect against foliar attack by these pests. For example, use of plant codon optimized Cry1Ab and Cry1Ac under control of maize ubiquitin, CAMV35S and Brassica Bp10 gene promoters produced high levels of tissue-specific expression of the toxins (Cheng *et al.*, 1998). Expression levels of up to 3% soluble protein were achieved, leading to between 97 and 100% mortality of the two main rice pests within 5 days. Similar wound-inducible expression was demonstrated by Breitler *et al* (2001). In this study, truncated Cry1B was fused to the -689 to +197 (C1) fragment of the maize proteinase inhibitor (mpi) gene and inserted into rice plants. Mechanical wounding produced Cry1B expression to reach up to 0.2% of total soluble proteins in leaves

within 12-16 hours of the damage. It was also demonstrated that there was no activity in pollen and seeds. Bioassays revealed that 100% mortality of second instar striped stem borer larvae feeding on leaves was achieved.

3.4.1.2 Stem

Constitutive promoters, such as CaMV35S, will result in expression in virtually all parts of the plant, including the stem. This is certainly the case for rice where stem-borers are vulnerable both during leaf feeding (see 3.4.1.1) and after they have entered the stem. Studies using constitutive (CaMV35S and Actin-1 from rice) and tissue-specific (pith tissue and PEPC) promoters to introduce Cry1Ab toxin into rice gave high expression in leaves and stem, especially with the CaMV35S and Actin-1 promoters (Datta *et al.*, 1998). Results from the tissue specific promoters indicated that differential expression, avoiding seeds could be achieved. Bioassays also confirmed that, at least for the constitutive expression, there was 100% mortality of yellow stem borer, *Scirpophaga incertula*. There have been many other studies of transformation of rice to include expression in both leaves and stems and indicating considerable promise for management of the majority of stem borers (Arencibia *et al.*, 1997; Ghareyazie *et al.*, 1997; Nayak *et al.*, 1997; Alam *et al.*, 1998; Cheng *et al.*, 1998; Alinia *et al.*, 2000; Ye *et al.*, 2001). Recently, research has concentrated on expression of combinations of Cry genes (Cry1Ac and Cry2A) and the snowdrop lectin (gna) (Maqbool *et al.*, 2001). Transformation to give single or combination expression of the genes gave up to 2.5% of total soluble protein (for the combination of genes). This strategy gave a higher level of protection against rice leaf folder, *Cnaphalocrocis medinalis*, yellow stem borer, *Scirpophaga incertulas* and brown plant hopper, *Nilaparvata lugens*, the latter only being reduced by 25% compared with 100% for the other two pests. This approach offers prospects for protecting against a range of pests and is also regarded as a viable long-term strategy to reduce the likelihood of resistance developing in the target pests.

3.4.1.3 Plant reproductive tissue

Attention to expression of *Bt* in reproductive tissues of crop plants has concentrated principally on corn where protection of the tassels, silk and kernels are paramount in producing a saleable commodity. Thus, some of the commercial varieties of *Bt*-corn have been engineered to ensure that, apart from the leaves, there is adequate expression in the stages leading up to kernel formation (Dowd, 2001). In this study, it was noted that expression of *Bt* was high in the kernels and in silk tissue which, in some cases, led to fortuitous reductions in mycotoxin (fumonisins) as a result of lower attack levels from *Helicoverpa zea*. Crops, such as Novartis BT-11, have also been shown to provide protection in green tissues and, specifically, the reproductive tissues of corn (tassel, silk and kernel) (Burkness *et al.*, 2001). Field assessments of the protection against *Ostrinia nubilalis* and *H. zea* conferred by incorporation of Cry1Ab in event BT-11, indicated very high (99-100%) kill of the former species and up to 88% kill of the latter. Assessment of *Bt* hybrids indicated that increases in the proportion of marketable corn ears could be achieved with improved levels of expression in the plants.

3.4.2 Expression in pollen

Expression of *Bt* in the pollen of GM plants was noted in the early work on transformation of a range of crop plants. This was particularly the case for Monsanto Event 176 corn when it was reported that monarch butterflies, *Danaus plexippus*, that fed on milkweed plants in the vicinity were susceptible to transgenic *Bt*-pollen in the larval stage (Losey *et al.*, 1999; Jesse & Obrycki, 2000). Event 176 produces relatively large quantities of *Bt* in corn, consequent on the use of a pollen-specific promoter (Koziel *et al.*, 1993). Comparison of the levels of expression in different tissues in a range of hybrids, including Event 176, indicated that pollen produced as high, or higher, amounts of *Bt* per g fresh weight as the leaves, especially at anthesis (pollen shed) (Bell & Whitmore, 1997). Comparative field studies with event BT-11 and Event-176 did not indicate any significant difference in mortality of *O. nubilalis* in adjacent rows of non-*Bt* corn (Pilcher *et al.*, 2001). This was not surprising for BT-11 where there is no pollen-specific promoter but it also indicated that pollen with high levels of expression appeared to have no effect, at least on a direct pest of corn, in a field situation.

3.5 Expression in roots

Although not usually mentioned specifically, it can be assumed that plants engineered to express *Bt* toxins constitutively or even by wound induction will have measurable quantities of the toxin in their roots. For example, Monsanto Event 176 corn expressed 80 ng/g of Cry1Ab fresh weight of root compared with up to 300 ng/g in leaves (Plant Health and Production Division, 2001b). Another transgenic corn crop, DBT418, using Cry1Ac against European corn borer (Dekalb Genetics) expresses 58-125 ng/g dry weight of the toxin in roots compared with 460-1195 ng/g in leaves (Plant Health and Production Division, 2001a). The results of research carried out by Stotzky and co-workers, which is the main subject of this review and is discussed in more detail in 3.6, also provides direct and indirect evidence for expression of *Bt* in roots (Stotzky, 2002b). It would appear, however, that engineering specifically to express high levels of *Bt* in the roots in order to combat root feeding pests, such as cutworms (Lepidoptera: Noctuidae), has not been carried out.

3.6 Bt *in root exudates*

3.6.1 Background

Complexity and the difficulty of extrapolating from laboratory microcosm studies to field situations make it difficult to interpret patterns in root exudation and interaction with the rhizosphere and bulk soil. Clearly, this is an important part of assessing the potential consequences of *Bt* toxins delivered to the soil by root exudation and by other means, such as degradation of *Bt*-plants and application of conventional *Bt* sprays. Factors, such as quantitative and qualitative rates of exudation with plant age are under-researched. For example, it is not known whether the *Bt* demonstrated to be

present in root exudates (Stotzky, 2002b) is part of water-soluble passive exudation or whether it is higher molecular weight secretion dependent on metabolic processes to take place. Investigations on amino-acid efflux and influx in corn by Jones & Darrah (1994) indicated that efflux of proteinaceous amino acids and sugars is a passive process, whereas resorption of amino acids is by active transport. Non-proteinaceous amino-acids (phytosiderophores) and organic acids are released by active transport mechanisms. Most amino acid exudation takes place near the root tip. Factors such as these may help to predict the true role of exudation of *Bt* but, as indicated in Chapter 4.3.2, the quantitative contribution of root exudates may be relatively insignificant compared with other pathways of entry of *Bt* to soil.

3.6.2 Studies of *Bt* exudation by roots

The finding that root exudates of transgenic corn, engineered to express truncated Cry1Ab, contained significant quantities of *Bt* was first reported by Saxena *et al* (1999). This initial report indicated that in hydroponic culture, where the roots were not in contact with soil and, therefore, were unlikely to be damaged during the course of experimentation, *Bt* was present in the hydroponic solution and was still toxic to larvae. The 66 kD Cry1Ab protein was confirmed by SDS-PAGE. Confirmation of the potential role of root exudation of *Bt* was provided by assessment of soil from the rhizosphere of seedlings that had been grown in either sterile or non-sterile soil. Both immunological and bioassay tests confirmed the presence of *Bt* with high levels of toxicity to tobacco hornworm, *M. sexta*, larvae. The authors expressed some concerns about possible effects of the addition of truncated *Bt* to soil and have since carried out a considerable amount of work on the interactions between root exudate *Bt*, soil composition, rates of degradation and impacts on non-target organisms. This has been comprehensively reviewed, along with a broader assessment of the inter-relationships between *Bt*, regardless of source, and the soil environment (Stotzky, 2002b). In recognition of the purpose of the current review, the work of Stotzky and his co-workers will be described in some detail.

A summary, based on a list of key findings provided by Stotzky (2002b) and later additions of *in press* data and discussion with Professor Stotzky and Dr Saxena, is provided in Table 3.3 and is described in this section.

Table 3.3: Summary of work on interactions between *Bt* and soil carried out by Professor G. Stotzky and co-researchers, with particular reference to exudation of *Bt* from the roots of *Bt*-crops.

Finding	Inter-relationship to expression of *Bt* in plants	References
Bt toxins (Cry1 & Cry3 complexes) bind rapidly and tightly to clays, humic acids and clay-humic acid complexes and is pH dependent	Solubilised protoxin to 66 kD toxin fragment (analogous to that expressed in transgenic plants)	(Venkateswerlu & Stotzky, 1992; Tapp *et al.*, 1994)
Bt-clay/soil complexes retain structure, immunological characteristics and toxicity	Applies both to wild-type and transgenically derived *Bt* toxins (full and truncated)	(Tapp & Stotzky, 1995; Koskella & Stotzky, 1997; Crecchio & Stotzky, 1998)
Binding leads to slower biodegradation than unbound toxin;	Free toxins readily mineralized by microbial	(Crecchio & Stotzky, 2001)

Finding	Inter-relationship to expression of *Bt* in plants	References
latter linked particularly to microbial utilisation of carbon.	utilisation of C and N	
Binding is relatively long term with Cry1 still detectable after 234 days (longest period studied)	Declines steadily but still present and active	(Tapp & Stotzky, 1998)
Toxicity retained longer in acid soils (lower antagonistic microbial activity)	Interaction with microbial activity	(Stotzky, 1986; Tapp & Stotzky, 1998)
Soil physical conditions had little effect on persistence; aerobic/anaerobic, wet/dry or freeze/thaw cycles	Binding is only slightly affected by physical alteration of soil	(Koskella & Stotzky, 1997)
Transgenic corn (Cry1Ab) debris incorporated into soil reduced metabolic activity (CO_2 production) and affected enzyme activity	Cry1Ab or isogenic corn leaves and stems added to soil +/- glucose +/- NH_4NO_3	Flores, Saxena & Stotzky unpublished (cited in Stotzky (Stotzky, 2000; Stotzky, 2002b))
Cry1Ab toxin is released in root exudates and persists in hydroponic culture and in soil	Initial results with event Bt11 (NK4640bt)	(Saxena *et al.*, 1999; Saxena & Stotzky, 2000)
Bt is present in exudates from 12 *Bt*-hybrids, confirming that phenomenon is not restricted to NK4640Bt hybrid (event Bt11)	Three transformation events; Bt11, 176, MON810	(Saxena *et al.*, 2002a)
Root exudates containing Cry1Ab have no effect on earthworms, nematodes, protozoa, bacteria and fungi	Cry1Ab from *Bt*-corn (NK4640bt) added to soil as exudates or as dried plant material. Test organisms added and assessed again after 40 or 45 days exposure.	(Saxena & Stotzky, 2001a)
Transgenic corn has higher lignin content than normal corn, implying a physiological trade-off between toxicity and growth	Events Bt11 and MON810 examined for lignin content (microscopy and chemical tests)	(Saxena & Stotzky, 2001b)
Bt from exudates is not taken up by corn, carrot, radish and turnip plants grown in soil previously planted with *Bt*-corn or amended with residues from *Bt*-corn plants	Immunological tests and bioassays did not detect any Cry1Ab presence in the plants	(Saxena & Stotzky, 2001c; Saxena & Stotzky, 2002)
Vertical movement of *Bt* in soils depends on the clay content and is greatest in soils with the lowest clay concentration	Both root exudate *Bt* and incorporation of *Bt*-corn debris gave same result. Strong binding, especially to higher concentrations of montmorillonite	(Saxena *et al.*, 2002b)
Cry1Ab is not present in exudates from *Bt*-cotton, *Bt*-canola (oilseed rape) and *Bt*-tobacco but Cry3A is present in the exudates from *Bt*-potato	Links to proximity of endoplasmic reticulum to plasma membrane of root cells	(Stotzky, 2002a)

The strong and rapid binding to soil components of spores and crystals of *Bt* has already been described (2.2.3.2). This is a well documented phenomenon and is further supported by the increasing diversity of *Bt* strains being found in virtually all locations where surveys have been carried out. Binding and retention of spores and crystals, both protoxin and toxin, is linked to the presence of surface-active particles, primarily clays and humic acids (Venkateswerlu & Stotzky, 1992). When protoxins (132 kDa) and toxins (solubilised and cleaved to the 66 kDa portion for Cry1Ab and

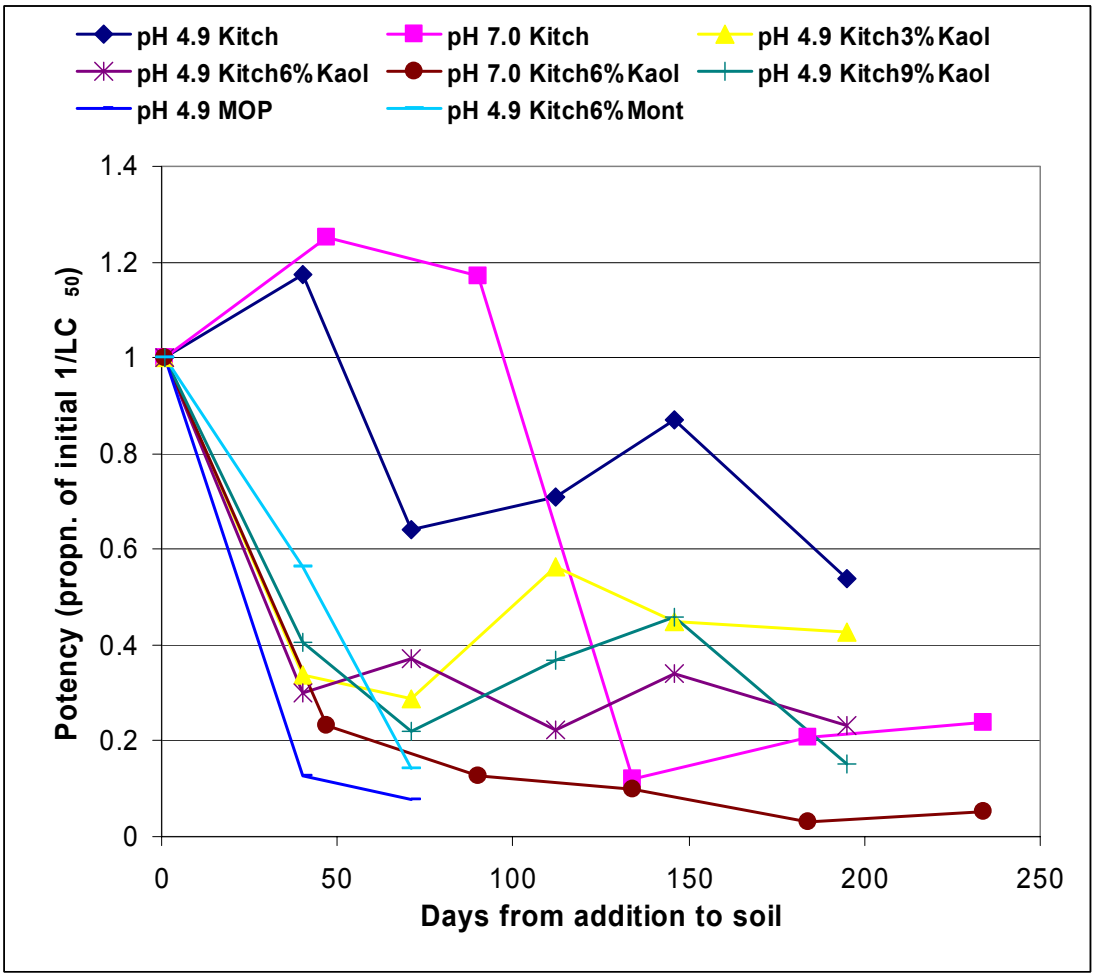
to the 68 kDa portion for Cry3A) were added to soils, adsorption to the clay minerals, montmorillonite and kaolinite, took place in less than 30 min, the shortest period studied. Adsorption of toxin was approximately twice that of protoxin for montmorillonite and three-times for kaolinite (probably linked to molecular mass of the smaller toxin) and, in all cases, adsorption to montmorillonite was greater (3x for toxin and 5x for protoxin) than kaolinite. Similar data were provided by Sundaram (1996) using solubilised *Btk* added to sandy or clay loams and where maximum adsorption was reached in up to 4 hours. Other factors, such as pH (Tapp *et al.*, 1994), moisture content and porosity (linked to the degree of aggregation of clay and other components (Hattori & Hattori, 1976; Hattori & Hattori, 1993) have a compounding effect on total retention. In particular, the porosity of soil components has a major effect on the degree of protection of bound toxins in relation to the activities of mineralizing bacteria and fungi. For example, there has been considerable study on the fate of genetically modified microorganisms, especially *Pseudomonas* spp. as model systems, in soil (for review see Dighton *et al* (1997)). Studies of *Pseudomonas fluorescens* modified to contain genes for bioluminescence and antibiotic resistance, indicated that pore size was critical in providing protection from the protozoan *Colpoda steinii* (Wright *et al.*, 1995). Soils with smaller pores (< 6µm neck diameter) provided the greatest protection against predatory activity by *C. steinii*. Other factors affecting retention of *Bt* included lower adsorption in soils with increased valency of cations and maximal adsorption of toxins at pH 6-8 on clean clays and pH 5-9 on dirty clays (coated with polymeric oxyhydroxides of iron to be more representative of field clay soils) (Venkateswerlu & Stotzky, 1992; Stotzky, 2002b).

Retention of the toxin, demonstrated both immunologically and by bioassay, was relatively long-term (at least 234 days in laboratory studies (Tapp & Stotzky, 1998)) and there was little vertical or horizontal movement in undisturbed soil (Saxena *et al.*, 2002b). The rate of decay of the bound toxins varied with both pH and with the nature of the soil clay fractions. This is illustrated for toxicity to *Manduca sexta* larvae in standardised bioassays in Figure 3.2 drawn from summary data in Tapp & Stotzky (1998) and Stotzky (2002b).

Retention was greatest in kaolinite rich soil (pH 4.9) compared with montmorillonite soil (pH 5.8 -7.3) and when the pH of the K soil was adjusted to pH 7.0 (closer to M-rich soil) retention decreased. As indicated in Figure 3.2, relative retention was greatest for normal K soil (around 54% of the original LC_{50} (concentration of toxin leading to 50% mortality in test insects)) and least for K6K adjusted to pH 7.0 (3-5% of the original LC_{50}). By contrast, montmorillonite soil (natural – MOP or enriched with M – K6M) lost activity relatively quickly to reach < 10% in 71 days in the unamended MOP soil. These data indicate that toxicity can be retained for at least 234 days but at declining rates determined by a complex interaction between soil structure and pH. Data of this nature can be used to aid prediction of the likelihood of retention of *Bt* toxins in soil and should be linked to other factors, such as degree of water percolation and possible run-off, in assessing movement of applied *Bt* in the soil ecosystem.

Interactions between any applied or natural *Bt* and soil microbiota depends on the degree of binding (i.e. the quantity of toxin retained after initial adsorption and any possible losses due to water percolation and microbial antagonism at the unbound interface) and, therefore, surface exposure to soil microbiota is an important

Figure 3.2: Rates of retention/decay of *Bt* toxin added to Mopala (MOP – montmorillonite dominant) or Kitchawan (Kitch – kaolinite dominant) soils with pH adjusted and addition of 3%, 6% or 9% of kaolinite or montmorillonite (6% only). Presence of *Bt* was determined by standardised bioassay using larvae of *Manduca sexta* (adapted from Tapp & Stotzky (1998) and Stotzky (2002b).



determinant of survival. Koskella & Stotzky (1997) addressed this aspect in studies of toxin utilization by a range of soil microbes (see Chapter 2.2.3.2 for more detail) and showed that bound toxins were not utilized as sources of C and only slightly as sources of N, irrespective of the nature (K or M homoionic to Na, Ca or Al) of the soil.

The fact that *Bt* spores and toxins bind to soil components and, thereby, retain activity for long periods has been known for many years (Pruett *et al.*, 1980; Meadows, 1993). However, the demonstration that root exudates from *Bt*-corn contained significant concentrations of truncated toxin presented a new pathway and form for delivery of the toxin to soil (Saxena *et al.*, 1999). The implications of this are discussed fully in Chapter 4, where the ecological interactions between different sources of *Bt* and organisms in the environment are considered. In summary, the initial studies leading to discovery that *Bt* was present in exudates arose from research carried out on the fate of *Bt*-corn in soil by Stotzky and co-workers. *Bt*-corn plants grown in hydroponic culture, which reduced the likelihood that damaged roots could be acting as a source of *Bt*, indicated that significant quantities of the toxin were exuded and these retained

toxicity to test larvae (Saxena & Stotzky, 2000). The toxin was shown to be the 66 kDa fragment of Cry1Ab that had been engineered and expressed in the corn plants. Experiments carried out over a period of 25 days indicated that, at the end of the period, *Bt* toxin was degraded by microbial proteases coinciding with the phase when the hydroponic culture began to support growth of microbial contaminants (i.e. non-sterile). Toxin was still detectable after 25 days in both sterile and non-sterile soil, confirming the earlier findings that toxin is strongly adsorbed to surface-active particles in soil and, once bound, is protected from microbial degradation.

Further studies of root exudation were carried out using solids amended with montmorillonite or kaolinite at 3, 6, 9 or 12% by volume (a similar series to those illustrated in Figure 3.2) (Saxena & Stotzky, 2000). Seeds of *Bt*-corn (Event Bt11, NK4640Bt) and the isogenic non-*Bt* corn were grown in batches of non-sterile amended soil and also in a sandy loam soil in the field. Samples from the soil of laboratory grown plants were assessed at 10, 20, 30 and 40 days post planting, whereas soil from the field-grown corn was sampled initially after corn had produced ears and also several months after the plants had died. All samples of rhizosphere soil from *Bt*-corn were positive for the toxin by immunological and bioassay testing using standard dosage-mortality tests developed by Saxena & Stotzky (2000). Both mortality of test larvae and, for the survivors, significantly reduced weights were noted, especially for the soils amended with montmorillonite. This finding was thought to correlate with the fact that montmorillonite has a greater cation-exchange capacity and specific surface area than kaolinite and, hence, retained more of the *Bt* root exudates, particularly during the early stages of plant growth. Significantly, although the M-amended soil resulted in more rapid adsorption than K-amended soil, the latter eventually accumulated similar quantities of toxin and both resulted in 100% mortality in test larvae.

These data clearly represent an extreme case of accumulation of *Bt* via root exudates because the plants were grown in only 15 g of soil and were not subject to normal cycles of rain and other field biophysical influences. Nevertheless, they are significant in indicating that toxins from root exudates accumulate in the immediate rhizosphere when leaching from the zone is limited. *Bt*-corn grown in the field also gave rise to significant mortality in test larvae, but was highly variable (38-100%), a result that would be expected in relation to the greater physico-chemical variability of natural field soil. *Bt* was still present and gave rise to high mortality (75-88 %) in soil below *Bt*-corn that had died and where the soil had been subject to frost. In this case, it would not have been possible to distinguish between *Bt* entering the soil by root exudation and by degradation of the dead plant tissues. However, in the overall assessment of potential impacts to soil-dwelling organisms, the net effect is likely to be similar, regardless of the precise pathway of entry to the soil. Immunological tests carried out by Saxena & Stotzky (Saxena & Stotzky, 2000) confirmed that the samples were the truncated 66 kDa toxic cores from Cry1Ab expressed by the corn plants. Vertical movement of *Bt* through soil profiles confirmed that the toxin was bound rapidly and, depending on the clay content of the soil, between 16% (high clay content) and 76% (low clay content) was leached through soil columns (2002b). These data provide valuable information on rates of movement of *Bt* toxin through soil and have been used to aid quantitative evaluation of toxin loads in soil in Chapter 4.4.1.3.

Assessment of exudation from other corn events confirmed that the phenomenon occurred in 12 different transformations, representing three *Bt* events, Bt11, Event 176 and MON 810, in both laboratory and field tests (Saxena *et al.*, 2002a). Extension of the work to other transformed plant species provided data on exudation in cotton, canola (oilseed rape), potato and tobacco (Stotzky, 2002a). There was no release of Cry1Ac from the roots of cotton, canola and tobacco, but Cry3A was released from the roots of potato, confirmed by both immunological tests and bioassays. Mortality in the bioassay of potato exudates was 35% and 38% in soil and hydroponic culture, respectively. The authors speculated on the reason for the lack of exudation of *Bt* from cotton, canola and tobacco and attributed the result to the fact that the endoplasmic reticulum is not so intimately associated with the root plasma membrane in these genera, whereas it is in corn and potato. This would aid active transfer of the large 66 kDa truncated *Bt* toxin. Further work is being carried out to investigate this finding, which would aid further risk assessment for other plant transformations.

Root exudation is normally a two-way event, allowing both egress and ingress of a range of molecules and water through the roots (see next section) and there has been some concern that other crops grown in soil previously used to grow *Bt*-crops could take up the toxin during growth. Saxena & Stotzky (2002) investigated this by growing corn, carrot (*Daucus carota*), radish (*Raphanus sativus*) and turnip (*Brassica rapa*) in either hydroponic culture (for corn) or soil in which *Bt*-corn had been grown previously. Presence of root exudate derived *Bt* was demonstrated in the growing media of all plants, but none took up *Bt* as indicated by immunological and bioassay testing.

In relation to potential impacts of root exudate derived *Bt*, both the persistence and the availability of the toxin are important. Studies of persistence have already been described and further data on this aspect were provided in assessment of the rate of decay of plant debris. *Bt*-corn was shown to have higher lignin content in the stem than isogenic corn (33% to 97% higher), with event Bt11 being higher than event MON 810 (Saxena & Stotzky, 2001b). Other studies of lignin content provided different results. Stotzky (2002b) quoted work by Faust (unpublished conference proceedings) that indicated no difference in lignin content. Foliar lignin content was found to be lower than transgenic corn in studies of Cry1Ab corn in relation to more rapid degradation by the woodlouse *Porcellio scaber* (Isopoda) (Escher *et al.*, 2000). The latter study also indicated a higher content of soluble carbohydrates in the leaves, which was thought to contribute to the greater nutritional value of transgenic corn to *P. scaber*. Such data are not incompatible with the findings of Saxena & Stotzky because it is possible that the *Bt*-corn is trading off carbon for growth against carbon for storage. However, this requires further investigation to determine the impacts on both agronomic performance and on prospects for longer term persistence of *Bt* corn in soil.

Effects on soil organisms have also been addressed specifically by Saxena & Stotzky (2001a). Laboratory assays with earthworms (*Lumbricus terrestris*), nematodes and culturable protozoa, fungi and bacteria, including actinomycetes, were carried out in soil that contained both root exudate *Bt* and debris from *Bt*-corn. There were no significant differences in mortality or weight of earthworms or in total presence of the other soil organisms. Toxin was present in worm casts, indicating that truncated *Bt*

passed through the digestive tract without affecting the worms and was completely voided by 3 days after they were removed from a *Bt* source.

3.7 Root exudation of Bt *and interaction with the rhizosphere*

Exudation of *Bt* from the roots of corn and potato, but not from cotton, canola (rape) and tobacco indicates that the phenomenon is not universal among transgenic crop plants (Stotzky, 2002a). Even though research on *Bt* corn is the main subject of this review, the process of exudation in this crop, particularly information on whether it is active, requiring a "signal peptide", or passive is uncertain. However, the work of Stotzky and his colleagues has indicated that this route of entry of *Bt* to soil should be considered among the several pathways that contribute to presence of the toxins in soil.

Rates of production of *Bt* toxin will vary with the growth stage of the plant and the fate of the exudates will be determined mainly by adsorption in the rhizosphere itself. Definition of the rhizosphere is vague and, over the years, has extended from a very narrow zone around the root, characterised by intense bacterial activity, to the current accepted meaning, which is "the zone of soil surrounding the root which is affected by it" (Darrah, 1993). Accompanying this widening of the term, the zone of influence of the rhizosphere has extended from 1-2 mm to reach several centimetres, depending on the nature of the substances or processes affected by root activity. There is no doubt that the rhizosphere is different to bulk soil, particularly in relation to the concentrations of nutrients and secondary chemicals that, in turn, influence a wide range of soil organisms. Among the many sources of information on the subject, there is consensus on the fact that microorganism presence and activity is much greater in the rhizosphere zone whereas larger organisms tend to be located in both rhizosphere and, especially, bulk soil. Table 4.5 in Chapter 4.4.1.3 provides a summary of the main groupings of soil organisms in the rhizosphere and bulk soil, with indications of the densities recorded during surveys for their presence.

It has frequently been pointed out that it is difficult to determine precisely both the physical and chemical interactions of roots with soil because any disturbance during sampling may actually influence the observed structures and functions. Corn, which is the main "model" for the current review has been well studied and there is now more confidence in description of the physical structure of the root system and this can aid interpretation of root exudation over time as the plant matures. An excellent review by McCully (1999) provides detailed descriptions of the root system of corn, which is summarised below.

The root system is divided into the seminal system, derived from the seed and maintained throughout the life of the plant, contributing about 20% of the total water supply, and the nodal system, which produces 40-70 axile roots developing from the stem. The latter system consists of roots developing from each node of the stem starting when the 3 leaf stage is reached and then at intervals of 2 leaves during corn development. Just before flowering, roots are produced from the younger nodes above ground and two tiers, termed prop roots, grow into the ground. The final two tiers (6 and 7) produce around 50% of the total and contribute 3 x more large xylem vessels than the rest of the root system. Water flow through mature roots is 8 times greater

than from the oldest nodal roots and 21 times more than in the primary root. Branch roots are usually ≤ 3 cm long and persist for the life of the crop, usually at a density of 7-12/cm, totalling 30 times the length of the main framework roots; they are the major site of water uptake. There are major differences in local pH at the root/soil interface between fine roots (pH around 1.5 units below bulk soil) and axile roots (pH about 1.5 units above bulk soil). Framework roots consist of a shiny white tip, up to 5 cm long, then a rhizosheath covered with soil (15-30+ cm long), the remainder being mainly free of soil. Exudation of mucilage in the outer two layers of cells at the flanks and tip of the root cap is credited with forming soil aggregates in the rhizosphere and are important in nutrient acquisition. An interesting finding from field observation is that, although the mucilage has the capacity to swell dramatically when in contact with water (e.g. in hydroponic culture), this does not take place in the field, even at field capacity (McCully & Sealey, 1996). Mucilage is only produced at the root tip and is retained as a sheath with cap cells in the soil as the root grows, forming a layer over the mature root surface. There is no evidence that mucilage is produced by more mature root cells. Root hairs grow through the rhizosheath, which is approximately the same thickness as the length of the hairs themselves.

The rhizosphere is, therefore, the site where the key processes affecting nutrient flow and availability to and from roots takes place. Mineralization of soil organic matter and solubilisation of minerals takes place in this zone but can be carbon limited (Grayston *et al.*, 1997). Quantities of carbon released through the roots of plants vary between annual and perennial plants but can constitute the largest element of carbon flux in these plants. This can be influenced strongly by the presence of vesicular arbuscular mycorrhizal (VAM) fungi. For example, corn plants grown with and without their normal VAM fungi affected shoot/root ratios and uptake of P, Zn and Cu in roots (Axaizeh *et al.*, 1995). However, the root exudates produced by the plants were similar, regardless of the presence or absence of VAM fungi (72-73% reducing sugars, 17-18% phenolics, 7% organic acids and 3% amino acids). When microorganisms associated with the rhizosphere were killed using antibiotics, the recovery of carbohydrates, phenolics and amino acids increased by up to six times, indicating that microbial activity in the zone of influence of the rhizosphere affects the utilisation of exudate components by the roots.

Exudates from the roots are important in stimulating microbial growth and activity as well as aiding the formation of soil aggregates around the roots. Grayston *et al* (1997) reviewed root exudation between perennial plants (trees) and annual plants (corn), making the point that carbon flux is key in the rhizosphere interaction and distinguishing several modes of carbon release to the soil:

- Water soluble exudates - low molecular weight substances exuded passively without the involvement of metabolic activity and takes place along a concentration gradient.
- Higher molecular weight secretions depending on metabolic processes for their release and which can take place against electrochemical and chemical potential gradients.
- Lysates - released from cells after autolysis and include the contents of sloughed-off cells and eventually the whole root.
- Gases e.g. ethylene, CO_2 and hydrogen cyanide, usually of low molecular weight.

