AN IMPROVED METHOD TO EXTRACT CRY PROTEINS FROM SOIL AND ITS USE IN QUANTIFYING THE RELEASE AND PERSISTENCE OF Cry3Bb1 and Cry1Ab PROTEINS FROM Bt CORN HYBRIDS IN THE FIELD

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ABSTRACT

Transgenic corn expressing the insecticidal crystalline (Cry) proteins derived from the bacterium, *Bacillus thuringiensis* (*Bt*; Bt corn), has been engineered to render plants resistant to Western corn root worm (CRW; Cry3Bb1) and/or European corn borer (ECB; Cry1Ab). As a result of the many agricultural and environmental benefits accrued, rapid adoption and widespread use of transgenic Bt corn has occurred over the past ten years and will likely continue to expand, potentially resulting in the accumulation and persistence of plant-produced Bt proteins in soil. In this investigation, I worked to resolve some analytical issues, such as low recovery of Cry3Bb1 and Cry1Ab proteins from field soils, and to quantitatively measure these proteins released in agricultural soils where many different varieties of Bt corn were being grown. First, non-Bt agricultural field soils were spiked with environmentally relevant concentrations of the two Cry proteins. The proteins were then recovered from the soil to evaluate the effects of physical and chemical modifications to the standard extraction methods until an improved protocol was developed for optimal soil-protein recovery. Poor protein recoveries from soil were reconciled when soil extraction protocols were modified to include bead-beating with two glass beads, a 2 h ELISA (enzyme-linked immunosorbant assay) plate incubation, and the use of an extraction buffer developed by Palm et al. in 1994. Second, soils were collected from the rhizosphere of Bt corn hybrids being grown in varietal trials at five field sites around New York (NY) State. The concentration(s) of the Cry3Bb1 and Cry1Ab proteins in these soils was measured using the modified protocol. The effects of climate, crop and soil factors on the release of these proteins from Bt corn were evaluated. In one soil from a field site in Aurora, NY, where Cry3Bb1 corn was grown for three consecutive years, the persistence of the Cry3Bb1 protein was evaluated.

Results showed that Bt corn expressing Cry3Bb1 releases this protein into soil from its roots and residues under NY environmental conditions. Cry3Bb1 was rapidly biodegraded in soils sampled at pre-planting and after-harvest during the 2006 growing season at Aurora, NY, indicating a low potential for persistence. Concentrations of Cry3Bb1 at the six NY sites varied. However, where soils contained a higher percentage of clay, much higher concentrations of both Cry3Bb1 and Cry1Ab were detected, compared to sites with lower clay contents. Bt corn plants without the construct for Round-up resistance (RR) had significantly higher concentrations of Cry3Bb1 and/or Cry1Ab compared to plants with the RR construct. The concentrations of Cry3Bb1 in rhizosphere soils of the different Bt corn varieties tested varied considerably, but were significantly greater for the variety TA5859, which contained the stacked construct, CB-RW (corn borer-root worm). Cry1Ab concentrations in rhizosphere soils also varied between corn varieties, even when they were grown under similar environmental conditions. Lastly, Cry3Bb1 concentrations in rhizosphere soils were significantly higher than those of Cry1Ab. In summary, Cry3Bb1 protein was rapidly biodegraded and did not accumulate or persist in field soil under NY State conditions. Thus, Cry3Bb1 is unlikely to pose any significant ecological risks to soil organisms in these locations.

BIOGRAPHICAL SKETCH

Benjamin R. Diaz was born in El Paso, Texas on April 28, 1972, to parents Robert D. Diaz and Virginia A. Diaz. He began his education at the Edgemere Elementary School in El Paso, Texas. His interests and passion for nature and the environment started as a young child during many camping trips in New Mexico. He also attended Socorro High School and graduated in 1990. Benjamin enrolled in El Paso Community College and transferred after two years to the University of Texas at El Paso (UTEP). Benjamin's passion for the environment continued while he traveled and camped at various locations in the Southwest and Western United States. During his travels, he experienced much environmental degradation and neglect for the environment. While at UTEP, he pursued animal and environmental activism with Dr. Steven Best. During his time at UTEP he met his future companion, the former Joana Jolene Jones and later married her. He graduated from UTEP in 1999 with a B.S. degree in Biology. His children are Matashia Paige Jones and Angelo Tobias Diaz. Benjamin also received an M.S. in Interdisciplinary Studies-Environmental Science from the University of Texas at El Paso, 2004 under his mentor Dr. Dirk Schulze-Makuch. His studies in Environmental Toxicology and Soil Biology continued at Cornell University with Dr. Janice Thies.

For Dad,

Your persistence and unending love have provided me the courage I need to fly!

I dedicate this work to you!

The soil you see is not ordinary soil –it is the dust of our ancestors....You will have to dig down through the surface before you can find nature's earth, as the upper portion is Crow. The land as it is, is my blood and my dead; it is consecrated....
Shes-his (late 19th century) Reno Crow

The earth and myself are of one mind. The measure of the land and the measure of our bodies are the same....
Joseph [Hinmaton Yalatkit (1830-1904) Nez Perce chief

Those who dwell among the beauties and mysteries of the earth are never alone or weary of life.
Rachel Carson

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 Literature Review and Objectives

1.1 Introduction

Breakthroughs in crop biotechnology have opened up enormous new possibilities in agriculture (Fukuda-Parr, 2007). With genetic engineering, new crop varieties are being developed that offer a range of benefits beyond those possible with traditional breeding. Thus, transgenic technology permits plant breeders to assemble, in one plant, valuable genes from a wide array of organisms foreign to the plant, enabling researchers to generate more useful and productive crop varieties containing new combinations of genes. Genetically engineered crops expand the possibilities beyond the limitations imposed by traditional cross-pollination and selection techniques (Byrne, 2004).

The acreage planted to transgenic crops (TCs) has increased substantially over the decade since their first commercial releases. The strong interest and popularity of TCs amongst farmers, resulting from success of the traits conferred, has encouraged their increased and widespread use. Transgenic crops have been developed to control various crop pests, resist disease, tolerate herbicides, improve nutritional content, resist drought and improve nitrogen (N) uptake when N availability is low (Sanvido et al., 2006). Rapid adoption of TCs has resulted in sustained increases in agricultural productivity, contributed to economic growth and ensured an abundance of food (Fernandez-Cornejo, 2002). Hence, TCs are likely to remain a very significant part of agriculture in the future (James, 2007). However, public concerns continue to be expressed that their rapid adoption and widespread use could have adverse effects on

the environment; notably, on soil organisms that are vital components of the soil subsystem.

Transgenic, insecticidal crops represent a new means of insect control that may result in a substantial decrease in pesticide use and thus would represent a "public good". Transgenic crops that express the *cry* (crystal) protein gene from *Bacillus thuringiensis* (*Bt*), a common soil bacterium, are toxic to select groups of herbivorous insect pests. These are referred to as Bt crops. Some research indicates that the Cry1Ab protein, active against the Lepidoptera, could potentially accumulate and persist in soil where Bt crops are grown repeatedly and where their residues are incorporated into the soil (Tapp and Stotzky, 1995; Crecchio and Stotzky, 1998). Thus, the intensity and duration of exposure of soil organisms to Cry proteins may be high and last longer (USEPA, 2000). Recently published data on the persistence of the Cry3Bb1 protein, active against the Coleoptera, suggest that this protein may be degraded more rapidly in soil than the Cry1Ab protein (Ahmad et al., 2005; Icoz and Stotzky, 2007). The Environmental Protection Agency (EPA) recently approved the registration of another Bt corn construct, YieldGard™ Plus, a new commercial variety that contains two "stacked' genes, one for Cry3Bb1 and one for Cry1Ab tissue expression, which provides farmers control of both the European corn borer (ECB) and the Western corn rootworm (CRW). One report on the decomposition of residues from YieldGard™ Plus indicates that they decompose readily in the field (Lehman et al., 2008). However, there are as yet no published reports on whether the two proteins expressed in YieldGard™ Plus corn are released in root exudates or from crop residues; and no information on the subsequent persistence of these proteins in soil. Since transgenic corn is grown in New York (NY) State, it is important to understand the extent of Cry protein release into the rhizosphere and whether these proteins

accumulate or persist in the diverse soils and agricultural field conditions found in NY State.

1.2 Transgenic Bt corn

Numerous Cry protein genes have been identified that code for various insecticidal proteins. These genes derived from *B. thuringiensis*, have been transformed into corn and other commodity crop plants as a means of controlling economically destructive corn pests (Table 1.1). Corn engineered to produce the Bt Cry protein(s) (Bt corn) is toxic to either CRW larvae (Cry3Bb1) or ECB (Cry1Ab) or both ("stacked"). Bt corn has gained popularity with farmers because it provides unprecedented control of these insect pests and represents economic and environmental advantages over using conventional insecticides (Munkvold et al., 1999, Munkvold and Hellmich, 2000, Shelton et al., 2002).

The first generation Bt corn incorporated genes coding for the Cry1Ab or Cry9C protein. TCs in which Cry1Ab is expressed are the most widely grown Bt crops today (Benedict and Ring, 2004). In early 2003, a new transgenic corn hybrid (YieldGard™ Rootworm) became commercially available to control the Western CRW (Payne et al., 2003; Rice, 2004; USEPA, 2007). Since their adoption, significant benefits to growers, the public and the environment have resulted from adopting Bt corn (Glaser and Matten, 2003). In addition, international competitiveness and environmental issues such as sustainable agro-ecosystems have also been linked to technological innovation and adoption (Stoneman, 1995).

Table 1.1 Major transgenic Bt crops commercially produced in the U.S. since 1995 (Ware and Whitacre, 2004).

Product (Company)	Year	Crop	Bt Protein	Target pests
New leaf (Monsanto)	1995	Potato	Cry3A	Colorado potato beetle
Bollgard (Monsanto)	1996	Cotton	Cry3Ac	Cotton bollworm, tobacco budworm
Attribute (Novartis)	1995 1996	Corn, sweetcorn	Cry1Ab	Corn earworm, European corn borer, other Lepidoptera
Yieldgard (Monsanto)	1996	Corn	Cry1Ab	Corn earworm, European corn borer
Yieldgard (Dekalb)	1997	Corn	Cry1Ab	Corn earworm, European corn borer,
Star Link (Aventis)	1997	Corn	Cry9c	Corn earworm, European corn borer,
New Leaf Plus (Monsanto)	1999	Potato	Cry3A	Colorado potato beetle & potato leaf roller virus
Herculex (Mycogen)	2001	Corn	Cry1F	European corn borer
Bollard (Monsanto)	2002	Cotton	Cry2Ab Cry1Ac	Stacked genes for cotton bollworm, tobacco budworm, pink bollworm & army worm
Yieldgard Rootworm (Monsanto)	2003	Corn	Cry3Bb1	Corn rootworm complex

1.2.1 Benefits of Bt Corn

Studies are limited because this technology is new, but significant benefits are expected from the use of Bt crops (Alton et al., 2002; Miller, 2000; Mitchell, 2002; US EPA, 2001; Ward, 2002). Notably, new benefits include reduced insecticide use, highly effective pest control, higher crop yields, supplemental pest control by beneficial non-target organisms, and reduced levels of fungal toxins (Betz et al., 2000).

Major environmental benefits are beginning to accrue from reduced use of synthetic insecticides. Six TCs planted in the U.S. were found to produce an additional four billion pounds of food and fiber, improve farm income by $1.5 billion and reduce pesticide use by 46 million lbs (Glaser and Matten, 2003). Greater grower satisfaction and consistent and substantial benefits, such as higher productivity and a safer environment, have resulted in higher adoption rates of Bt crops (Glaser and Matten, 2003). Table 1.2 shows the distribution of transgenic crop plantings in 2007 for the top nine countries that have adopted TCs.

Adoption rates have increased substantially in many developed and developing countries and the land area planted to TCs now exceeds 2.0 million ha. In effect, farmers have continued to plant Bt corn every year (James 2007). In the U.S., transgenic corn use also continues to increase each year (Fernandez-Cornejo and McBride, 2002). Bt crops such as Bt corn, potato and rice potentially contribute to the presence of Cry proteins in soil via root exudates throughout their growth (Saxena, 2004) and after harvest as corn residues, left in the field, decompose (Zwahlen et al., 2003a; Stotzky, 2002, 2004). The very rapid adoption of Bt crops has outpaced the monitoring work necessary to assure that increasing rates of input and longer-term

persistence of these proteins in soil has no lasting adverse environmental consequences.

1.3 Target pests of Bt corn

Two insects are the primary pests of corn in the U.S.; the European corn borer (*Ostrinia nubilalis*) in the Lepidoptera and the Western corn rootworm (*Diabrotica* spp.) in the Coleoptera.

The ECB is among the main yield-limiting factors in the U.S. Corn Belt. It causes damage when larvae feed on all above ground tissues of the corn plant. ECB bores into, feeds, and tunnels within the tassel, ear, ear shank and stalk. This feeding behavior forms cavities that interfere with the translocation of water and nutrients. These cavities also reduce the strength of the stalk and ear shank, thereby predisposing the corn plants to stalk breakage, lodging and ear drop (Kalisch, 1997). Losses resulting from ECB and the cost of its control can exceed $1 billion each year (Krattiger, 1997). Insecticides applied to control ECB account for the major proportion of corn insecticide acre-treatments and these sprays may be ineffective because the insect bores into the corn tissue and can escape contact with the insecticide (Comis, 1997).

The CRW is also a major pest of corn in the U.S. As larvae, CRW feed on corn roots and reduce the plant's ability to absorb water and nutrients from the soil. Since CRW larvae feed belowground, insecticide sprays are not very effective. Farmers have suffered significant financial losses ($1 billion dollars annually) from reduced yields and increased chemical insecticide use due to this pest (Metcalf, 1986; Agricultural Research Service, 2001). It is widely accepted that CRW is a much more damaging pest than ECB (Gray and Luckman, 1994).

Table 1.2 Distribution of transgenic crops (TCs) planted in the top nine adopting countries (James, 2007).

Country	Area (M ha)	TCs
USA	57.70	Soybean, corn, cotton, canola
Argentina	19.10	Soybean, corn, cotton
Brazil	15.00	Soybean, cotton
Canada	7.00	Canola, corn, soybean
India	6.20	Cotton
China	3.80	Cotton
South Africa	1.80	Corn, cotton
Philippines	0.30	Corn
Australia	0.10	Cotton

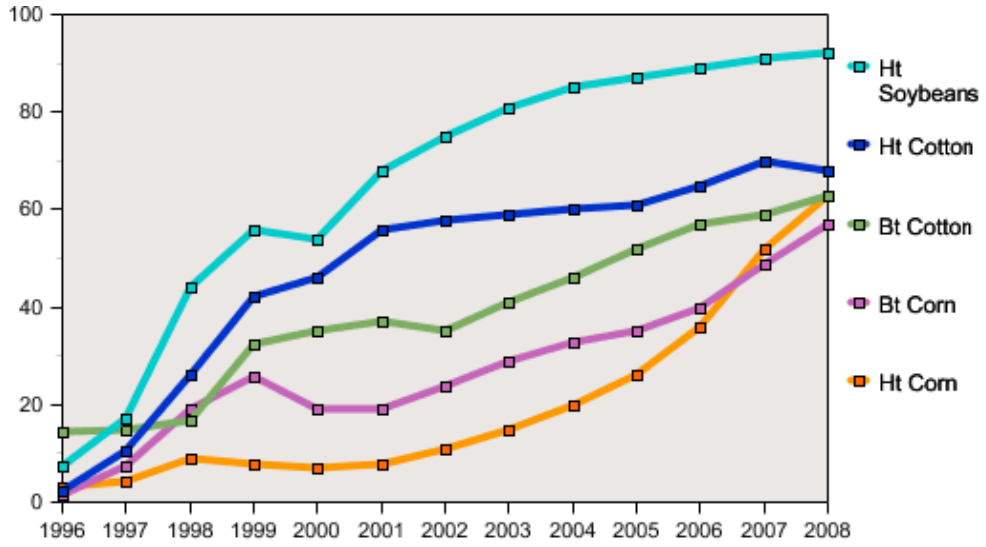

Figure 1.1 Rapid growth in the adoption of genetically engineered crops continues in the U.S. (Fernandez-Cornejo and McBride, 2002).

1.3.1 Pest management: Pesticides and Bt sprays as insecticidal toxins

Many acres planted with corn are treated annually with organophosphates, carbamate and pyrethroid insecticides to control CRW and ECB. These and other pest-management options that were effective at managing ECB and CRW, such as crop rotation, have turned out to be only partially effective and occasionally fail at controlling these corn pests (Chandler et al., 2000; Duan et al., 2002). Prior to the early 1980's, most farmers rotated corn with other crops in order to control insect pests. Corn was commonly rotated with soybeans and this eliminated the need to use insecticides on first-year corn. However, recent changes in the behavior of CRW populations have decreased the efficacy of crop rotation as a pest management tool (Gray and Luckman, 1994; Ostlie, 2001). This has resulted in increased insecticide use. Insecticides to control CRW are usually applied to the soil at planting, a management strategy designed to minimize the exposure of above-ground, non-target organisms to it and reduce insecticide residues after harvest (USDA, NASS/ERS, 1998). However, environmental problems can arise when insecticide residues are leached from soil and move to surface and ground waters through field drainage systems. Many pesticides are toxic to birds, fish and mammals and their increased use may result in their accumulation in the environment (USDA, NASS/ERS, 1998). Pesticides such as atrazine, metolachlor, cyanazine (CRW) and chloropyrifos (ECB) are routinely applied to corn (EPA, 1994; Carpenter and Gianessi, 2001).

1.4 *Bacillus thuringiensis* and its insecticidal proteins

Before the advent of Bt crops, pesticides were the choice of most conventional growers; whereas, *Bt* microbial products were, and continue to be, the preferred insect control choice for organic growers. *Bt* microbial products contain *Bacillus*

thuringiensis, a Gram positive, aerobic, spore-forming bacterium that produces a parasporal crystalline-protein inclusion during sporulation (Fiorito et al., 2008). It is a common soil bacterium with a worldwide distribution, as strains have been isolated from stored plant products, deciduous and coniferous leaves, insects and soil from forests, agricultural fields, steppes and the tundra (Martin and Travers, 1989; Iriarte et al., 1998; Schnepf et al., 1998). *Bacillus thuringiensis* is considered to be a soil bacterium; however, since only spores are found commonly in soil, its principal ecological niche is likely to be the insect integument (Jensen et al., 2003).

In *B. thuringiensis*, the *cry* gene is expressed only during the stationary growth phase as a parasporal crystal protoxin. In nature, Cry proteins are produced as protoxins, which are non-toxic until they are proteolytically cleaved into their active form in the alkaline environment of the insect integument. In contrast, TCs typically express the *cry* gene in all of their tissues throughout the growth cycle of the plant in its truncated, active form (74 kDa); thus, it is not necessary to solubilize or cleave the protein enzymatically to activate this protein toxin (Stotzky 2000; Icoz and Stotzky 2007).

The parasporal crystalline-protein inclusion that is produced during sporulation is known to contain several types of insecticidal crystal proteins (ICPs) which, after ingestion by susceptible insect larvae, cause larval death (Fiorito, 2008). Since the 1950's, this insecticidal bacterium has been the most successful and widely used commercial biological control agent of insect pests in the world (Federici, 1999). *Bacillus thuringiensis* has been used mainly to suppress numerous lepidopteran and coleopteran pests of forests and vegetable and field crops; and has been used to control the larvae of mosquitoes and black flies. Its commercial importance is recognized for many reasons. *Bacillus thuringiensis* produces a wide range of endotoxins that vary in

their activity against specific genera of important pest insects. Although toxic to certain pests, these protein toxins are considered to be "reasonably safe" to non-target organisms because their toxicity is a result of recognition of and binding to specific cadherin receptors found only in target pests. *Bacillus thuringiensis,* as a biocontrol agent, is highly adaptable to conventional formulations and application technologies (Federici, 1999) and is approved for use in organic cropping systems.

