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DEVENIR DES TOXINES Cry DE *BACILLUS THURINGIENSIS* DANS LE SOL

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LIST OF ABBREVIATIONS

Aa	*Anopheles albimanus*
Ae	*Aedesa egypti*
Ag	*Anopheles gambiae*
Ag	*Anthonomusgrandis*
ALP-GPI	ALkaline Phosphatase – GlycosylPhosphatidyl-Inositol
APN-GPI	AminoPeptidase N – GlycosylPhosphatidyl-Inositol
Aq	*Anopheles quadrimaculatus*
Bm	*Bombyxmori*
Bt	*Bacillus thuringiensis*
Bta	*Bacillus thuringiensis aizawai*
Bti	*Bacillus thuringiensis israelensis*
Btk	*Bacillus thuringiensis kurstaki*
Btt	*Bacillus thuringiensis tenebrionis*
BSA	Bovine Serum Albumin
CADR	CADherin-like Receptor
CAPS	3-(CyclohexylAmino)-1-PropaneSulfonic acid
CHAPS	3-[(3-CHolamidopropyl)dimethylAmmonio]-1-PropaneSulfonate
LC50	Lethal Concentration 50
Cry	Crystal Protein
Cyt	Cytolitic Protein
ELISA	Enzyme-Linked ImmunoSorbent Assay
GCR	GlycoConjugate Receptor
GM	Genetically Modified
GMO	genetically modified organism
Ha	*Helicoverpa armigera*
Hv	*Heliothis virscens*
ICP	Insecticidal Crystal Protein
IEP	IsoElectric Point
ISAAA	International Service for the Acquisition of Agri-biotech Applications
kDa	kiloDalton
M. sexta	*Manduca sexta*
Mha	Million of hectares
MOPS	4-MOrpholinePropaneSulfonic acid
Ms	*Manduca sexta*
Ld	*Limantriadispar*
Lde	*Leptinotarsa decemlineata*
O. nubilalis	*Ostrinia nubilalis*
Pg	*Pectinophora gossypiella*
P. interpunctella	*Plodia interpunctella*
P. xylostella	*Plutella xylostella*
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
ssp.	subspecies
UV	Ultra violet
Tm	*Tenebrio molitor*
Dv	*Diabrotica virgifera*

SUMMARY

The insecticidal properties of *Bacillus thuringiensis*, discovered by Shigentane Ishiwatari, have been used for decades as biopesticides and this use has been increasing rapidly because of concerns about the negative environmental effects of chemical pesticides. Currently, Bt toxin in the form of both biopesticides and Bt transgenic plants may supplement or replace chemical pesticides. There is little evidence to demonstrate that Bt toxin has any harmful effect to the environment or to human health. Nevertheless, there are concerns that commercial transgenic crops may have harmful impacts on the environment. After release into soil via root exudation and the breakdown of plant residues, Bt toxin interacts with soil particles. The interactions of Bt toxin with soil particles influence its mobility, its bioavailability, its persistence and its toxicity. In this study, we aim to establish the relative importance of biological and physicochemical factors in the determination of the dynamics of detectable Cry proteins in soils, to clarify if adsorbed protein maintains its insecticidal properties and to identify the soil properties that determine the fate of Cry proteins in soil.

The results show that Cry proteins have strong affinity on soil surface. However, there was little relationship between affinity for soil or the extraction yield and soil properties including clay content, organic carbon content and soil pH. The proteins differ in both their affinity for soil and their extraction yields. There was little relationship between the affinity and the extraction yield.

An assessment of role of soil and environmental factors in the fate of Cry protein from commercial biopesticide formulation showed a rapid decline of detectable Cry protein subjected to direct sunlight under the laboratory condition, whereas little effect was observed under field conditions. The half-life of proteins in soil under natural conditions was about one week. Strong temperature effects were observed, but they differed for biopesticide and purified protein, indicating different limiting steps. For biopesticide, the observed decline was due to biological factors, possibly including sporulation. In contrast for purified proteins, increased temperature enhanced conformational changes of the soil-adsorbed protein, leading to fixation and hence extraction efficiency that decreased with time. Moreover, the study of the persistence of various Cry proteins in contrasting soils, carried out by immuno-detection and bioassay, showed that extractable toxin decreased with incubation for up to four weeks. Insecticidal activity was retained in the adsorbed state, but lost after two weeks of incubation at 25°C. The decline in extractable protein and toxicity was much lower at 4°C than 25°C. There was no significant effect of soil sterilization to persistence of Cry toxin indicating that decrease in detectable Cry toxin in soil may be time-dependent fixation of adsorbed protein as well as decreasing solubilization in larva midgut, but not microbial breakdown.

Exposition to Cry in the adsorbed form could have a significant impact on target and even non target insects and should be investigation to determine the potential impact.

RESUME

Les propriétés insecticides du *Bacillus thuringiensis*, découvert par Shigentane Ishiwatari, ont été utilisées pendant des décennies comme biopesticides et cette utilisation a augmenté rapidement en raison des préoccupations au sujet des effets environnementaux négatifs des pesticides chimiques. Actuellement, la toxine Bt dans la forme de biopesticides et de plantes transgéniques Bt peut compléter ou remplacer les pesticides chimiques. Il y a peu d'indication que la toxine Bt a un effet nocif pour l'environnement ou la santé humaine. Néanmoins, il y a des préoccupations que les cultures transgéniques commerciales peuvent avoir des effets néfastes sur l'environnement. Après son introduction dans le sol par l'exsudation racinaire et la dégradation des résidus végétaux, la toxine Bt interagit avec les particules de sol. Les interactions de la toxine Bt avec des particules de sol influencent sa mobilité, sa biodisponibilité, sa persistance et sa toxicité. Dans cette étude, nous visons à établir l'importance relative des facteurs biologiques et physico-chimiques dans la détermination de la dynamique des protéines Cry détectables dans les sols, de clarifier si la protéine adsorbée conserve ses propriétés insecticides et d'identifier les propriétés du sol qui déterminent le devenir des protéines Cry dans le sol.

Les résultats montrent que les protéines Cry ont une forte affinité sur la surface du sol. Cependant, il y a peu de relation entre l'affinité pour le sol ou le rendement d'extraction et les propriétés du sol, y compris la teneur en argile, teneur en carbone organique et le pH du sol. Les protéines diffèrent à la fois dans leur affinité pour les sols et leurs rendements d'extraction. Il y a peu de rapport entre l'affinité et le rendement d'extraction.

Une évaluation du rôle du sol et des facteurs environnementaux dans le devenir des protéines Cry de la formulation de biopesticides commerciale a montré un déclin rapide de la protéine Cry détectable soumise aux rayons du soleil sous la condition de laboratoire, alors que peu d'effet a été observé dans des conditions de terrain. La demi-vie des protéines dans le sol dans des conditions naturelles était d'environ 1 semaine. Des effets de la température forts ont été observés, mais ils diffèrent pour les biopesticides et la protéine purifiée, indiquant différentes étapes limitantes. Pour le biopesticide, la baisse observée était ralentie par des facteurs biologiques, y compris éventuellement sporulation. En revanche pour des protéines purifiées, augmentation de la température augmentait des changements de conformation de la protéine adsorbée du sol, conduisant à une fixation et, par conséquent, l'efficacité d'extraction diminuait avec le temps. En outre, l'étude de la persistance de diverses protéines Cry dans les sols contrastés, réalisée par immuno-détection et par biotests a montré que la toxine extractible diminue avec une incubation allant jusqu'à quatre semaines. L'activité insecticide était maintenue à l'état adsorbé, mais a disparue après deux semaines d'incubation à 25°C. La baisse de la protéine extractible et la toxicité était beaucoup plus faible à 4°C qu'à 25°C. La stérilisation du sol n'a pas eu d'effet significatif sur la persistance de la toxine Cry indiquant que le déclin observé était provoqué par la fixation en fonction du temps de la protéine adsorbée, ce qui diminue la proportion de toxine Cry extractible, la dégradation de la protéine par l'activité microbienne jouant un rôle plus mineur.

L'exposition des insectes aux protéines Cry sous la forme adsorbé pourrait avoir un impact significatif sur les insectes cibles et même les insectes non cibles, et devrait être plus étudiée afin de déterminer son impact potentiel.

SUMMARY

Résumé Etendu en Français

Contexte Général

La protection des cultures contre des insectes améliore quantitativement et qualitativement les récoltes. De même, la lutte antivectorielle est souvent la meilleure arme contre le développement et l'émergence de maladies vectorielles graves, surtout en l'absence fréquente de vaccins comme dans le cas du paludisme, de l'onchocerchose, de la dengue ou encore du chikungunya. Plusieurs stratégies existent pour limiter les dégâts causés par des insectes phytophages ou vecteurs mais tous ont des limites et des désavantages. L'approche conventionnelle, de très loin la plus répandue, est basée sur l'usage de molécules de synthèse. Toutefois, ces insecticides chimiques présentent à la fois une grande rémanence, une toxicité conséquente et une absence de spécificité et s'accumulent dans l'environnement et la chaine alimentaire. Les problèmes sanitaires et environnementaux associés aux pesticides chimiques entraînent un rejet de plus en plus important, notamment dans l'opinion public. En outre, leur efficacité est de plus en plus limitée par le développement de résistances. Les biopesticides représentent une alternative généralement bien perçue par le public. Toutefois ces agents de lutte biologique qui constituent l'arsenal principal de l'agriculture biologique ne représentent encore qu'une faible proportion du marché phytosanitaire (1%). Toutefois, ils représentent une part considérable dans certains marchés comme par exemple la lutte antivectorielle et notamment la lutte antimoustiques. Les formulations contenant les spores et des cristaux de *Bacillus thuringiensis*, dite Bt, une bactérie du sol, représentent environ 80 % de ces biopesticides (Whalon & Wingerd, 2003). Enfin, plus récemment des gènes de diverses souches de cette bactérie ont été introduits dans des plantes pour leur conférer des propriétés insecticides. Ces cultures génétiquement modifiées (GM) ont été commercialisé pour la première fois en 1996, et leur usage a considérablement augmenté pour atteindre une surface cultivée au niveau mondial de près de 80 Mha en 2013, donc plus de la moitié sont des plantes contenant aussi un trait de tolérance aux herbicides(James, 2014). L'EPA (Environmental Protection Agency) américain a conclu que les cultures Bt de coton et de maïs ne posait pas de risques pour l'environnement (Mendelsohn *et al.*, 2003). Malgré l'efficacité des cultures GM dites Bt, leur usage suscite des questionnements et des oppositions pour des raisons socio-économiques, environnementaux et sanitaires. Ceci est exacerbé par la rapidité de l'expansion, laissant peu de recul pour évaluer l'impact de ces cultures malgré leur similarité avec les traitements de biopesticides, utilisés depuis des décennies.

Mode d'action des insecticides Bt

La bactérie *Bacillus thuringiensis* produit pendant sa sporulation des corps d'inclusions protéiques, pouvant représentées 20-30 % de leur masse. Ces protéines insecticides, baptisées Cry pour « Crystal » ou corps d'inclusion, ont une très grande spécificité pour leur cible et présentent un mode d'action très particulier. Ce mode d'action est en fait une cascade d'évènements jouant chacun un rôle dans la spécificité de ces toxines. Les corps d'inclusion produits par la bactérie et présents dans les biopesticides sont solubilisés par le pH spécifique du tube digestif de l'insecte cible. De façon concomitante, les protoxines libérées par la solubilisation sont activées par les enzymes digestives de l'insecte cible, à la suite de quoi les toxines activées doivent obligatoirement reconnaître un site récepteur très spécifique à la surface de l'intestin moyen de l'insecte. Après fixation sur ce récepteur, les toxines s'internalisent dans la membrane cellule et créent des pores qui entraînent la destruction des cellules de l'intestin et la mort rapide de l'insecte. Ces récepteurs n'existent pas chez les vertébrés et leur spécificité est telle que chaque protéine Cry est active dans un nombre très

limité d'espèces d'insecte. C'est ce trait spécifique qui confère la sûreté pour des organismes non-cible et d'autant plus pour des vertébrés. En outre, les toxines Cry sont très rapidement détruites par hydrolyse acide dans l'estomac des vertébrés.
Les protéines utilisées en lutte biologique dans les biopesticides et dans les plantes Bt se répartissent en trois familles: Cry, Cyt et Vip. On répertorie à l'heure actuelle 59 familles de protéines Cry qui groupent un total de 468 toxines, 2 familles de toxines Cyt comprenant 33 toxines différentes et 3 familles de protéines Vip totalisant 29 toxines différentes (Crickmore *et al.*, 2010). Tous sont séquencés et caractérisés et les toxines sont classées selon des critères de similarité de séquence. A ce jour la structure tri-dimensionelle de seules 6 protéines Cry ont été décrites (Cry1Aa, Cry3Aa, Cry4Aa, Cry2Aa, Cry3Bb, Cry4Ba). Malgré des différences entre séquence génétique, certaines protéines Cry possèdent une grande similarité de structure. Les homologies de structure sont de 50% à 90% dans une même classe et de 20% à 30% entre classe. Ces similarités peuvent être reflétées par des similarités de fonction. Les protoxines sont composées de deux domaines, un domaine C-terminal et un domaine N-terminal. Certains traits de structure sont responsables pour la stabilité des cristaux des protoxines, et sont très conservés entre Cry, notamment le domaine C-terminal. Le domaine N-terminal est plus largement responsable pour la toxicité, et donc les différences de structure contrôlent la spécificité des propriétés insecticides, notamment la reconnaissance et fixation des protéines sur les récepteurs des membranes de l'insecte-cible. A notre connaissance aucune étude n'a essayé de mettre en relation la structure et l'adsorption des protéines Cry, et encore moins leur persistance dans l'environnement.

Toxine Bt : B. thuringiensis et culture GM

Les toxines produites par les plantes transgéniques sont presque identiques à celles issues de bactéries après activation et donc les données de sécurité biologique rassemblées sur les biopesticides sont appliquées ainsi aux protéines insecticides GM et facilitent l'homologation. Le mode de l'action de ces protéines insecticides est un élément important de sécurité biologique. Les protéines insecticides de Bt sont des poisons stomacaux agissant à une dose très basse compatible avec le niveau prévu de l'expression des plantes, qui est également très basse. Contrairement aux insecticides chimiques qui agissent par simple contact, les protéines insecticides de *B. thuringiensis* doivent obligatoirement être ingérées pour devenir par la suite actives. L'ingestion est une étape obligatoire du mode d'action des toxines de Bt. Une conséquence directe est que seuls les insectes s'alimentant sur la plante, en d'autres termes les ravageurs de cette plante, peuvent être affectés. Les concepts de spécificité et de sûreté se situent à ce niveau et sont la conséquence du mécanisme particulier d'action en cascade des protéines insecticides Cry de *B. thuringiensis*.
Une différence majeure entre les biopesticides à *B. thuringiensis* et certaines des plantes Bt est que ces dernières produisent directement des toxines Cry activées et solubles, n'ayant donc pas besoin des étapes de solubilisation et activation protéolytique. Ceci pourrait diminuer la forte spécificité des protéines pour leur cible mais de façon limitée, le facteur majeur de spécificité étant la reconnaissance du récepteur qui est un mécanisme présent dans les deux cas de figure. La première génération de cultures Bt ne produisait qu'une seule toxine, par exemple Cry1Ab dans le maïs de Monsanto, MON810 et Cry1Ac dans le cotonnier Bollgard. Les nouvelles générations de plantes GM sont basées sur le concept de pyramidage, c'est à dire de production simultanée de plusieurs protéines Cry reconnaissant des sites récepteurs différents avec pour double objectif d'augmenter l'effet toxique aiguë et de retarder l'apparition de résistance. De plus, les variétés plus récentes produisent des protéines non tronquées (Mendelsohn *et al.*, 2003).
Une variété de formulations de biopesticides Bt existe contenant différents mélanges de spores et de cristaux avec des adjuvants pour protéger contre une dégradation trop rapide

(notamment après exposition au rayonnement UV) et pour permettre un meilleur dépôt foliaire ou même une pénétration foliaire. Les biopesticides sont très dépendants pour leur efficacité d'une fenêtre de traitement souvent étroite mais aussi du comportement alimentaire des ravageurs cibles. Les toxines de Bt devant être ingérées pour être efficaces, la présence des biopesticides uniquement en surface limite leur action aux ravageurs phyllophages. A l'inverse, les cultures GM présentent l'avantage de présenter la toxine dans la plante à protéger, et peuvent donc cibler des insectes foreurs ou carpophages non touchés par les biopesticides et qui sont souvent les ravageurs les plus dommageables. L'impact économique de la protection est donc meilleur par rapport aux biopesticides non efficaces ou aux insecticides chimiques de moins en moins efficaces du fait de résistances, avec comme conséquences une augmentation de rendement et de profit pour les agriculteurs. Certaines études constatent une réduction de l'usage de traitements phytosanitaires chimiques, mais ceci n'est pas toujours le cas. Par exemple, dans le cas du maïs et de la pyrale du maïs aux USA, l'adoption de maïs Bt s'est surtout traduite par une hausse du rendement, très peu de traitements chimiques étant fait auparavant sur cet insecte (EPA, 2001). Par contre des augmentations de qualité de récolte sont parfois notées avec les cultures GM par rapport aux variétés non GM. Ceci provient du risque d'infection fongique des cultures exposées aux insectes phytophages. Des mycotoxines, des métabolites secondaires des champignons, peuvent rendre une récolte impropre à la consommation humaine (Phipps & Park, 2002; Brookes, 2008). L'amélioration de la qualité des cultures varie selon les conditions climatiques, plus ou moins propice aux infections fongiques (Wu, 2007).
Une autre différence entre biopesticides *B. thuringiensis* et plantes Bt est liée au mode de délivrance. La pulvérisation des biopesticides dans une fenêtre de temps étroite entraine une présence de toxines limitée dans le temps. A l'inverse, la production systémique et constitutive de protéines insecticides dans les plantes GM conduit à leur présence permanente tout au long de la culture. Les toxines sont exsudées dans le sol par les racines mais également libérées lors de la décomposition des résidus de culture (Saxena *et al.*, 1999; Saxena & Stotzky, 2000; Zwahlen *et al.*, 2003; Saxena *et al.*, 2004; Stotzky, 2004). Cette source continue pourrait entraîner une plus grande rémanence dans le sol et permettre une exposition à des organismes non cibles, qui augmenterait la probabilité d'effet non désirés.

Effets non désirés de protéines Cry dans le sol

Un autre champ de recherche significatif est la détection de l'impact négatif de la toxine Bt sur non des insectes de cible. L'examen le plus à jour et le plus complet des données actuellement disponibles sur cette question est celui de Stotzky (Icoz & Stotzky, 2008). D'autres revues incluent cela du Département Fédéral Suisse des affaires économiques (Sanvido *et al.*, 2006) et de l'opinion scientifique de l'EFSA (EFSA, 2008) en réponse à la décision française de suspendre la culture du maïs Bt de Monsanto 810. Bien que certaines des conclusions de ces études soient contradictoires, en général aucun effet négatif des récoltes Bt qui peuvent être directement attribuées à la production de la toxine n'a été trouvé. Par exemple, aucun effet négatif de la toxine n'a été trouvé pour des vers de terre, bien que de petites différences de croissance aient été notées et attribuées à la digestibilité du maïs Bt, qui a un contenu plus élevé de lignine que le maïs non-Bt équivalent. Ni la survie ni la croissance des microarthopodes et des macroarthopodes, également prise comme indicateurs de qualité de sol, ne semblent ne pas être affectées par le maïs Bt. Aucun effet significatif n'a été trouvé sur le nombre ou la biodiversité des nématodes dans la rhizosphère du maïs Bt, bien qu'on ait rapporté que les proportions relatives de différents types de nématodes (bactériophages, mycophages et phytophages) varient, et encore ceci peut être un effet indirect. Il est plus difficile d'évaluer les données sur des effets sur des micro-organismes et l'activité microbienne, à cause de la grande variation spatiale et de l'influence forte de l'âge et de la

variété de plante cultivée. Néanmoins, la plupart des études indiquent peu ou pas d'effet des récoltes Bt sur la structure et activité des populations microbiennes (Donegan *et al.*, 1995; Saxena & Stotzky, 2001; Koskella & Stotzky, 2002; Ferreira *et al.*, 2003; Blackwood & Buyer, 2004; Brusetti *et al.*, 2004; Devare *et al.*, 2004; Wu *et al.*, 2004a; Flores *et al.*, 2005; Griffiths *et al.*, 2006; Naef *et al.*, 2006; Shen *et al.*, 2006; Devare *et al.*, 2007). En revanche quelques études ont indiqué que les récoltes Bt modifient les communautés bactériennes (Brusetti *et al.*, 2004; Castaldini *et al.*, 2005; Rui *et al.*, 2005; Xue *et al.*, 2005) et peut de manière significative réduire le niveau des mosseae,champignons symbiotiques d'un Glomus (Turrini *et al.*, 2004). Bien que quelques études indiquent que l'activité enzymatique de sol est modifiée par des toxines de Bt (Wu *et al.*, 2004a; Wu *et al.*, 2004b; Sun *et al.*, 2007), d'autres ne trouvent aucun changement des processus importants de sol qui sont contrôlés enzymatiquement (Devare *et al.*, 2004; Flores *et al.*, 2005; Cortet *et al.*, 2006; Shen *et al.*, 2006).

Bt et le sol

Les protéines Cry, en commun avec d'autres protéines (Haynes & Norde, 1994), s'adsorbe fortement et souvent irréversiblement sur les argiles de référence, les fractions argileuses de sol, les sols et des acides humiques (Pagel-Wieder *et al.*, 2007; Icoz & Stotzky, 2008; Helassa *et al.*, 2009). Hopkins et Gregorich (Hopkins & Gregorich, 2003) ont détecté des concentrations de toxine Bt plus élevées dans des sols organiques et de texture sableux que dans un sol argileux, mais ceci pourrait en partie refléter l'efficacité de l'extraction avant l'analyse. Les travaux de Stotzky et son équipe indique que la toxine adsorbée gardent ses propriétés insecticide, et qu'elle est protégée contre la dégradation microbienne (Crecchio & Stotzky, 2001; Lee *et al.*, 2003). Cette observation, qui mérite d'être étudiée en plus ample détail, suggère que la toxine maintient sa conformation à l'état adsorbé.

Les études au champ et en microcosme ont indiquent une perte rapide de toxine et de protoxine de Bt dans des sols cultivés avec des plantes Bt (Head *et al.*, 2002; Shan *et al.*, 2008) et dans des sols amendés avec des résidus de culture de plantes Bt (Donegan *et al.*, 1995; Palm *et al.*, 1996; Sims *et al.*, 1996; Sims & Ream, 1997; Saxena *et al.*, 2002; Hopkins & Gregorich, 2003; Muchaonyerwa *et al.*, 2004; Wang *et al.*, 2006; Icoz & Stotzky, 2008). De même, une perte rapide de protéine détectable est observé quand un sol est amendé avec des protéines Bt solubilisées (Donegan *et al.*, 1995; Palm *et al.*, 1996; Tapp & Stotzky, 1998) ou à l'état solide (Herman *et al.*, 2002; Marchetti *et al.*, 2007). Pour de nombreux cas laperteinitialeest rapide, pendant quelques jours ou semaines, et puisun déclin beaucoup plus progressif est observé. L'adsorption sur des minéraux de sol augmente la persistance de la toxine du Bt probablement dû à la protection contre la dégradation microbienne (Icoz & Stotzky, 2008). Cependant, à notre connaissance, seulement trois études ont essayé de comparer la persistance de la toxine Bt sur le sol stérile et non stérile. Palm et al (Palm *et al.*, 1996) a rapporté un déclin plus lent en toxine Bt d'un sol γ-stérilisé que du même sol sans stérilisation. De même, Accinelli et al. (Accinelli *et al.*, 2008) signalent que la stérilisation à l'autoclave ralentissait la cinétique de minéralisation de la protoxine Bt marqué au ^{14}C et que le taux initial de minéralisation était augmenté par l'addition d'un substrat organique. Plus récemment l'effet opposé a été observé (Helassa *et al.*, 2011). Pour quatre sols, il n'y a eu aucune modification de la cinétique de déclin de Cry1Aa en stimulant ou *a contrario* en inhibant l'activité microbienne par diverses méthodes chimiques et physiques (apport de carbone assimilable, $HgCl_2$, autoclavage, stérilisation-γ). Ces auteurs avaient conclu à l'importance des interactions physico-chimiques entre protéines et surfaces de sol, sans avoir pu distinguer entre fixation progressive de la protéine ou dégradation.

Objectifs généraux dela thèse

L'objectif général de ce travail de thèseétait de mieux comprendre le devenir des protéines insecticides issues de *Bacillus thuringiensis* dans les sols dans le but de prévoir leurs effets à long terme et de renseigner des stratégies de monitoring. Une question importante adressée en priorité était centrée sur la pertinence et la validité des tests immunochimiques pour la surveillance environnementale. Ces tests sont plus rapides et plus faciles à mettre en œuvre que des tests de toxicité, et sont par conséquent préférés pour les surveillances *in campo*. Néanmoins, une protéine (ou des résidus de cette protéine) peut être détectée par un test immunochimique, sans pour autant avoir gardé son caractère insecticide. Nous avons donc comparé la détection des protéines par biotest basé sur leurs propriétés insecticides et par extraction chimique suivie de détection immunochimique. Notre hypothèse était que la nature des surfaces organo-minérales des sols allait déterminer les modifications de conformation des protéines, et donc le maintien ou non des propriétés insecticides des protéines.

Il ne suffit pas de détecter les protéines dans le sol, il faut aussi connaître la cinétique de perte d'activité en fonction du temps, et en relation avec les propriétés du sol et de son activité biologique. Notre hypothèse de travail était que la persistance des protéines Cry dépend à la fois de leur interaction avec les surfaces organo-minérales du sol qui détermine l'évolution des changements de conformation et confère une protection contre l'activité catalytique des protéases du sol. Nous avons donc étudié la persistance de deux protéines Cry en fonction des sols et de leur activité microbiologique. En particulier, nous avons supposé que l'activité de la faune du sol, influe sur l'activité protéasique, et nous avons pris les vers de terre comme modèle de faune de sol.

Nous avons comparé des protéines produites par des plantes génétiquement modifiées qui sont actuellement commercialisées, et des protéines produites par des souches sauvages utilisées dans des formulations de biopesticides. Cette comparaison est importante, car seul un très petit nombre de protéines Cry sont actuellement produites par des plants GM, mais ce nombre va croître et il sera essentiel de s'appuyer sur des bases scientifiques pour extrapoler des propriétés entre classes de Cry. Cette étude comparative expérimentale était renforcée par une réflexion et des formations centrées sur les différences et les similarités entre les protéines Cry d'origine différentes (culture GM et biopesticides).

Pour résumer, les questions posées étaient

- la détection immunochimique des protéines Cry dans les sols informe-t-elle sur leur toxicité ?
- quelles propriétés physicochimiques des sols déterminent l'adsorption des protéines Cry sur les sols et le rendement d'extraction chimique après adsorption ?
- quelles propriétés physicochimiques et biologiques des sols déterminent la persistance des protéines et leur toxicité à l'état adsorbé ?
- est-ce que la persistance dépend d'une dégradation ou la fixation des protéines les rendant plus difficile à désorber ?
- quelles sont les différences et les similarités entre les protéines Cry (culture GM et biopesticides) ?
- quelles sont les différences réelles et perçues des protéines produites par des cultures GM et les souches sauvages de *B. thuringiensis* ?

La démarche expérimentale était de suivre les protéines Cry dans des sols, soit en conditions contrôlées soit *in campo,* et ceci pour des protéines purifiées obtenue par culture bactérienne, soit contenu dans une formulation commerciale de biopesticide. Les protéines Cry, Cry1Ac, Cry2A et Cry1C, ont été obtenues par culture des bactéries qui les produisent en grandes quantités (des centaines de milligrammes par culture) ensuiteelles ont été purifiées

par chromatographie. La formulation de biopesticide étudiée était de type HD-1, contenant plusieurs Cry, dont Cry1Ac,

Un premier travail était le criblage de l'affinité des trois Cry, en quantité trace, pour des sols ayant des propriétés contrastées (pH, teneur en matière organique et en argile). Ces mesures ont été faites sous conditions contrôlées. La désorption des protéines adsorbées a été mesurée, utilisant une solution d'extraction mis au point dans une étude précédente (Helassa et al, 2011) et l'affinité comparée avec la désorbabilité. La quantité de protéine apportée aux sols était aussi près que possible aux teneurs attendues dans les sols contaminés, et donc plusieurs ordres de grandeur moins que les quantités nécessaires pour mesurer la capacité d'adsorption des sols.

Le devenir à plus longue terme a été suivi en incubant un petit nombre de sols avec deux protéines Cry, Cry1Ac et Cry2A, dans des conditions contrôlées, pendant jusqu'à 30 jours. Deux méthodes ont été utilisé pour suivre le devenir de Cry1Ac, extraction chimique suivi de dosage immunochimique et la toxicité envers les larves de l'insecte *Manduca sexta*. Cry2A a été suivi uniquement pas extraction chimique et dosage ELISA. *Manduca sexta*est non seulement sensible à Cry1Ac, mais les larvesont une taille suffisante pour ingérer le sol. La mortalité a été suivie pendantsept jours.

Enfin la protéine Cry1Ac, issue de biopesticide ou bien sous forme purifié, a été suivi *in campo* (pour le biopesticide) ou sous conditions contrôlées de laboratoire. Ceci est la première étude qui compare directement les protéines purifiées avec les mêmes dans une formulation commerciale de biopesticide.

Résultats obtenus

Adsorption de protéine Cry en fonction de propriétés des sols

Au vu de la pauvreté des données dans la littérature sur les paramètres du sol qui déterminent l'adsorption des protéines Cry, nous avons choisi d'étudier un nombre de sols assez important. La plupart des sols ont été choisis dans la collection du Réseau de Mesures de la Qualité des Sols (RMQS) avec quelques sols de la collection de l'UMR Eco&Sols, disponibles en plus grande qualité. Les sols ont été choisis en fonction (i) de leur occupation (sous grande culture, susceptibles de soutenir des cultures de plantes génétiquement modifiées et portant le trait Bt dans l'éventualité de l'autorisation de culture GM en France ; et culture non intensive, y compris les jardins et les zones humides, susceptibles d'être traités avec les biopesticides contenant Bt) et (ii) pour chacun de ces groupes en fonction de leur composition (teneur en argile, teneur en matière organique et pH). L'affinité des sols pour les protéines est estimée par le coefficient de distribution, Kd, mesurée en suspension diluée (1 g/l). Une gamme très importante de valeurs de Kd a été observée, allant de 10^3 à 3 10^4 dm^3 kg^{-1}. Aucune corrélation significative n'a été observée par régression simple entre l'affinité et les propriétés de sol considérées. Une analyse par ANOVA avec régression par étape (stepwise) confirme qu'il n'y a aucun effet de l'usage des terres, mais indique des effets significatifs de la teneur en argile (positive) et de la teneur en matière organique et du pH (négatifs) pour les Cry1. Plus surprenant, les affinités des trois Cry pour les sols ne suivaient pas les mêmes tendances.

Comme la plupart des protéines, les protéines Cry s'adsorbent de façon quasi irréversible sur les sols. Ceci a des implications importantes à la fois pour leur devenir, étant plus protégées contre la dégradation microbienne à l'état adsorbé qu'en solution, et pour leur suivi, car leur dosage nécessite une désorption. La solution d'extraction que nous avons mise au point précédemment, à pH alcalin, contenant un surfactant et la protéine albumine de sérum de bœuf (BSA), n'avait pas été testée pour un grand nombre de sols. Contrairement à ce qu'on pouvait attendre, il n'y a qu'une très faible relation entre fixation et affinité. Le taux d'extraction d'une des protéines, Cry1C, variait considérablement moins que les deux autres,

indiquant que la méthode était mieux adaptée pour cette protéine, comme pour la Cry1Aa pour laquelle elle a été initialement mise au point.