- Mucilage - covers the roots of many plants and is composed mainly of insoluble polysaccharides and high molecular weight polygalacturonic acids. Under non-sterile conditions typically found in the field, the mucilage is a mixture of plant and microbial origin, and has been termed the mucigel.

Rates of carbon deposition in the rhizosphere vary from 10-60% in annual plants (e.g. *Zea mays*) to 60-73% in perennial plants such as *Pinus sylvestris* and *Pseudotsuga menziesii* (summarised in Grayston *et al* (1997)). Decomposition of the fine roots contributes a significant proportion of the carbon to the plants via the remaining root system and it has been estimated that a combination of death of fine roots and mycorrhizal associates recycles 2-5 times more organic matter to soil than aerial parts of plants (Fogel & Hunt, 1983). Root exudation is a common phenomenon in both annual and perennial plants and has an important function in both nutrient cycling and in protection and movement by growth of the vulnerable root tip through soil. The plant itself will be an important determinant of the qualitative and quantitative nature of exudation leading to great diversity in chemical composition of exudates. With specific reference to exudation from transgenic corn, it has been shown consistently that the amount of exudation in corn plants is greatest at the seedling stage and then tails off during later plant growth (Gransee & Wittenmayer, 2000). Using radioactive ^{14}C, it was demonstrated that sugars declined by about 50% from the 4 leaf to the 6 leaf stage of growth and by a further 50% to the 8 leaf stage. Amino acids declined by around 65% from 4 leaf to 6 leaf but remained at the same level thereafter. There were no differences in the quantities of carboxylic acids in relation to plant age. Other plants, such as trees, have similar profiles during early growth but can differ considerably in amino acid, amide and organic acid production during maturation.

Genotypic factors are clearly important in determining the composition of root exudates, but both the composition and the quantity of exudates are subject to environmental conditions that can make it extremely difficulty to interpret the significance of exudation. Experiments carried out in sterile conditions in hydroponic cultures, while they avoid problems of root damage, can lead to abnormal rates of exudation. For example, increases in mechanical impedance results in increased root exudation in corn, possibly as a result of mechanical damage (Schönwitz & Ziegler, 1982). However, re-adsorption of exudates can also be a significant factor such that corn plants exuded eight times less carbon when they were grown in continuous culture than when the culture media were changed daily (Jones & Darrah, 1993). Other factors include deficiencies in key nutrients, especially N, P and K, which might result in altered rates of exudation of amino acids, especially increased exudation and stimulation of mycorrhizal growth when P is lacking (Rousseau & Reid, 1990). Soil pH affects exudation directly by potentially altering the ionic balance of substances released and subsequently re-adsorbed. Loss of C from *Lolium perenne* was shown to increase 2.5 fold when pH was increased from 4.3 to 6.0 (Meharg & Killham, 1990). Conversely, the pH of the rhizosphere itself may be affected by exudates, such that a difference of more than 2 units has been noted between the rhizosphere and bulk soil (Marschner, 1991). Other co-varying factors include water availability (+ve and –ve effects on exudation), temperature (changes in temperature, rather than temperature *per se*, tend to lead to higher exudation (Vancura, 2002)) and oxygen availability (anoxia leads to increased exudation (Whipps & Lynch, 1986)).

Microorganisms in the rhizosphere zone have an important influence on the process of root exudation and can also be influenced themselves by the composition of the exudates. In general, the presence of microorganisms tends to increase root exudation, which has been demonstrated for both free-living microorganisms and for mycorrhizae (Bonkowski *et al.*, 2000). This is an area of considerable uncertainty and complexity because of the diverse nature of the soil fauna, particularly in the rhizosphere itself. Apart from nutrient utilization and mineralization by soil bacteria and fungi, the interaction with Protozoa is particularly important, because they are strong microbial grazers and are known to selectively decrease the numbers of gram-negative and to increase numbers of gram-positive bacteria (Griffiths *et al.*, 1999). Release of phytohormones by microbial activity is also important because of their effects on root cell permeability and hence rates of exudation (Bowen, 1994).

Studies of the interactions between abiotic factors, including cultivation procedures and soil fauna, has been studied as part of risk assessment for genetically modified microorganisms added to soil (Dighton *et al.*, 1997). The authors considered the movement of microorganisms through soil and reviewed information on the main groups (viruses, bacteria, actinomycetes and fungi) and their interaction both with the soil physico-chemical environment and with other soil fauna. In gathering data from a number of sources, the abundance of different soil fauna constituents was compared and it was shown that arable soil tended to contain the greatest densities of Protozoa (1-10 x 10^9 per m^2) and, to a less marked degree, Nematoda (1-10 x 10^6 per m^2) and Lumbricidae (earthworms) (30-300 per m^2). It was interesting to note that there was little information on other soil faunal groups from arable ecosystems even though these were clearly important and diverse components of the soil assemblages in other ecosystems, particularly deciduous forests (Jones & Darrah, 1993).

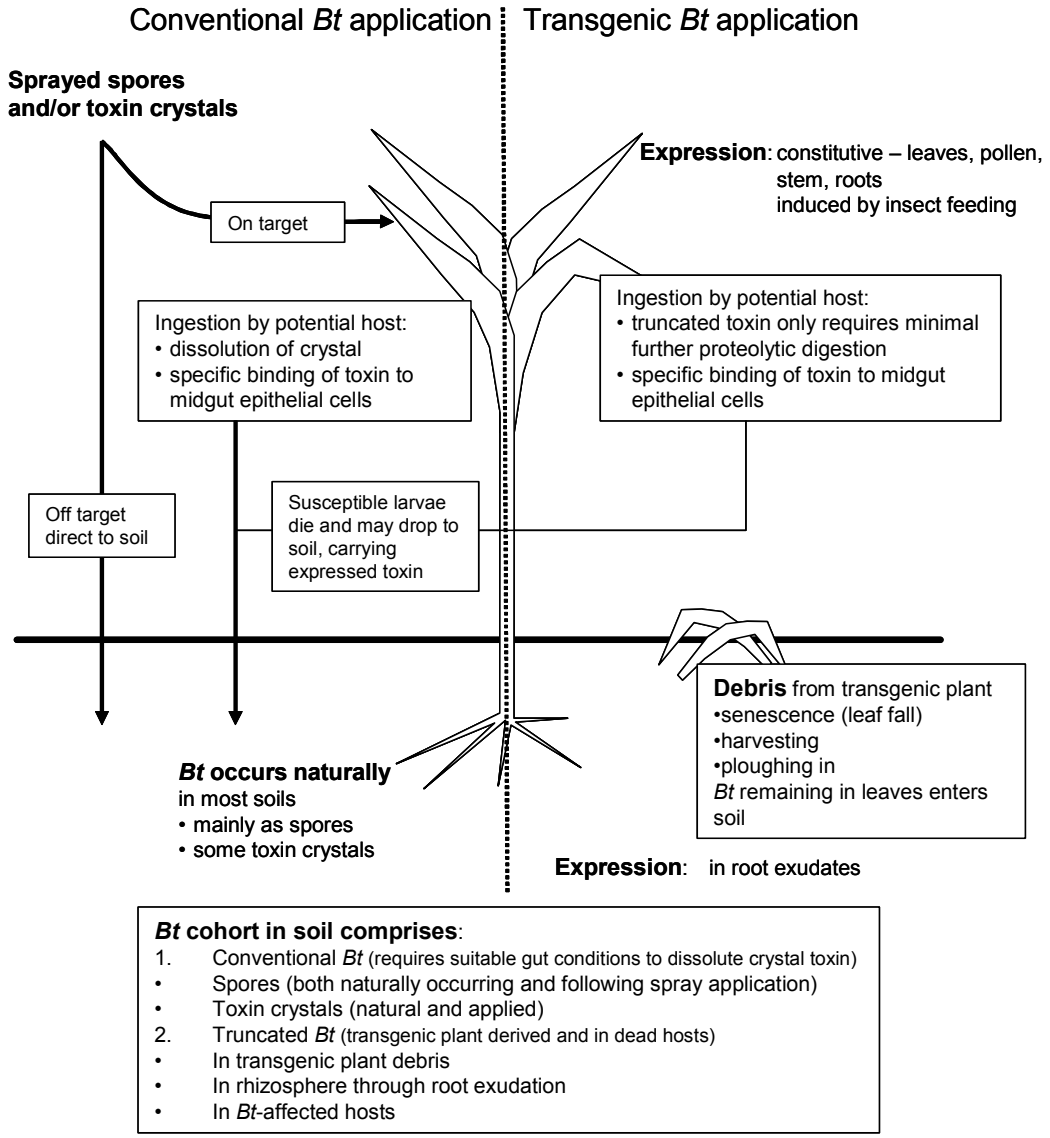
4 Interaction effects

The work of Stotzky and his co-workers has shown clearly, not only that root exudates from *Bt*-plants can accumulate in soil and persist in significant quantities, but also that the toxin is acting in an identical manner to solubilised and/or activated toxins entering the soil from other routes. In terms of potential impacts on soil dwelling organisms, at a range of trophic levels, the critical questions therefore relate to the process of encounter between the toxin and those organisms. In this context, the key components of the system must be identified and assessed in relation to how the toxin source might affect the outcome of any *Bt*-organism interaction.

4.1 The process of encounter between potential hosts and expressed Bt – introduction

Figure 4.1 illustrates schematically the principal components of *Bt* entry to the soil ecosystem, whether it is applied conventionally or through the transgenic plant route.

Figure 4.1: Conceptual representation of the routes of entry of *Bt* to soil from conventional and transgenic plant sources of the toxin.



Although it has been shown conclusively that *Bt* is already present in the great majority of soils, this route of encounter is not considered explicitly in Figure 4.1. In essence, this reflects the "base-line" for *Bt*-putative host encounter and a broad assumption is made, at this stage, that organisms present in soil are already exposed to *Bt* spores and to a much lesser extent, vegetative stages and crystals. This is an important consideration but, unfortunately, there is still no consensus on the true ecological role of *Bt* and, therefore, no means of assessing precisely the effects of adding toxins to an existing pool of inoculum/whole toxins.

The components of Figure 4.1 provide a framework for assessing risk in a step-wise manner that should enable the key issues to be identified and their **field** roles assessed. An evaluation of the likelihood of encounter between soil organisms and the *Bt* entering the soil is at the centre of any risk investigation and is discussed in more detail below. A number of authors have expressed concern about the fact that the *Bt* entering the environment after being engineered into crop plants is a truncated, "active" form of the toxin (Obrycki *et al.*, 2001; Hilbeck, 2002; Stotzky, 2002b). This is a valid concern in relation to the ultimate potential to induce toxicity, but in terms of risk assessment the concerns about direct entry of "active" toxin to the environment identify hazard rather than risk. Therefore, both the intrinsic hazard of the truncated toxin and the likelihood of that perceived hazard being realised (i.e. the risk) must be evaluated. In terms of encounter frequency, the ecological framework in Figure 4.1, although it distinguishes the different sources of *Bt*, represents a continuum that encompasses all methods of entry of *Bt* to the soil ecosystem.

4.2 Encounter between target and non-target organisms and wild type and truncated Bt

On the basis of existing assessments of the direct and indirect effects of *Bt* on target and non-target organisms, the likelihood of effects being realised is greatest in invertebrates, particularly insects. In relation to the crop that is the main subject of the current review and which represents the greatest area over time and space of any transgenic crop expressing *Bt*, the discussion below will concentrate on the fauna associated with corn and its immediate environment and will be used as a "model system" to consider potential environmental impacts.

Table 4.1 lists the main organisms that are directly or indirectly associated with corn in the North American corn belts, principally as consumers of both above and below ground parts of the plant.

Table 4.1: The main consumers of corn in North America

Consumer group	Order	Example	Part of crop consumed	Other information
Insects	Coleoptera	Wireworms e.g. *Conoderus falli* (Southern potato wirewom), *C. vespertinus* (Tobacco wireworm)	Roots, Seeds	Larvae of click beetles, attack corn root system through out the growing season. Feed on germinating seeds and attack underground stem and roots.
		Flea beetles e.g. *Chaetocnema pulicaria* (Corn flea beetle)	Foliage, Roots	Attack foliage and the larvae feed on roots of corn. However economic damage is primarily due to overwintering beetles carrying bacterial wilt of corn (Stewarts disease).
		Seedcorn beetles	Seed	Only adults cause economic injury, larvae predate on other insects and are regarded as beneficial
		Weevils e.g. *Spenophorus callosus* (Billbug)	Stalk	Adults feed on the tender inner tissue of seedling corn, and larvae develop in and around the underground portion of the stalk.
		Rootworms e.g. *Diabrotica undecimpunctata howardi* (Southern corn rootworm)	Roots, stem	Both adults and larvae feed on corn plants, but larvae are more destructive; they cause injury during late spring by chewing holes in growing points, killing terminal blades. Extensive feeding on roots may occur late in season.
	Coleoptera: Scarabaeidae	Grubs e.g. *Phyllophaga* sp. (White Grubs)	Roots and decaying matter	Immature larval stage of scarab beetles e.g. Japanese beetles, eggs hatch and larvae feed on plant root or decaying vegetation
	Diptera	Fly larvae e.g. *Hylema platura* (Seedcorn maggots)	Seed, Stem	Larvae of small flies that are attracted to germinating seeds, eggs hatch and larvae feed on germinating seed contents, and occasionally tunnel into seedling stems.
		Midge larvae e.g. *Contarina sorghicola* (sorghum midge)	Ovary	Larvae feed on the corn plant ovary, thereby preventing normal seed development.
	Hemiptera	Aphids e.g. *Rhopalosiphum maidis* (Corn leaf aphid)	Leaves, corn tassels, silks	Feeds on the leaves, the corn tassels and the silks of corn.
		Aphids e.g. *Amuraphis maidiradicis* (Corn root aphid)	Roots	Pierces roots and extracts sap
		Chinch bugs; *Blissus leucopterus leucopterus*	Stem	Pierces plant stem and sucks out sap, can cause lodging, dwarfing and yield reductions.

Consumer group	Order	Example	Part of crop consumed	Other information
	Lepidoptera	Cutworms e.g. *Agrotis ipsilon* (black cutworm)	Stalk, Roots, Underground stem	Feed both above and below ground on corn crops. Larvae sever plants near soil line, some feed on the roots and underground stems of cut plants.
		Webworms e.g. *Crambus* sp. (sod webworm)	Roots, Stalk	Feed both above and below ground on corn crops. Attack roots of seedling corn plants, and feed on stalk above or below soil surface.
		Stalkborer e.g. *Elasmopalpus lignosellus* (lesser cornstalk borers)	Stalk, Foliage	Bore into stalk above ground, and develop inside stalk, larvae feed on foliage when young.
		Armyworms e.g. *Pseudaletia unipuncta* (Armyworm)	Foliage, Stems	In corn, characteristically feed on the lower leaves and progress to the top of plants. Leaves of seedlings are completely eaten.
		Armyworms e.g. *Spodoptera frugiperda* (Fall Armyworm)	Whorl, Late pre-tassel	Usually attack late corn and cause damage to the whorl or pre-tassel.
		Earworms e.g. *Helicoverpa zea* (Corn earworm).	Foliage	Attacks foliage of corn plants.
		Corn borers e.g. *Ostrinia nubilalis* (European Corn Borer or ECB)	Whorl, leaf and stalk	Initial feeding occurs on the leaf surface, generally in the whorl; later larvae bore down the midribs of leaves into stalks. Major economic pest in some areas.
		Cornstalk borers e.g. *Diatraea crambidoides* (Southern cornstalk borer), *Elasmopalpus lignosellus* (Lesser cornstalk borer).	Whorl, Stalk	Young caterpillars feed within the plant whorl, larger caterpillars tunnel into stalk. There may be extensive tunnelling just above the soil level.
	Orthoptera	Grasshoppers e.g. *Melanophus* sp.	Leaf, Ear	Can cause extensive damage in large numbers to corn crops.
Mites	Acari: Prostigmata	Spider mites e.g. *Tetranychus urticae*, *T. cinnabarinus*	Leaves	Feed on underside of leaves by piercing the epidermis and extracting sap.
Nematodes	Nematoda	Species from nematode families such as: *Meloidogyne* (Root knot nematode), *Pratylenchus* (Lesion nematode), *Heterodera zea* (Corn cyst nematode), *Tylenchorhynus* (Stunt nematode) plus others.	Roots	Some species of nematodes are known to parasitise and feed on corn roots of living corn plants. Few species are agronomically important.
Gastropods	Mollusca	Slugs and snails.	Dead vegetation	Slugs and snails feed on rotting vegetation.

Consumer group	Order	Example	Part of crop consumed	Other information
Birds		Common crow (*Corvus brachyrhynchos*), blackbirds, grackles, gulls, housefinches, jays/bluejays, pigeons.	Seed, grain, ripening corn.	A range of bird species will feed on corn plants, the majority eating seed and grain, other such as crows will target newly sprouting grains.
Mammals		Racoons	Ripened ears	Racoons have been known to eat ripened corn ears.
Decomposers		Bacteria and Fungi	Roots, dead plant material	Bacteria and fungi are the fundamental decomposers, and about 90% of all material produced by plants is broken down by decomposers, therefore any remains of corn crops left or incorporated into the soil will ultimately be affected by this group.
Soil microfauna	Acari	Mites	Plant material in soil.	Mites feed on virtually everything in the soil environment, including plant material.
	Isopoda	Woodlice	Dead vegetation	Woodlice feed on dead plant material.
	Diplopoda	Millipedes	Dead vegetation	Millipedes feed on dead plant material.

In relation to the widespread use of transgenic corn expressing Cry1Ac and Cry1Ab (both primarily Lepidoptera-active, see Figure 2.1), likelihood of ingestion and susceptibility to the *Bt* toxin is greatest for the Lepidoptera in Table 4.1. The list is, of necessity, abbreviated and, actually represents only a very small proportion of the Lepidoptera associated with corn globally. Letourneau *et al* (2002) have comprehensively reviewed the benefits and risks from potential escape of transgenes in the wild and have employed the Natural History Museum, London HOSTS database (Robinson *et al.*, 2002). Lists of the lepidopteran species associated with corn (*Zea mays*) were supplied and have been summarised by family and by the maximum and minimum recorded host plants by families and genera in Appendix 3 (Chapter 8.2). This list includes a total of 382 species in 23 families of Lepidoptera and includes species with very wide host ranges.

Within each lepidopteran family there may be extremes of plant host range such that, in the largest family Noctuidae, which contains 166 species known to feed on corn, *Spodoptera litura* feeds on 166 plant genera in 69 families. At the other extreme, there are 12 species of Noctuidae that feed on 1 genus (*Z. mays*) only. Letourneau *et al* (2002) summarised the data from the HOSTS search and made the point that the species with recorded host ranges restricted to a single genus represent the extremes of the risk continuum in relation to potential outcrossing of the *Bt* genes from the manipulated crop plant to a wild relative. They identified 13 Lepidoptera species in 4 families that were at risk of extinction if *Bt* crops were able to cross successfully with the only known host of the listed species. Since many of the listed lepidopteran species were Nearctic in distribution, they would clearly be within the region where the greatest usage of transgenic corn has taken place. They also make the significant point that, for the Poaceae, three out of four species on non-crop relatives also feed on the crop plant, *Zea mays*. Extrapolating from this and also emphasising that the HOSTS database itself clearly under-represents both the total numbers of Lepidoptera globally and also the actual feeding patterns on many non-host plant species, the authors speculate that, overall, “40 to 100% of the herbivores recorded from the non-economic relatives of crop plants also feed on the crop plant species. The inference seems fair that a similar proportion of the species on the crop plant might feed on the non-crop relatives (given geographical feasibility) but sufficient data have simply not been recorded”.

4.2.1 The potential effects of *Bt* in the corn environment

4.2.1.1 Phylloplane and stem

Bt-corn is engineered mainly to target European corn borer, *O. nubilalis* which feeds initially on the leaves and later in the stalks, thus giving its common name. Other Lepidoptera feed in a similar way (leaves then stem) and are, therefore, subject to the same prospects of ingesting *Bt*. Armyworm and earworms tend to attack leaves or, in the case of *S. frugiperda*, the later stages of corn growth up to the pre-tassel stage. Table 4.2 provides a summary of recent research into the relative efficacies of several *Bt*-corn events in relation to damage by four of the principal corn pests in North America.

Table 4.2: Summary of the effects of various *Bt*-corn transformation events on four of the principal pests of corn in North America.

ECB	CEW	SWCB	FAW	Comments	Reference
✓	✓			Mon810, Bt11, CBH354: good control of corn borers in shanks and ears. Event 176: poor control. All poor for CEW control in kernels.	(Archer *et al.*, 2001)
	✓		✓	Mon810, Bt11 effective in reducing damage to kernels but depended on population density.	(Buntin *et al.*, 2001)
✓	✓			Bt11. ECB control: 99-100%; CEW control: 85-88%. Marketable ears: 98-100% for *Bt*-hybrids, 37-46% for isolines.	(Burkness *et al.*, 2001)
✓				Event 176: Analysis of diapausing larvae surviving exposure to Cry1Ab indicated no difference in fitness in subsequent generation.	(Siegfried *et al.*, 2001)
	✓			Mon810, Bt11: Mortality of larvae steady, but 15-40% survival to pupae, then further mortality to give overall reduction in adult emergence of 65-95%. Concerns that this sub-lethal effect would lead to resistance development in extensive field planting.	(Storer *et al.*, 2001)
✓		✓		Mon810, Bt11, 176, CBH354 (Cry9c): 1st generation of ECB and SWCB controlled by all. Event 176 did not protect against 2nd generation. No significant difference in yields from any of the *Bt*-hybrids.	(Archer *et al.*, 2000)
✓				Cry1Ab, Cry1Ac, Cry9C: Interaction with nitrogen fertilization. *Bt*-corn + manure (N-source) gave 19% and 7% greater yields in outbreak and endemic years, respectively.	(Singer *et al.*, 2000)

ECB	CEW	SWCB	FAW	Comments	Reference
✓				Bt11, Event 176, Mon810, Mon802 (all Cry1Ab), DBT418 (Cry1Ac) and CBH351 (Cry9C): infested with ECB at different corn developmental stages. Bt11, Mon810, Mon802 and CHB351 were effective, Event 176 efficacy declined in later corn development stages. Event DBT418 not effective in controlling late-instar ECB during vegetative or reproductive stages of development.	(Walker *et al.*, 2000)
	✓		✓	Bt11 (Cry1ab): high protection of leaves and silk to neonate CEW feeding. Negligible ear damage in the field. Moderate protection against FAW.	(Lynch *et al.*, 1999)

ECB: European Corn Borer, *Ostrinia nubilalis*
CEW: Corn Ear Worm, *Helicoverpa zea*
SWCB: South Western Corn Borer, *Diatraea grandilosella*
FAW: Fall Armyworm, *Spodoptera frugiperda*

As a typical example of the evaluation effort that is being put into assessing impacts of *Bt*-corn on the major pests, the recent work by Archer *et al* (2000) provides insights into efficacy for several *Bt* hybrids. In a study of 28 *Bt*-corn hybrids producing Cry1Ab (Mon810, Bt11, Event 176) or Cry9c (CBH354) the impacts on Southwestern corn borer, *Diatraea grandiosella* and European corn borer, *O. nubilalis* were evaluated in several geographic locations within the Texas Panhandle. After artificial infestation with each corn borer species at the mid-whorl (representing the first borer generation) and tassel (the second borer generation) stages, mortality and plant protection were recorded. All *Bt* hybrids protected against the first generation for both borer species, whereas the second generation borers were only controlled by Mon810, Bt11 and CBH354 events and not by event 176, which does not express *Bt* in the appropriate tissue. Further downstream protection against pests not included in Table 4.2 has also been demonstrated for some *Bt*-corn hybrids. In particular, the longer-term expression of *Bt* in harvested corn against Indian mealmoth, *Plodia interpunctella* and Angoumois grain moth, *Sitotroga cerealella* have been investigated. Giles *et al* (2000) compared survival, development time, adult body lengths and sex ratios of *P. interpunctella* reared on corn expressing Cry1Ab, Cry1Ac or Cry9C, compared with non-transgenic isolines. When Cry1Ab and Cry9C were expressed using the constitutive CaMV35S promoter, survival decreased and any adults emerging were smaller than non-*Bt* adults. When Cry1Ab was expressed with the PEPC promoter (green tissue specific) there were no significant differences in survival or growth parameters. Cry1Ac did not give any enhanced protection of kernels. The positive effects of Cry1Ab and Cry9C were retained up to 5 months after harvest.

Similar studies on the effects of Cry1Ab expression in corn on *P. interpunctella* and *S. cerealella* were carried out using whole corn or separated (cracked) corn by Sedlacek *et al* (2001). Both moth species had reduced emergence and fecundity when reared on corn derived from three events P33V08 anti N6800Bt, MON 810 and Bt11. The authors concluded that storage of *Bt*-corn grain may offer potential as a management tactic for suppression of *P. interpunctella* and *S. cerealella* without the use of chemical pesticides.

These data provide valuable insights into the longer-term persistence of transgenically expressed *Bt*, particularly when, as is the case in the stored product environment, there are limited environmental degradation factors present. Ultimately, however, the impacts of *Bt* corn can only be measured in terms of the field environmental attributes of the corn while it is still growing and, after harvest, during the degradation phase of any debris left on site. The additional factor of continuous expression of *Bt* and its delivery to the soil via root exudates is discussed in the next section.

4.2.2 Rhizosphere and surrounding soil

Table 4.1 includes several groups of organisms that are predominantly associated with the soil environment. Among these, the Lepidoptera such as cutworms (*Agrotis* spp.) and webworms (*Crambus* spp.) are most likely to be affected by use of the current commercial varieties of corn, all of which express the Lepidoptera-active Cry1 or Cry9 genes. Black cutworm (BCW), *Agrotis ipsilon* is a serious pest of corn and other crops (including all commercial *Bt* crops); it feeds on both stems and roots and moves between the soil and the lower parts of the crop. Mortality of BCW arising from feeding on *Bt*-corn 176 was, however, low regardless of whether the larvae fed on leaf or silk tissue in both laboratory and, particularly, field tests (Pilcher *et al.*, 1997b). The typical feeding pattern for this pest in the field was on leaves, by stalk cutting (usually at the soil interface) and by feeding on ear tips and there were no significant differences between *Bt*-corn and isogenic corn. These data were consistent with information on susceptibility to Cry1Ab confirming that *A. ipsilon* is not affected by this particular Cry gene (MacIntosh *et al.*, 1990), thus suggesting that specific engineering against pests with known susceptibility profiles will require incorporation of a cocktail of genes. The recent discovery of a toxicity factor termed Vip3A secreted by *Bt*-HD1 during vegetative growth and which resulted in enhanced toxicity of Cry1 against *A. ipsilon* (Donovan *et al.*, 2001) also shows promise as a potential component of specific engineering against secondary pests.

True soil dwelling organisms, including those in Table 4.1, will encounter *Bt* directly by feeding on roots of *Bt*-corn or will ingest/encounter toxin exuded directly from the roots or entering the soil with plant debris. Among the soil-associated Coleoptera, the wireworms, flea beetles and rootworms all feed on the roots. *A priori* the possibility of toxicity by the Coleoptera-active Cry3 genes that are currently being investigated for incorporation into transgenic corn is greatest with respect to impacts on target and, particularly, non-target soil Coleoptera. There is little information available on the effects of Cry3 *Bt* on Coleoptera encountering the toxin in soil and effects must be inferred from studies of systems other than transgenic corn. Studies of the effects of incorporation of Cry3A in the nodule forming bacterium *Rhizobium leguminosarum*

biovar *viciae* and tests of white clover and pea plants with nodules formed by the transgenic bacteria indicated some toxicity against the root weevil *Sitona flavescens* (Skot *et al.*, 1994). Tests of the effects of transgenic corn expressing new *Bt* toxins of 14 and 44 kDa size, indicated that western corn rootworm larvae (*Diabrotica virgifera virgifera*) were susceptible to the toxins in the roots (Moellenbeck *et al.*, 2001). There is, however, a gap in information on soil dwelling Coleoptera in relation to exposure to *Bt*.

4.3 Factors affecting the likelihood of expression and retention of lethal/sub-lethal dosages of Bt

Expression of *Bt* within the tissues of transgenic plants and the possible retention of toxin temporally and spatially can be regarded as a pool of inoculum for potential encounter with target and non-target organisms. As with all crop systems, the concept of a target host represents a fine line between ascribing pest status to the organism or designating it as a valuable component of the biodiversity of the ecosystem into which the *Bt* is introduced. The inoculum pool will impact on many of the organisms in the crop system, either directly or indirectly through, for example, removal of competition for other herbivores or removal of prey in the case of some natural enemies. Knowledge of the relative contributions of different sources of *Bt* and their statuses (full toxin, part activated or fully activated) are essential components in developing a rational risk assessment for as many organisms as possible.

4.3.1 Expression during the life of the transgenic plant – a whole life budget for *Bt* (with emphasis on the relative quantities from root exudates and expression in other tissues, debris, etc.)

Quantitative evaluation of the expression of *Bt* in transgenic plants has been carried out for most of the commercial crops already in use and this information provides a basis for comparison with the quantities of the bacterium applied conventionally and also with *Bt* already in the soil. However, the data are not always expressed in the same way and there is considerable inconsistency in methods of reporting (e.g. fresh weight or dry weight, percentage of total protein, a mixture of units, including pg, ng and μg). It is, therefore, difficult to determine a whole life budget for *Bt* in transgenic plants under field conditions. Nevertheless, available data, concentrating on corn, are summarised in Table 4.3, which includes a breakdown by tissue type and plant growth stage, if these are available.

Table 4.3: Expression of *Bt* in different tissues of transgenic crops, predominantly corn (expressed as µg/g of plant tissue).

Active Ingredient	Leaf	Root	Pollen	Pith/Stalk	Seed	Whole Plant
Cry1Ab Bt11	3.3	2.2-37.0	< 90	-	1.4	-
Cry1Ab (MON 810)	10.34	-	< 90		0.19-0.39	4.65
Cry9C (StarLink)	44	25.87	0.24	2.8	18.6	250
Cry1Ac (DeKalb)	2.04		0.0115		1.62	
Cry3A (potato)	28.27	0.39 tuber				3.3
Cry3B (MON 863)	81 (65-93)	41 (25-56)	62 (30-93)		70 (49-86)	37 (24-45)
Cry1F (hybrid)	0.111 (0.057-0.149)		0.136 (0.113-0.168)	0.55 (0.356-0.737)	0.09 (0.071-0.115)	1.064 (0.803-1.573)
Cry1F (inbred)	0.17 (0.079-0.21)		0.208 (0.186-0.231)	0.638 (0.481-0.849)	0.112 (0.067-0.142)	1.358 (1.284-1.428)
Cry1Ac* (DeKalb)	0.46-1.2	0.058-0.125	-	0.041-0.124	0.036-0.043	
Cry1Ab (Event 176)	4.4	0.08	7.1		0.001	0.6
Cry1Ab~ (Event 176)	0.596-1.159 (seedling) 0.53-3.03 (anthesis) 0.44-0.47 (maturity) 0.066-0.225 (senescence)	0.008	1.137-2.348	<0.008	<0.005	0.00025-0.0014% of fresh weight
Cry1Ab Event 176 hybrid (Fearing *et al.*, 1997)	0.86 (seedling) 3.0 (anthesis) 0.43 (seed maturity) 0.21 (senescence)	<0.008	2.36 (anthesis)		<0.005	0.32 (seedling) 0.13 (anthesis) 0.08 (seed maturity)
Cry2Aa2 (tobacco) Full protoxin expressed in chloroplasts (Kota *et al.*, 1999)	2-3% of total soluble leaf protein ≡ up to 30,000 µg/g					

All data are for transgenic corn, apart from Cry3A (potato) and Cry2Aa2 (tobacco).
All transformations, other than Cry2Aa2 (full protoxin) are for the truncated toxin.
Unless otherwise stated, all data from US EPA on-line information sources (www.epa.gov)
* Dry weights (Data from Canadian Plant Health and Production Division – www.inspection.gc.ca)
~ Fresh weights (Data from Canadian Plant Health and Production Division – www.inspection.gc.ca)

The recent finding that overexpression of *Bt* can be achieved by using transformation of chloroplasts is included in the table, to indicate the current upper limits for expression in transformed plants (Kota *et al.*, 1999). Data on expression in roots, which has relevance to the potential for direct effects on soil dwelling herbivores that consume roots and root debris, is not always provided but does indicate that quantities of *Bt* toxin are lower than in leaves or stems in most cases, the exceptions being Cry3B expression in MON 863 corn and Cry9C expression in Starlink corn. In relation to whole life expression of *Bt* in corn, there is a progression from relatively low in the seedling stages (5-6 leaf stage, 3 weeks post planting), through maximum expression at anthesis (pollen shed, 10-11 weeks post planting) then declining through seed maturity (18-20 weeks post planting) to senescence (23 weeks post planting) (Fearing *et al.*, 1997). There are no equivalent data for root-expressed *Bt*, but it is likely that expression with constitutive promoters would parallel the pattern of expression in other major tissues.