Bacillus thuringiensis is placed phylogenetically in a bacterial clade with numerous subspecies recognized for their entomopathogenic properties. Under environmental conditions insufficient for continued growth, *B. thuringiensis* produces a spore and parasporal bodies (insecticidal crystal proteins, ICPs). The principal ICPs are the Cry and Cyt δ-endotoxins. The δ-endotoxins have been characterized and are known to have molecular weights that range from 130-140 kDa. These protoxins do not have insecticidal activity until they are solubilized in the alkaline environment of the insect mid-gut (pH 8-11), which activates proteolytic enzymes that cleave the protoxin to a biologically active form of molecular weight 60-70 kDa (Hofte and Whiteley, 1989; Schnepf et al., 1998) (Figure 1.2).

1.4.1 Mode of action

The general model for the mode of action of the Cry proteins has been known for some years, but it has not yet been resolved at the molecular level. What is generally known is that the ICPs are consumed by the target insect, solubilized in the insect mid-gut and cleaved by proteases. As a result, the disulfide bridges that stabilize the protoxin break, yielding a 60 to 70 kDa activated protein (Federici, 1999). Proteolytic processing and binding to specific receptors in the mid-gut are critical steps in toxin activation and determine insect specificity (Saraswathy and Kumar, 2004). Accordingly, proteolytic processing typically involves removal of an N-terminal

peptide by insect proteases (Figure 1.2). The activated toxin then binds to specific cadherin receptors on the mid-gut epithelial cells of the susceptible insects before inserting itself into the apical membrane (Bravo and Soberon, 2005). Galitsky et al. (2001) proposed that binding of the activated protein toxin to specific receptors also concentrates the toxin in the brush border cells and may allow for the proteins to associate together and penetrate the membrane lipid bilayer. Insertion causes the formation

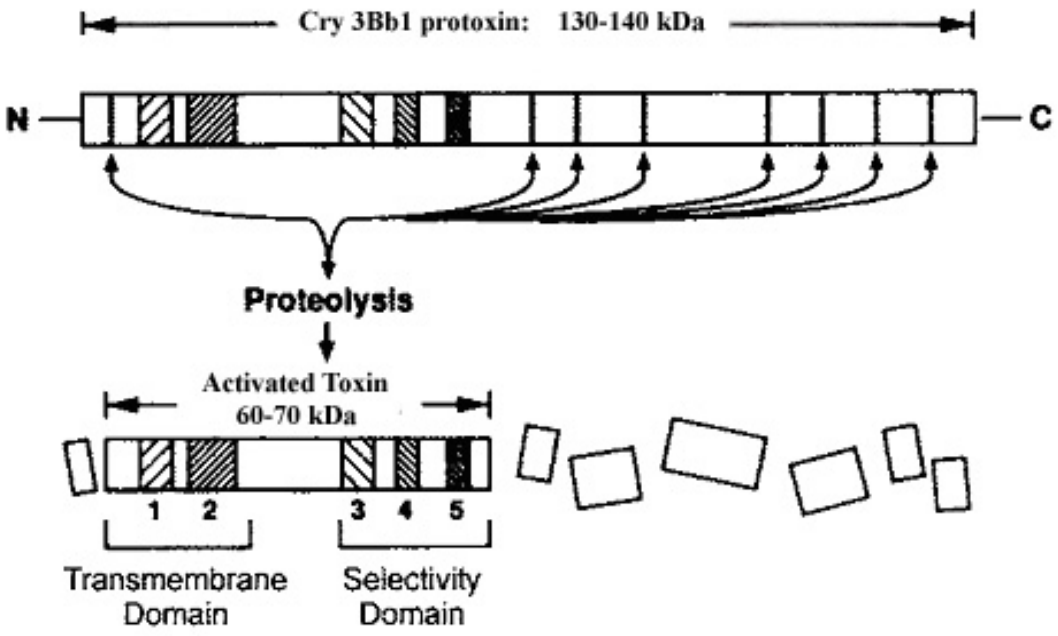

Figure 1.2 Dissolution and proteolytic cleavage of the Cry3Bb1 protoxin results in an activated toxin with a molecular weight of 60-70 kDa. Modified from Hofte and Whiteley (1989).

of lytic pores in the gut membrane, disrupting the membrane potential and resulting in cell lysis, severe septicemia and insect death (Figure 1.3; Schepf et al., 1998; de Maagd et al., 2001).

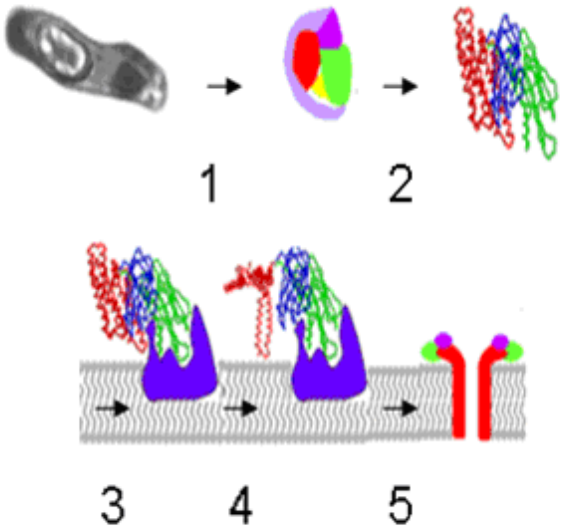

Figure 1.3 Proposed mode of action for Cry proteins. 1. Ingestion and solubilization of the protoxin; 2. Proteolytic activation at N- and C- termini; 3. Interaction with cell surface binding protein; 4. Conformational change exposing α 4-5 helices hairpin; 5. Oligomerization and insertion in membrane to form a pore (N. Crickmore; available at: www3.imperial.ac.uk/people/d.wright/research**)**.

1.4.2 Cry protein structure

Illuminating the three-dimensional structure of the Cry δ-endotoxins has helped us understand their function, specificity and mode of action. Li et al. (1991) showed that Cry proteins are basically wedge-shaped, globular proteins. Holfte and Whiteley (1989) showed that the active portion of the Cry toxin contains five blocks of conserved amino acids distributed along the molecule and a highly variable region within the C-terminal half. The first structural determination of a Cry protein by X-ray crystallography revealed a three-domain structure (Li et al., 1991). Each of the three discrete domains of the Cry δ-endotoxin has independent and inter-related functions in the larval mid-gut (Knowles, 1994). Domain I consist of 7-bundles of

antiparallel α-helices (residues 64-294) and is responsible for the formation of lytic pores or ion channels in the insect mid-gut. Domain II consists of three antiparallel β-sheets (residues 295-502) containing hydrophobic, surface-exposed loops and is involved in receptor binding and insect specificity (Sarawathy and Kumar, 2004). Domain III consists of two-twisted antiparallel β-sheets (residues 503-652) that form a β-sandwich with a "jellyroll" topology (Figure 1.4). This domain has a number of key

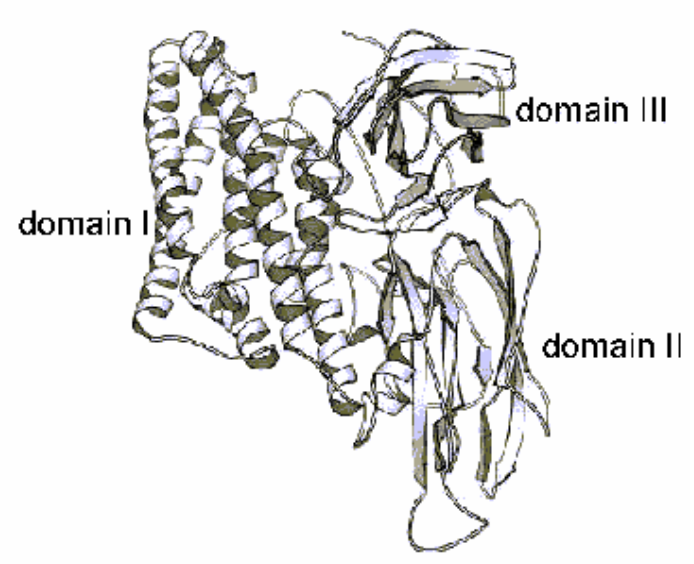

Figure 1.4 Schematic ribbon representation of Cry3Bb1 showing its three-domain organization: I, II and III (Galitsky, 2001).

roles in the biochemistry, structural integrity, receptor binding, membrane penetration, and ion channel functions (Schnepf et al., 1998; Galitsky et al., 2001). Thus, the structure of the Cry protein is important for the pathogenicity of *B. thuringiensis* to certain target insect species. Galitsky et al. (2001) determined that the tertiary structure of Cry3Bb1 is similar to the Cry3A and Cry1Aa proteins and also has three discrete domains.

1.4.3 Specificity

The value of Cry protein toxins lies in the characteristic that they are only effective against specific classes of insect pests. In order for *B. thuringiensis* to be effective as a biocontrol agent it must be ingested during the feeding stage of insect development, i.e., when they are larvae. Hence, Cry toxins are effective only against specific insects at a susceptible stage of their development (Parker and Feil, 2005). Thus, *B. thuringiensis* is not effective against adult insects. Another condition for activity is that the Cry crystal protein must be solubilized and dissolved in the insect gut, cleaved by insect proteases and then bound to specific receptors found only in specific target pests. Thus, the Cry toxins are highly specific to insects belonging to the Lepidoptera (butterflies and moths), Diptera (mosquitoes and black flies), or Coleoptera (beetles and weevils) because of their requirement to bind to specific receptors in order to effect activity (Knowles, 1990; Parker and Feil, 2005). To date, more than three-hundred Cry protein toxins have been sequenced and characterized (Crickmore et al., 2007). They are classified according to their sequence and insect specificity: toxins of the Cry1 class are specific for Lepidoptera, Cry2 proteins target Lepidoptera or Diptera, Cry3 proteins are Coleoptera-specific and Cry4 proteins are Diptera-specific (Knowles, 1994). For example, target pests that are controlled effectively with *B. thuringiensis* var. *kurstaki* (*Btk*) include common caterpillar pests, such as the ECB, fall webworm, Indian meal moth in stored grain, *Mimosa* webworm, cabbage looper, bagworms, imported cabbageworm, spring and fall cankerworm, diamondback moth, tomato/tobacco hornworm, red-humped caterpillar, tent caterpillars, sod webworms,

cutworms, loopers, and the Oleander moth. Caterpillars not affected by *Btk* include corn earworm, squash vine borer, and cutworms (Weinzierl et al., 1997).

1.4.4 Insecticidal activity of *B. thuringiensis* Cry proteins

The insecticidal properties of *B. thuringiensis* were first recognized in 1901 by Ishawata, a Japanese scientist, and then in 1911 by Berliner, a German scientist (Lambert and Peferoen, 1992; Glare and O'Callaghan, 2000). The first commercial applications of *B. thuringiensis* as an insecticide (*Bt*) were in France in 1938 (Federici, 1999). The U.S. entered the commercial market in the 1950's (Camilla, 2000). Today, *Bt* microbial insecticides are produced world-wide and now constitute a few thousand tons annually (Federici, 1999). The main strains used to produce *Bt* insecticides are *B. thuringiensis* subspecies *kurstaki* (active against Lepidoptera), *morrisoni* (active against Coleoptera) *tenebrionis* (active against Coleoptera) and *israelensis* (active against mosquitoes and flies or Diptera). The microbial insecticide, *Bt*, is applied much like most synthetic insecticides. However, *Bt* is essentially nontoxic to wildlife, humans and most other organisms not related to the target organisms, with no toxicities known to have occurred. Because of this, they can be applied at almost any stage of vegetative growth and are approved for use in organic agricultural systems. Trade names of commercial products include Dipel®, Javelin®, Thuricide®, Worm Attack®, Caterpiller Killer®, Bactospeine® and SOK-Bt® (Weinzierl et al., 1997).

There are limitations to the effectiveness of the *Bt* microbial insecticides. First, *Bt* does not persist or establish itself in the environment at populations necessary to provide continuous control of agricultural pests (Weinzierl et al., 1997). Second, they are rapidly degraded in the environment by UV radiation, heat and desiccation. Spraying generally results in poor and incomplete coverage leading to more rapid development

of insect resistance, and applications tend to have moderate to low effectiveness in controlling pest insects (Benedict and Altman, 2001).

1.5 Environmental fate

Naturally-occurring *B. thuringiensis* cells and commercial *Bt* spray formulations have three possible fates in soil: (i) consumed by larvae, (ii) degraded by phyllosphere or soil microorganisms, or (iii) destroyed by UV, heat and sunlight (Stotzky, 2000; Zwahlen et al., 2003). However, when *Bt* proteins bind to soil particles, they are moderately persistent, but are also rapidly inactivated in soils with a pH below 5.1 (EXTOXNET, 1996). *In vitro* studies with purified (Cry1Ab) proteins have shown that *Bt* insecticidal toxins bind rapidly and tightly to clays, humic acids, and clay-humic acid complexes. Bound toxins were also shown to retain their structure and insecticidal activity, and resist biodegradation (Crecchio and Stotzky, 1998; Stotzky, 2000, 2004). In water, *Bt* is effective for up to 48 h as it gradually settles out or adheres to organic matter. On plants, under normal sunlight, it has a half-life of 3.8 h. When *Bt* protein does not bind to soil, it is rapidly degraded by microbial exoenzymes and used as a source of carbon (C) and nitrogen (N) for microorganisms (Fiorito, 2008). Sunlight (UV) breaks down the proteins and rain washes them from the plants (Shelton et al., 2002; de Maagd et al., 2004; Benedict and Ring, 2004). In nature, *Bt* vegetative cells do not survive or grow well in the environment (Griego and Spence, 1978; Ignoffo and Garcia, 1978).

1.5.1 Environmental fate and persistence of Bt corn products

Bt corn has the same insecticidal traits as *Bt* microbial insecticides. However, there are some critical differences between them. Primarily, the insecticidal toxin from Bt corn

enters the soil by different pathways. For example, Bt corn expresses the Cry1Ab protein gene throughout the plant and throughout most of the growth cycle. The Cry1Ab protein has been shown to be introduced into soil through root exudates and via plant residue decomposition (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995; Koskella and Stotzky, 1997; Tapp and Stotzky, 1998; Crecchio and Stotzky, 1998; Stotzky, 2000; Lee et al., 2003; Stotzky, 2004).

Soil-bound proteins are protected against biodegradation and inactivation since microbial hydrolytic enzymes cannot get at the sites needed to break the bonds holding the molecule together. Several studies have reported that Cry1Ab protein added to soil binds to soil constituents within 30 min. Since roots are in constant contact with the soil, binding of the protein toxin to soil particles and their build-up and movement beyond the rhizosphere are possible. Clark et al. (2005) considered the soil fate of Cry1Ab protein a key parameter governing exposure of non-target organisms in the environment. Cry1Ab protein from transgenic corn can persist, accumulate and remain insecticidal in soil as a result of binding, thus, the concentrations in soil from Bt corn can be significantly greater than those introduced into soil by *Bt* microbial insecticides (Venkateswerlu and Stotzky, 1992).

Another difference is that the genes introduced in Bt corn express the protein in the active form, which does not require an insect midgut to solubilize the protoxin and activate specific protease enzymes to cleave the protoxin to an active form (Federici, 1999). Lastly, in studies cited by Monsanto, the Cry3Bb1 protein toxin in

YieldGard™ corn is expressed at concentrations higher than that of other Bt corn lines (EcoStrat, 2002).

1.5.2 Accumulation in soil

Corn is the highest acreage crop grown in the U.S., accounting for 80 million acres planted and 20% of total agricultural cropland (Scott and Pollak, 2005). As a result, there is a potential for Bt proteins to accumulate where commercial insect resistant transgenic corn is grown. Such accumulation may be potentially hazardous to non-target organisms. A second concern is that the analytical methods used to quantify Bt protein(s) in soil are actually designed to detect Bt protein in plant tissues. Commercial kits are not yet available for use with soils. Extraction of Bt protein from soil has proven to be difficult and extraction efficiencies have been relatively low (Palm et al., 1994; Head et al., 2002; Ahmad et al., 2005; Baumgarte and Tebbe, 2005; Shan et al., 2005; Ahmad, 2006; Prihda and Coates, 2008; Wang et al., 2006). The preferred method for extracting proteins from environmental matrices has been that of Palm et al. (1994), but a reliable, accurate and universal analytical method is lacking and has hindered the adequate quantification of these proteins in soils.

The introduction of the Cry3Bb1 gene into Bt corn (MON863) is a fairly new technology and there are few environmental studies in the literature to date that report on this genetic event. Laboratory and field studies have revealed differences in the persistence of Cry proteins in soils (Icoz and Stotzky, 2008). A summary of these results by Icoz and Stotzky (2008) indicates that the degradation and persistence of Bt protein in soils depend on microbial activity, soil type, pH, temperature, and other physicochemical and biological characteristics of the soil. In addition, the production of Bt protein in Bt crops varies with season, is different in different plant parts, and can be influenced by numerous environmental factors. These aspects of Bt protein

expression emphasize the importance of studying its production and persistence in soils under local climatic conditions with local varieties (Jehle, 2007; Icoz and Stotzky, 2008) and on a case-by-case basis. Not much is known about Cry3Bb1 protein concentrations in soils and, most notably, the environmental persistence of Bt crop-associated Bt proteins in soil is not well-described under NY conditions and merits investigation. This study serves as a model for studying the potential fate and effects of other transgenic technologies and products (biomolecules) that will eventually reach the soil and other environmental compartments in vegetation and root exudates (Stotzky, 2000).

1.6 Study objectives and hypotheses tested

In this study, I worked to resolve analytical issues, such as low recovery of Cry3Bb1 protein from soil, and measured Cry3Bb1 protein remaining in agricultural soils where field studies were conducted previously. My objectives were as follows.

Objective 1: Modify and optimize current protocols for extracting Cry proteins from soil and crop residues in order to increase extraction efficiencies and provide reliable and accurate quantification of Bt proteins in agricultural soils.

Objective 2: Investigate the effects of climate, crop and soil factors on the release of Cry3Bb1 and Cry1Ab proteins from Bt corn into rhizosphere soils and its persistence in one soil where Cry3Bb1 (MON863) corn was grown for three consecutive years.

Cry3Bb1 protein was extracted directly from Bt corn residues (leaves, cobs, stalks, and roots) to estimate its concentration in these tissues and follow its loss through decomposition in litterbags placed in field soils for two years. The Cry3Bb1 protein was also extracted from field soils in which MON863 was grown for three consecutive

years to estimate its persistence. Finally, Cry1Ab and Cry3Bb1 proteins were also extracted from rhizosphere soils sampled from cultivar field trials in five locations in NY State. Cry protein concentrations were measured in plant tissues and soil by extracting them in an alkaline solution and using a commercial kit (PathoScreen, Agdia, Elkhart, IN) to detect them by use of an enzyme-linked immunosorbent assay (ELISA).

1.6.1 Hypotheses

H1. Current methods used to quantify Cry proteins in soil seriously underestimate their concentrations.

H2. Cry proteins are released from roots and residues of transgenic Bt corn, but do not persist or accumulate in soils where these crops are grown.

H3. Bt corn hybrids vary in the amount of protein released by their roots into their rhizosphere at flowering.

H4. Soil texture affects the efficiency of Cry protein extraction and leads to differences in Cry protein residence time in soils.

H5. The Cry proteins Cry3Bb1 and Cry1Ab differ in their persistence in different soils.