Persistance des protéines Cry en fonction de propriétés des sols et des conditions d'incubation

La durée de présence des protéines Cry dans le sol impacte sur la probabilité d'apparition d'effets non désirés, tel que l'exposition d'insectes non cibles ou l'acquisition de résistance, et a une importance majeure pour la surveillance de l'environnement. Les données publiées dans la littérature indiquent une grande variabilité de la durée de détectabilité des protéines Cry soit *incampo* soit sous des conditions contrôlées. Dans une étude précédente, nous avons conclu que la disparition de Cry1Aa dans quatre sols contrastés n'était pas déterminée par l'activité microbienne. Par contre, la décroissance de la quantité de protéine détectable était moins rapide à 4°C qu'à 25°C, et nous avions émis l'hypothèse que ceci résultait de la favorisation d'interactions hydrophobes à température ambiante et que la fixation qui en résulte était ralentie à basse température. Nous avons donc étudié les cinétiques de disparition de deux protéines Cry, Cry1Ac et Cry2A, 4°C et à 25°C. L'effet de la teneur en eau des sols pendant l'incubation de Cry1Ac a également été suivi. Comme le criblage n'a pas pu mettre en évidence des propriétés de sol qui détermine l'affinité des protéines Cry, trois sols ont été choisis pour cette étude, avec des textures et teneurs en carbone organique contrastées. Le premier est un Chromic Cambisoléchantillonné sous forêt (Cazevielle, Cz) est un sol argileux, avec un pH neutre et une teneur en matière organique de 31.7 g/kg. Les deux autres sols sont des sols de vigne (Cruscades, Cc), limoneux-argileux, calcaires, (pH = 8.3) avec des teneurs en matière organique contrastées à cause des pratiques agricoles : 16.5 g/kg pour Cc_{org} après 17 ans de culture biologique et 7.2 g/kg pour l'autre, Cc_{con} maintenu en culture conventionnelle. Leur affinité pour les deux protéines Cry ainsi que lerendement d'extraction chimique des protéines se situent dans la gamme observée pour les sols RMQS, décrite précédemment. La quantité de protéine détectable décroît rapidement au cours de la première semaine, et puis lentement ensuite. La période de demi-vie à 25°C se situe à environ 3 jours pour chacun des sols et indépendamment de la teneur en eau. L'humidité n'a pas d'effet significatif sur le devenir de Cry1Ac dans les sols étudiés. Pour chacun des sols, et à des degrés différents, la réfrigération accorde une protection, les pertes de protéine étant environ 50% pour chacun des sols contre 90% à 25°C. Le constat est remarquablement similaire pour Cry2A avec un déclin rapide au cours des 3 premiers jours pour chacun des sols, pour atteindre environ 5% de la quantité initiale après deux semaines, tandis que 40% est resté après incubation à 4°C.

Persistance de la toxicité de Cry1Ac à l'état adsorbé

La quantification des protéines Cry par détection avec un test ELISA après désorption du sol, a l'avantage d'être rapide. Par contre cette approche ne donne aucune information sur la réelle toxicité à l'état adsorbé et ne permet pas d'affirmer que la protéine reste intacte. Seule des biotests, utilisant des insectes cibles, permettent d'affirmer que les protéines adsorbées conservent leur conformation biologiquement active. Ces bioessais sont chronophages et sujets à une grande variabilité biologique et des artefacts. Peu de données existent sur la toxicité des protéines Cry à l'état adsorbées. Des manipulations préliminaires ont trouvé que les larves de *Manduca sexta* acceptaient d'ingérer 5% de sol mélangé dans leur alimentation, qui est à base de gélose et de farines. Cette ingestion n'avait aucune conséquence sur leur comportement ou leur croissance ultérieure. Des essais préliminaires ont servi au choix du stade de croissance des larves et de la quantité de Cry apportée. Les larves ont été nourries avec 1 g de leur alimentation habituelle contentant (i) pas de Cry (ii) une solution de Cry, 200 ng protéine, ou (iii) la même quantité de Cry préalablement adsorbée sur 50 mg de sol. Il y a

avait 10 larves par traitement et les larves étaient placées dans des boites individuelles pendant la période de l'essai, l'alimentation changée quotidiennement et la mortalité suivie pendant 7 jours. Les larves qui ingèrent la toxine Cry1Ac meurent à partir de 2 jours après la présentation de la toxine. La toxine préalablement adsorbée sur le sol garde ses propriétés toxiques. L'observation le plus surprenante et inattendue est que la toxicité semble encore plus importante quand la toxine est adsorbée. L'explication semble être que les larves consomment plus d'aliment contaminé quand la toxine était adsorbée que quand elle était apportée en solution. Il est possible que l'adsorption diminue la détection de Cry et donc limite leur aversion, elles mangeraient donc plus en présence de sol. L'explication le plus probable est que la Cry sous forme soluble induit une paralysie des mâchoires et l'arrêt de l'alimentation, mais que ceci intervient moins quand la protéine est sous forme adsorbée.

Persistance des protéines Cry issues de biopesticides commerciaux et de protéines purifiées

Un des objectifs de ce projets était de comparer et le comportement et la perception du public des protéines insecticides issues des biopesticides et des cultures biotechnologiques. Bien que les protéines soient quasiment identiques dans les deux cas, le conditionnement des protéines dans les formulations biopesticides, y compris la présence de spores de *Bacillus thuringiensis*, peut influer sur le devenir des protéines dans l'environnement du sol. Dans cette série d'expériences nous avons comparé le devenir de la protéine Cry1Ac soit dans la formulation commerciale, Vi-Bt ®, pulvérisée en plein champ, soit directement sur le sol, soit sur le feuillage de la culture de patate douce, ou bien pulvérisée sur les plateaux de sol, puis incubé avec exposition directe à la lumière du soleil, ou bien à l'obscurité à 25°C ou à 4 °C. Le même sol a aussi été incubé avec la protéine Cry1Ac purifiée, comme dans les expérimentations précédentes et incubé dans des conditions contrôlées de température (4 ou 25 °C), et teneur en eau, et avec ou sans une préincubation après humectation avant l'apport de la solution content la protéine Cry, pour épuisé le flush microbien avant l'ajout de Cry. Toutes les incubations ont été faites en triplicata, et après des périodes d'incubation allant d'une heure à 28 jours des échantillons de sol sont été pris, la protéine extraite et dosé par la méthode ELISA.

Des essais préliminaires ont permis de déterminer la dilution et la quantité de spray à apporter pour pouvoir suivre les incubations pendant environ 1 mois. Le spray a été dirigé soit directement sur les feuilles, d'où une partie atteignait le sol ou sur le sol inter-rang. Ces traitements ont eu lieu le matin, Dans un autre cas indiqué « afternoon » où le traitement a eu lieu l'après-midi pour réduire l'action initiale du rayonnement solaire. Le mode de d'application du spray a eu un effet sur la quantité de Cry1Ac détectable immédiatement dans le sol, avec plus de protéine dans le sol quand le spray a été dirigé vers le sol directement. La perte de protéine était initialement plus rapide quand le sol a été la cible directe du spray. Après un mois d'incubation, aucune différence significative ne restait entre les modes d'apport du spray. Environ 10% de la protéine contenue dans la formulation commerciale était encore détectable après un mois.

Pour pallier les variabilités dues à une expérimentation au champ et l'hétérogénéité d'échantillonnage, une autre incubation a été menée au laboratoire. Le sol a été échantillonné au champ, séché à l'aire, homogénéisé, humecté à 10% (pondérale) pendant 3 jours avant le début de l'expérience, puis une suspension spray commercial Vi-Bt® pulvérisée sur le sol, et ensuite la teneur en eau des sols ajustée avec de l'eau distillée. Les incubations ont été menées à l'obscurité à 25°Cou à 4°C, ou incubée avec ensoleillement (SL). Le masse des sols était vérifiée tous les jours et ajustée si nécessaire en pipetant de l'eau distillée sur la surface du sol. Tous les traitements ont été faits en trois répétitions, et les sols échantillonnés en faisant des échantillons composites qui étaient homogénéisés avant extraction et dosage de Cry1Ac en trois répétitions. L'exposition au soleil provoque une décroissance plus rapide de la

protéine détectable. Ceci est cohérent avec les observations montrant que les cristaux de protéines sont sensibles aux rayonnements UV, et les formulations commerciales contiennent des adjuvants pour les protéger. L'observation la plus surprenante est la disparition très rapide de Cry à 4°C, en contraste avec les observations avec la protéine purifiée. Si des interactions hydrophobes ont été invoquées pour expliquer la protection de la Cry purifiée, en contact avec des sols, un autre mécanisme doit expliquer l'effet de température sur la Cry dans des formulations commerciales contenant de spores de Bt, des cristaux de la protoxine et pas de toxine tronquée.
Pour élucider cette différence, nous avons incubé le même sol avec une solution de Cry1Ac purifiée. Pour vérifier que la différence ne provenait pas de la réhumectation partielle du sol avant l'apport de Cry, nous avons comparé, pour l'humidité standard (40%) l'effet de préhumecter ou non le sol à 10% 3 jours avant l'apport de Cry, et ceci pour les températures d'incubation de 4 et de 20°C. La protection à 4°C est observée pour ce sol, et nullement modifiée par la préhumectation. Par contre, à 25°C la perte de Cry lors du premier jour de l'incubation est légèrement moins importante après une préhumectation, ce qui serait cohérent avec une perte de protéine accrue lors du flush microbien.
Nous émettons l'hypothèse que la différence entre le devenir de Cry purifiée et celle issue de biopesticide vient de l'importance des processus biologiques pour le dernier. Quand le biopesticide était incubé avec une solution du sol, une solution nutritive ou de l'eau, soit à 25°C ou à 4°C, la cinétique de Cry indiquait que les spores de Bacillus continuaient à produire de la protéine Cry à 25°C en présence de nutrimentsmais pas dans de l'eau et pas à 4°C.

Conclusions et Perspectives

Les conclusions majeures de cette étude sont que les protéines Cry varient considérablement dans leur affinité pour des sols et donc il n'est pas possible de prédire exactement l'adsorption, le rendement d'extraction, ni même la persistance au cours du temps des protéines Cry dans le sol. Néanmoins, il semble claire que toutes les protéines Cry seront fortement adsorbées par des sols, et ceci limitera leur mobilité dans l'environnement.
La dynamique des protéines Cry purifiées, comme celles produites par des plantes GM, dans le sol est déterminée par les processus de fixation Cette adsorption protègent peut-être également de la dégradation microbienne et enzymatique. La comparaison entre protéines Cry issues d'un biopesticide commercial montre que des processus différents déterminent leur devenir. Les formulations commerciales contiennent, en plus de la protéine sous forme de protoxine, des spores et donc la quantité de protéine peut croître après application, ralentissant le déclin net de protéine détectable.
Les protéines Cry maintiennent leur toxicité à l'état adsorbé, et donc si un insecte sensible consomme du sol contaminé par Cry, il sera exposé à la toxine. Par définition, les insectes cibles de Cry sont phytophages et non pas géophages, mais des phytophages peuvent être géophages involontaires, notamment quand la pluie fait projeter du sol sur les végétaux. Le risque potentiel pour des insectes cibles est que l'exposition à des doses sub-léthales accélère l'acquisition de résistance. En ce qui concerne le risque éventuel pour des organismes non cible, géophages volontaires ou non volontaires, il faudrait qu'ils possèdent les récepteurs membranaires nécessaires pour le processus complexe de toxicité. Plus de recherche serait nécessaire pour élucider le rôle des protéases du sol et du faune du sol, comme les vers de terre, dans la dégradation des protéines Cry.

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GENERAL INTRODUCTION

Global food requirements are increasing and are predicted to continue to increase this century with the ever increasing world population. One of solutions for global demand is to increase food crop yields using improved seed varieties and by optimizing agronomic management (Huang *et al.*, 2002). However, the yields are decreased because of damage to crops by animal pests, especially insects. Potential crop loss has been estimated to be between 16 and 18 %. But in reality, the crop yield loss due to these pests was reduced to about 10 percent because of pest control measures (Oerke, 2006). Among the pest control measures, chemical pesticides have been widely used to protect crops. However, there are a lot of problems relating to use chemical pesticides in agriculture. Chemical pesticides are not only become less effective as target insect populations develop resistance, but also kill non-target predators and parasites that otherwise keep pest insects in balance. Moreover, using chemical pesticides over long periods will lead to accumulate in environmental of chemical toxin, leading to ecological impact in the environment as well as deleterious effects on human health. Therefore, it is necessary to find new pesticides that have narrow target spectra and fewer long term hazards.

The insecticidal properties of the soil-endemic bacteria, *Bacillus thuringiensis* (*Bt*), can be exploited in both biopesticides and biotechnological products to provide alternatives to chemical pesticides. In contrast to chemical pesticides, Bt products have high target specificity and so can be selected so as not to have any effect on non-target insects. Bt is considered to be environmentally friendly, although there is concern about Bt of biotechnological origin.

Biopesticides containing Bt have been used successfully worldwide for decades and the market share of such products is expected to increase to supplement or replace chemical pesticides.Currently, formulations containing the bacterium *B. thuringiensis* account for about 70% of the market (Lacey *et al.*, 2001).

Bacillus thuringiensis is gram-positive aerobic spore-forming bacteria. Bt produces insecticidal proteins during the sporulation phase as parasporal crystal.

B. thuringiensis is an ubiquitous Gram-positive bacterium that produces large quantities of insecticidal proteins during sporulation under nutrient-limiting conditions (de Maagd *et al.*, 2001; Crickmore, 2005; Sanchis, 2011). Insecticidal proteins used formulated biopesticides are contained in parasporal inclusion bodies also known as “Crystal”, and so are given the name Cry (δ-endotoxin). Each of the many strains of *B. thuringiensis* produce a small number of Cry proteins, usually between one and five, and these proteins have a large degree of specificity for target insects at the larval stage. δ-endotoxins are in fact protoxins of around 135 kDa (Sanchis & Bourguet, 2008). Protoxin is not toxic to insect. They must be solubilized after ingestion by larvae in the alkaline midgut (pH>10) and proteolytically activated into toxins by specific proteases (Höfte & Whiteley, 1989). The active toxins interact with receptors on midgut epithelial cells, where the toxins form pores and destroy cells. To date, many strains of *B. thuringiensis* have been used commercially as biopesticides, and small number of modified genes from *B. thuringiensis* has been inserted into plants. Cry genes are expressed and Cry protein produced in plant tissues continually during plant growth. During the crop cycle protein can be released from into soil by root exudates (Saxena *et al.*, 2002), by pollen (Losey *et al.*, 1999) as well as from crop residues (Zwahlen *et al.*, 2003a). Cry protein also can be released into soil by the spraying of Bt formulation to protect crops against insects. In the soil environment, Cry protein from Bt spray or Bt plants may be inactivated or removed from soil environment by UV activation and degradation of microorganisms. Soil acts as an efficient UV-filter, thereby potentially prolonging the maintenance of Cry protein. There is evidence that the persistence of Cry proteins in soil may be enhanced when the proteins are bound to solid particles (e.g., clays and humic substances), thus rendering it less

accessible for microbial degradation while still retaining insecticidal activity (Stotzky, 2000; 2004). Previous studies showed that Cry protein may persist for long periods in soil -up to180 days (Feng *et al.*, 2011b), 120 days (Helassa *et al.*, 2011b). 200 days (Saxena *et al.*, 2002; Zwahlen *et al.*, 2003b), 9 months (Zurbrüegg *et al.*, 2010). The long term persistence could constitute a hazard to non-target organisms, soil microbiota or beneficial insects (e.g., pollinators, predators and parasites of insect pests) (Flexner *et al.*, 1986). However, some studies have shown that Cry proteins do not persist, on the contrary they may decrease rapidly in soil (Head *et al.*, 2002; Dubelman *et al.*, 2005; Icoz & Stotzky, 2008b). Bt has been used in biopesticides with no evidence of harmful side-effects and little concern for negative environmental impact. In contrast, there are concerns that commercial transgenic plant may have harmful impacts on the environment. The level of concern is exacerbated by the rapid increase in agricultural land used worldwide for the production of genetically modified crops, leaving insufficient time for an appreciation of long-term, unexpected side effects.

Soil plays important roles in the persistence of Cry protein in soil. The interaction between protein and surface of soil leads to adsorption. The mechanism of interaction includes enthalpic forces and entropic effects that may also promote conformational modification of adsorbed protein (Quiquampoix, 2000).

Studies of the adsorption and persistence of Cry proteins in soil are very important. They should allow the long-term effect of Cry toxin in the environment to be predicted. Moreover, a better understanding of the fate of *B. thuringiensis* insecticidal proteins in soil are necessary for the monitoring of the use of Bt pesticides as well as Bt transgenic plants in agriculture.

In this thesis we have chosen two approaches to the monitoring of the fate of Cry proteins in soil -chemical extraction followed by immunodetection using ELISA and biotests. Extraction-ELISA is a very sensitive method. This method is fast and easy to implement. However, a protein (or residues of this protein) might be detected by immunodetection, while insecticidal properties had been lost. We propose to compare the detection of proteins by bioassays based on their insecticidal properties with chemical extraction followed by immunochemical detection. Our aims are to establish the relative importance of biological and physicochemical factors in the determination of the decline of detectable Cry proteins in soils, to clarify if adsorbed protein maintains its insecticidal properties and to identify the soil properties that determine the fate of Cry proteins in soil. For the first time different Cry proteins will be compared in this thesis to determine how different their fate in soil will be. We also aim to compare the persistence in soil of detectable purified protein and Cry toxins applied in the commercial Bt biopesticides where the persistence was followed under both field and laboratory conditions to test to what extent observations can be extrapolated from one protein to another.

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CHAPTER 1. LITERATURE REVIEW

1.1. General characteristics of *Bacillus thuringiensis*

Bacillus thuringiensis (*Bt*) is a ubiquitous gram positive, aerobic-spore forming, soil bacteria that produces parasporal crystal during the stationary stage of its growth cycle. *B. thuringiensis* has a rod shape with 3-5μm in length and 1-1.2 μm in wide and is provided with flagella (Figure 1.1a). Cry protein from *B. thuringiensis* is potent and highly insect specific. Each strain of *Bt* produces a small number of toxin that each type of toxin affect a narrow group of insect (Sanahuja *et al.*, 2011). *Bt* is member of *Bacillus* group that includes *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis and Bacillus weihenstephanensis.* However *Bt* is unique in the *Bacillus* group in the production during the sporulation process of protein crystals that are toxic to insects including *Coleoptera*, *Diptera* and *Lepidoptera* (Andrews *et al.*, 1987; De Barjac, 1981b) and even other organism such as nematodes, mites and protozoa (Feitelson *et al.*, 1992)).

B. thuringiensis was first discovered by the Japanese scientist Shigentane Ishiwatari who was studying wilt disease in silk worms. That bacterium was named *Bacillus sotto (Ishiwata, 1901).* Ten years letter, a Germen biologist, Ernst Berliner isolated the same bacteria from diseased Mediterranean flour moth (*Ephestia kuehniella*) in the province of Thuringe and this bacterium was called *Bacillus thuringiensis*. Neither Ishiwata nor Berliner described the bacterium, and it was not until 1960 that the first application of *B. thuringiensis* was reported and tested with European corn borer (*Ostrinia lubilalis*) by Husz in 1982 (Kumar *et al.*, 1996). Since then, because of concern about effects of chemical pesticides to the environment, *B. thuringiensis* is increasingly considered to be an environmentally friendly pesticide.

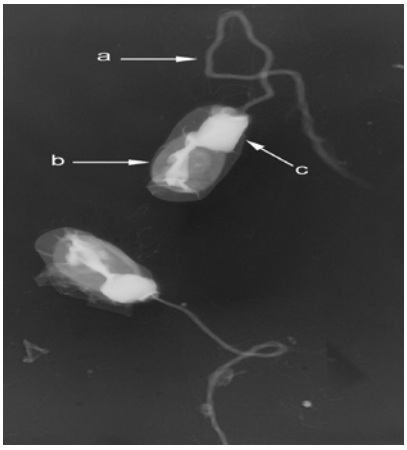
B. thuringiensis can be isolated on simple media such as nutrient agar from many habitats including soil (Martin & Travers, 1989; Carozzi *et al.*, 1991; Hastowo *et al.*, 1992), dead insects (Carozzi *et al.*, 1991), plant surfaces (Kaelin *et al.*, 1994) and insect faeces (Federici *et al.*, 2006). If nutrient and environmental conditions are suitable, the spores of *Bt* will germinate and produce vegetative cell that grow and reproduce by binary fission. The cell will continue to multiply until the nutrient and environmental conditional are limiting for growth, the bacterium then sporulates and produces one or more insecticidal protein in the form of crystalline inclusions (Figure 1.1b). In order that proteins (protoxins) become toxic, protoxins must be activated by proteolytic cleavage. Today, *B.thuringiensis* represents about 2% of the total insecticidal market (Bravo 2011).

1.2. The δ- endotoxin of *Bacillus thuringiensis*

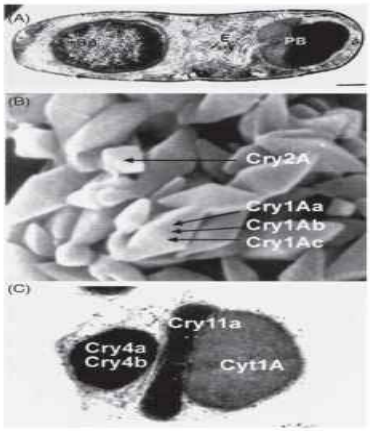
1.2.1. Structural features of Cry protein

During sporulation, *Bacillus thuringiensis* synthesizes various insecticidal proteins including one or more proteins Crystals (Cry) and Cytolytic (Cyt). They are termed δ-endotoxins and can make up to 25% of the dry weight of the sporulated cells (Agaisse & Lereclus, 1995). Although sequences of Cry proteins are diverse, they have a similar overall tertiary structure. Parasporal inclusion bodies containing the protein resemble crystals and so the name Cry of crystal is used. These proteins are protoxins generally have two different lengths (approximately 130 or 70kDa). The C-terminal extension in the larger protoxins is not important for toxicity. When protoxins are ingested by sensitive insects, they are dissolved in the alkaline conditions present in the midgut of insects, and the solubilized inactive protoxins are cleaved at the long C-terminal by midgut protease and is removed from N-terminus. The N-terminal portion is the toxin itself, and it comprises three domains (de Maagd *et al.*, 2001). Domain I is a bundle of seven helices that the seventh hydrophobic helix is circled by six

other helices. Domain I has important role in membrane insertion and pore formation. Domain II includes three anti-parallel δ -sheets with exposed loop regions and domain III is δ sandwich. Domain II and III is related to both larval receptor binding and pore function (Boonserm *et al.*, 2006).



Fingure 1-1a. Electron Micrograph of *Bt* strain namely Bt2-56. A Transmission Electron Micrograph of negatively stained spores from Bt2-56 containing a filament (a), and a sac-like structure containing a spore (b) and parasporal body (c). (Rampersad & David Ammons, 2005)



Fingure 1-1b. Crystals of insecticidal proteins.
(A), Sporulating cell of *B. thuringiensis* subsp. *israelensis*.(B), Scanning electron micrograph of purified crystals produced by the HD1 isolate of *B. thuringiensis* subsp. *kurstaki.* (C), Transmission electron micrograph of the parasporal body of *B. thuringiensis* subsp. *Israelensis* (Federici *et al.*, 2010)

To date, the structure of some Cry proteins have been solved by X-ray crystallography such as Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba and Cry8Ea (Grochulski *et al.*, 1995; Galitsky *et al.*, 2001; Li *et al.*, 2001; Morse *et al.*, 2001; Boonserm *et al.*, 2005; Boonserm *et al.*, 2006). For example, Figure 1.2 shows the three-dimensional structure of Cry2Aa from *Bacillus thuringiensis* subsp *kustaki* (Morse et al., 2001)

The mature structure of Cry2Aa includes three domains. Domain I extends from residues 1-272, it is seven – α helical bundle (Figure 1.2d). This domain is important for the formation of complexes with the biological membrane in the insect gut. Domain II extends from residues 273-473, it is a receptor binding β-prism, a three-fold symmetric arrangement of β sheets, each with a Greek key fold (Figure 1.1e). Cry2Aa is specific against both *Lepidoptera* and *Diptera* (Schnepf *et al.*, 1998), the specific residues to *Lepidoptera and Dipteran* are 278-340 and 341-412 respectively (Lospez Pazos & Cerón Salamanca, 2007). Domain III (residues 474-633) is related to both larval receptor binding and pore function and is a C-terminal β sandwich. Morse stated that a candidate toxin-receptor binding surface on Cry2Aa that is comprised of a distribution of hydrophobic residues across the solvent-exposed surface of the middle and C-terminal domains (Morse *et al.*, 2001). The removal of 49 N-terminal amino acids and exposure of residues comprising this putative toxin-receptor binding surface are involved proteolytic activation and removal of the 49 N-terminal amino residues would not affect the structure of the seven-helical membrane insertion domain.

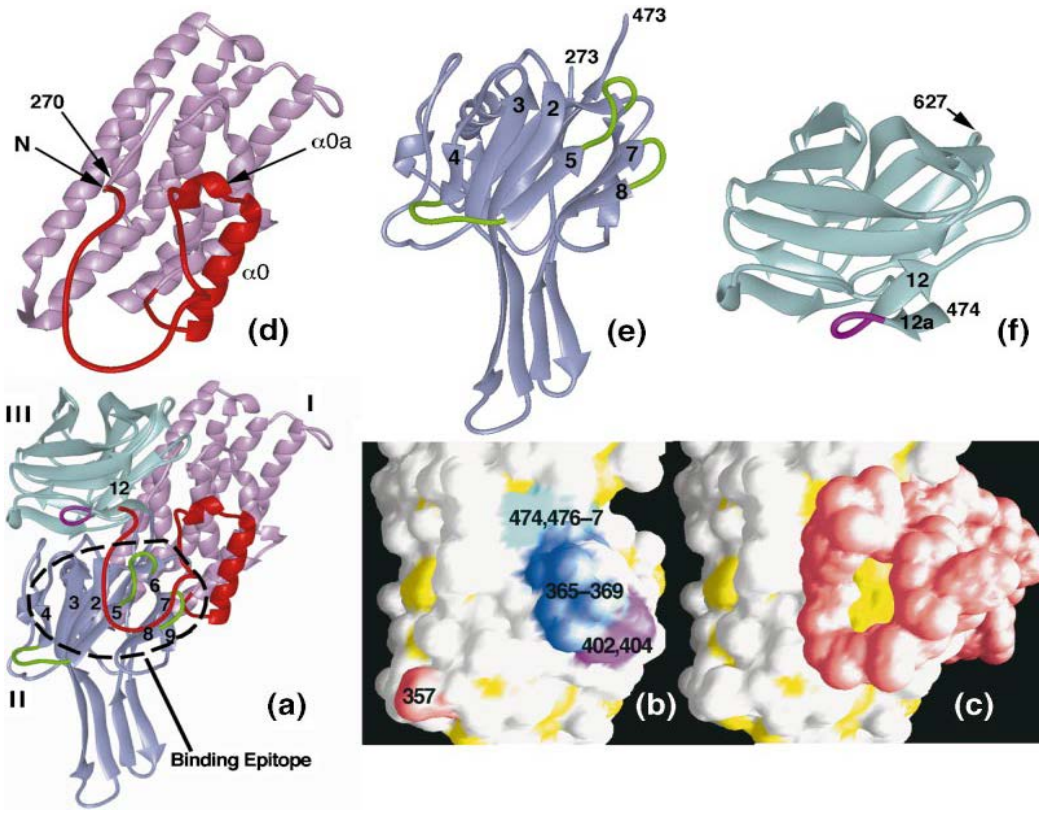


Fig 1.2. The three- dimensional structure of Cry2Aa protein (Morse *et al.*, 2001).
a: Structure of Cry2Aa with domain I (magenta), domain II (blue), domain III (cyan), N-terminus (red) and loops (green).
b,c: The solvent accessible surface of domain II and III with Portions of the hydrophobic surface contributed by residues 474, 476, and 477 are shown in cyan, 365–369 (blue), 402 and 404 (magenta), portion of residue hydrophobicity (yellow), non hydrophobic (white) and N terminus (red). d-f: The three domains of Cry2Aa

1.2.2. Diversity of the δ-endotoxin of *Bacillus thuringiensis*

Since the first gene was sequenced in 1985 (Schnepf *et al.*, 1985), about 500 genes that encode δ-endotoxins have been sequenced (Crickmore *et al.*, 2010). δ-endotoxins are classified based on the sequence homology of amino acids and they have been divided into classes (1, 2, 3, 4, etc.) (Figure 1.3), each class includes subclasses (Cry1A, Cry 1B, Cry1C, etc.), these subclasses are subdivided into subfamilies (Cry1Aa, Cry1Ab, Cry1Ac, etc.). To date, 67 classes of Cry proteins are known (Cry1 to Cry67) (Crickmore *et al.*, 2010). δ-endotoxins also include the Cyt family of toxins and VIP toxin. Unlike Cry toxin, VIP toxin are produced during the vegetative growth phase, At least three VIP toxins have been characterized, VIP1/VIP2, a binary toxin, and VIP3 (Estruch *et al.*, 1996.; Bravo *et al.*, 2011). Cry toxins are encoded by *cry* genes and genes of each class have more than 45 percent identical to other classes. Cry toxins are specific to target insects and have very restricted spectrum activity, limited to the larval stages of small number species. However there is not an exact relationship between the identity of proteins and the level of spectrum activity. For example, only Cry1Aa is toxic to *Bombyx mori*, although Cry1Aa and Cry 1Ac have 84 percent homology, whereas both Cry3Aa and Cry7Aa are toxic to *Leptinotarsa decemlineata*, although they have only 33 percent homology. Some Cry proteins do not affect insects but they are toxic to nematodes, for example Cry5A and Cry6A (Sanchis & Bourguet, 2008).

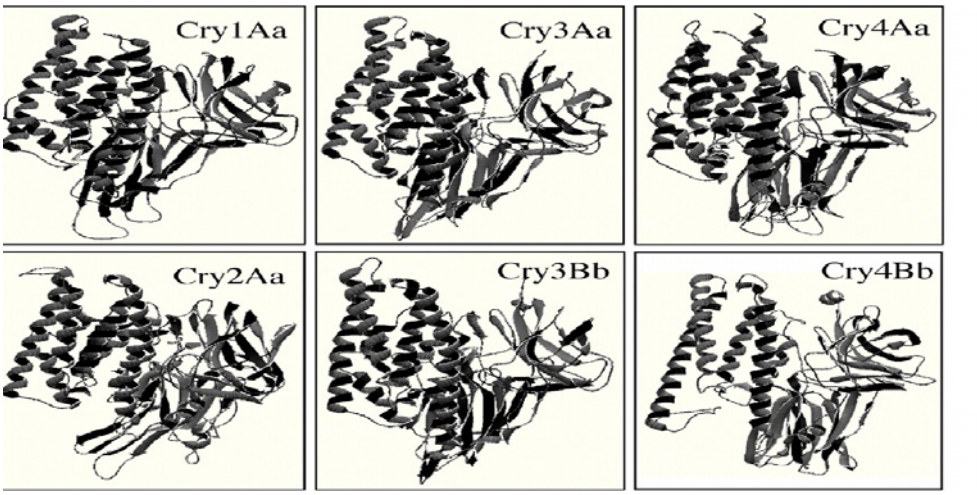


Figure 1.3. Three-dimensional structures of insecticidal toxins produced by *Bacillus thuringiensis* Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa,Cry4Bb (Bravo *et al.*, 2007)

1.2.3. Mode of action of cry protein

Unlike chemical pesticides, Cry proteins must be ingested by insects to have an effect. The mode of action of Cry protein includes solubilisation of Crystal in the midgut of insect, proteolytic cleavage of Cry protoxin in midgut, binding of Cry toxin to receptors on the midgut of insect and creation of pores when Cry toxins are inserted into membrane. Protoxin must ingested by susceptible insects to become active, under the alkaline condition, protoxin will be solubilized. A difference of solubilisation between insects is one cause of differences in the degree of toxicity among Cry protein to sensitive insects (Aronson *et al.*, 1991; Du *et al.*, 1994). Protoxins are cleaved in the insect midgut by proteases to become activated toxins (Tojo et al., 1983).

Activated Cry toxin has two functions, including receptor binding and ion channel activity. Activated Cry toxin binds to specific receptors on the brush border of the midgut of susceptible insects. For example, in case of *lepidopteran* insect, Cry toxin can be bound to receptors on midgut of insect such as Cadherin, Glycosylphotphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), (GPI)- anchored alkaline phosphatase (ALP), a 270 kDa glycolconjugate and a 250 kDa protein called P252. Table 1.1 shows some receptors binding protein in three different insects (Bravo *et al.*, 2011). Binding is a two stage process, with a reversible (Hofmann & Lüthy, 1986; Hofmann *et al.*, 1988) and an irreversible step (Ihara *et al.*, 1993). The latter step can involve a tight binding between the toxin and receptor, insertion of toxin into the apical membrane, or both.

Following toxins are solubilized and activated in the midgut, toxins disrupt the ion balance of midgut cells. The cell is made permeable to small ions through the pore formation allowing a net uptake of ions into cells, followed by water, which results in cell swelling and eventual lysis (Schwartz *et al.*, 1993)

Note: Manduca sexta (Ms), Heliothis virscens (Hv), Ostrinia nubilalis (On), Helicoverpa armigera (Ha), Bombyx mori (Bm), Pectinophora gossypiella (Pg), Limantria dispar (Ld); Diptera, Anopheles gambiae (Ag), Anopheles quadrimaculatus (Aq), Anopheles albimanus (Aa), Aedes aegypti (Ae). Coleoptera, Tenebrio molitor (Tm), Diabrotica virgifera (Dv), Anthonomus grandis (Ag), Leptinotarsa decemlineata (Lde).