In developing the concept of a whole life budget for expressed *Bt*, the additional factor of continuous exudation of truncated toxins from the roots of crops, including corn, has not been included in published totals for *Bt* commercial production. The only data available are, therefore, those produced by Stotzky and colleagues (summarised in Chapter 3.6). Quantification of the exuded toxins has been mainly through immunological testing to confirm presence and by bioassay to determine the level of toxicity. Extrapolating from data on the biological activity of free and bound toxins, either derived from exudates (Saxena & Stotzky, 2000) or by purification of the 66 kDa toxic core from commercial sources of *Btk* (Tapp & Stotzky, 1998), provides an estimate of quantities of *Bt* exuded into the rhizosphere. Bioassay methods used by Stotzky and colleagues have been standardised over all experiments and, therefore, it is possible to compare assays of toxins from different origins and, thus, estimate the relative toxin loads. Tapp & Stotzky (1998) examined persistence of toxin in different soils and, from bioassays with susceptible larvae, provided data on initial LC_{50}s for free and soil-bound toxins. Back-extrapolation from volumes of toxin-soil mixtures used to feed the test larvae to the initial quantities of soil and the concentrations of toxins added, it appears that a range of initial LC_{50}s from 15-178 ng/g soil can be used as baseline data for comparison with root-exudate derived values. Free toxin (not bound to soil aggregates) gave an initial LC_{50} of 225 ng/g (≡ 90 ng/100 µl), indicating that when toxins are bound and concentrated on soil clay micelles they are up to 15 times more potent per unit volume/weight than when they are provided as free toxin. This might also reflect the possible degradation of free toxin, even in the relatively short period of ingestion during bioassay.

Assuming that mortality of larvae carried out during tests of root exudate-derived *Bt* is quantitatively the same as assays with purified toxins, concentrations that produce equal mortalities (measured as LC_{50}) with either bound or free toxin should provide an estimate of the concentration of exudate *Bt* per g of soil in the rhizospheres of *Bt* plants. Taking the data in Saxena & Stotzky (2000), the initial mortalities noted in the majority of larvae fed defined quantities of soils subject to root exudation 10 days after germination of test plants were between 25% and 100%. Those with values of 50% mortality can be assumed to have received the LC_{50} dose of equivalent to a maximum of 178 ng/g soil. Mortality tended to increase over time up to 40 days post germination and, therefore, the LC_{50}s would also be expected to decline (i.e. implying a greater dosage is present in the soil). Preliminary experiments reported in the same

paper indicated that LC_{50}s at days 15 and 25 were 78.3% and 69.6% lower than the 7 day value, confirming accumulation of toxin to give increased potential potency ratios of 1.28 and 1.44 respectively. These extrapolations would, therefore, imply that field dosage of exuded toxin would rise over time to at least 256 ng/g of soil and probably higher.

Clearly, there is a need to determine more precisely the actual quantity of *Bt* that is entering and accumulating in soil over time. Significantly, in terms of the relative potential of Cry3 toxins to affect soil Coleoptera, the insect order most likely to be present in that environment, the degree of binding of Coleoptera-active Cry3 toxin is considerably lower than for Cry1, both for initial adsorption to soil aggregates and in terms of retention during desorption (Stotzky, 2000).

4.3.2 Accumulation and retention of activity of transgenic *Bt* vs. wild type *Bt* in soil

Quantities of *Bt* entering soil can be estimated from the values in Table 4.3 and from the potential rates of exudation derived from work by Stotzky and colleagues. This allows comparison with the use of conventionally applied *Bt* in some systems but does not necessarily apply to insecticide regimes used on genetically modified crops where there might not be a prior history of *Bt* application.

There are relatively few data on extrapolated total yields of expressed *Bt* per ha. It is also difficult to determine from the available information precisely how the yields have been calculated, bearing in mind the wide range of different units that have been used to quantify *Bt* yields. Table 4.4 includes examples where values for total *Bt* production in modified corn have been provided, although in no cases were the methods of calculation provided.

Table 4.4: Published and estimated quantities of *Bt* entering soil per ha. Sources, include various tissues of *Bt*-corn and root exudates.

	Yield of *Bt* per ha (g) and per plant (µg)							
Published *Bt* yields	Vegetative		Anthesis		Silage (maturity)		Harvest (grain)	
	g/ha	µg/plant	g/ha	µg/plant	g/ha	µg/plant	g/ha	µg/plant
Cry9C (Yieldgard)	254.5	4105	825.3	13312	1223.1	19728	244.6	3946
Cry1Ac (Bt11)			3105	50082	-	-	-	-
Cry1Ab (Event 176)*	0.6	10.0	10.3	164.5	6.2	99.6	0.5	7.6
Cry1F (Herculex/Pioneer)	-	-	-	-	20.5	331	-	-
Estimated *Bt* yields					g/ha			
Cry3B (MON 863) (maximum)	-	-	-	-	13412	-	-	-
Estimated maximum concentration ng/g soil	ng/g soil		ng/g soil		ng/g soil		ng/g soil	
Cry9C (Yieldgard)	1144		3712		5500		1100	
Cry1Ac (Bt11)	-		13964		-		-	
Cry1Ab (Event 176)*	2.70		46.32		27.88		2.25	
Cry3B (MON 863) (maximum)	-		60316		-		-	
Cry1F (Herculex/Pioneer)	-		-		23.0		-	
Estimated concentration in soil of exudates from *Bt*-corn	ng/g soil							
Cry1Ac (Bt11)	250		-		-		-	
Estimated concentration of intact toxin from conventionally applied *Bt* formulations	g/ha	ng/g soil	g/ha	ng/g soil				
Applied per ha	111*	-	40˜	-	-		-	
Estimated concentration reaching soil	-	1790	-	645	-		-	

Data calculated on an average planting density of 62000 plants per ha and soil depth for incorporation of *Bt* plant material of 15 cm.

All data from http://www.epa.gov/scipoly/sap/, unless stated.
*(Fearing *et al.*, 1997)
˜(Bernhard & Utz, 1993)

Corn crop agronomic yield is usually expressed in bushels per acre or per ha, with the expected weight per bushel being approximately 56 lbs (25.4 Kg). This does not provide broad equivalence to total weight or, particularly, to leaf weight of corn plants which is the tissue normally expressing the greatest quantity of *Bt*. However, it is possible to make approximate comparisons in relation to harvesting of corn for silage purposes, where the total weight of the plant is the quoted parameter. There are numerous on-line extension services offering expected yield tables for farmers in the USA and these also include information on the relative agronomic performances of *Bt*-corn relative to conventional corn. For example, useful information on the yields of silage corn crops is provided by Penn State University (Roth & Adams, 2002). These data have been used to provide an approximate converter for assessing yields per ha from published information. Applying the published yield of approximately 12000 lb/A (13450 Kg/ha) dry weight and allowing for the normal 65%-70% moisture content, the weight of a corn plant at the silage stage approximates to 0.75 Kg. Further extrapolation to the other stages of growth is provided in Table 4.4. The data are used to provide upper bounds for yields of *Bt* in corn tissues, thus giving a comparative indicator of potential toxin delivery to the soil. In reality, the quantity of *Bt* on site declines once anthesis has taken place and much of the toxin load is removed from site during harvesting for grain or, particularly, silage.

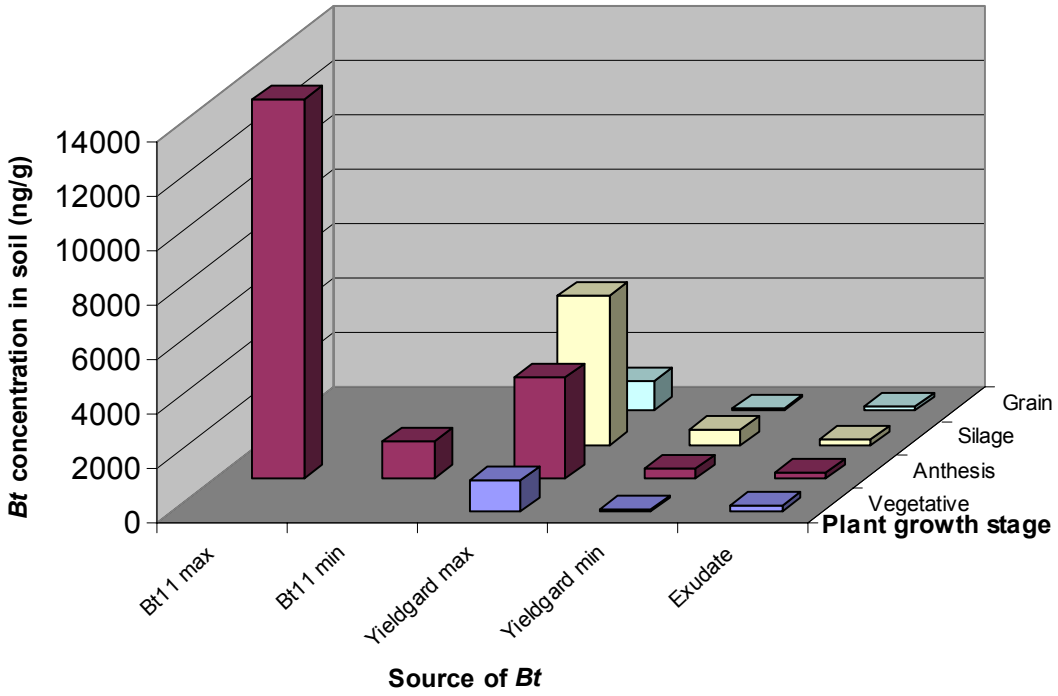
Taking a worse-case option of full incorporation of the entire corn plant into the upper layer of soil with full mixing (e.g. by ploughing), a series of potential *Bt*-soil concentrations can be calculated. These are also included in Table 4.4, based on the dry weight of 67.5 g per cc of soil used in the studies of Cry9C incorporation into soil included in the Table. The estimated concentrations range from as low as 46 ng/g for Event 176 to over 60,000 ng/g for MON 863, reflecting the enormous variation in degree of expression between different transgenic events.

The rate of exudation into the soil is even more difficult to quantify but must be linked to both the growth stage of corn and the mode of expression of *Bt*. Studies by Saxena *et al* (2002a) indicated that there is *Bt* exudation in corn hybrids variously expressing Cry1Ab toxin as Bt11 (6 hybrids), Event 176 (1 hybrid) and MON810 (6 hybrids), which all resulted in mortality to tobacco hornworm larvae. Although variable, (37% - 81% mortality), results from field-grown corn confirmed that exudation took place under outdoor conditions. Using the extrapolated data on quantities of *Bt* in root exudates (Chapter 4.3.1), the quantity of toxin accumulated in the immediate rhizospheres of corn was estimated to be at least 250 ng/g soil. In relation to the potential delivery of *Bt* toxin from various tissues of the corn plant, the quantities of toxin that might be present in soil on a weight basis are summarised in Figure 4.2.

The largest values for each growth stage represent the worst-case extrapolation on the basis that the entire plant is ploughed into the soil. In reality, it can be assumed that <10% of plant components are actually incorporated into the soil, either accidentally or as part of normal agronomic practice and these extrapolations are also shown in Figure 4.2. The quantities from root exudation remain the same as long as the plant is growing and actively exuding material through the roots. Although these data are based on some broad assumptions, they indicate that quantities of *Bt* entering soil from normal agronomic practice may be similar for *Bt*-corn debris and for root exudates but this depends on the nature of the promoters used in the transformed

plants. In all cases quantities of *Bt* lethal to fully susceptible test species enter the soil and, subsequently, are subject to a range of factors that will determine the rate of degradation of the toxin (see Figure 3.2 showing the rate of degradation of Cry1Ab in soil aggregates as an indication).

Figure 4.2: Estimated quantities of truncated *Bt* in the rhizosphere of soil in relation to corn event and growth stage. Maximum and minimum values refer to incorporation of the entire plant or of 10% (the more likely figure).



As a final measure of the relative contributions of various sources of toxin to the soil reservoir *Bt*, Table 4.4 also includes an estimate of the quantity of toxin likely to reach the soil from commercial applications of the bacterial toxin. This assumes that up to 50% of the applied *Bt* reaches the soil, either directly from the spray application or indirectly by being washed off leaves, etc. Clearly, the more efficient the spray application system, the lower the volume of spray reaching the ground, but it is often the case, especially with high volume sprayers, that a significant proportion of the applied agent misses its target. The estimates of conventional deposition rates are based on the maximum dosages of commercially applied products, such as Foray 76B, at the recommended rate of 60 BIU (Billion Infectious Units) per ha. Unfortunately, the precise breakdown between spores and toxin crystals is not provided on the product labels but it is assumed that around 2% of the undiluted volume of the product is made up of toxin (Bernhard & Utz, 1993). Data from Fearing *et al* (1997) are also included for comparison. From these estimates, it would appear that quantities of *Bt* applied conventionally fall within the range estimated for introduction to the soil from incorporation of debris from transgenic corn but exceed the quantities estimated for root exudation. However, these data have to be treated with considerable caution and serve to emphasise the need for more accurate and consistent quantification of life budgets for *Bt*, whether applied conventionally or through transgenic routes.

4.4 *Conceptual framework for encounter between* Bt *toxin and putative hosts*

Presence of *Bt* in soil is virtually universal, although in most cases the toxin is not directly available for immediate encounter by organisms in the soil. Data presented in Chapter 4.3 provide estimates of quantities of toxin likely to enter soil from transgenic plants and from conventional applications of *Bt*. These provide a baseline for likely encounter by the diverse range of soil-dwelling organisms, particularly those in the rhizosphere. The remainder of this section is devoted to assessing likelihood of encounter between putative hosts and the pool of *Bt* active ingredient (≡ inoculum) in the soil. A framework for the discussion is provided in the conceptual model for encounter proposed by Evans (1999) in relation to genetically modified bioinsecticides. Modifications have been made to the original framework to allow for the emphasis of the current review on *Bt* toxin, which does not have the ability to replicate exhibited by the parental organism. Figure 4.3 shows the full conceptual framework that determines many of the biological, ecological and physical parameters that have to be considered in determining encounter frequency. For the purposes of the present review, discussion will concentrate on Sub-Model C (Figure 4.4), dealing with the final encounter determinants between organisms, whether target or non-target, and a given concentration of *Bt* toxin.

Figure 4.3: Conceptual framework for pathogen host interaction Evans (1999). Original framework for bioinsecticides capable of replication. This would apply to wild-type *Bacillus thuringiensis* including a spore as well as the toxic crystal

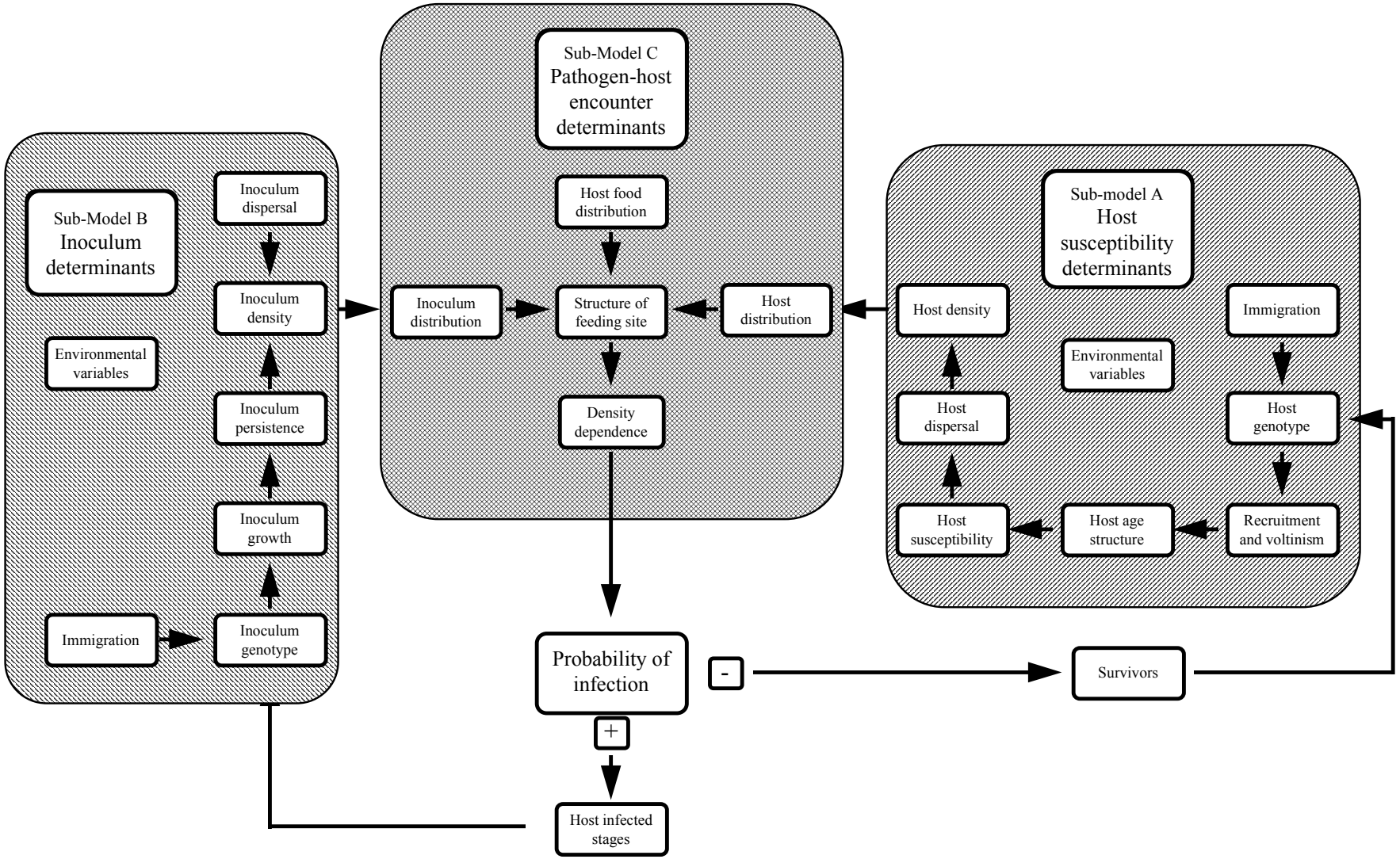
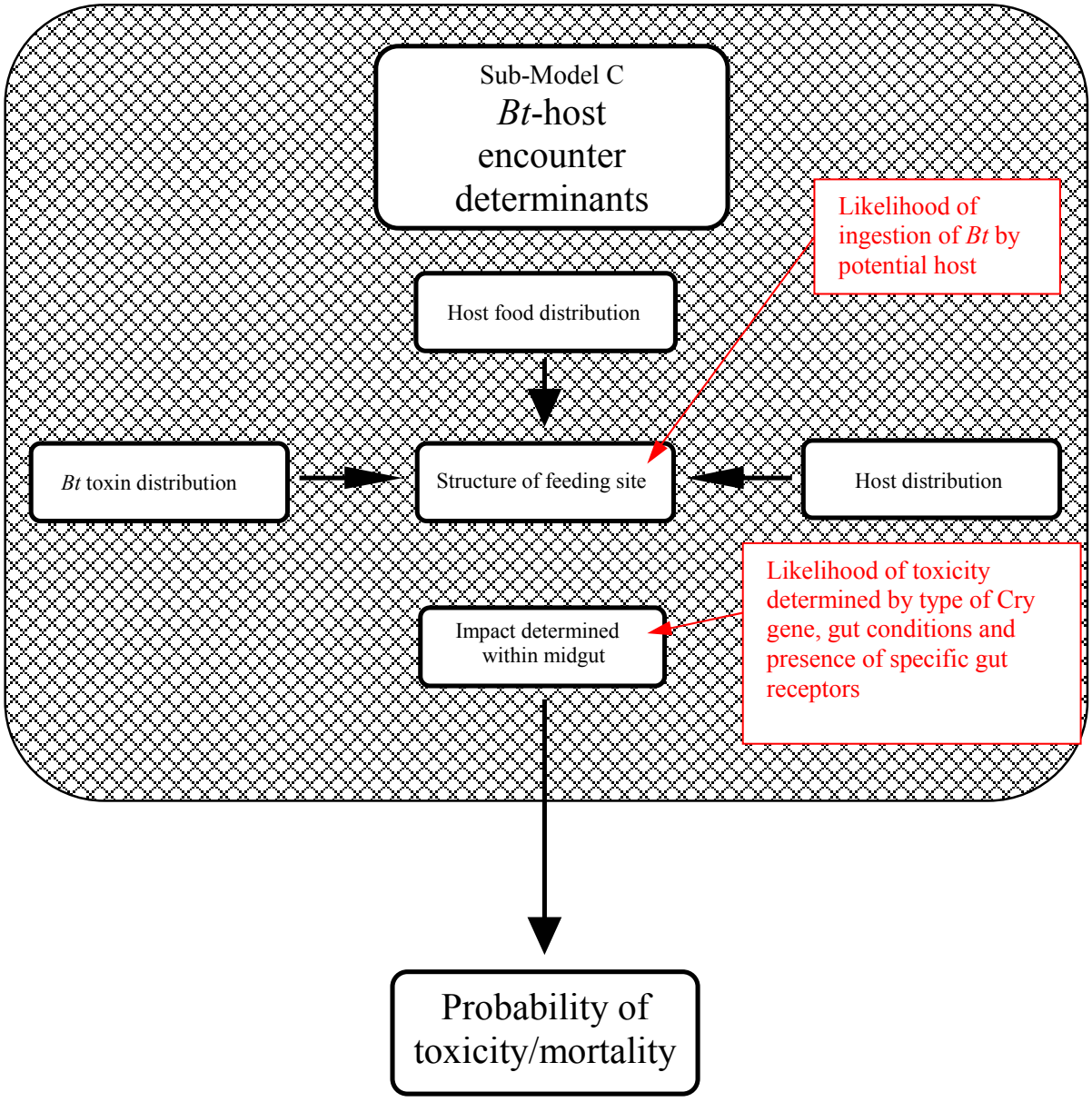


Figure 4.4: Sub-Model C has been modified to take account of the encounter determinants when *Bt* is present only as the toxin moiety, either as intact crystals or as truncated toxin typically expressed in transgenic plants.



4.4.1 Risk assessment in relation to encounter between hosts and wild type or truncated *Bt* toxins

Ingestion by target pest organisms of plant material expressing lethal concentrations of *Bt* is the primary purpose of engineering crop plants in agriculture, horticulture and forestry. The listing in Table 4.1, indicates the range of primary pests that could ingest plant material in corn systems in North America. Depending on the construct, protection of the crop from target hosts has been shown to be effective, particularly at higher population densities (Shelton *et al.*, 2002). In terms of environmental impact, concerns have been expressed over whether dosages of toxin remain sufficiently high over the lifetime of the crop to sustain the high dose strategy that is part of resistance management in corn and other systems (Ferré & Van Rie, 2002). This paradox is highlighted by the data in Table 4.3, where it is apparent that the concentration of expressed *Bt* toxins tails off with increasing plant age and is generally much higher in aerial plant parts rather than in the root. However, it seems unlikely that this will affect the majority of target pests because these will generally be active during the early period of plant growth before the plant reaches maturity.

4.4.1.1 Encounter in the phylloplane

Assessment of the effects of whole-life *Bt* budgets must take account of both above and below ground expression of the toxin. Ecosystem function is a dynamic balance and there are direct interactions between above and below-ground herbivory, although this has not been explored in great depth (Hooper *et al.*, 2000). Impacts of transgenically expressed *Bt* will also be a function of concentration and availability and, therefore, it is important to assess phylloplane activity both for direct effects on those organisms associated with that zone, and also for the indirect effects of further entry to the soil of *Bt* through cadavers of those killed by the toxin and by leaf debris and frass, both of which will contain *Bt* toxin. Both host and non-host organisms will also contribute to dispersal of *Bt* through surface contamination (when the organism is applied conventionally) and by survival and passage in the gut and subsequent excretion. In model systems, transmission of pseudomonads between plants by *Mamestra brassicae* larvae (Lepidoptera: Noctuidae) (Lilley *et al.*, 1997) and by grasshopper adults, *Melanoplus femurrubrum*, (Snyder *et al.*, 1999) have been demonstrated.

Transmission and impact on non-target organisms are important parameters in risk assessment. Specific studies of effects of transgenically delivered *Bt* over time are lacking, but there have been some studies of the non-target effects of feeding on transgenic plants. For example, Pilcher *et al* (1997b) assessed how *Bt*-corn, expressing Cry1Ab (Ciba 3906X) to target European corn borer (ECB), affected four other species of Lepidoptera in the family Noctuidae. Mortality of ECB was high, as expected, whereas effects on the non-target species were lower. No effects were observed on larval survival, pupal weights or days to adult emergence in black cutworm, *Agrotis ipsilon* and the stalk borer, *Papaipema nebris* during laboratory feeding trials. A slight decrease in the armyworm *Pseudaletia unipuncta* and corn earworm *Helicoverpa zea* larval survival and pupal weight was noted. These data were generally confirmed in the field but there were more marked effects on all bar

A. ipsilon, when results were expressed as reduced damage to corn. The lack of effects on *A. ipsilon* mirrored those found by MacIntosh *et al* (1990) who carried out feeding tests with purified Cry1Ab and demonstrated similar toxicity effects to *H. zea* to those demonstrated by Pilcher *et al* (1997b).

More recent work has confirmed the low impacts of Cry1Ab expressed in cotton plants (MON 810) on secondary plant feeders (Head *et al.*, 2001). In addition to the Lepidoptera *O. nubilalis*, *H. zea* and *A. ipsilon*, studies on the effects on the corn leaf aphid, *Rhopalosiphum maidis* were also carried out. Apart from assessment of impact on the test organisms, the authors also quantified the amount of Cry1Ab in the various insect stages. After feeding on artificial diet containing up to 100 ppm of toxin, concentrations 142 times lower than the original food source were recorded in *O. nubilalis* larvae, 34 times lower for *H. zea* and 14 times lower for *A. ipsilon*. Laboratory assay of the aphid *R. maidis* indicated that toxin was taken up in the artificial system but at levels 250-500 times less than in the diet itself. Bioassays with lyophilised aphids from these treatments confirmed that the toxin was active against *O. nubilalis* larvae. By contrast, no toxin was detected in aphids that had fed on transgenic corn expressing the Cry1Ab gene.

Insects in other orders would not be expected to succumb to the Lepidoptera-specific Cry1 toxins and this has generally been confirmed during tests on various crops. Tarnished plant bugs (*Lygus lineolaris*; Hemiptera, Heteroptera) were not affected by feeding on transgenic cotton expressing Cry1Ac (Hardee & Bryan, 1997). As part of risk assessment prior to incorporation of Cry2A into cotton, representatives of Hymenoptera (honeybee and three parasitoids), Coleoptera (5 species), Homoptera (3 species), Orthoptera (2 species), Collembola (2 species), Isoptera (1 species), Neuroptera (1 species) and Isoptera (1 species) were shown to be unaffected in laboratory assay (Sims, 1997). The latter was a significant result in that Cry2A is known to have dual specificity to Lepidoptera and Diptera (Moar *et al.*, 1994). Toxin administered in the bioassays was truncated to mimic the 634 amino acid protein produced in transgenic cotton plants as closely as possible.

One of the aspects of ingestion of transgenic plant material by susceptible herbivores is the possible "knock-on" effect on higher trophic levels (Schuler *et al.*, 1999a; Schuler *et al.*, 1999b). Studies by Hilbeck *et al* (Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1998b) indicated a possible negative effect on lacewing larvae, *Chrysoperla carnea* (Neuroptera) after feeding on *Spodoptera littoralis* or on *O. nubilalis* larvae that had consumed Cry1Ab engineered corn. Although there is a degree of uncertainty about whether the effects on predator survival were directly as a result of toxin remaining in the larvae or whether the affected *S. littoralis* were nutritionally unsuitable, the data provide a note of caution in relation to tritrophic impacts. In particular, it is important to assess directly whether toxin binding to mid-gut receptor sites takes place, which would be a large shift in host range that may not have been predicted on the basis of the domain tree in Figure 2.1: . Unpublished work described by Hilbeck (2002) suggests that Cry1Ab binds to isolated brush border membranes of *C. carnea* and this warrants further investigation in relation to presence of binding sites in predatory insects that would otherwise be regarded as having unsuitable gut conditions for solubilisation and activation of toxins (see 4.4.2).

Investigation of the feeding activity of predators with sucking mouthparts presents difficulties in carrying out controlled bioassays, as indicated in the studies on *C. carnea* (Hilbeck, 2002). Another predatory group with sucking mouthparts that is commonly associated with crop systems are the Heteroptera, of which *Orius insidiosus* (Anthocoridae) is a common, generalist predator. Like *C. carnea*, this species is an opportunist that can take advantage of prey availability on both transgenic crops and on other plant sources. Tests of feeding by *O. insidiosus* nymphs on *O. nubilalis* larvae that had been fed on either normal *Bt* (Dipel - spores and Cry1Aa, Cry1Ab, Cry1Ac, Cry2Ac, Cry2B crystals) or silk from transgenic corn expressing Cry9C (Yieldgard) were carried out by Al Deeb *et al* (Al Deeb *et al.*, 2001). Apart from mortality arising from feeding on either transgenic or normal silk (an unsuitable food source), there were no effects on *O. insidiosus*, which was also confirmed by field observations in transgenic MON 810 (Cry1Ab) corn and the non-*Bt* isoline. A two year study of the effects of laboratory feeding or field-exposure to corn pollen and other components of Cry1Ab *Bt*-corn revealed no effects on the three predator species *Coleomegilla maculata* (Coleoptera: Coccinellidae), *O. insidiosus* and *C. carnea* (Pilcher *et al.*, 1997a).

4.4.1.2 Indirect effects on natural enemies by removal of key prey or host organisms

Indirect effects on natural enemies by removal of key prey or host organisms, are further factors that have, as yet, not been studied intensively, but which might be significant both in terms of natural enemy dynamics and on their potential to regulate non-target prey within the spectrum of pests in *Bt*-crops. For example, laboratory studies of performance by the parasitoid *Diaeretiella rapae* (Hymenoptera: Braconidae) attacking the aphid *Myzus persicae* on *Bt*-oilseed rape (*Brassica napus*) indicated no effects on the natural enemy (Schuler *et al.*, 2001). Further studies of this nature are needed to assess both indirect effects but also the beneficial interactions that would be expected through more specific targeting of key host and prey organisms using *Bt*-crops.

4.4.1.3 Encounter in the rhizosphere and bulk soil

The high diversity of organisms in the soil, particularly in the rhizosphere, and the demonstrated natural occurrence and persistence of applied *Bt* indicates that encounter frequency with *Bt* will be high (Table 4.5).

Table 4.5: Summary of the main soil dwelling organisms likely to be influenced by rhizosphere and immediate bulk soil incorporation of *Bacillus thuringiensis* applied conventionally or via genetically modified plants.

Organism Grouping	Organism	Body width	Nos. in rhizosphere per g	Non-rhizosphere no. per g	Density		Diet	Action in Soil
					per cm^2	per m^2		
Microflora	Bacteria	1µm-10µm	1.2×10^9	5×10^7	6-10 million		Organic matter, especially simple carbon compounds.	Decompose organic matter, immobilise nutrients in the rooting zone, some genera fix nitrogen from air. Conversion of ammonium to nitrate, and nitrate to nitrogen gases. Decomposition. Create substances which help to bind the soil.
	Fungi	<10µm	1200000	100000	1 to 2km of hyphae		Organic matter, especially simple carbon compounds, and living plants.	Decompose organic matter, immobilise nutrients in the rooting zone, mycorrhizal fungi form mutually beneficial associations with roots. Help stabilise soil aggregates.
Microfauna	Protozoa	5-500µm	24000	10000	10 million		Mainly bacteria.	Stimulate and control growth of bacteria, release ammonium.
	Nematodes	<50µm			10-30 million	>1g	Bacteria, fungi, protozoa, other nematodes, and roots.	Control many disease causing organisms, also cause a range of plant root diseases. Release ammonium.
Mesofauna	Collembola	0.1-2mm				40,000-500,000	Fungi and bacteria	Important in soil decomposition.
	Acari	0.1-2mm				100,000-600,000 (or 2g)	Most soil organisms, and plants.	Important in soil decomposition.
	Enchytraeidae	0.1-2mm				200,000-300,000 (or 35-50g)	Dead plant material.	Important in soil structure, through plant decomposition, and burrowing and transport of organic and mineral material
Macrofauna	Diplopoda (millipedes)	2-20mm				20-100+	Fungi and dead insect and plant material.	Decomposition of plant material.