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CHAPTER 2 Methods Development and Optimization of Extraction Methods

2.1 Abstract

Current methods used to extract Cry insecticidal proteins from soil fail to completely recover and do not reliably estimate Cry3Bb1 and Cry1Ab protein contents in soil. Chemical, physical and biological modifications to existing methods were explored by using the Palm et al. (1994) and/or Shan et al. (2005) extraction methods as the basis for optimizing Cry protein extraction from soil in order to achieve a robust, simple, rapid, effective and complete recovery of Bt Cry proteins from diverse NY State agricultural field soils. For this purpose, several site soils were spiked with known concentrations of Cry3Bb1 or Cry1Ab protein and repeatedly extracted with fresh extractant, until the bulk of extractable Cry protein was recovered. Several techniques were explored individually which resulted in low protein recovery. A combination of (i) the Palm extraction buffer (pH =8.80 for Cry3Bb1 and pH =10.50 for Cry1Ab), (ii) bead-beating with two, 1/8 inch, glass beads in a 2 ml Eppendorf tube containing 0.5 g soil, (iii) a 2 h incubation with Palm extraction buffer and (iv) using the Palm buffer to prepare the Cry protein standards yielded an accurate quantification of the Cry proteins, Cry3Bb1 and Cry1Ab, in NY field soils. The optimized Palm extraction method was found to be a more convenient, inexpensive, rapid and efficient technique to estimate unknown concentrations of Cry proteins in field soils.

2.2 Introduction

The extent of adsorption of the insecticidal proteins from Bt crops on soil particles is important for assessing the environmental risk associated with TC's (Pagel-Wieder et al., 2007). Methods for extracting proteins from soil aim to (i) achieve quantitative recovery of the protein from the complex matrix of soil particles and (ii) purify the extract in order to minimize interference with downstream analyses (Ogunseitan, 2006). Accordingly, optimal recovery of proteins from soil is crucial to accurately quantify protein concentrations in soil. However, proteins, unlike environmentally relevant metals and organic pesticides, undergo molecular conformational structural changes that contribute to their complex adsorption on soil surfaces (Quiquampoix and Burns, 2007). *In vitro* and *in situ* studies indicate that Cry1Ab protein released in root exudates and from corn biomass adsorb and bind rapidly (<30 min) on surface-active particles, such as clays and humic substances in soil (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995, 1998; Crecchio and Stotzky, 1998, 2001; Saxena and Stotzky, 2002; Lee et al., 2003; Zhou et al., 2005; Florito et al., 2007). Binding can be reversible or irreversible, has high specificity for the adsorbate, and usually involves only one layer of adsorbate (Stotzky, 1986; Fiorito et al., 2008). Consequently, proteins have a strong affinity for all types of surfaces found in soil. This affinity originates in the flexibility of the polypeptide chain and in the diversity of the 20 amino acids that are classified, on an electrical scale, as positively, neutrally or negatively charged and, on a hydrophobic scale, from polar to non-polar. These properties give rise to a large variety of interactions with soil surfaces. Thus, the adsorption of enzymes on mineral surfaces is a complex phenomenon (Quiquampoix and Burns, 2007). A survey of the peer-reviewed literature showed that Cry protein recovery from soil is poor (Table 2.1).

Table 2.1 Summary of published extraction efficiencies for Cry proteins from soil.

Protein	Recovery (%)	Reference
Cry1Ab	27 - 60	Palm et al. (1994)
	34 - 114	Baumgarte and Tebbe (2005)
	66 - 75	Shan et al. (2005)
	4 - 82	Wang et al. (2006)
Cry1Ac	24 - 39	Head et al. (2002)
	83 - 87	Shan et al. (2005)
Cry1F	82 - 112	Baumgarte and Tebbe (2005)
	46 - 92	Shan et al. (2005)
Cry3Bb1	17 - 66	Ahmad et al. (2005)
	15 - 49	Ahmad (2006)
	15 - 41	Prihda and Coates (2008)

Accordingly, protein-soil complexes have made the study of Cry proteins in soil difficult. Methods have been developed to follow the fate and persistence of various proteins in soil, but the quantity, fate and persistence of the Cry protein toxins in soil is still poorly known. Current methods cannot reliably extract Cry proteins from soil (Clark et al., 2005) and little has been published on the quantitative recovery of protein from soil (Palm et al., 1994). Therefore, current methods used to quantify Cry proteins in soil seriously underestimate their concentrations and, thus, hinder the environmental fate studies needed by U.S. regulatory agencies.

2.2.1 Extraction buffers and extraction techniques

Palm et al. (1994) and Shan et al. (2005) developed chemical extraction methods that have been used in studies to examine Cry protein behavior in soil both in laboratory and field trials. Extraction buffers have basically been designed to mimic conditions inside larval insect guts in order to solubilize soil-bound protein and disrupt hydrophobic and ionic interactions that have been found to be important in protein binding to soil (Palm et al., 1994). Extraction buffers are typically composed of high salt and moderate to high pH solutions that include a surfactant or detergent (Palm et al., 1994; Clark et al., 2005; Shan et al., 2005). The surfactant is analogous to the insect gut fluid that is capable of solubilizing the protein from soil (Clark et al., 2005). The primary role of surfactants or detergents is to form hydrophobic interactions, through micelles, that effect protein solubilization (Neubauer, 1990). The surfactants used to extract Cry proteins from soil are Tween 20 and sodium taurocholate (Palm et al., 1994; Clark et al., 2005; Shan et al., 2005).

All protein extraction methods aim to achieve quantitative recovery of protein from the complex matrix of soil particles and to estimate the concentration of protein molecules in the extract (Ogunseitan, 2006). Therefore, an ideal procedure for recovering protein from environmental samples should meet several criteria (Hurt et. al., 2001). One, protein extraction from soil should give high yields of extracted protein (Nannipieri, 2006). Second, the extraction and purification protocol should be kept as simple as possible so that the recovery process is rapid and inexpensive. Third, the extraction and purification protocol should be robust and reliable; and should perform well on many diverse environmental samples (Hurt et. al., 2001). Most extracellular proteins in soil are associated with either the clay or organic colloidal fractions and the persistence and stability of proteins in soils can be attributed to their association with clays and humic acids (Nielson et al., 2006). In many instances, these

protein-soil complexes interfere with protein detection and quantification methods. Fourth, methods should not strip proteins of their active functions (Ogunseitan, 2006). Fifth, many techniques have been published for extracting protein molecules from soil but some case-by-case evaluation is necessary (Ogunseitan, 2006). Last, risk assessment studies for TC's should be performed under a variety of environmental conditions (Donegan et al., 1995).

One of the most common methods for breaking apart soil aggregates to release bound proteins is sonication. Sonication or ultrasound (typically 20-50 kHz) is the process of applying high frequency oscillation to a sample that results in cavitations and impaction, ultimately breaking apart soil aggregates and releasing any bound protein. However, sonication may potentially denature protein when too much heat is generated. Another common laboratory-scale mechanical method for disrupting the interaction between protein and soil particles is bead-beating. Bead-beating uses small ceramic, glass or steel beads and a high level of agitation by stirring or shaking the mixture. It works well for extracting DNA and releasing cells from soil and is inexpensive. Lastly, in order to enhance protein recovery and purify extracted proteins, the sample is extracted several times by repeated addition of extraction buffer and subsequent centrifugation to recover the supernatant.

Palm et al. (1994) reported the importance of considering ionic and hydrophobic interactions between Bt protein and soil particles to improve toxin protein recovery. Their findings showed that high pH, use of a surfactant and high concentrations of various salts were necessary to recover Bt protein from soil. Also, protein adsorption appeared to be reversible to a limited extent and was pH dependent. Knowledge of the isoelectric point (pI) of the protein is crucial for adjusting the pH of extraction solution to account for the desorption or the maximal amount adsorbed in the range of the

protein's pI (Nielsen et al., 2006; Palm et al., 1994). Fiorito et al. (2008) and Dubelman have reported the pI for several important Cry proteins: Cry3Bb1 = 5.90 and Cry1Ab = 5.50 (Table 2.2). Stotzky (1986), Boyd and Mortland (1990), Palm et al. (1994) and Fiorito et al. (2008) have suggested that ion exchange is the main mechanism of adsorption and that hydrophobic effects have also been shown to be important (Quiquampoix et al., 2002; 2007). Thus, in developing suitable extraction methods, the pH of the buffer should be far away from the pI of the Bt protein to achieve optimal protein recovery.

Table 2.2 The pH of soils from six field sites in NY and the pH of the isoelectric point for two Cry proteins.

Soil site / Protein	**pH**	**pI**
Cry3Bb1		5.90
Cry1Ab		5.50
Albion	6.23	
Aurora	7.71	
Avon	6.44	
New Hope	6.09	
Kingston	5.26	
Pittsford	5.17	
Scipio	6.94	

A summary of selected studies on the persistence of Bt proteins in the soil (Clark et al., 2005; Icoz and Stotzky, 2008) shows that measuring Bt protein concentrations in soils typically starts with a high pH extraction (Palm et al.,1994; Clark, 2005) followed by protein detection by use of the ELISA (Enzyme-Linked Immunosorbant Assay). However, current chemical extraction methods, such as the Palm buffer, are not satisfactory since extraction efficiencies are relatively low (27-60%) and underestimate their actual concentrations in soil (Clark et al., 2005).

Given the necessity for an efficient, accurate and a quantitative assay for the routine measurement of Bt protein in soil, Shan et al. (2005) developed a biomimetric approach using "artificial insect gut fluid" (AGF) that effectively extracted Bt protein from soil. Moreover, this approach correlated very well with insect bioavailability assays. However, the AGF extract has not yet been evaluated for its ability to extract Cry3Bb1 protein from soil.

2.2.2 Soil texture affects adsorption

There are many soil constituents to which Bt proteins can adhere or adsorb. Proteins could adsorb on pH independent surface-active clay mineral surfaces and/or bind to organic matter, which is another likely surface-active adsorption surface that is pH dependent. Other surface-active particles in soil are clay minerals coated with Fe-hydroxides or metal oxides, for which activity is also pH dependent. Tapp et al. (1994) found that adsorption on surface-active particles in soil is rapid (less than 30 min) and highly irreversible. Laboratory and field studies have shown that Cry1Ab proteins bind to clay minerals and humic substances because they possess high surface area and have a high cation exchange capacity (Sposito, 1984; McBride, 1994; Stotzky, 1986). Muchaonyerwa et al. (2004) found that the clay fraction in field soils absorbed more Cry1Ab protein than the silt fractions or bulk soils. Adsorption on montmorillonite

clay (M) was greater than on kaolinite clays (K) (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Lee et al., 2003). Moreover, the adsorption of Bt protein on humic acids increased with increasing amounts of humic acid (Crecchio and Stotzky, 1998) and was highest at pH values near the isoelectric point of the protein (Venkateswerlu and Stotzky, 1992; Crecchio and Stotzky, 2001). Chevallier et al. (2003) and Lee et al. (2003) reported that less than 10% of the adsorbed Bt protein could be desorbed and there was essentially no desorption of the Bt protein after extensive washing of organomineral complexes with double distilled water or sodium chloride (NaCl) (Crecchio and Stotzky, 2001; Pagel-Wieder et al., 2007). Therefore, the forces that bind Bt protein to soil particles appear to be ionic and hydrophobic interactions (Quiquanmpoix and Burns, 2007).

2.2.3 Immunoassay for Cry3Bb1

The state of the art biochemical technique for the analysis of Bt proteins in environmental matrices has been the ELISA. ELISA has been routinely used for biological analyses for many years because it is a rapid immunoassay that is considered to be reliable, less costly and time-consuming. ELISA also provides an alternative means for detecting and quantifying Cry proteins in environmental samples (Wang et al., 2007). Furthermore, it is a sensitive analytical method and has been proven to be the best quantification method for Bt proteins in environmental matrices when compared to other methods, such as flow cytometry, dot-blot techniques or HPLC (high pressure liquid chromatography) with UV detection (Clark et al., 2005).

In order to utilize ELISA, polyclonal antibodies are developed by immunizing rabbits or goats with a specific Cry protein. The animal's immunological response generates polyclonal antibodies, which are then extracted and purified. Purified polyclonal antibodies are coated onto test wells of a microplate to develop a direct Double-

Antibody Sandwich (DAS) ELISA. In this test, a sample is added to the plate, along with an enzyme conjugate containing polyclonal antibodies specific to the Cry protein. If the specific Cry protein is present in the sample, the antibodies bind to it and are captured on the microplate. The plate is then washed to remove any unbound protein and enzyme conjugate. Tetramethyl benzidine (TMB) is then added to each well. If peroxidase conjugate is present, a color will develop signifying the presence of Cry protein (Agdia, 2007). Moreover, Cry protein can be quantified from a sample when purified Cry protein is used to prepare a standard curve. A commercial DAS-ELISA kit was used in this study (Agdia, Elkhart, IN). However, none of the commercial DAS-ELISA kits available were designed to detect Cry protein in soil. Protocol modifications were made to enable detection and quantification of Cry protein in soil.

2.2.4 Study objective: Modifying and optimizing extraction method(s)

Current methods used to extract Cry insecticidal proteins from soil fail to completely recover and do not reliably estimate Cry protein contents in soil (Palm et al., 1994; Sims and Holden, 1996; Hopkins and Gregorich, 2003; Clark et al., 2005). The objectives of this study were to evaluate current Cry protein extraction methods and then to optimize these methods to achieve a robust, simple, rapid, effective and complete recovery of Cry protein(s) from diverse NY State agricultural field soils.

Rhizosphere soil samples were gathered from corn varietal field trials in five locations in NY State where transgenic corn transformed by event MON863 (Cry3Bb1) and MON810 (Cry1Ab) and their non-transgenic parental lines were grown. Site soils differed in their type and texture and sites differed in their seasonal climate.

In order to improve Cry protein recoveries from these soils, various protocol modifications were investigated. Since protein adsorption to soil is influenced by factors such as soil type, pH, temperature, and other physicochemical and biological

characteristics, these variables were modified and a variety of chemical and mechanical techniques was examined. Chemical modifications included evaluating the effects of changes in pH, extraction buffers and preparations of standards in selected extraction buffers. Mechanical techniques included sonication and bead-beating. Temporal modifications included changes in incubation and extraction times and in sample plate incubation time.

2.3 Materials and Methods

2.3.1 Soils

Characteristics of the seven soils used for spike-and-recovery studies are given in Table 2.3. Soil properties were determined by standard protocols.

Table 2.3 Textural classification, pH and particle size distribution for the seven NY soils tested.

Site	Classification	pH	Sand (%)	Silt (%)	Clay (%)
Albion	clay-loam	6.23	37	31	32
Aurora	clay-loam	7.71	33	35	32
Avon	silty-clay	6.44	4	41	55
Kingston	loam	5.26	49	31	20
New Hope	clay-loam	6.09	37	31	32
Pittsford	clay-loam	5.17	35	31	34
Scipio	clay-loam	6.94	41	31	28

2.3.2 Spike-and-recovery of Cry3Bb1 and Cry1Ab from test field soils

2.3.2.1 *Soil description and Cry protein spike-and-recovery*

In order to determine the efficiency of Cry3Bb1 and Cry1Ab protein extraction from the different soils, soil samples were taken from non-transgenic corn plots at each site (Table 2.3). Each soil was spiked with purified Cry3Bb1 protein in solution at different concentrations (0, 3, 10, and 80 ng g^{-1} soil) and the efficiency of different extraction methods to recover the spiked protein was evaluated (Figure 2.1).

The Cry3Bb1 protein solutions were vortexed for 1 min to distribute the protein uniformly, added to soil, vortexed again and incubated overnight at 4ºC. The samples were vortexed again the following day, then centrifuged and the supernatants removed. Cry3Bb1 and Cry1Ab protein concentrations in the sample supernatants were determined using a DAS-ELISA and PathoScreen kit for each protein type (Agdia, Elkhart, IN). The supernatant (100 µl per test well), along with enzyme-conjugate (100 µl per test well), was dispensed into an ELISA plate pre-coated with antibodies directed to the Cry protein of interest. The plate was incubated for 2 h at room temperature in a humid box to prevent evaporation of the samples. When the incubation was complete, the contents of the test wells were emptied with a quick flipping motion and washed 6 times by adding 1x PBST (phosphate-buffered saline-Tween 20) solution. After the washing step, the plates were tapped firmly on paper towels in order to remove any remaining liquid. Then, 100 µl of tetramethyl benzidine (TMB) substrate solution was dispensed into each test well and the plate incubated for 20 min.

2.3.2.2 *Soil protein extraction*

Corn tissue to buffer ratios used in commercial detection kits are 1:10 (w/v). For this study, the soil to buffer ratio used was 1:2 (w/v).

2.3.2.3 *Cry protein quantification*

Cry3Bb1 protein concentration in the supernatant(s) was determined by measuring optical density on a spectrophotometer and comparing readings against a standard curve developed by using purified Cry3Bb1 protein prepared in the same buffer. The optical density of the samples was determined by a V_{max} enzyme kinetic microplate reader (μQuant- Bio-Tek Instruments, Inc., KC Junior software) set at 620 nm. Purified Cry3Bb1 protein was provided by Monsanto Company (St. Louis, MO) at 83% purity. A five-point standard curve was established using purified Cry3Bb1 protein and linear regression and subsequently used to estimate the Cry3Bb1 protein in supernatants from the soil recovery assays.

2.3.3 Optimization of extraction methods

2.3.3.1 *Screening of extraction buffers: Biomimetic approach vs. the Palm extraction method*

Two extraction buffers were prepared; one using the Biomimetic approach (artificial gut fluid, AGF) developed by Shan et al. (2005) and one using the extraction method developed by Palm et al. (1994). The AGF consisted of 0.088 *M* NaCl (sodium chloride), 0.006 *M* Na_2SO_4 (sodium sulfate), 0.002 *M* KCl (potassium chloride), 0.003 *M* $CaCl_2$ (calcium chloride), 0.26 *M* $MgCl_2$ (magnesium chloride), 0.0033 *M* sodium taurocholate and 2.5 g bovine serum albumin (BSA) in 250 ml dH_2O. The AGF was adjusted to pH 9.00 or pH 10.50 using sodium hydroxide (NaOH). The Palm extraction buffer consisted of 50 m*M* sodium borate, 0.75 *M* KCl, 10 m*M* ascorbic

acid and 0.075% Tween 20 in 1.0 L dH_2O. The Palm extraction buffer was adjusted with NaOH to pH 9.00 and pH 10.50.

2.3.3.2 *Evaluation of pH on extraction efficiency using Palm buffer*

Buffers were prepared to extract Cry3Bb1 protein from soil using the Palm extraction method (Palm et al., 1994). The Palm extraction buffer consisted of 50 m*M* sodium borate, 0.75 *M* KCl, 10 m*M* ascorbic acid and 0.075% Tween 20 in 1.0 L dH_2O. The Palm extraction buffer was adjusted with NaOH to pH 7.50, pH 9.00 and pH 10.50. Two soil types, Aurora and Pittsford, were evaluated for Cry protein recovery. Each soil was spiked with purified Cry3Bb1 protein at different concentrations (0, 10 and 80 ng g^{-1} soil).

2.3.3.3 *Evaluation of incubation/extraction time using Palm buffer: 2 h vs. overnight*

The Palm extraction buffer (Palm et al., 1994) was prepared and used to extract Cry3Bb1 or Cry1Ab protein from various NY soils. Seven soil types: Aurora, New Hope, Pittsford, Albion, Avon, Kingston and Scipio were evaluated for protein soil recovery efficiency. Each soil was spiked with purified Cry3Bb1 or Cry1Ab protein at varying concentrations (0, 10, and 80 ng g^{-1} soil).

2.3.3.4 *Evaluation of the effects of sonication, bead-beating and incubation time on the recovery of Cry3Bb1 and Cry1Ab protein from soil using the Palm buffer*

The Palm extraction buffer (Palm et al., 1994) was used to extract Cry3Bb1 or Cry1Ab protein from soil. Three NY soil types that differed in their texture, Aurora, New Hope and Pittsford, were evaluated for Cry protein recovery efficiency. Each soil was spiked with purified Cry3Bb1 or Cry1Ab protein at different concentrations (0, 10 and 80 ng g^{-1} soil). Sonicated samples were placed into 1 L of dH_2O and sonicated for

10 min. After sonication, the soil solution was transferred to Lysing Matrix E tubes (FastDNA® SPIN kit for soil, QBiogene, MP Biomedicals, OH) for bead-beating and mixed at full speed for 1 min using a standard mini-vortexer.