Table 1.1. Midgut Cry toxin binding proteins in three insect order (Bravo *et al.*, 2011)

Insect order	Insect species	Cry-binding protein
Lepidoptera	Ms, Hv, On, Ha, Bm, Pg	Cadherin
	Ld	270 Glycoconjugate
	Bm	P252
	Ms, Bm, Hv, Ld, Px	APN
	Ms, Hv	ALP
Diptera	Ag, Ae	Cadherin
	Ag, Ae, Aq	APN
	Ag, Ae, Aq	ALP
	Aa	Alpha-glucosidase
Coleoptera	Tm, Dv	Cadherin
	Lde	ADAM 3 metalloprotease
	Ag	ALP

***** Unlike chemical pesticides, endotoxin Crystal must be ingested by susceptible organisms to become toxic. Thus sucking insects and other invertebrates such as spiders or mites are not sensitive to Cry toxin.

* Under alkaline conditions in midgut lumen of insect, where pH is 8 or higher, Bt endotoxin crystals are activated. Activation requires that the protein be solubilized. Thus Cry toxins have no effect on invertebrates, including human, since the crystal protoxin is sparing soluble in neutral or highly acids conditions in their digestive systems. When protein does solubilize it is rapidly degraded to non-toxic peptides by gastric juice.

(1) Crystal solubilization; (2) protoxin proteolytic activation; (3) monomer binding to Bt-R1 and cleavage of helix a-1; (4) pre-pore oligomeric structure formation; (5) oligomer binding to APN and mobilization to DRM; (6) pore formation in DRM.

* After dissolution, proteases in insect midguts will cleave Cry protoxin protein to produce activated toxin.

* Activated toxin binds to receptor on the midgut of insect before entering the cell membrane and forming a cation-selective channel. Insects must have appropriate receptors for binding Cry protein, this is one of the reasons for strong insect species specificity of Cry proteins.

* After binding to a midgut receptor, the toxin must enter the cell membrane and form a cation-selective channel. This requires a change in the conformation of the active Cry molecule and oligomerization to form the channel.

The mechanism of Cry toxin (Figure 1.3) can be summarized below:

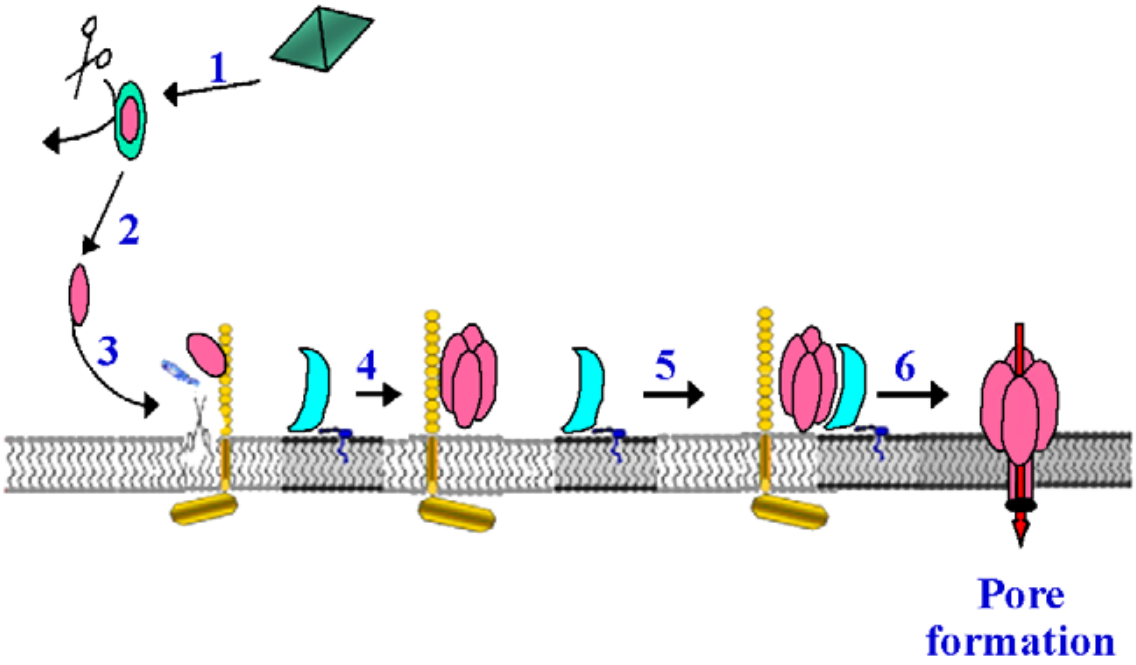


Figure 1.4. Model of the mode of action of Cry toxins. (Bravo *et al.*, 2007).

1.3. *B. thuringiensis* toxins in biopesticides and GM crop

1.3.1. Global status of commercialized transgenic Crop

Although there is much debate about the impact of transgenic plants in the environment as well as for human health, the area planted with transgenic plants has been increasing rapidly throughout the world. Since the transgenic plant was first commercialized in the USA in 1996 with 1.7 million hectares, today GM crops currently account for 180 million hectares representing an increase of 3% compared to 2012 and 100-fold increase in hectares since 1996 (James, 2013). In 2013, biotech crops were planted in 27 countries (Table 1.2 and Figure 1.1) including 19 developing and 8 industrial countries (James, 2013)

Most of biotech crops are grown in mega-countries (countries growing 50,000 hectares, or more). Five principal countries grew nearly 90% of global transgenic crop area including 70,1 million hectares (40%) in the USA, followed by Brazil with 40,3 million hectares(23%), Argentina 24,4 million hectares (13,9%), India 11 million hectares (6,3%) and Canada 10,8 million hectares (6.2%).

In 2013, the increase of biotech crop harvest including both area planted and productivity per hectare with reduction in production costs (reduction of chemical pesticides cost and associated labour costs) has brought much benefit to farmers. For example, the global value of biotech seed was nearly 15.6 billion dollars in 2013 compared to 14.6 billion in 2012. This contributed to the alleviation of poverty and hunger, especially in developing country. For example, biotech cotton have brought the income more than 16.5 million dollars to small resource-poor farmer in developing countries such as China, India, Pakistan, Myanma, Burkina, Faso and South Africa (James, 2013). In 2013 was also second consecutive year that biotech crop was grown more in developing countries than in industrial countries, namely 94 million hectares or 54% of 175 million hectares compared to 81 million hectares or 46% in industrial countries.

The value of GM crops reached 116.9 billion dollars from 1996 to 2012. Beside the benefit about economic, GM crops also contribute to protect environment by saving pesticide and

reducing CO2 namely reducing 26.7 billion kg CO_2 emissions in 2012 alone or saving 123 million hectares land from 1996 to 2012. GM crops also contributed to reduce poverty for small farmer namely more than 16.5 million from 1996 to 2012.

Table 1.2. Global area of Biotech crops in 2013: by countries (million hectares) (James, 2013)

Rank	Country	Area (million hectares)	Biotech Crops
1	USA*	70.1	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash
2	Brazil*	40.3	Soybean, maize, cotton
3	Argentina*	24.4	Soybean, maize, cotton
4	India*	11	Cotton
5	Canada*	10.8	Canola, maize, soybean, sugar beet
6	China*	4.2	Cotton, papaya, poplar, tomato, sweet pepper
7	Paraguay*	3.6	Soybean, maize, cotton
8	South Africa*	2.9	Maize, soybean, cotton
9	Pakistan*	2.8	Cotton
10	Uruguay*	1.5	Soybean, maize
11	Bolivia*	1	Soybean
12	Philippines*	0.8	Maize
13	Australia*	0.6	Cotton, canola
14	Burkina Faso*	0.5	Cotton
15	Myanmar*	0.3	Cotton
16	Spain*	0.1	Maize
17	Mexico*	0.1	Cotton, soybean
18	Colombia*	0.1	Cotton, maize
19	Sudan*	0.1	Cotton
20	Chile	<0.1	Maize, soybean, canola
21	Honduras	<0.1	Maize
22	Portugal	<0.1	Maize
23	Cuba	<0.1	Maize
24	Czech Republic	<0.1	Maize
25	Costa Rica	<0.1	Cotton, soybean
26	Romania	<0.1	Maize
27	Slovakia	<0.1	Maize
	Total	**175.2**	

* 19 biotech mega-countries growing 50,000 hectares, or more, of biotech crops

** Rounded off to the nearest hundred thousand

Source: Clive James, 2013.

However, there are concerns that commercial biotech crops that could have negative impacts on the environment including exposition of non-target organisms, out crossing of herbicide

tolerant varieties to produce uncontrollable weeds, or adverse effects on wildlife (Dale *et al.*, 2002).

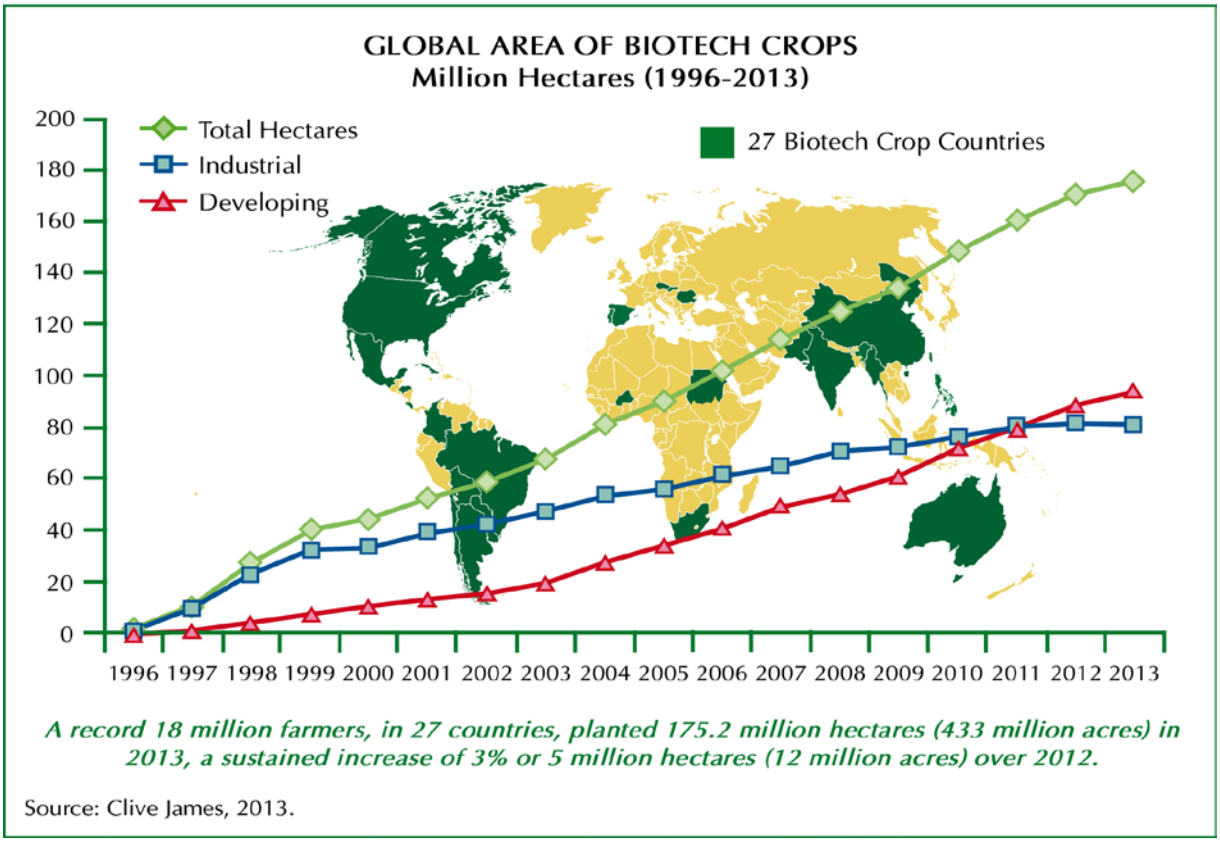


Figure 1.5. Global area of biotech crops million hectares (1996 -2013) (James, 2013)

1.3.2. B. thuringiensis toxins are used as biopesticide and Bt transgenic plant

Bt pesticides may partially replace chemical pesticide whose damaging effects, such as resistance, environmental degradation and human health problems, are increasingly recognised. Bt is useful in agriculture, forest management, control of mosquitos and against other insects. Unlike chemical pesticides, Bt pesticides have highly specific toxicity against target insects. Cry proteins from Bt are released with endospore when the sporangium is lysed under suitable conditions. Commercial Bt products often contain a mixture of crystals and spores of Bt, although only crystal of Bt can kill insect but the spores can enhance activity of the crystal (Crickmore, 2005). The effect of spore to activity of crystal toxicity has been known for a long time although its mechanism has remained unclear. (Asano *et al.*, 2000)

Bt pesticide products have been used since 1950s (Navon, 2000) and during 1970, one of the most successful applications of Bt has been the control of *Lepidoptera* in the N. American forests, using the strain HD-1, or *Bt* subsp. *Kurstaki*, that produces the toxins Cry1Aa, Cry1Ab, Cry1Ac and Cry2A, thereby significantly reducing the use of chemical insecticides (Navon, 2000; Lacey *et al.*, 2001; Bauce *et al.*, 2004; Crickmore, 2006). *Btk* products have been commercialized by many companies (Table 1.3) to protect against the Gypsy moth (*Lymantria dispar*) and the Spruce Budworm (*Choristoneura* fumiferana). *Btk* is often used to supplement chemical pesticides especially when there is insect resistance (Watkinson, 1994). Between the 1970s and 1990s, *B. thuringiensis* subsp *aizawai* that is specific against armyworms such as *Spodoptera* spp., and subspecies subspecies *tenebrionis* and *san-diego*

(Hernstadt *et al.*, 1986), active against beetles were produced commercially. Later more *Bt* strains with different toxicity spectra toxic have been introduced. For example, four novel *Bt* strain have been deposited at the BCCM-LMG under accession numbers. LMG P-12592, LMG P-12593, LMG P-12594, and LMG P-13493 have been found to active against *Lepidoptera* (Rosas-García. 2009).

Biotechnology has developed various trains of bacteria transformed to express gene of insecticidal Cry protein. Genetic manipulation has simplified and improved pest control mainly in crops**,** where they enabled a single genetically modified Bt product to control all the lepidopterous pests infesting the plant.

As outlined above, Bt pesticides are useful to protect against insects and can complement or substitute for chemical pesticide. However, some studies report the development of resistance by several species of insect exposed to Cry toxin of *B. thuringiensis* (Ferré *et al.*, 1995; Bates *et al.*, 2005). The resistance to the Bt reduces the efficiency of insect control. The mechanism of resistance to Bt toxin can be identified at each steps of the action of Bt toxin including solubilisation, proteolytic processing, passage through the peritrophic membrane, receptor binding, membrane insertion, pore formation, and osmotic lysis of midgut cells (Ferré & Van Rie, 2002). Some studies demonstrated a genetic linkage between decreased susceptibility to Cry1Ac and the absence of a major gut protease. Competition-binding studies also showed the reason of the resistance of insect to Cry toxin. For example, Cry1Aa binds to receptor A, Cry1Ab binds to both receptor A and B, and Cry1Ac recognizes both of these sites, as well as receptor C. Thus, Cry1Ac and Cry1Ab also bind to the Cry1Aa binding site. Consequently, it was proposed that the altered Cry1Aa binding site causes resistance to all three Cry1A proteins and that the additional binding sites recognized by Cry1Ab and Cry1Ac may not be involved in toxicity (Ferré & Van Rie, 2002).

To date, due to the limitations of biopesticides (short effective period after application and poor protection against stem borers), Bt transgenic crops have been developing and using widely throughout the world. Bt transgenic plant produces insecticidal proteins active against target insects with no harmful effects to the environment and human health. The first modified plant was a tobacco plant produced in 1983 using *Agrobacterium tumefasciens* (Barton *et al.*, 1987). Some years later, modified cry genes that protect tomato, tobacco and cotton plants were expressed into plants (Perlak *et al.*, 1990). In 1995, Potatoes producing Cry3A to control Colorado potato were first cultivated commercial in USA and both maize and cotton producing Cry1A to protect against various *Lepidoptera* were also cultivated in the following years (Mendelsohn *et al.*, 2003). After that many crops such as rice, maize, cotton, corn, tomato have been genetically transformed by Cry genes from Bt bacteria that are active against many important harmful insects (Perlak *et al.*, 1990; Fujimoto *et al.*, 1993; Yu *et al.*, 2011). For example, Bt gene (*Cry1Ac, Cry1Ab, Cry2Ab, and Cry1F*) of cotton were expressed and commercialized in 11 countries in 2009 (Naranjo, 2010). To reduce the development of resistance of insects to Cry protein, genes may be stacked, for example was two *cry* genes (*cry1Ac* and *cry 2Ab*) have been inserted in cotton and adopted by some countries (Naranjo, 2010). In 2009, maize Bt expressing several cry *genes* (*Cry1Ab, Cry1F, Cry3Bb1, VIP3A, Cry34Ab1/Cry35Ab, Cry2Ab*) was commercialized in 16 countries.

Table 1.3. Natural and genetically modified Bt products registered for agricultural use. Reproduced from (Navon, 2000).

Bt strain	Company	Product	Target insects	Crop
(a) Natural				
Kurstaki HD-1	Abbott Laboratories Chicago IL, US	Biobit, Dipel, Foray,	*Lepidoptera*	Field and vegetable crops, greenhouse, orchard fruits and nuts, ornamentals, forestry, stored products
Kurstaki HD-1	Thermo Trilogy Corp. Columbia MD, US	Javelin, Steward, Thuricide, Vault	*Lepidoptera*	
Kurstaki	Abbott	Bactospeine, Futura,	*Lepidoptera*	
Kurstaki	Thermo Trilogy	Able, Costar,	*Lepidoptera*	
Aizawai	Abbott	Florbac, Xentari	*Lepidoptera armyworms*	Row crops
Tenebrionis	Abbott	Novodor	*Colorado Potato Beetle,*	Potato, tomato, eggplant
Tenebrionis	Thermal Trilogy	Triden	*Coleoptera*	Potato, tomato, eggplant
Kurstaki	BioDalia, Dalia, Israe	Bio-Ti	*Lepidoptera*	Avocado, tomato, vineyards, pine forests
Kurstaki	Rimi, Tel Aviv Israel	Bitayon (granular feeding baits)	*Btrachedra amydraula*	Date palms
Galleriae	Tuticorin Alkali Chemicals & Fertilizers Ltd. India	Spicturin	*Lepidoptera*	Cruciferous crop plants
YB-1520	Huazhong Agric. University, China	Mainfeng pesticide	*Lepidoptera*	Row crops, fruit trees
-	Scient. & Technol. Develop. China	Bt 8010 Rijin	*Lepidoptera*	Row crops, rice, maize, fruit trees, forests, ornamentals
CT-43	Huazhong Agric. University, China	Shuangdu	*Lepidoptera, Coleoptera, Diptera*	Row crops, garden plants, forests
(b) Genetically modified				
Aizawai recipient *kurstaki* donor	Thermo Trilogy	Agree, Design (transconjugant)	Lepidoptera (Resistant *P.xylostella*)	Row crops
Kurstaki recipient *aizawai* donor	Ecogen, Inc. Langhorne PA, US	Condor, Cutlass (transconjugant)	Lepidoptera	Row crops
Kurstaki	Ecogen	CRYMAX, Leptinox,	Lepidoptera	Vegetables, horticultural, ornamental
Kurstaki	Ecogen	Leptinox (recombinant)	Lepidoptera armyworms	Turf, hay, row crops, sweet corn
Kurstaki recipient	Ecogen	Raven (recombinant)	Lepidoptera Coleoptera	Row crops
d-endotoxin encapsulated in *Pseudomonas yuorescens*	Mycogen, Corp. San Diego, CA, USA	MVP MATTCH MTRACK (CellCap')	 Lepidoptera Lepidoptera Coleoptera	 Potato, tomato, eggplant Row crops - armyworms Potato, tomato, eggplant

Corn, cotton and potato are common Bt transgenic crops that be used in agriculture to give protection against insects. They are of great economic importance. In 2010, the global area of Bt cotton was about 19.6 million hectares, an increase of 4.6 million hectares from 2009 (James, 2010). China and India are major cotton-growing countries and India is the country that has the largest area cultivated cotton growing with 8.4 million hectares in 2009. In 2010, Bt maize was planted on 39 million hectares, up by 3.0 million hectares. After USA, Brazil becomes the second country that grows the largest Bt-maize with 5 million hectares in 2009 (Marshall 2010). The top 5 countries are USA, Brazil, Argentina, India and Canada, together they account for over 90% of the area planted with Bt crops. USA is still largest Bt growing country with global market share of 45 % resulting in an increase of 4.5 billion in farm income. In 2010, the area planted with transgenic crops in the USA increased to 66.8 million hectares include maize, soybean, cotton, canola, sugar beets, alfalfa, papaya and squash (James, 2010).

At the end of 2013, Bt crop was planted on 28.8 million hectares. Table 1.4 shows the countries that have commercialized Bt crops from 1996 to 2013 (James, 2013). From 1996 to 2012, the value of Bt crop reached 68.9 billion dollars making up 60% of global value GM crops of 116.9 billion dollars. In 2012 alone, the value of Bt crops reached 12 billion dollars compared to 18.7 billion dollars of global GM crops.

1.4. Advantages and disadvantages of biopesticides and Bt-GM Crops

Bt pesticides are very useful for protection against insects. A significant advantage of the use of Bt products is to contribute reduction of insect resistance to chemical pesticides because of the more limited application. For example, in Australia, the United States and Far East, the risk of resistance was decreased because of the use of Bt products to protect crops. Therefore, chemical pesticides were less often used and these products can continue to be used effectively when required (Watkinson, 1994). However they have some constraints. For example, Bt pesticides have narrow spectrum to insects, Bt sprays cannot kill bollworm and borer larvae, and Bt sprays are inactivated under some environmental conditions including direct sunlight (Navon, 2000). Moreover, some studies have found evidence of resistance to Bt toxin. For example, *Bti* (*israelensis*) which is used for 80% of the control of Dipterous such as Black flies (*Simulium*) and mosquito, which are vectors of many diseases in the African countries because of their resistance to the organophosphates (Bravo *et al.*, 2007).

Compared to Bt pesticide,the incorporation of Bt genes in plants are more useful than biopesticides and chemical pesticides (Betz *et al.*, 2000; Shelton *et al.*, 2002). For example, Bt biopesticides containing different mixtures of spores and crystals are rapidly inactivated under environmental condition, especially under UV exposure. Moreover, Bt toxins of biopesticide are only limited their action on the surface of plant. In contrast, modified cry genes are inserted into plant, cry genes are expressed in tissue of plant and Cry toxins are protected against effect of environmental conditions, and can target borers that are largely unaffected by biopesticides, which are often the most damaging pests.

The other difference between biopesticide of *B. thuringiensis* and Bt crops is that Bt crops often produce activated Cry toxins directly, the steps of solubilisation and proteolytic activation are not required. This could potentially lead to the reduction of the high target specificity of the proteins. An important difference between Bt biopesticide and Bt crops is that while biopesticide is limited in time, whereas the protein is produced throughout the crop growth. Bt transgenic crops also have important role in reducing the use of chemical pesticides, they are environmentally friendly and allow an increase in farmer’s income (Betz *et al.*, 2000). For example, the average increase in farm income worldwide from Bt cotton is estimated to be 2.9 billion dollars in 2008. Within this sum, 65% was derived from increased

yield and 35% from decreased the cost related to the spraying of insecticides (Brookes & Barfoot, 2010).

Table 1.4. The countries that have commercialized Bt Crop from 1996 to 2013 (James, 2013)

Bt crop	Country
Cotton	Argentina, Australia, Brazil, Burkina Faso, Canada, China, Colombia, Costa Rica, European Union (EU), India, Japan, Mexico, Myanmar, New Zealand, Pakistan, Paraguay, Philippines, Singapore, South Africa, South Korea, United States of America (USA)
Eggplant	Bangladesh
Maize	Argentina, Australia, Brazil, Canada, Chile, China, Colombia, Egypt, El Salvador, EU, Honduras, Indonesia, Japan, Malaysia, Mexico, New Zealand, Panama, Paraguay, Philippines, Russian Federation, Singapore, South Africa, South Korea, Switzerland, Taiwan, Thailand, Turkey, USA, Uruguay
Poplar	China
Potato	Australia, Canada, Japan, Mexico, New Zealand, Philippines, Russian Federation, South Korea, USA
Rice	China, Iran
Soybean	Argentina, Australia, Brazil, Canada, China, Colombia, EU, Japan, Mexico, New Zealand, Paraguay, South Korea, Taiwan, Thailand, USA, Uruguay
Tomato	Canada, Chile, USA

Since the first insect-resistant transgenic plants were produced in 1987, the area of Bt plants has increased continuously. Use of Bt crops has led to a reduction in the use of chemical pesticides on crops and chemical pesticides are only used as complements. Therefore, production costs have been reduced and there is less harmful impact to the environment as well as human health (Shelton *et al.*, 2002). For example, in 1998, the chemical pesticides used to protect against bollworm/budworm had been reduced by approximately 1 million kg from 1995 in the USA (Williams *et al.*, 1998a). The use of Bt cotton led to a reduction of approximately 80 % in the average number of applications of insecticides in USA and a reduction of 60-80 % in China between 1995 and 2001 (Romeis *et al.*, 2006b; a).
Bt transgenic crops also have positive environmental impacts such as saving of raw materials needed to manufacture chemical insecticides. Bt crops are also effect highly to insects. Most

European and south western corn borer larvae that feed on Bt corn was died within 72 hours. Plants express Bt genes throughout the growing season and thus benefit from full protection against European and south western borer larvae (Betz *et al.*, 2000).

The use of Bt transgenic crops also contributes to yield increase, European estimates of losses due to the corn borer fluctuate between 30 and 300 million bushels per year (USDA, 1975). In 1997 Bt corn was planted on 4 million acres and area of Bt corn was increased up 14 million acres one year later. In 1995, before the introduction of Bt cotton in the USA, the average yield loss about 4% nationwide and 29% in Alabama because of tobacco budworm and cotton bollworm. Three years later, Bt cotton accounted for total 17 % of cotton in the USA and over 90 % in Alabama. The reduction of the impact of corn borer has allowed an increase in total yield of 85 million pounds (nearly 40 thousand tonnes) (Betz *et al.*, 2000).

Transgenic crops, especially Bt plants have been brought many benefits. However, there are concerns that commercial transgenic plant may result potential impact to environment such as the exposition of non-target organisms and a change in microbe-mediated processes and functions in soil, as well as development of insect resistance. Most studies report no direct effect of Bt toxin from transgenic plants on predators. For example, Zhang et al stated that the Cry1Ab/Ac fusion toxin had no direct effect on young larva of *P. japonica* (Zhang *et al.*, 2006), while in study of the impact of toxin from Bt plants to *P. subpiraticus* and *C. medinalis*, Chen et al stated that *P. subpiraticus* does not have binding receptors in its midgut to Cry1Ab, while *C. medinalis* does and Bt rice did not significantly affect the density of *P. subpiraticus* (Chen *et al.*, 2009). However, some author stated that Bt plant impact to non-target insects such as predators (Ponsarda *et al.*, 2002; Zhang *et al.*, 2006; Chen *et al.*, 2009; Lawo *et al.*, 2010). Ponsard stated that the longevity of *Orius tristicolor* White and *Geocoris punctipes* decreased significantly (by 28 and 27% of the control value, respectively) (Ponsarda *et al.*, 2002).

The influence of Bt toxin from transgenic plants has also been studied for earthworms, nematodes, protozoa, bacteria, and fungi. Saxena stated that *Bt* toxin (Cry 1Ab) released from Bt plants had no effect on any of these classes of soil biota (Saxena *et al.*, 2010). The presence of Bt toxin in the gut of earthworm suggests that toxin released from Bt plants can be adsorbed and bound to the surface of soil particles giving the toxin some protection from degradation with no apparent toxicity to earthworms. Although most studies report no significant differences of mortality and weight of earthworms as well as other organisms, but most that studies only focus to single organisms and do not determine the effect of Cry toxin to non-target organisms over a long period. In addition, Shelton reported that transgenic plant can outcross with wild or weedy relatives(Shelton *et al.*, 2002). One of the major environmental biosafety concerns regarding the release of transgenic plant is the consequence of the transgenic escape of into wild and weedy plant populations. This is an important potential impact of Bt transgenic crops to the environment.

1.5. Cry Proteins and soil

1.5.1. Release of Bt toxin from modified plant on soil

The expression of cry genes from *Bacillus thuringiensis* into plant that code insecticidal protein has many benefit in control of insects and reducing the chemical pesticides. However, there is concern about the potential impact of transgenic plants to nature and agriculture ecosystems because Bt toxin can be released from transgenic plants into soil and may affect the environment. The Cry proteins from Bt crops are released into soil by different pathways such as root exudates (Saxena & Stotzky, 2001; Saxena *et al.*, 2002), by pollen (Losey *et al.*, 1999) as well as the decomposition of crop residues (Zwahlen *et al.*, 2003a). Losey and co-workers found Bt toxin to be expressed in the pollen of transgenic crops and that this pollen can be transported for up to 60 m by wind. This could effect to non-target organisms. Losay

stated that larvae of the monarch butterfly, *Danaus plexippus* that fed with Bt corn pollen ate less, grew more slowly than larvae that fed untransformed corn (Losey *et al.*, 1999). Zwahlen stated that the large amount of *B. thuringiensis* corn plant residues are left in the field after harvest could have an effect on soil organisms. Bt toxin from plant residues degrades slowly and can persist until 200 days (Zwahlen *et al.*, 2003a). Cry toxins are also released into soil through root exudates of transgenic plant. Bt corn that expresses*cry1Ab* genes releases Cry1Ab into the rhizosphere soil in root exudates from Bt corn. The presence of Cry proteins in the rhizosphere soil which was detected by Western Blot assay and found to be toxic to larvae of *M. sexta* (Saxena & Stotzky, 2000).

Cry3Bb1 toxin has been found to be released as root exudates of Bt corn into sterile hydroponic culture, 7.5 ± 1.12 ng/ml after 28 days of growth and into nonsterile rhizosphere soil throughout growth of the plants (2.2 ± 0.62 ng/g after 63 days of growth) (Icoz & Stotzky, 2008a). The release into soil of various Cry protein from various transgenic have been determined such as Cry1Ab protein from corn (*Zea mays* L.) and rice (*Oryyza sativa* L.), Cry3A protein from potato (*Solanum tuberosum* L.), and Cry3Bb1 protein from corn. This Cry proteins were released in root exudates whereas Cry1Ac protein from canola (*Brassica napus L.*), cotton (*Gossypium hirsutum L.*), and tobacco (*Nicotiana tabacum L.*) were not determined in root exudates.

1.5.2. Detection of Bt and Cry toxin in soil

Bacillus thuringiensis can persist in various habitats such as water, leaf, insect's dead body but they were found particularly in soil habitats (Vilas-Bôas *et al.*, 2007; Raymond *et al.*, 2010). Normally, Bt persists in soil in the sprore form. When environmental conditions become favourable, spore will germinate. The previous studies showed that spores of Bt appear to be able to survive in soil for at least 1 to 2 years (Smith & Barry, 1998). Bt was often detected in soil by isolation and culture in the nutrient medium based on observation of shape of colony in petri dishes or Crystal under light microscope (Tohidi *et al.*, 2013). More recently, DNA from *B. thuringiensis* has been extracted from soil and analysed by various PCR reactions (Polymer Chain Reactions) to monitor the presence of Bt.

The detection and quantification of Bt protein in soil can be performed by many methods but in most case, Cry protein must be extracted from soil before quantitative assaying. The extraction method was described by (Palm *et al.*, 1994) and was improved by some other authors. In study of Palm, extraction solution was combined by mixture of a high pH, a strong concentration of salts and a surfactant. Recently, Helassa el al have improved extraction of Cry protein in soil by using zwitterionic and nonionic surfactants such as CHAPS, Triton-X-100, and Tween 20 (Helassa *et al.*, 2009). After extraction Cry protein from soil, detection can be performed by using various methods. For example, Cry protein can be detected by SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis). Gel was stained by Coomasie Blue and separated on the basis of molecular weight. Cry proteins then were estimated by comparison with marker (protein molecular weight standards).