Organism Grouping	Organism	Body width	Nos. in rhizosphere per g	Non-rhizosphere no. per g	Density		Diet	Action in Soil
					per cm^2	per m^2		
	Chilopoda (centipedes)	2-20mm				120-150+	Insects and other soil arthropods.	Predate other organisms.
	Isopoda (woodlice)	2-20mm				500-3000	Fungi and dead insect and plant material.	Decomposition of plant material.
	Insects e.g. Orthoptera, Coleoptera,. Hymenoptera, Lepidoptera, Hemiptera, Dermaptera,	2-20mm				720	Other arthropods, plant material, fungi, bacteria, some e.g. ants, feed on plant secretions (nectar),	Decomposition of plant material, important in soil micro-organism interactions and food webs. Some shred plant material making it more accessible to bacteria and fungi. Others enhance soil structure by creating faecal pellets, and by burrowing. Control populations of other organisms.
	Molluscs	2-20mm				15-450	Rotting vegetation.	Organic matter decomposition.
	Earthworms	2-20mm				180	Bacteria, fungi and organic matter	Shred plant tissue residues, and enhance soil structure by burrowing, mixing and creating faecal pellets. Transport and stimulate the growth of bacteria.

Various sources, including:

http://www.earthlife.net/insects/soileco.html
http://www.extension.umn.edu/distribution/cropsystems/components/7403_02.html
http://www.tu-cottbus.de/BTU/Innov/e_sp_6_1.htm
http://www.dpw.wageningen-ur.nl/ssg/publ/didden.htm
http://www.herper.com/Millipede.html
http://www.earthlife.net/insects/chilopod.html
http://www.biotech-info.net/woodlice.html
http://ohioline.osu.edu/hyg-fact/2000/2067A.html
http://www.tu-cottbus.de/BTU/Innov/e_sp_6_1.htm
http://www.dpw.wageningen-ur.nl/ssg/publ/didden.htm
http://www.cals.ncsu.edu/course/ent525/soil.html :

Activity of the microflora, microfauna and mesofauna components of the table will be particularly high in the rhizosphere zone where they are numerically extremely abundant. Decomposition of plant material, for example, can lead to enormous increases in Protozoa and nematodes in the rhizosphere as they take advantage of the large increase in bacterial activity associated with ingress of biomass. This was measured by Christensen *et al* (1992) who showed that protozoa and nematodes were 80 and 30 times higher in soil immediately surrounding decomposing roots compared with bulk soil. In turn, these densities were up to 20 times higher than around live root systems. This has been recognised as a zone of high encounter, especially following the first extensive plantings of commercial *Bt*-crops and some studies have addressed the interaction specifically. Donegan *et al* (1996) assessed the effects of *Bt*-potato (Cry3A) on a wide range of soil bacteria and fungi. Assessments were made on both phylloplane and rhizosphere populations and, apart from greater longevity of the transgenic potato plants which provided a longer period of availability for the fungus *Verticillium dahliae*, there were no significant effects on microflora. Studies to specifically address the potential effects to soil organisms of *Bt* delivered by root exudation have also been carried out by Saxena & Stotzky (2001a) (see Chapter 3.6 for full details). No effects on a range of soil organisms were noted in assays involving *Bt* exudates or incorporation of *Bt*-corn into soil. In relation to exposure profiles, the finding that *Bt*-corn has a higher lignin content than isogenic corn suggests that, as a consequence of slower degradation of *Bt*-corn plant tissue, the toxin might persist for longer periods than is currently estimated for transgenic corn (Saxena & Stotzky, 2001b). The authors speculated that this could have both positive effects, by extended impact on soil dwelling pests (e.g. webworms or cutworms for Lepidoptera active *Bt* and rootworms for Coleoptera active *Bt*) and negative effects, by impact on non-target organisms or development of resistance in susceptible organisms. There appears to be little evidence for negative effects but definitive studies are still lacking.

Studies of impacts of soil *Bt* on mesofauna (Collembola, Acari and Enchytraeidae) have mainly indicated no adverse effects, although there are some indications of direct transient negative impacts. Addison (Addison, 1993) reviewed persistence and non-target effects of *Bt* in soil with emphasis on conventional, rather than transgenic, delivery of toxin/spores to the soil. Prior to attention being focussed on the toxin itself, most studies of *Bt* impact in soil did not distinguish between spores and crystals and were dominated by spore survival and degradation by soil microorganisms. Addison quotes a number of papers where effects of conventional application of *Bt* on non-target organisms have been demonstrated. These included Hymenoptera (Mück *et al.*, 1981), mites (Acari) (Chapman & Hoy, 1991) and nematodes (Meadows *et al.*, 1990). In all cases, the effects were small and it was not possible to distinguish directly between spores and crystals, although a study by Bottjer *et al* (1985) using a range of doses of *Bti* and other crystal toxins in liquid culture resulted in mortality of eggs of a number of soil dwelling nematode species.

More recent studies on specific Cry toxins, truncated to mimic transgenic expression or by actual expression in *Bt*-plants, provide a direct measure of potential impacts of transgenically expressed *Bt*. Studies on the collembolan *Folsomia candida*, and the orbatid mite *Oppia nitens* fed on transgenic cotton (Cry1Ab or Cry1Ac) or potato (Cry3A – *F. candida* only) indicated no impact on the two soil-dwelling organisms (Yu *et al.*, 1997). Cry1Ab, Cry1Ac, Cry2A and Cry3A truncated toxins were fed to

the Collembola *F. candida* and *Xenylla grisea* (Sims & Martin, 1997). There was no effect on survival or reproduction of the two Collembola species even though the dose of 200 µg/g of diet was considerably in excess of the estimated soil dosages shown in Table 4.4 (maximum of 13.9 µg/g for Cry1Ac (Bt11) and 60.3 µg/g for Cry3B (MON 863)). The significantly greater expression of Cry3B in MON863 is of particular significance for risk assessment in soil because of the increased likelihood of encounter with Coleoptera, both pest and beneficial, in soil than in the phylloplane. The lack of effect of Cry3A on Collembola in the Yu *et al* (1997) and Sims & Martin (1997) papers is encouraging in relation to non-Coleoptera hosts from Cry3 expression. Direct studies of soil-dwelling Coleoptera are, however, surprisingly limited in view of the move towards transgenic crops expressing Cry3 genes. Carabidae (Coleoptera) are important predators and scavengers in all ecosystems and exhibit a range of specificity from generalists through to virtually specific to a single prey species. They are relatively abundant at the soil surface and immediate sub-surface and would, therefore, be expected to have a high encounter frequency with *Bt* in soil. A study of Cry3A-potato in different proportions with isogenic potatoes indicated that the main target, *L. decemlineata* was subject to very high mortality that had a knock-on effect on the carabid *Lebia grandis* but not on the coccinellid *C. maculata* (Riddick *et al.*, 1998). The reduction in *L. grandis* in the *Bt*-potato plots was attributed to absence of the primary prey, *L. decemlineata*, whereas *C. maculata* was able to switch to other prey items and was unaffected. Follow-up studies indicated that the *L. decemlineata* larvae affected by Cry3A during feeding on transgenic potato were palatable to *L. grandis* and had no effect on predator survival (Riddick & Barbosa, 2000). Confirmation of the general lack of effect of Cry3A-potato deployment was provided in a season-long study that indicated no negative impacts on either foliar-dwelling (Heteroptera and Coccinellidae) or ground-dwelling (Carabidae, ants and spiders) generalist predators (Riddick *et al.*, 2000). *O. insidiosus* (Heteroptera) and spiders were significantly more abundant in the transgenic crops during one of the years studied. Although investigating *Bt*-corn expressing Cry1Ab, Lozzia (1999) also found no significant differences in carabid assemblages within and between years that could be attributed to the presence of the transgenic crop.

4.4.1.4 Conclusions on likelihood of encounter with *Bt*

Observations on both target and non-target effects in relation to *Bt* delivered conventionally or by engineering into crop plants have, so far, not indicated any serious departures from expectation in relation to non-target effects. In areas dominated by a single crop system, the rate of encounter with phylloplane organisms is likely to be relatively limited, simply because there is limited faunal diversity in the already ecologically impoverished agro-ecosystem. This is a consequence of intensive agricultural production and not of modification of the crop itself. However, as indicated in the diversity data in Letourneau *et al* (2002), there are many Lepidoptera with both wide and extremely narrow host plant ranges that are associated with corn and it would be prudent to take account of the presence of some of these, particularly those restricted only to corn, in impact assessment.

In many cases, whether the organisms are mono- or poly-phagous, the impacts of transgenic delivery of *Bt* will be considerably smaller than conventional practice where relatively broad spectrum chemical insecticides are employed routinely

(Shelton *et al.*, 2002). Taking all these factors into account, it would appear that the consequences of large-scale usage of transgenic agricultural crops on the majority of organisms occupying the phylloplane will be closely related to the likelihood of direct mortality from consumption of a lethal quantity of tissue. Significantly, the greater potential consequences, but still largely unquantified risk factor, stem from the potential for increased resistance of target and non-target organisms to *Bt*. Data discussed in Chapters 3.4, 4.2.1 and 4.3.1 point to the variable and plant age-related expression of *Bt* in different tissues of corn, in particular. A decline in expression levels to partial lethality at the host population level removes one of the cornerstones of the resistance management strategy of high dose delivery (Ferré & Van Rie, 2002). Complementary strategies, such as incorporation (stacking) of multiple Cry genes, targeting different receptor sites in the guts of pest organisms offer prospects of overcoming resistance development (Liu *et al.*, 2001), but these will also be susceptible to whole life toxin budgets and particular attention must be paid to the decline phase of expression.

Data on the rhizosphere and bulk soil encounter between the very wide range of organisms present and the accumulated and relatively persistent pool of *Bt* present are relatively scant. However, studies so far carried out to address general questions of soil encounter have not indicated significant deleterious effects. However, the close inter-relationships between soil organisms and crop/ecosystem functioning, which are rather poorly understood in any event, must now include the added factor of presence of *Bt* in a truncated form, rather than the spore/intact crystal presence that is already ubiquitously present. Aspects of interactions between soil ecosystem processes and the many organisms that contribute to them are already subject to intense research (Lavelle *et al.*, 1997; Brussaard, 1998; Couteaux & Darbyshire, 1998; Rusek, 1998; Foissner, 1999; Kennedy, 1999; McCully, 1999; Bonkowski *et al.*, 2000; Gange, 2000; Berg *et al.*, 2001; Scheu, 2001). These provide useful guidance on the initial encounter frequencies between *Bt* in soil and, particularly, those organisms likely to ingest the toxin either while it is still in a free state (inside or released from transgenic plant material) or after it has become adsorbed to soil particles. The initial steps of encounter and ingestion then lead to a further set of processes that determine the eventual impact of the ingested *Bt* toxins. These are discussed below.

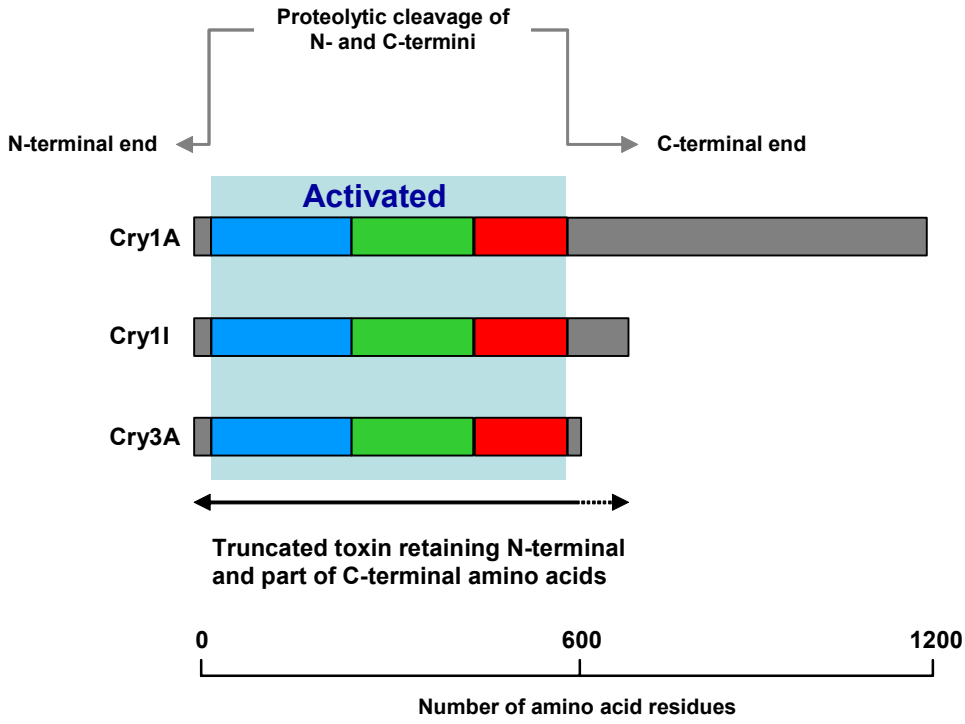
4.4.2 Survival of ingested toxin under gut conditions

After ingestion by potential hosts, *Bt* toxins enter the midgut environment and encounter a set of conditions determined by the taxonomic grouping of the organism and of its feeding strategy. Conventional *Bt* toxin interaction with the gut environment with respect to pH solubilisation, proteolysis to activate the toxin and penetration of the peritrophic membrane to enable encounter with the surfaces of midgut cells was discussed in Chapter 2.1.6. Conventionally applied *Bt*, whether it is present on foliage or in the soil, is subject to the full set of activation processes before it has the opportunity to encounter gut cell membranes. There is general consensus that gut barriers, such as the need for highly alkaline pH to initiate solubilisation of the protoxin, contribute to specificity and likelihood of toxicity in a given organism (Schnepf *et al.*, 1998). Although some Cry genes have now been shown to have dual (Bradley *et al.*, 1995) and triple (Zhong *et al.*, 2000) insect order toxicity, the majority shown in Figure 2.1: are restricted to one order or even family of organisms.

However, the data on which such assumptions are made are generally derived from studies of full protoxin activation processes and do not necessarily apply to transgenic sources of *Bt* that express truncated toxins.

One of the main concerns in assessing risks from truncated *Bt* toxins is the fact that the protoxin is already cleaved to virtually the toxic core of the given construct. However, the toxin is not fully active and it is more accurate to describe it as truncated with reference to its status in transgenic plant tissues. Further processing to remove the final amino acids at the N- and C-termini of the truncated toxin is, therefore, required. The concepts are illustrated in Figure 4.5, modified from de Maagd *et al* (1999).

Figure 4.5: Conceptual illustration of the structure of truncated genes incorporated into transgenic plants, based on de Maagd *et al* (1999).



Truncation takes place from the C-terminal end and the degree of truncation varies with the length of the existing protoxin. Thus Cry 1A is longer than Cry 1I but some Cry genes, such as Cry2 and Cry3 have very short C-termini that may, as in Cry6, lack some or all of the five conserved blocks present in many of the Cry genes (Schnepf *et al.*, 1998). Further processing of the truncated Cry genes is not necessarily pH-dependent but certainly requires the presence of proteolytic enzymes to provide the final cleavage steps to expose the activated toxin.

There is evidence for specific enzyme requirements to ensure that the proteolysis proceeds to a suitable end-point and to prevent inactivation by excessive cleavage. This was demonstrated for Cry1Ac activity in *Pieris brassicae* (a susceptible butterfly in the family Pieridae) and *Mamestra brassicae* (2000 time less susceptible, in the

family Noctuidae) (Lightwood *et al.*, 2000). The proteases in the gut of *M. brassicae* resulted in insoluble products with molecular masses of around 58, 40 and 20 kDa, whereas the proteases from *P. brassicae* produced only a toxic product of 56 kDa. The difference occurred in N-terminal sequencing, which took place at an additional arginine site in Domain II in *M. brassicae.* This finding was confirmed *in vivo* and by replacement of the Arg sequence with Gln or Ser. However, it was also shown that potency did not increase when the Arg sequence was replaced and, therefore, it was thought that other factors, probably differential toxin proteolysis during membrane binding, were also involved in the low toxicity in *M. brassicae.*

Differential proteolysis and its influence on both toxicity and specificity has been postulated (e.g. complete degradation of *Btk* by *Spodoptera litura* gut proteases (Inagaki *et al.*, 1992)). Effects on host range have also been linked to differential proteolysis so that Cry1Ab exposed to lepidopteran gut enzymes produced a 55 kDa protein toxic only to Lepidoptera but if this fragment was further treated with dipteran gut proteases, a 53 kDa toxin active against Diptera was produced (Haider *et al.*, 1986).

One of the key areas in relation to risk assessment of encounter within the guts of potential hosts is, therefore, whether truncated toxins are more or less likely to survive and to be fully activated by further, albeit minor, proteolytic cleavage. In particular, the question of whether proteolysis contributes to changes in host range must be addressed specifically. Coleoptera-active Cry3 genes have already been highlighted as being intrinsically more likely to pose a risk because of the high probability of encounter with non-pest Coleoptera in the soil environment (Chapter 4.2.2). Gut pH conditions differ considerably between the Coleoptera (neutral to acid) and Lepidoptera or Diptera (highly alkaline) but, because the truncated toxins do not require solubilisation, this may not be a critical factor. In any event, Cry3A in conventional application is rather restricted in its activity profile and is particularly active in the family Chrysomelidae (Keller & Langenbruch, 1993), which have midgut pH values of 4.5-6.6. Proteolysis in Coleopteran midguts is driven mainly by cysteine proteases that are optimal at slightly acid pH. Studies of solubilisation and proteolytic cleavage of Cry3A suggest that the protoxin is not easily solubilised at neutral to acid pH conditions, even in susceptible larvae (Koller *et al.*, 1992). Solubilisation of Cry3A revealed that polypeptides were soluble in alkaline and acid conditions but precipitated under acid conditions likely to be encountered in the gut of *L. decemlineata* (Carroll *et al.*, 1997). Proteolytic treatments showed that a chymotrypsinized product was soluble under neutral pH and was fully active. Despite the enzyme cleaving the toxin into three polypeptides of 49, 11 and 6 kDa, they remained associated in solution, suggesting that all three components are needed for membrane binding (Hofte & Whiteley, 1989).

Studies of the development of resistance to *Bt* have indicated linkage between altered proteolytic processing and susceptibility to the bacterial toxin (Ferré & Van Rie, 2002). A resistant colony of *Plodia interpunctella* was found to lack a major gut protease required for activation of Cry1Ac (Oppert *et al.*, 1997), but this is generally not regarded as a primary factor in altered susceptibility (Liu *et al.*, 2000).

This level of complexity makes it difficult to predict in advance whether the truncated toxins have a competitive advantage over protoxins in a given gut environment. The

fact that target host species appear to be as susceptible to truncated *Bt* as to conventional *Bt* tends suggests that pH and gut enzyme conditions are not highly detrimental to the truncated toxin. However, considerably more study is needed of the survival of truncated *Bt* in the guts of non-target organisms before it is possible to be more definitive in assessing relative survival in this part of the encounter model.

4.4.3 Likelihood of Cry toxin binding to midgut receptors

Assuming that toxin has survived the gut pH and enzyme milieu and has been further cleaved to a fully activated toxic core, the next step in the possible development of toxicity is binding to midgut receptors (Aronson & Shai, 2001). This process was described in Chapter 2.1.6.3 and further discussion here will concentrate on the relative performance of truncated toxin. As indicated previously and illustrated in Figure 2.3: , the fully activated toxin binds to receptor sites, principally through Domains II and III (de Maagd *et al.*, 2001). Binding at this stage is reversible and it is only the final stage of insertion of Domain I into the membrane that leads to irreversible binding, pore formation and ultimate toxicity.

The question of whether truncated toxins that have undergone further proteolysis to become potentially fully active behave in a different way to protoxins that have been solubilised and enzyme digested in the same gut environment has not been addressed specifically. This is a key component determining the final irreversible binding discussed in the next section. Studies of the general question of binding specificity may, however, throw some light on this important final stage in the encounter model. Schnepf *et al* (1998) summarised research into the effects of mutations of domain II in relation to various parameters such as effects on binding and changes in toxicity. In some cases, such as mutations in Cry1Ac, increases in toxicity of 1000 fold accompanied large reductions in competition for binding sites (Borgonie *et al.*, 1996). Mutagenesis experiments are increasing our understanding of the determinants of toxicity and specificity so that, for example, swapping of domain II between different toxins has demonstrated changes in host range and in membrane binding (Rang *et al.*, 1999). With respect to prediction of the effects of *Bt* transgenic plants, knowledge of the precise composition of the inserted toxin gene, particularly any alteration in domains II and/or III is critical in assessing whether host range shifts are likely. This level of sophistication is not yet available but would be a key element of future research into environmental risk from transgenic plants expressing *Bt*.

On the basis of current information, particularly the lack of demonstrated host range effects in the relatively limited laboratory and field evaluations that have been carried out, all current transformations appear to conform with the expected receptor binding dynamics of the parental *Bt*.

4.4.4 Likelihood of pore formation and cell disruption – toxicity.

As indicated in Chapter 2.1.6.3, toxicity and direct effects of *Bt* in host organisms is ultimately dependent on insertion of domain I and the formation of ion channels, leading to cell lysis. This is intimately linked to risk assessment of the reversible binding steps discussed in the previous section and, therefore, the same arguments

concerning putative host range and final expression of the *Bt* toxin apply. The linkage between the expanding number of Cry genes recognised and registered in the Crickmore *et al* (2002) electronic database and actual host ranges in field situations is tenuous. Initial correlation between Cry gene nomenclature and host range (Hofte & Whiteley, 1989) has been superseded by new nomenclature based on sequence homology (Crickmore *et al.*, 1998). Groupings within the domain tree in Figure 2.1: suggest that some genes are still restricted to particular insect orders or named hosts but that others, such as Cry1Ba1 are now known to extend to two or more orders. With increased understanding of receptor binding (domain II and III) and, particularly, insertion characteristics (domain I), attention should now be paid to whether it is possible to predict changes of host range on the basis of detailed Cry structure. This is made even more urgent by the need to understand and manage the development of resistance to *Bt* under both conventional and transgenic delivery systems.

5 Conclusions and research needs

The purpose of this review has been to assess the environmental consequences of a newly identified pathway for entry of *Bt* into soil via root exudates. Within this specific remit, the approach taken has deliberately widened the scope of the background information and discussion in order to place the exudates pathway into the wider context of natural and man-assisted presence of *Bt* in the environment. In doing so, it has gone beyond the ecological aspects of encounter frequency at the gross organism level implicit in the conceptual model described in Chapter 4.1 and has taken the encounter analogy down to the gut and gut cell level, reflecting the critical role of this "environment" in determining likelihood of a perceived hazard being realised. The key elements and the need for further research are, therefore, based on the evaluations in the main part of this review, particularly Chapter 4.

5.1 Hazard – Cry toxin effects on non-target organisms and possible development of resistance

Bt as a microbial control agent delivers its effects through inducing toxicity in target organisms and, as such, it has a long-standing and safe record of use (1993). Its credentials are such that it is regarded as safe to use, even in organic crop production and, therefore, there is a high level of sensitivity to any potential long-term threat to its availability. Conventional methods of application, usually by spray application from ground or air, introduce locally high concentrations of a spore/toxin combination that has to be ingested by the target host leading to solubilisation and proteolysis and, finally, to receptor binding and pore formation in gut cells. Despite the large quantities of *Bt* that are applied in this way, the environmental persistence is generally regarded as low and so the ecological hazard of retained inoculum and potential effects on non-target hosts has been regarded as acceptable.

Production of transgenic crops to deliver *Bt* toxin directly to the site of feeding by target hosts would appear to offer gains in selective delivery of the microbial agent and potential reductions in the use of broad spectrum chemical insecticides (Shelton *et al.*, 2002). At the same time, new hazards from delivery of *Bt* through this novel pathway have been identified:

1. Continuous expression of *Bt* toxin through the life of the plant may lead to development of resistance to the toxin in target organisms as a result of continuous exposure and selection of tolerant individuals. There is already evidence for development of resistance where *Bt* has been over-used in conventional application systems.

2. The use of a truncated toxin removes some of the steps that contribute to host specificity (pH, proteolysis, penetration of the peritrophic membrane) and could result in extension of host range to non-target organisms.

3. Although *Bt* is primarily expressed in the above-ground tissues of transgenic crops, recent research has indicated that the truncated toxin is delivered to the

soil through root exudates. This has been shown to persist for long periods by adsorption to clays and humic acids and could present a threat to non-target organisms encountering the toxin in soil.

The second and third hazards are the main subject of this review and are closely inter-related.

5.2 Risk – likelihood of hazard being realised

As with all risk assessment exercises, it is important to distinguish between recognition of a potential hazard and the likelihood of that hazard actually being realised. The Precautionary Principle can be applied to all new situations where there is little information available on the identified hazard and there have certainly been calls to reduce or ban the introduction of transgenic crops on this basis. However, the fact that a considerable body of research and field information exists and that introduction of transgenic crops has already progressed through a precautionary case by case approach, indicates that a "do nothing" option is not appropriate to the current reality of 11.4 million ha of *Bt* crops worldwide. A rational approach to risk assessment based on sound principles is, therefore, required and this review has attempted to identify the sequence of events that would enable actual risk, as opposed to hazard, to be identified.

5.2.1 Risk is dependent on survival of toxin through a sequence of events

The hazards identified in Chapter 5.1 are key to risk assessment and can be considered together, reflecting the novel nature of the toxin and one among several modes of delivery of that toxin. Taking the conceptual model of encounter in Chapter 4.1, risk assessment requires a step-wise evaluation of survival of the *Bt* toxin and relative likelihood of encounter at each stage. Quantification of toxin loads during the lifetime of the crop and in the soil environment is important in this process. It has not been easy to obtain comparative data on quantities of toxins in either of these situations. This reflects the wide range of data sources and the lack of standardisation in providing the information. This is particularly true for information provided by the main agro-chemical companies in support of their applications for release and/or marketing to regulatory agencies. Data have, therefore, been converted to metric units and, where no direct information was available, extrapolated from related sources. This inevitably introduces possible sources of error if some of the assumptions made in extrapolation are erroneous.

Despite the uncertainties, it is possible to consider, at least in outline, the relative survival of Cry toxins in the three principal zones of encounter; the plant, rhizosphere and bulk soil. Hazards in relation to non-target effects can then be related to potential encounter with sources of *Bt* by organisms in these zones, both in the wider environment and also in the "gut environment", which is the final determinant of toxicity.

Table 5.1 provides a budget for *Bt* delivery and retention in the "model" system of *Bt*-corn expressing Cry1Ab, the toxin for which most information is available. The table uses data presented in Table 4.4, Chapter 4.3.2 and extrapolates from data on retention of activity provided by Tapp & Stotzky (1998).

Table 5.1: Budget for *Bt*-corn (Cry1Ab) in relation to risk profiles. Data are based on Table 4.4, Chapter 4.3.2. Assumptions: leaching rate is 75% for sandy loam and 16% for montmorillonite enriched soil (Saxena *et al.*, 2002b). Decline rate over time based on Tapp & Stotzky (1998), minimum and maximum loss rates for sandy and clay-amended soils.

		Concentration of *Bt* by location over time (ng/g)	
Above ground	Whole plant (peak)	31301	
Below ground			
Rhizosphere	Initial values		
	Plant debris	13964	
	Exudate	250	
	Total for *Bt*-corn	14214	
	Conventional *Bt*	1790	
	After leaching		After 200 days
Sandy loam	Plant debris	3491	175
	Exudate	63	3
	Total for *Bt*-corn	3554	178
	Conventional *Bt*	448	22
Clay-rich (12%M)	Plant debris	11730	5865
	Exudate	210	105
	Total for *Bt*-corn	11940	5970
	Conventional *Bt*	1504	752
Bulk soil*	After leaching		
Sandy loam	Plant debris	10473	524
	Exudate	188	9
	Total for *Bt*-corn	10661	533
	Conventional *Bt*	1343	67
Clay-rich (12%M)	Plant debris	2234	1117
	Exudate	40	20
	Total for *Bt*-corn	2274	1137
	Conventional *Bt*	286	143

*No allowance made for the dilution factor of moving from the small volume of the rhizosphere to the very large volume of the bulk soil. Actual concentration per g soil is likely to be much lower.

Although many of the assumptions are subject to considerable potential error, the message in relation to total toxin loads, both during peak delivery and over time, is clear. *Bt* entering soil through the medium of root exudates is lower than either bulk incorporation of *Bt*-corn or through spray application of conventional *Bt*. It is difficult to estimate the profile of exudation over the life of the crop, but the average value

used in the table is probably a reasonable estimate since it uses the quantity delivered to soil from young plants. At this stage of growth, exudation is at its maximum and, thereafter, declines significantly (Gransee & Wittenmayer, 2000). Saxena *et al* (2002b) also measured the rate of leaching of *Bt* toxin from soil columns, either in normal sandy loam field soil or in the same soil amended with montmorillonite. These data point to movement of *Bt* from the immediate rhizosphere into the bulk soil, the rate depending on the proportion of clay available for rapid adsorption of the toxin. Although not included in Table 5.1, it can be assumed that the dilution factor on entry to the bulk soil will also contribute to reducing the concentration of *Bt* per gram of soil in that zone. Active *Bt* is still present up to 234 days after incorporation into soil (the longest time measured) (Tapp & Stotzky, 1998). As indicated in Figure 3.2, Chapter 3.6, rates of decline in soil vary with the proportion of clay in the soil and, at best might still be around 50% (after approximately 200 days, the value used as a maximum measure in Table 5.1). Thus, quantitatively, exudate *Bt* does not pose a higher risk than *Bt* entering the soil from transgenic plants or, to a lesser extent, conventional application.

When considering overall risk, therefore, it is not the source of *Bt*, whether by exudation or incorporation of transgenic plant material, but the interaction with soil biota that is important. In this respect the risk profile from *Bt* derived from transgenic plants does differ from that posed by conventional application. The removal of elements of the gut barrier to toxicity is an important consideration. Although reference to activated toxin being present in plant tissue is not strictly correct, the fact that the toxin is truncated to a greater or lesser extent does change the risk profile. Direct transfer from ingested food to allow encounter with the membranes of gut epithelial cells with only minimal further proteolytic processing is, therefore, possible. This leaves the remaining specificity determinants of receptor binding (reversible) and domain I insertion (irreversible) as the only remaining barriers to toxicity. There has been surprisingly little work to assess this aspect of risk, especially in the soil environment where intimate contact with *Bt* is possible over relatively longer periods than in the phylloplane. It is encouraging that the studies that have been carried out so far have shown no significant negative effects on higher and lower trophic levels. Indeed, it would appear that the specificity profiles of the original sources of *Bt* are retained in the truncated forms, suggesting that the key determinant of specificity is receptor binding rather than either pH or enzyme constituents in the gut (Schnepf *et al.*, 1998). Mutagenesis studies in relation to specific functions of the various Cry genes would appear to bear this out, especially in developing an understanding of the alteration in binding sites noted in insects known to be resistant to *Bt* (Ferré & Van Rie, 2002). Age related change in susceptibility to *Bt* in larvae, which is a well known phenomenon in relation to targeting conventional *Bt* applications, is now being studied in relation to the gut environment and has also shown that loss of binding receptors are important contributors to declining susceptibility. For example, final instar larvae of pine processionary moth *Thaumetopoea pityocampa* and nun moth *Lymantria monacha* were shown to lose high affinity binding sites for Cry1Ab, which was correlated with declining susceptibility to the toxin (Rausell *et al.*, 2000).

5.3 Key research needs

A number of gaps in current knowledge have already been mentioned in assessing the overall impact of root exudation of *Bt* in the wider context of total delivery of *Bt* in crop systems. The key research needs are summarised below.

5.3.1 Relative fitness of truncated toxins

The role and fate of truncated toxins is one of the key aspects of risk assessment in comparing transgenic plants to conventional *Bt* applications. The mode of delivery of the toxin does not appear to alter the quantitative aspects and so research needs to address both exudation and entry to the soil from debris of transgenic plants. Research needs are addressed by posing a series of questions and, where appropriate, suggesting methods of approach or of presentation of data.

5.3.1.1 Environment

Do the truncated toxins differ in survival characteristics once they have entered the environment?

This question covers aspects of competitive performance of the truncated forms of Cry genes and relates to both "free" toxins delivered in root exudates or by "incorporated" toxins in whole or decomposing plant material. An essential part of this process is accurate quantification of toxins. This aspect is poorly developed in current research into transgenic plants, not aided by inconsistencies in expressing toxin loads. Considering that up to 11 million ha of transgenic crops were planted world-wide in 2000, it is surprising that so little opportunity has been taken to carry out statistically valid observation and field experimentation. The collaborative studies to address the risks posed by pollen from transgenic corn on monarch butterflies indicate that well thought out quantitative risk assessments can be carried out (Hellmich *et al.*, 2001; Oberhauser *et al.*, 2001; Pleasants *et al.*, 2001; Sears *et al.*, 2001; Stanley-Horn *et al.*, 2001; Zangerl *et al.*, 2001).