2.3.3.5 *Evaluation of the effect of autoclaving using AGF buffer on Cry3Bb1 and Cry1Ab protein recovery from soil*

The Palm extraction buffer (Palm et al., 1994) was used to extract Cry3Bb1 and Cry1Ab protein from soil. Seven NY soil types, Aurora, New Hope, Pittsford, Albion, Avon, Kingston and Scipio were sterilized by autoclaving at 126°C for 30 min. Sterilized and non-sterilized soils were evaluated for protein recovery efficiency. Each soil was spiked with purified Cry3Bb1 or Cry1Ab protein at different concentrations (0, 10 and 80 ng g^{-1} soil).

2.3.3.6 *Evaluation of the effect of combining protein plate incubation for 2 h and undergoing treatment with bead-beating using Palm extraction buffer*

The Palm extraction buffer (Palm et al., 1994) was used to extract Cry3Bb1 and Cry1Ab protein from soil. To determine the efficiency of Cry3Bb1 and Cry1Ab protein extraction, soil was sampled from a non-transgenic corn plot and evaluated for protein recovery efficiency. Each soil was spiked with purified Cry3Bb1 or Cry1Ab protein at different concentrations (0, 10, and 80 ng g^{-1} soil) and vortexed to distribute the protein uniformly. Spiked soils were incubated for 30 min at room temperature. A 1000 µl aliquot of Palm extraction buffer was dispensed into a 2 ml Eppendorf tube containing 0.3 g soil and two, 1/8 inch glass beads and incubated for 30 min. Samples were then homogenized at full speed for 1 min using a standard mini-vortexer with a tube adapter. The suspension was then separated by centrifuging at 10,000 x g for 2 min after which, the supernatant was removed. The soil was extracted twice more and supernatants from each extraction were analyzed separately.

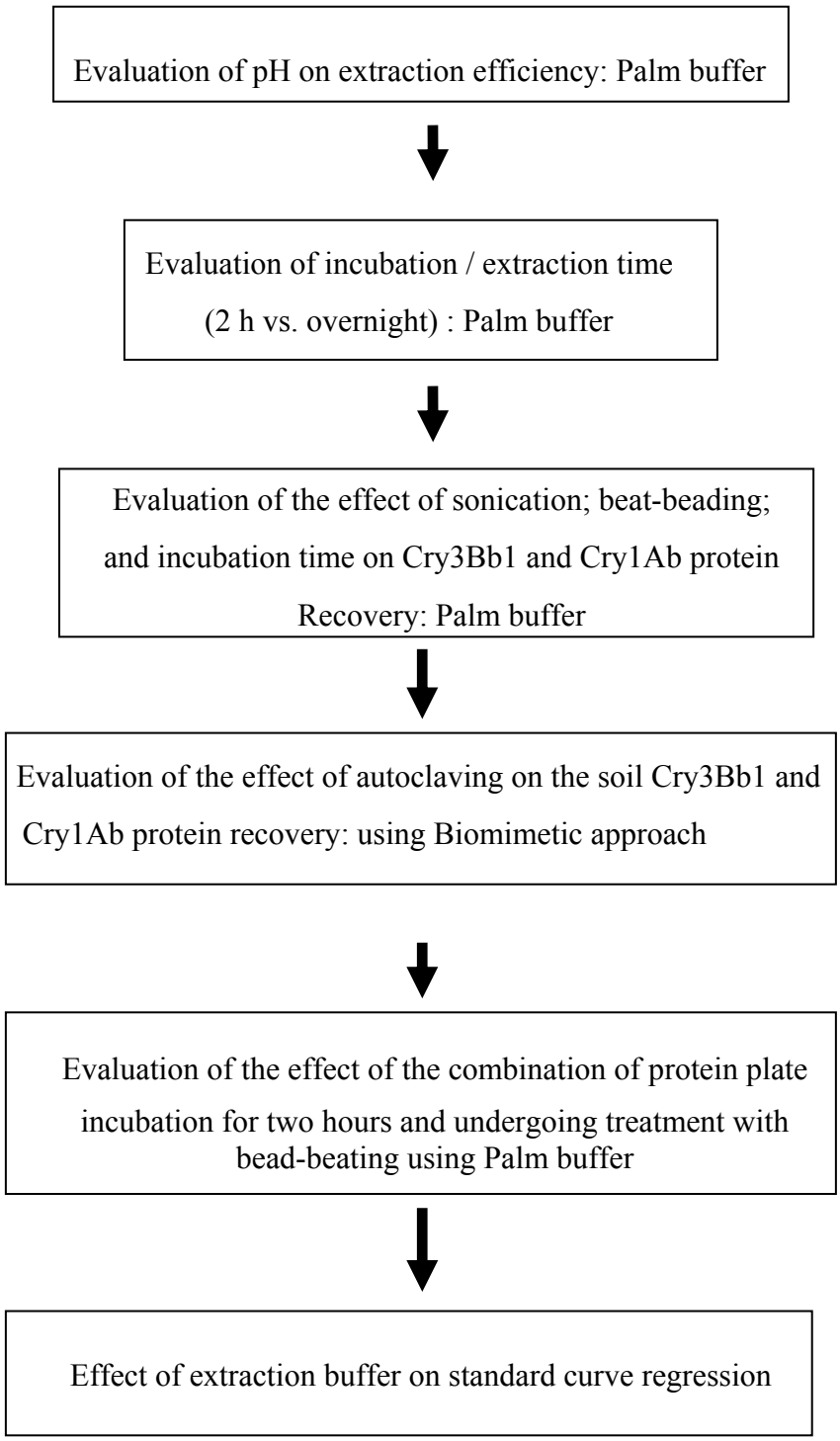

Figure 2.1 Schematic summary of the methods development and optimization of extraction methods.

2.3.4 Statistical analysis

The data are expressed as percent (%) protein recovery. Generalized least-square fit and analysis of variance (ANOVA) were used to examine significance of differences between treatments, where $p < 0.05$ was considered significant. All statistics were performed with the S-PLUS® 8.0 software package (Insightful Corp.).

2.4 Results

2.4.1 Soils

In general, the pH of the soils at the different sites ranged between 5.26 (Kingston) to 7.71 (Aurora) and the clay content ranged between 20 and 55%. The soils at the different sites were mostly classified as clay-loams, except for one loam (Kingston) and a silty-clay soil (Avon).

2.4.2 Optimization of extraction methods

The optimal conditions for improved recovery of Cry protein from soil were determined for the seven soil types used in this study. An array of chemical and mechanical techniques was examined as summarized in Table 2.4.

2.4.2.1 *Determination of a linear standard curve*

Standard curves for determining Cry protein concentrations in sampled soils were developed using purified Cry3Bb1 protein. However, when standards in the range of 0.1 to 8.0 ng 100 μL^{-1} were used, a curvilinear, rather than a linear, relationship resulted (Figure 2.2). In order to remain in the linear range, only standards equal to and below 0.6 ng 100 μl^{-1} were used (Figure 2.3).

Table 2.4 Summary of procedures to develop an optimal extraction method for the recovery of Cry protein from soil.

Procedure	Method	Variable modified
1	Biomimetic vs. Palm	Extraction buffer screening
2	Palm extraction	pH
3	Palm extraction	Incubation/extraction time
4	Biomimetic extraction	Sterilization (autoclaving)
5	Biomimetic extraction	Sonication, bead-beating, incubation time, and all 5 variables combined.
6	Palm extraction	Incubation time and bead-beating

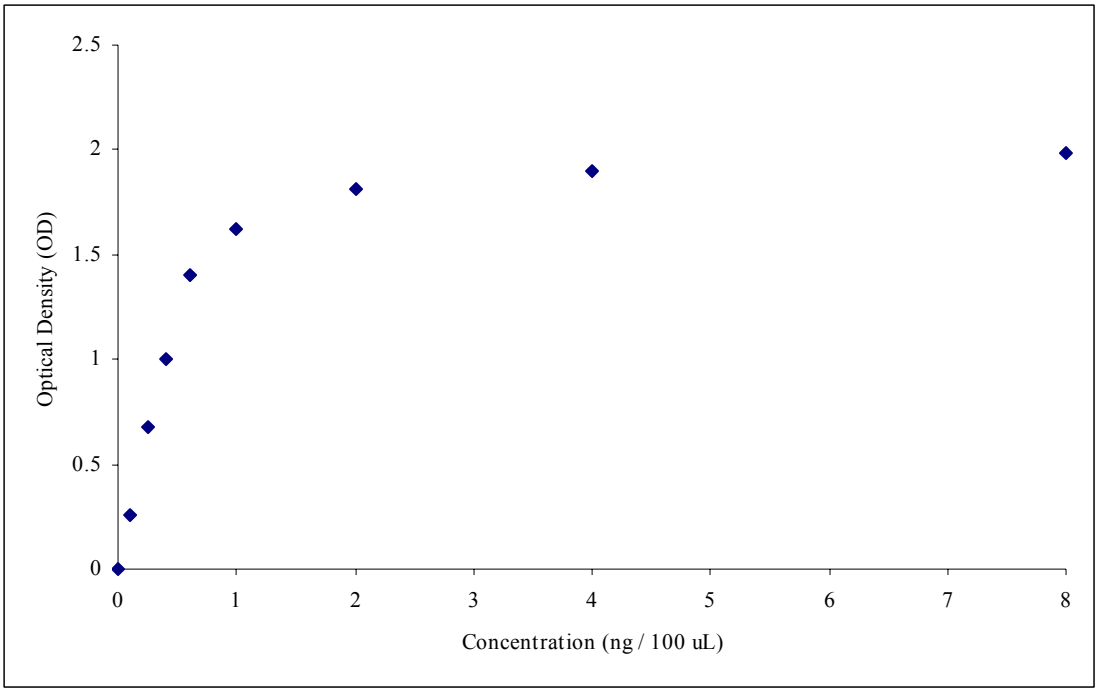

Figure 2.2 Curvilinear standard curve for quantifying Cry3Bb1 protein in soil.

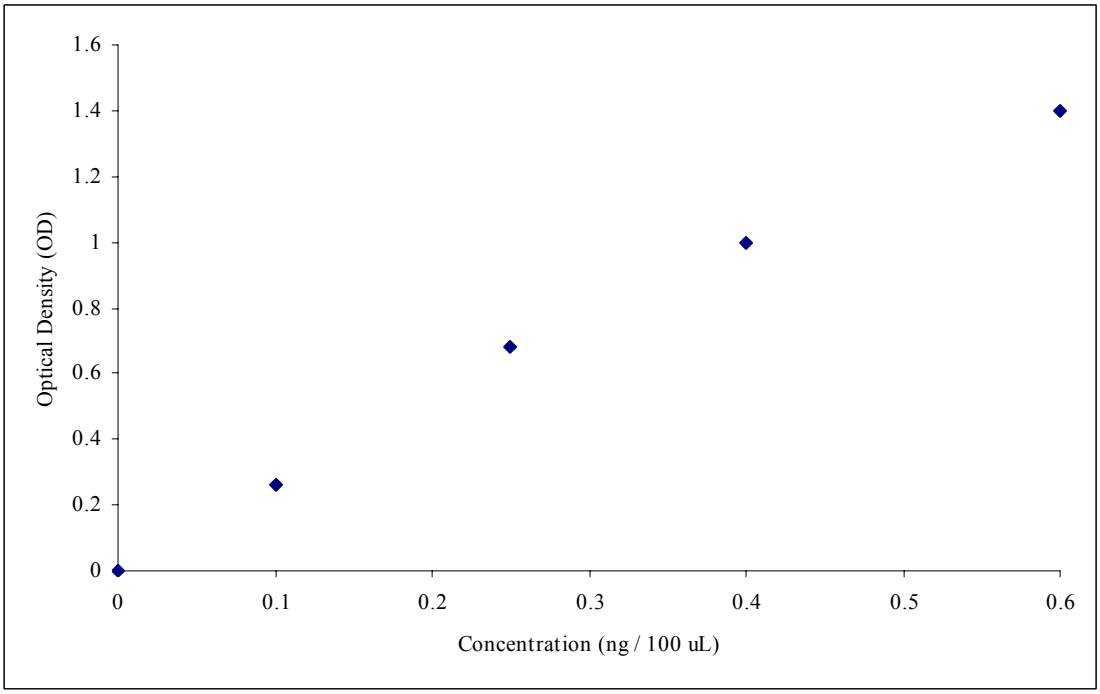

Figure 2.3 Corrected linear standard curve for quantifying Cry3Bb1 protein in soil.

2.4.3 Recovery of extractable Cry proteins

2.4.3.1 *Screening of extraction buffers: Biomimetic approach vs. the Palm extraction method*

The Cry proteins in soil were detected by ELISA. The extraction buffers, AGF and Palm, were compared on the basis of % recovery of spiked Cry protein from the tested soils. There was no Cry3Bb1 protein detected in non-spiked soil or in blank samples. Cry3Bb1 protein recovered was highest, at 25% recovery, for Avon soil (55% clay content) spiked with 10 ng g^{-1} using the Palm extraction buffer at pH 9.00. Higher recovery of Cry3Bb1 protein was generally obtained with use of the Palm extraction buffer compared to the Biomimetic approach (AGF). Moreover, higher recovery of Cry3Bb1 protein was obtained for the lower concentration (10 ng g^{-1}) compared to 80 ng g^{-1} spiked Cry3Bb1 protein.

2.4.3.2 *Evaluation of pH on extraction efficiency using the Palm buffer*

Recovery rate increased with a decrease in the concentration of spiked Cry3Bb1 protein (Table 2.6). Furthermore, recovery rate increased when the pH of the extraction buffer was further away from the pI of Cry3Bb1 protein (pI=5.9). Aurora and Pittsford soils were spiked with 80 ng g^{-1} Cry3Bb1 protein; and the highest recovery of Cry3Bb1 protein was at pH 9.00 (1.41% and 9.14%, respectively). The highest recovery of Cry3Bb1 protein was in the Pittsford soil (34% clay content) at 10 ng g^{-1} at pH 7.50. There were no significant differences in recovery due to changes in buffer pH.

Table 2.5 Comparative extraction efficiencies for Cry3Bb1 for different extraction buffers.

		Palm method						AGF method			
Cry3Bb1 added g^{-1}		**0 ng**	**3 ng**	**10 ng**	**80 ng**			**0 ng**	**3 ng**	**10 ng**	**80 ng**
Soil site	**pH**					**Soil site**	**pH**				
					% recovery						
Kingston	9.00	0.00		7.17	0.32	Kingston	9.00	0.00		0.14	0.04
Avon	9.00	0.00		24.49	0.21	Avon	9.00	0.00		0.14	
Aurora	10.50	0.00	6.61			Aurora	10.50	0.00	1.47		
Kingston	10.50	0.00		12.93	14.68	Kingston	10.50	0.00		4.59	8.59

Table 2.6 Effect of pH on extraction efficiencies for Cry3Bb1 protein on soil.

		Soil spiked with Cry3Bb1 (g^{-1} wet soil)		
pH	**Soil site**	**0 ng**	**10 ng**	**80 ng**
		% recovery		
7.50	Aurora	0.00	1.82	1.25
9.00	Aurora	0.00	1.82	1.41
7.50	Pittsford	0.00	11.84	6.18
9.00	Pittsford	0.00	0.00	9.14
10.50	Pittsford	0.00	0.00	2.45

2.4.3.3 *Evaluation of incubation/extraction time using Palm buffer: 2 h vs. overnight*

A longer incubation time for extraction resulted in a greater percent recovery of Cry3Bb1 protein (Table 2.7). Higher recovery rates were observed for lower concentrations of Cry3Bb1 spiked. Highest recoveries were noted in soils containing lower clay content (Kingston soil - 20% clay). No significant differences were reported for incubation / extraction time. Significant differences were reported for soils at Aurora ($F=7.61$; $df=6$; $\rho=0.0218$), Kingston ($F=7.61$; $df=6$; $\rho=0.0016$), and New Hope ($F=7.61$; $df=6$; $\rho=0.0145$).

Table 2.7 Effect of incubation / extraction time (two hours vs. overnight) on extraction efficiencies for Cry3Bb1 protein on soil.

	Two hours			Overnight		
	Soil spiked with Cry3Bb1 (g^{-1} wet soil)					
Soil site	**0 ng**	**10 ng**	**80 ng**	**0 ng**	**10 ng**	**80 ng**
	% recovery					
Albion	0.00	0.00	0.00	0.00	0.30	0.89
Aurora	0.00	3.64	1.91	0.00	6.91	2.55
Avon	0.00	0.20	0.50	0.00	0.10	0.45
Kingston	0.00	9.71	4.31	0.00	8.82	6.04
New Hope	0.00	4.73	3.18	0.00	6.91	4.68
Pittsford	0.00	4.00	2.09	0.00	3.27	1.91
Scipio	0.00	4.95	0.40	0.00	0.89	0.59

For Cry1Ab protein, no differences were observed between the 2 h and overnight incubations, except for Kingston and Scipio soils, which had substantially higher % recoveries after the overnight incubation. Overall, protein recovery rates were less than 4% (Table 2.8). Highest recoveries were again noted in the soil with the lowest clay content (Kingston soil - 20% clay). There was no significant effect of incubation-extraction time.

Table 2.8 Effect of incubation-extraction time (2 h vs. overnight) on extraction efficiencies of Cry1Ab protein from soil.

	2 h			Overnight		
	Soil spiked with Cry1Ab (g^{-1} wet soil)					
Soil site	**0 ng**	**10 ng**	**80 ng**	**0 ng**	**10 ng**	**80 ng**
	% recovery					
Albion	0.00	2.26	6.24	0.00	2.02	4.79
Avon	0.00	1.82	3.15	0.00	1.35	3.49
Kingston	0.00	7.18	12.51	0.00	27.79	17.89
Scipio	0.00	0.15	1.92	0.00	1.03	2.73

2.4.3.4 *Evaluation of the effect of autoclaving using AGF extraction buffer on the soil Cry3Bb1 and Cry1Ab protein recovery*

Soils were autoclaved to evaluate the effect of sterilization on the recovery of spiked Cry3Bb1 protein from soil using the Biomimetic extraction method. Only Kingston soil showed any recovery in non-sterilized soil, given the low clay content of this soil. In general, recovery of Cry3Bb1 protein from autoclaved and non-autoclaved soil was poor and below 4%. No significant difference was observed between non-sterilized and sterilized soil when dosed at 10 ng g^{-1}. However, average values from non-sterilized soils were observed to have a slightly greater portion of protein recovery compared to average values from sterilized soils (0.50 vs. 0.27 ng g^{-1}). This treatment did not improve protein recovery from soils (Table 2.9).

Table 2.9 Effect of sterilized (autoclaving) soil vs. viable (non-sterilized) soil on extraction efficiencies for Cry3Bb1 protein on soil using Biomimetic approach (AGF extraction buffer prepared at pH 7.20).

	Non-sterilized			Sterilized		
	Soil spiked with Cry3Bb1(g^{-1} wet soil)					
Site soil	**0 ng**	**10 ng**	**80 ng**	**0 ng**	**10 ng**	**80 ng**
	% recovery					
Albion	0.00	0.00	0.00	0.00	0.00	0.00
Aurora	0.00	0.00	0.00	0.00	0.00	0.00
Avon	0.00	0.00	0.00	0.00	0.00	0.00
Kingston	0.00	3.92	3.27	0.00	0.00	0.89
New Hope	0.00	0.00	0.00	0.00	2.04	0.42
Pittsford	0.00	0.00	0.00	0.00	1.66	0.47
Scipio	0.00	0.00	0.00	0.00	0.00	0.10

In general, recovery of Cry1Ab protein from autoclaved and non-autoclaved was poor (below 5.9%), but was slightly higher than for Cry3Bb1. Average values from sterilized soils were observed to have a slightly greater portion of protein recovery compared to average values from non-sterilized soils (2.49 vs. 1.31 ng g^{-1}). However, this treatment did not enhance or improve protein recovery from soils (Table 2.10) since the treatments was not significantly different.