Today, there have been a lots of methods approached to improve detection of Cry protein, Most authors now prefer immunological detection such as western blot (Sims *et al.*, 1996) especially extraction-Elisa. This method is very sensitive. It allows to detect protein in soil at low concentrations (< ng g^{-1} soil). Kits may detect various proteins from the same class, for example, Cry1Aa, Cry 1Ab and Cry1Ac.These tests are faster and easier to implement than other methods. However, this method can only determine the presence of extractable Cry proteins in soil but not their insecticidal activity. Biotests are expensive, labour intensive, time consuming and subject to biological variability, however they require no sample pre-treatment and detect the active toxin which may not be the case for chemical and biochemical tests that may detect inactive fragments of the protein (Sims & Holden, 1996).

1.5.3. Interaction of Cry proteins with soils

1.5.3.1. Mechanisms of interaction of protein on surfaces

Adsorption of proteins on surfaces depends on enthalpic forces and entropic effects that promote conformational modifications of adsorbed protein. The modification toward a more disordered structure may contribute to the driving forces of adsorption of protein on surface. The increase of entropy of the system will cause decrease the Gibbs energy. The change of conformation of protein may affect the area occupied by single protein on the soil surface, thus effect to the maximal of adsorbed protein (Quiquampoix, 2000).

* ***Enthalpic effects***

- Coulombic interaction: Electrostatic forces between proteins and surface are among the factors that effect to adsorption of protein on soil. Proteins and mineral surfaces carry electrical charges. The electrical charges of proteins are caused by the ionization of carbonxylic, tyrosyl, amine, imine, and imidazole groups of the side chains of some amino acids, whereas the electrical charges of mineral surfaces are caused by the pH-independent isomorphic substitutions in the crystal lattice, or by pH- dependent ionization of hydroxyl groups (Quiquampoix, 2000). The sorbent surface can be positively or negatively charge depending on the mineralogy and pH. Coulombic forces are very strong and long-range intermolecular forces. All electrical charges of the given sign are compensated by an equal number of electrical charges of opposite sign, giving rise to a diffuse double layer model of repartition of counterions and co-ions in the surrounding solution, thus a screening of the interaction occurs, and the electric field in solution decreases rapidly
- Lifshitz-van der Waals interaction: Van der Waals forces act on all molecules. They are short-range force that comprise three different components with dispersion forces, which originate from the instantaneous dipolar moment result fluctuation of the electric around the nuclear protons and induction force, and the orientation force, relate to the interaction between two polar molecules (Quiquampoix, 2000; 2002).

**** Entropic Effects***

- Hydrophobic interaction: both protein and sorbent have non-polar group which are exposed to aqueous solution. The shielding of amino acids with a hydrophobic side chain in the core of the protein in contact with water and stabilises protein in solution. The water molecules establish more hydrogen bonds among themselves in the presence of a non-polar group than the presence of the polar group. Water molecules only form hydrogen bonds with polar groups but not non-polar groups, thus rearranging themselves around the non-polar group, to maximize their mutual association by hydrogen bonding. Two hydrophilic entities will tend to retain a hydrated layer between them, whereas two hydrophobic entities will expel water from their surface of interaction and leads to the increase in the entropy of the system and increase adsorption.
- Modifications in protein molecular conformation: The modification of protein structure can result entropy changes that contribute to adsorption. The increase of the rotational freedom in the secondary structure of peptide bonds such as α-helices and β-sheets induce modifications of protein structure. The ordered secondary structures are important part of the densely packed hydrophobic core of protein. When a protein is adsorbed on a surface, internal hydrophobic amino acids can reach more external positions in contact with the surface, these amino acids remain shielded from contact with the water molecules of the surrounding solvent phase. The breaking of hydrogen bonds that maintain the peptide chain in a given conformation, which is associated with decrease of ordered secondary structures, results in an increase of conformational entropy (Quiquampoix, 2000; 2002).

1.5.3.2. Interaction between Bt toxin and soil particles

Adsorption is phenomenon of attracting and retaining the molecules of a substance on the surface of a liquid or a solid resulting into a higher concentration of the molecules on the surface. Adsorption results in removal of solutes from solution and their concentration at a surface, until the amount of solute remaining in solution is in equilibrium with that on the surface or that the surface is fully saturated.

Proteins are macromolecules with pH dependant charge that usually have a great affinity for surfaces. The adsorption of Cry proteins depends on the pH - adsorption of Cry toxins reaches maximum near their isoelectric point on charged surfaces such as clays and the isoelectric point of toxins have been reported to be approximately pH 4.4 and pH 5.5 (Bietlot *et al.*, 1989; Venkateswerlu & Stotzky, 1992) This happened because at values that above the isoelectric point, the negative charge protein and negative charge clay surface will be repulsive each other, and for values below the isoelectric point the attractive interaction between the protein and the surface may lead to increase the points of contact and can change the proteins conformation, thus spreading of the protein on the surface and therefore the adsorption of Cry proteins were decreased (Quiquampoix, 2008). The adsorption decreases with increasing pH above isoelectric point have been reported by some authors, for example adsorption capacity of Cry1Aa on montmorillonite and kaolinite decreased as pH increased above 6.5 (Helassa *et al.*, 2009) and adsorption of Cry toxins on rectorite decreased in the pH range from 9 to 11 carbonate buffer) (Zhou *et al.*, 2007).

Cry toxins are released on soil and can be persisted on soil during a long period when the toxins are bound on the environmental surfaces (e.g., clays and humic substances). The adsorption of Cry protein is influenced by soil type and environmental conditions (Clark *et al.*, 2005). Toxins from *B.thuringensis* are adsorbed and tightly bound on reference clays such as homoionic montmorillonite and kaolinite (Venkateswerlu & Stotzky, 1992; Tapp *et al.*, 1994; Fiorito *et al.*, 2008; Helassa *et al.*, 2009).The adsorption of Cry toxin on montmorillonite is higher than on kaolinite. The clay size fractions adsorbed more Bt toxin than the silt size fractions or bulk soil (Muchaonyerwa *et al.*, 2004). This because of their large specific surface area and high cation-exchange capacity leading adsorption of Cry protein in soil is quickly. Adsorption is rapid; about 70 % of the total of adsorption of the toxin occurred within the first hour of contact and maximum adsorption of Bt toxin occurred within <8h (Crecchio & Stotzky, 2001) whereas maximum adsorption of Bt toxin on a sandy soil and clay was occur after 3 and 4 hours (Sundaram, 1996b).

The adsorption of Cry protein is also affected by the concentration of the clay particles. Increase of concentration of clay resulted decrease of the amount of the adsorbed Cry protein per unit weight of clay (Tapp *et al.*, 1994; Chevallier *et al.*, 2003). This could be explained that the increase of concentration of clay leads to increase aggregation of the particles. This leads reduce the specific external surface area available for the adsorption of Cry protein (Chaplain *et al.*, 1995).

Temperature may affect adsorption of Cry protein in soil. Zhou stated that temperature had no significant influence on the toxin adsorption between 10 and 50^{o}C (Zhou *et al.*, 2007). Venkateswerlu and Stotzky also report that the adsorption of protoxin and toxin on montmorillonite and kaolinite was not considerably different between 7 and 50 °C (Venkateswerlu & Stotzky, 1992). This result was contrary to Helassa who reported that temperature effect on both adsorption and persistence of Cry protein on soil. Adsorption was found to be less at lower temperature for all the soils (Helassa et al, 2009).

It is also necessary to understand the reversibility of adsorption but there are few studies of the desorption of Cry protein and the conclusions are conflicting. In most case authors reported that no desorption was observed after washing with either water or neutral buffer (Crecchio & Stotzky, 2001). In contrast, some authors showed that Bt toxins can be desorbed

from soils. For example, about 10 and 30 %Cry toxin from *B. thuringiensis* subsp *kustaki* and *B thuringiensis* subsp *tenebrionis* could be desorbed from montmorillonite and kaolinite respectively by washing with water, but most could not, indicating that the Cry proteins were tightly bound on the clays (Saxena *et al.*, 2010). Some other studies also showed the desorption of Cry protein from soil by water with different results such as 8±4% (Chevallier *et al.*, 2003), 2-12% (Lee *et al.*, 2003), 50% (Sundaram, 1996a; Muchaonyerwa *et al.*, 2006; Zhou *et al.*, 2007) or more than 90% of Cry protein adsorbed on kaolinite were desorbed with 0,2% Na_2CO_3 and distilled water (Venkateswerlu & Stotzky, 1992).

Changing pH or increasing ionic strength can lead to the desorption of proteins. Desorption of protein occur to different extents. This difference may arise from the nature of the surface investigated and the experimental conditions. Zhou reported that the desorption efficiency of water was higher than that of carbonate buffer at pH 9 (Zhou *et al.*, 2005). Crecchio and Stotzky stated that the desorption of toxin from *B.thuringiensis* subsp *kustaki* adsorbed on humic acids from four soils varied from 20 to 50 % by two washes with water and no more adsorbed toxin was desorbed with additional washings (Crecchio & Stotzky, 1998) indicating that Cry protein tightly bound on soil. However, Helassa et al reported that fraction of adsorbed toxin could be desorbed by water (14 %) and more adsorbed toxins were desorbed by alkaline pH buffer (36±7 %) indicating that the toxin was not tightly bound and the desorption of Cry protein could be improved when using zwitterionic and nonionic surfactants such as CHAPS, Triton-X-100, and Tween 20 (Helassa *et al.*, 2009).

The insecticidal activity has been reported to be preserved after the toxin adsorption on soil (Crecchio & Stotzky, 1998; 2001; Lee *et al.*, 2003; Zhou *et al.*, 2007). The insecticidal activity may still be observed for months indicating that the Cry toxin that be released into soil can be bound on the surface and maintain its insecticidal activity. The insecticidal activity of the toxin from *Btk* and *Btt* bound on kaolinite montmorillonite or the clay-size fraction were studied with hornworm *Manduca sexta* or potato beetle (*Leptinotarsa decemlineata*) (Tapp & Stotzky, 1995). The 50% lethal concentration of free toxin from *Btk* is higher than toxin adsorbed on soil, indicating that the adsorbed toxin increase toxicity to insect. The adsorbed toxin can retain insecticidal activity after exposure to microbes, whereas the toxicity of free toxins was decreased by microbial activity, this result indicated that the toxins are released from transgenic plants can accumulate in soil as a consequence of binding on surface-active soil particles (Crecchio & Stotzky, 2001). In a recent study Sander and co-workers reported that Cry toxin retained insecticidal activity when toxin adsorbed to SiO_2, indicating high protein conformational stability during adsorption process (Sander *et al.*, 2010). The binding of Cry toxin on soil does not change protein conformation since the insecticidal activity is retained, thus adsorption con soil is counteracting the biodegradation and increasing potential hazards for non – target organisms. There are few data relating to the adsorption of Cry proteins on different soils so it is impossible to state which soil properties determine affinity, desorbability (and hence mobility) and persistence.

Adsorption – desorption of Cry proteins on soils have been studied by many authors, but the results are conflicting. In most cases, no desorption was observed after washing with either water or buffer (Tapp *et al.*, 1994; Crecchio & Stotzky, 2001; Chevallier *et al.*, 2003; Fu *et al.*, 2007). In contrast, other authors reported a considerable proportion of *Bt* toxin to be desorbed from soils and soil components by resuspension in water (Koskella & Stotzky, 1997; Crecchio & Stotzky, 1998; Zhou *et al.*, 2005; Zhou *et al.*, 2007). In addition, there have never been comparative studies of adsorption on various soils of various Cry proteins. The adsorption of Cry proteins on soils should be studied in more detail, in particular more information is required on the precise nature of the chemical entities detected by ELISA tests since inactive protein or fragments of partially degraded protein can be detected by an immunochemical test, without having kept its insecticide. Moreover, although the few

published data suggest that insecticidal activity is maintained after adsorption, the results are highly variable, trends not always logical and at least one recent study by Sanders showed changes in growth, not mortality, even the non adsorbed toxin was not lethal.

1.5.4. Persistence and degradation of *B. thuringiensis* toxins in soil

Increasingly Bt crops are used in agriculture thus leading to increasing probability of Cry protein in soil. Cry toxin from Bt transgenic crops such as Bt maize, Bt cotton, or Bt rice can be released into soil through different ways such as through root exudates from plant (Saxena *et al.*, 1999; Saxena & Stotzky, 2000; Saxena *et al.*, 2004; Icoz & Stotzky, 2008a) or in pollen (Losey *et al.*, 1999; Obrycki *et al.*, 2001)or from crop residues after harvest (Zwahlen *et al.*, 2003a; Stotzky, 2004). This release leads to potential accumulation of Cry toxin in soil. Previous studies showed that Cry protein are strongly adsorbed to reference clay minerals, natural clay fractions from soils, and humic acids (Hopkins & Gregorich, 2003; Pagel-Wieder *et al.*, 2007; Icoz & Stotzky, 2008a). The binding of Cry proteins on soil components may protect them against decay and so allow their long term accumulation leading to the possibility of low-level exposition control of target pests or exposition to nontarget organisms, such as soil microbiota and beneficial insects.

There are many studies about the persistence and biodegradation of Cry protein in soils, summarized in Table 1.5. Most authors state that Cry protein displayed a rapid degradation in the early stages of incubation and then more slowly. This pattern has been reported for the degradation of *Btk* purified toxin (Palm *et al.*, 1996), Cry 1Aa purified protein (Helassa *et al.*, 2011), Cry protein released from Bt corn (Sims & Holden, 1996; Herman *et al.*, 2002; Hopkins & Gregorich, 2003; Wang *et al.*, 2003a), Bt rice (Bai *et al.*, 2005; Wang *et al.*, 2006), Bt cotton (Palm *et al.*, 1996) in soil. Most authors stated that almost all of Cry toxin was degraded in the first week and only a small proportion was still retained in soil after long periods such as 180 days (Feng *et al.*, 2011b), 120 days (Helassa *et al.*, 2011b). 200 days (Saxena *et al.*, 2002; Zwahlen *et al.*, 2003b), 350 days, 9 months (Zurbrüegg *et al.*, 2010) or 23 weeks. The rapid decline quickly in the early stages has been attributed microbial activity and the slower decline in the second stage is due to protection of Cry protein by clays and organic matter (Palm *et al.*, 1996). Any free toxin would be rapidly used as source of carbon and energy by soil micro-organisms, in contrast to adsorbed toxin. Cry1Ab has been reported to have a shorter half-life in water than in soil as would be expected (Douville *et al.*, 2005).

In contrast to conclusion of long-tern persistence of Cry protein in soil, other studies reported that Cry proteins do not persist and Cry protein was decreased rapidly in soil (Head *et al.*, 2002; Dubelman *et al.*, 2005; Icoz & Stotzky, 2008b). (Head *et al.*, 2002) found no detectable CrylAc protein was present in any of the soil samples collected from within or outside the fields. The level of CrylAc protein in these samples was evaluated using both enzyme-linked immuno sorbent assays (ELISA) and bioassays with a susceptible insect species, *Heliothis virescens* (F.), the tobacco budworm. (Hopkins & Gregorich, 2003) reported that no detectable difference in the decomposition of plant material from the two lines of maize (Bt-maize and non-Bt-maize). Cry protein in the decomposing plant material and soil mixtures declined rapidly with time during the incubation and no Cry protein was detected after 14 days. The rapid disappearance of the Cry protein occurred at a rate similar to that of the water-soluble components of the maize residues indicating that much of the delta-endotoxin in crop residues is highly labile. (Dubelman *et al.*, 2005) reported that Cry 1Ab did not persist or accumulate in fields planted for at least 3 consecutive years with Bt maize. (Icoz *et al.*, 2007) reported that no detectable Cry3Bb1 was found in the rhizosphere of Bt maize transgenic plant grown for 4 consecutive years. (Ahmad *et al.*, 2005) also found no persistence of Cry3Bb1in the field for 3 consecutive years with Bt maize indicating that

Cry3Bb1 protein released from root exudates of plant transgenic does not persist and degrades rapidly in soil.

The differences in the persistence of Cry protein in soil may be related to the different Cry protein that used in experiments as well as the ecological factors in environment such as temperature, pH, and types and amount clay minerals and organic matter present in the soil. For example, temperature in laboratory experiments is constant, which is different to field condition.(Zwahlen *et al.*, 2003a) reported that no Cry 1Ab protein was detected in the first months of trial and Cry 1Ab protein decreased 20 % during the second month. During winter, there was no further degradation. When temperatures rose again in spring, the toxin continued to degrade slowly. (Feng *et al.*, 2011a) stated that soil temperature had significant effects on the degradation of Cry1Ab protein, with a higher degradation rate at higher temperature. This conclusion was similar with result of (Helassa *et al.*, 2011) who reported that the decline of Cry 1Aa was much slower at lower temperature. The effect of temperature to persistence of Cry protein in soil may be explained by the role of microbial activity (Tapp & Stotzky, 1998; Bai *et al.*, 2005). In this case, low temperature decreases microbial activity, leading to slow degradation of Cry protein in soil. However, other methods used to either enhance or suppress microbial activity or to inhibit the activity of extracellular soil protease did not have any significant effects for Cry1Aa (Helassa *et al.*, 2011a). This leads the hypothesis that the driving forces underlying the decline in detectable Cry are not microbial in origin, but are more probably due to conformational changes of the protein, induced by hydrophobic interactions with soil organo-mineral surfaces. These conformational changes could result in fixation of the protein, hence a decrease in the extraction yield. They might also irreversible modify the zone of the protein detected by the immune-chemical assay, leading to a decrease in detectability of the desorbed protein in solution.
Stotzky and coworkers report faster loss of detectable protein at alkaline pH due to the pH dependency of both microbial activity and adsorption (Venkateswerlu & Stotzky, 1992; Tapp *et al.*, 1994; Tapp & Stotzky, 1998; Crecchio & Stotzky, 2001; Lee *et al.*, 2003). (Wang *et al.*, 2006) reported that Cry 1Ab protein from biomass of Bt rice degraded with the half-life of 11.5d in an alkaline soil and half-life of 34.3 d in acidic soil. (Icoz & Stotzky, 2008b) reported that persistence of Cry 3Bb1 that was released from the root exudates of transgenic Bt corn varied with the type and amount of clay mineral and the pH of the soils. Persistence of Cry proteins increased with increasing concentration of kaolinite but decreased with increasing concentration of montmorillonite. Furthermore insecticidal properties were lost more in pH-neural soils than acid soil and this was attributed to pH-dependent microbial activity. However, (Helassa *et al.*, 2011) found no significant effect on the dynamics of Cry1Aa in 4 contrasting soils was observed when the pH was adjusted. It should be noted that the soils were not identical apart from their pH and so the observed difference could have resulted from their microbial populations or their texture, mineralogical and chemical properties.

Table 1.5. Summary of persistence of Cry proteins in soil (Icoz & Stotzky, 2008b)

Protein	Study location	Experimental variable	Persistence of proteins in soil	References
Cry1Ab Cry1Ac Cry3Aa	Laboratory	Soil amended with biomass of Bt maize, cotton, and potato	No persistence of proteins in soil; proteins degraded in soil with a half-life of 20 d	Ream et al. (1994)
Cry1Ab Cry1Ac	Laboratory	Soil amended with purified protein or biomass of Bt cotton	Purified proteins and Cry proteins from cotton tissue decreased rapidly, with a half-life of approximately 4 and 7 d, respectively, by ELISA	(Palm *et al.*, 1996)
Cry1Ab Cry1Ac	Laboratory	Soils amended with purified protein or biomass of Bt cotton	Purified protein was detected up to 28 d, and the protein from Bt cotton was detected up to 56 d	(Donegan *et al.*, 1995)
Cry1Ab	Laboratory	Soil amended with purified protein	Protein still detectable in soil after 234 d by larvicidal assay	(Tapp & Stotzky, 1998)
Cry1Ab	Laboratory	Soil amended with biomass of Bt maize	50% decrease (half-life) in the insecticidal activity of Cry1Ab protein in 1.6 d and a 90% decrease in 15 d	(Sims & Holden, 1996))
Cry2A	Laborator Field	Soil amended with biomass of Bt cotton Bt cotton cultivation	Half-life of bioactivity was estimated at 15.5 d by insect bioassay Half-life of bioactivity was estimated at 31.7 d by insect bioassay	(Sims & Ream, 1997)
Cry1Ab	Laboratory	Soil with Bt maize or amended with biomass of Bt maize	Cry1Ab protein from root exudates and in plant biomass persisted for at least 180and 350 d, respectively, in soil	(Saxena *et al.*, 2002)
Cry1Ab	Laboratory Field	Laboratory Soil amended with biomass of Bt maize or cultivation of Bt maize for 4 y	No persistence of protein in soil	(Hopkins & Gregorich, 2003)

Cry1Ab	Laboratory	Soils amended with biomass of Bt maize	Protein persisted for several weeks	(Muchaonyerwa *et al.*, 2004)
Cry3Bb1	Laboratory	Soils amended with biomass of Bt maize	Protein was detected only 21 d in soils amended with montmorillonite and 40 d in soils amended with kaolinite (K); after adjustment of pH of the K soils to ca. 7, protein was detected for only 21 d	(Icoz & Stotzky, 2008a)
Cry1Ac	Field	Bt cotton cultivation	No detectable level of protein in soil for 3–6 consecutive years	(Head *et al.*, 2002)
Cry1Ab	Field	Bt maize cultivation	No persistence for 3 years	(Dubelman *et al.*, 2005)
Cry3Bb1	Field	Bt maize cultivation	No detectable level of protein in soil during 3 consecutive years	(Ahmad *et al.*, 2005)
Cry1Ab	Field	Bt maize cultivation	Protein detected in soils during 4 consecutive years	(Icoz *et al.*, 2007)
Cry3Bb1	Field	Bt maize cultivation	No detectable level of protein in soil during 3 consecutive years Protein detected in soils during 4 consecutive years	
Cry1Ab	Field	Bt maize cultivation	Protein in Bt maize litter persisted at least 8 months	(Zwahlen *et al.*, 2003a)
Cry1Ab	Laboratory Field	Bt maize cultivation	Protein persisted through winter but no accumulation	(Baumgarte & Tebbe, 2005)
Cry1Ab	Laboratory Field	Soils amended with biomass of Bt rice and Bt rice cultivation	The half-lives of the protein in soils amended with Bt rice straw (4%) was estimated at 11.5 for alkaline soils and 34.3 d for acidic soils	(Wang *et al.*, 2006)

In conclusion, there are many contradictions in the conclusions of studies about adsorption and persistence of Cry proteins in soil. Some authors reported that Cry proteins do not persist but degrade rapidly in soil and other authors reported that Cry protein persisted for long periods after adsorption. In addition, there have been no comparisons of detection by extraction-Elisa and biotests. No previous studies of persistence have compared Cry proteins (Tapp & Stotzky, 1998; Head *et al.*, 2002; Vettori *et al.*, 2003; Muchaonyerwa *et al.*, 2004; Dubelman *et al.*, 2005). Bioassay method is used to detect the toxicity of proteins that is not assessed immunochemical methods. However this method is expensive, costly labour and time consuming. There are also a very limited understanding of the relationship between protein structure and its environmental fate including the maintenance of biological activity when adsorbed on soil.

The aim of this investigation is to compare the detection of various Cry proteins from different classes on various soils to determine whether they have the same adsorption behaviour and insecticidal activity, and whereby provide the prediction of the persistence of insecticidal Cry protein in soil. Differences in protein structure may lead to radical contrasts in persistence of toxicity. The understanding of persistence of Cry protein in soil is important to establish monitoring programmes of the effect of GM plants as well as to determine one route of possible exposure of non-target insects to the toxins. As for other protein, adsorption of Cry protein on soil is likely to be a determining factor for their fate in the environment. The adsorption limits the mobility of proteins, it may increase persistence of Cry protein and may maintain its insecticidal activities. There are only a few Cry protein have been studied whereas many Cry proteins will be used in current and future crop. Therefore, it is important to know whether small change in structure or conformation of Cry protein have important consequences on the stability of interaction of Cry protein in soil and whereby have a better understanding about the fate of different Cry protein in soil.

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CHAPTER 2.

COMPARISON OF THE AFFINITY AND EXTRACTION YIELD OF TRACE AMOUNTS OF THREE CRY PROTEIN FROM *BACILLUS THURINGIENSIS* IN CONTRASTING TYPES OF SOIL

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Running title : *Soil affinity and extraction yield of Cry proteins*

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Summary

The use of insecticidal proteins known as Cry or Bt, either as biopesticides used in agriculture or as vector control or originating from commercial genetically modified crops (GM), is increasing rapidly. The fate of these proteins in the environment depends strongly on their adsorption on the organo-mineral complexes of soil. Environmental monitoring requires the quantification of the proteins and this entails their chemical extraction from soil. Three Cry proteins, Cry1Ac, Cry1C and Cry2A, present in commercial biopesticides formulations or synthesized by GM plants or both were studied. The adsorption of trace amounts of Cry proteins on over forty types of soil with contrasting properties was measured in dilute suspension. After a short incubation the extraction yield was measured with a previously tested alkaline solution that contained surfactant and another protein. Each of the proteins had a strong affinity for soil. No soil property was observed to determine either the affinity for soil or the extraction yield. There was no simple relation between the affinity (assessed from the distribution coefficient, K_d) and the extraction yield, although there was a significant inverse relation ($P<0.05$) for two of the proteins, Cry1Ac and Cry2A. The proteins differ in both their affinity for soil and their extraction yields. We conclude that these insecticidal proteins will be largely immobile in soil, but that routine environmental monitoring can give only semi-quantitative values for protein in soil.

Résumé étendu

Le devenir des protéines dans les sols dépend en grande partie de leur adsorption. De même leur détection dans le but de surveillance requiert une désorption sans perte de structure pour que la protéine soit toujours reconnaissable par un test immunochimique. L'objectif de cette recherche était de cribler un grand nombre de sols et trois protéines Cry différentes pour tenter d'identifier les propriétés des sols qui déterminent l'adsorption et le rendement d'extraction des protéines. Jusqu'alors les propriétés d'adsorption ont été étudiées pour un petit nombre de sols ou minéraux, à chaque fois pour une protéine. Il en était de même pour le rendement d'extraction des protéines Cry adsorbées. Seul un petit nombre de sols et de de cocktails d'extraction ont été étudiés.

Dans cette étude jusqu'à 41 types de sols ont été étudiés avec trois protéines : Cry1Ac, Cry1C et Cry2A. Les sols provenaient de la collection RMQS (Réseau de Mesure de la Qualité des Sols) tenue par Infosol, INRA, Orléans qui a mis à disposition les analyses des sols. Les sols provenaient de sites susceptibles de recevoir des traitements de biopesticide Bt, avec des usages des terres peu intensifs, comprenant des prairies, des zones humides et des forêts, et des sols susceptibles d'être support de culture de maïs Bt si ces cultures étaient acceptées à l'avenir en France. Les protéines ont été obtenues par culture bactérienne et purifiées avant l'usage. L'adsorption et l'extraction des protéines ont été mesurées sous des conditions contrôlées de laboratoire en suspension diluée. La quantité de protéine apportée aux sols était aussi proche que raisonnable des quantités mesurées dans des sols soumis aux cultures de plantes GM. Il s'agit donc de mesurer l'affinité d'une quantité trace de protéine, et non pas la capacité d'adsorption des sols. Un seul cocktail d'extraction a été utilisé, car son efficacité avait été testée pour une autre protéine Cry, (Cry1Aa) avec quatre sols de teneurs en argile et en matière organique différentes. Les résultats sont exprimés par le coefficient de distribution, Kd, défini comme le rapport des concentrations à l'état adsorbé et restant en solution. Ceci correspond à la pente de l'isotherme d'adsorption en tendant vers zéro. Ce calcul n'implique pas que le système soit en équilibre dynamique. Le rendement d'extraction est exprimé comme la fraction de protéine préalablement adsorbée qui est solubilisée. Les comparaisons statistiques ont été effectuées par régression simple et par analyse de variance avec une approche itérative (stepwise) qui élimine des paramètres n'ayant pas d'effet ou ceux dont l'effet est dû à leur lien avec un autre paramètre.

Chacune des protéines avait une affinité forte pour tous les sols. Des régressions simples n'ont pas indiqué de relation forte avec des propriétés de sol ou avec l'usage des sols. Une relation inverse non linéaire existe entre Kd et le rapport de la teneur en carbone organique et de la teneur en argile pour Cry1Ac (Figure 2.1). Les valeurs de Kd pour les trois protéines ne suivaient pas les mêmes tendances (Figure 2.2).Par contre l'analyse itérative est plus puissante, les résultats des analyses sont donnés dans le Tableau 2.1. Cette analyse permet d'identifier des relations entre Kd et teneur en argile (pour Cry1Ac), la teneur en matière organique (inverse pour Cry1Ac et Cry1C) et pH (inverse pour Cry1Ac et Cry1C). La relation qui était visible pour Cry1Ac et le rapport entre les teneurs en carbone organique et argile n'a pas été mise en évidence pour Cry1Ac, mais l'a été pour Cry2A.

Les rendements d'extraction étaient plus variables que ceux mesuré pour Cry1Aa dans une étude précédente. Des relations inverses, faibles mais significatives, sont observées entre l'affinité Kd et le rendement d'extraction pour Cry1Ac et Cry2A. Bien que ces relations soient faibles, elles confirment l'attente intuitive que plus une protéine est attirée pour une surface, plus difficile serait sa désorption (Figures 2.3a &2.3b. L'analyse itérative identifie des relations entre le rendement d'extraction et la teneur en argile (inverse pour Cry1Ac), la teneur en carbone organique (inverse pour Cry2A), la capacité d'échange cationique (pour

Cry1Ac) et le rapport entre les teneurs en carbone organique et en argile pour les trois protéines (inverse pour Cry1Ac et Cry1C).
En conclusion, les protéines Cry sont fortement adsorbées par l'ensemble des sols. Cette adsorption va limiter leur mobilité et donc la zone contaminée. Le devenir des protéines va donc être déterminé par les interactions à l'état adsorbé. Cet aspect est repris dans la section suivante. Il n'a pas été possible de trouver les propriétés des sols qui déterminent l'affinité des protéines pour les surfaces organo-minérales des sols. Les implications des résultats de cette étude pour le suivi environnemental sont que les mesures de routine ne donneront que des estimations qualitatives de la quantité de protéine Cry dans un sol.

2.1. Introduction

Bacillus thuringiensis, or Bt, is an endemic soil dwelling bacteria that produces large amounts of insecticidal proteins during sporulation. Some of these proteins are used for pest control in both biopesticide formulations and more recently in genetically modified crops, known as Bt-crops. A good understanding of their interaction with soil is essential to predict their environmental fate and impact and to optimize monitoring programmes. Each of the many strains of the bacterium (over 170 are known to date) produces a small number of these proteins, known as Cry because they aggregate to form parasporal bodies that resemble crystals. Only a few of these proteins are used for pest control in the form of biopesticides or genetically modified crops. The mechanism of toxicity is complex and comprises several steps that each has a large degree of species specificity, so that the toxins are remarkably species specific. To summarize, the parasporal bodies contain protoxins that must first be solubilised at high pH, the pH of insect larval guts, then enzymatically cleaved to produce toxins, proteins with molecular weight of about 67 kDa, these may then bind with specific receptor sites in the mid-gut, probably as tetramers that form pores in gut membrane leading to cell lysis and death of the insect (Schnepf *et al.*, 1998; Shelton *et al.*, 2002; de Maagd *et al.*, 2003).

Spores and parasporal inclusion bodes of Bt have been used successfully for decades as biopesticides and account for a large share of the current, growing market for biological pest control (Crickmore, 2006; Sanchis, 2011). The use of Bt in this context has not given rise for concern and little research has been carried out on the environmental fate of the proteins (Vilas-Bôas *et al.*, 2000; Tetreau *et al.*, 2012). However the genetic modification of commercial crops to introduce a trait so that the plants synthesise one or more Cry proteins has led to speculation on the dangers of these proteins in soil. The major difference between the proteins produced by bacteria and by genetically modified (GM) so-called Bt crops, is that the latter produce toxins directly, thereby reducing to some extent the species specificity that contributes to safety. Bt proteins are released from GM crops into soil by root exudation and during the degradation of crop residues (reviewed by Clark *et al.*, 2005; Icoz & Stotzky, 2008). Many studies have been devoted to the development of resistance of the target insect and to the impact on non-target species. The interactions and fate of a small number of Cry proteins, Cry1Aa, Cry1Ab and Cry3Bb, have been studied in the field, in soil microcosms and in contact with reference minerals and humic substances (Crecchio & Stotzky, 2001; Herman *et al.*, 2002; Hopkins & Gregorich, 2003; Marchetti *et al.*, 2007; Helassa *et al.*, 2009).