Quantitative data on *Bt* budgets in soil before, during and after planting *Bt*-crops, accompanied by parallel assessments in non-planted or alternative crop systems would add immeasurably to the knowledge base on field performance and assessment of truncated *Bt*. Sampling procedures to provide quantitative descriptions of key soil fauna and flora in both the rhizosphere and bulk soil would also be valuable in assessing risk. However, it is always difficult to assess small changes in ecosystem dynamics and, therefore, evaluation of alternative research approaches, outlined below, might be a better approach to targeting which organisms to assess in the field.

5.3.1.2 Within the guts of putative hosts in relation to pH, enzymes and receptor binding

Does truncated Bt have a wider host range than the protoxin from which it is derived?

There is already excellent and painstaking work being carried out world-wide on the gut environment and its implications for *Bt* performance. Many of the approaches use mutagenesis to probe the precise nature of amino acid composition on the relative contributions of domains I, II and III in receptor binding. However, there has been little direct study on the relative performance of different truncated Cry genes as incorporated in transgenic plants. What exactly are the dynamics of truncated *Bt* Cry toxins from the time they enter the guts of potential hosts to either their successful binding/pore formation or elimination from the host? Studies of a range of potential hosts to represent organisms with wide extremes of gut conditions, particularly pH and enzyme constitution, are essential in determining whether specificity has been compromised by truncation. Only then will a more considered assessment of risks be possible.

5.3.1.3 Identification of vulnerable organisms in relation to risk profile

If gut conditions imply that changes in specificity can be expected from employment of truncated Bt, what are the implications for organisms in the field?

This aspect is deliberately placed after determination of the gut specificity questions. Identification of putative changes in specificity would pose a new hazard and risk assessment would then proceed to determining the likelihood of that hazard being realised. It is not realistic to assess the entire very wide array of organisms present in an agro-ecosystem, especially in the soil and, therefore, a step-wise approach based on hazard identification would provide a more rational framework to maximise research resources.

5.4 Ecological framework for rational risk assessment

The interactions that will determine whether *Bt* delivered to the environment pose an unacceptable threat to one or more non-target organisms take place at a number of trophic levels. However, the key effects are driven initially by encounter frequency between a defined *Bt* load and the array of organisms likely to consume the toxin. Such encounters only "count" if the gut environment is suitable for binding and pore formation, leading to mortality of the host. Ecological impact will, therefore, be driven both at the population level, requiring assessment of species diversity and abundance, and at the organ level within putative hosts, requiring broad risk categorisation of those groups that might be vulnerable to the truncated *Bt*. This poses a dilemma in determining which is the dominant factor in determining risk. In relation to return from effort expended, it would appear that accelerated effort into determining interactions at the gut level will provide the framework necessary for

designing and interpreting field sampling programmes to assess both efficacy and unintentional impacts of transgenic crops.

5.5 Concluding summary

Despite a relative lack of detailed information on the interactions between putative hosts and truncated toxins, the balance of probability indicates that truncation does not substantially change the host range of such toxins. In relation to the specific objective of assessing the relative risks from the root exudation pathway of entry of *Bt* to soil, data indicate that quantities of toxin introduced in this way are no greater than from other pathways. Impacts of root exudation must, therefore, be considered as part of a total *Bt* load entering the soil as a consequence of planting transgenic *Bt* crops.

There is a surprising lack of quantitative information on the total load of *Bt* in soil beneath transgenic crops, thus pointing to the need for more research in this area. Methods are available for accurate quantification and also for assessment of potential impacts, both at the gross population level and by direct study of the effects of reversible and irreversible binding to gut receptors. At the same time, indirect effects involved in tritrophic interactions can also be considered, although these do not appear to be influenced directly by the *Bt* toxin and are more the result of change in abundance of prey or host items for natural enemies.

The planting of large areas of transgenic *Bt*-crops should be taken as an opportunity for quantitative assessment of impacts on agro-ecosystem function in comparison with isogenic lines of the same crops. Opportunities exist for fully replicated tests, taking advantage of the refuge strategies being adopted for management of potential resistance to transgenically delivered *Bt*. Use of molecular and biological methods for assessing effects are essential in such studies, but the approaches adopted should be optimised in relation to investigations of the interactions in the gut environments of putative hosts. Greater collaboration between research communities embracing studies of crop dynamics linked to soil flora and fauna carried out at both the organism and molecular levels are necessary to establish a framework for risk assessment.

6 Acknowledgements

I would like to express my sincere appreciation to Professor G. Stotzky and Dr D. Saxena (New York University) for their generosity in devoting time and resources during my visit to their department in November 2001. The provision of unpublished or in press material is particularly appreciated. Thanks are also due to Dr J. Losey and Dr D Pimentel (Cornell University) for useful discussions on risks from transgenic plants and to the Union of Concerned Scientists for their generous welcome to their meeting on GM plants held at Cornell University. Dr D Ellar (Cambridge University) provided the images for Figure 2.2 and also useful comments on aspects of *Bt* specificity for which I am most grateful. Dr N Crickmore also generously provided useful views on receptor binding and I would like to extend my thanks to him.

Miss Sara Brough worked with me on this task and carried out the bulk of the literature scanning and procurement as well as great help in summarising information, particularly for some of the tables in the report. She showed patience and interest throughout and I am very grateful for her assistance.

The task of scanning the great array of literature on *Bt* and writing the report often spilled into my home and my wife and family showed considerable forbearance throughout, for which I am give them heartfelt thanks.

7 References

Adang, M. J. & Spence, K. D. (1983). Permeability of the peritrophic membrane of the Douglas fir tussock moth (*Orgyia pseudotsugata*). *Comparative Biochemistry and Physiology, A* **75**, 233-238.

Adang, M. J., Staver, M. J., Rocheleau, T. A., Leighton, J., Barker, R. F., & Thompson, D. V. (1985). Characterized full length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene* **36**, 289-300.

Addison, J. A. (1993). Persistence and nontarget effects of *Bacillus thuringiensis* in soil - a review. *Canadian Journal of Forest Research-Revue Canadienne de Recherche Forestiere* **23**, 2329-2342.

Aer'aqua Medicine Ltd & (formerly Jenner Consultants Ltd). (2001) Health Surveillance following Operation Ever Green: A programme to eradicate the white-spotted tussock moth from the eastern suburbs of Auckland. 1-85. Aer 'aqua R Medicine Ltd (formerly Jenner Consultants Ltd). Unpublished Report

Al Deeb, M. A., Wilde, G. E., & Higgins, R. A. (2001). No effect of *Bacillus thuringiensis* corn and *Bacillus thuringiensis* on the predator *Orius insidiosus* (Hemiptera : Anthocoridae). *Environmental Entomology* **30**, 625-629.

Alam, M. F., Datta, K., Abrigo, E., Vasquez, A., Senadhira, D., & Datta, S. K. (1998). Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiensis* cryIA(b), gene with enhanced resistance to yellow stem borer. *Plant Science* **135**, 25-30.

Alinia, F., Ghareyazie, B., Rubia, L., Bennett, J., & Cohen, M. B. (2000). Effect of plant age, larval age, and fertilizer treatment on resistance of a cry1Ab-transformed aromatic rice to lepidopterous stem borers and foliage feeders. *Journal of Economic Entomology* **93**, 484-493.

Angus, T. A. (1954). A bacterial toxin paralysing silkworm larvae. *Nature* **173**, 545-546.

Ankarloo, J., Caugant, D. A., Ankarloo, J., Caugant, D. A., Hansen, B. M., Berg, A., Kolsto, A. B., & Lovgren, A. (2000). Genome stability of *Bacillus thuringiensis* subsp *israelensis* isolates. *Current Microbiology* **40**, 51-56.

Appel, H. M. & Schultz, J. C. (1994). Oak tannins reduce effectiveness of thuricide (*Bacillus thuringiensis*) in the gypsy moth (Lepidoptera, Lymantriidae). *Journal of Economic Entomology* **87**, 1736-1742.

Archer, T. L., Patrick, C., Schuster, G., Cronholm, G., Bynum, E. D., & Morrison, W. P. (2001). Ear and shank damage by corn borers and corn earworms to four events of *Bacillus thuriengiensis* transgenic maize. *Crop Protection* **20**, 139-144.

Archer, T. L., Schuster, G., Patrick, C., Cronholm, G., Bynum, E. D., & Morrison, W. P. (2000). Whorl and stalk damage by European and Southwestern corn borers to four events of *Bacillus thuringiensis* transgenic maize. *Crop Protection* **19**, 181-190.

Arencibia, A., Vazquez, R. I., Prieto, D., Tellez, P., Carmona, E. R., Coego, A., Hernandez, L., delaRiva, G. A., & SelmanHousein, G. (1997). Transgenic sugarcane plants resistant to stem borer attack. *Molecular Breeding* **3**, 247-255.

Aronson, A. I. & Shai, Y. (2001). Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *Fems Microbiology Letters* **195**, 1-8.

Atwood, D. W., Young, S. Y., & Kring, T. J. (1997). Development of *Cotesia marginiventris* (Hymenoptera: Braconidae) in tobacco budworm (Lepidoptera: Noctuidae) larvae treated with *Bacillus thuringiensis* and thiodicarb. *Journal of Economic Entomology* **90**, 751-756.

Axaizeh, H. A., Marschner, H., Romheld, V., & Wittenmayer, L. (1995). Effects of a vesicular-arbuscular mycorrhizal fungus and other soil microorganisms on growth,

mineral nutrient acquisition and root exudation of soil-grown maize plants. *Mycorrhiza* **5**, 321-327.

Bajwa, W. I. & Aliniazee, M. T. (2001). Spider fauna in apple ecosystem of western Oregon and its field susceptibility to chemical and microbial insecticides. *Journal of Economic Entomology* **94**, 68-75.

Barton, K. A., Whiteley, H. R., & Yang, N. S. (1987). *Bacillus thuringiensis* delta endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiology* **85**, 1103-1109.

Bauer, L. S. (1995). Resistance: A threat to the insecticidal crystal proteins of *Bacillus thuringiensis*. *Florida Entomologist* **78**, 414-443.

Becker, N. & Margalit, J. (1993). Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 147-170. John Wiley & Sons Ltd, Chichester.

Bell, J. L. & Whitmore, R. C. (1997). Bird populations and habitat in *Bacillus thuringiensis* and dimilin-treated and untreated areas of hardwood forest. *American Midland Naturalist* **137**, 239-250.

Berenbaum, M. (1980). Adaptive significance of midgut pH in larval Lepidoptera. *American Naturalist* **115**, 138-146.

Berg, M., de Ruiter, P., Didden, W., Janssen, M., Schouten, T., & Verhoef, H. (2001). Community food web, decomposition and nitrogen mineralisation in a stratified Scots pine forest soil. *Oikos* **94**, 130-142.

Berliner, E. (1915). Über die Schlaffsucht der Mehlmottenraupe. *Zeitschrift fur Angewandte Entomologie* **2**, 29-56.

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D. J., Roberts, G. M., Pauli, S., Rodgers, P., & Burges, H. D. (1997). Natural isolates of *Bacillus thuringiensis*: Worldwide distribution, characterization, and activity against insect pests. *Journal of Invertebrate Pathology* **70**, 59-68.

Bernhard, K. & Utz, R. (1993). Production of *Bacillus thuringiensis* insecticides for experimental and commercial uses. In *Bacillus thuringiensis, an environmental biopesticide: Theory and practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 255-267. John Wiley & Sons, Chichester.

Beveridge, N. & Elek, J. A. (1999). *Bacillus thuringiensis* var. *tenebrionis* shows no toxicity to the predator *Chauliognathus lugubris* (F.) (Coleoptera : Cantharidae). *Australian Journal of Entomology* **38**, 34-39.

Bonkowski, M., Cheng, W. X., Griffiths, B. S., Alphei, G., & Scheu, S. (2000). Microbial-faunal interactions in the rhizosphere and effects on plant growth. *European Journal of Soil Biology* **36**, 135-147.

Borgonie, G., Claeys, M., Leyns, F., Arnaut, G., DeWaele, D., & Coomans, A. (1996). Effect of a nematicidal *Bacillus thuringiensis* strain on free- living nematodes .3. Characterization of the intoxication process. *Fundamental and Applied Nematology* **19**, 523-528.

Bottjer, K. P., Bone, L. W., & Gill, S. S. (1985). Nematoda: susceptibility of the egg to *Bacillus thuringiensis* toxins. *Experimental Parasitology* **60**, 239-244.

Bowen, G. D. (1994). The ecology of ectomycorrhiza formation and functioning. *Plant and Soil* **159**, 61-67.

Bradley, D., Harkey, M. A., KIM, M. K., Biever, K. D., & Bauer, L. S. (1995). The insecticidal CryIB crystal protein of *Bacillus thuringiensis* ssp *thuringiensis* has dual specificity to coleopteran and lepidopteran larvae. *Journal of Invertebrate Pathology* **65**, 162-173.

Breitler, J. C., Cordero, M. J., Royer, M., Meynard, D., San Segundo, B., & Guiderdoni, E. (2001). The-689/+197 region of the maize protease inhibitor gene directs high level, wound-inducible expression of the cry1B gene which protects transgenic rice plants from stemborer attack. *Molecular Breeding* **7**, 259-274.

Briggs, S. P. & Koziel, M. (1998). Engineering new plant strains for commercial markets. *Current Opinion in Biotechnology* **9**, 233-235.

Brownbridge, M. (1991). Native *Bacillus thuringiensis* isolates for the management of lepidopteran cereal pests. *Insect Science and Its Application* **12**, 57-61.

Brussaard, L. (1998). Soil fauna, guilds, functional groups and ecosystem processes. *Applied Soil Ecology* **9**, 123-135.

Bryant, J. E. (1994). Commercial production and formulation of *Bacillus thuringiensis* . *Agriculture Ecosystems & Environment* **49**, 31-35.

Buntin, G. D., Lee, R. D., Wilson, D. M., & McPherson, R. M. (2001). Evaluation of yieldgard transgenic resistance for control of fall armyworm and corn earworm (Lepidoptera : Noctuidae) on corn. *Florida Entomologist* **84**, 37-42.

Burges, H. D.Burges, H. D. Ed. (1981). *Microbial control of pests and plant diseases 1970-1980*, pp. 1-949. Academic Press, London.

Burges, H. D.Burges, H. D. Ed. (1998). *Formulation of Microbial Biopesticides, Beneficial Micro-organisms and Nematodes*, pp. 1-412. Kluwer Academic, Dordrecht.

Burges, H. D. & Hurst, J. A. (1977). Ecology of *Bacillus thuringiensis* in storage moths. *Journal of Invertebrate Pathology* **30**, 131-139.

Burges, H. D. & Hussey, N. W. E. (1971). *Microbial Control of Insects and Mites*, pp. 1-861. Academic Press, London.

Burkness, E. C., Hutchison, W. D., Bolin, P. C., Bartels, D. W., Warnock, D. F., & Davis, D. W. (2001). Field efficacy of sweet corn hybrids expressing a *Bacillus thuringiensis* toxin for management of *Ostrinia nubilalis* (Lepidoptera : Crambidae) and *Helicoverpa zea* (Lepidoptera : Noctuidae). *Journal of Economic Entomology* **94**, 197-203.

Burton, S. L., Ellar, D. J., Li, J., & Derbyshire, D. J. (1999). N-acetylgalactosamine on the putative insect receptor aminopeptidase N is recognised by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *Journal of Molecular Biology* **287**, 1011-1022.

Buse, A., Good, J. E. G., Dury, S., & Perrins, C. M. (1998). Effects of elevated temperature and carbon dioxide on the nutritional quality of leaves of oak (*Quercus robur* L.) as food for the winter moth (*Operophtera brumata* L.). *Functional Ecology* **12**, 742-749.

Cadogan, B. L. & Scharbach, R. D. (1993). Efficacy of Foray 48B (*Bacillus thuringiensis* Berliner) applications against the spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera, Tortricidae), timed for phenological development of balsam fir and black spruce. *Canadian Entomologist* **125**, 479-488.

Cardinal, P. & Marotte, P. M. (1987) Persistance des spores de *Bacillus thuringiensis* dans le sol forestier suite auz pulvérisations aériennes contre la tordeuse des bourgeons de l'epinetter au Québec en 1984. Québec, Gouvernement du Québec, Ministère de l'Energie et des Ressources, Direction de la Conservation. Report

Carpenter, J. E. & Gianessi, L. P. (2001) Agricultural biotechnology: updated benefit estimates. http://www.ncfap.org/biotech/updatedbenefits.pdf, 1-48. National Center for Food and Agricultural Policy, Washington, DC. Electronic Citation

Carroll, J., Convents, D., VanDamme, J., Boets, A., Van Rie, J., & Ellar, D. J. (1997). Intramolecular proteolytic cleavage of *Bacillus thuringiensis* Cry3A delta-endotoxin may facilitate its coleopteran toxicity. *Journal of Invertebrate Pathology* **70**, 41-49.

Chapman, M. & Hoy, M. A. (1991). Relative toxicity of *Bacillus thuringiensis* var. *tenebrionis* to the two-spotted spider mite (*Tetranychus urticae* Koch) and its predator *Measeiulus occidentalis* (Nesbitt) (Acari, Tetranychidae and Phytoseiidae). *Journal of Applied Entomology* **111**, 147-154.

Cheng, X. Y., Sardana, R., Kaplan, H., & Altosaar, I. (1998). *Agrobacterium*-transformed rice plants expressing synthetic cryIA(b) and cryIA(c) genes are highly toxic to striped stem borer and yellow stem borer. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2767-2772.

Chilcott, C. N. & Wigley, P. J. (1993). Isolation and toxicity of *Bacillus thuringiensis* from soil and insect habitats in New Zealand. *Journal of Invertebrate Pathology* **61**, 244-247.

Chilcutt, C. F. & Tabashnik, B. E. (1997a). Host-mediated competition between the pathogen *Bacillus thuringiensis* and the parasitoid *Cotesia plutellae* of the diamondback moth (Lepidoptera: Plutellidae). *Environmental Entomology* **26**, 38-45.

Chilcutt, C. F. & Tabashnik, B. E. (1997b). Independent and combined effects of *Bacillus thuringiensis* and the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) on susceptible and resistant diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology* **90**, 397-403.

Chilcutt, C. F. & Tabashnik, B. E. (1999). Effects of *Bacillus thuringiensis* on adults of *Cotesia plutellae* (Hymenoptera : Braconidae), a parasitoid of the diamondback moth, *Plutella xylostella* (Lepidoptera : Plutellidae). *Biocontrol Science and Technology* **9** , 435-440.

Christeller, J. T., Liang, W. A., Markwick, N. P., & Burgess, E. P. J. (1992). Midgut protease activities in 12 phytophagous lepidopteral larvae - dietary and protease inhibitor interactions. *Insect Biochemistry and Molecular Biology* **22**, 735-746.

Christensen, S., Griffiths, B. S., Ekelund, F., & Ronn, R. (1992). Huge increase in bacterivores on freshly killed barley roots. *Fems Microbiology Ecology* **86**, 303-309.

Cloutier, C. & Jean, C. (1998). Synergism between natural enemies and biopesticides: a test case using the stinkbug *Perillus bioculatus* (Hemiptera : Pentatomidae) and *Bacillus thuringiensis tenebrionis* against Colorado potato beetle (Coleoptera : Chrysomelidae). *Journal of Economic Entomology* **91**, 1096-1108.

Cooke, B. J. & Regniere, J. (1999). Predictability and measurability of *Bacillus thuringiensis* efficacy against spruce budworm (Lepidoptera : Tortricidae). *Environmental Entomology* **28**, 711-721.

Couteaux, M. M. & Darbyshire, J. F. (1998). Functional diversity amongst soil protozoa. *Applied Soil Ecology* **10**, 229-237.

Crecchio, C. & Stotzky, G. (1998). Insecticidal activity and biodegradation of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound to humic acids from soil. *Soil Biology & Biochemistry* **30**, 463-470.

Crecchio, C. & Stotzky, G. (2001). Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp *kurstaki* bound on complexes of montmorillonite-humic acids-Al hydroxypolymers. *Soil Biology & Biochemistry* **33**, 573-581.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., & Dean, D. H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* **62**, 807-+.

Crickmore, N., Zeigler, D. R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A., & Dean, D. (2002) *Bacillus thuringiensis* toxin nomenclature. http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html. Electronic Citation

Damgaard, P. H., Abdel-Hameed, A., Eilenberg, J., & Smits, P. H. (1998). Natural occurrence of *Bacillus thuringiensis* on grass foliage. *World Journal of Microbiology & Biotechnology* **14**, 239-242.

Darrah, P. R. (1993). The rhizosphere and plant nutrition - a quantitative approach. *Plant and Soil* **156**, 1-20.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M. F., Oliva, N., Abrigo, E., Khush, G. S., & Datta, S. K. (1998). Constitutive and tissue-specific differential expression of the cryIA(b) gene in transgenic rice plants conferring resistance to rice insect pest. *Theoretical and Applied Genetics* **97**, 20-30.

de Maagd, R. A., Bosch, D., & Stiekema, W. (1999). *Bacillus thuringiensis* toxin-mediated insect resistance in plants. *Trends in Plant Science* **4**, 9-13.

de Maagd, R. A., Bravo, A., & Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics* **17**, 193-199.

Dighton, J., Jones, H. E., Robinson, C. H., & Beckett, J. (1997). The role of abiotic factors, cultivation practices and soil fauna in the dispersal of genetically modified microorganisms in soils. *Applied Soil Ecology* **5**, 109-131.

Ding, X. F., Gopalakrishnan, B., Johnson, L. B., White, F. F., Wang, X. R., Morgan, T. D., Kramer, K. J., & Muthukrishnan, S. (1998). Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Research* **7**, 77-84.

Donegan, K. K., Schaller, D. L., Stone, J. K., Ganio, L. M., Reed, G., Hamm, P. B., & Seidler, R. J. (1996). Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var *tenebrionis* endotoxin. *Transgenic Research* **5**, 25-35.

Donovan, W. P., Donovan, J. C., & Engleman, J. T. (2001). Gene knockout demonstrates that vip3A contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*. *Journal of Invertebrate Pathology* **78**, 45-51.

Dowd, P. F. (2001). Biotic and abiotic factors limiting efficacy of Bt corn in indirectly reducing mycotoxin levels in commercial fields. *Journal of Economic Entomology* **94**, 1067-1074.

Downing, K. J., Leslie, G., & Thomson, J. A. (2000). Biocontrol of the sugarcane borer *Eldana saccharina* by expression of the *Bacillus thuringiensis* cry1Ac7 and *Serratia marcescens* chiA genes in sugarcane-associated bacteria. *Applied and Environmental Microbiology* **66**, 2804-2810.

Dulmage, H. T. (1970). Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti*. *Journal of Invertebrate Pathology* **15**, 232-239.

Dupont, C. & Boisvert, J. (1986). Persistence of *Bacillus thuringiensis* serovar *israelensis* toxic activity in the environment and interaction with natural subtrates. *Water Air and Soil Pollution* **29**, 425-438.

Ellar, D. J. (1990) Pathogenic determinants of entomopathogenic bacteria. 298-302. Society for Invertebrate Pathology. Proceedings Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia. Conference Proceedings

Ely, S. (1993). The engineering of plants to express *Bacillus thuringiensis* δ-endotoxins. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 105-124. John Wiley & Sons, Chichester.

Entwistle, P. F., Cory, J. S., Bailey, M., & Higgs, S. Eds. (1993). *Bacillus thuringiensis, an Environmental Biopesticide: Theory and Practice*, pp. 1-311. John Wiley & Sons, Chichester.

Escher, N., Käch, B., & Nentwig, W. (2000). Decomposition of transgenic *Bacillus thuringiensis* maize by microorganisms and woodlice *Porcellio scaber* (Crustacea: Isopoda). *Basic and Applied Ecology* **1**, 169.

Estruch, J. J., Carozzi, N. B., Desai, N., Duck, N. B., Warren, G. W., & Koziel, M. G. (1997). Transgenic plants: An emerging approach to pest control. *Nature Biotechnology* **15**, 137-141.

Evans, H. F. (1997). The role of microbial insecticides in forest pest management. In *BCPC Symposium Proceedings No. 68: Microbial Insecticides: Novelty or Necessity?*, ed. Evans, H. F., pp. 29-40. British Crop Protection Council, Farnham.

Evans, H. F. (1998). Principles of Dose Acquisition for Bioinsecticides. In *Biopesticides: Use and Delivery*, eds. Hall, F. R. & Menn, J., pp. 553-573. Humana Press, Totowa, NJ.

Evans, H. F. (1999). Genetically Modified Bioinsecticides. *DETR Research Report* **13**, 1-131.

Farrar, R. R., Martin, P. A. W., & Ridgway, R. L. (1996). Host plant effects on activity of *Bacillus thuringiensis* against gypsy moth (Lepidoptera: Lymantriidae) larvae. *Environmental Entomology* **25**, 1215-1223.

Fearing, P. L., Brown, D., Vlachos, D., Meghji, M., & Privalle, L. (1997). Quantitative analysis of CryIA(b) expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. *Molecular Breeding* **3**, 169-176.

Federici, B. A. & Bauer, L. S. (1998). Cyt1Aa protein of *Bacillus thuringiensis* is toxic to the cottonwood leaf beetle, *Chrysomela scripta*, and suppresses high levels of resistance to Cry3Aa. *Applied and Environmental Microbiology* **64**, 4368-4371.

Ferré, J. & Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology* **47**, 501-533.

Fogel, R. & Hunt, G. (1983). Contribution of mycorrhizae and soil fungi to nutrient cycling in a Douglas fir ecosystem. *Canadian Journal of Forest Research* **12**, 219-232.

Foissner, W. (1999). Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. *Agriculture Ecosystems & Environment* **74**, 95-112.

Fuxa, J. R. (1989). Fate of released entomopathogens with reference to risk assessment of genetically engineered microorganisms. *Bulletin of the Entomological Society of America* **35**, 12-24.

Fuxa, J. R. (1995). Ecological factors critical to the exploitation of entomopathogens in pest control. *Biorational Pest Control Agents* **595**, 42-67.

Gaertner, F. H., Quick, T. C., & Thompson, M. A. (1993). CellCap: an encapsulation system for insecticidal biotoxin proteins. In *Advanced engineered pesticides*, ed. KIM, L., pp. 73-83. Marcel Dekker, Inc, New York.

Gange, A. (2000). Arbuscular mycorrhizal fungi, Collembola and plant growth. *Trends in Ecology & Evolution* **15**, 369-372.

Gelernter, W. D. & Evans, H. F. (1999). Factors in the Success and Failure of Microbial Insecticides. *Integrated Pest Management Reviews* **4**, 279-316.

Gelernter, W. D. & Lomer, C. J. (2000). Measures of success in biological control of insects by pathogens. In *Measures of Success in Biological Control*, eds. Gurr, G. & Wratten, S., pp. 297-322. Kluwer, Dordrecht.

Ghareyazie, B., Alinia, F., Menguito, C. A., Rubia, L. G., dePalma, J. M., Liwanag, E. A., Cohen, M. B., Khush, G. S., & Bennett, J. (1997). Enhanced resistance to two stem borers in an aromatic rice containing a synthetic cryIA(b) gene. *Molecular Breeding* **3** , 401-414.

Giles, K. L., Hellmich, R. L., Iverson, C. T., & Lewis, L. C. (2000). Effects of transgenic *Bacillus thuringiensis* maize grain on *B.thuringiensis*-susceptible *Plodia interpunctella* (Lepidoptera : Pyralidae). *Journal of Economic Entomology* **93**, 1011-1016.

Gleave, A. P., Mitra, D. S., Markwick, N. P., Morris, B. A. M., & Beuning, L. L. (1998). Enhanced expression of the *Bacillus thuringiensis* cry9Aa2 gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth. *Molecular Breeding* **4**, 459-472.

Gonzales, J. M., Brown, B. J., & Carlton, B. C. (1982). Transfer of *Bacillus thuringiensis* plasmids coding for delta endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **79**, 6951-6955.

Gonzales, J. M. & Carlton, B. C. (1984). A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. *Plasmid* **11**, 28-38.

Gonzales, J. M., Dulmage, H. T., & Carlton, B. C. (1981). Correlation between specific plasmids and delta endotoxin production in *Bacillus thuringiensis*. *Plasmid* **5**, 351-365.

Gransee, A. & Wittenmayer, L. (2000). Qualitative and quantitative analysis of water-soluble root exudates in relation to plant species and development. *Journal of Plant Nutrition and Soil Science-Zeitschrift fur Pflanzenernahrung und Bodenkunde* **163**, 381-385.

Grayston, S. J., Vaughan, D., & Jones, D. (1997). Rhizosphere carbon flow in trees, in comparison with annual plants: The importance of root exudation and its impact on microbial activity and nutrient availability. *Applied Soil Ecology* **5**, 29-56.

Griffiths, B. S., Bonkowski, M., Dobson, G., & Caul, S. (1999). Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa. *Pedobiologia* **43**, 297-304.

Guihard, G., Vachon, V., Laprade, R., & Schwartz, J. L. (2000). Kinetic properties of the channels formed by the *Bacillus thuringiensis* insecticidal crystal protein Cry1C in the plasma membrane of Sf9 cells. *Journal of Membrane Biology* **175**, 115-122.

Guttmann, D. M. & Ellar, D. J. (2000). Phenotypic and genotypic comparisons of 23 strains from the *Bacillus cereus* complex for a selection of known and putative *B. thuringiensis* virulence factors. *Fems Microbiology Letters* **188**, 7-13.

Haider, M. Z., Knowles, B. H., & Ellar, D. J. (1986). Specificity of *Bacillus thuringiensis* var *colmeri* insecticidal delta endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *European Journal of Biochemistry* **156**, 531-540.

Hall, F. R., Chapple, A. C., Taylor, R. A. J., & Downer, R. A. (1995). Modeling the dose acquisition process of *Bacillus thuringiensis* - influence of feeding pattern on survival. *Biorational Pest Control Agents* **595**, 68-78.

Hall, F. R. & Menn, J. J. Eds. (1998). *Biopesticides: Use and Delivery*, pp. 1-626. Humana Press, Totowa, NJ.

Hannay, C. L. & Fitz-James, P. (1955). The protein crystals of *Bacillus thuringiensis* Berliner. *Canadian Journal of Microbiology* **1**, 694-709.

Hansen, G. & Wright, M. S. (1999). Recent advances in the transformation of plants. *Trends in Plant Science* **4**, 226-231.

Hardee, D. D. & Bryan, W. W. (1997). Influence of *Bacillus thuringiensis*-transgenic and nectariless cotton on insect populations with emphasis on the tarnished plant bug (Heteroptera: Miridae). *Journal of Economic Entomology* **90**, 663-668.

Hattori, R. & Hattori, T. (1993). Soil aggregates as microcosms of Bacteria Protozoa biota. *Geoderma* **56**, 493-501.

Hattori, T. & Hattori, R. (1976). The physical environment in soil microbiology: an attempt to extend principles of microbiology to soil microorganisms. *Crc Critical Reviews in Microbiology* 423-461.

Head, G., Brown, C. R., Groth, M. E., & Duan, J. J. (2001). Cry1Ab protein levels in phytophagous insects feeding on transgenic corn: implications for secondary exposure risk assessment. *Entomologia Experimentalis et Applicata* **99**, 37-45.

Hellmich, R. L., Rice, M. E., Pleasants, J. M., & Lam, W. K. (2000) Of monarchs and men: possible influences of Bt corn in the agricultural community. 85-94. Ames, Iowa State University Extension Service. Proceedings of the Integrated Crop Management Conference. Conference Proceedings

Hellmich, R. L., Siegfried, B. D., Sears, M. K., Stanley-Horn, D. E., Daniels, M. J., Mattila, H. R., Spencer, T., Bidne, K. E., & Lewis, L. C. (2001). Monarch larvae sensitive to Bacillus thuringiensis-purified proteins and pollen. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11925-11930.

Hilbeck, A. (2002). Transgenic host plant resistance and non-target effects. In *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*, eds. Letourneau, D. K. & Burrows, B. E., pp. 167-185. CRC Press, Boca Raton, Fla.

Hilbeck, A., Baumgartner, M., Fried, P. M., & Bigler, F. (1998a). Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera : Chrysopidae). *Environmental Entomology* **27**, 480-487.

Hilbeck, A., Moar, W. J., Pusztai-Carey, M., Filippini, A., & Bigler, F. (1998b). Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera : Chrysopidae). *Environmental Entomology* **27**, 1255-1263.

Hofmann, C., Luthy, P., Hutter, R., & Pliska, V. (1988a). Binding of the delta endotoxin from *Bacillus thuringiensis* to brush border membrane vesicles of the cabbage butterfly (*Pieris brassicae*). *European Journal of Biochemistry* **173**, 85-91.