Table 2.10 Effect of sterilized (autoclaving) soil vs. viable (non-sterilized) soil on extraction efficiencies for Cry1Ab protein on soil using Biomimetic approach (AGF extraction buffer prepared at pH 7.20).

	Non-sterilized			**Sterilized**		
	Soil spiked with Cry1Ab (g^{-1} wet soil)					
Soil site	**0 ng**	**10 ng**	**80 ng**	**0 ng**	**10 ng**	**80 ng**
	% recovery					
Albion	0.00	2.63	2.30	0.00	2.63	2.96
Aurora	0.00	3.28	2.96	0.00	2.63	2.30
Avon	0.00	3.28	1.97	0.00	5.91	4.60
Kingston	0.00	3.92	2.30	0.00	3.94	2.63
New Hope	0.00	0.00	2.30	0.00	3.28	2.96
Pittsford	0.00	0.00	2.30	0.00	5.25	4.27
Scipio	0.00	0.00	0.30	0.00	5.90	2.96

2.4.3.5 *Evaluation of the effect of sonication; bead-beating; and incubation time using AGF extraction buffer on soil Cry3Bb1 and Cry1Ab protein recovery*

Cry3Bb1 protein recovery was poor (less than 3%) from soils that were treated by sonicating; sonicating + bead-beating; or sonicating + bead-beating + increased incubation time. There were no significant differences between these treatments. Moreover, recovery rates were greater, on average, when 10 ng of Cry3Bb1 protein g^{-1} soil was added, compared to soils receiving 80 ng Cry3Bb1 protein g^{-1} soil when soils were sonicated or treated by sonicating + bead-beating + increased incubation time (Table 2.11).

Cry1Ab protein recovery was also poor (less than 3%) from soils that were treated by sonicating + bead-beating or a combination of sonicating + bead-beating + increased incubation time. Recovery rates increased with a decrease in the concentration of spiked Cry1Ab protein (Table 2.12). Recovery rates were greater at 10 ng Cry1Ab protein g^{-1} soil, compared to 80 ng Cry1Ab protein g^{-1} soil when soils were treated by sonicating, sonicating + bead-beating, or a combination of sonicating + bead-beating + increased incubation time (Table 2.12). These treatments had a greater effect on Cry1Ab protein recovery compared to Cry3Bb1 protein recovery, but were not significantly different from each other.

2.4.3.6 *Evaluation of the effect of the combination of protein plate incubation for 2 h and undergoing treatment with bead-beating using Palm extraction buffer*

Seven soils were used to evaluate the effect protein plate incubation for 2 h and treatment with two, 1/8 inch glass-beads had on Cry3Bb1 protein recovery (Table 2.13). Cry3Bb1 protein was extracted from the soils with Palm buffer at pH 8.80. Average protein recoveries ranged from 11 ± 8% to 97 ± 3% (Figure 2.4). Moreover, protein recovery for soils correlated well with percent clay content of the soils

Table 2.11 Effect of sonication; sonication + bead-beating; and sonication + bead-beating + incubation time on extraction efficiencies for Cry3Bb1 protein on soil (pH = 9.00).

	Sonication			Sonication + Bead-beating			Sonication + Bead-beating + incubation		
	Soil spiked with Cry3Bb1 (ng g^{-1} wet soil)								
Soil site	**0**	**10**	**80**	**0**	**10**	**80**	**0**	**10**	**80**
	% recovery								
Aurora	0.00	2.55	0.95	0.00	0.00	0.00	0.00	1.82	0.59
New Hope	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.36	0.36
Pittsford	0.00	0.73	1.05	0.00	0.00	0.90	0.00	1.09	0.45

Table 2.12 Effect of sonication; sonication + bead-beating; and sonication + bead-beating + incubation time on extraction efficiencies for Cry1Ab protein on soil (pH = 9.00).

	Sonication			Sonication + Bead-beating			Sonication + Bead-beating + incubation		
	Soil spiked with Cry1Ab (ng g^{-1} wet soil)								
Soil site	**0**	**10**	**80**	**0**	**10**	**80**	**0**	**10**	**80**
	% recovery								
Aurora	0.00	8.30	1.76	0.00	5.81	2.24	0.00	4.98	1.04
New Hope	0.00	4.10	2.07	0.00	6.69	1.58	0.00	4.15	0.10
Pittsford	0.00	10.79	2.39	0.00	10.79	2.54	0.00	4.15	0.83

Table 2.13 Effect of the combination of incubation time, bead-beating using Palm buffer and determination of the Limit of detection (LOD) (pH=8.80) on the soil Cry3Bb1 protein recovery spiked with different concentrations of the Cry3Bb1 purified protein.

	Soil spiked with Cry3Bb1 (ng g^{-1} wet soil)						
Soil site	**0.1**	**0.2**	**1.0**	**4.0**	**10.0**	**20.0**	**80.0**
				% recovery			
Albion	100	100	51	70	69	85	67
Aurora	ND	ND	25	31	38	42	40
Avon	ND	ND	ND	12	15	17	19
Kingston	100	100	100	100	100	100	80
New Hope	ND	ND	51	45	69	56	61
Pittsford	ND	ND	100	100	100	100	74
Scipio	ND	100	100	63	96	84	67

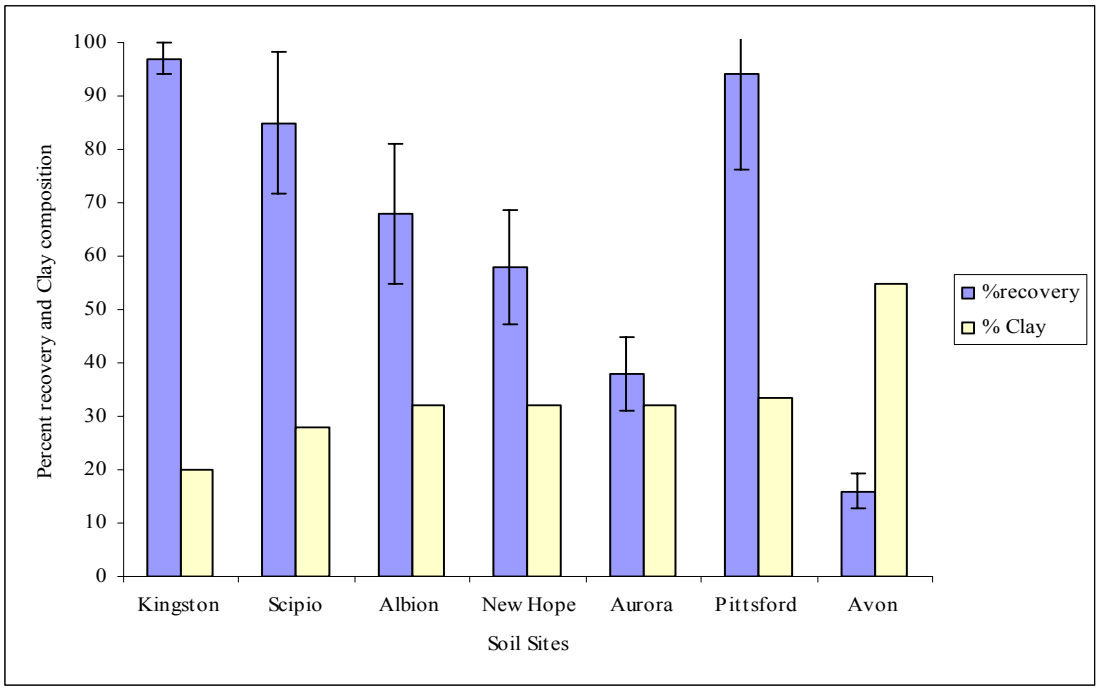

Figure 2.4 Percent (%) Cry3Bb1 Protein recovery and % clay composition of soil.

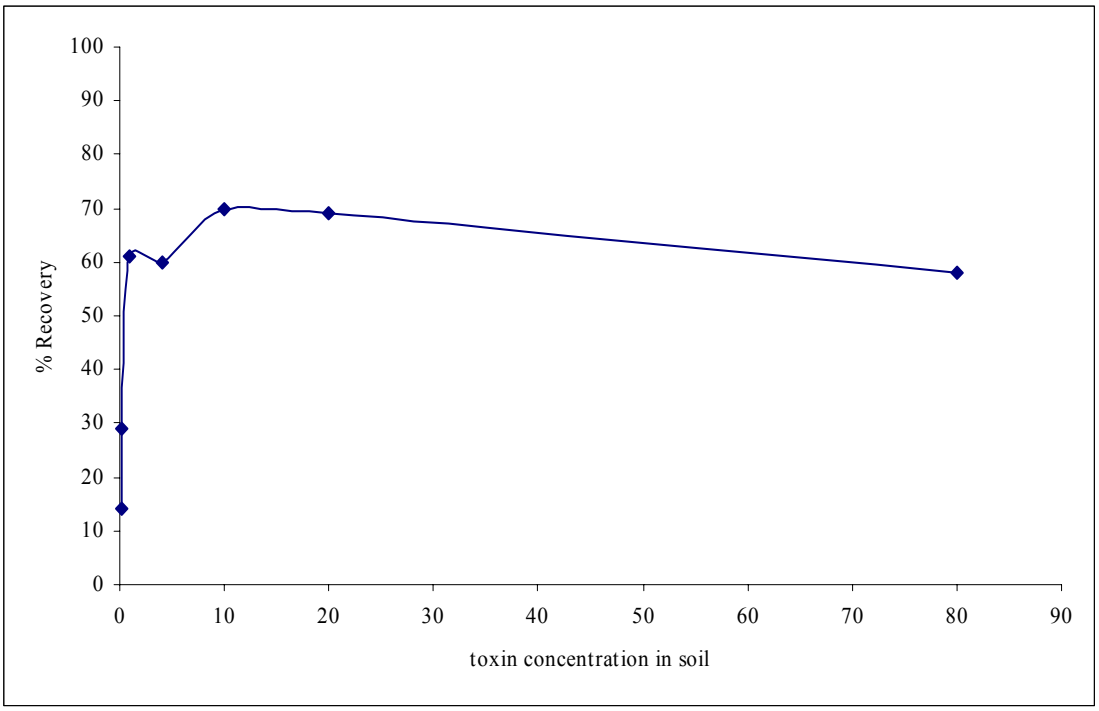

Figure 2.5 Percentage (%) recovery as a function of Cry3Bb1 concentration in soil.

(R^2=0.68) (see Appendix A). The limit of detection for this assay was 0.5 ng Cry3Bb1 protein g^{-1} soil. A decrease in protein recovery corresponded with a decrease in the amount of Cry3Bb1 protein spiked into the soils (Figure 2.5).

Seven soils were chosen to evaluate the effect protein plate incubation for 2 h and treatment with two, 1/8 inch glass-beads had on Cry1Ab protein recovery (Table 2.14). Cry1Ab protein was extracted with Palm buffer at a pH of 10.50. The average protein recoveries ranged from 40 ± 17% to 72 ± 15%. However, protein recovery from soil did not correlate well with percent soil clay content (R^2 = 0.3011) (Figure 2.6) (see Appendix A). The limit of detection for this assay was 0.5 ng Cry1Ab protein g^{-1} soil. A decrease in protein recovery corresponded with a decrease in Cry1Ab protein spiked into the soils (Figure 2.7).

Table 2.14 Effect of the combination of incubation time, bead-beating using Palm buffer and determination of the Limit of detection (LOD) (pH=10.50) on the soil Cry1Ab protein recovery spiked with different concentrations of the Cry1Ab purified protein.

	Soil spiked with Cry1Ab (ng g^{-1} wet soil)						
Site soil	**0**	**0.05**	**0.10**	**0.50**	**2.0**	**4.0**	**8.0**
	% recovery						
Albion	ND	100	100	100	100	50	50
Avon	ND	ND	100	100	100	40	40
Kingston	ND	ND	ND	100	100	54	54
New Hope	ND	ND	ND	100	100	34	43
Pittsford	ND	50	50	46	46	50	50
Scipio	ND	100	100	100	100	51	53

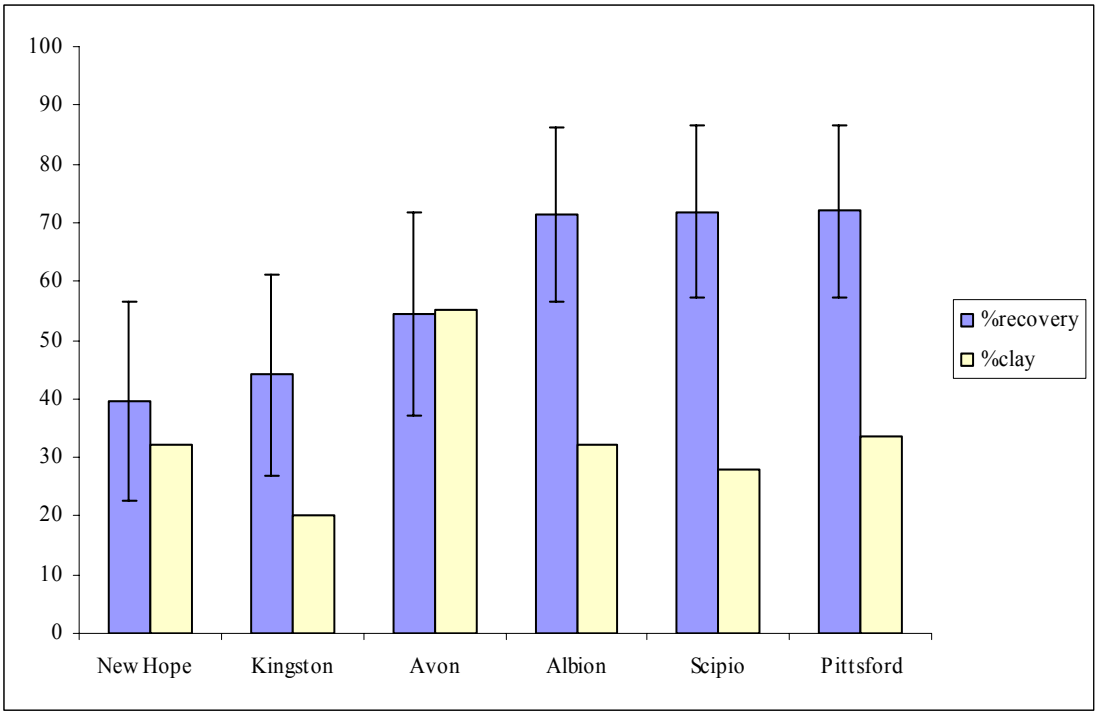

Figure 2.6 Percent (%) Cry1Ab Protein recovery in relation to % soil clay content.

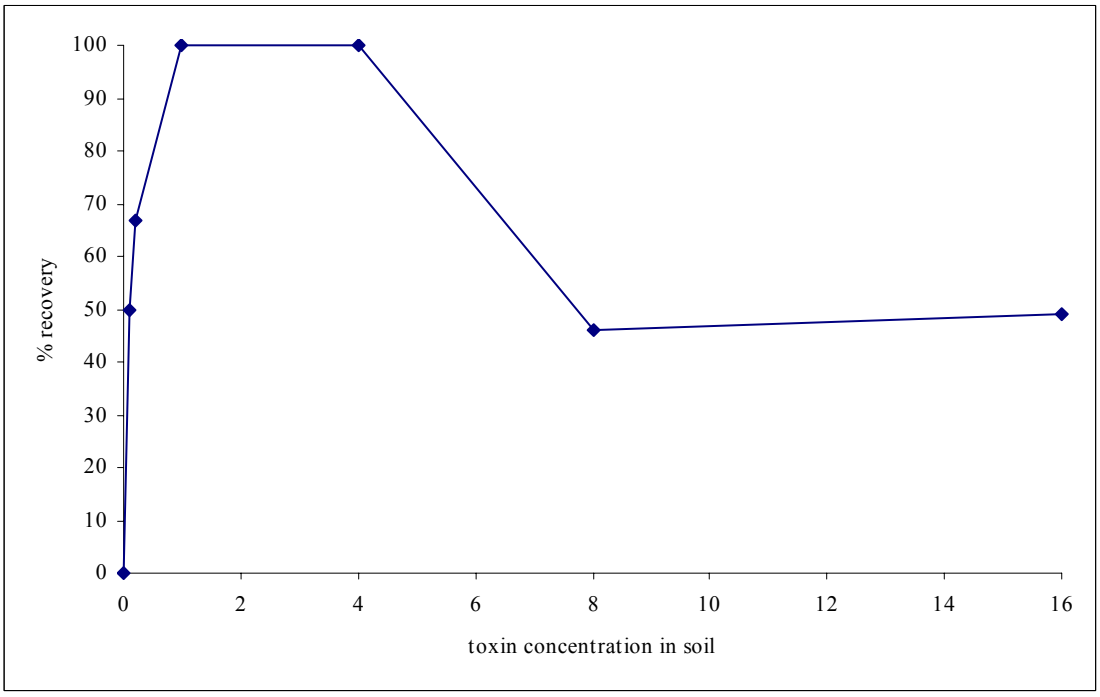

Figure 2.7 Percent recovery as a function of Cry1Ab protein concentration in soil.

2.4.3.7 *Effects of extraction buffer on standard curve regression analysis (correlation coefficient)*

Initially, PBST was used as the solvent for preparing a standard curve to estimate concentrations of Cry protein in recovery assays. This protocol produced a correlation coefficient of R^2= 0.9143 (Figure 2.4). However, when Cry3Bb1 protein was added to the Palm buffer, a correlation coefficient of R^2= 0.9801 was obtained.

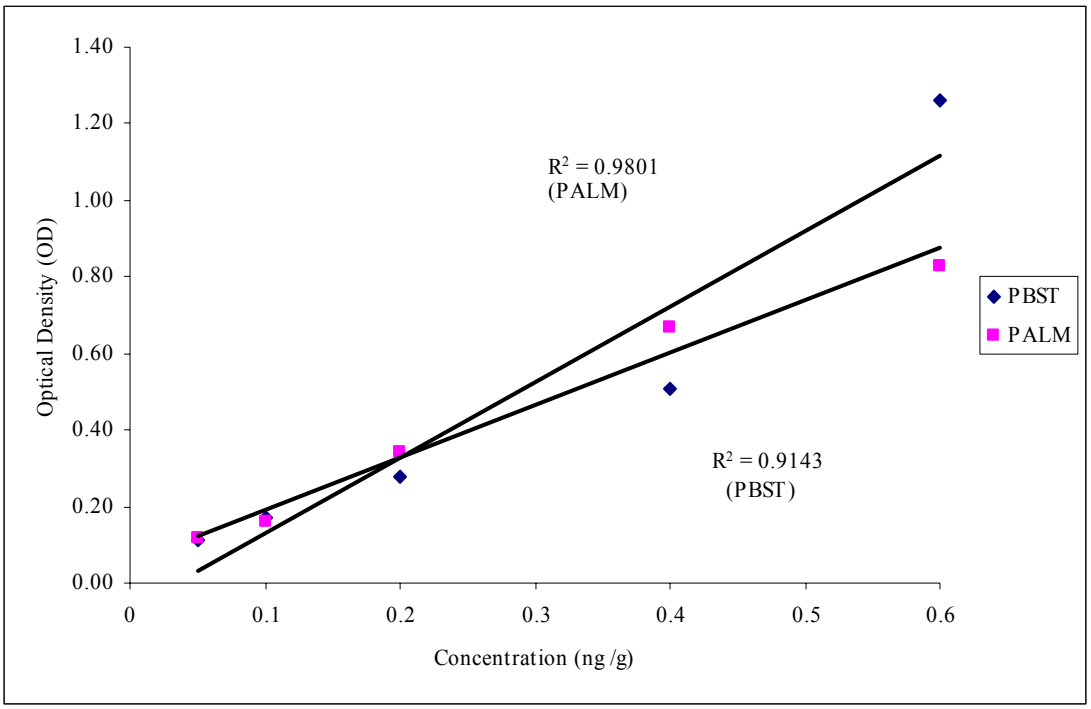

Figure 2.8 Effect of extraction buffer on standard curve regression analysis.