In general, soils and clays have a large affinity for Cry proteins and a large adsorption capacity, adsorption is found to be rapid, and importantly, adsorption is not easily reversible (Fu *et al.*, 2007; Helassa *et al.*, 2009). Adsorption of proteins has several consequences, and these are all pertinent for the fate of Bt in soil. Firstly adsorbed proteins are immobilised, thereby limiting their zone of influence (Quiquampoix, 2008; Helassa *et al.*, 2010). Secondly, adsorption may confer some protection against microbial breakdown, thus increasing their life-span in soil (Nannipieri & Eldor, 2009). Thirdly, conformational changes may follow adsorption and there may be preferred orientation of the macromolecules on the organo-mineral surfaces of soil and these could modify biological activity. Some studies suggest that toxicity of Cry protein is conserved, at least for some days or weeks, in the adsorbed state (Crecchio & Stotzky, 2001; Zhou *et al.*, 2007). Finally the protein is most easily detected in a solubilised form, and so must be extracted from soil prior to quantification, the most common method being immuno-detection. Various extraction cocktails have been proposed and tested. Greatest extraction efficiency is found, as for other soil proteins, including enzymes, when extraction solutions are at alkaline pH and contain surfactants, high salt concentration and a competitive protein (Palm *et al.*, 1994; Fornasier &Margon, 2007; Helassa *et al.*, 2011; Mueting *et al.*, 2014). The role of the competitive protein, in this case bovine serum albumin

(BSA), is to replace and prevent the Cry proteins readsorption on the soil surfaces by a mechanism of heteromolecular exchange. The BSA is well suited to be used as a competitive protein since it is a "soft" protein with a very high affinity for solid surfaces and has been found to enhance enzyme extraction from soils without loss of catalytic activity (Fornasier & Margon, 2007). Extraction efficiency of proteins from various surfaces is often reported to decrease with time due to time dependent fixation (Nakanishi *et al.*, 2001; van der Veen *et al.*, 2007). Detectable Cry proteins have been reported to decrease with increasing period of contact with soils, however it has yet not been possible to distinguish between chemical or microbial breakdown of the proteins, and time-dependent fixation (Hopkins & Gregorich, 2003; Marchetti *et al.*, 2007; Helassa *et al.*, 2011).

In order to establish relevant monitoring programmes and risk assessment of Cry proteins in soil, it is necessary to better understand and predict the interaction of these proteins with soils. Ideally the affinity of Cry proteins for soil, the extractability and their persistence in soil could be predicted from soil composition.

The aim of this study was to carry out a large scale screening of the affinity and extractability of contrasting Cry proteins from a large range of soils. The soils were chosen from the collection of the French Network for the Monitoring of the Quality of Soils (RMQS) and a full analysis of their composition was available. They varied in their land use, either agricultural soils under intensive cereal farming, likely to be used for the growth of GM-Bt crops in the event of a suspension of the current restrictions of the growth of GM crops in France, or soils in non-agricultural land or woodlands liable to receive Bt biopesticides. For each group, there was a wide range of clay content, organic matter content and pH. The Cry proteins were Cy1Ac, Cry2A and Cry1C, chosen because they are present in commercial biopesticides and current or future GM crops. All could be obtained by bacterial culture of natural or genetically modified bacterial strains. Adsorption and extraction were measured in dilute suspension of soil under controlled conditions of temperature. The distribution coefficient, Kd, defined as the ratio of adsorbed protein concentration to that remaining in solution was used as a proxy for the affinity of protein for soil. This facilitates comparison with other studies, avoiding the effect of soil:solution ratio on the fraction adsorbed , there is no implied assumption that adsorption is reversible. The extraction cocktail used had alkaline pH and contained surfactant and a competitive protein to optimize extraction efficiency. The composition has some similarity to the chemical conditions in the mid-gut of target insects, but was developed and tested by Helassa and co-workers without consideration of physiological media (Helassa *et al.*, 2011). This is the first study to compare the interactions of several Cry proteins and many soils.

2.2. Materials and methods

2.2.1. Preparation of purified Cry proteins

A natural strain of *B. thuringiensis* subsp. *kurstaki* HD-74 producing only Cry1Ac protoxin, and genetically modified strains of *B. thuringiensis* producing either only Cry2A or Cry1C protoxin provided by CIRAD, Montpellier, France were cultivated in 500 ml Erlenmeyer flasks with baffles to maximise aeration containing 100 ml autoclaved Nutrient Broth (containing erythromycine for the GM strains)+ 4 ml of 25% glucose sterilized by filtration. After sporulation (about 48 hours), the medium was centrifuged to obtain pellets containing spores and crystals, washed in 1 mNaCl to eliminate spores in the foam formed. The pellets were resuspended in distilled water and ultrasonicated for 1 minute in ice and centrifuged again. The protoxin thus obtained was solubilised in solution containing 50 mM Na_2CO_3 and 10 mMDL-Dithiothreitol (DDT) for 1 hour at 37^0C, then centrifuged and filtered at 0.45µm. This protoxin was activated using trypsin for 45 minutes, at 37^0C and pH 8. Activation was stopped with phenylmethanesulfonyl fluoride (PMSF) 1mM and was centrifuged for 30

minutes at 15 000 g at 4^0C and filtered to 0.45 µm. The toxin was purified by fast flow chromatography on a strong anion exchanger (Q-HP Sepharose, Amersham) using an AKTA purifier (Amersham Pharmacia Biotech) at pH 10.4 as described by Vié et al (2001). Stock solutions of the protein of about 20 g l^{-1} were stored at 4°C until required in a N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer at pH 10.4 containing 350 mMNaCl to prevent polymerisation (Helassa et al., 2008; Masson et al., 2002). Immediately prior to preparation of soil suspensions, the background electrolyte was exchanged for 0.01M $Ca(NO_3)_2$ by repeated cycles of dilution and concentration using Amicon ® filter devices until pH was neutral. These proteins have a strong tendency to oligomerise at neutral pH and high concentration (Helassa *et al.*, 2011), therefore it is essential to dilute solutions after electrolyte exchange and to use them immediately. Initial protein concentrations were 3 mg dm^{-3} for Cry1Ac and Cry1C and 40 mg dm^{-3} for Cry2A.

2.2.2. Soils

Forty-one soils were selected from the 2200 soils in the French RMQS collection supplied by Infosol, INRA, Orléans. Soils were air-dried and sieved < 200 µm. This size fraction was preferred to the classical < 2 mm to reduce sample heterogeneity, given the small sample sizes. Land use categories were (i) soils used for intensive agriculture, including short rotations with grassland and (ii) natural areas and parks and gardens including meadows, wooded areas and wetlands. For each land use category soils were randomly selected to cover the full range of clay content, then within each third of the sample, selected for highest, intermediary and lowest organic matter contents, ensuring that there was a large range of the ratio of organic carbon:clay contents, and finally within each group high, intermediate and low pH. All analyses were carried out by the Laboratoired'Analyses des Sols, LAS, Arras, INRA and made available by Infosol.

2.2.3. Adsorption and extraction

Triplicate suspensions were made by weighing 0.1 g of each soil into Eppendorf tubes then adding 1 ml of solution containing the Cry protein to be studied, at the initial concentration indicated above in preparation of purified Cry proteins, in a background electrolyte of 0.01 M$Ca(NO_3)_2$solution. Suspensions were shaken end-over-end at 25°C for 2 hours. Phases were separated by centrifugation at 19 000 *g* for 30 minutes, then 0.8 ml removed for analysis. The soil was then resuspended in 0.8 ml extraction buffer and shaken vigorously to resuspend. The new suspensions were shaken again end-over-end at 25°C for 2 hours. Phases were again separated by centrifugation at 19 000 *g* for 30 minutes and 0.8 ml supernatant solution removed for analysis. The extraction buffer contained 10mM CAPS, 140 mMNaCl, 1% Tween 20, 4% Bovine serum albumin, at pH 11 (Helassa *et al.*, 2011). Soils were not allowed to dry and were not incubated between the adsorption and extraction steps. Adsorption and extraction supernatant solutions were diluted as required and the Cry protein quantified using Elisa microplate kits purchased from Envirologix following the manufacturer's instructions. Tubes and pipette tips were always "low-binding" to prevent loss of protein during manipulations, this was verified with control samples not containing soil. ELISA determinations were calibrated by comparison with the optical density at 290 nm and were found to be linear in the range 1-15 µg l^{-1} for Cry1Ac and Cry1C and in the range 10-200 µg l^{-1} for Cry2A. Each sample was analysed after at least two different dilutions to ensure that the concentration was within the linear range.

2.2.4. Data treatment

Affinity was assessed from the value of the distribution coefficient, Kd defined as the ratio of concentrations in the adsorbed and solution phases after 2 hours incubation, calculated by depletion with respect to the initial solution prior to addition of soil. The extraction yield was calculated from the ratio of protein desorbed with respect to that initially adsorbed. Stepwise linear regression was performed, involving an iterative construction with automatic selection of independent variables, to obtain the most simple and robust relationships between both affinity and extraction yield and soil variables.

2.3. Results and Discussion

2.3.1. Affinity of Cry1Ac and soil properties and land-use

Soil properties covered a wide range as seen in Table 2.1. The full set of 41 soils was studied for the protein Cry1Ac. The affinity of the protein for soil was assessed from the value of the distribution coefficient, Kd. The range of Kd values observed and the average values are given in Table 2.2. A large range of Kd values was measured, $1.6x10^3 – 3.8x10^4$, with an average of $1.2\ x10^4$. The values measured for used for cereal culture (liable to be used for Bt commercial crops) and natural or semi-natural lands covered similar ranges and were not significantly different. The large values of Kd show that the protein has strong affinity for soil surfaces, as is often observed for proteins on many surfaces.

There was no significant relationship between Kd and soil clay content considered alone, although the stepwise correlation identified a weakly significant relationship (Table 2.3 for analysis of variance). It is well known that proteins are strongly adsorbed on mineral surfaces (Demanèche *et al.*, 2009). The adsorption capacity of a surface for a protein would thus be expected to vary as a function of surface area, and hence for a soil, to some extent, as a function of clay content. Helassa *et al.* (2009) found that the difference in adsorption capacity of two reference clays, montmorillonite and kaolinite, for Cry1Aa which is similar to Cry1Ac, was in line with their specific surface areas. However the situation is different for the adsorption of a trace amount of protein, orders of magnitude less than the amount required to saturate soil surfaces, as is the case in this study. There is no simple relationship between affinity of a trace amount of protein and the adsorption maximum. The adsorption isotherms of Cry1Aa protein on reference mineral surfaces has been found to be of the L-affinity type, but this does not preclude that affinity of trace amounts would be high, as observed for these soils (Helassa *et al.*, 2009).

There was no significant relationship between Kd and organic carbon content, considered alone, although larger values of Kd tended to be observed for soils with low Corg contents, and smaller values for organic-rich soils. The stepwise analysis identified a significant, inverse relationship between Kd and organic carbon content. Previous studies of the adsorption of Cry proteins on soils have included only small numbers of soils. Pagel-Wieder *et al.* (2007) noted that adsorption of Cry1Ab decreased with increasing organic carbon content. However both increases and decreases in adsorption of Cry proteins have been observed after chemical removal of soil organic matter (Crecchio & Stotzky, 2001; Muchaonyerwa *et al.*, 2002; Muchaonyerwa *et al.*, 2006; Mueting *et al.*, 2014). When the ratio of organic matter to clay is low, most organic matter will be in the form of clay-organic matter complexes (Dexter *et al.*, 2008), and organic coatings might compete with protein for adsorption. However, this is more likely to influence affinity for large additions of protein, near surface saturation and may be less relevant for the adsorption of trace amounts of protein. When the organic carbon content is high, more organic matter will be un-complexed (Dexter *et al.*, 2008) and so the protein is more likely to be in contact with particulate organic matter. Cry protein is also adsorbed or complexed with humic acids, but there have been few

studies of the strength of the interaction (Crecchio & Stotzky, 1998; Sander *et al.*, 2012; Tomaszewski *et al.*, 2012).

Table 2.1.Maximum, minimum and average values (in brackets) of some soil chemical and physical properties.

	Clay content	**Corg content**	**pH (H_2O)**	**CEC**	**Corg/Clay**
	g kg^{-1}	g kg^{-1}		meq_c/100 g	%
All soils (N=41)					
Range (average) (17)	16-707 (249)	0.6-243 (38)	4.3-8.6 (6.2)	0-39 (11.4)	1-70
Soils under Cereal culture (16 soils)					
Range (average)	78-480 (247)	6.9-33 (16.8)	4.6-82 (6.5)	2.3-31.6 (12.9)	3-22 (8)
Soils from (semi-)natural systems (25 soils)					
Range (average) (23)	16-707 (250)	.59-243 (51)	4.3-8.6 (6.1)	0-39 (10.5)	1-70
Sub-set of soils studied for all proteins (N=19)					
Range (average) (17)	16-707 (295)	059-243 (45)	4.3-8 (6.3)	0-39 (12.6)	1-58

Simple linear regression found no significant relation between affinity and soil pH. In contrast affinity was found to be highly significantly related to pH when a stepwise approach was used. In common with other proteins, the adsorption capacity of minerals for Cry1Aa has been observed to decrease with increasing pH above the isoelectric point of the protein (Helassa *et al.*, 2009). Zhou *et al.* (2007) observed a decrease in affinity of a reference mineral, rectorite, for Cry with increasing pH, but at a range of pH that is not relevant for soils, 9-11. Sander *et al.* (2010) also report an inverse relationship between Cry adsorption on silica and pH above the isoelectric point, but at saturation concentrations. There was no significant relation between Cry1Ac affinity and soil cation exchange capacity. There was a weak inverse curvilinear relationship between Kd and the ratio of organic carbon to clay contents, as seen in Figure 2.1, although no correlation was identified by the stepwise analysis, certainly because the ratio depends on both clay and organic carbon contents. The relationship was strongest for cereal soils, that are depleted in organic matter and in which organic matter is mostly complexed. There was no effect for the soils from (semi-)natural systems with Corg:clay ratios above 0.20, indicating that non-complexed organic matter did not contribute to the affinity for Cry1Ac. Thus for lower ratios of Corg:clay, where organic matter is complexed, there is some evidence that organic matter coatings contribute to lowering the affinity of minerals for Cry.

Table 2.2. Minimum-maximum values and average (in brackets) of affinity (Kd / dm^3 kg^{-1}) for each of the proteins on the soils

Protein	Cry1Ac	Cry2A	Cry1C
Full sample set (41 soils)			
Range (average)	1630-38400 (12100)	-	-
Soils under Cereal culture (16 soils)			
Range (average)	1630-28600 (10100)	-	-
Soils under (semi-)natural land-use (25 soils)			
Range (average)	2820-38400 (13200)	-	_
Soils studied for all proteins (19 soils)			
Range (average)	1630-24400 (11300)	1560-29300 (16100)	837-54600 (18300)
Soils under Cereal culture (7 soils)			
Range (average)	-	1550-26700 (4700)	5000-54600 (19150)
Soils under (semi-)natural land-use (12 soils)			
Range (average)	-	1560-29300 (13700)	837-42900 (17700)

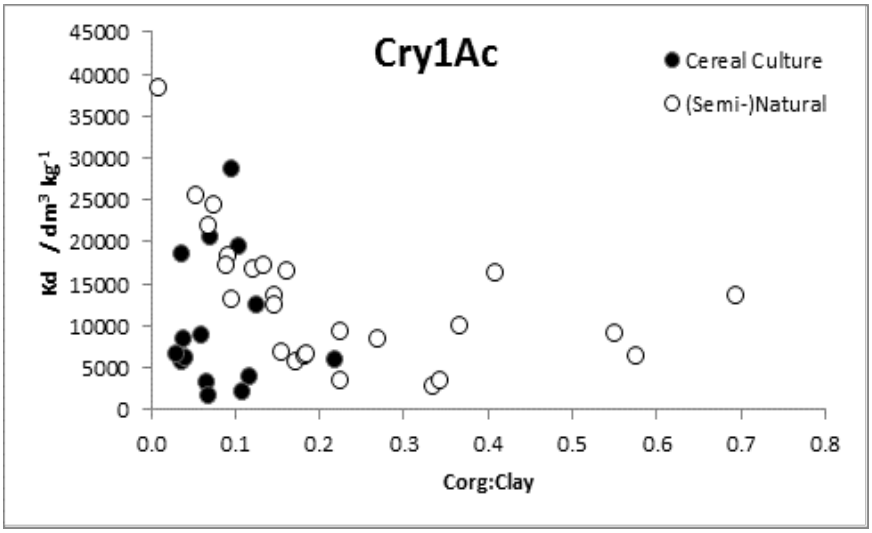

Figure 2.1. Distribution coefficient, Kd, of Cry1Ac as a function of the ratio of soil organic matter content to clay content, Corg:Clay for all soils. Closed symbols are for soils under intensive agriculture of cereal crops and open symbols for soils under natural or semi-natural land-use.

2.3.2. Comparison of affinities of Cry1Ac, Cry2A and Cry1C

A smaller number of soils (19) were retained to study the adsorption-desorption properties of the other two Cry proteins, Cry2A and Cry1C. The range and average values of the soil clay content, organic matter content, pH and the ratio between Corg and clay were similar to those

of the full sample set (Table 2.1). The range and average value of Kd for Cry2A was the same as for Cry1Ac, and no difference was observed with respect to land-use.

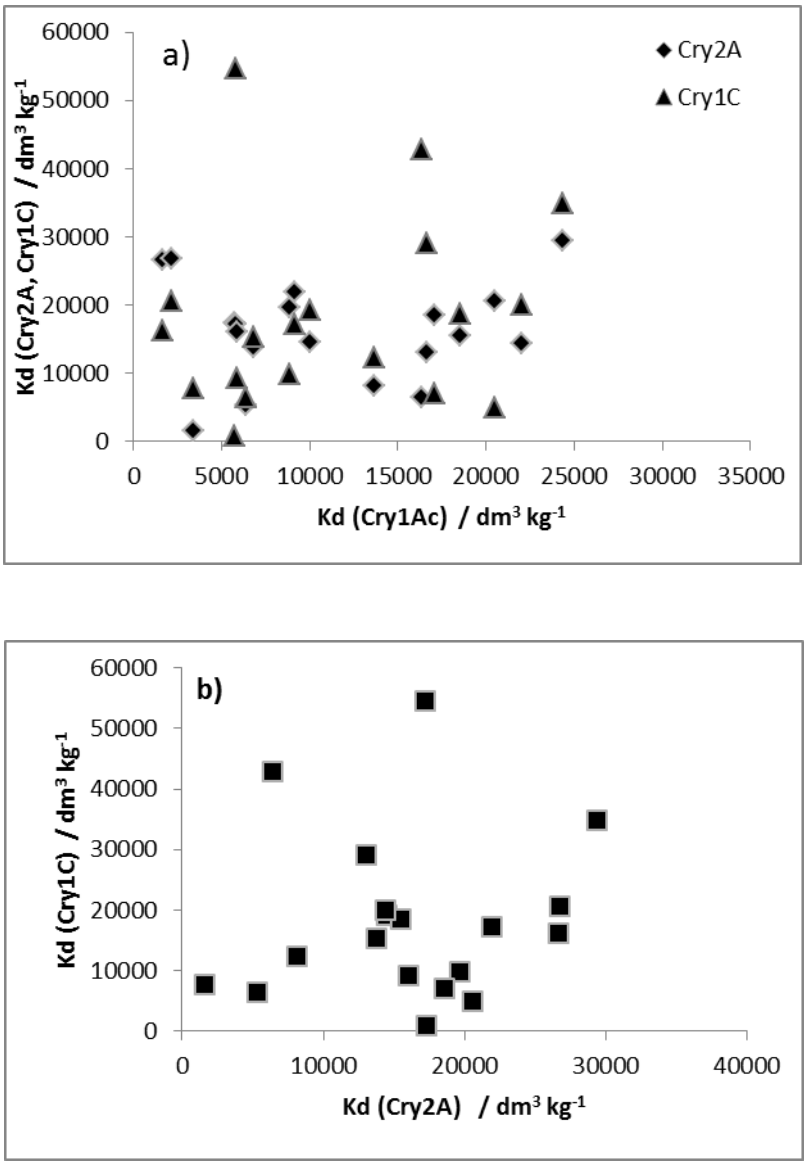

Figure 2.2. Distribution coefficient, Kd, of a) Cry2A or Cry1C as a function of Kd of Cry1Ac and b) of Cry2A as a function of that of Cry1C.

The range of Kd values measured for Cry1C was larger than for the other proteins, but the average value was similar and again, no effect of land-use was observed. As for Cry1Ac, no strong significant relations were observed between affinity of Cry2A or Cry1C and the soil properties. The inverse relation between Kd and the ratio Corg:clay was not as strong for either Cry2A or Cry1C as for Cry1Ac, but this simply reflect the smaller number of soils studied. There was no significant relationship between the affinities measured on each of the soils, as illustrated by Figure 2.2. This demonstrates that rather small differences in the three-domain structure between proteins can led to large differences in affinity of trace amounts, even if at saturation levels similar adsorption capacities might be observed.

2.3.3. Extraction yields for Cry1Ac

The extraction cocktail used in this study was developed and tested by Helassa *et al.* (2011) and found to give very similar extraction yields, about 60%, for Cry1Aa on four soils with contrasting texture and organic matter content. The range and average values of extraction yields for each of the proteins from the soils were between 43 to 100%, with an average of 74%. The range in extraction yield was much larger than expected. No land-use effect on extraction yield was observed. Given the highly alkaline pH of the extraction solution, no effect of soil pH was expected and none was found. However, given the strength of interaction between proteins and mineral and organo-mineral surfaces, which leads to adsorption being quasi-irreversible, we expected extraction yields to decrease with increasing clay content of soils. There was no influence of clay content, organic matter content nor CEC (which is strongly related to the former properties) on extraction yield of Cry1Ac. Extraction yield tended to increase with decreasing affinity (Figure 2.3a) ($P<0.05$). This demonstrates that affinity is to some extent linked with the strength of interaction and hence difficulty in desorbing adsorbed protein.

2.3.4. Extraction yields for Cry2A and Cry1C

The extraction yields of Cry2A were somewhat lower than for Cry1Ac, in the range 30-100% and an average of 60%. Part of the reason for this may lie in the strong tendency of this protein to oligomerize, making detection less precise. There was no land-use effect on extraction yield. The yield tended to increase with decreasing clay content, although outlying points decreased the significance of the correlation. There was a significant ($P<0.05$) inverse relation between extraction yield and affinity for Cry2A (Figure 2.3b). There was no significant relation between Cry2A extraction yield and other soil properties.

The extraction yields of Cry1C vary little between soils, in the range 33-70%, with an average of 49±11%. Because of this very constant extraction yield it is not surprising that there was no significant correlation between extraction yield and soil properties, land-use or affinity (not shown).

2.3.5. Environmental implications of affinity and extraction yields of Cry proteins

The starting premise for this study was that the fate and the environmental monitoring of Cry proteins, whether from GM crops or biopesticides are determined by their adsorption on soil organo-mineral surfaces. This study demonstrates that three contrasting Cry proteins are strongly adsorbed on a wide range of soils. Even the lowest Kd measured would result in only about

0.02% of protein in soil remaining in solution. Cry proteins would therefore be effectively completed immobilized very close to the point at which they were released into soil. Movement would only occur by bioperturbation or soil particle transport. Microbial and catalytic degradation would therefore depend on the degree of protection afforded by adsorption. Since Cry proteins are stomacal poisons, and because the mechanism of toxicity requires the protein to be in solution, only organisms that consume soil, either deliberately or involuntarily, and whose digestive systems were sufficiently alkaline to desorb the protein could be exposed to the toxin. Adsorption would thus protect many species, and certainly mammals, whose digestive systems are acidic, from exposure.

The other major consequence of Cry adsorption on soils is the difficulty in environmental monitoring. Routine detection using immunochemical tests requires the protein to be in solution. The extraction solutions provided with commercial ELISA kits are adapted for extraction from plant material, not soils. The range of extraction yields measured in this study for Cry1Ac and Cry2A indicate that absolute quantification of Cry proteins may be subject to caution. Without controlled studies it would not be possible to predict the extraction yield and hence the true amount of protein in the sample. A further limitation is that detectable amounts of Cry proteins are observed to decline with time, but it is not yet known if this decline is due to progressive fixation of the protein, decreasing the extraction yield, or to degradation of the

adsorbed protein. Nevertheless, for practical purposes, fixation would result in a loss of toxicity, since toxicity requires the protein to be desorbed in the intestinal tracts of sensitive animals.

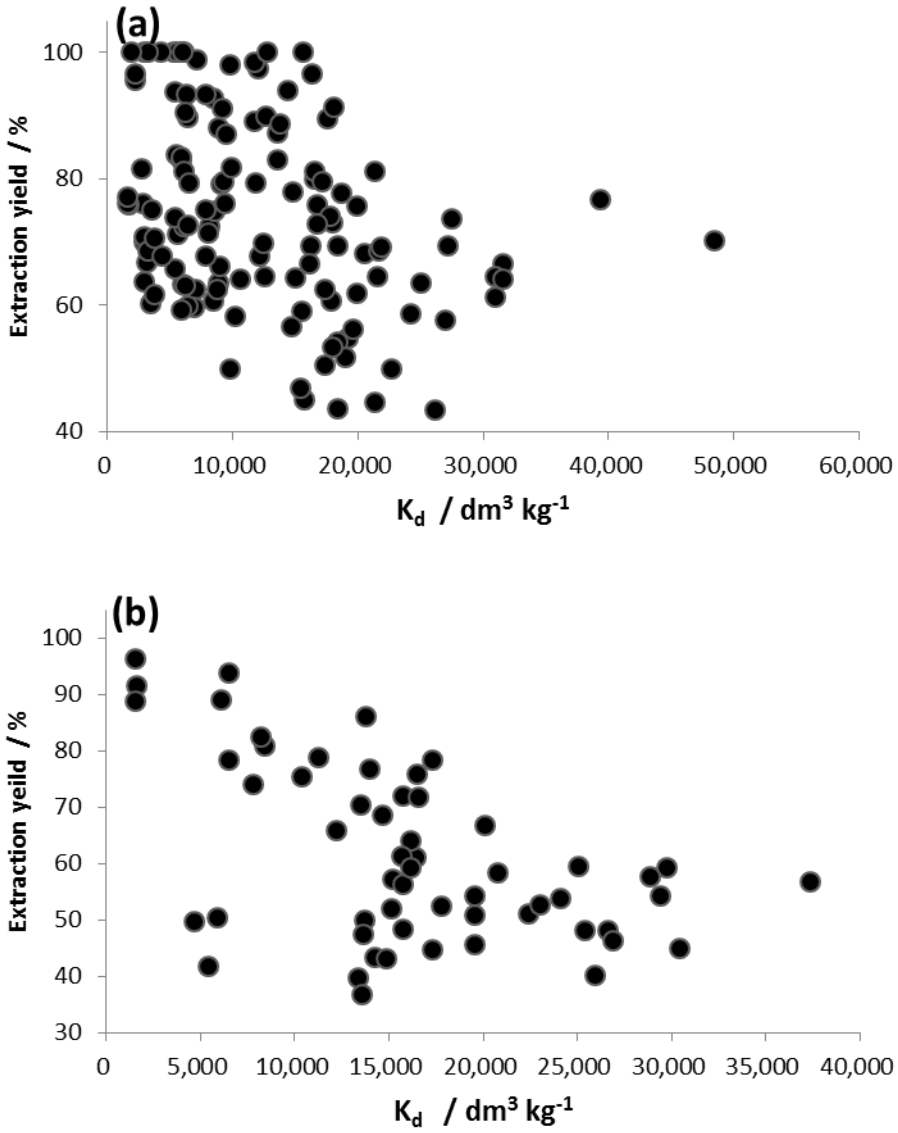

Figure 2.3. Extraction yield as a function of affinity (Kd) for a) Cry1Ac and b) Cry2A

Conclusion

Cry proteins are all strongly adsorbed by soil and will be effectively immobilized after their release into soil. They differ in their affinity for soil with different textures, organic matter contents and mineralogy. Both affinity and extraction yield vary between soils, and extraction yield is not determined by affinity for all soils and each protein. There is no simple relation between soil properties and either affinity or extraction yield. The amount of Cry protein detected in soil by extraction followed by immunodetection is not an absolutemeasure of the protein present in soil because of differences in extraction yield.

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CHAPTER 3.

FATE OF *BACILLUS THURINGIENSIS* CRY PROTEIN IN SOIL: DIFFERENCES BETWEEN PURIFIED TOXIN AND BIOPESTICIDE FORMULATION

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ABSTRACT

BACKGROUND
Bacillus thuringiensis produces insecticidal proteins known as Cry and its efficiency and absence of side-effects make it the most widely used biopesticide. There is little information on the role of soils in the fate of Cry proteins from commercial biopesticide formulations, unlike toxins from genetically modified crops that have been intensively studied in recent years. The persistence of Cry in soil was followed under field and laboratory conditions.
RESULTS
Sunlight accelerated loss of detectable Cry under laboratory conditions but little effect of shade was observed under field conditions. The half-life of biopesticide proteins in soil under natural conditions was about one week. Strong temperature effects were observed, but they differed for biopesticide and purified protein, indicating different limiting steps.
CONCLUSION
For the biopesticide the observed decline in detectable protein was due to biological factors, possibly including the germination of *B. thuringiensis* spores and was favoured by higher temperature. In contrast for purified proteins, the decline in detectable protein was slower at low temperature, probably because the conformational changes of the soil-adsorbed protein that cause fixation and hence reduced extraction efficiency, are temperature dependent.

Résumé étendu

Les protéines insecticides issues de biopesticides et de plantes génétiquement modifiées pour posséder le trait Bt sont de quasiment identiques. Mais les craintes de la société envers l'ingénierie biotechnologique relatives àl'expansion rapide des cultures GM au niveau mondial font que les recherches sur le devenir des protéines Cry issues de plantes GM dominent largement les recherches sur les biopesticides Bt. La plupart des recherches sur les biopesticides Bt concerne les formulations pour améliorer l'adhésion du produit aux récoltes à protéger et optimiser la période effective après application. Il y a eu peu de recherches sur le devenir des protéines Cry issues de biopesticides dans le sol. Avec l'expansion du marché des biopesticides comme alternative aux pesticides de synthèse, il est probable que les protéines Bt seront présentes à des concentrations plus importantes dans des zones de plus en plus étendues. Les connaissances obtenues sur les biopesticides pourraient enrichir les connaissances sur les protéines Bt-GM et vice versa. L'objectif de ce volet de recherche était de comparer le devenir de protéines Cry issue de biopesticide et sous forme purifiée, modèle des protéines Bt-GM.

Les essais avec la formulation biopesticide commerciale ont été menés au champ et en laboratoire sous des conditions contrôlées. L'effet de l'ensoleillement, connu pour accélérer le déclin de Bt dans les biopesticides par le biais du rayonnement UV, a été suivi. Pour cerner l'origine des différences entre les biopesticides et la protéine purifiée résultant de leur mode d'application, l'effet de préhumectation du sol, pour crée un flush microbien avant l'ajout de protéines a été étudié. Comme la température a été identifiée comme le paramètre de l'incubation qui influe le plus sur le devenir de la protéine Cry purifiée, ce paramètre a aussi été pris en compte au laboratoire, et de façon indirecte en changeant l'heure d'application de la biopesticide, au champ. Dans tous les cas, le devenir de la protéine Cry1Ac a été suive par extraction chimique, utilisant le même cocktail chimique que dans les volets précédents, suivie de quantification immunochimique avec des kits ELISA commerciaux.

Au champ le déclin de Cry1Ac était graduel pendant un mois. Le déclin très rapide observé au laboratoire pour les protéines purifiées n'a pas été observé. Le feuillage a accordé une protection faible mais significative pendant quelques jours. Aucune différence significative sur le devenir de Cry entre la pulvérisation le matin ou l'après-midi, pour changer l'exposition initiale au rayonnement solaire (Figure 3.1), n'a été observée. Au laboratoire trois conditions ont été suivies pour le biopesticide pulvérisé sur une couche de sol sur un plateau: (i)ensoleillement direct, (ii) température de 25°C à l'obscurité ou (iii) de 4°C à obscurité. L'exposition au soleil a provoqué un déclin rapide de Cry détectable au cours d'une semaine (Figure 3.2). L'effet de la température était l'inverse de celui observé dans les études précédentes pour les protéines purifiées – un déclin lent à 25°C et un déclin rapide, encore plus rapide qu'observé avec l'ensoleillement direct. Il y a deux différences majeures entre les essais menés avec la protéine purifiée et les formulations de biopesticide ; (i) le biopesticide ne contient pas que la protéine, il y a aussi des spores de B. thuringiensis et (ii) la protéine purifiée a été ajoutée à des sols sec, le flush microbien qui suit la réhumectation du sol pourrait accélérer la dégradation de la protéine Cry. Le fait de réhumecter le sol trois jours avec l'ajout d'une solution contenant Cry1Ac n'a eu aucun effet significatif sur le devenir de Cry incubé dans le sol à 4°C. La préhumectation a ralenti le déclin de Cry dans le sol à 25°C, mais l'effet n'est pas suffisamment important pour expliquer le contraste entre la dynamique de la Cry purifiée et celle contenue dans le biopesticide (Figure 3.4). L'explication semble être que la germination de spores dans le biopesticide conduit à la production de plus de protéine au cours de l'incubation avec le sol après pulvérisation. Ceci a été confirmé par une expérience sans sol où le biopesticide a été incubé en suspension soit à 4°C soit à 25°C dans de l'eau, une solution du sol ou bien de la solution nutritive utilisée pour la culture de B.

thuringiensis à trois dilutions (Figure 3.3). A 4°C et dans de l'eau pure, la concentration de protéine ne variait guère au cours des deux semaines d'incubation. Par contre, à 25°C en présence de nutriments, soit d'une solution nutritive soit d'un extrait aqueux de sol, une augmentation de la concentration de protéine a été observée pendant la première semaine d'incubation environ, suivie par une diminution.