Hofmann, C., Vanderbruggen, H., Hofte, H., Van Rie, J., Jansens, S., & Van Mellaert, H. (1988b). Specificity of *Bacillus thuringiensis* delta endotoxins is correlated with the presence of high affinity binding sites in the brush border membrane of target insect

midguts. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 7844-7848.

Hofte, H. & Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**, 242-255.

Hooper, D. U., Bignell, D. E., Brown, V. K., Brussaard, L., Dangerfield, J. M., Wall, D. H., Wardle, D. A., Coleman, D. C., Giller, K. E., Lavelle, P., van der Putten, W. H., De Ruiter, P. C., Rusek, J., Silver, W. L., Tiedje, J. M., & Wolters, V. (2000). Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: Patterns, mechanisms, and feedbacks. *Bioscience* **50**, 1049-1061.

Hossain, M. A., Ahmed, S., & Hoque, S. (1997). Abundance and distribution of *Bacillus thuringiensis* in the agricultural soil of Bangladesh. *Journal of Invertebrate Pathology* **70**, 221-225.

Hunter, M. D. (1992). A variable insect-plant interaction: the relationship between tree budburst phenology and population levels of insect herbivores among trees. *Ecological Entomology* **17**, 91-95.

Ichimatsu, T., Mizuki, E., Nishimura, K., Akao, T., Saitoh, H., Higuchi, K., & Ohba, M. (2000). Occurrence of *Bacillus thuringiensis* in fresh waters of Japan. *Current Microbiology* **40**, 217-220.

Ignoffo, C. M. (1992). Environmental factors affecting persistence of entomopathogens. *Florida Entomologist* **75**, 516-525.

Inagaki, S., Miyasono, M., Ishiguro, T., Takeda, R., & Hayashi, Y. (1992). Proteolytic processing of delta-endotoxin of *Bacillus thuringiensis* var *kurstaki* HD-1 in insensitive insect, *Spodoptera litura*: unusual proteolysis in the presence of sodium dodecyl sulfate. *Journal of Invertebrate Pathology* **60**, 64-68.

Jenkins, J. L., Lee, M. K., Sangadala, S., Adang, M. J., & Dean, D. H. (1999). Binding of *Bacillus thuringiensis* Cry1Ac toxin to *Manduca sexta* aminopeptidase-N receptor is not directly related to toxicity. *Febs Letters* **462**, 373-376.

Jenkins, J. L., Lee, M. K., Valaitis, A. P., Curtiss, A., & Dean, D. H. (2000). Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. *Journal of Biological Chemistry* **275**, 14423-14431.

Jenkins, J. N. (1999). Transgenic plants expressing toxins from *Bacillus thuringiensis*. In *Biopesticides: Use and Delivery*, eds. Hall, F. R. & Menn, J. J., pp. 211-232. Totowa, NJ, Humana Press Inc.

Jesse, L. C. H. & Obrycki, J. J. (2000). Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia* **125**, 241-248.

Jin, R. G., Liu, Y. B., Tabashnik, B. E., & Borthakur, D. (2000). Development of transgenic cabbage (*Brassica oleracea* var. *Capitata*) for insect resistance by *Agrobacterium tumefaciens*- mediated transformation. *In Vitro Cellular & Developmental Biology-Plant* **36**, 231-237.

Johnson, C. & Bishop, A. H. (1996). A technique for the effective enrichment and isolation of *Bacillus thuringiensis*. *Fems Microbiology Letters* **142**, 173-177.

Johnson, D. E. & McGaughey, W. H. (1996). Contribution of *Bacillus thuringiensis* spores to toxicity of purified cry proteins towards Indianmeal moth larvae. *Current Microbiology* **33**, 54-59.

Johnson, K. S., Scriber, J. M., Nitao, J. K., & Smitley, D. R. (1995). Toxicity of *Bacillus thuringiensis* var. *kurstaki* to 3 nontarget Lepidoptera in field studies. *Environmental Entomology* **24**, 288-297.

Jones, D. L. & Darrah, P. R. (1993). Re-sorption of organic compounds by roots of *Zea mays* and its consequences in the rhizosphere. II. Experimental and model evidence for simultaneous exudation and re-sorption of soluble C compounds. *Plant and Soil* **153**, 47-57.

Jones, D. L. & Darrah, P. R. (1994). Amino-acid influx at the soil-root interface of *Zea mays* L and its implications in the rhizosphere. *Plant and Soil* **163**, 1-12.

Jurat-Fuentes, J. L. & Adang, M. J. (2001). Importance of Cry1 delta-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). *Applied and Environmental Microbiology* **67**, 323-329.

Kaelin, P., Morel, P., & Gadani, F. (1994). Isolation of *Bacillus thuringiensis* from stored tobacco and *Lasioderma serricorne* (F). *Applied and Environmental Microbiology* **60**, 19-25.

Kaelin, P., Zaugg, L., Albertini, A. M., & Gadani, F. (1999). Activity of *Bacillus thuringiensis* isolates on *Lasioderma serricorne* (F.) (Coleoptera : Anobiidae). *Journal of Stored Products Research* **35**, 145-158.

Keller, B. & Langenbruch, G. A. (1993). Control of coleopteran pests by *Bacillus thuringiensis*. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 171-191. John Wiley & Sons Ltd, Chichester.

Kennedy, A. C. (1999). Bacterial diversity in agroecosystems. *Agriculture Ecosystems & Environment* **74**, 65-76.

Kim, H. S., Lee, D. W., Woo, S. D., Yu, Y. M., & Kang, S. K. (1998). Seasonal distribution and characterization of *Bacillus thuringiensis* isolated from sericultural environments in Korea. *Journal of General and Applied Microbiology* **44**, 133-138.

Kim, S. Y., Kang, M. H., Choi, H. B., Lee, J. U., Charles, J. F., Dumanoir, V. C., Lecadet, M. M., & Lee, H. H. (1999). Characteristics of thirty-six *Bacillus thuringiensis* isolates and a new serovar of *B. thuringiensis* subsp *kim* (serotype H52). *Journal of Microbiology and Biotechnology* **9**, 534-540.

Knight, P. J. K., Crickmore, N., & Ellar, D. J. (1994). The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Molecular Microbiology* **11**, 429-436.

Knowles, B. H. (1994). Mechanism of action of *Bacillus thuringiensis* insecticidal delta endotoxins. *Advances in Insect Physiology* **24**, 275-308.

Knowles, B. H., Blatt, M. R., Tester, M., Horsnell, J. M., Carroll, J., Menestrina, G., & Ellar, D. J. (1989). A cytolytic delta endotoxin from *Bacillus thuringiensis* var *israelensis* forms cation-selective channels in planar lipid bilayers. *Febs Letters* **244**, 259-262.

Koller, C. N., Bauer, L. S., & Hollingworth, R. M. (1992). Characterization of the pH-mediated solubility of *Bacillus thuringiensis* var *San Diego* native delta endotoxin crystals. *Biochemical and Biophysical Research Communications* **184**, 692-699.

Koskella, J. & Stotzky, G. (1997). Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. *Applied and Environmental Microbiology* **63**, 3561-3568.

Kota, M., Daniell, H., Varma, S., Garczynski, S. F., Gould, F., & Moar, W. J. (1999). Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 1840-1845.

Koziel, M. G., Beland, G. L., Bowman, C., Carozzi, N. B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M. R., Merlin, E., Rhodes, R., Warren, G. W., Wright, M., & Evola, S. V. (1993). Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. *Bio-Technology* **11**, 194-200.

Koziel, M. G., Carozzi, N. B., & Desai, N. (1996). Optimizing expression of transgenes with an emphasis on post- transcriptional events. *Plant Molecular Biology* **32**, 393-405.

Kramer, K. J. & Muthukrishnan, S. (1997). Insect chitinases: Molecular biology and potential use as biopesticides. *Insect Biochemistry and Molecular Biology* **27**, 887-900.

Lacey, L. A. Ed. (1997). *Manual of Techniques in Insect Pathology*, pp. 1-409. Academic Press, San Diego.

Lacey, L. A., Frutos, R., Kaya, H. K., & Vail, P. (2001). Insect pathogens as biological control agents: Do they have a future? *Biological Control* **21**, 230-248.

Lacey, L. A., Horton, D. R., Chauvin, R. L., & Stocker, J. M. (1999). Comparative efficacy of *Beauveria bassiana*, *Bacillus thuringiensis*, and aldicarb for control of Colorado

potato beetle in an irrigated desert agroecosystem and their effects on biodiversity. *Entomologia Experimentalis et Applicata* **93**, 189-200.

Lavelle, P., Bignell, D., Lepage, M., Wolters, V., Roger, P., Ineson, P., Heal, O. W., & Dhillion, S. (1997). Soil function in a changing world: the role of invertebrate ecosystem engineers. *European Journal of Soil Biology* **33**, 159-193.

Lee, M. K., Jenkins, J. L., You, T. H., Curtiss, A., Son, J. J., Adang, M. J., & Dean, D. H. (2001). Mutations at the arginine residues in alpha 8 loop of *Bacillus thuringiensis* delta-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and aminopeptidase N. *Febs Letters* **497** , 108-112.

Lee, M. K., Rajamohan, F., Jenkins, J. L., Curtiss, A. S., & Dean, D. H. (2000). Role of two arginine residues in domain II, loop 2 of Cry1Ab and Cry1Ac *Bacillus thuringiensis* delta-endotoxin in toxicity and binding to *Manduca sexta* and aminopeptidase N. *Molecular Microbiology* **38**, 289-298.

Lereclus, D., Delécluse, A., & Lecadet, M. M. (1993). Diversity of *Bacillus thuringiensis* toxins and genes. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 37-69. John Wiley & Sons Ltd, Chichester.

Letourneau, D. K., Hagen, J. A., & Robinson, G. S. (2002). *Bt*-crops: Evaluating benefits under cultivation and risks from escaped transgenes in the wild. In *Genetically engineered organisms: assessing environmental and human health effects*, eds. Letourneau, D. K. & Burrows, B. E., pp. 33-98. CRC Press, Boca Raton.

Li, J., Derbyshire, D. J., Promdonkoy, B., & Ellar, D. J. (2001). Structural implications for the transformation of the *Bacillus thuringiensis* delta-endotoxins from water-soluble to membrane- inserted forms. *Biochemical Society Transactions* **29**, 571-577.

Lightwood, D. J., Ellar, D. J., & Jarrett, P. (2000). Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin. *Applied and Environmental Microbiology* **66**, 5174-5181.

Lilley, A. K., Hails, R. S., Cory, J. S., & Bailey, M. J. (1997). The dispersal and establishment of pseudomonad populations in the phyllosphere of sugar beet by phytophagous caterpillars. *Fems Microbiology Ecology* **24**, 151-157.

Liu, Y. B., Tabashnik, B. E., Masson, L., Escriche, B., & Ferré, J. (2000). Binding and toxicity of *Bacillus thuringiensis* protein Cry1C to susceptible and resistant diamondback moth (Lepidoptera : Plutellidae). *Journal of Economic Entomology* **93**, 1-6.

Liu, Y. B., Tabashnik, B. E., Meyer, S. K., & Crickmore, N. (2001). Cross-resistance and stability of resistance to *Bacillus thuringiensis* toxin Cry1C in diamondback moth. *Applied and Environmental Microbiology* **67**, 3216-3219.

Losey, J. E., Rayor, L. S., & Carter, M. E. (1999). Transgenic pollen harms monarch larvae. *Nature* **399**, 214.

Lozzia, G. C. (1999). Biodiversity and structure of ground beetle assemblages (Coleoptera: Carabidae) in *Bt* corn and its effects on non-target insects. *Bollettino di Zoologia Agraria e di Bachicoltura* **31**, 37-58.

Luo, K., Banks, D., & Adang, M. J. (1999). Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry1 delta-endotoxins using brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. *Applied and Environmental Microbiology* **65**, 457-464.

Lynch, R. E., Wiseman, B. R., Plaisted, D., & Warnick, D. (1999). Evaluation of transgenic sweet corn hybrids expressing CryIA(b) toxin for resistance to corn earworm and fall armyworm (Lepidoptera : Noctuidae). *Journal of Economic Entomology* **92**, 246-252.

Ma, D. L., Gordh, G., & Zalucki, M. P. (2000). Toxicity of biorational insecticides to *Helicoverpa* spp. (Lepidoptera : Noctuidae) and predators in cotton field. *International Journal of Pest Management* **46**, 237-240.

MacIntosh, S. C., Stone, T. B., Sims, S. R., Hunst, P. L., Greenplate, J. T., Marrone, P. G., Perlak, F. J., Fischhoff, D. A., & Fuchs, R. L. (1990). Specificity and efficacy of

purified *Bacillus thuringiensis* proteins against agronomically important insects. *Journal of Invertebrate Pathology* **56**, 258-266.

Maeda, M., Mizuki, E., Hara, M., Tanaka, R., Akao, T., Yamashita, S., & Ohba, M. (2001). Isolation of *Bacillus thuringiensis* from intertidal brackish sediments in mangroves. *Microbiological Research* **156**, 195-198.

Malvar, T. & Baum, J. A. (1994). TN5401 disruption of the spoOF gene, identified by direct chromosomal sequencing, results in CryIIIA overproduction in *Bacillus thuringiensis*. *Journal of Bacteriology* **176**, 4750-4753.

Manonmani, A. & Balaraman, K. (2001). A highly mosquitocidal *Bacillus thuringiensis* var. *thompsoni*. *Current Science* **80**, 779-781.

Manonmani, A. M., Rajendran, G., & Balaraman, K. (1991). Isolation of mosquito-pathogenic *Bacillus sphaericus* and *B. thuringiensis* from the root surface of hydrophytes. *Indian Journal of Medical Research* **93**, 111-114.

Maqbool, S. B., Riazuddin, S., Loc, N. T., Gatehouse, A. M. R., Gatehouse, J. A., & Christou, P. (2001). Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Molecular Breeding* **7**, 85-93.

Margalit, J. & Dean, D. (1985). The story of *Bacillus thuringiensis* var *israelensis* (Bti). *Journal of the American Mosquito Control Association* **1**, 1-7.

Marschner, H. (1991). Plant-soil relationships: acquisition of mineral nutrients by roots from soils. In *Plant growth: Interactions with nutrition and environment*, eds. Porter, J. R. & Lawlor, D. W., pp. 125-155. Cambridge University Press, Cambridge.

Martin, P. A. W. & Travers, R. S. (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Applied and Environmental Microbiology* **55**, 2437-2442.

Martin, W. F. & Reichelderfer, C. F. (1989) *Bacillus thuringiensis*: persistence and movement in field crops. 25. University of Maryland, USA, Proceedings and Abstracts Society for Invertebrate Pathology XXIInd Annual Meeting. Conference Proceedings

McCully, M. E. (1999). Roots in soil: Unearthing the complexities of roots and their rhizospheres. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 695-718.

McCully, M. E. & Sealey, L. J. (1996). The expansion of maize root-cap mucilage during hydration. 2. Observations on soil-grown roots by cryo-scanning electron microscopy. *Physiology of Plants* **97**, 454-462.

McGaughey, W. H. (1985). Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science* **229**, 193-194.

McGaughey, W. H. & Beeman, R. W. (1988). Resistance to *Bacillus thuringiensis* in colonies of Indian meal moth and almond moth (Lepidoptera: Pyralidae). *Journal of Economic Entomology* **81**, 28-33.

McLinden, J. H., Sabourin, J. R., Clark, B. D., Gensler, D. r., Workman, W. E., & Dean, D. H. (1985). Cloning and expression of an insecticidal K-73 type crystal protein gene from *Bacillus thuringiensis* var *kurstaki* into *Escherichia coli*. *Applied and Environmental Microbiology* **50**, 623-628.

Meade, T. & Hare, J. D. (1994). Effects of genetic and environmental host-plant variation on the susceptibility of 2 noctuids to *Bacillus thuringiensis*. *Entomologia Experimentalis et Applicata* **70**, 165-178.

Meadows, J., Gill, S. S., & Bone, L. W. (1990). *Bacillus thuringiensis* strains affect population growth of the free-living nematode *Turbatrix aceti*. *Invertebrate Reproduction & Development* **17**, 73-76.

Meadows, M. P. (1993). *Bacillus thuringiensis* in the Environment: Ecology and Risk Assessment. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 193-220. John Wiley & Sons, Chichester.

Meadows, M. P., Ellis, D. J., Butt, J., Jarrett, P., & Burges, H. D. (1992). Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Applied and Environmental Microbiology* **58**, 1344-1350.

Meharg, A. A. & Killham, K. (1990). The effect of soil pH on rhizosphere carbon flow of *Lolium perenne*. *Plant and Soil* **123**, 1-7.

Milner, R. J. (1994). History of *Bacillus thuringiensis*. *Agriculture Ecosystems & Environment* **49**, 9-13.

Miranda, R., Zamudio, F. Z., & Bravo, A. (2001). Processing of Cry1Ab delta-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. *Insect Biochemistry and Molecular Biology* **31**, 1155-1163.

Miyasono, M., Inagaki, S., Yamamoto, M., Ohba, K., Ishiguro, T., Takeda, R., & Hayashi, Y. (1994). Enhancement of delta-endotoxin activity by toxin-free spore of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella*. *Journal of Invertebrate Pathology* **63**, 111-112.

Mizuki, E., Ichimatsu, T., Hwang, S. H., Park, Y. S., Saitoh, H., Higuchi, K., & Ohba, M. (1999). Ubiquity of *Bacillus thuringiensis* on phylloplanes of arboreous and herbaceous plants in Japan. *Journal of Applied Microbiology* **86**, 979-984.

Moar, W. J., Trumble, J. T., Hice, R. H., & Backman, P. A. (1994). Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf-colonizing strain of *Bacillus cereus*. *Applied and Environmental Microbiology* **60**, 896-902.

Moellenbeck, D. J., Peters, M. L., Bing, J. W., Rouse, J. R., Higgins, L. S., Sims, L., Nevshemal, T., Marshall, L., Ellis, R. T., Bystrak, P. G., Lang, B. A., Stewart, J. L., Kouba, K., Sondag, V., Gustafson, V., Nour, K., Xu, D. P., Swenson, J., Zhang, J., Czapla, T., Schwab, G., Jayne, S., Stockhoff, B. A., Narva, K., Schnepf, H. E., Stelman, S. J., Poutre, C., Koziel, M., & Duck, N. (2001). Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. *Nature Biotechnology* **19**, 668-672.

Mohaghegh, J., de Clercq, P., & Tirry, L. (2000). Toxicity of selected insecticides to the spined soldier bug, *Podisus maculiventris* (Heteroptera : Pentatomidae). *Biocontrol Science and Technology* **10**, 33-40.

Mück, O., Hassan, S., Huger, A. M., & Krieg, A. (1981). Effects of *Bacillus thuringiensis* Berliner on the parasitic Hymenoptera *Apanteles glomeratus* L. (Braconidae) and *Pimpla turionellae* (L) (Ichneumonidae). *Zeitschrift fur Angewandte Entomologie* **92**, 303-314.

Nagamatsu, Y., Koike, T., Sasaki, K., Yoshimoto, A., & Furukawa, Y. (1999). The cadherin-like protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. *Febs Letters* **460**, 385-390.

Nayak, P., Basu, D., Das, S., Basu, A., Ghosh, D., Ramakrishnan, N. A., Ghosh, M., & Sen, S. K. (1997). Transgenic elite indica rice plants expressing CryIAc delta- endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proceedings of the National Academy of Sciences of the United States of America* **94**, 2111-2116.

Novillo, C., Castanera, P., & Ortego, F. (1997). Characterization and distribution of chymotrypsin-like and other digestive proteases in Colorado potato beetle larvae. *Archives of Insect Biochemistry and Physiology* **36**, 181-201.

Oberhauser, K. S., Prysby, M. D., Mattila, H. R., Stanley-Horn, D. E., Sears, M. K., Dively, G., Olson, E., Pleasants, J. M., Lam, W. K. F., & Hellmich, R. L. (2001). Temporal and spatial overlap between monarch larvae and corn pollen. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11913-11918.

Obrycki, J. J., Losey, J. E., Taylor, O. R., & Jesse, L. C. H. (2001). Transgenic insecticidal corn: Beyond insecticidal toxicity to ecological complexity. *Bioscience* **51**, 353-361.

Ogiwara, K., Indrasith, L. S., Asano, S., & Hori, H. (1992). Processing of delta endotoxin from *Bacillus thuringiensis* subsp *kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. *Journal of Invertebrate Pathology* **60**, 121-126.

Ohba, M. (1997). Three *Bacillus thuringiensis* flagellar serovars widely occurring in natural environments of Japan. *Journal of Basic Microbiology* **37**, 71-76.

Oppert, B., Kramer, K. J., Beeman, R. W., Johnson, D., & McGaughey, W. H. (1997). Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *Journal of Biological Chemistry* **272**, 23473-23476.

Oppert, B., Kramer, K. J., Johnson, D. E., MacIntosh, S. C., & McGaughey, W. H. (1994). Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. *Biochemical and Biophysical Research Communications* **198**, 940-947.

Peferoen, M. (1997). Progress and prospects for field use of Bt genes in crops. *Trends in Biotechnology* **15**, 173-177.

Perez, C. J. & Shelton, A. M. (1997). Resistance of *Plutella xylostella* (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* Berliner in central America. *Journal of Economic Entomology* **90**, 87-93.

Perlak, F. J., Fuchs, R. L., Dean, D. A., McPherson, S. L., & Fischhoff, D. A. (1991). Modification of the coding sequence enhances plant expression of insect control protein genes. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 3324-3328.

Perlak, F. J., Stone, T. B., Muskopf, Y. M., Petersen, L. J., Parker, G. B., McPherson, S. A., Wyman, J., Love, S., Reed, G., Biever, D., & Fischhoff, D. A. (1993). Genetically improved potatoes: protection from damage by Colorado potato beetles. *Plant Molecular Biology* **22**, 313-321.

Picot, J. J. C. & Kristmanson, D. D. (1997). *Forestry Pesticide Aerial Spraying: Spray Droplet Generation, Dispersion, and Deposition*, pp. 1-213. Kluwer Academic Publishers, Dordrecht.

Pilcher, C. D., Obrycki, J. J., Rice, M. E., & Lewis, L. C. (1997a). Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environmental Entomology* **26**, 446-454.

Pilcher, C. D., Rice, M. E., Higgins, R. A., & Bowling, R. (2001). Pollen drift from *Bacillus thuringiensis* corn: Efficacy against European corn borer (Lepidoptera : Crambidae) in adjacent rows of non-Bt corn. *Environmental Entomology* **30**, 409-414.

Pilcher, C. D., Rice, M. E., Obrycki, J. J., & Lewis, L. C. (1997b). Field and laboratory evaluations of transgenic *Bacillus thuringiensis* corn on secondary lepidopteran pests (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **90**, 669-678.

Plant Health and Production Division, P. B. O. (2001a) Decision Document 98-23; Determination of Environmental Safety of Dekalb Genetics Corporation's European Corn Borer (ECB) Resistant Corn (Zea mays L.) Line DBT418. Plant Health and Production Division and the Animal Health and Production Division, Canadian Food Inspection Agency. Electronic Report

Plant Health and Production Division, P. B. O. (2001b) Decision Document DD96-09: Determination of Environmental Safety of Event 176 Bt Corn (*Zea mays* L.) Developed by Ciba Seeds and Mycogen Corporation. Plant Health and Production Division, Canadian Food Inspection Agency. 10-4-2001b. Electronic Report

Pleasants, J. M., Hellmich, R. L., Dively, G., Sears, M. K., Stanley-Horn, D. E., Mattila, H. R., Foster, J. E., Clark, P., & Jones, G. D. (2001). Corn pollen deposition on milkweeds in and near cornfields. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11919-11924.

Prasertphon, S., Areekul, P., & Tanada, Y. (1973). Sporulation of *Bacillus thuringiensis* in host cadavers. *Journal of Invertebrate Pathology* **21**, 205-207.

Pruett, C. J. H., Burges, H. D., & Wyborn, C. H. (1980). Effect of exposure to soil on potency and spore viability of *B. thuringiensis*. *Journal of Invertebrate Pathology* **35**, 168-174.

Rang, C., Vachon, V., de Maagd, R. A., Villalon, M., Schwartz, J. L., Bosch, D., Frutos, R., & Laprade, R. (1999). Interaction between functional domains of *Bacillus thuringiensis* insecticidal crystal proteins. *Applied and Environmental Microbiology* **65**, 2918-2925.

Rausell, C., Martinez-Ramirez, A. C., Garcia-Robles, I., & Real, M. D. (2000). A binding site for *Bacillus thuringiensis* Cry1Ab toxin is lost during larval development in two forest pests. *Applied and Environmental Microbiology* **66**, 1553-1558.

Reed, G. L., Jensen, A. S., Riebe, J., Head, G., & Duan, J. J. (2001). Transgenic Bt potato and conventional insecticides for Colorado potato beetle management: comparative efficacy and non-target impacts. *Entomologia Experimentalis et Applicata* **100**, 89-100.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., Ginzberg, I., KonczKalman, Z., Koncz, C., Schell, J., & Zilberstein, A. (1996). Synergistic activity of a *Bacillus thuringiensis* delta- endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Applied and Environmental Microbiology* **62**, 3581-3586.

Regniere, J. & Cooke, B. (1998). Validation of a process-oriented model of *Bacillus thuringiensis* variety *kurstaki* efficacy against spruce budworm (Lepidoptera: Tortricidae). *Environmental Entomology* **27**, 801-811.

Richards, A. G. & Richards, P. A. (1977). The peritrophic membranes of insects. *Annual Review of Entomology* **22**, 219-240.

Riddick, E. W. & Barbosa, P. (2000). Cry3A-intoxicated *Leptinotarsa decemlineata* (Say) are palatable prey for *Lebia grandis* Hentz. *Journal of Entomological Science* **35**, 342-346.

Riddick, E. W., Dively, G., & Barbosa, P. (1998). Effect of a seed-mix deployment of Cry3A-transgenic and nontransgenic potato on the abundance of *Lebia grandis* (Coleoptera: Carabidae) and *Coleomegilla maculata* (Coleoptera: Coccinellidae). *Annals of the Entomological Society of America* **91**, 647-653.

Riddick, E. W., Dively, G., & Barbosa, P. (2000). Season-long abundance of generalist predators in transgenic versus nontransgenic potato fields. *Journal of Entomological Science* **35**, 349-359.

Robinson, G. S., Ackery, P. R., Kitching, I. J., Beccaloni, G. W., & Hernández, L. M. (2002) HOSTS - The Natural History Museum's Database of Caterpillar Hostplants and Foods. http://flood.nhm.ac.uk/cgi-bin/perth/hosts/index.dsml Electronic Citation

Roth, G. W. & Adams, R. S. (2002) Corn silage production and management. Agronomy Facts 18, 1-4. PennState College of Agricultural Sciences, Cooperative Extension. Ref Type: Electronic Citation

Rousseau, J. V. D. & Reid, C. P. P. (1990). Effects of phosphorus and ectomycorrhizas on the carbon balance of loblolly pine seedlings. *Forest Science* **36**, 101-112.

Rusek, J. (1998). Biodiversity of Collembola and their functional role in the ecosystem. *Biodiversity and Conservation* **7**, 1207-1219.

Sample, B. E., Butler, L., Zivkovich, C., Whitmore, R. C., & Reardon, R. (1996). Effects of *Bacillus thuringiensis* Berliner var *kurstaki* and defoliation by the gypsy moth [*Lymantria dispar* (L) (Lepidoptera: Lymantriidae)] on native arthropods in West Virginia. *Canadian Entomologist* **128**, 573-592.

Sampson, M. N. & Gooday, G. W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology-Uk* **144**, 2189-2194.

Saxena, D., Flores, S., & Stotzky, G. (1999). Transgenic plants - Insecticidal toxin in root exudates from Bt corn. *Nature* **402**, 480.

Saxena, D., Flores, S., & Stotzky, G. (2002a) Bt toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events. Soil Biology & Biochemistry In Press

Saxena, D., Flores, S., & Stotzky, G. (2002b) Vertical movement in soil of insecticidal CryIAb protein from *Bacillus thuringiensis*. Soil Biology & Biochemistry . In Press

Saxena, D. & Stotzky, G. (2000). Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. *Fems Microbiology Ecology* **33**, 35-39.

Saxena, D. & Stotzky, G. (2001a). *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Biology & Biochemistry* **33**, 1225-1230.

Saxena, D. & Stotzky, G. (2001b). Bt corn has a higher lignin content than non-Bt corn. *American Journal of Botany* **88**, 1704-1706.

Saxena, D. & Stotzky, G. (2001c). Bt toxin uptake from soil by plants. *Nature Biotechnology* **19**, 199.

Saxena, D. & Stotzky, G. (2002) Bt toxin is not taken up from soil or hydroponic culture by corn, carrot, radish, or turnip. Plant and Soil. In Press

Scheu, S. (2001). Plants and generalist predators as links between the below- ground and above-ground system. *Basic and Applied Ecology* **2**, 3-13.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., & Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* **62**, 775-+.

Schnepf, H. E. & Whiteley, H. R. (1981). Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **78**, 2893-2897.

Schnepf, H. E. & Whiteley, H. R. (1985). Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. *Journal of Biological Chemistry* **260**, 6273-6280.

Schnepf, H. E., Wong, H. C., & Whiteley, H. R. (1985). The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *Journal of Biological Chemistry* **260**, 6264-6272.

Schönwitz, R. & Ziegler, H. (1982). Exudation of water-soluble vitamins and of some carbohydrates by intact roots of maize seedlings (*Zea mays* L.) into a mineral nutrient solution. *Pflanzenphysiol* **107**, 7-14.

Schuler, T. H., Denholm, I., Jouanin, L., Clark, S. J., Clark, A. J., & Poppy, G. M. (2001). Population-scale laboratory studies of the effect of transgenic plants on nontarget insects. *Molecular Ecology* **10**, 1845-1853.

Schuler, T. H., Poppy, G. M., Kerry, B. R., & Denholm, I. (1999a). Potential side effects of insect-resistant transgenic plants on arthropod natural enemies. *Trends in Biotechnology* **17**, 210-216.

Schuler, T. H., Potting, R. P. J., Denholm, I., & Poppy, G. M. (1999b). Parasitoid behaviour and Bt plants. *Nature* **400**, 825-826.

Schwartz, J. L., Lu, Y. J., Sohnlein, P., Brousseau, R., Laprade, R., Masson, L., & Adang, M. J. (1997). Ion channels formed in planar lipid bilayers by *Bacillus thuringiensis* toxins in the presence of *Manduca sexta* midgut receptors. *Febs Letters* **412**, 270-276.

Sears, M. K., Hellmich, R. L., Stanley-Horn, D. E., Oberhauser, K. S., Pleasants, J. M., Mattila, H. R., Siegfried, B. D., & Dively, G. P. (2001). Impact of Bt corn pollen on monarch butterfly populations: A risk assessment. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11937-11942.

Sedlacek, J. D., Komaravalli, S. R., Hanley, A. M., Price, B. D., & Davis, P. M. (2001). Life history attributes of Indian meal moth (Lepidoptera : Pyralidae) and Angoumois grain moth (Lepidoptera : Gelechiidae) reared on transgenic corn kernels. *Journal of Economic Entomology* **94**, 586-592.

Shelton, A. M., Zhao, J. Z., & Roush, R. T. (2002). Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology* **47**, 845-881.

Shibano, Y., Yamagata, A., Nakamura, N., Iizuka, T., Sugisaki, H., & Takanami, M. (1985). Nucleotide sequence coding for the insecticidal fragment of the *Bacillus thuringiensis* crystal protein. *Gene* **34**, 243-251.

Siegel, J. P., Smith, A. R., & Novak, R. J. (2001). Recovery of commercially produced *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* from tires and

prevalence of Bacilli in artificial and natural containers. *Journal of the American Mosquito Control Association* **17**, 33-41.

Siegfried, B. D., Zoerb, A. C., & Spencer, T. (2001). Development of European corn borer larvae on Event 176 Bt corn: influence on survival and fitness. *Entomologia Experimentalis et Applicata* **100**, 15-20.

Sims, S. R. (1997). Host activity spectrum of the CryIIA *Bacillus thuringiensis* subsp. *kurstaki* protein. Effects on Lepidoptera, Diptera, and non-target arthropods. *Southwestern Entomologist* **22**, 395-404.

Sims, S. R. & Martin, J. W. (1997). Effect of the *Bacillus thuringiensis* insecticidal proteins CryIA(b), CryIA(c), CryIIA, and CryIIIA on *Folsomia candida* and *Xenylla grisea* (Insecta: Collembola). *Pedobiologia* **41**, 412-416.

Singer, J. W., Heckman, J. R., Ingerson-Mahar, J., & Westendorf, M. L. (2000). Hybrid and nitrogen source affect yield and European corn borer damage. *Journal of Sustainable Agriculture* **16**, 5-15.