2.4.3.8 *Effect of percent (%) clay on regression analysis (correlation coefficient)*

Regression analysis was carried out to evaluate the relationship between % soil clay content and % recovery of Cry proteins from soil. Cry3Bb1 showed a stronger linear

relationship (R^2 = 0.836), which suggests that % clay in site soils may be a good predictor of % protein recovery from these different soil types. However, with Cry1Ab, there was a weaker linear relationship (R^2 = 0.3011), which suggests that % soil clay content in soils may not be a good predictor of % Cry protein recovery from these soil types.

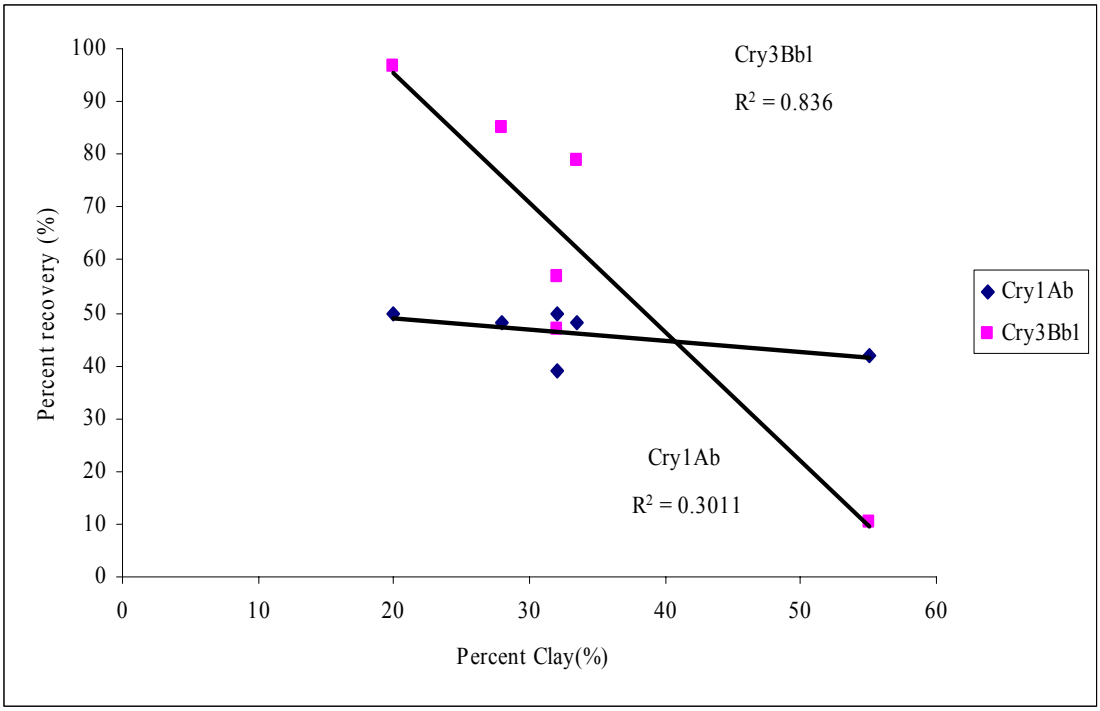

Figure 2.9 Relationship between percent recovery (%) as a function of percent clay content (%) in soil.

2.5 Discussion

ELISA is a sensitive analytical method and provides better quantification of Cry3Bb1 in environmental matrices than other methods; however recovery from soils is poor (27-60%) (Clark et al .2005). ELISA has been widely used as a qualitative and quantitative method for detecting Cry proteins in environmental samples (Shan et. al 2005). Commercial ELISA kits have been developed primarily for detecting Cry proteins in plant leaves and seeds (e.g., Agdia and Envirologix kits) and not in soil. Moreover, these kits do not quantify the Cry protein concentration, but indicate only the presence of the Cry protein in the sample. These limitations were overcome by modifying the ELISA kit protocol to quantify Cry3Bb1 protein in soil using known concentrations of purified Cry3Bb1 protein and modifying the extraction buffer developed by Palm et al. (1994). Poor soil recoveries were improved by modifications in chemical, physical and temporal factors, such as adjustments in buffer pH, plate incubation time and extraction time; the addition of glass beads; and correcting for irreversible adsorption. The limit of detection for quantifying Cry3Bb1 or Cry1Ab protein in soil was 0.5 ng Cry protein g^{-1} soil.

The optimized Palm extraction buffer was tested in seven different soil types spiked with Cry3Bb1 or Cry1Ab protein. The seven soils included in the study were from various geographical locations in NY State and had diverse properties that influence protein recovery, including pH (5.51 to 7.71) and varying soil textures (Table 2.3).

The adsorption of insecticidal Cry proteins from transgenic Bt corn on soil particles is important for assessing the environmental risk associated with TCs (Pagel-Wieder et al., 2007). Studies have shown that solid soil surfaces, such as humic substances and clay minerals, are the most important adsorbents of Cry1Ab protein in soils (Sposito, 1984; Stotzky, 1986). Moreover, Cry1Ab protein has a high affinity for solid soil

surfaces, is not readily desorbed, and percent recoveries are low (Clark, 2005; Stotzky, 2004). Muchaonyerwa et al. (2006) reported that adsorption increased with increasing amounts of clay minerals in soil Binding was also shown to be pH dependent and greatest near the isoelectric point (pI) of the proteins.

Considering these factors, poor soil recoveries were corrected by modifications in chemical, physical and temporal factors using the Palm et al. (1994) extraction buffer for protein extraction. The efficiency of extraction for Cry3Bb1 and Cry1Ab proteins appeared to depend on soil texture, pH and the chemical characteristics of the extraction buffer. The differences in the extraction efficiencies from different site soils were largely explained by soil texture. Low extraction efficiencies for certain soil types was related to the greater amounts of clay found in these soils (Avon = 55%, Aurora = 32% and New Hope = 32% clay content). Percent clay in soil was a good predictor of % Cry3Bb1 protein recovery (Figure 2.8). Soils containing high surface-active particles, such as clay, are already known to adsorb Cry1Ab protein (Crecchio and Stotzky, 2001). Soils with higher clay content also have a greater cation exchange capacity (CEC) than soils that are silty or sandy. This implies that soils with greater clay contents have more surface-area and more positions on surface-active sites to adsorb proteins. Accordingly, proteins have multiple contact points to bind to surface-active sites. Hence, variations in the optimal protein recoveries reported from the soils examined (Table 2.3) likely results from difference in soil texture. Optimal recoveries were improved over those of other studies reported in Table 2.1.

Another important factor controlling Cry protein adsorption to soil is pH. According to Chevallier et al. (2003), the adsorption of proteins is influenced by electrostatic interactions and is thus, pH dependent. Hence, adsorption of proteins to soil is greatest close to the isoelectric point of the Cry protein (Stotzky, 1986). A decrease in the

adsorption of Cry1Ab with an increase in pH was the result of the electrostatic repulsion between the negatively charged protein and the negatively charged surface (Pagel-Wieder et al., 2007). In effect, raising the pH of the extraction buffer solution above the isoelectric point of the Cry protein of interest causes a change to the net charge of the proteins, which become negative, thus disassociating the protein complex from the clay (Palm et al., 1994; Fiorito et al., 2008). Studies have shown that adsorption of Cry1Ab, Cry3Aa and Cry4 on clays decreased with an increase in pH (Palm et al., 1994; Tapp et al., 1994; Tapp and Stotzky, 1998; Crecchio and Stotzky, 2001; Lee et al., 2003).

One physical factor controlling adsorption of Cry protein to soil is soil aggregation. The use of bead-beating with glass beads was important for breaking apart the soil aggregates that form when root exudates containing Cry protein are released onto the rhizoplane or when purified Cry protein is spiked into soil microcosms or field soils. Quiquampoix and Burns (2007) stated that the rearrangement of the protein structure on the clay surface can subsequently be facilitated when hydrophobic amino acids come in contact with the hydrophobic siloxane layer and remain shielded from the water molecules of the solution. As a result, bead-beating with glass beads promoted the release or exposure of bound-Cry protein to the ionic solution and detergent. The combination could then promote disaggregation, increase exposure and then enhance solubilization with newly available Cry protein (Neugebauer, 1990; Shan et al., 2005).

There were some exceptions to improved recovery of protein from soil. Low recoveries were reported for Cry3Bb1 (16-58%) and Cry1Ab (39-50%). Many studies have demonstrated that Cry protein adsorbs to soil components, such as clay minerals, and that a portion is not readily desorbed (Venkateswerlu and Stotzky, 1992; Tapp and Stotzky, 1998; Tapp et al., 1994; Tapp and Stotzky, 1995; Koskella and Stotzky,

1997). Soil with more clay contents may have more surface active sites in which Cry proteins could adsorb. Furthermore, irreversible binding may be a result of multiple contact points between the macromolecules and the adsorbent surface (Theng, 1979; Pagel-Wieder et al., 2007). Theng (1979) stated that very limited desorption is characteristic of macromolecules because of their multiple binding sites and, once adsorbed, it is unlikely that all the segments will detach simultaneously (Pagel-Wieder et al., 2007). Depending on soil texture, binding can be reversible or irreversible, has a high specificity for the adsorbate, and usually involves only one layer of adsorbate (Stotzky, 1986). Other examples of poor protein recovery are shown in Figures 2.5 and 2.7. Protein recovery decreased with a decrease in the amount of both Cry3Bb1 and Cry1Ab protein spiked into soil (on average). Similar results were observed by Palm et al. (1994), where they suggested percent recovery was dependent on initial protein toxin concentration. They observed that lower initial concentrations of Cry protein resulted in lower percent recoveries when extracted. This lower recovery at lower concentrations spiked into soil could be a result of proteins at lower concentration having less competition for binding sites and greater electrostatic attractions with multiple binding sites.

Cry protein recovery from soil can be optimized fin order to accurately quantify Cry proteins (3Bb1 and 1Ab) in environmental samples, particularly in the field (Palm et al., 1994; Head et al., 2002; Baumgarte and Tebbe, 2005; Shan et al., 2005; Ahmad et al., 2005; Wang et al., 2006; Ahmad, 2006; Prihda and Coates, 2008). This study demonstrated that it was possible to overcome extraction difficulties and advance analytical methods to improve recovery of and quantify Cry proteins in agricultural soils (16-97% for Cry3Bb1 protein and 39-50% for Cry1Ab protein). Furthermore, these modifications are compatible for use with ELISA and allow protein concentrations to be estimated. This study has shown that the current chemical

extraction technique developed by Palm et al. (1994), coupled with the ELISA immunological method can be modified to yield improved recoveries, which account for the % Cry protein that is irreversibly retained by the soil matrix and cannot be extracted. Moreover, the high recoveries obtained by use of the method developed in this study was not compromised by other Cry proteins in the soils used, a problem that occurs with the use of bioassays (Shan et al., 2005). Lastly, chemical methods are still the least expensive analytical methods for quantifying Cry protein concentration in soil, once extraction difficulties are overcome, and can be used with confidence with field soils.

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CHAPTER 3 Persistence of Cry3Bb1 protein in crop residues and presence of Cry3Bb1 and Cry1Ab protein in soils planted to Bt corn

3.1 Abstract

There are few published, quantitative estimates of Bt protein load in soil and little is known about Cry3Bb1 protein concentrations in soils. Most notably, the environmental persistence of Bt crop residues and Cry proteins in soil is not well described under NY State conditions and merited investigation. In this investigation, I employed an improved analytical extraction method to better understand the effects of climate, and crop and soil factors on the release of Cry3Bb1 and Cry1Ab proteins from Bt corn roots into rhizosphere soils and their persistence in one soil where Cry3Bb1 (MON863) corn was grown for three consecutive years. Rhizosphere soils were collected from fifteen different Bt corn varieties at mid-season and concentrations of Cry proteins in them were quantified. Data gathered were averaged and the effects of soil site, construct, variety (hybrid) and soil texture on Cry protein recovery were compared. Data on Cry protein contents of soil are needed to determine the potential loads in soil to which non-target organisms may be exposed. The production and release of Cry3Bb1 protein in rhizosphere soil is common and widespread as determined in this study under NY State field conditions, and the Cry3Bb1 protein does not appear to accumulate or persist in soil in concentrations that might constitute a hazard to beneficial soil-dwelling organisms. This is the first field study reporting the presence of Cry3Bb1 protein, mid-season, in rhizosphere soils of different Bt corn varieties. More importantly, Cry3Bb1 protein was accurately quantified in soil and did not accumulate or persist in soils under NY conditions from

mid-season to after harvest in the same growing year. These results suggest that under various NY State climatic, crop and soil conditions, that there is a low level of hazard to most groups of non-target organisms, since there is little to no potential for Cry3Bb1 proteins to accumulate or persist in rhizosphere soils.

3.2 Introduction

Environmental risk assessment of transgenic crops (TCs) remains the subject of many studies, especially the persistence and effects of insecticidal proteins from Bt crops (Clark et al, 2005). There are clearly many beneficial effects from growing transgenic crops that contribute to development of sustainable agricultural systems. One, they have been shown to provide an environmentally safe and effective control of certain insects (USEPA, 2001). Their commercialization and use has resulted in a reduction in pesticide use and, thus, a decline in the impact of conventional chemical insecticides on the environment, non-target organisms and human health (James, 2007; Vaugh et al., 2001). Second, Bt crops increase profits from increases in productivity (Duffy, 2000; Mitchell, 2002) and yield (Betz et al., 2000). Farmers save money when they do not apply wide spectrum insecticides to control corn root worm (CRW) (Shelton et al., 2002) or European corn borer (ECB). Yet, the short- and long-term consequences of commercial Bt transgenic corn use on agricultural land is not well known (i.e., exposure and hazard). Field study data are limited in regard to information on soil Cry3Bb1 protein levels, especially under NY State climatic conditions.

3.2.1 Environmental fate studies

A major introduction of Cry protein into soil occurs during pollen deposition (Losey et al., 1999), and during the vegetative period of Bt corn growth as a result of root exudation and root senescence (Saxena et al., 1999, 2002; Saxena and Stotzky, 2000).

Studies have also reported the release of Cry protein during and after harvest (Saxena and Stotzky, 2000; Zwahlen et al., 2003). Furthermore, Cry1Ab protein is rapidly adsorbed and bound on clay minerals and humic substances in soil (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995a,b, 1998; Koskella and Stotzky, 1997; Crecchio and Stotzky, 1998, 2001; Lee et al., 2003). Repeated and large-scale planting of Bt corn could lead to an accumulation and persistence of plant-produced Cry proteins in soil (Tabashnik, 1994; Crecchio and Stotzky, 1998; Tapp and Stotzky, 1998; Saxena and Stotzky, 2001a,b; Saxena et al., 2002a,b; Zwahlen et al., 2003a; Muchaonyerwa et al., 2004; Stotzky, 2004). Furthermore, the concentration of the Cry3Bb1 protein expressed in the tissues of Bt corn was reported to be significantly higher (81 μg g^{-1} in leaves, 41 μg g^{-1} in roots) than in other transgenic Bt corn lines (i.e., events 176, Bt11, and MON810) expressing the Cry1Ab protein (3–10 μg g^{-1} of leaves, negligible in roots) (EcoStrat, 2002; EPA, 2000). The maximum concentration of Cry3Bb1 protein in corn was reported to be 93 μg g^{-1} fresh weight pollen (USEPA, 2007) and, therefore, the Cry3Bb1 protein in soil may potentially pose ecological and environmental risks.

3.2.2 Cry protein persistence

Organic molecules may persist in soil as a result of their inherent chemical recalcitrance, low accessibility, or stabilization due to intermolecular interactions with minerals, inorganic solutes and other organic compounds (Christensen, 1992; Quiquampoix and Burns, 2007).

Most extracellular proteins are short-lived in soil environments, unless they are protected from various abiotic and biotic factors. The year 1996 marked the beginning of the first large-scale, commercial use of TCs. It also marked the first surge of public interest and scientific research with the realization that some classes of soil-released

proteins may have a major and possibly a deleterious effect on our environment. Since then, cultivation of TCs continues to expand worldwide due to the demands of intensive agriculture and is another concern in the public mind. The use of TCs expressing an insecticidal protein suggests the possibly that the toxin could be released, persist and accumulate in the soil (Quiquampoix and Burns, 2007). Thus, Cry1Ab proteins have been shown to rapidly bind to soil and become "stabilized" remaining "active" as an insecticidal protein toxin (Zwahlen et al., 2003; Muchaonyerwa et al., 2004). Studies have shown that soil texture influences adsorption of Cry1Ab protein to soil; hence, its persistence and stability can be attributed to its association (complexing) with clays and humic substances.

Persistence of Bt proteins in soils also depends on soil type, pH, temperature, and other physicochemical and biological characteristics of soil (Clark et al., 2005). Icoz and Stotzky (2008) stated that the persistence and biodegradation of Cry1Ab proteins in soil depends on the level of soil microbial activity. When Cry1Ab protein is rapidly adsorbed and becomes bound on clay minerals and humic substances, it is resistant to microbial degradation and is thereby potentially able to persist in soil over time. A review of the literature by Clark et al. (2005) indicates that there is potential for longer-term persistence of the Cry1Ab protein in the soil environment. Thus, when production, by way of root exudation and degradation of plant residues in soil exceeds the inactivation and/or degradation by both abiotic and biotic factors, Cry1Ab proteins may accumulate in soil. Yet, there are conflicting reports on the persistence of Cry1Ab protein in soil (Clark et al., 2005; Icoz and Stotzky, 2008).

Studies have shown that there are three factors that make persistence possible. One, Cry1Ab protein is rapidly adsorbed onto clay and humic substances in the soil matrix (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994; Tapp and Stotzky, 1995; Saxena and Stotzky, 2001b). Second, adsorbing and binding to soil enhances protein stability

and makes it resistant to microbial degradation (Koskella and Stotzky, 1997; Zwahlen et al., 2003). This process renders the Cry1Ab protein less accessible to microbial degradation, implying the potential for it to accumulate (Koskella and Stotzky, 1997). Third, Cry proteins remain "active" and retain their insecticidal activity, even when they are adsorbed to soil minerals or entrapped by humic colloids (Tapp and Stotzky, 1995; Stotsky, 2004). The combination of these factors increases the potential for Cry proteins to persist in soil.

Inasmuch as the persistence of Cry1Ab protein in soil and the effects on the environment have not been studied adequately, their potential hazards are not fully known and cannot be predicted (Saxena et al., 1999). In addition, since studies have shown that Cry1Ab proteins can potentially accumulate to concentrations in soil that may constitute a hazard to non-target organisms (Saxena and Stotzky, 2000), knowledge of the persistence of Cry3Bb1 (and Cry1Ab) protein in soil are needed.

Icoz and Stotsky (2008) suggested a potential for long-term persistence and thereby longer exposure of target and non-target organisms to Cry1Ab protein in soil. However, not many studies have been conducted with Cry3Bb1 protein to determine its persistence in soil (Ahmad et al, 2005; Icoz and Stotzky, 2007; Zwahlen et al., 2003; Prihoda and Coats, 2008). Hence, the development of an accurate risk assessment begins with the identification of a potential hazard that could arise from the release of Cry3Bb1 protein from roots into rhizosphere soils where these transgenic corn crops are grown. Thus, a risk assessment on transgenic Cry3Bb1 corn should start with an estimation of the potential rhizosphere loads that transgenic Bt roots release into field soil to accurately determine the potential for target and non-target soil organisms to be exposed.