En conclusion, les comportements de Cry issue de biopesticide et purifiée pour imiter celles produites par des plantes génétiquement modifiées diffèrent de façon importante. Le devenir des protéines purifiées résulte de l'interaction de la protéine avec des surfaces organo-minérales du sol. Elles sont exposées à l'activité microbienne et peuvent être dégradée par des protéases. En grande partie le déclin observé provient de la fixation progressive des protéines sur les surfaces du sol. Par contre pour les biopesticides plusieurs autres processus interviennent avant que les protéines puissent être adsorbées. En particulier, la germination des spores actives contenues dans la formulation de biopesticides dans des conditions favorables peut augmenter la concentration de protéine qui s'oppose au déclin de la protéine sous l'influence de la fixation sur les surfaces et de la dégradation. Les dynamiques de Bt issues de biopesticide et de cultures GM seront donc très différentes au moins pendant les premiers jours qui suivent leur introduction dans le sol.

3.1. Introduction

The realization that chemical pest control may have negative impacts on the environment and human health has led to an increase in the use of biopesticides for crop protection, particularly in organic farming, and vector control (*1, 2*). The market for biopesticides has increased and is expected to continue to increase. Currently, formulations containing the bacterium *Bacillus thuringiensis* account for up to 90% of the market (*3*). *B. thuringiensis* is an ubiquitous Gram-positive bacterium that produces large quantities of insecticidal proteins during sporulation under nutrient-limiting conditions (*4-6*). Insecticidal proteins used in formulated biopesticides are contained in parasporal inclusion bodies also known as "Crystal", and so are given the name Cry. Each of the many strains of *B. thuringiensis* produces a small number of Cry proteins, usually between one and five, and these proteins have a large degree of specificity for target insects at the larval stage. The proteins in the parasporal inclusion bodies are protoxins that must be solubilized at the high pH of larval mid-gut, then activated by enzymatic cleavage to form lower molecular weight proteins, that are the toxins. The activated proteins then react with specific receptors in the insect mid-gut forming pores leading to rapid death of the insect (*6, 7*).

Bt toxins are stomach poisons that must be ingested, unlike chemical pesticides that are often contact poisons. This fact, along with the highly specific mechanisms that lead to toxicity, gives them clear advantages over non-specific chemical pesticides (*2, 8*). Only a small number of Bt strains are used as biopesticides, although over 100 different commercial formulations exist (*8*). Formulations usually contain crystals and spores and are sprayed onto crops. The presence of spores is known to enhance the toxicity of the protein, although the reasons are not clearly understood and may include protection of the crystals against degradation by UV-light (*7*). Commercial formulations also contain adjuvants to improve the adhesion of the Bt active ingredients to plants and to protect against photolytic degradation.

There have been few studies of the persistence of biopesticide-derived spores and toxins in the environment (*9, 10*). Bt has been found to persist in soils and waters for days or months, and in some favorable circumstances spores may germinate (*10, 11*). Although there is no mechanism by which Bt protoxins or toxins may be harmful to mammals, the persistence has two conflicting consequences. Firstly, the longer the toxin remains intact and in contact with the plant to be protected, the longer is the period of protection. Secondly, the persistence of the protoxin or toxin at sub-lethal levels could increase the probability of acquisition of resistance and possibly the exposure of non-target insects, via soil or crop residues.

In contrast to biopesticides Bt, there have been many studies of the environmental fate of Bt toxins (and not protoxins) derived from genetically modified crops since their commercialization in 1996 (reviewed by *12, 13*). The protoxins and toxins are usually observed to decline rapidly in soil, but may remain detectable for months. The roles of soil in determining the fate of Cry proteins are potentially very important. Soil acts as an efficient UV-filter, thereby potentially prolonging the conservation of crystals. The microbial activity of soil, including catalytic activity of extracellular proteases, contributes to the decline in insecticidal protein. Although the solubilization of crystals in soils may be slow, given that soil pH is rarely strongly alkaline, crystals will eventually be solubilized and truncated. As for other proteins, soluble Cry proteins released into soil are rapidly adsorbed on soil organo-mineral surfaces (*14-18)*. Adsorbed proteins are largely immobilized (*19*) and adsorption has various consequences. Adsorption is thought to confer both physical and chemical protection against microbial breakdown, although recent studies of fungal phosphatases indicate that this may not always be the case (*20, 21*). Conformational changes due to electrostatic and hydrophobic interactions may modify the biological properties of the proteins. Conformational changes may change with time and this may cause the extraction efficiency to decrease, a phenomenon known as aging or fixation. No published studies have successfully

distinguished between breakdown of Cry protein and fixation as causes of the observed decline in extractable-detectable protein in soil (*15*).
The aim of this study was to follow the persistence in soil of detectable Cry toxins applied in a commercial formulation of *B. thuringiensis* var. *kurstaki* crystals and spores. The persistence was followed under field conditions, varying the mode of application (canopy protection or exposure to direct sunlight, application morning or afternoon). The persistence was also monitored in soil with no crop under controlled laboratory conditions, varying temperature and exposure to sunlight and in aqueous solution without soil as a function of temperature and nutrient supply. For comparison, the persistence of purified Cry1Ac toxin applied to the same soil was monitored under controlled conditions. We were interested in the effect of temperature on persistence since previous studies with purified Cry proteins showed a strong temperature effect, despite no effect on soil microbial activity from which we concluded that protein conformational changes following adsorption were temperature dependent (*15*). However for biopesticides there are no data on the temperature effect on persistence. Prewetting soil prior to the application of Bt was another variable chosen to modify microbial activity and so to distinguish between physico-chemical and microbial driving forces.

3.2. Materials and methods

3.2.1. B. thuringiensis biopesticide spray

A commercial spray, Vi-Bt, was purchased from Vietnam Pesticide Joint Stock Company and used according to the suppliers recommendations, by dilution in water. This spray is commonly used in Vietnam and the potency unit was given as 16000 IU/mg. It was composed of *B. thuringiensis* var. *kurstaki* (isolate HD-1) crystals and spores. The HD-1 strain produces various Cry proteins including Cry1Ac.

3.2.2. Cry1Ac purified protein

Cry1Ac protein from *B. thuringiensis* strains HD73 was cultivated in shaken Erlenmeyer flasks at 28°C until sporulation (about 48 hours). The sterile nutrient solution was composed of $MgSO_4.7H_2O$ at 500 µM, $MnSO_4.H_2O$ at 10 µM, $ZnSO_4.7H_2O$ at 50 µM, $Fe_2(SO_4)_3$ at 50 µM, $CaCl_2.2H_2O$ at 1 mM, KH_2PO_4 at 50 mM, H_2SO_4 at 30 µL L^{-1}, bacteriological peptone (Sigma P0556) at 7.5 g L^{-1}, glucose 1%, at pH 7.4. The protoxin solubilized and enzymatically truncated and the resulting Cry1Ac toxin purified as previously described (*18, 22*). The protein solution stored at 4°C in CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffer at pH 10.4 containing 350 mMNaCl to avoid oligomerization of the protein. Immediately prior to addition to soil the storage solution was removed and replaced by 0.01 M $Ca(NO_3)_2$ solution by repeated dilution and concentration in Amicon filter devices.

3.2.3. Soils and study site

The study site for the field experiment and from which soils were sampled for the controlled laboratory experiment was situated in North Vietnam, near Hanoi, in the Plant Protection Research Institute. The climate is subtropical, with most of the annual rainfall of 1700 mm during the rainy season (May-October) and average daily mean temperatures between 16.5°C (January) and 29.5°C (July). The study plot is used for the cultivation of sweet potato (*Ipomoea batatas*). For laboratory studies and soil analysis, triplicate composite soil samples were collected from the top layer of soil (0-5 cm). The soil samples were air-dried, sieved < 200 µm, thoroughly mixed and stored until required. The soil was a sandy loam containing 1.1% organic carbon, with a C/N ratio of 13, a cation exchange capacity of 8.2 and a pH of 8.4.

3.2.4. Persistence of biopesticide Cry proteins under field conditions

This experiment was carried out over one month in winter (December 2013 to January 2014). The temperature ranged from 14 to 17ºC at night and from 24 to 26ºC in the day, rainfall was low (5-15 mm in the period) and did not occur soon after spray application. Light intensity was low for Vietnam, with about 70 hours of sunshine per month. A sweet potato crop had been planted 21 days prior to spraying. Fertilizer and pesticide treatments were usual for this crop in Vietnam to protect against the *lepidopteran* pest *Agrius convolvuli*. This plot had not previously received any Bt treatment and Cry1Ac was not detectable. Spray was prepared by dilution in water (5 g dm^{-3}), then sprayed at a rate of 0.2 dm^3 m^{-2} to give an application rate of $1.6x10^7$ IU m^{-2}). Spraying was carried out in the morning, except for one treatment when the crop was sprayed in the afternoon when sun intensity was less. Three spray application and soil-sampling variables were chosen to follow the persistence of Cry1A proteins from Bt spray in the field: (i) soil sampled under leaf canopy; (ii) soil sprayed directly and sampled from inter-row (iii) spray applied directly to inter-row soil in the afternoon of the first day, when light intensity was lower and soil sampled inter-row. Three replicate rows were sprayed. Soil was sampled after various time intervals (between 1 hour and 28 days). Composite samples from each of the three positions were taken to obtain about 5 g soil which was placed in plastic bags and returned to the laboratory for analysis. Moisture content was determined by oven-drying of a sub-sample. Seven repetitions of about 0.2 g equivalent dry soil were accurately weighed into Eppendorf tubes and protein extracted with 1 ml of a solution containing 10 mM CAPS, 140 mM NaCl, 1% Tween 20, 4% bovine serum albumin (BSA), pH 11 (*15*). The suspensions were shaken end-over-end for 30 minutes, then centrifuged for 30 minutes at 19 000 *g* to separate aqueous and solid phases. Supernatant solution was removed, diluted as required and Cry proteins assayed using ELISA kits (Qualiplate Combo Kit for Cry1Ab/1Ac, Envirologix) following manufacturer's instructions. Low binding plastics (Eppendorf tubes and pipette tips) were used to handle solutions containing Cry.

3.2.5. Persistence of biopesticide Cry proteins under controlled laboratory conditions

Commercial Bt preparation was suspended in distilled water (50 g dm^{-3}). Ten g soil was weighed into Petri dishes and Bt suspension was sprayed onto the soils to give a moisture content of 20%, the amount of solution added was determined by weighing the Petri dishes. Moisture content was adjusted to 40% by pipetting distilled water onto the soils. The soils were incubated under the required conditions and weight checked daily and adjusted for moisture loss as required. The incubation variables were temperature (4°C, 25°C) in darkness or direct sunlight. At intervals, soil was sampled in 3 places from each sample to give composite samples of about 1 g soil from which 5 replicates of 0.1 g were weighed into Eppendorf tubes, protein was extracted and assayed as described above. All incubations were carried out in triplicate.

3.2.6. Persistence of purified Cry1Ac protein in soil under controlled laboratory conditions

Purified Cry1Ac was added to soil by pipetting solution onto soil in Eppendorf tubes. Four treatments, with 3 repetitions of each were made. Soil was either wetted directly with Cry solution, or prewetted with water 3 days prior to addition of Cry to allow a microbial flush to dissipate. Moisture content was adjusted to 40% with distilled water after addition of the required volume of Cry solution. Soils were incubated at either 25°C or 4°C. At the end of the required incubation period, samples were destructively sampled, extraction solution was added (to give a soil:solution ratio of 1:5), the suspension shaken then centrifuged (as above) and the Cry1Ac content assayed by ELISA detection.

3.2.7. Effect of nutrients and soluble soil components on biopesticide Cry proteins under controlled laboratory conditions

Detectable Cry protein from Bt commercial formulation was monitored in solution for up to 14 days at either 25 or 4°C. The solutions were either i) distilled water, ii) the nutrient solution used for Bt culture at three dilutions, 1:1, 1:10 or 1:100 or iii) an aqueous extract of the soil. The soil aqueous extract was obtained by shaking a suspension of soil (1g:10 ml) for 30 minutes end-over-end, then separating phases by centrifugation at 19 000 *g*. At the end of each incubation period, an aliquot of each solution was taken and Cry1 proteins were assayed by ELISA test.

3.3. Results

3.3.1. Persistence of commercial formulation of Bt crystal proteins in soil under natural conditions

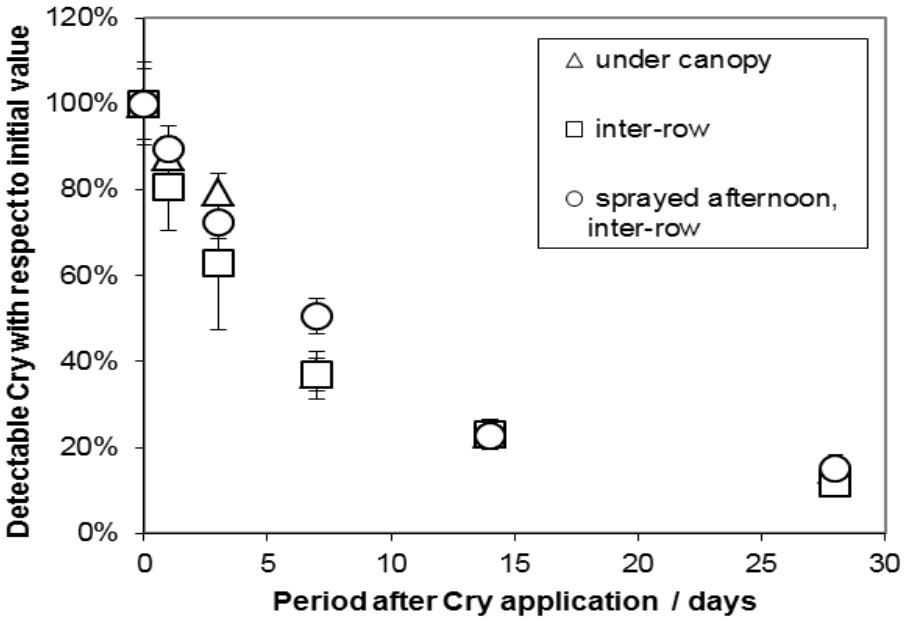

Figure 3.1. Persistence of Cry1A toxins from a commercial Bt formulated biopesticide under field conditions as a function of period after spraying, for three conditions of spraying-sampling. Average of three repetitions of spraying (Coefficient of variation about 15% between subsamples within each area sprayed and between areas sp

rayed). Error bars (not always visible) show variation between the three areas sprayed.

Soil samples collected after field spraying of the commercial preparations of HD-1 Bt formulated biopesticide were assayed. Soil was either collected under leaf canopy, spayed directly and sampled between rows, or was sprayed and sampled in the afternoon, between rows to give contrasting exposure to sunlight in comparison to the previous treatment. Average data for the three rows are shown in Figure 3.1 (the coefficient of variation was about 10%). The decrease of detection of Bt toxins in soils as given by anti-Cry1 ELISA tests was similar whatever the sample. There was no coherent effect of canopy protection from sunlight. Detectable Cry tended to be greater for afternoon sprayed soil for the first week, but this was not observed for each row and the effect was not significant when the full data set was considered and compared with either of the other treatments ($P > 0.05$). Similarly

detectable Cry was lower for the morning-sprayed inter-row samples than for the other treatments during the first week after spraying, but taking the full data set, the effect was not significant (P>0.05). The decline in detectable Cry1A followed approximately first order kinetics (although curvature in the log-linear plot of concentration *vs* time indicates that this is at best an approximate mathematical fitting procedure). The half-life of detectable protein was about one week by visual appraisal and calculated to be 9-10 days by linear regression after log transformation of data.

3.3.2. Persistence of commercial formulation of Bt crystal proteins in field soil under laboratory conditions

Figure 3.2 shows the decline in detectable Cry1A toxins (average of three repetitions) after application of biopesticides spray to replicate soil samples under laboratory conditions. At 25°C in the dark there was a slow, gradual decline with about 70% of the initially detectable protein remaining after one week. In some cases an increase in Cry was initially observed before a net decline. In contrast, at 4°C there was a fast initial decrease of detectable toxins, reaching less than 20% of the initial load after only one day followed by a slower decline. Sunlight accelerated the rate of decrease of detectable Cry1A toxins with respect to the soil maintained at 25°C in the dark. However, this effect was less than that of low temperature. Time dependence of detectable Cry was significantly different for each of the three treatments (P<0.05).

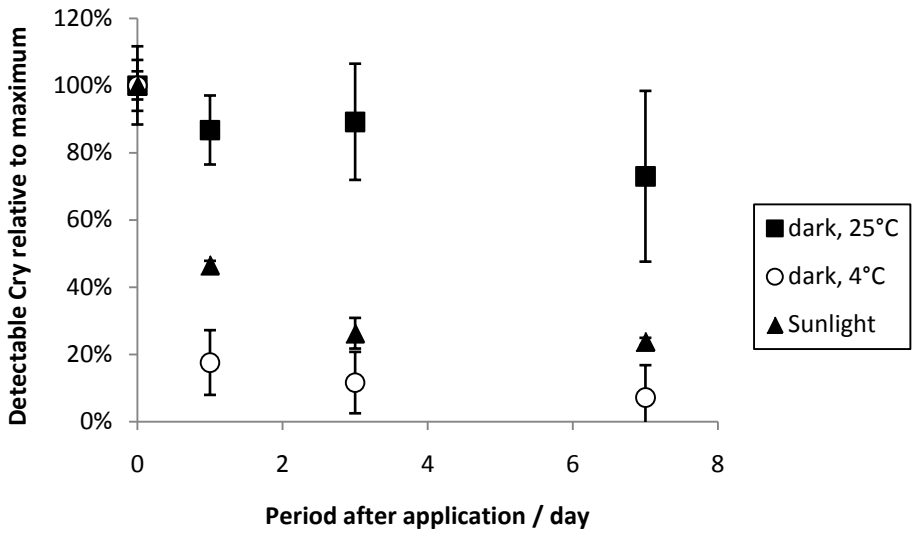

Figure 3.2. Persistence of Cry1A toxins from a commercial Bt formulated biopesticides in field soil under laboratory conditions as a function of period after application of spray with soil incubated at either 25°C or 4°C in the dark, or exposed to direct sunlight. Bars show variation between replicates

3.3.3. Effect of soil solution (SS) and nutrient solution (NS) on the persistence of Bt crystal proteins from the commercial formulation

Figure 3.3 shows the time dependence of detectable Cry incubated in various aqueous solutions at either 25°C or 4°C. At 4°C, the amount of detectable toxin remained fairly constant throughout the 14 days of the experiment, for all the solution compositions, although a small increase after day 3 was observed in presence of soil extract and nutrient solutions. Similarly at 25°C in water, there was no change in the amount of detectable Cry protein.

However, when maintained at 25°C in the presence of either nutrient solution or soil solution, the amount of detectable Cry1A toxins increased. In both 100% nutrient solution and soil solution the maximum Cry was detected after 1 week and then decreased by about 20% in the following week. The level of maximum detectable Cry was smaller and the time taken to reach this maximum was greater for Cry incubated in diluted nutrient solution. After 7 days, when the contrast between nutrient solutions was greatest, the concentration of Cry in 100% NS was 1.3 times that in 10% NS and 1.6 times that in 1% NS. The average rate of increase in detectable Cry was thus 30% less in 10% NS and 40% less in 1% NS than in 100% NS.

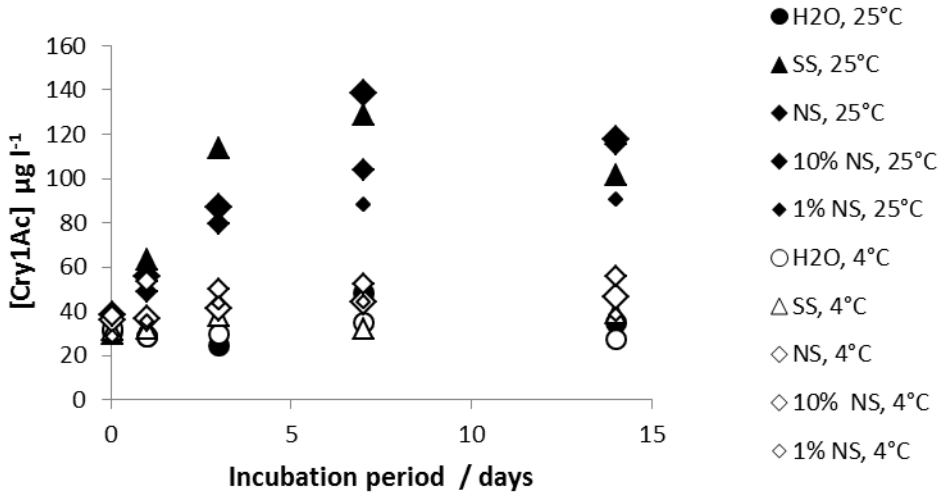

Figure 3.3. Effect of various aqueous solutions and temperature on the persistence of commercial formulated Bt crystal proteins (without soil) as a function of incubation period. The abbreviations in the legend refer to the composition of the solutions: H_2O: distilled water; SS: Soil solution; NS: nutrient solution; 10% NS: 10 fold-dilution of nutrient solution; 1% NS: 100-fold dilution of nutrient solution. Closed symbols - incubation at 25°C, open symbols – incubation at 4°C. Coefficients of variation between triplicates were about 7%, not shown for clarity.

3.3.4. Persistence of purified Cry1Ac toxin in soil under laboratory conditions

Figure 3.4 shows the results of the control experiment that monitored purified Cry1Ac toxin incubated with soil under similar conditions as the Bt biopesticides. The amount of detectable protein decreased rapidly at 25°C in contact with soil. However, prewetting the samples to induce a microbial flush prior to addition of Cry protein limited the decrease so that about twice the amount of Cry remained detectable in the prewet sample in comparison to the soil wet directly with Cry solution. The decline in the amount of purified Cry1Ac toxin was markedly less when incubated at 4°C, in comparison to 25°C. At 4°C, Cry only fell to about 60% of the initial value, in comparison to about 15% at 25°C. At low temperature, soil prewetting had no significant effect on the subsequent rate of decline of detectable Cry.

3.4. Discussion

There are very few studies of the persistence of Bt spores and proteins in the environment. This is largely due to the assumption that being a natural product, there is no danger associated with its use. Field observations indicate that insecticidal properties persist for a few days (*23, 24*), and this information is sufficient for users to time applications with respect to the presence of insects. Industrial research has aimed to optimize efficiency by protecting crystals from UV-light, improving the adhesion of the product to plant parts to minimize run-

off and optimizing spray storage and utilization (*5, 24*). Spores and the bacterium may survive in soil and water for weeks or even longer (*9-11, 25, 26*). One study has shown that the Cry1Ab protein from the commercial product Dipel ® remains detectable in soil for a few days (*27*). The paucity of data contrasts with the number of studies of the toxin produced by genetically modified (GM) crops in field studies and the purified protein in soil microcosms (*12* and references therein). These studies are prompted by the fears of exposure of non-target insects and the risk of acquisition of resistance by the exposure of target insects to non-lethal levels of the toxin. The longer the protein remains in the environment, the greater will be the probability of both undesired effects.

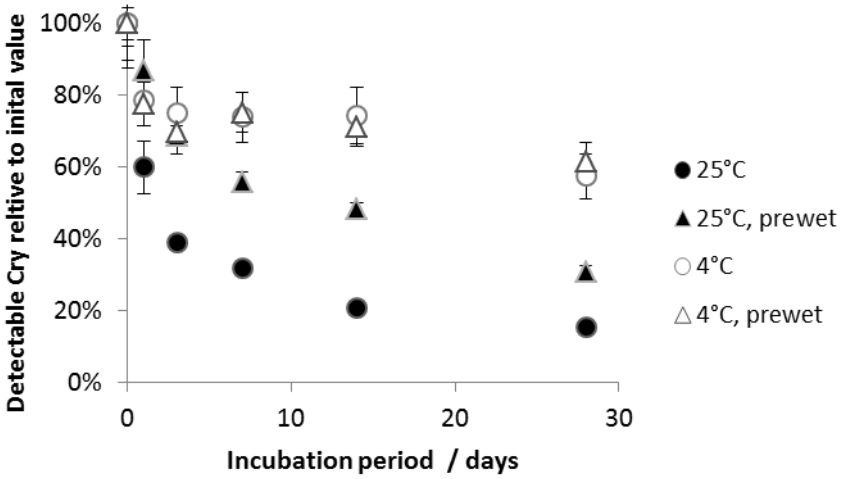

Figure 3.4. Persistence of purified Cry1Ac toxin in field soil under laboratory conditions as a function of period after addition of Cry solution, with or without prewetting of soil 3 days prior to Cry addition and incubation at either 25°C or 4°C. Bars show variation between triplicates.

In the present study, detectable Cry1A proteins from biopesticide decreased gradually with time under field conditions. The half-life was about one week and the protein remained detectable after one month, the maximum period of the trial. We are not aware of any similar field data with which these findings can be compared. However the kinetics of decline are markedly different to those usually reported for purified protein in soil microcosms or resulting from GM crops (*15, 28-30*). GM or purified Bt toxins in soil usually decline rapidly in the first few days and then more slowly over the following weeks, with half-lives of between less than one day and up to one week. This was the pattern observed for the present soil contaminated with purified Cry1Ac protein, incubated at 25°C. The difference did not therefore arise because of any inherent difference between the soils studied in this and other studies.

The comparison between the field study and the application of biopesticide under laboratory conditions (25°C in darkness) shows marked difference in the fate of Cry proteins. Under laboratory conditions in the dark, the level of detectable Cry decreased more slowly than in the field. It should be noted than the extraction method used would not only desorb protein from soil but would also solubilize any protein remaining in crystal form. Douville et al. (*27*) compared extraction of Cry1Ab from soil using extraction solutions at pH 7.4 and 10.5 and assumed that the former extracted only truncated protein whereas the later solubilized the protoxin. In fact, at alkaline pH both proteins would be solubilized/desorbed and the extraction yield of truncated Cry protein would be more efficient at pH 10.4 than at pH 7.4. In

a previous study we observed poor extraction yields at neutral pH, particularly in the absence of surfactants (*31*). This is in accordance with the very low extraction yields reported at pH 7.4 or by water (*27, 32*). The differences between laboratory and field conditions include temperature, sunlight and application conditions. In the field, suspension containing the Cry crystals could percolate in depth, allowing some dilution, whereas the layer of soil in the Petri dishes used in the laboratory trial was only about 5 mm thick. However this is unlikely to be the major cause of the observed difference since there was no rainfall in the first week of the field sampling. Preliminary studies showed that there was no effect of moisture content on the dynamics of either biopesticide or purified Cry under laboratory conditions suggesting that the difference was not due to the surface layer of the soil in the field being drier than for the laboratory study. Feng et al. (*29*) also report no effect of moisture content on the release of Cry1Ab from transgenic straw and its subsequent decline in soil.

Effect of sunlight on persistence. It is known that sunlight degrades Cry proteins (*24*). This was confirmed in the present study by the strong decrease in detectable protein in the laboratory experiment where soil was exposed to direct sunlight, whereas in soil kept in the dark at 25°C the decrease was slower. The greater effect of sunlight in the laboratory by comparison with conditions of shade in the field, suggests that in the field differences in sunlight intensity are not sufficient to cause differences in the rate of loss of detectable Cry, Protein content of soils sampled between rows of plants, exposed to more sunlight, relative to the initial content tended to be lower for the first week, but this was not observed when soil had been sprayed directly. There was no significant effect of spraying in the afternoon, to ensure a shorter and less intense exposure to sunlight during the first day, than under standard conditions when the spray was applied in the morning. The variability of the field study prevented the sunlight effect to be established. Other effects were stronger and dominated. The larger effect of sunlight in the laboratory conditions may be in part due to the shallow soil layer, 5 mm, affording less protection to Cry.

Effect of temperature on persistence in soil. Previous studies have shown that low incubation temperature, 4°C, of purified Cry1Aa toxin with four contrasting soils conserved protein more than incubation at 25°C (*15*). Two mechanisms for this temperature effect were considered to explain the observation. The first was that lower microbial activity at low temperature slowed microbial breakdown of the protein. However this was discounted because stimulation of microbial activity or inhibition by chemical methods or sterilization did not have marked effects. The other, preferred, hypothesis was that conformational changes of the adsorbed protein led to increasing fixation on the soil surface and hence decreasing extraction yield. We postulated that the fixation was dominated by hydrophobic interactions since they are known to decrease with decreasing temperature. Feng et al. (*29*) reported an increase in the rate of decline of Cry1B in soil with increasing temperature, but it is impossible to distinguish between the temperature effect on release of protein from straw and its subsequent degradation or fixation on soil. The same strong temperature effect was observed for purified protein on this soil, however the reverse effect, namely a more rapid decline was observed in the laboratory for biopesticide. One of the important differences between biopesticide and purified protein is the presence of spores as well as crystal protein in the former.

We postulate that the temperature effect for biopesticide in the laboratory is predominantly biological, with the possibility that spores could produce more protein, thus counteracting the decline that dominates the trend in the field. This hypothesis is strengthened by the fate of biopesticide in aqueous solutions which was designed to test the effect of temperature without the effect of adsorbing surfaces. Detectable biopesticide protein increased with time when

incubation conditions favored microbial development, namely at 25°C rather than at 4°C and in solutions containing nutrients, either aqueous soil extract or nutrient solution, rather than pure water. The temperature effect of soil and nutrient solutions on the increase in Cry concentration could be interpreted as the solubilization of crystals, but this would not explain the effect of nutrient solution concentration and the absence of an effect in water. Spore germination with the protein production occurring at 25°C in the presence of nutrients seems a more likely explanation. The nutrient solution contained bactopeptone which supplies L-alanine which is a very efficient germinant for *Bacillus* spore germination (*34*). Dilution of nutrient solution caused the increase in protein to be slower and the maximum value lower, which is consistent with bacterial growth. In the presence of the soil solid phase the increase would be tempered by protein adsorption, the activity of soil proteases and competition with other bacteria and so the net effect is a constant level of protein or a slow decline. When low temperature inhibited bacterial growth in the laboratory the decline in protein was rapid, as observed in the field. The fact that protein dynamics at low temperature are different for biopesticide and purified protein shows that the two processes do not have the same rate limiting factors. We postulate that the limiting factor for purified protein is the on-going fixation of protein on soil surfaces leading to increasing irreversibility of adsorption. For the biopesticide formulation, protein fixation is counteracted by an induction of the spore germination. The fact that the soil solution is as effective for this process as nutritive solution means that some soil solution compounds can act as germinants.

In conclusion, the persistence of Cry proteins in the field results from average conditions of sunlight and temperature. The rate of decline of detectable protein from biopesticide differs from that of purified protein due to additional processes of spore germination and probably the protective effects of commercial additives.

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CHAPTER 4.

PERSISTENCE OF DETECTABLE INSECTICIDE PROTEIN FROM *BACILLUS THURINGIENSIS* (CRY) AND TOXICITY AFTER ADSORPTION ON CONTRASTING SOILS

In Press, *Environmental Pollution*

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Abstract

Insecticidal Cry, or Bt, proteins are produced by the soil-endemic bacterium, *Bacillus thuringiensis* and some genetically modified crops. Their environmental fate depends on interactions with soil. Little is known about the toxicity of adsorbed proteins and the change in toxicity over time. We incubated Cry1Ac and Cry2A in contrasting soils subjected to different treatments to inhibit microbial activity. The toxin was chemically extracted and immunoassayed. *Manduca sexta* was the target insect for biotests. Extractable toxin decreased during incubation for up to four weeks. Toxicity of Cry1Ac was maintained in the adsorbed state, but lost after 2 weeks incubation at 25°C. The decline in extractable protein and toxicity were much slower at 4°C with no significant effect of soil sterilization. The major driving force for decline may be time-dependent fixation of adsorbed protein, leading to a decrease in the extraction yield in vitro, paralleled by decreasing solubilisation in the larval gut.