Skibbe, U., Christeller, J. T., Callaghan, P. T., Eccles, C. D., & Laing, W. A. (1996). Visualization of pH gradients in the larval midgut of *Spodoptera litura* using P-31-NMR microscopy. *Journal of Insect Physiology* **42**, 777-790.

Skot, L., Timms, E., & Mytton, L. R. (1994). The effect of toxin-producing *Rhizobium* strains on larvae of *Sitona flavescens* feeding on legume roots and nodules. *Plant and Soil* **163**, 141-150.

Smith, R. A. & Barry, J. W. (1998). Environmental persistence of *Bacillus thuringiensis* spores following aerial application. *Journal of Invertebrate Pathology* **71**, 263-267.

Smith, R. A. & Couche, G. A. (1991). The phylloplane as a source of *Bacillus thuringiensis* variants. *Applied and Environmental Microbiology* **57**, 311-315.

Snyder, W. E., Tonkyn, D. W., & Kluepfel, D. A. (1999). Transmission of a genetically engineered rhizobacterium by grasshoppers in the laboratory and field. *Ecological Applications* **9**, 245-253.

Stanley-Horn, D. E., Dively, G. P., Hellmich, R. L., Mattila, H. R., Sears, M. K., Rose, R., Jesse, L. C. H., Losey, J. E., Obrycki, J. J., & Lewis, L. (2001). Assessing the impact of Cry1Ab-expressing corn pollen on monarch butterfly larvae in field studies. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11931-11936.

Stoger, E., Williams, S., Keen, D., & Christou, P. (1999). Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Research* **8**, 73-82.

Storer, N. P., Van Duyn, J. W., & Kennedy, G. G. (2001). Life history traits of *Helicoverpa zea* (Lepidoptera : Noctuidae) on non-Bt and Bt transgenic corn hybrids in Eastern North Carolina. *Journal of Economic Entomology* **94**, 1268-1279.

Stotzky, G. (1986). Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. In *Interactions of soil minerals with natural organics and microbes*, eds. Huang, P. M. & Schnitzer, M., pp. 305-428. SSSA, Madison, WI.

Stotzky, G. (2000). Persistence and biological activity in soil of insecticidal proteins from *Bacillus thuringiensis* and of bacterial DNA bound on clays and humic acids. *Journal of Environmental Quality* **29**, 691-705.

Stotzky, G. (2002a) Clays and humic acids affect the persistence and biological activity of insecticidal proteins from *Bacillus thuringiensis* in soil. Unpublished Work

Stotzky, G. (2002b). Release, persistence and biological activity in soil of insecticidal proteins from *Bacillus thuringiensis*. In *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*, eds. Letourneau, D. K. & Burrows, B. E., pp. 187-222. CRC Press, Boca Raton, Fla.

Strongman, D. B., Eveleigh, E. S., van Frankenhuyzen, K., & Royama, T. (1997). The occurrence of two types of entomopathogenic Bacilli in natural populations of the spruce budworm, *Choristoneura fumiferana*. *Canadian Journal of Forest Research* **27**, 1922-1927.

Subramanyam, B. & Cutkomp, L. K. (1985). Moth control in stored grain and the role of *Bacillus thuringiensis*: An overview. *Residue Reviews* **94**, 1-47.

Sundaram, K. M. S. (1996). Sorptive interactions and binding of delta-endotoxin protein from *Bacillus thuringiensis* subsp *kurstaki* in forest soils. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes* **31**, 1321-1340.

Tabashnik, B. E., Cushing, N. L., Finson, N., & Johnson, M. W. (1990). Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera, Plutellidae). *Journal of Economic Entomology* **83**, 1671-1676.

Tabashnik, B. E., Malvar, T., Liu, Y. B., Finson, N., Borthakur, D., Shin, B. S., Park, S. H., Masson, L., DeMaagd, R. A., & Bosch, D. (1996). Cross-resistance of the diamondback moth indicates altered interactions with domain II of *Bacillus thuringiensis* toxins. *Applied and Environmental Microbiology* **62**, 2839-2844.

Tang, J. D., Shelton, A. M., Van Rie, J., De Roeck, S., Moar, W. J., Roush, R. T., & Peferoen, M. (1996). Toxicity of *Bacillus thuringiensis* spore and crystal protein to resistant diamondback moth (*Plutella xylostella*). *Applied and Environmental Microbiology* **62**, 564-569.

Tapp, H., Calamai, L., & Stotzky, G. (1994). Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subsp *kurstaki* and subsp *tenebrionis* on clay minerals. *Soil Biology & Biochemistry* **26**, 663-679.

Tapp, H. & Stotzky, G. (1995). Insecticidal activity of the toxins from *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* adsorbed and bound on pure and soil clays. *Applied and Environmental Microbiology* **61**, 1786-1790.

Tapp, H. & Stotzky, G. (1998). Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biology & Biochemistry* **30**, 471-476.

Terra, W. & Ferreira, C. (1994). Insect digestive enzymes: properties, compartmentalization and function. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **109**, 1-62.

Thamthiankul, S., Suan-Ngay, S., Tantimavanich, S., & Panbangred, W. (2001). Chitinase from *Bacillus thuringiensis* subsp *pakistani*. *Applied Microbiology and Biotechnology* **56**, 395-401.

Thomas, W. E. & Ellar, D. J. (1983). Mechanism of action of *Bacillus thuringiensis* var *israelensis* insecticidal delta endotoxin. *Febs Letters* **154**, 362-368.

Thorogood, R., Broadwell, A., Chilcott, C., & Wigley, P. (1996) Aerial Spraying of Btk (*Bacillus thuringiensis* var. *kurstaki*) in Auckland; I. Btk spore penetration into dwellings II. Persistence of air-borne Bk spores. 1-13. Unpublished Report

Tran, L. B., Vachon, V., Schwartz, J. L., & Laprade, R. (2001). Differential effects of pH on the pore-forming properties of *Bacillus thuringiensis* insecticidal crystal toxins. *Applied and Environmental Microbiology* **67**, 4488-4494.

Travers, R. S., Martin, P. A. W., & Reichelderfer, C. F. (1987). Selective process for the efficient isolation of soil *Bacillus* species. *Applied and Environmental Microbiology* **53**, 1263-1266.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., DeBeuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., & Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* **328**, 33-37.

Valaitis, A. P., Jenkins, J. L., Lee, M. K., Dean, D. H., & Garner, K. J. (2001). Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that binds *Bacillus thuringiensis* Cry1A toxins with high affinity. *Archives of Insect Biochemistry and Physiology* **46**, 186-200.

Van Aarssen, R., Soetaert, P., Stam, M., Dockx, J., Gossele, V., Seurinck, J., Reynaerts, A., & Cornelissen, M. (1995). CryIA(b) transcript formation in tobacco is inefficient. *Plant Molecular Biology* **28**, 513-524.

van Frankenhuyzen, K. (1990). Development and current status of *Bacillus thuringiensis* for control of defoliating forest insects. *Forestry Chronicle* **66**, 498-507.

van Frankenhuyzen, K. (1993). The Challenge of *Bacillus thuringiensis*. In *Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice*, eds. Entwistle, P. F., Cory, J. S., Bailey, M. J., & Higgs, S., pp. 1-35. John Wiley & Sons Ltd, Chichester.

van Frankenhuyzen, K. & Nystrom, C. (1999) The Bacillus thuringiensis toxin specificity database. http//www.glfc.cfs.nrcan.gc.ca/Bacillus/btsearch.cfm Electronic Citation

van Frankenhuyzen, K., Nystrom, C., & Dedes, J. (2000). Mortality, feeding inhibition, and recovery of spruce budworm (Lepidoptera : Tortricidae) larvae following aerial application of a high-potency formulation of *Bacillus thuringiensis* subsp *kurstaki*. *Canadian Entomologist* **132**, 505-518.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D., & Van Mellaert, H. (1989). Specificity of *Bacillus thuringiensis* delta-endotoxins: importance of specific receptors on the brush border membrane of the mid gut of target insects. *European Journal of Biochemistry* **186**, 239-247.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D., & Van Mellaert, H. (1990a). Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* delta endotoxins. *Applied and Environmental Microbiology* **56**, 1378-1385.

Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D., & Van Mellaert, H. (1990b). Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* **247**, 72-74.

Vancura, V. (2002). Root exudates of plants III. Effect of temperature and 'cold shock' on the exudation of various compounds from seeds and seedlings of maize and cucumber. *Plant and Soil* **27**, 319-328.

Venkateswerlu, G. & Stotzky, G. (1992). Binding of the protoxin and toxin proteins of *Bacillus thuringiensis* subsp *kurstaki* on clay minerals. *Current Microbiology* **25**, 225-233.

Visser, B., Munsterman, E., Stoker, A., & Dirkse, W. G. (1990). A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. *Journal of Bacteriology* **172**, 6783-6788.

Walker, K. A., Hellmich, R. L., & Lewis, L. C. (2000). Late-instar European corn borer (Lepidoptera : Crambidae) tunneling and survival in transgenic corn hybrids. *Journal of Economic Entomology* **93**, 1276-1285.

Watkinson, I. (1994). Global view of present and future markets for *Bt* products. *Agriculture Ecosystems & Environment* **49**, 3-7.

West, A. W., Burges, H. D., White, R. J., & Wyborn, C. H. (1984a). Persistence of *Bacillus thuringiensis* parasporal crystal insecticidal activity in soil. *Journal of Invertebrate Pathology* **44**, 128-133.

West, A. W., Burges, H. D., & Wyborn, C. H. (1984b). Effect of incubation in natural and autoclaved soil upon potency and viability of *Bacillus thuringiensis*. *Journal of Invertebrate Pathology* **44**, 121-127.

Whipps, J. M. & Lynch, J. M. (1986). The influence of the rhizosphere on crop productivity. *Advances in Microbial Ecology* **6**, 187-244.

Whiteley, H. R. & Schnepf, H. E. (1986). The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annual Review of Microbiology* **40**, 549-576.

Williams, S., Friedrich, L., Dincher, S., Carozzi, N., Kessmann, H., Ward, E., & Ryals, J. (1992). Chemical regulation of *Bacillus thuringiensis* delta endotoxin expression in transgenic plants. *Bio-Technology* **10**, 540-543.

Wirth, M. C., Federici, B. A., & Walton, W. E. (2000). Cyt1A from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti* (Diptera : Culicidae). *Applied and Environmental Microbiology* **66**, 1093-1097.

Wiwat, C., Thaithanun, S., Pantuwatana, S., & Bhumiratana, A. (2000). Toxicity of chitinase-producing *Bacillus thuringiensis* ssp *kurstaki* HD-1 (G) toward *Plutella xylostella*. *Journal of Invertebrate Pathology* **76**, 270-277.

Woods, H. A. & Kingsolver, J. G. (1999). Feeding rate and the structure of protein digestion and absorption in Lepidoptera midguts. *Archives of Insect Biochemistry and Physiology* **42**, 74-87.

Wright, D. A., Killham, K., Glover, L. A., & Prosser, J. I. (1995). Role of pore-size location in determining bacterial activity during predation by Protozoa in soil. *Applied and Environmental Microbiology* **61**, 3537-3543.

Yara, K., Kunimi, Y., & Iwahana, H. (1997). Comparative studies of growth characteristic and competitive ability in *Bacillus thuringiensis* and *Bacillus cereus* in soil. *Applied Entomology and Zoology* **32**, 625-634.

Ye, G. Y., Shu, Q. Y., Yao, H. W., Cui, H. R., Cheng, X. Y., Hu, C., Xia, Y. W., Gao, M. W., & Altosaar, I. (2001). Field evaluation of resistance of transgenic rice containing a synthetic cry1Ab gene from *Bacillus thuringiensis* Berliner to two stem borers. *Journal of Economic Entomology* **94**, 271-276.

Yu, L., Berry, R. E., & Croft, B. A. (1997). Effects of *Bacillus thuringiensis* toxins in transgenic cotton and potato on *Folsomia candida* (Collembola: Isotomidae) and *Oppia nitens* (Acari: Orbatidae). *Journal of Economic Entomology* **90**, 113-118.

Zangerl, A. R., McKenna, D., Wraight, C. L., Carroll, M., Ficarello, P., Warner, R., & Berenbaum, M. R. (2001). Effects of exposure to event 176 Bacillus thuringiensis corn pollen on monarch and black swallowtail caterpillars under field conditions. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 11908-11912.

Zhong, C. H., Ellar, D. J., Bishop, A., Johnson, C., Lin, S. S., & Hart, E. R. (2000). Characterization of a *Bacillus thuringiensis* delta-endotoxin which is toxic to insects in three orders. *Journal of Invertebrate Pathology* **76**, 131-139.

8 Appendices

8.1 Appendix 1: Table of Cry genes taken from (Crickmore et al., 2002)

Website accessed in March 2002. The references can be accessed on the website.

Name	Acc No.	Authors	Year	Source Strain	Comment
Cry1Aa1	M11250	Schnepf et al	1985	*Bt kurstaki HD1*	
Cry1Aa2	M10917	Shibano et al	1985	*Bt sotto*	
Cry1Aa3	D00348	Shimizu et al	1988	*Bt aizawai IPL7*	
Cry1Aa4	X13535	Masson et al	1989	*Bt entomocidus*	
Cry1Aa5	D17518	Udayasuriyan et al	1994	*Bt Fu-2-7*	
Cry1Aa6	U43605	Masson et al	1994	*Bt kurstaki NRD-12*	
Cry1Aa7	AF081790	Osman et al	1999	*Bt C12*	
Cry1Aa8	I26149	Liu	1996		
Cry1Aa9	AB026261	Nagamatsu et al	1999	*Bt dendrolimus T84A1*	
Cry1Aa10	AF154676	Hou and Chen	1999	*Bt kurstaki HD-1-02*	
Cry1Aa11	Y09663	Tounsi et al	1999	*Bt kurstaki*	
Cry1Aa12	AF384211	Yao et al	2001		No NCBI link
Cry1Ab1	M13898	Wabiko et al	1986	*Bt berliner 1715*	
Cry1Ab2	M12661	Thorne et al	1986	*Bt kurstaki*	
Cry1Ab3	M15271	Geiser et al	1986	*Bt kurstaki HD1*	
Cry1Ab4	D00117	Kondo et al	1987	*Bt kurstaki HD1*	
Cry1Ab5	X04698	Hofte et al	1986	*Bt berliner 1715*	
Cry1Ab6	M37263	Hefford et al	1987	*Bt kurstaki NRD-12*	
Cry1Ab7	X13233	Haider & Ellar	1988	*Bt aizawai IC1*	
Cry1Ab8	M16463	Oeda et al	1987	*Bt aizawai IPL7*	
Cry1Ab9	X54939	Chak & Jen	1993	*Bt aizawai HD133*	
Cry1Ab10	A29125	Fischhoff et al	1987	*Bt kurstaki HD1*	
Cry1Ab11	I12419	Ely & Tippett	1995	*Bt A20*	
Cry1Ab12	AF059670	Silva-Werneck et al	1998	*Bt kurstaki S93*	
Cry1Ab13	AF254640	Tan and Zhang	2000		No NCBI link
Cry1Ab14	U94191	Meza-Basso & Theoduloz	2000	*Native Chilean Bt*	
Cry1Ab15	AF358861	Li, Zhang et al	2001		No NCBI link
Cry1Ab-like	AF327924	Nagarathinam et al	2001	*Bt kunthala RX24*	uncertain sequence
Cry1Ab-like	AF327925	Nagarathinam et al	2001	*Bt kunthala RX28*	uncertain sequence
Cry1Ab-like	AF327926	Nagarathinam et al	2001	*Bt kunthala RX27*	uncertain sequence
Cry1Ac1	M11068	Adang et al	1985	*Bt kurstaki HD73*	
Cry1Ac2	M35524	Von Tersch et al	1991	*Bt kenyae*	
Cry1Ac3	X54159	Dardenne et al	1990	*Bt BTS89A*	
Cry1Ac4	M73249	Payne et al	1991	*Bt kurstaki PS85A1*	
Cry1Ac5	M73248	Payne et al	1992	*Bt kurstaki PS81GG*	
Cry1Ac6	U43606	Masson et al	1994	*Bt kurstaki NRD-12*	
Cry1Ac7	U87793	Herrera et al	1994	*Bt kurstaki HD73*	
Cry1Ac8	U87397	Omolo et al	1997	*Bt kurstaki HD73*	
Cry1Ac9	U89872	Gleave et al	1992	*Bt DSIR732*	

Name	Acc No.	Authors	Year	Source Strain	Comment
Cry1Ac10	AJ002514	Sun and Yu	1997	*Bt kurstaki YBT-1520*	
Cry1Ac11	AJ130970	Makhdoom & Riazuddin	1998		
Cry1Ac12	I12418	Ely & Tippett	1995	*Bt A20*	
Cry1Ac13	AF148644	Qiao et al	1999	*Bt kurstaki HD1*	
Cry1Ad1	M73250	Payne & Sick	1993	*Bt aizawai PS81I*	
Cry1Ad2	A27531		1995	*Bt PS81RR1*	
Cry1Ae1	M65252	Lee & Aronson	1991	*Bt alesti*	
Cry1Af1	U82003	Kang et al	1997	*Bt NT0423*	
Cry1Ag1	AF081248	Mustafa	1999		
Cry1Ah1	AF281866	Tan et al	2000		No NCBI link
Cry1A-like	AF327927	Nagarathinam et al	2001	*Bt kunthala nags3*	uncertain sequence
Cry1Ba1	X06711	Brizzard & Whiteley	1988	*Bt thuringiensis HD2*	
Cry1Ba2	X95704	Soetaert	1996	*Bt entomocidus HD110*	
Cry1Ba3	AF368257	Zhang et al	2001		
Cry1Bb1	L32020	Donovan et al	1994	*Bt EG5847*	
Cry1Bc1	Z46442	Bishop et al	1994	*Bt morrisoni*	
Cry1Bd1	U70726	Kuo et al	2000	*Bt wuhanensis HD525*	
Cry1Be1	AF077326	Payne et al	1998	*Bt PS158C2*	
Cry1Bf1	AX189649	Arnaut et al	2001		
Cry1Ca1	X07518	Honee et al	1988	*Bt entomocidus 60.5*	
Cry1Ca2	X13620	Sanchis et al	1989	*Bt aizawai 7.29*	
Cry1Ca3	M73251	Feitelson	1993	*Bt aizawai PS81I*	
Cry1Ca4	A27642	Van Mellaert et al	1990	*Bt entomocidus HD110*	
Cry1Ca5	X96682	Strizhov	1996	*Bt aizawai 7.29*	
Cry1Ca6 [1]	AF215647	Yu et al	2000	*Bt AF-2*	
Cry1Ca7	AY015492	Aixing et al	2000		No NCBI link
Cry1Ca8	AF362020	Chen, Wu et al	2001		No NCBI link
Cry1Cb1	M97880	Kalman et al	1993	*Bt galleriae HD29*	
Cry1Cb2	AY007686	Song et al	2000		No NCBI link
Cry1Da1	X54160	Hofte et al	1990	*Bt aizawai HD68*	
Cry1Da2	I76415	Payne & Sick	1997		
Cry1Db1	Z22511	Lambert	1993	*Bt BTS00349A*	
Cry1Db2	AF358862	Li et al	2001	*Bt B-Pr-88*	
Cry1Ea1	X53985	Visser et al	1990	*Bt kenyae 4F1*	
Cry1Ea2	X56144	Bosse et al	1990	*Bt kenyae*	
Cry1Ea3	M73252	Payne & Sick	1991	*Bt kenyae PS81F*	
Cry1Ea4	U94323	Barboza-Corona et al	1998	*Bt kenyae LBIT-147*	
Cry1Ea5	A15535	Botterman et al	1994		
cry1Ea6	AF202531	Sun et al	1999		No NCBI link
Cry1Eb1	M73253	Payne & Sick	1993	*Bt aizawai PS81A2*	
Cry1Fa1	M63897	Chambers et al	1991	*Bt aizawai EG6346*	
Cry1Fa2	M73254	Payne & Sick	1993	*Bt aizawai PS81I*	
Cry1Fb1	Z22512	Lambert	1993	*Bt BTS00349A*	
Cry1Fb2	AB012288	Masuda & Asano	1998	*Bt morrisoni INA67*	
Cry1Fb3	AF062350	Song & Zhang	1998	*Bt morrisoni*	
Cry1Fb4	I73895	Payne et al	1997		
Cry1Fb5	AF336114	Li et al	2001		No NCBI link
Cry1Ga1	Z22510	Lambert	1993	*Bt BTS0349A*	
Cry1Ga2	Y09326	Shevelev et al	1997	*Bt wuhanensis*	
Cry1Gb1	U70725	Kuo & Chak	1999	*Bt wuhanensis HD525*	

Name	Acc No.	Authors	Year	Source Strain	Comment
cry1Gb2	AF288683	Li et al	2000		No NCBI link
Cry1Ha1	Z22513	Lambert	1993	*Bt BTS02069AA*	
Cry1Hb1	U35780	Koo et al	1995	*Bt morrisoni BF190*	
Cry1H-like	AF182196	Srifah et al	1999	*Bt JC291*	insufficient sequence
Cry1Ia1	X62821	Tailor et al	1992	*Bt kurstaki*	
Cry1Ia2	M98544	Gleave et al	1993	*Bt kurstaki*	
Cry1Ia3	L36338	Shin et al	1995	*Bt kurstaki HD1*	
Cry1Ia4	L49391	Kostichka et al	1996	*Bt AB88*	
Cry1Ia5	Y08920	Selvapandiyan	1996	*Bt 61*	
Cry1Ia6	AF076953	Zhong et al	1998	*Bt kurstaki S101*	
Cry1Ia7	AF278797	Porcar	2000		No NCBI link
Cry1Ia8	AF373207	Song et al	2001		
Cry1Ib1	U07642	Shin et al	1995	*Bt entomocidus BP465*	
Cry1Ic1	AF056933	Osman et al	1998	*Bt C18*	
Cry1Ic2	AAE71691	Osman et al	2001		
Cry1Id1	AF047579	Choi	2000		
Cry1Ie1	AF211190	Song et al	2000	*Bt BTC007*	
Cry1I-like	I90732	Payne et al	1998		insufficient sequence
Cry1Ja1	L32019	Donovan et al	1994	*Bt EG5847*	
Cry1Jb1	U31527	Von Tersch & Gonzalez	1994	*Bt EG5092*	
Cry1Jc1	I90730	Payne et al	1998		
Cry1Jd1	AX189651	Arnaut et al	2001		
Cry1Ka1	U28801	Koo et al	1995	*Bt morrisoni BF190*	
Cry1-like	I90729	Payne et al	1998		insufficient sequence
Cry2Aa1	M31738	Donovan et al	1989	*Bt kurstaki*	
Cry2Aa2	M23723	Widner & Whiteley	1989	*Bt kurstaki HD1*	
Cry2Aa3	D86064	Sasaki et al	1997	*Bt sotto*	
Cry2Aa4	AF047038	Misra et al	1998	*Bt kenyae HD549*	
Cry2Aa5	AJ132464	Yu & Pang	1999	*Bt SL39*	
Cry2Aa6	AJ132465	Yu & Pang	1999	*Bt YZ71*	
Cry2Aa7	AJ132463	Yu & Pang	1999	*Bt CY29*	
Cry2Aa8	AF252262	Guo	2000		No NCBI link
Cry2Aa9	AF273218	Zhang et al	2000		No NCBI link
Cry2Ab1	M23724	Widner & Whiteley	1989	*Bt kurstaki HD1*	
Cry2Ab2	X55416	Dankocsik et al	1990	*Bt kurstaki HD1*	
Cry2Ab3	AF164666	Chen et al	1999	*Bt BTC002*	
Cry2Ab4	AF336115	Li et al	2001		No NCBI link
Cry2Ab5	AF441855	Yao et al	2001		No NCBI link
Cry2Ac1	X57252	Wu et al	1991	*Bt shanghai S1*	
Cry2Ac2	AY007687	Song et al	2000		No NCBI link
Cry2Ad1	AF200816	Choi et al	1999	*Bt BR30*	
Cry3Aa1	M22472	Herrnstadt et al	1987	*Bt san diego*	
Cry3Aa2	J02978	Sekar et al	1987	*Bt tenebrionis*	
Cry3Aa3	Y00420	Hofte et al	1987		
Cry3Aa4	M30503	McPherson et al	1988	*Bt tenebrionis*	
Cry3Aa5	M37207	Donovan et al	1988	*Bt morrisoni EG2158*	
Cry3Aa6	U10985	Adams et al	1994	*Bt tenebrionis*	
Cry3Aa7	AJ237900	Zhang et al	1999	*Bt 22*	
Cry3Ba1	X17123	Sick et al	1990	*Bt tolworthi 43F*	
Cry3Ba2	A07234	Peferoen et al	1990	*Bt PGSI208*	

Name	Acc No.	Authors	Year	Source Strain	Comment
Cry3Bb1	M89794	Donovan et al	1992	*Bt EG4961*	
Cry3Bb2	U31633	Donovan et al	1995	*Bt EG5144*	
Cry3Bb3	I15475	Peferoen et al	1995		
Cry3Ca1	X59797	Lambert et al	1992	*Bt kurstaki BtI109P*	
Cry4Aa1	Y00423	Ward & Ellar	1987	*Bt israelensis*	
Cry4Aa2	D00248	Sen et al	1988	*Bt israelensis HD522*	
Cry4Ba1	X07423	Chungjatpornchai et al	1988	*Bt israelensis 4Q2-72*	
Cry4Ba2	X07082	Tungpradubkul et al	1988	*Bt israelensis*	
Cry4Ba3	M20242	Yamamoto et al	1988	*Bt israelensis*	
Cry4Ba4	D00247	Sen et al	1988	*Bt israelensis HD522*	
Cry5Aa1	L07025	Narva et al	1994	*Bt darmstadiensis PS17*	
Cry5Ab1	L07026	Narva et al	1991	*Bt darmstadiensis PS17*	
Cry5Ac1	I34543	Payne et al	1997		
Cry5Ba1	U19725	Foncerrada &Narva	1997	*Bt PS86Q3*	
Cry6Aa1	L07022	Narva et al	1993	*Bt PS52A1*	
Cry6Ba1	L07024	Narva et al	1991	*Bt PS69D1*	
Cry7Aa1	M64478	Lambert et al	1992	*Bt galleriae PGSI245*	
Cry7Ab1	U04367	Payne & Fu	1994	*Bt dakota HD511*	
Cry7Ab2	U04368	Payne & Fu	1994	*Bt kumamotoensis 867*	
Cry8Aa1	U04364	Narva & Fu	1992	*Bt kumamotoensis*	
Cry8Ba1	U04365	Narva & Fu	1993	*Bt kumamotoensis*	
Cry8Ca1	U04366	Ogiwara et al.	1995	*Bt japonensis Buibui*	
Cry9Aa1	X58120	Smulevitch et al	1991	*Bt galleriae*	
Cry9Aa2	X58534	Gleave et al	1992	*Bt DSIR517*	
Cry9Ba1	X75019	Shevelev et al	1993	*Bt galleriae*	
Cry9Ca1	Z37527	Lambert et al	1996	*Bt tolworthi*	
Cry9Da1	D85560	Asano et al	1997	*Bt japonensis N141*	
Cry9Da2	AF042733	Wasano & Ohba	1998	*Bt japonensis*	
Cry9Ea1	AB011496	Midoh & Oyama	1998	*Bt aizawai SSK-10*	
cry9Ea2	AF358863	Li et al	2001		No NCBI link
Cry9Eb1	AX189653	Arnaut et al	2001		
Cry9 like	AF093107	Wasano et al	1998	*Bt galleriae*	insufficient sequence
Cry10Aa1	M12662	Thorne et al	1986	*Bt israelensis*	
Cry10Aa2	E00614	Aran & Toomasu	1996	*Bt israelensis ONR-60A*	
Cry11Aa1	M31737	Donovan et al	1988	*Bt israelensis*	
Cry11Aa2	M22860	Adams et al	1989	*Bt israelensis*	
Cry11Ba1	X86902	Delecluse et al	1995	*Bt jegathesan 367*	
Cry11Bb1	AF017416	Orduz et al	1998	*Bt medellin*	
Cry12Aa1	L07027	Narva et al	1991	*Bt PS33F2*	
Cry13Aa1	L07023	Narva et al	1992	*Bt PS63B*	
Cry14Aa1	U13955	Narva et al	1994	*Bt sotto PS80JJ1*	
Cry15Aa1	M76442	Brown & Whiteley	1992	*Bt thompsoni*	
Cry16Aa1	X94146	Barloy et al	1996	*Cb malaysia CH18*	
Cry17Aa1	X99478	Barloy et al	1998	*Cb malaysia CH18*	
Cry18Aa1	X99049	Zhang et al	1997	*Paenibacillus popilliae*	
Cry18Ba1	AF169250	Patel et al	1999	*Paenibacillus popilliae*	
Cry18Ca1	AF169251	Patel et al	1999	*Paenibacillus popilliae*	
Cry19Aa1	Y07603	Rosso & Delecluse	1996	*Bt jegathesan 367*	
Cry19Ba1	D88381	Hwang et al	1998	*Bt higo*	
Cry20Aa1	U82518	Lee & Gill	1997	*Bt fukuokaensis*	
Cry21Aa1	I32932	Payne et al	1996		

Name	Acc No.	Authors	Year	Source Strain	Comment
Cry21Aa2	I66477	Feitelson	1997		
Cry22Aa1	I34547	Payne et al	1997		
Cry22Ab1	AAK50456	Baum et al	2000	*Bt EG4140*	
Cry23Aa1	AAF76375	Donovan et al	2000	*Bt*	Binary with Cry37Aa1
Cry24Aa1	U88188	Kawalek and Gill	1998	*Bt jegathesan*	
Cry25Aa1	U88189	Kawalek and Gill	1998	*Bt jegathesan*	
Cry26Aa1	AF122897	Wojciechowska et al	1999	*Bt finitimus B-1166*	
Cry27Aa1	AB023293	Saitoh	1999	*Bt higo*	
Cry28Aa1	AF132928	Wojciechowska et al	1999	*Bt finitimus B-1161*	
Cry28Aa2	AF285775	Moore and Debro	2000	*Bt finitimus*	
Cry29Aa1	AJ251977	Delecluse et al	2000		No NCBI link
Cry30Aa1	AJ251978	Delecluse et al	2000		No NCBI link
Cry31Aa1	AB031065	Mizuki et al	2000	*Bt 84-HS-1-11*	
Cry32Aa1	AY008143	Balasubramanian et al	2001	*Bt yunnanensis*	
Cry32Ba1	BAB78601	Takebe et al	2001	*Bt*	
Cry32Ca1	BAB78602	Takebe et al	2001	*Bt*	
Cry32Da1	BAB78603	Takebe et al	2001	*Bt*	
Cry33Aa1	AAL26871	Kim et al	2001	*Bt dakota*	
Cry34Aa1	AAG50341	Ellis et al	2001	*Bt PS80JJ1*	Binary with Cry35Aa1
Cry34Aa2	AAK64560	Rupar et al	2001	*Bt EG5899*	Binary with Cry35Aa2
Cry34Ab1	AAG41671	Moellenbeck et al	2001	*Bt PS149B1*	Binary with Cry35Ab1
Cry34Ac1	AAG50118	Ellis et al	2001	*Bt PS167H2*	Binary with Cry35Ac1
Cry34Ac2	AAK64562	Rupar et al	2001	*Bt EG9444*	Binary with Cry35Ab2
Cry34Ba1	AAK64566	Rupar et al	2001	*Bt EG4851*	Binary with Cry35Ba1
Cry35Aa1	AAG50342	Ellis et al	2001	*Bt PS80JJ1*	Binary with Cry34Aa1
Cry35Aa2	AAK64561	Rupar et al	2001	*Bt EG5899*	Binary with Cry34Aa2
Cry35Ab1	AAG41672	Moellenbeck et al	2001	*Bt PS149B1*	Binary with Cry34Ab1
Cry35Ab2	AAK64563	Rupar et al	2001	*Bt EG9444*	Binary with Cry34Ac2
Cry35Ac1	AAG50117	Ellis et al	2001	*Bt PS167H2*	Binary with Cry34Ac1
Cry35Ba1	AAK64566	Rupar et al	2001	*Bt EG4851*	Binary with Cry34Ba1
Cry36Aa1	AAK64558	Rupar et al	2001	*Bt*	
Cry37Aa1	AAF76376	Donovan et al	2000	*Bt*	Binary with Cry23Aa
Cry38Aa1	AAK64559	Rupar et al	2000	*Bt*	
Cry39Aa1	BAB72016	Ito et al	2001	*Bt aizawai*	
Cry40Aa1	BAB72018	Ito et al	2001	*Bt aizawai*	
Cyt1Aa1	X03182	Waalwijk et al	1985	*Bt israelensis*	
Cyt1Aa2	X04338	Ward & Ellar	1986	*Bt israelensis*	
Cyt1Aa3	Y00135	Earp & Ellar	1987	*Bt morrisoni PG14*	
Cyt1Aa4	M35968	Galjart et al	1987	*Bt morrisoni PG14*	
Cyt1Ab1	X98793	Thiery et al	1997	*Bt medellin*	
Cyt1Ba1	U37196	Payne et al	1995	*Bt neoleoensis*	
Cyt2Aa1	Z14147	Koni & Ellar	1993	*Bt kyushuensis*	
Cyt2Aa2	AF472606	Promdonkoy	2001	*Bt darmstadiensis73E10*	
Cyt2Ba1	U52043	Guerchicoff et al	1997	*Bt israelensis 4Q2*	
Cyt2Ba2	AF020789	Guerchicoff et al	1997	*Bt israelensis PG14*	
Cyt2Ba3	AF022884	Guerchicoff et al	1997	*Bt fuokukaensis*	
Cyt2Ba4	AF022885	Guerchicoff et al	1997	*Bt morrisoni HD12*	
Cyt2Ba5	AF022886	Guerchicoff et al	1997	*Bt morrisoni HD518*	
Cyt2Ba6	AF034926	Guerchicoff et al	1997	*Bt tenebrionis*	
Cyt2Ba7	AF215645	Yu & Pang	2000	*Bt T301*	
Cyt2Ba8	AF215646	Yu & Pang	2000	*Bt T36*	

Name	Acc No.	Authors	Year	Source Strain	Comment
Cyt2Bb1	U82519	Cheong & Gill	1997	*Bt jegathesan*	
Cyt2Ca1	AAK50455	Baum et al	2001	*Bt*	

8.2 Appendix 2: Table of host ranges for Bt taken from van Frankenhuizen & Nystrom (1999)

This is a severely abbreviated listing and there is no doubt that many insect associations and demonstrated toxicities are not included in the table. However, the data are a useful indication of activity and, in the context of risk assessment, of non-activity despite feeding the test organisms with very high toxin dosages. It should be noted that the host ranges indicated in the Table are based primarily on toxicity demonstrated using WT *Bt* in which solubilisation and proteolytic dissolution of the protoxin in the guts of the hosts is necessary for the toxin to become active.