The adoption and widespread use of transgenic Bt crops represents a new method of

delivery for insect control and a shift in how this insect control is carried out (Benbrook et al., 1996). Furthermore, Bt corn transformed with event MON863 released in 2003, expresses significantly higher concentrations of Cry3Bb1 protein throughout plant tissue compared to other Bt corn lines. The strong interest and popularity of TCs amongst farmers has resulted in their increased and widespread use. One main concern is that Cry3Bb1 protein may potentially accumulate in soil via decomposition of plant residues and/or through the release in root exudates and that they may persist and therefore accumulate in soil over time, possibly presenting ecological and environmental risks. Unfortunately, research findings are limited in regard to the fate, persistence and stability of Cry3Bb1 protein in field soils (Icoz and Stotzky, 2007; Ahmad, 2005; Zwahlen et al., 2003; Prihoda and Coats, 2008). There are few published estimates of the amount of Bt protein that could be added to soil by TCs. Moreover, not much is known about Cry3Bb1 protein activity in soils and most notably, the environmental fate and persistence of CRW resistant Bt crops and associated Cry3Bb1 proteins in soil is not well described under NY State conditions and merits investigation. Because NY State is a major grower of corn for silage and feed grain nationally and has differences in biota, soil and climatic conditions, an investigation into the fate, persistence and stability of Cry proteins under NY State environmental conditions was necessary. Further, the fate and persistence of Bt proteins in NY field soils has not yet been explored.

In order to evaluate the protein load that root exudates containing Cry3Bb1 protein could represent in the field, the objective of this study was to estimate the concentrations of Cry3Bb1 and Cry1Ab insecticidal proteins in rhizosphere soils of Bt corn varieties grown at different locations in NY. To estimate potential exposure of soil organisms to these proteins, two main factors were investigated 1. production in the field; and 2. persistence of protein in field soil over time. The concentrations of

Cry3Bb1 and Cry1Ab proteins in the field were evaluated as a function of site (soil type); construct; variety (hybrid) and soil textural classification.

3.3 Methods

3.3.1 Quantitative Cry toxin detection methodology

The residues of Cry3Bb1 Bt corn and soils were analyzed for Cry3Bb1 and Cry1Ab protein concentration using a double-antibody sandwich (DAS) ELISA and PathoScreen Kit (Agdia, Elkhart, IN). The optical density was determined by a Vmax enzyme kinetic microplate reader (μQuant- Bio-Tek Instruments, Inc./KC Junior software) set at 620 nm. A five-point standard curve developed using purified Cry3Bb1 protein was established by linear regression for use in estimating the Cry3Bb1 protein concentrations in soil and corn residues.

3.3.1.1 *Quantification of Cry3Bb1 Protein in corn residues*

Cob, leaf, stalk and root samples of Bt and non-Bt corn plants that were collected at anthesis and harvest were oven-dried and ground to pass through a 2 mm sieve. Ground corn residues were weighed (0.1 g) into 2 ml Ependorf tubes, and 1 ml of 1x PBST extraction buffer was used to extract Cry3Bb1 protein from ground corn tissues. Eppendorf tubes were vortexed for 15 sec and then centrifuged at 5000 x g for 20 min. A 1 ml aliquot of each supernatant was transferred into a new tube. The supernatants were analyzed for Cry3Bb1 protein concentration using a DAS-ELISA kit (Agdia, Elkhart, IN.) A 100 μl aliquot of peroxidase enzyme-conjugate was dispensed into the appropriate test wells of the ELISA plate. Next, 100 μl of supernatant(s) was dispensed into test wells of the ELISA plate and incubated for 2 h at 27ºC in a humid box. The contents of the plate were removed by quickly flipping it over into a sink. The plate was then washed 6 times with 1x PBST extraction buffer. One-hundred

microliters of tetramethyl benzidine (TMB) substrate solution was then dispensed into each test well and incubated for 20 min. Samples were analyzed in duplicate. The DAS-ELISA plate was read at 620 nm by use of a μQuant-Biotek Instruments, Inc. Plate reader operated by the KC Junior software package. Purified Cry3Bb1 protein was provided by Monsanto Co. (St. Louis, MO).

3.3.1.2 *Experimental site and design (2004-2007): Aurora Farm (2006)*

Long-term field trials and a three-year litterbag study with transgenic corn transformed by event MON863 were carried out along with non-Bt isogenic hybrids without the Cry3Bb1 gene at the Musgrave Research Farm in Aurora, NY, from May 2004-2007. The experiment was designed as a randomized complete block with two field histories: continuous corn and an alfalfa rotation with three treatments (Bt, non-Bt and Non-Bt corn with Tefluthrin insecticide applied, Table 3.2) and three replicates. A total of 15 plots were sampled, with each plot measuring 51 m wide by 46 m long. Bulk and rhizosphere soils were sampled 3 times during the season (at pre-planting, mid-season and after-harvest). In addition, cob, leaf, stalk, and root samples of Bt and non-Bt corn plants were also collected at anthesis and harvest and were oven-dried, ground and tested for their Cry3Bb1 protein concentration.

3.3.1.3 *Experimental site and design (2006)*

Field trials were carried out to compare corn cultivar yield performance at multiple sites in NY State in 2006. The experiments were designed as randomized complete blocks with 15 corn varieties and 3 replicates. Fifteen transgenic Bt corn varieties (TA Seeds: TA5524, TA5854, TA6704, TA5859, TA67657, TA552-13, TA675-13; Pioneer: PIO34H39, PIO36N73, PIO38P-10; and DeKalb: DKC46-22, DKC46-24, DKC50-20, DKC51-39, DKC6168, respectively) (Table 3.2) were compared with non-isogenic, non-Bt varieties (TA Seeds: TA5510, TA6750, and Pioneer: PIO 38P-

05, respectively). Bt corn varieties (TA Seeds: TA5524, TA5854, TA5859, TA6704, TA67657, TA552-13, TA675-13; Pioneer: PIO34H39, PIO36N73; and DeKalb: DKC6168) were grown at the following sites: Kingston, Avon, and Pittsford, NY; while Bt corn varieties (DeKalb: DKC46-22, DKC46-24, DKC50-20, DKC51-39;

Table 3.1 Genetic constructs used in this study (treatments).

Constructs (genetic traits)	
1. Roundup Ready™ corn (Non-*Bt* corn)	RR
2. YieldGard™ (corn borer resistance-Cry1Ab)	CB
3. YieldGard™ corn rootworm. resistance (Cry3Bb1)	RW
4. YieldGard™ Plus (for both corn borer and corn rootworm resistance-	CB + RW

Table 3.2 Hybrids grown at various sites containing various genetic constructs of the *cry*3Bb1 and *cry*1Ab genes.

Constructs	**Hybrid(s)**					
1. RW	TA5524	TA5854	TA6704			
2. RR + RW	TA67657	DKC46-24	DKC61-68			
3. RR + CB	DKC50-20					
4. CB + RW	TA5859	PIO 36H39				
5. RR + CB + RW	TA552-13	TA675-13	DKC46-22	DKC51-39	PIO 36N73	PIO-38P10

and Pioneer: PIO38P-10) were grown at the Albion, New Hope and Scipio, NY, sites. In addition, transgenic Bt corn varieties grown at the Kingston, Avon, and Pittsford, NY, sites contained stacked transgenic events for Cry3Bb1 and Cry1Ab proteins (PIO36N73, TA552-13, TA 675-13 and DKC 51-39), whereas at the Albion, New Hope and Scipio, NY, sites, stacked transgenic Bt corn varieties for Cry3Bb1 and Cry1Ab proteins were grown (DKC 46-22, , DKC 51-36, PIO 38P-10, respectively). Rhizosphere soil samples from Bt and non-Bt cultivar field trials were collected during mid-season, 2006.

3.3.2 Quantification of Cry3Bb1 and Cry1Ab proteins in soil

Rhizosphere soils (0.5 g soil) sampled from the field sites were weighed into 2 ml Eppendorf tubes and 1.0 ml Palm buffer (pH= 8.80 for Cry3Bb1 and pH=10.5 Cry1Ab) was dispensed into each tube and incubated overnight. Two glass beads were added to the samples, which were vortexed for 1 min at full speed and then centrifuged at 10,000 x g for 2 min. The supernatant was removed and placed into a new tube. Two and three extractions were repeated with 1000 µl of Palm buffer, with vortexing for 1 min at full speed and then centrifugation at 10,000 x g for 2 min. One-hundred microliters of each extraction sample were then dispensed into the ELISA plate and incubated for 1 h. The plate was then washed 6 times with 1x PBST buffer. One-hundred microliters of peroxidase enzyme-conjugate were dispensed into the appropriate test wells of the ELISA plate and incubated for 2 h. The plate was washed again 6 times with 1x PBST buffer. One-hundred microliters of tetramethyl benzidine substrate solution was dispensed into each test well and incubated for 20 min. Optical density of samples in the DAS-ELISA plate was analyzed at 620 nm with a µQuant-Biotek Instruments, Inc., and was operated by KC Junior software package. Purified Bt Cry3Bb1 protein was provided by Monsanto Co. (St. Louis, MO).

3.3.3 Soil recovery conversions

A seven-point standard curve was established to determine corrected amounts of Cry3Bb1 protein in soil on the basis of its extraction efficiency from the seven soil sites. Soil recovery conversions were determined to account for adsorption interference with protein recovery from soil samples (Table 3.3 and Appendix B).

Table 3.3 Soil recovery conversion factors.

	Cry3Bb1	**Cry1Ab**
Kingston	0.45	0.54
Pittsford	0.39	0.52
Scipio	0.35	0.53
Albion	0.36	0.52
New Hope	0.31	0.41
Aurora	0.21	N/A
Avon	0.11	0.40

3.3.4 Statistical analysis

The data are expressed as means ± SE. Generalized least square fit and analysis of variance (ANOVA) were used to examine significance of differences between soils, genetic constructs, plant varieties, soil textures, and between Cry proteins (Cry3Bb1

vs. Cry1Ab). A $\rho < 0.05$ was considered significant. All statistics were performed with S-PLUS® 8.0 software package (Insightful Corp.)

3.4 Results

3.4.1 Quantification of Cry3Bb1 protein in corn residues

Cry3Bb1 protein was below the limit of detection (LoD< 0.05 ng g^{-1}) in the control non-Bt corn isoline, but was present at concentrations 1.10 ± 0.59 ng g^{-1}, 5.54 ± 1.7 ng g^{-1}, 1.36 ± 0.68 ng g^{-1}, and 0 ng g^{-1}, respectively in 2005 cob, leaves, stalk, and root Bt corn residues (Table 3.4). Leaves had a significantly higher concentration ($F=6.69$; $df=3$; $\rho=0.0020$) of Cry3Bb1 compared to other plant residues.

Table 3.4 Cry3Bb1 concentrations in Bt corn residues.

	(ng Cry3Bb1 protein g^{-1} plant residue)			
	Cobs	**Leaves**	**Stalks**	**Roots**
MON863 Bt corn	1.10 ± 0.59	5.54 ± 1.70	1.36 ± 0.68	0
Non-Bt	<LoD			

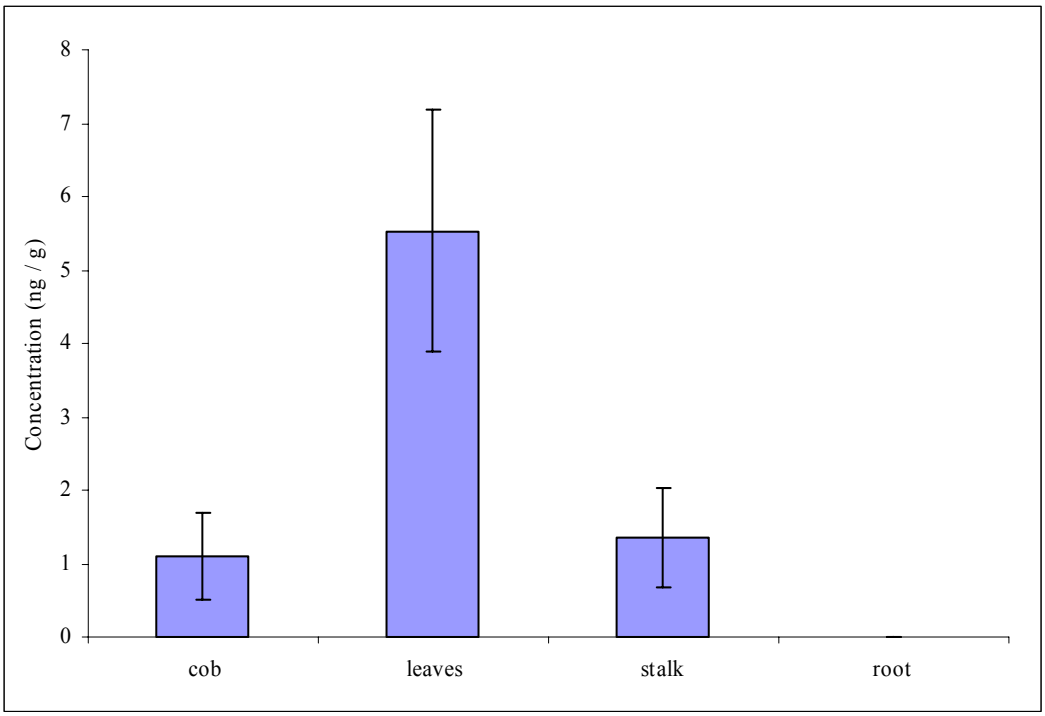

Figure 3.1 Cry3Bb1 concentrations in Bt corn residues (ng Cry3Bb1 protein g^{-1} plant residue).

3.4.2 Experimental site and design (2004-2007): Aurora Farm (2006)

Only first extractions were analyzed for this study. Actual Cry3Bb1 concentration in soil was estimated after correcting for protein adsorption using the soil recovery conversion factors given in Table 3.3. The Cry3Bb1 protein was not detected by ELISA in any of the non-Bt corn soils at the Aurora, NY, site. The release of Cry3Bb1 protein in root exudates was examined in Bt corn fields on three different occasions during the growing season (pre-planting; mid-season; after-harvest). Cry3Bb1 protein was not detected in any of the plots where Bt corn was grown when sampled during pre-planting, 2006. At mid-season, Cry3Bb1 protein was detected in all of the plots except one where Bt corn was grown. The average concentration mid-season was 7.22

ng g^{-1} soil (Table 3.5). The highest concentration (non-average) measured mid-season was 36 ng g^{-1} soil. Cry3Bb1 protein was detected in only one plot (AH-11) after harvest in 2006 at 0.48 ng g^{-1} soil.

Table 3.5 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at the Aurora, NY field site (2006) as determined by ELISA.

Sampling time		
Pre-planting	**Mid-season**	**After Harvest**
0.00	7.22±4.82	0.07±0.07

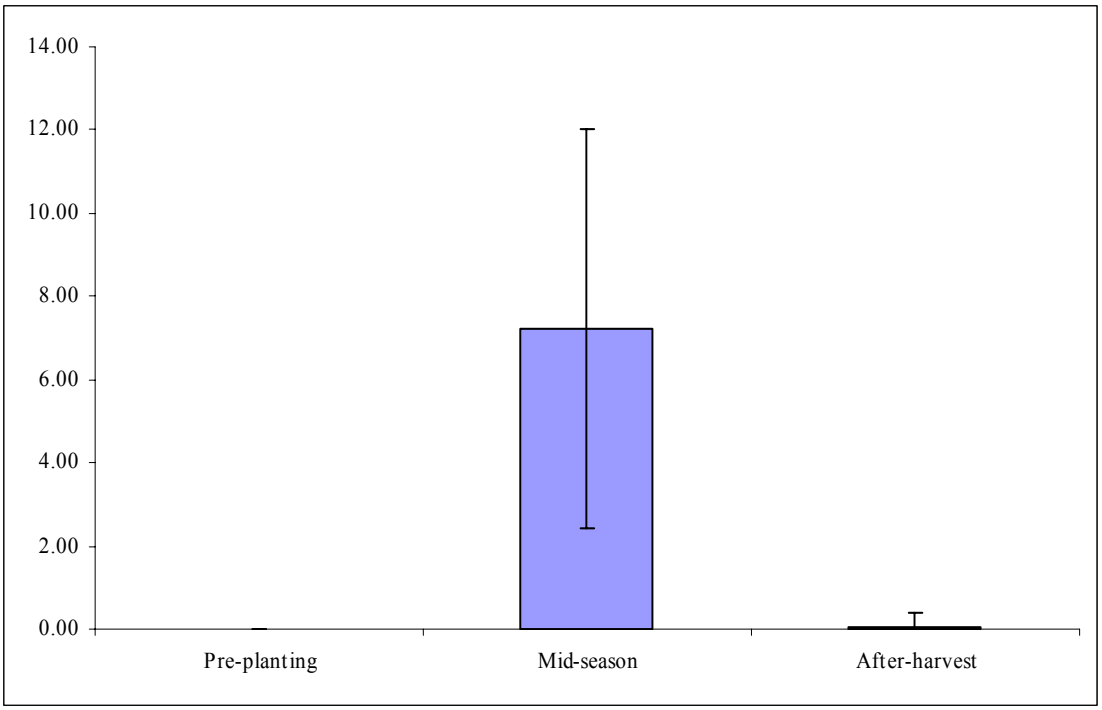

Figure 3.2 Temporal expression data: Cry3Bb1 protein concentrations at different sampling times in Aurora, NY, soil in 2006 (ng Cry3Bb1 protein g^{-1} plant residue).

3.4.3 Experimental site and design (2006)

Cry3Bb1

Only first extractions were analyzed for this study. Actual Cry3Bb1 concentration in the soil was estimated after correcting for protein adsorption using the soil recovery conversion factors given in Table 3.3. Cry3Bb1 protein was detected in all of the soils from the six sites. The highest Cry3Bb1 concentration was reported at Avon, NY, 78.64 ng g^{-1} soil (55% clay).

Corrected values were then averaged and tested for responses to the variables soil type (site), construct, variety (hybrid) and soil texture. When the averages were compared for the sites, the highest reported average concentration was for Pittsford, NY, soil at 10.40 ng g^{-1} (Figure 3.3). Pittsford (clay-loam, 34% clay) had a significantly higher concentration of Cry3Bb1 protein compared to other sites sampled in the 2006 growing season ($F=2.86$; $df=5$; $\rho=0.0188$).

Cry3Bb1 concentrations in the rhizosphere soil fo different constructs were examined. The highest concentrations recorded were for the stacked construct CB + RW and in RW (12.39 ng g^{-1} and 5.21 ng g^{-1}, respectively), compared to the constructs containing RR (RR & RW and RR & CB & RW) (Figure 3.4). Significant differences were reported for CB + RW ($F=3.12$; $df=3$; $\rho=0.0287$).

Cry3Bb1 concentrations in the rhizosphere soil of different corn varieties (hybrids) were examined (Figure 3.4). The highest concentration recorded was for the stacked variety, TA5859 (construct: CB + RW) at 18.94 ng g^{-1} soil. Significant differences were reported for the hybrid TA5859 ($F=2.17$; $df=13$; $\rho=0.0136$).