Keywords : Soil; adsorption; persistence; *Bacillus thuringiensis*; toxicity

Capsule

Toxicity was initially maintained after adsorption on soil and both extractable Cry and toxicity declined rapidly, more slowly at low temperature, due to different fixation dynamics.

Résumé étendu

Il est connu que les protéines Cry persistent pendant des mois dans le sol, mais à ce jour on ne sait pas quelles propriétés physico-chimiques, minéralogiques ou biologiques déterminent cette persistance. L'étude précédente a confirmé que les protéines Cry, malgré des différences entre elles, sont fortement adsorbées sur l'ensemble des sols. Le devenir des protéines Cry dépend donc de leur comportement à l'état adsorbé. L'adsorption est réputée protéger des protéines contre la dégradation microbienne et enzymatique, mais on ne connait pas la part relative des processus biotique et abiotique dans de devenir. Trois questions restent en suspens : (i) quelle est la nature du composé extrait du sol, une protéine entière ou des fragments partiellement dégradés, sans activité biologique ? (ii) quelles propriétés du sol déterminent le devenir des protéines adsorbées ? et(iii) est-ce que la protéine adsorbée reste potentiellement toxique ? L'objectif de cette partie de l'étude est de répondre à ces questions.
Il n'a pas été possible d'identifier des sols ayant des affinités très contrastées pour les protéines Cry. Les sols choisis pour cette étude avaient des teneurs en argile et en matière organique contrastées. Deux protéines Cry ont été choisies pour cette étude puisqu'elles sont produites par des plantes Bt actuellement cultivées à grande échelle et une des deux est toxique pour l'insecte cible idéal pour des biotests avec sol. Les protéines sont issues de culture bactérienne, activées et purifiées comme décrit dans l'annexe. Des microcosmes de sol avec Cry ont été incubés sous des conditions contrôlées en laboratoire. Pour explorer les parts relatives des processus physicochimiques et microbiologiques, nous avons fait varier lors de l'incubation du sol avec la Cry, la teneur en eau, la température et la stérilité du sol avec autoclavage répété. Les teneurs en Cry dans les sols ont été mesurées par immunochimie après extraction chimique des sols après des périodes d'incubation allant de 2 heures à 30 jours. Pour un petit nombre de conditions, la toxicité de Cry1Ac envers les larves d'insectes cibles a été mesurée. L'insecte cible est *Manduca sexta* qui présente l'avantage d'être de taille suffisante pour consommer du sol sans en être gêné et d'être relativement facile à élever et à manipuler. Vingt larves dans des compartiments individuels ont été nourries avec une alimentation artificielle avec ou sans Cry, adsorbée ou en solution. L'alimentation a été changée tous les jours et la mortalité suivie pendant 7 jours.
L'étude de persistance des protéines Cry1Ac et Cry2A à 25°C montre que les quantités de protéines décroissent rapidement au cours des premiers jours de contact, et plus lentement ensuite (Figure 4.1). Ce résultat s'accorde avec des observations déjà publiées pour quelques protéines Cry en contact avec un petit nombre de sols, de minéraux de référence et sont cohérentes avec des observations faites au champ. Les dynamiques de Cry différaient avec peu de différences entre les sols. Le déclin initial de Cry2A était moins rapide et le changement de vitesse de déclin un peu moins marqué que pour Cry1Ac. En ce qui concerne les variables choisis pour influer sur l'activité microbienne, la teneur en eau des sols lors de l'incubation n'a eu aucun effet significatif. De même, l'autoclavage des sols à trois reprises n'a pas changé la dynamique de Cry1AC ou de Cry2A dans les sols (Figure 4.2). Par contre, à température basse, les deux protéines ont été conservées plus longtemps dans chacun des sols. Sans l'absence des autres variables censées agir sur l'activité microbienne, et les conclusions d'une étude précédente sur Cry1Aa, il serait facile de conclure que ceci est une preuve du rôle déterminant des processus microbiens. Nous postulons au contraire que des interactions physicochimiques entre les protéines Cry et les surfaces organo-minérales des sols interviennent et dépendent de la température. Ces interactions pourraient être hydrophobes. Elles conduisent à une fixation progressive de la protéine et donc une baisse du rendement d'extraction.

Les biotests ont donné des résultats intéressants. Le constat le plus important est que Cry1Ac adsorbée sur un sol reste toxique. La toxicité ne variait pas significativement entre les trois sols, ayant des textures et des teneurs en carbone organique contrastées. Plus étonnant, la toxicité à l'état adsorbé semble être plus élevée qu'en solution. Pour que ceci soit vrai, il faudrait que l'adsorption accorde à la protéine une confirmation favorable à la toxicité qui serait maintenue lors de l'extraction et pendant l'ensemble des réactions dans le tractus intestinal de la larve. Nous avons constaté que les larves consommaient moins d'aliment quand il contenait la Cry, surtout sous forme soluble. L'explication plus plausible serait que la Cry soluble provoquerait une paralysie des mâchoires des larves menant à une cessation d'alimentation. Ce phénomène est connu pour des doses sub-létales de Cry, mais encore mal expliqué. Quand la Cry est adsorbée sur un sol, sachant que les conditions physicochimique dans la cavité buccale de la larve ne favorisent pas la désorption, la protéine reste adsorbée et incapable de produire cette réaction physiologique. La toxicité des Cry adsorbées requiert la solubilisation de la protéine dans l'intestin moyen de la larve. Le cocktail chimique utilisé pour l'extraction est à pH alcalin et contient un surfactant et une protéine, le rendant similaire, mais pas identique aux sucs digestifs des larves. La protéine adsorbée est toxique car elle demeure intacte à la surface du sol et peut être désorbée.

Quand le sol avait été incubé à 25°C avant le biotest, aucune toxicité n'était détectable. Soit la protéine avait été dégradée, soit elle était trop fortement adsorbée pour être solubilisé dans l'intestin des larves. Au contraire, quand le sol avait été incubé à 4°C, la toxicité avait diminué, mais la protéine était clairement encore toxique. Ce déclin de toxicité est en parfait accord avec le déclin de détection par extraction-immunochimie. Le facteur limitant pour la toxicité semble être le même que pour les tests ELISA. Ceci valide l'utilisation des tests ELISA pour quantifier les protéines Cry, avec toujours la limitation soulignée dans la section précédentes, qui fait que l'efficacité de l'extraction varie de façon non prévisible entre protéines et entre sols. Néanmoins le cocktail d'extraction utilisé semble refléter la capacité du milieu intestinal des larves à désorber la protéine des particules de sol.

La conclusion environnementale de ce travail est que les effets insecticidesdes protéines Cry décroissent en parallèle avec leur détectabilité immunochimique dans le sol. Il est possible alors que des organismes consomment du sol contaminé par Cry et soit ainsi exposés. Ceci concerne bien sur des organismes géophages, mais d'autres peuvent consommer le sol non intentionnellement, par exemple quand la pluie projette des particules de sol sur la couverture végétale. Si l'animal n'est pas sensible à Cry, cette consommation ne peut pas avoir un effet négatif. Si l'animal est sensible, la voie d'exposition par le sol se doit être prise en compte. Pour les organismes cible, la possibilité d'exposition via ce mécanisme pendant la période de décomposition des résidus de culture pourrait éventuellement créer une expositionsub-léthale qui contribuerait au développement de résistance

4.1. Introduction

The area of agricultural land planted with genetically modified (GM) crops has increased continually since their first commercialization in 1996. In 2013, about 430 Mha were planted with GM crops worldwide, of which about 200 Mha with crops containing the insecticidal Bt (*Bacillus thuringiensis*) trait, often in combination with the herbicide tolerance trait (James, 2013). While biopesticides containing spores and parasporal inclusion bodies of *Bacillus thuringiensis* have been used for vector control and in agriculture, including organic farming, for decades, the use of GM crops gives rise to environmental concern (Raymond et al., 2010; Sanchis, 2011). Proponents of Bt crops point to the increase in crop yield and quality, including decreases in mycotoxin levels of some crops (James, 2013; Wu, 2007). It is claimed that the strong similarities between the proteins produced by *Bacillus thuringiensis* that is an endemic soil-dwelling bacterium, and by Bt plants is a guarantee of safety (Sanchis, 2011; Shelton et al., 2002). Furthermore, the highly species-specific mechanism of toxicity of the proteins should preclude any adverse effects on non-target species. Nevertheless there are significant differences between GM and natural bacterial proteins.

There are thousands of known strains of *Bacillus thuringiensis* and each produces between one and five protein protoxins in parasporal bodies during sporulation. The proteins are called Cry proteins because of their resemblance to crystals. These proteins are stomach poisons, unlike chemical pesticides that are often contact poisons. They must be ingested to be toxic. Each protein has a very small range of target hosts. The mechanism of toxicity is a cascade of events, each of which contributes to the species specificity. To summarize the complex series of reactions, described in greater detail elsewhere (de Maagd et al., 2003; Schnepf et al., 1998) the protoxins produced by bacteria are solubilized in the alkaline midgut of target insects, then enzymatically cleaved by digestive proteases to form toxins, proteins with molecular masses of about 70 kDa. These toxins attach to specific binding sites in the insect midgut to form pores leading to cell lysis and death. Some Bt GM crops produce the toxin directly rather than the protoxin (Mendelsohn et al., 2003), and the protein is released into soil by root exudation and by turn-over of crop residues (Icoz and Stotzky, 2008).

Soil plays important roles in the fate of Cry proteins in the environment. Cry proteins, in common with other proteins are strongly adsorbed on soil organo-mineral surfaces (Chevallier et al., 2003; Crecchio and Stotzky, 2001; Helassa et al., 2009). Adsorption leads to immobilization and may confer some protection against microbial breakdown (Nannipieri et al., 1996; Quiquampoix and Burns, 2007). Both field and laboratory based soil microcosm experiments indicate that detectable Cry proteins decline rapidly in soil, but may remain detectable for weeks or months (Helassa et al., 2011; Herman et al., 2002; Hopkins and Gregorich, 2003; Marchetti et al., 2007; Palm et al., 1996). The period during which proteins may persist in soil has important consequences for the probability that adverse effects could occur. These adverse effects could be non-lethal exposure to target insects accelerating the acquisition of resistance, or the exposure of non-target insects. It would also be important for the purposes of environmental monitoring to predict how long residues of GM crops or contamination from neighbouring land could persist. The studies to date on the persistence of Cry proteins in soil have not identified the soil properties that determine persistence (Dubelman et al., 2005; Head et al., 2002; Shan et al., 2008). We recently showed that the rate of decline of detectable Cry1Aa in four contrasting soils did not depend on soil microbial activity (Helassa et al., 2011). We postulated that the observed decline was due to conformational changes in the adsorbed protein, possibly leading to decreasing extractability of the protein, prior to quantification by ELISA-detection. The slower rate of decline at low temperature (4°C rather than 25°C) agrees with the hypothesis that the driving forces were predominantly hydrophobic interactions between the protein and the organo-mineral surfaces. The importance of hydrophobic interactions between Cry1Aa and mineral surfaces was

illustrated by (Janot et al., 2010), although only electrostatic forces were identified in the interaction of Cry1Ab and quartz (Sander et al., 2010).There is some evidence that insecticidal properties are conserved after adsorption, but there are relatively few data available (Crecchio and Stotzky, 2001; Madliger et al., 2011). Most studies rely on chemical extraction of the protein from soil followed by quantification using ELISA assays. Bioassays using target insects are less frequent since they are time and labour consuming, and results may be highly variable due to biological variability and differences in feeding patterns. There have been few studies of the time dependence of the toxicity of adsorbed Cry proteins, one exception being the study of Tapp and Stotzky (1998) who found that the toxicity of Cry proteins adsorbed on soil decreased with increasing incubation period.

The aims of this study were to attempt to establish the relative importance of biological and physicochemical factors in the determination of the decline of detectable Cry proteins in soils, to clarify if adsorbed protein maintains its insecticidal properties and to identify the soil properties that determine the fate of Cry proteins in soil. We compared two contrasting Cry proteins, Cry1Ac and Cry2A, both present in commercial GM crops. The extractable proteins were monitored in three soils with contrasting organic matter content and texture/mineralogy for up to one month. The microbiological factors were varied in three ways: (i) different moisture contents, (ii) incubation at either 25°C or 4°C and (iii) sterilization by autoclaving or not. The temperature difference was also chosen since chemical interactions may be temperature dependent. In particular, hydrophobic interactions, which we postulate play important roles in the fixation of the adsorbed protein, are entropy-led and hence decrease with decreasing temperature. The change in toxicity of Cry1Ac was also monitored as a function of the period and the temperature of incubation with each of the soils using the target insect, *Manduca sexta*, at the larval stage.

4.2. Materials and methods

4.2.1. Preparation of purified Bt toxins

A natural strain of *B. thuringiensis* subsp. *kurstaki* HD-74 producing only Cry1Ac protoxin, and a genetically modified strain of *B. thuringiensis* producing only Cry2A protoxin provided by CIRAD, Montpellier, France were cultivated in 500 ml Erlenmeyer flasks containing 100 ml autoclaved Nutrient Broth (containing erythromycin for the GM strain) + 4 ml of 25% glucose sterilized by filtration to each Erlenmeyer flasks + 1 ml of inoculum for each Erlenmeyer flasks, shaken at 400 rpm, 28°C until sporulation (about 48 hours). The medium was centrifuged at 8000 *g* at 4°C and the pellets containing spores and crystals resuspended and shaken in 300 ml of 1 MNaCl to eliminate spores in the foam formed. The remaining solution was centrifuged at 8000 *g* at 4°C for 15 minutes. The pellets were resuspended in distilled water and ultrasonicated for 1 minute in ice. After centrifugation the protoxin was solubilized in 50 mM Na_2CO_3 and 10 mMdithiothreitol (DTT) for 1 hour at 37°C, then centrifuged for 30 minutes at 15 000 *g* at 4°C and filtered at 0.45µm. This protoxin was activated using trypsin for 45 minutes, 37°C at pH 8. Activation was stopped with 1mM phenylmethanesulfonylfluoride (PMSF) and was centrifuged for 30 minutes at 15 000 *g* at 4°C and filtered to 0.2 µm. The activated protein was purified using an anion exchanger (Q-HP Sepharose, Amersham) on an AKTA purifier (Amersham Pharmacia Biotech) at pH 10.4, and the toxin was eluted by a NaCl gradient ranging from 0 to 1 M. The stock solution of purified toxin in CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid) 10 mM, NaCl 350 mM, pH 10.4, was stored for short periods at 4°C or for longer periods at -20°C.

4.2.2. Soils and soil treatments

Three soils from Southern France were used for this study, a woodland clayey soil, Cazevieille (Cz), containing 31 g kg^{-1} organic carbon with a pH of 6.7 and two silty clay

vineyard samples from Cruscades with differing organic matter content due to 17 years of organic farming or conventional management, containing 16 (Cc_{org}) and 7.2 (Cc_{con}) g kg^{-1} organic carbon respectively and with a pH of 8.3. Each soil sample was air-dried and sieved < 200 µm. When required, soils were sterilized by autoclaving (20 minutes at 121°C, followed by air-drying) three times at 24-hour intervals.

4.2.3. Soil microcosm experiments

Immediately prior to the adsorption experiments (to avoid polymerization) the background electrolyte of the stock solution containing either Cry1Ac or Cry 2A was diluted and exchanged with 5 mM $Ca(NO_3)_2$ solution by repeated centrifugation and solution addition using an Amicon Ultra - 15 filter devices. The pH of solution was verified and taken as proof that storage buffer at pH 10.5 had been removed. For each treatment and incubation period, triplicate microcosms were prepared by weighing soil into low-binding Eppendorf tubes then adding the appropriate volume of either protein solution to give gravimetric moisture contents of 20%, 40% or 60% for Cry1Ac, and 40% for Cry2A. Microcosms were sealed and incubated at either 25°C or 4°C in the dark. After the required incubation period, microcosms were destructively sampled by chemical desorption. The desorption solution contained 10 mM CAPS, 140 mMNaCl, 1% Tween 20, 4% bovine serum albumin, at pH 11(Helassa et al., 2011). The soil:solution ratio was 1:20 and suspensions were shaken end over end at 25°C for 30 minutes, then centrifuged at 19 000 *g* for 10 minutes and supernatant solution removed for analysis, after appropriate dilution. Both proteins were analysed using ELISA kits purchased from Envirologix following the manufacturer's instructions. Assays of Cry1Ac were found to give a linear response in the range 1-15 µg dm^{-3} and Cry2A in the range 10-200 µg dm^{-3}. Tubes and pipette tips were always "low-binding" to prevent loss of protein during manipulations. ELISA determinations were calibrated by comparison with the optical density at 280 nm. Each sample was analysed after at least two different dilutions to ensure that the concentration was within the linear range. Preliminary experiments showed that adsorption was strong and that protein remaining in soil solution was negligible.

4.2.4. Bioassays

Bioassays were performed using the target insect *Manduca sexta* at the L3 larval stage. Eggs were purchased from the University of Bath, UK. The eggs were hatched and the larvae reared in plastic boxes containing moist absorbent paper and Poitou artificial feed and maintained under controlled conditions, 60% humidity, 25°C, 12-hour day-night cycle. Poitou feed was composed of corn flour, yeast extract, wheat germ and agar and supplied by N. Volkoff, LDGIM, INRA-University of Montpellier. Boxes were cleaned and feed replaced every 2 days. For the bioassays, larvae were placed in individual compartments of Perspex boxes and presented with about 1 g of artificial feed that had been mixed with Cry (200 ng) or not (control). Cry was added either in solution or previously adsorbed on soil (5 % soil by weight in feed) as required. The soil used for the bioassays was either contaminated with Cry immediately prior to mixing with feed (t=0) or incubated for 14 days at either 25 or 4°C prior to mixing with feed. Preliminary experiments had determined that larvae fed and grew normally with feed containing this proportion of soil. This amount of toxin was chosen so as not to be in large excess of the toxic dose so that decreases would be detectable. Preliminary experiments had shown that Cry1Ac was toxic at half this dose but that less than 10% mortality was observed at a quarter of the dose (50 ng Cry1Ac). The same number of repetitions was made for feed containing neither soil nor Cry. The number of larvae used for each treatment was 20 and each assay carried out three times. Feed was changed daily and consumption noted. Mortality was monitored for 7 days and expressed as the cumulative mortality.

4.2.5. Data treatment

Detectable Cry protein was expressed as a percentage of that extracted and detected after 2-hour contact period with soil. Time trends of detectable Cry in soil were non-linear and attempts to linearize the data gave either poor fits or gave unequal weight to individual time points and had no mechanistic justification. We therefore preferred pair-wise comparison of data sets for each treatment or soil. The deviations of the gradients of the correlations obtained from unity is given as the difference in overall rates of decline for the two treatments or soils and the P values of the linear regression gives the significance of the difference. Since coefficients of variation between replicate treatments were about 5%, only differences greater than 5% were considered to be significant. Mortality in the bioassays was expressed as the number of larvae dead after 7 consecutive days of exposure to Cry (or no Cry for the controls) expressed as a percentage of the number of larvae in each treatment (20). Treatments were compared using a one-way ANOVA followed by Tukey-Kramer method using Excel software.

4.3. Results

4.3.1. Toxin affinity for soils

The affinity of protein for soil organo-mineral surfaces was assessed, by the value of the distribution coefficient, Kd, defined as the ratio of concentrations in the adsorbed state and that remaining in solution after 2 hours of contact between protein and soil. The Kd values of Cry2A were greater than those of Cry1Ac for the vineyard soils, but all were in the range reported by Hung et al.(2015)) for a large number of contrasting soils. The Kd values measured for Cry1Ac were very similar for all the soils studied, $1.6x10^4$ dm^3 kg^{-1} for the clayey woodland soil (Cz) and 1.3 and $1.5x\ 10^4$ dm^3 kg^{-1} for the silty clay soils under organic (Cc_{org}) and conventional management (Cc_{con}), respectively. In contrast, much larger differences were measured between soils for Cry2A, with Kd being 1.5×10^4 dm^3 kg^{-1} for Cz and 3.5 and 2.7×10^4 dm^3 kg^{-1} for Cc_{org} and Cc_{con} respectively. Extraction yield was about 80% for Cry2A in each of the soils, but for Cry1Ac varied between 47% for Cz and about 64% from Cc_{org} and Cc_{con}). These extraction yields are in the range reported by Hung et al. (2015)) for a larger group of soils.

4.3.2. Persistence of Cry toxins in different types of soil

The persistence of Cry1Ac followed similar trends in each of the three soils (Figures 4.1 a-c). When incubated at 25°C, ELISA-detectable Cry1Ac decreased rapidly, with about a 50% loss in the first 24-h period and a decrease to about 20% of the initial level over the first seven days. The decrease of the amount of ELISA-detectable toxin continued at a lower rate until day 28 to reach the level of 10-15% of the initial level. The type of soil did not affect the dynamics of protein persistence (Figures 4.1 a-c). The soil had small effects on the time-trend of Cry1Ac detection. Pairwise linear regressions of detectable Cry1Ac at each moisture content showed small differences (5-8 %) between soils, with decline increasing in the order $Cc_{org}<Cz<Cc_{con}$, but only differences between Cc_{con} and the other two soils were significant.

When soils were incubated at 4°C there was less contrast between the rates of initial and subsequent decline than at 25°C. A greater proportion of detectable Cry1Ac was conserved at 4°C than at 25°C. About 60% of initially detectable Cry1Ac remained for the woodland soil at the end of the 28-day incubation, and about 40% for the two vineyard soils with contrasting organic matter contents, in comparison to about 10% at 25°C. Data at 4°C gave a better fit to first order kinetics, which are often used to describe such data. Although there is no mechanistic origin for this fit, it gives a numerical basis for the calculation of a half-life. This

half-life was calculated to be 36 days for the clayey woodland soil and 18 and 24 days for the organic and conventionally managed vineyard silty clay soils.
Similar trends were observed for the toxin Cry2A (Figures 4.1d, 4.1e, 4.1f), namely a rapid initial decrease in detectable protein at 25°C, followed by a slower decrease. Pairwise comparison of soils incubated at 25°C showed no difference in the rate of decline of Cry between the two vineyard soils, but a slightly slower decline (8%) in the clayey woodland soil (Cz). As for Cry1Ac, the temperature had a major effect on persistence. At 4°C the percentage of detectable protein declined gradually and did not show the sharp initial decline observed at 25°C and so could be fitted to a first order decline. The calculated half-lives were between 11 and 12 days.

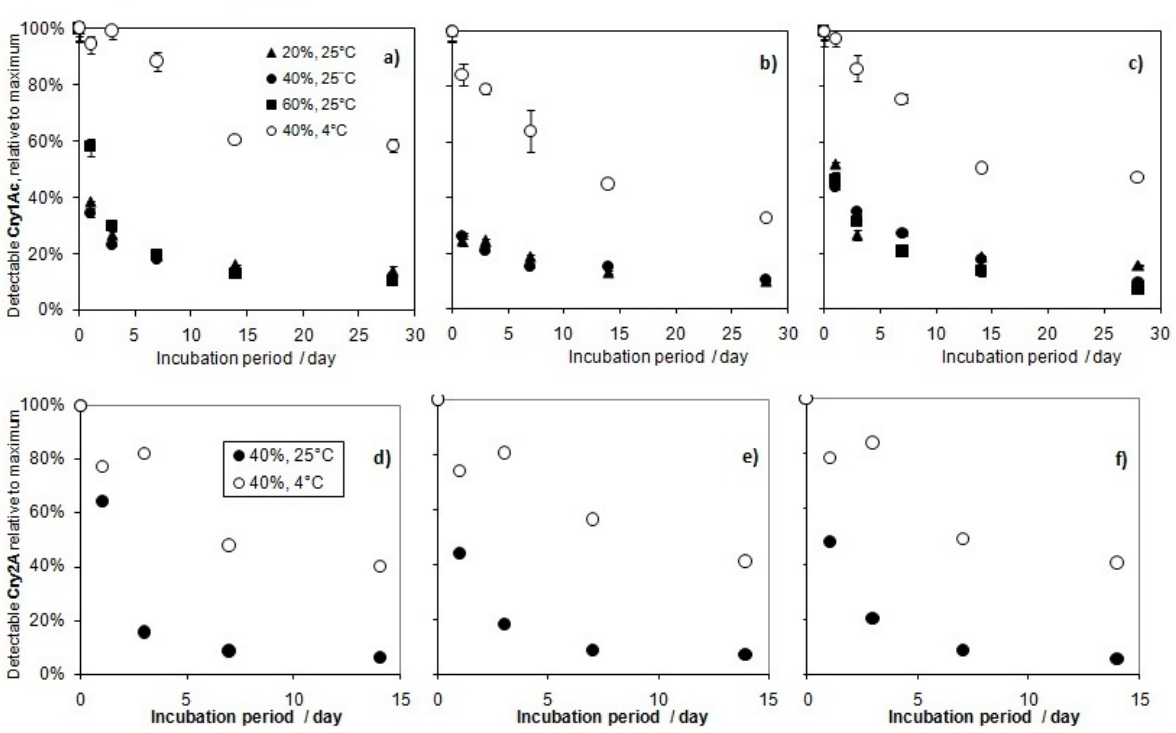

Figure 4.1. Detectable Cry protein, relative to that after 1-h contact with soil, as a function of incubation period in soil for soils incubated at either 25°C or 4°C and for 25°C, at various moisture contents (20%, 40% and 60%). a) – c) Cry1Ac; d) – f) Cry2A. a) & d) - Soil Cz; b) & e) - Soil Cc_{org} and c) & f) - Soil Cc_{con}. Vertical bars (not always visible) show the variation between triplicates.

4.3.3. Effect of the soil microbial activity on the persistence of Cry toxin

The first variable used to influence microbial activity was moisture content (for incubation of Cry1Ac at 25°C); Pairwise linear regressions of detectable protein confirmed visual appraisal (Figures 4.1a-4.1c) that there was no significant difference on the decline of detectable Cry between moisture content treatments for any of the soils. The second variable used to influence microbial activity was incubation temperature. Temperature had a large and significant effect on the persistence of both Cry1Ac (Figures 4.1a-4.1c) and Cry2A (Figures 4.1d-4.1f). For each soil and for both proteins, the decline of extractable-detectable Cry was slower at 4°C. The initial sharp decrease observed at 25°C was not present and the decline was more gradual. Finally, sterilization by repeated autoclaving (Figure 4.2) had no significant effect on the observed decline of Cry1Ac (no significant difference of detectable protein between the correlation between sterilized and non-sterilized soils). For Cry2A sterilization had no effect on the decline of detectable protein incubated at 25°C, however at

4°C the decline was markedly slower due to a short time lag (0-3 days) in the onset of decline in sterilized soils, leading to detectable protein content being 7% greater in sterilized soil than in non-sterilized soil over the 14-day monitoring period. The marked temperature difference was still significant after sterilization for both Cry1Ac and Cry2A (comparison of Figures 4.1 and 4.2).

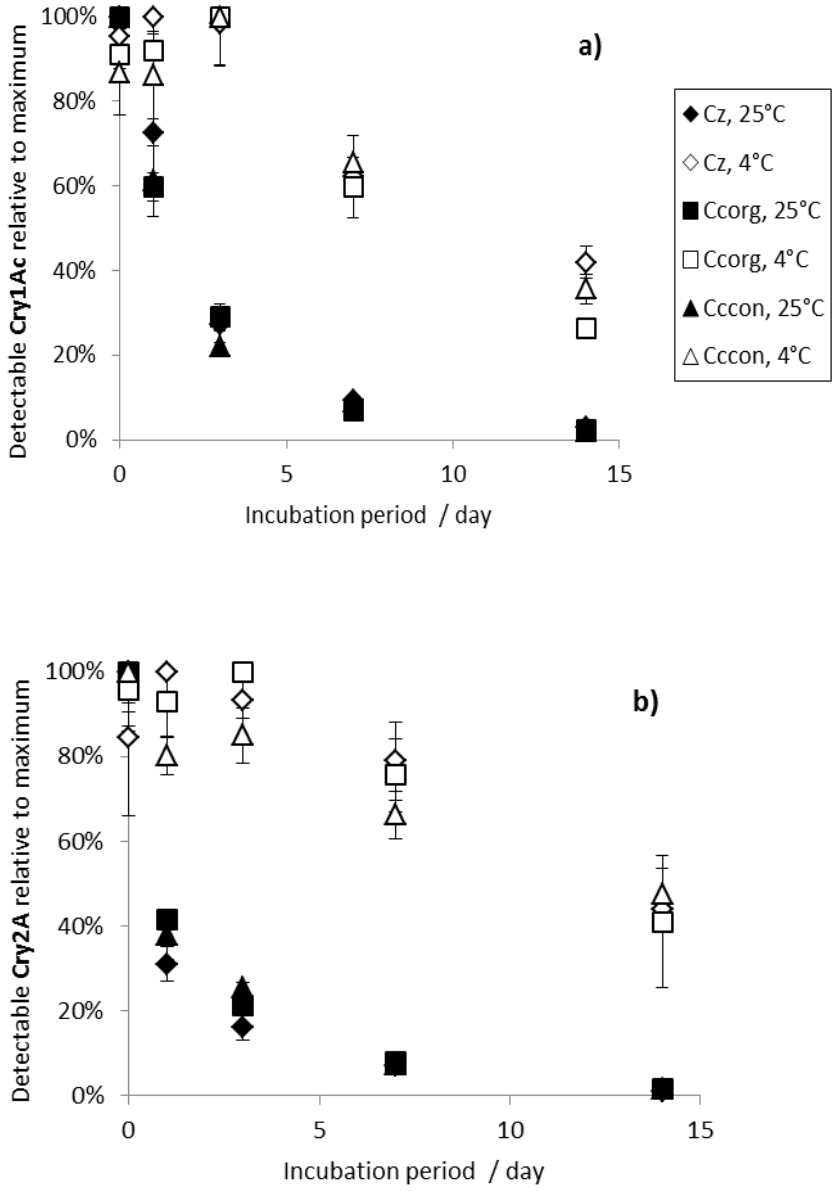

Figure 4.2. Detectable Cry protein, relative to that after 1-h contact with soil, as a function of incubation period in soil at either 25°C or 4°C for soils that were sterilised by repeated autoclaving. a) Cry1Ac and b) Cry2A. Vertical bars (not always visible) show the variation between triplicates.

4.3.4. Insecticidal activity of soil-adsorbed Cry toxins

Bioassays were conducted using *Manduca sexta* larvae as target insects to ascertain if toxicity was maintained in the adsorbed state and if so how it declined with respect to immunodetectable protein. Data are summarized in Figure 4.3.There was a time lag of 2 days

before mortality was observed. When Cry1Ac adsorbed on soil immediately prior to the bioassay (no incubation) the observed mortality after the cut-off point of seven days incubation was about 55%, with no significant difference (P>0.05) between soils. These mortality rates were significantly (P<0.05) greater than for soluble Cry (22%). We noted a parallel marked decrease in the amount of feed consumed between the control and feed+Cry. After two weeks of incubation of Cry1Ac with soils at 4°C, the respective mortality for woodland, organic culture and conventional culture soils was 40%, 21.5% and 28.5% at the cut-off point of seven days (Figure 4.3). After two weeks of incubation of Cry1Ac with soil at 25°C prior to the bioassay, no mortality was observed after seven days for woodland and conventional vineyard soils and a single larva died when exposed to the organic vineyard soil, giving a mortality of 3.3%. Mortality of larvae when exposed to soils incubated at 25°C was not significantly different to the controls (P>0.05). No mortality was observed in the Cry-free control after seven days of bioassay.

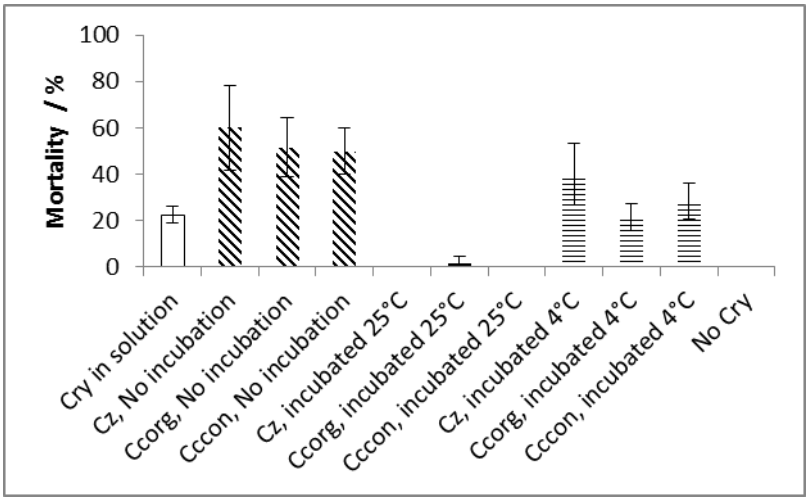

Figure 4.3. Average mortality after 7 days of target insect larvae, *Manduca sexta*, induced by Cry1Ac in solution or adsorbed on each of the soils with no incubation, adsorbed then incubated at either 25°C or 4°C for two weeks and a control with no Cry. Each assay was carried out in triplicate using 20 individuals for each treatment, and error bars show the standard deviation between replicate assays.