The shaded rows indicate those species where the *Bt* tests were regarded as non-active.

http//www.glfc.cfs.nrcan.gc.ca/Bacillus/btsearch.cfm
(accessed February 2002)

Gene	Genus	Species	Order/Taxon	AssayType	InstarAge	Resistance	ED50	Units	Parameter	Qualitative
cry01Aa	*Argyrotaenia*	*citrana*	Lepidoptera	Diet surface	neonate		32.0	ng/cm2	mortality	
cry01Aa	*Chilo*	*suppressalis*	Lepidoptera	Diet surface	L2		1690(1350-2030)	ng/cm2	mortality	
cry01Aa	*Choristoneura*	*rosaceana*	Lepidoptera	Diet surface	neonate		1.8(0.4-4.3)	ng/cm2	mortality	
cry01Aa	*Cydia*	*pomonella*	Lepidoptera	Diet surface	neonate		97000(46500-154000)	ng/ml	mortality	active
cry01Aa	*Earias*	*vittella*	Lepidoptera	Leaf surface	neonate		0.599(0.380-0.929)	ng/cm2	mortality	active
cry01Aa	*Helicoverpa*	*armigera*	Lepidoptera	Diet incorp.	neonate		370(140-950)	ng/ml	mortality	
cry01Aa	*Pandemis*	*pyrusana*	Lepidoptera	Diet surface	neonate		1.1(0.4-2.5)	ng/cm2	mortality	
cry01Aa	*Platynota*	*stultana*	Lepidoptera	Diet surface	neonate		0.7(0.4-1.1)	ng/cm2	mortality	
cry01Aa	*Plutella*	*xylostella*	Lepidoptera	Diet surface	L3		1.4(0.8-2.9)	ng/cm2	mortality	active
cry01Aa	*Spodoptera*	*exigua*	Lepidoptera	Diet incorp.	neonate		275000(210000-443000)	ng/ml	mortality	
cry01Aa	*Trichoplusia*	*ni*	Lepidoptera	Diet incorp.	neonate		6670(4870-10260)	ng/ml	mortality	
cry01Aa1	*Bombyx*	*mori*	Lepidoptera	Leaf surface			370(0.7-729)	ng/larva	mortality	
cry01Aa1	*Choristoneura*	*fumiferana*	Lepidoptera	Diet incorp.	L2		6000(3000-11000)	ng/ml	mortality	
cry01Aa1	*Ephestia*	*kuehniella*	Lepidoptera	Diet incorp.	neonate		>100	OD565/ml	mortality	
cry01Aa1	*Helicoverpa*	*zea*	Lepidoptera	Diet surface	neonate		2070(1668-2528)	ng/cm2	mortality	
cry01Aa1	*Heliothis*	*virescens*	Lepidoptera	Diet surface	neonate		157(43-574)	ng/cm2	mortality	
cry01Aa1	*Hyphantria*	*cunea*	Lepidoptera	Diet surface	L1		100(16-364)	ng/cm2	mortality	
cry01Aa1	*Lymantria*	*dispar*	Lepidoptera	Force feed	L4		151(100-190)	ng/larva	weight	
cry01Aa1	*Mamestra*	*brassicae*	Lepidoptera	Diet surface	L1		165	ng/cm2	mortality	
cry01Aa1	*Manduca*	*sexta*	Lepidoptera	Diet surface	neonate		23(20-27)	ng/cm2	mortality	
cry01Aa1	*Ostrinia*	*nubilalis*	Lepidoptera	Diet surface	neonate		1247(273-5694)	ng/cm2	mortality	
cry01Aa1	*Perileucoptera*	*coffeella*	Lepidoptera	Leaf dip	neonate		>1000000	ng/ml	mortality	not active
cry01Aa1	*Pieris*	*brassicae*	Lepidoptera	Leaf dip	L3		800	ng/ml	mortality	
cry01Aa1	*Pseudoplusia*	*includens*	Lepidoptera	Diet surface	neonate		7.7(6.6-8.8)	ng/cm2	mortality	
cry01Aa1	*Sciropophaga*	*incertulas*	Lepidoptera	Diet incorp.	L1		1.32+/-1.19	ng/ml	mortality	
cry01Aa1	*Spodoptera*	*frugiperda*	Lepidoptera	Diet surface	neonate		>5555	ng/cm2	mortality	
cry01Aa1	*Spodoptera*	*littoralis*	Lepidoptera	Diet surface	L2	cry01C	>4.7X10**6	ng/cm2	mortality	
cry01Aa1	*Thaumetopoea*	*pityocampa*	Lepidoptera	Leaf dip	neonate		956(586-1552)	ng/ml	mortality	
cry01Aa3	*Spodoptera*	*litura*	Lepidoptera	Diet incorp.	L4		0.3-1.0X10**6	ng/ml	mortality	
cry01Aa6	*Actebia*	*fennica*	Lepidoptera	Force feed	L5		>3500	ng/larva	frass	

Gene	Genus	Species	Order/Taxon	AssayType	InstarAge	Resistance	ED50	Units	Parameter	Qualitative
cry01Aa6	*Choristoneura*	*occidentalis*	Lepidoptera	Force feed	L6		11.6(6.2-19.5)	ng/larva	frass	
cry01Aa6	*Choristoneura*	*pinus pinus*	Lepidoptera	Force feed	L6		9.2(1.8-19.8)	ng/larva	frass	
cry01Aa6	*Elasmolpalpus*	*lignosellus*	Lepidoptera	Diet incorp.	neonate		54000(31000-116000)	ng/ml	mortality	
cry01Aa6	*Malacosoma*	*disstria*	Lepidoptera	Force feed	L5		36.9(12.5-83.2)	ng/larva	frass	
cry01Aa6	*Orgyia*	*leucostigma*	Lepidoptera	Force feed	L4		52.6(36.2-76.3)	ng/larva	frass	
cry01Ab	*Mamestra*	*configurata*	Lepidoptera	Diet incorp.	L2		1410(240-3190)	ng/cm2	mortality	
cry01Ab10	*Agrotis*	*ipsilon*	Lepidoptera	Diet incorp.	neonate		>80000	ng/ml	mortality	
cry01Ab5	*Plodia*	*interpunctella*	Lepidoptera	Diet surface	L2-L3	Dipel	26300	ng/larva	mortality	
cry01Ab6	*Chrysoperla*	*carnea*	Neuroptera	Diet incorp.	L1,L2,L3				mortality	active
cry01Ab7	*Aedes*	*aegypti*	Diptera	Free ingestion	L2				mortality	active
cry01Ac	*Folsomia*	*candida*	Collembola	Diet incorp.	immature				mortality	not active
cry01Ac	*Xenylla*	*grisea*	Collembola	Diet incorp.	immature				mortality	not active
cry01Ac	*Epiphyas*	*postvittana*	Lepidoptera	Diet incorp.	neonate		14700	ng/ml	mortality	
cry01Ac7	*Eldana*	*saccharina*	Lepidoptera	Diet incorp.	14d				mortality	active
cry01Ac8	*Glossina*	*mortisans mortisans*	Diptera	Free ingestion	Adult		740	ng/ml	mortality	active
cry01Ac9	*Ctenopsuestis*	*obliquana*	Lepidoptera	Diet incorp.	L1		144(96-191)	nL-lysate/ml	mortality	
cry01Ac9	*Planotortrix*	*octo*	Lepidoptera	Diet incorp.	L1		149(109-194)	nL-lysate/ml	mortality	
cry01B	*Diatraea*	*grandiosella*	Lepidoptera	Diet incorp.	neonate		5200(3600-5500)	ng/ml	mortality	
cry01B	*Diatraea*	*saccharalis*	Lepidoptera	Diet incorp.	neonate		113600(45,800-318,900)	ng/ml	mortality	
cry01Ba	*Wiseana*	*cervinata*	Lepidoptera	Diet incorp.	neonate		6400	ng/ml	mortality	
cry01Ba1	*Chrysomela*	*scripta*	Coleoptera	Leaf surface	L2	cry03Aa	277,000(130,300-853,000)	ng/cm2	mortality	
cry01Ba1	*Chrysomela*	*scripta*	Coleoptera	Leaf surface	L2	cry03Aa	29,240(18,000-45,000)	ng/cm2	mortality	
cry01Ba1	*Leptinotarsa*	*decemlineata*	Coleoptera	Leaf surface	neonate		1.0(0.8-1.3)X10**6	ng/ml	mortality	
cry01Ba1	*Artogeia*	*rapae*	Lepidoptera		neonate		58(30-117)	ng/cm2	mortality	active
cry01Ba2	*Lambdina*	*fiscellaria fiscellaria*	Lepidoptera	Force feed	L5		>208	ng/larva	frass	
cry01Bc1	*Musca*	*domestica*	Diptera	Diet incorp.	neonate				mortality	active
cry01Ca	*Anopheles*	*gambiae*	Diptera	Free ingestion	6d		283000(245000-346000)	ng/ml	mortality	
cry01Ca	*Culex*	*quinquefasciatus*	Diptera	Free ingestion	3d		126000(109000-151000)	ng/ml	mortality	
cry01Ca4	*Spodoptera*	*exempta*	Lepidoptera	Leaf dip	L1		380(230-550)	ng/ml	mortality	
cry01Ia2	*Tenebrio*	*molitor*	Coleoptera	Diet incorp.	L1				mortality	not active
cry01Ia2	*Culex*	*pervigalans*	Diptera	Free ingestion	L3				mortality	not active
cry01Ia3	*Agelastica*	*coerulae*	Coleoptera	Leaf dip	L3		>1900000	ng/ml	mortality	not active
cry01Ia3	*Phaedon*	*brassicae*	Coleoptera	Leaf dip	L3		>190000	ng/ml	mortality	not active
cry01Ia4	*Diabrotica*	*spp.*	Coleoptera	Diet surface					mortality	not active
cry01Jb1	*Diabrotica*	*undecimpunctata*	Coleoptera	Diet surface	neonate				mortality	not active
cry01Jb1	*Anticarsia*	*gemmatalis*	Lepidoptera	Diet surface	neonate				mortality	not active
cry02A	*Folsomia*	*candida*	Collembola	Diet incorp.	immature				mortality	not active
cry02A	*Xenylla*	*grisea*	Collembola	Diet incorp.	immature				mortality	not active
cry02Aa	*Anthonomus*	*grandis*	Coleoptera	Diet surface	L1				mortality	not active
cry02Aa	*Diabrotica*	*undecimpunctata*	Coleoptera	Diet surface	neonate				mortality	not active
cry02Aa	*Folsomia*	*candida*	Collembola	Diet incorp.	immature				mortality	not active
cry02Aa	*Hippodamia*	*convergens*	Collembola	Free ingestion	Adult				mortality	not active
cry02Aa	*Leptinotarsa*	*decemlineata*	Collembola	Diet surface	neonate				mortality	not active
cry02Aa	*Aedes*	*aegypti*	Diptera	Free ingestion	L3		65.5(41.1-100)	ng/ml	mortality	
cry02Aa	*Aedes*	*triseriatus*	Diptera	Free ingestion	L3-L4		2840	ng/ml	mortality	
cry02Aa	*Anopheles*	*quadrimaculatus*	Diptera	Free ingestion	L3-L4		370	ng/ml	mortality	

Gene	Genus	Species	Order/Taxon	AssayType	InstarAge	Resistance	ED50	Units	Parameter	Qualitative
cry02Aa	*Culex*	*pipiens*	Diptera	Free ingestion	L2-L4		>200000	ng/ml	mortality	
cry02Aa	*Drosophila*	*melanogaster*	Diptera	Free ingestion	L1		>100000	ng/ml	mortality	
cry02Aa	*Musca*	*domestica*	Diptera	Free ingestion	neonate		>100000	ng/ml	mortality	
cry02Aa	*Rhopalosiphum*	*padi*	Hom: Aphididae	Free ingestion	L1-L4				mortality	not active
cry02Aa	*Oncopeltrus*	*fasciatus*	Hom: Lygaeidae	Diet incorp.	L2-L3				mortality	not active
cry02Aa	*Bemisia*	*tabaci*	Homoptera	Free ingestion	Adult				mortality	not active
cry02Aa	*Macrocentrus*	*ancylivorus*	Hym: Braconidae	Free ingestion					mortality	not active
cry02Aa	*Meteorus*	*pulchricornis*	Hym: Braconidae	Free ingestion					mortality	not active
cry02Aa	*Nasonia*	*vitripennis*	Hym: Pteromalidae	Free ingestion					mortality	not active
cry02Aa	*Apis*	*mellifera*	Hymenoptera	Free ingestion	Adult				mortality	not active
cry02Aa	*Porcellio*	*scaber*	Isopoda	Diet incorp.	Adult				mortality	not active
cry02Aa	*Reticulitermes*	*flavipes*	Isoptera	Diet incorp.	Adult				mortality	not active
cry02Aa	*Actebia*	*fennica*	Lepidoptera	Diet incorp.	neonate		6	ng/ml	weight	
cry02Aa	*Agrotis*	*ipsilon*	Lepidoptera	Diet incorp.	neonate		>200000	ng/ml	mortality	
cry02Aa	*Anticarsia*	*gemmatalis*	Lepidoptera	Diet incorp.	neonate		16	ng/ml	mortality	
cry02Aa	*Helicoverpa*	*armigera*	Lepidoptera	Diet incorp.	neonate		710(16-1960)	ng/ml	mortality	
cry02Aa	*Heliothis*	*virescens*	Lepidoptera	Diet incorp.	neonate		1140	ng/ml	mortality	
cry02Aa	*Lymantria*	*dispar*	Lepidoptera	Force feed	L4		102(77-181)	ng/larva	weight	
cry02Aa	*Manduca*	*sexta*	Lepidoptera	Diet incorp.	neonate		1860	ng/ml	mortality	
cry02Aa	*Ostrinia*	*nubilalis*	Lepidoptera	Diet incorp.	neonate		60	ng/ml	mortality	
cry02Aa	*Plutella*	*xylostella*	Lepidoptera	Diet surface	L3		19480(12.987-32467)	ng/cm2	mortality	not active
cry02Aa	*Pseudoplusia*	*includens*	Lepidoptera	Diet incorp.	neonate		55	ng/ml	mortality	
cry02Aa	*Spodoptera*	*exigua*	Lepidoptera	Diet incorp.	neonate		79200	ng/ml	mortality	
cry02Aa	*Spodoptera*	*frugiperda*	Lepidoptera	Diet incorp.	neonate		>200000	ng/ml	mortality	
cry02Aa	*Trichoplusia*	*ni*	Lepidoptera	Diet incorp.	neonate		1280	ng/ml	mortality	
cry02Aa	*Chrysoperla*	*carnea*	Neuroptera	Diet surface	immature				mortality	not active
cry02Aa	*Acheta*	*domesticus*	Orthoptera	Diet incorp.	L3-L4				mortality	not active
cry02Aa	*Blattella*	*germanica*	Orthoptera	Diet incorp.	L3-L4				mortality	not active
cry02Aa1	*Chilo*	*suppressalis*	Lepidoptera	Diet incorp.	L1		67.2+/-40.0	ng/ml	mortality	
cry02Aa1	*Helicoverpa*	*zea*	Lepidoptera	Diet surface	neonate		113	ng/cm2	mortality	
cry02Aa1	*Sciropophaga*	*incertulas*	Lepidoptera	Diet incorp.	L1		5.45+/-3.5	ng/ml	mortality	
cry02Aa2	*Culex*	*quinquefasciatus*	Diptera	Free ingestion	L2		1630	ng/ml	mortality	
cry02Aa2	*Cnaphalocrocis*	*medinalis*	Lepidoptera	in planta	L2				mortality	active
cry02Aa2	*Cydia*	*pomonella*	Lepidoptera	Diet surface	neonate		52200(30200-74400)	ng/ml	mortality	active
cry02Aa2	*Elasmolpalpus*	*lignosellus*	Lepidoptera	Diet incorp.	neonate		1340(900-2000)	ng/ml	mortality	
cry02Aa2	*Plodia*	*interpunctella*	Lepidoptera	Diet surface	L3		1400(600-3300)	ng/larva	mortality	
cry02Aa2	*Tryporyza*	*incertulas*	Lepidoptera	in planta	L2				mortality	active
cry02Aa3	*Bombyx*	*mori*	Lepidoptera	Force feed	L4		620(470-830)	ng/larva	mortality	
cry02Ab	*Anopheles*	*gambiae*	Diptera	Free ingestion					mortality	active
cry02Ab	*Pieris*	*brassicae*	Lepidoptera	Leaf dip					mortality	active
cry03A	*Folsomia*	*candida*	Collembola	Diet incorp.	immature				mortality	not active
cry03A	*Xenylla*	*grisea*	Collembola	Diet incorp.	immature				mortality	not active
cry03Aa	*Phaedon*	*cochleariae*	Coleoptera	Leaf dip	L2				mortality	Highly active
cry03Aa	*Tenebrio*	*molitor*	Coleoptera	Force feed					mortality	low activity
cry03Aa1	*Attagenus*	*unicolor*	Coleoptera	Diet incorp.	L3				mortality	not active
cry03Aa1	*Callosobruchus*	*maculatus*	Coleoptera	Diet incorp.	Larva				mortality	low activity
cry03Aa1	*Carpophilus*	*hemipterus*	Coleoptera	Diet incorp.	Larva				mortality	not active

Gene	Genus	Species	Order/Taxon	AssayType	InstarAge	Resistance	ED50	Units	Parameter	Qualitative
cry03Aa1	*Diabrotica*	*balteata*	Coleoptera	Diet incorp.	Adult				mortality	low activity
cry03Aa1	*Diabrotica*	*undecimpunctata*	Coleoptera	Leaf surface	Adult,L1,L2				mortality	low activity
cry03Aa1	*Gibbium*	*psylliodes*	Coleoptera	Diet incorp.	Adult				mortality	not active
cry03Aa1	*Haltica*	*tombacina*	Coleoptera	Leaf surface	Adult,L2,L3				mortality	Highly active
cry03Aa1	*Hypera*	*brunneipennis*	Coleoptera	Leaf surface	Larva				mortality	Highly active
cry03Aa1	*Leptinotarsa*	*decemlineata*	Coleoptera	Leaf surface	L1,L2				mortality	Highly active
cry03Aa1	*Otiorhynchus*	*sulcatus*	Coleoptera	Diet incorp.	L2,L3				mortality	Intermediate active
cry03Aa1	*Pyrrhalta*	*luteola*	Coleoptera	Leaf surface			120	ng/cm2	mortality	Highly active
cry03Aa1	*Tribolium*	*castaneum*	Coleoptera	Diet incorp.	Adult,L3				mortality	Intermediate active
cry03Aa1	*Aedes*	*aegypti*	Diptera	Free ingestion	L1				mortality	not active
cry03Aa1	*Musca*	*domestica*	Diptera	Diet incorp.	Larva				mortality	not active
cry03Aa1	*Spodoptera*	*exigua*	Lepidoptera	Diet incorp.	L1				mortality	not active
cry03Aa1	*Trichoplusia*	*ni*	Lepidoptera	Diet incorp.	L1				mortality	not active
cry03Aa4	*Hypera*	*postica*	Coleoptera	Leaf dip	L1				mortality	not active
cry03Aa4	*Phyllotreta*	*armoraciae*	Coleoptera	Leaf dip	L1				mortality	not active
cry03Aa4	*Popillia*	*japonica*	Coleoptera	Leaf dip	L1				mortality	not active
cry03Ba2	*Heliothis*	*virescens*	Lepidoptera	Diet surface					mortality	not active
cry03Ba2	*Manduca*	*sexta*	Lepidoptera	Diet surface					mortality	not active
cry03Ba2	*Spodoptera*	*littoralis*	Lepidoptera	Diet surface					mortality	not active
cry04Aa	*Aedes*	*aegypti*	Diptera	Free ingestion	L4		1600(1300-2000)	ng/ml	mortality	
cry04Aa	*Anopheles*	*stephensi*	Diptera	Free ingestion	L3		7400(6600-8400)	ng/ml	mortality	
cry04Aa	*Culex*	*pipiens*	Diptera	Free ingestion	L4		400(300-500)	ng/ml	mortality	
cry04Aa1	*Anopheles*	*gambiae*	Diptera	Free ingestion	3d		1070(770-1530)	ng/ml	mortality	
cry04Aa1	*Culex*	*quinquefasciatus*	Diptera	Free ingestion	3d		910(640-1320)	ng/ml	mortality	
cry04Ba	*Tipula*	*oleracae*	Diptera	Leaf surface	L1		3.0X10**7	CFU/cm2	mortality	
cry04Ba1	*Manduca*	*sexta*	Lepidoptera	Diet surface	L1				mortality	not active
cry05Aa1	*Caenorhabditis*	*elegens*	Rhabditida	Free ingestion					mortality	active
cry05Aa1	*Pratylenchus*	*spp.*	Rhabditida	Free ingestion					mortality	active
cry05Ab1	*Fasciola*	*hepatica*	Prosostomata (liver fluke)							active
cry06Aa1	*Panagrellus*	*redivivus*	Rhabditida	Free ingestion					mortality	active
cry06Aa1	*Pratylenchus*	*scribneri*	Rhabditida	Free ingestion					mortality	active
cry06Ba1	*Pratylenchus*	*spp.*	Rhabditida	Free ingestion					mortality	active
cry08Aa1	*Leptinotarsa*	*decemlineata*	Coleoptera	Leaf dip	L2				mortality	active
cry08Ba1	*Cotinis*	*spp.*	Coleoptera	Diet incorp.	L3				mortality	active
cry08Ba1	*Cyclocephala*	*borealis*	Coleoptera	Diet incorp.	L3				mortality	active
cry08Ba1	*Cyclocephala*	*pasadenae*	Coleoptera	Diet incorp.	L3				mortality	active
cry08Ba1	*Popillia*	*japonica*	Coleoptera	Diet incorp.	L3				mortality	active
cry08Ca1	*Anomala*	*cuprea*	Coleoptera	Diet incorp.	L1				mortality	active
cry09A	*Helicoverpa*	*armigera*	Lepidoptera	Diet incorp.	neonate				mortality	not active
cry09Aa1	*Galleria*	*mellonella*	Lepidoptera	Diet incorp.			30000	ng/ml	weight	low activity
cry09Aa1	*Lymantria*	*dispar*	Lepidoptera	Diet incorp.			>10000	ng/ml	mortality	not active
cry09Aa2	*Ctenopsuestis*	*obliquana*	Lepidoptera						mortality	Intermediate active
cry09Aa2	*Epiphyas*	*postvittana*	Lepidoptera						mortality	Intermediate active

Gene	Genus	Species	Order/Taxon	AssayType	InstarAge	Resistance	ED50	Units	Parameter	Qualitative
cry09Aa2	*Phthorimaea*	*operculella*	Lepidoptera	Diet incorp.			80	ng/ml	mortality	
cry09Aa2	*Planotortrix*	*octo*	Lepidoptera						mortality	Intermediate active
cry09Aa2	*Spodoptera*	*litura*	Lepidoptera						mortality	not active
cry09Aa2	*Wiseana*	*cervinata*	Lepidoptera						mortality	not active
cry09Ca	*Plutella*	*xylostella*	Lepidoptera	Diet surface	L3		3.3(2.9-3.7)	ng/cm2	mortality	active
cry09Ca1	*Actebia*	*fennica*	Lepidoptera	Force feed	L5		>4691	ng/larva	frass	
cry09Ca1	*Agrotis*	*segetum*	Lepidoptera	Diet surface	neonate		234(165.2-327.1)	ng/cm2	mortality	
cry09Ca1	*Bombyx*	*mori*	Lepidoptera	Force feed	L4		13.4(8.1-23.4)	ng/larva	mortality	
cry09Ca1	*Choristoneura*	*fumiferana*	Lepidoptera	Force feed	L6		7.5(6.1-9.2)	ng/larva	frass	
cry09Ca1	*Heliothis*	*virescens*	Lepidoptera	Diet surface	neonate		51.8(39.2-64.9)	ng/cm2	mortality	
cry09Ca1	*Mamestra*	*brassicae*	Lepidoptera	Diet surface	neonate		78.8(53.5-111.4)	ng/cm2	mortality	
cry09Ca1	*Manduca*	*sexta*	Lepidoptera	Diet surface	neonate		83.0(66.9-103.4)	ng/cm2	mortality	
cry09Ca1	*Ostrinia*	*nubilalis*	Lepidoptera	Diet surface	neonate		96.6(77.7-119.8)	ng/cm2	mortality	
cry09Ca1	*Spodoptera*	*exigua*	Lepidoptera	Diet surface	neonate		133(96.7-180.4)	ng/cm2	mortality	
cry09Ca1	*Spodoptera*	*frugiperda*	Lepidoptera	Diet surface	neonate		>1350	ng/cm2	mortality	
cry09Ca1	*Spodoptera*	*littoralis*	Lepidoptera	Diet surface	neonate		65.5(44.9-93.7)	ng/cm2	mortality	
cry10Aa	*Aedes*	*aegypti*	Diptera	Free ingestion	L2				mortality	low activity
cry10Aa	*Anopheles*	*stephensi*	Diptera	Free ingestion	L2				mortality	not active
cry10Aa	*Culex*	*pipiens*	Diptera	Free ingestion	L2				mortality	not active
cry11Aa	*Aedes*	*aegypti*	Diptera	Free ingestion	L1		39.2(33.4-45.9)	ng/ml	mortality	
cry11Aa	*Anopheles*	*albimanus*	Diptera	Free ingestion	L1		675(507-900)	ng/ml	mortality	
cry11Aa	*Anopheles*	*stephensi*	Diptera	Free ingestion	L3		455(387-534)	ng/ml	mortality	
cry11Aa	*Culex*	*pipiens*	Diptera	Free ingestion	L4		268(240-300)	ng/ml	mortality	
cry11Aa	*Culex*	*quinquefasciatus*	Diptera	Free ingestion	L4		64(48-84)	ng/ml	mortality	
cry12Aa1	*Pratylenchus*	*spp.*	Rhabditida	Free ingestion					mortality	active
cry15Aa1	*Leptinotarsa*	*decemlineata*	Coleoptera							not active
cry15Aa1	*Aedes*	*aegypti*	Diptera							not active
cry15Aa1	*Cydia*	*pomonella*	Lepidoptera	Diet surface	neonate		129(36-246)	ng cells/cm2	mortality	active
cry15Aa1	*Manduca*	*sexta*	Lepidoptera	Diet surface	neonate		250	ng/cm2	mortality	
cry16Aa1	*Aedes*	*aegypti*	Diptera	Free ingestion	L2		185000+/-81000	ng/ml	mortality	
cry16Aa1	*Anopheles*	*stephensi*	Diptera	Free ingestion	L2		129000+/-10000	ng/ml	mortality	
cry16Aa1	*Culex*	*pipiens*	Diptera	Free ingestion	L2		156000+/-10000	ng/ml	mortality	
cry17Aa1	*Aedes*	*aegypti*	Diptera	Free ingestion	L2				mortality	not active
cry17Aa1	*Anopheles*	*stephensi*	Diptera	Free ingestion	L2				mortality	not active
cry17Aa1	*Culex*	*pipiens*	Diptera	Free ingestion	L2				mortality	not active
cry19A	*Aedes*	*aegypti*	Diptera	Free ingestion	L4		>4X10**5	ng/ml	mortality	
cry19A	*Anopheles*	*stephensi*	Diptera	Free ingestion	L2		24,926(19,580-31,732)	ng/ml	mortality	
cry19A	*Culex*	*pipiens*	Diptera	Free ingestion	L4		10,282(8,010-13,521)	ng/ml	mortality	
cry20Aa1	*Aedes*	*aegypti*	Diptera	Free ingestion	L2		6800(5300-8400)	ng/ml	mortality	
cry27Aa1	*Aedes*	*aegypti*	Diptera	Free ingestion	4day		>945000	ng/ml	mortality	not active
cry27Aa1	*Anopheles*	*stephensi*	Diptera	Free ingestion	4day		94300(81700-109000)	ng/ml	mortality	active
cry27Aa1	*Culex*	*pipiens*	Diptera	Free ingestion	4day		>850000	ng/ml	mortality	not active

8.3 Appendix 3: Table of Lepidoptera associated with corn, Zea mays*, from Letourneau* et al *(2002)*

The table includes numbers of Lepidoptera species recorded from *Zea mays*, including the maximum and minimum numbers of families and genera of plants colonised by members of each Lepidoptera family.

Family	Number of species	Maximum and minimum number of families/genera of plants – species
Acrolophidae	1	2/2 – *Acrolophus popeanella*
Arctiidae	28	49/100 – *Hyphantria cunea* 1/1 – *Alpenus pardalina*
Cosmopterigidae	6	16/18 – *Pyroderces badia* 2/3 – *Pyroderces amphisaris*
Eupterotidae	1	1/5 – *Eupterote petola*
Gelechiidae	3	6/14 – *Phthorimaea operculella* 1/1 – *Dichomeris granivora*
Geometridae	8	14/22 – *Thalassodes quadraria* 1/1 – *Eupithecia scopariata*
Hesperiidae	15	3/11 – *Pelopidas mathias* 1/2 – *Zenonia zeno*
Limacodidae	5	20/22 – *Acharia stimulea* 4/4 – *Darna ochracea*
Lycaenidae	1	31/67 – *Strymon melinus*
Lymantriidae	18	50/97 – *Olene mendosa* 2/2 – *Bembina isabellina*
Noctuidae	166	69/166 – *Spodoptera litura* 1/1 – 12 species
Nolidae	5	9/22 – *Earias insulana* 1/1 – *Nola spermophaga*
Notodontidae	2	1/4 – *Phalera combusta* 1/1 – *Phalera lydenburgi*
Nymphalidae	4	25/104 – *Vanessa cardui* 5/7 – *Acraea acerata*
Oecophoridae	3	6/6 – *Hofmannophila pseudospretella* 1/1 – *Autostichella solita* and *Borkhausenia minutella*
Psychidae	1	50/92 – *Thyridopteryx ephemeraeformis*
Pyralidae	90	35/61 – *Cadra cautella* 1/1 – 10 species
Saturniidae	8	35/85 – *Automeris io io* 1/1 – *Hemileuca mania*
Scythrididae	1	8/11 – *Eretmocera impactella*
Sphingidae	3	18/43 – *Hippotion celerio* 1/3 – *Leucophlebia lineate*
Tineidae	4	19/25 – *Opogona sacchari* 1/1 – *Tinea fictrix*
Tortricidae	8	43/87 – *Archips micaceana* 8/11 – *Platynota nigrocervina*
Yponomeutidae	1	11/22 – *Plutella xylostella*

Totals	**Families**	**Species**
	23	382