4.4. Discussion

4.4.1. Comparison of affinity for soils and extractability of Cry proteins

The affinities and the extraction yields of both proteins were in the range reported by Hung et al. (2015) for a large number of soils with contrasting properties. The similarity of both affinity and extractability of each of the proteins for the vineyard soils suggests that the organic matter content has little effect on the interaction between protein and soil. It is impossible to ascertain if differences in extractability were due to fixation, namely irreversible adsorption, or due to changes in the protein during adsorption or desorption that rendered it undetectable by the immunochemical test. However, ELISA tests may react positively to fragments of the original protein, so the former explanation appears more plausible. The extraction cocktail used had been found to give very similar extraction yields for Cry1Aa for contrasting soils of about 60% (Helassa et al., 2011), but showed more variability when tested for Cry1Ac, Cry2A and Cry1C against a larger number of soils (Hung et al., 2015). Although it is not intended to mimic the chemical conditions inside the larval midgut, the high pH, presence of surfactants and proteins resemble the conditions found in insect digestive systems

(Shan et al., 2005). This has important implications for the comparison of immunodetection and toxicity of adsorbed protein.

4.4.2. Dynamics of protein extraction-detection

The initial rapid decline in Cry followed by a more gradual decline observed for all soils at 25°C is similar to the trends previously reported for various Cry proteins (Cry1Aa, Cry1Ab and Cry3Bb1) (Helassa et al., 2011; Palm et al., 1996; Sims and Holden, 1996; Wang et al., 2006; Zwahlen et al., 2003). In contrast, Li and co-workers observed a more gradual decline that could be described by first order kinetics, for Cry1Ac in water, soil and sediment (Li et al., 2013). Although it is convenient to calculate half-lives, data usually give rather poor fits to first-order kinetics, particularly due to the sharp decline in the first days, as is the case for this study. Some authors have used empirical kinetic fits, such as a shift-log transformation, essentially an Elovich equation (Li et al., 2013), but this gives undue mathematical weight to the first point. For the present data, the time for 50% loss was about one day for Cry1Ac on each of the soils. The decline was more gradual, without a marked change in rate for Cry2A, although the 50% loss period was of a similar order, between one and three days. The fraction of detectable Cry1Ac was about 10% after one month, whereas for Cry2A a similar loss was observed in only 2 weeks. There were small differences between the rates of decline between soils, but differences were not large, and do not indicate a strong effect of soil chemical composition, nor microbiological activity. There was no significant effect of soil moisture content on the decline of Cry1Ac in any of the soils. It is well known that microbial activity decreases with decreasing soil moisture content (Davidson et al., 2000) and this was the reason for including this variable among the treatments. This accords with the conclusion of a previous study namely that microbial activity is not a major driving force in the decline of measureable Cry in soil (Helassa et al., 2011). Only one previous study has considered the effect of soil moisture content on the dynamics of Cry proteins and no effect of moisture content was observed (Feng et al., 2011).

Our previous study of Cry1Aa on four contrasting soils noted a marked effect of incubation temperature and concluded that the effect was not due to the temperature dependence of microbial activity (Helassa et al., 2011). In agreement with that previous study, the rate of decline was markedly slower at 4°C than at 25°C, for both proteins and for each of the soils. At 4°C, half-lives of Cry2A were about one week and those of Cry1Ac about two weeks on silty soils, Cc_{org} and Cc_{con}, and even longer, about one month, for the clayey, woodland soil, Cz. Li et al. observed small increases in the half-live of Cry1Ac in soil and sediment with sterility and decreasing temperature (34°C, 24°C and 4°C), but the temperature effect was much smaller than in our studies (Li et al., 2013). Temperature is classically varied in order to manipulate microbial activity and so it would be easy to conclude that the observed temperature effect was caused by a suppression of microbial activity at 4°C. However, the temperature effect was observed for sterilized and non-sterilized soils for each of the proteins and soils. Helassa and co-workers showed that in contrast to temperature, various treatments that either enhanced or suppressed microbial activity or extracellular protease activity had no significant effects on the dynamics of Cry1Aa in soil. This leads us to conclude that the driving forces underlying the decline in detectable Cry are not microbial in origin, but are more probably due to conformational changes of the protein, induced by interactions with soil organo-mineral surfaces. These conformational changes could result in fixation of the protein, hence a decrease in the extraction yield. They might also irreversibly modify the zone of the protein detected by the immunochemical assay, leading to a decrease in detectability of the desorbed protein in solution. It should be added that autoclaving has variable effects on the catalytic activity of extracellular enzymes in soil (Carter et al., 2007) and so it is possible that the protease activity of the soils was unaffected by sterilization and

that the temperature effect was, at least in part, due to slower catalytic activity at 4°C than at 25°C. However, in a previous study we noted no difference in the rate of decline of Cry1Aa with the addition of protease inhibitors (Helassa et al., 2011).

4.4.3. Dynamics of toxicity of adsorbed protein

Another question left unanswered by previous studies is the insecticidal activity of adsorbed Cry proteins. A small number of studies have suggested that adsorbed Cry proteins retain insecticidal activity when adsorbed on clay minerals (Crecchio and Stotzky, 1998; Lee et al., 2003; Madliger et al., 2011; Saxena and Stotzky, 2000). The major disadvantages of bioassays are that they are time- and labour consuming and that biological variability often leads to highly variable results, with large coefficients of variability or confidence limits. Stotzky and co-workers have suggested that toxicity may even be enhanced when toxin is adsorbed on clay or clay-organic matter complexes (Crecchio and Stotzky, 1998; Saxena and Stotzky, 2000; Stotzky, 2004). Madliger et al. observed no mortality of a target insect caused by 1 to 65 ng Cry1Ab g^{-1} diet and therefore used growth inhibition as a measure of toxicity and concluded that toxicity was maintained when adsorbed on silica particles (Madliger et al., 2011). The absence of mortality suggests that the dose was sub-lethal and the growth limiting effect could be due to cessation of feeding caused by paralysis of the mandibles. This is well known but poorly understood effect (Raymond et al., 2010).

In the present study the biological variability was reduced by selecting larvae at the same stage and of similar size, and most importantly by exposing them to Cry-contaminated feed individually. Preliminary experiments showed that 100 and 200 ng of Cry added to feed induced mortality in excess of 50%, whereas a lower level, 50 ng led to little mortality, therefore the level of 200 ng was chosen for bioassays. Preliminary experiments also showed that the larvae fed and grew normally when presented with feed containing up to 5% soil, as long as soil was homogeneously mixed with the jelly-like feed. If Cry is applied as a surface layer on top of feed, as described by (Tapp and Stotzky, 1995), larvae may avoid soil and hence not be exposed to the toxin. Mortality appeared to be greater when the Cry was adsorbed on soil, for each of the soils in comparison with the Cry in solution (Figure 4.3). However the larvae consumed less feed when the feed contained Cry without soil. Larvae consumed all the non-contaminated feed (1 g day^{-1}) but consumed much less Cry-contaminated feed. There were difference in the feed consumption when Cry was added in soluble form or adsorbed on soil: about 20% feed was consumed when Cry was added in soluble form and significantly more, 27% when Cry was adsorbed on soil (with no significant difference between soils), Consumption was variable, and the difference between soluble and soil-adsorbed Cry does not appear sufficient to account for the apparent increase in toxicity. There are cases where biological activity of proteins is enhanced in an immobilised state, but this is usually observed for membrane proteins that attain their active conformation in an immobilized state. Since *B. thuringiensis* does not produce Cry proteins to be active in soil, it seems unlikely that their active conformation would be favoured after adsorption on soil interfaces. We suggest that toxicity may not have been enhanced by adsorption, but rather that larvae consumed less feed when it contained soluble Cry than when Cry was adsorbed on soil. This could result from either (i) greater aversion to soluble Cry-contaminated feed leading to a larger decrease in consumption of feed, or (ii) less marked mandible paralysis and hence less cessation of feeding for adsorbed Cry with respect to soluble Cry in feed. The latter explanation implies that Cry must be in solute form to be biologically active, either to induce paralysis of larvae mandibles or inducing toxicity; adsorbed Cry would not be solubilized in the insect mouth, but could be desorbed in the alkaline solution of the midgut.

No significant difference in mortality of adsorbed Cry was observed between soils when the contact period between Cry and soil was short (No incubation), ($P<0.05$). When the Cry was

incubated with the soil at 25°C for 2 weeks prior to exposure to the larvae, toxicity disappeared, namely there was no significant difference between the toxicity of these soil+Cry mixtures and the control containing no Cry ($P<0.05$). However Cry incubated at 4°C after adsorption on soil retained some toxicity. On average toxicity decreased nearly two-fold from 54±5% to 30±10%. There was no significant difference in toxicities between soils ($P<0.05$). However the decrease in toxicity due to incubation at 4°C was only 50% in the clayey soil, Cz, and was not significantly different ($P<0.05$) whereas in the silty clay soils there was a significant 2-fold decrease.

4.4.4. Environmental implications of fate of adsorbed Cry proteins

Since the persistence of Cry proteins in soil or in the environment is more likely to be monitored by chemical extraction followed by immunodetection rather than bioassays, it is important to verify if the apparent trends in persistence are the same when monitored by both approaches. Only Cry1Ac was used for the bioassays, since *Manduca sexta* is not sensitive to Cry2A and we wished to use a large insect to ensure that soil particles could be consumed. When Cry-contaminated soil was incubated at 25°C mortality fell to that of the negative control, for each of the soils. In marked contrast, after incubation of Cry with soil at 4°C toxicity was reduced, but only by 30-50%. Toxicity therefore followed the same trends and most importantly showed the same temperature effect as Cry immunodetection following chemical extraction. It is reasonable to suppose that protein must be in a soluble form to induce toxicity, since the mechanism of pore formation in insect midgut involves the reaction with specific receptors and the formation of oligomers (Crickmore, 2005). Since the extraction buffer we used resembles the chemical conditions in the midgut, it is likely that the extraction efficiencies are similar *in vitro* and *in vivo*(Shan et al., 2005). It is thus not possible to distinguish between chemical fixation and conformational changes that reduce toxicity, either in bioassays or by immunodetection. However the important conclusion to this study is that controlled incubation followed by extraction and immunodetection give the same result: protein is maintained in a biologically active form when adsorbed on soil and that activity decreases with time faster at 25°C than at 4°C. Under environmental conditions, average temperatures would be intermediate and therefore the half-life of the toxin between one day (25°C) and two to four weeks (4°C), independently of the soil composition.

The persistence of detectable protein has important implications for soil monitoring. The rapid initial decrease confirms that Cry resulting from the culture of GM crops would not accumulate in soil and that the time trend of detectable protein would depend more on its release from growing crops and from the breakdown of crop residues than on the fate of the protein once adsorbed on soil. However the fact that the adsorbed protein retains its toxic properties means that target insects could be exposed to non-lethal levels of the toxin, thereby increasing the probability of the acquisition of resistance. In principle target insects are not geophagous, however after rain events or irrigation, soil particles are deposited by splash-back on leaves and so soil is unintentionally consumed. Non target animals would also be exposed to Cry in soil as well as in crop residues. Although exposure does not imply toxicity because of the highly specific mechanisms of toxicity of Cry proteins, the persistence of Cry proteins in soil increases the potential risk of sensitive, non-target animals being affected.

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GENERAL CONCLUSIONS

The objective of this thesis was to explore the possible impact of Cry toxins in the environment *via* the study of the adsorption and the persistence of purified Cry protein and Bt biopesticide in soil, including the toxicity of adsorbed Cry in soil. In this thesis, we have tried to elucidate some of the scientific questions that remain unanswered for Cry proteins.

Firstly, there have been few studies of the effect of soil properties on the adsorption – desorption properties of Cry proteins. No previous studies of the interaction of Cry proteins with soil have compared different Cry proteins. A few studies have focused on the extraction efficiency of various chemical extraction methods, but only for a small number of soils and usually for a single Cry protein.

Secondly, there have been no studies of the time dependence of the toxicity of adsorbed Cry proteins.

Thirdly, there have been very few studies of environmental fate of Bt toxin from biopesticide, and the persistence of biopesticide has never been compared to that of purified Cry protein used to mimic the fate of protein from genetically modified crops.

We have carried out studies of the affinity and extraction yield of three Cry proteins namely Cry1Ac, Cry 1C and Cry 2A for various soil types. We then studied we focussed on the relative importance of biological and physicochemical factors in the determination of the decline of detectable Cry proteins in soils, to clarify if adsorbed protein maintains its insecticidal properties and to identify the soil properties that determine the fate of Cry proteins in soil. Finally, the persistence of Cry toxin from biopesticide Bt in the field was compared to that of purified and biopesticide Bt under controlled laboratory conditions to determine the factors that determined the differences observed.

The affinity of three Cry proteins, assessed as the value of the distribution coefficient, Kd, was measuredforover forty soil types. Affinity was compared to land-use and soil properties. Each Cry protein was found to have strong affinity for all the soils. This confirms that Cry proteins will be very immobile in soil and that their fate depends entirely on their adsorbed forms.

The adsorption properties differed considerably between the three proteins studied. There was no observed effect of land-use. Simple linear regression found no relationship between Kd and any of the soil properties tested, including clay content, organic carbon content and soil pH. This is in contrast with findings of studies with a smaller number of soils and is contrary to our expectation that clay content and organic matter would have important influence on affinity. Stepwise ANOVA was able to detect some relationships, including as had been expected a positive relation between clay content and affinity (for Cry1Ac and Cry1C) and an inverse relationship between clay content and extraction yield (for Cry1Ac). The organic carbon content tended to decrease affinity for Cry1Ac, but the ration Corg:clay content was found to have a significant effect on either affinity or extraction yield, suggesting that the degree of complexation between clay and organic matter was a significant parameter.

The fact that the extraction yields of the proteins differed between soils and did not follow the same pattern has important consequences for the use of chemical extraction prior to ELISA detection for routine monitoring. Not only will the apparent concentration of protein reflect the residence time in soil, but will differ in an unpredictable fashion between soils.

The investigation of the persistence of two of the proteins, Cry1Ac and Cry2A, in soil showed remarkable similarities in the time trends of their decline in each of the three soils studied. The decline of Cry2A was slightly more gradual than that of Cry1Ac and no large or significant differences were observed between soils. This trend is similar to previously published data. There is little evidence of the importance of microbial activity. Moisture

content had no significant effect on the rate of decline of Cry1Ac in any of the soils. Furthermore sterilisation of the soils by repeated autoclaving had no significant effect on the rate of decline for either protein in any of the soils. However, as has previously been reported, temperature had a marked effect on the rate of decline which was much slower and more gradual when soil was incubated at 4°C rather than at 25°C. This effect could be attributed to microbial activity, but this would not be coherent with the other means of varying influence microbial activity. We hypothesise that the temperature dependence is due to temperature dependent fixation of the adsorbed protein leading to decreased extraction yield.

We have also addressed the essential question of the toxicity of adsorbed protein and found that toxicity of Cry1Ac towards *Manduca sexta* was initially retained by the proteins, and that toxicity declined in a similar manner to the extractable protein. This is a major finding with implications for the environmental impact of Cry proteins. Aside from detailed considerations of the mechanism for development of resistance and the probability of possible negative impacts, the longer the protein exists in a toxic form the greater is the probability of these events. One major difference between the proteins from GM crops and from biopesticide is that the former is produced and released for a much longer period. The period during which toxicity is maintained in the adsorbed state adds further to this possible exposure period. The surprising observation that the adsorbed toxin appeared to be even more toxic than in the solution state is ascribed to the feeding behaviour of the larvae. This has been reported by previous studies but never satisfactorily explained. Larvae consumed less feed when it contained Cry, particularly in the solution form. This certainly results from the well-known but poorly understood effect of sub-lethal doses of Cry that induce paralysis of larval mouth parts and cessation of feeding. Whatever the mechanism of this effect, it must require the protein to be in a soluble state, and is thus not observed when the protein is adsorbed since the physicochemical conditions in the mouth are not favourable for desorption, unlike in the midgut. This can be taken as further evidence that the limiting factor for toxicity is the resolubilisation of the protein after consumption; this can take place in the midgut but not in the insect mouth.

Similarly the parallel between the temperature dependence of toxicity and extraction-ELISA detection supports our hypothesis that in both cases the limiting factor is the extraction yield that decreases with time as the proteins is progressively fixed on the soils surface. Furthermore the validity of chemical extraction followed by ELISA detection is shown to be a relevant and pertinent approach and the composition of the chemical extractant used, is biologically relevant, despite the different extraction yields observed.

Finally we observed marked differences in the fate of Bt from biopesticides and in the purified state, which was used to mimic protein from GM crops. Cry from Bt biopesticide decreased gradually with time under field conditions with a half-life of about one week. The effects of temperature and sunlight were difficult to establish in the field. However under laboratory conditions sunlight was found to greatly increase the rate of decline of Cry in soil, as was expected. We conclude that under field conditions Cry was better protected from sunlight than in a thin layer of soil and that average conditions in the field did not vary enough between treatments to lead to a detectable effect.

A very interesting observation was that the temperature effect on the dynamics of biopesticide Cry was the reverse of that observed for purified Cry. Since biopesticide is applied to soil that is not usually air-dry, one possible cause of the difference observed could have been the microbial flush that follows rewetting of soil, but no effect of prewetting soil to allow the microbial flush to have finished before addition of protein was observed at 4°C and very little at 25°C. The crucial test to explain the difference between biopesticide and purified Cry was a study of the biopesticide product in soil-free incubation. At 4°C and in deionised water the level of Cry protein remained almost constant throughout the 2-week incubation period. In

marked contrast, when conditions were favourable for spore germination, thus at 25°C and with addition of nutrients, either from artificial nutrient solution or from soil solution obtained by suspending soil in water then discarding the solid phase, there was a marked increase in Cry during the first week of 10 days followed by a gradual decline. Clearly for the biopesticide the observed change in Cry results from two opposing trends, namely an increase in protein under biologically favourable conditions and the decrease observed for purified protein due to the interaction of the soluble protein with soil.

The environmental implications of our findings are considerable. **Firstly**, the difference in behaviour between even very similar Cry proteins means that it is not possible to extrapolate findings from one protein to another. Routine environmental monitoring can yield information on extractable protein which is only a qualitative estimate of the protein actually present in soil. **Secondly**, the persistence of Cry proteins appears to be remarkably similar for different soils and proteins, with a rapid decline followed by a slower decline, with half-lives of less than one week, but detectable levels being maintained for several weeks. The driving force for this decline appears to be mainly the chemical fixation of the protein on sol, rendering it less efficiently solubilised, either by chemical extraction prior to immunodetection or in vivo within the midguts of target insects. **Thirdly** and most importantly, toxicity also appears to be driven by solubilisation. Adsorbed toxin remains potentially toxic, the insecticidal properties being limited by solubilisation. We have also proposed a plausible explanation for the hitherto anomalous observation that adsorption may even enhance toxicity: adsorbed protein cannot cause paralysis of the insect mandibles leading to cessation of feeding and so the true exposure of feeding insects may be greater than for the same amount of Cry in soluble form. This route of exposition of insects to Cry toxins, whether they be geophageous or involuntary consumers of soil, constitutes an important mechanism that could lead either to exposition of non-target insects, or to low, non-lethal exposition of target insects thus contributing to the acquisition of resistance. More studies would be necessary to test if these risks are significant.

LIST OF FIGURES

LIST OF TABLES

ANNEXES

Communications presented at international scientific conferences

Is the decline in detectable Bt protein (Cry1Ac and Cry2A from *Bacillus thuringiensis*) in soil due to microbial breakdown or chemical fixation?

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Introduction

The endemic soil bacteria *Bacillus thuringiensis* produce many insecticidal proteins that show strong host specificity. These bacteria have been used for decades in both conventional and organic farming and to control mosquitos. More recently this trait has been genetically engineered into commercial crops to confer insecticidal properties. The so-called Bt genetically modified (GM) crops have been adopted worldwide and currently account for nearly 40% of all GM crops, grown on 170 million ha of agricultural land. This rapid use of GM, and in particular Bt crops has raised question about their safety.

Like most proteins, these toxins are strongly adsorbed on soil organo-mineral surfaces. This adsorption may induce changes in protein conformation and confer some protection against microbial degradation. Helassa et al. (2011) showed no effect on persistence of Cry1Aa in soils by enhancing or inhibiting microbial activity, suggesting that the fate of Cry proteins in soil is determined by abiotic factors.

Scientific question

Is the fate of Cry proteins in soil determined by microbial activity, protein conformational changes and fixation?

Materials & Methods

Soils

	Corg (g/kg)	pH	Texture
Woodland	31	6.7	Clay
Vine_Org	16.5	8.3	Silty clay
Vine_Conventional	7.2		

Variables

Protein: Cry1Ac, Cry 2A
Incubation **Temperature:** 4°C, 25°C
Incubation **moisture:** 20, 40, 60 % (by weight)
Incubation **period:** 1 – 28 days

Soil + Cry + H_2O

Incubated T=4° or 25°C

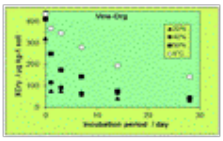
Extraction

alkaline buffer
+ BSA protein
+ Tween20 surfactant

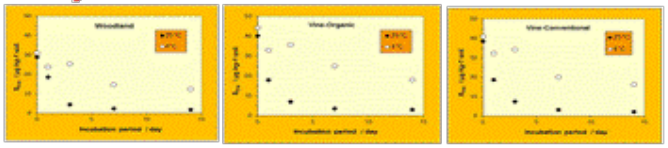
Detection Elisa assay

Results & Discussion

Cry1Ac: variable moisture at 25°C; 40% at 4°C



Cry2A: 40% moisture



- **Better extraction yield from silty clay (Vine) soils than clayey (Woodland)**
- **No effect organic matter contents (Vine soils)**
- **No Temperature effect on extraction yield**
- **Rapid decline extractable Cry, all soils, both Cry, all moisture contents**
- **Little effect moisture content**
- **Cry conserved at 4°C**

°Suppressed microbial activity

Less conformational changes and fixation

[1] Helassa et al. 2011, Soil Biol Biochem
No change in Cry1Ac fate in soil when microbial activity enhanced or reduced
($HgCl_2$, autoclaving, γ-irradiation, anti-protease)

Assessment of persistence of Cry1Ac protein from Bt spray in soil: comparison of field and controlled laboratory applications

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Introduction

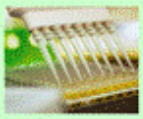
The harmful effect of chemical pesticides to the environment and to human health has led to increasing use of biopesticides in agriculture. *Bacillus thuringiensis* which produces insecticidal proteins known as Cry, is the most widely used biopesticide because of its efficiency and absence of side-effects. Of the many strains of *Bacillus thuringiensis*, Btk (*kurstaki*) that produces Cry1Ac is present in many commercial products. Most attention has been given to the fate of purified Cry proteins, in the context of genetically modified crops that are engineered to produce these proteins. There is less information on role of soils in the fate of Cry proteins from commercial biopesticide formulations that contain mixtures of protein crystals, spores and bacillus along with filler material. Adsorption on soil modifies the biological properties and persistence of proteins. We have compared the fate of Cry1Ac from a commercial formulation under field and laboratory conditions and also compared biopesticide Cry with the fate of purified protein in the same soils.

Materials and Methods

Soils
soil studied in the field or laboratory

Biopesticide / Cry1Ac
Biopesticide containing spores and crystals *B.kustaki* or purified Cry 1Ac protein

Extraction
Alkaline buffer
+ BSA protein
+ Tween 20 surfactant



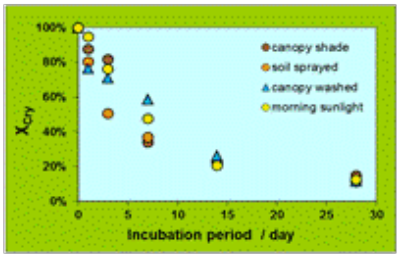
Detection Elisa assay

Variables

- **Protein**: spore and crystals of Bt protoxin **or** purified Cry 1Ac toxin
- **Conditions** : Field **or** laboratory
- **Temperature** of incubation: ambient temperature **or** 4°C
- **Sunlight** factor: dark (lab); shade **or** sunlight
- **Moisture** content: 20, 40, 60% (lab)
- **Period** of trial: 1-28 days

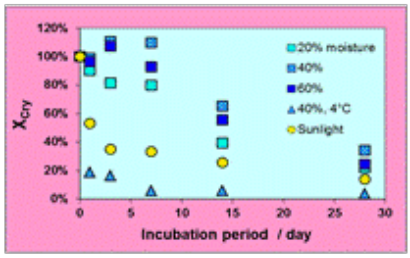
Results and Discussion

Biopesticide - field



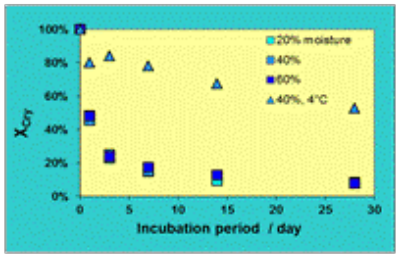
- Detectable Cry1Ac decreases rapidly (field and lab) still detectable after 28 days
- No longterm effect of spray conditions (field)
- Initial faster decrease when soil sprayed directly
  Some protection by canopy shade
  Protection decreased by canopy washing (remove spray additives)

Biopesticide - laboratory



- Little effect moisture content
- More rapid decline in sunlight
  Not observed in field
- Very rapid decline at 4°C
  not due to low-temperature
  decrease of microbial activity

Purified Cry1Ac toxin



- No effect moisture content
- Large protection at 4°C
  Contrast biopesticide spray
  Effect not microbial
  Hydrophobic interactions
  of adsorbed protein with soil
  (Helassa et al. 2011)

In conclusion,

marked differences between commercial biopesticide and purified Cry protein due to

(i) additives in spray that protect protein and spores (ii) microbial effects on spores
(iii) protein-soil interfacial interactions

Conservation of insecticidal activity of Cry1Ac adsorbed on three contrasting soils and persistence with time

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Siobhán STAUNTON

Résumé

Les propriétés insecticides du *Bacillus thuringiensis*, découvert par Shigentane Ishiwatari, ont été utilisées pendant des décennies comme biopesticides et cette utilisation a augmenté rapidement en raison de préoccupations au sujet des effets environnementaux négatifs des pesticides chimiques. Actuellement, la toxine Bt dans la forme de biopesticides et des plantes transgéniques Bt peut compléter ou remplacer les pesticides chimiques. Il y a peu d'indication que la toxine Bt a un effet nocif pour l'environnement ou la santé humaine. Néanmoins, il ya des préoccupations que les cultures transgéniques commerciales peuvent avoir des effets néfastes sur l'environnement. Après son introduction dans le sol l'exsudation racinaire et la dégradation des résidus végétaux, la toxine Bt interagit avec les particules de sol. Les interactions de la toxine Bt avec des particules de sol influencent sa mobilité, sa biodisponibilité, sa persistance et sa toxicité. Dans cette étude, nous visons à établir l'importance relative des facteurs biologiques et physico-chimiques dans la détermination de la dynamique des protéines Cry détectables dans les sols, de clarifier si la protéine adsorbée conserve ses propriétés insecticides et d'identifier les propriétés du sol qui déterminent le devenir des protéines Cry dans le sol. Les résultats montrent que les protéines Cry ont une forte affinité sur la surface du sol. Cependant, il y avait peu de relation entre l'affinité pour le sol ou le rendement d'extraction et les propriétés du sol, y compris la teneur en argile, teneur en carbone organique et le pH du sol. Il y avait peu de rapport entre l'affinité et le rendement d'extraction. Les protéines diffèrent à la fois dans leur affinité pour les sols et leurs rendements d'extraction.

Une évaluation du rôle du sol et des facteurs environnementaux dans le sort des protéines Cry de la formulation de biopesticides commerciale a montré un déclin rapide de la protéine Cry détectable soumise aux rayons du soleil sous la condition de laboratoire, alors que peu d'effet a été observé dans des conditions de terrain. La demi-vie des protéines dans le sol dans des conditions naturelles était d'environ 1 semaine. Des effets de la température forts ont été observés, mais ils diffèrent pour les biopesticides et la protéine purifiée, indiquant différentes étapes limitantes. Pour le biopesticide, la baisse observée était ralenties par des facteurs biologiques, y compris éventuellement sporulation. En revanche pour des protéines purifiées, augmentation de la température améliorée des changements conformation els de la protéine adsorbée du sol, conduisant à une fixation et, par conséquent diminué efficacité d'extraction qui a diminué avec le temps. En outre, l'étude de la persistance de diverses protéines Cry dans les sols contrastés a été réalisée par immuno-détection et dosage biologique a montré que la toxine extractible diminue avec incubation allant jusqu'à quatre semaines. L'activité insecticide était toujours maintenue à l'état adsorbé, mais a disparue après deux semaines d'incubation à 25°C. La baisse de la protéine extractible et la toxicité était beaucoup plus faible à 4°C à 25°C. La stérilisation du sol n'a pas eu d'effet significatif sur la persistance de la toxine Cry indiquant que le déclin observé était provoqué par la fixation en fonction du temps de la protéine adsorbée ce qui diminue la quantité de toxine Cry extractible, la dégradation de la protéine par l'activité microbienne jouant un rôle plus mineur.

L'exposition des insectes aux protéines Cry sous la forme adsorbé pourrait avoir un impact significatif sur les insectes cibles et même les insectes non cibles, et devrait être plus étudiée afin de déterminer son impact potentiel.

Mots clés : organismes génétiquement modifiés (OGM) ; *Bacillus thuringiensis* (*Bt*) ; sol ; adsorption ; extraction ; détection immunologique ; persistance

Summary

The insecticidal properties of *Bacillus thuringiensis*, discovered by Shigentane Ishiwatari, have been used for decades as biopesticides and this use has been increasing rapidly because of concerns about the negative environmental effects of chemical pesticides. Currently, Bt toxin in the form of both biopesticides and Bt transgenic plants may supplement or replace chemical pesticide. There is little evidence to demonstrate that Bt toxin has any harmful effect to the environment or to human health. Nevertheless, there are concerns that commercial transgenic crops may have harmful impacts on the environment. After release into soil via root exudation and breakdown of plant residues, Bt toxin interacts with soil particles. The interactions of Bt toxin with soil particles influence its mobility, its bioavailability, its persistence and its toxicity. In this study, we aim to establish the relative importance of biological and physicochemical factors in the determination of the dynamics of detectable Cry proteins in soils, to clarify if adsorbed protein maintains its insecticidal properties and to identify the soil properties that determine the fate of Cry proteins in soil. The results show that Cry proteins have strong affinity on soil surface. However, there was little relationship between affinity for soil or the extraction yield and soil properties including clay content, organic carbon content and soil pH. There was little relationship between the affinity and the extraction yield. The proteins differ in both their affinity for soil and their extraction yields.

An assessment of role of soil and environmental factors in the fate of Cry protein from commercial biopesticide formulation showed a rapid decline of detectable Cry protein subjected to direct sunlight under the laboratory condition, whereas, little effect was observed under field conditions. The half-life of proteins in soil under natural conditions was about one week. Strong temperature effects were observed, but they differed for biopesticide and purified protein, indicating different limiting steps. For biopesticide, the observed decline was due to biological factors, possibly including sporulation. In contrast for purified proteins, increased temperature enhanced conformational changes of the soil-adsorbed protein, leading to fixation and hence extraction efficiency decreased that decreased with time. Moreover, the study of persistence of various Cry proteins in contrasting soils was carried out by immuno-detection and bioassay showed that extractable toxin decreased with incubation of up to four weeks. Insecticidal activity was still retained in the adsorbed state, but lost after two weeks of incubation at 25°C. The decline in extractable protein and toxicity was much lower at 4°C than 25°C. There was no significant effect of soil sterilization to persistence of Cry toxin indicating that decrease in detectable Cry toxin in soil may be time-dependent fixation of adsorbed protein as well as decreasing solubilization in larva midgut, but not microbial breakdown.

Exposition to Cry in the adsorbed form could have a significant impact on target and even non target insects and should be investigation to determine the potential impact.

Keywords : Genetically modified organisms (GMO) ; *Bacillus thuringiensis* (*Bt*) ; soil ; adsorption ; extraction ; immunological detection ; persistence