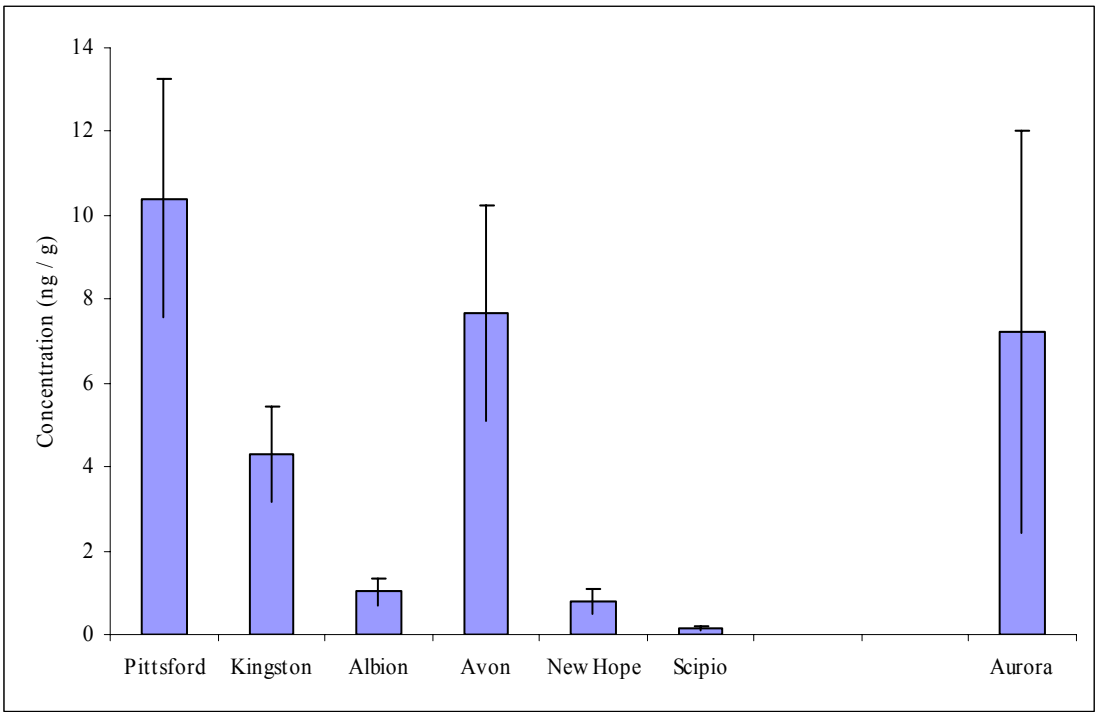

Figure 3.3 Cry3Bb1 concentrations in rhizosphere soils in mid-season 2006 at different locations (ng Cry3Bb1 protein g^{-1} soil).

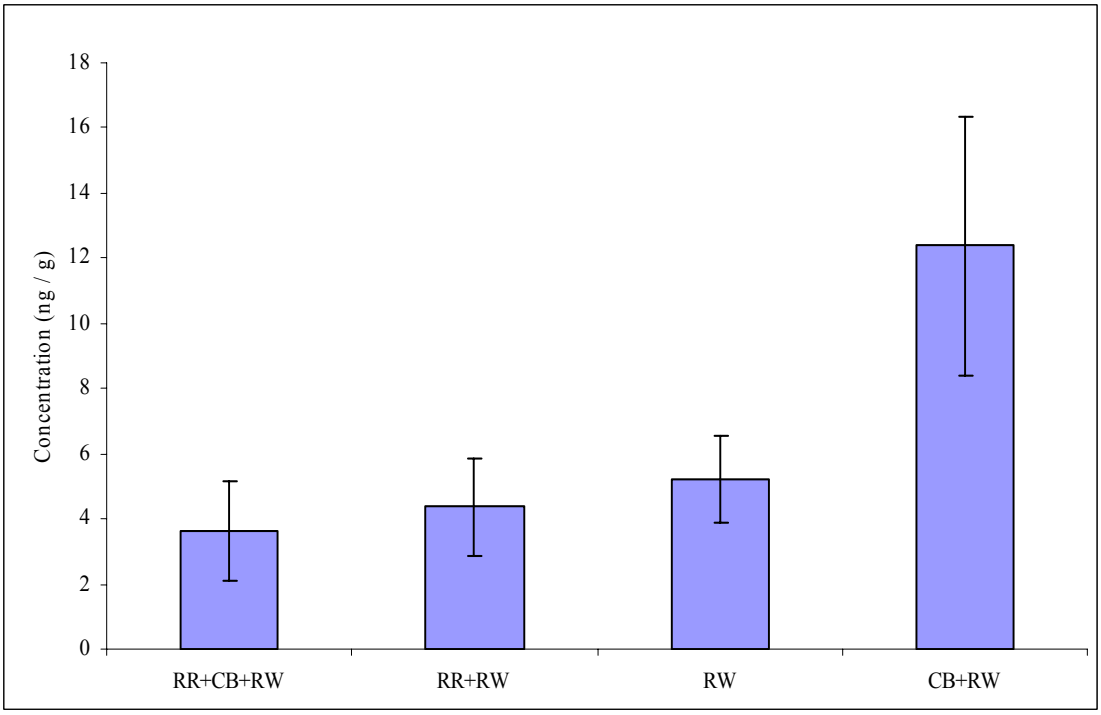

Figure 3.4 Cry3Bb1 concentrations in rhizosphere soil in mid-season 2006 in different Bt corn constructs (ng Cry3Bb1 protein g^{-1} soil).

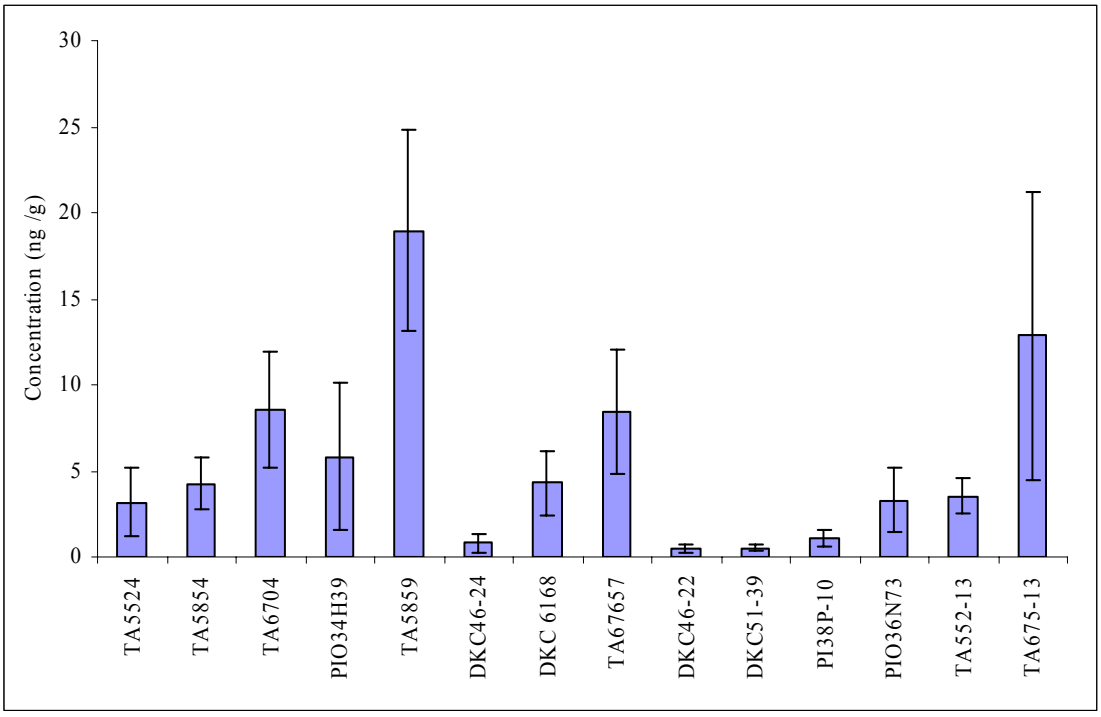

Figure 3.5 Cry3Bb1 concentrations in rhizosphere soil at mid-season 2006 in different Bt corn varieties (ng Cry3Bb1 protein g^{-1} soil).

Cry3Bb1 concentrations in rhizosphere soils of different textures were examined (Figure 3.6). The highest concentrations recorded were for the silty-clay soil (Avon, NY) at 7.67 ng g^{-1} soil. Cry3Bb1 concentrations in clay-loam and loam soils were between 4.3 and 4.87 ng g^{-1} soil and were not significantly different.

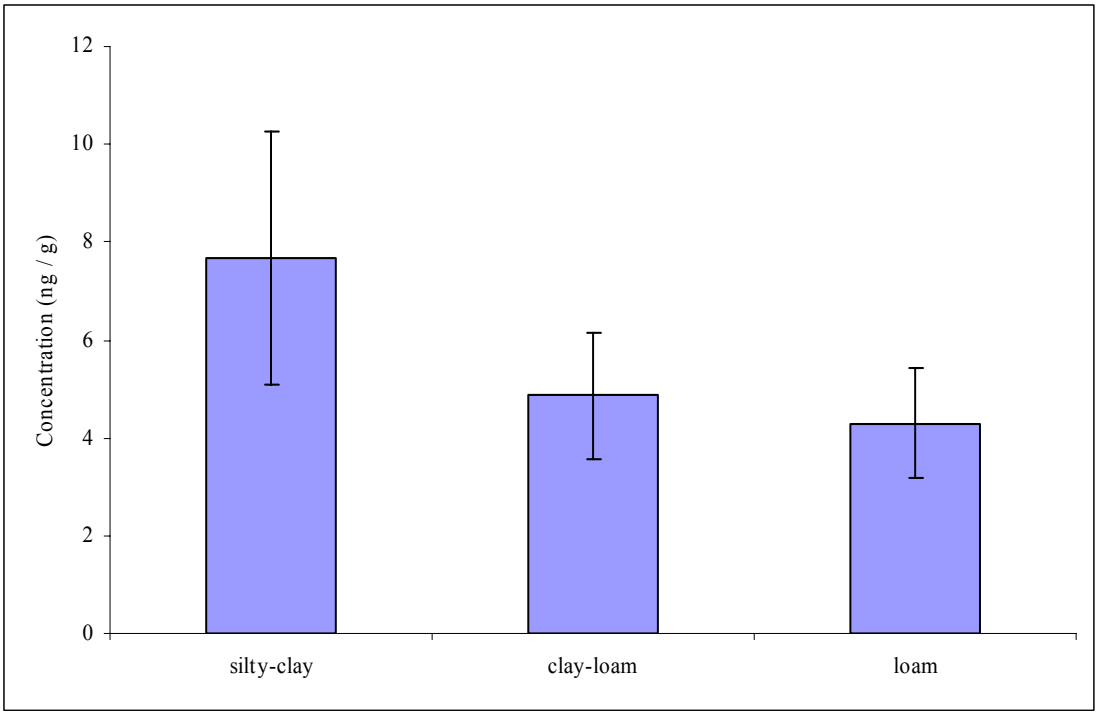

Figure 3.6 Cry3Bb1 concentrations in rhizosphere soils in mid-season 2006 in soils of differing texture (ng Cry3Bb1 protein g^{-1} soil).

Cry1Ab

Only first extractions were analyzed for this study. Actual Cry1Ab concentration in soil was estimated after correcting for protein adsorption using the soil recovery conversion factors given in Table 3.3. Cry1Ab protein was detected in all of the soils from the four sites (Kingston, Avon, Pittsford and Albion, NY). The highest Cry1Ab concentration was recorded for Albion, NY, at 7.88 ng g^{-1} soil (clay-loam, 32% clay composition).

Corrected values were then averaged and tested for responses to the variables soil type (site), construct, variety (hybrid) and soil texture..

The average Cry1Ab concentrations at different sites were examined. The highest concentrations recorded were for the Pittsford, NY, soil at 2.17 ng g^{-1} soil. The other sites had concentrations between 0.91 to 1.94 ng g^{-1} soil and were not significantly different (Figure 3.7).

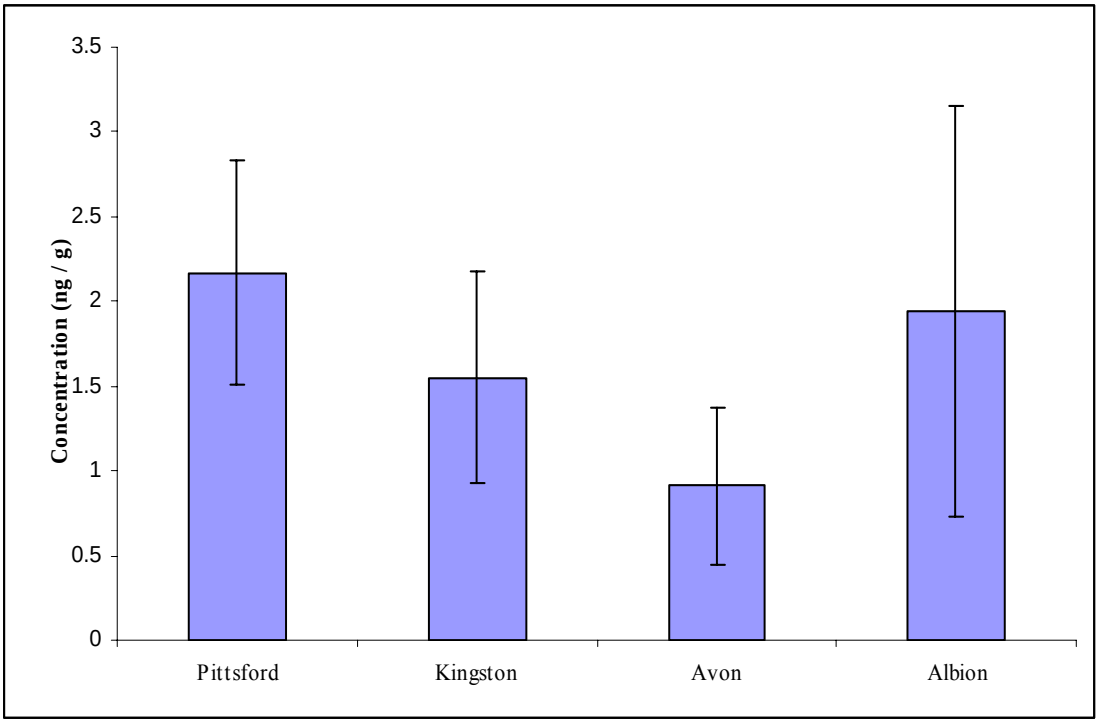

Figure 3.7 Cry1Ab concentrations in rhizosphere soil at mid-season 2006 at different locations (ng Cry1Ab protein g^{-1} soil).

Cry1Ab concentrations in rhizosphere soils from different constructs were examined. The highest concentrations recorded were for the stacked constructs RR + CB + RW, at 1.80 ng g^{-1} soil. There were no significantly differences between any of the constructs. The concentration of Cry1Ab protein in rhizosphere soil appeared to increase when the number of constructs increased in a Bt Cry1Ab corn variety (Figure 3.8).

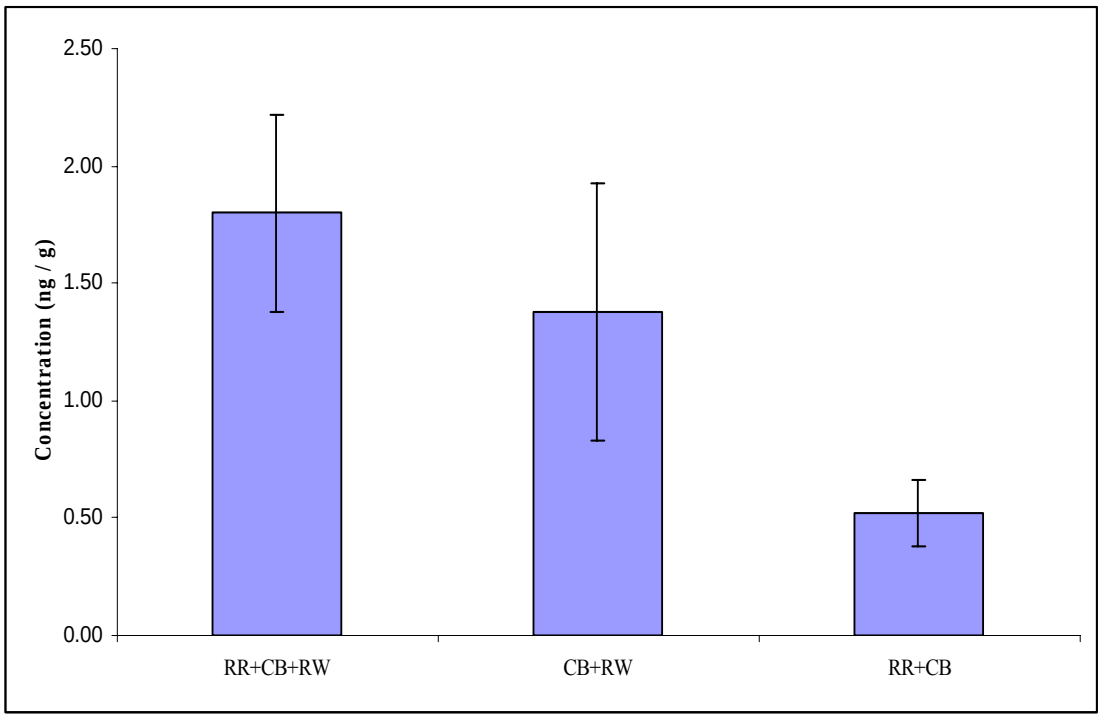

Figure 3.8 Cry1Ab concentrations in rhizosphere soil at mid-season 2006 in different Bt corn constructs (ng Cry1Ab protein g^{-1} soil).

Cry1Ab concentrations in rhizosphere soils from the different hybrids were examined. The highest concentrations recorded were in the stacked constructs RR + CB + RW and hybrid DKC51-39 at 4.83 ng g^{-1} soil (Figure 3.9). There were no significant differences between the different varieties.

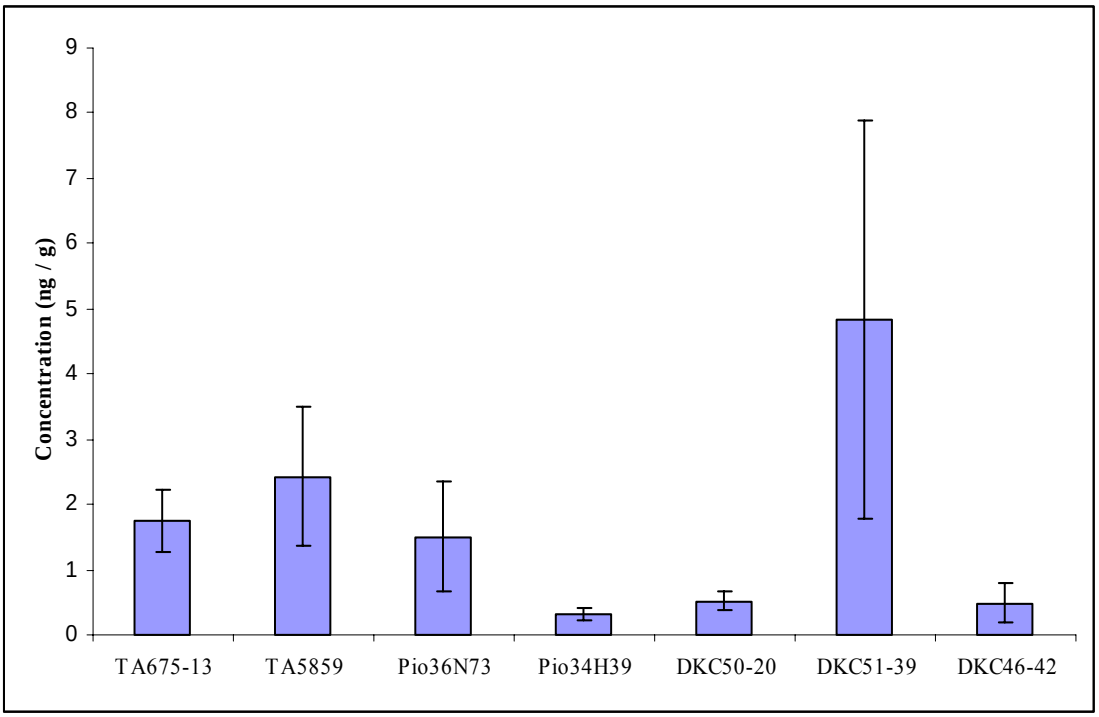

Figure 3.9 Cry1Ab concentrations in rhizosphere soil at mid-season 2006 in different Bt corn hybrids (ng Cry1Ab protein g^{-1} soil).

Cry1Ab concentrations in the rhizosphere soil of soils with different textures were examined. The highest concentrations recorded were in clay-loam soils at 2.1 ng g^{-1} soil (Figure 3.10). No significant differences were reported between the soil textures: clay-loam, silty-clay and loam ($F=1.22$; $df=2$; $\rho=0.3028$). The concentration of Cry1Ab in different soils appeared to decrease when the clay content increased in the soil sample (Figure 3.10).

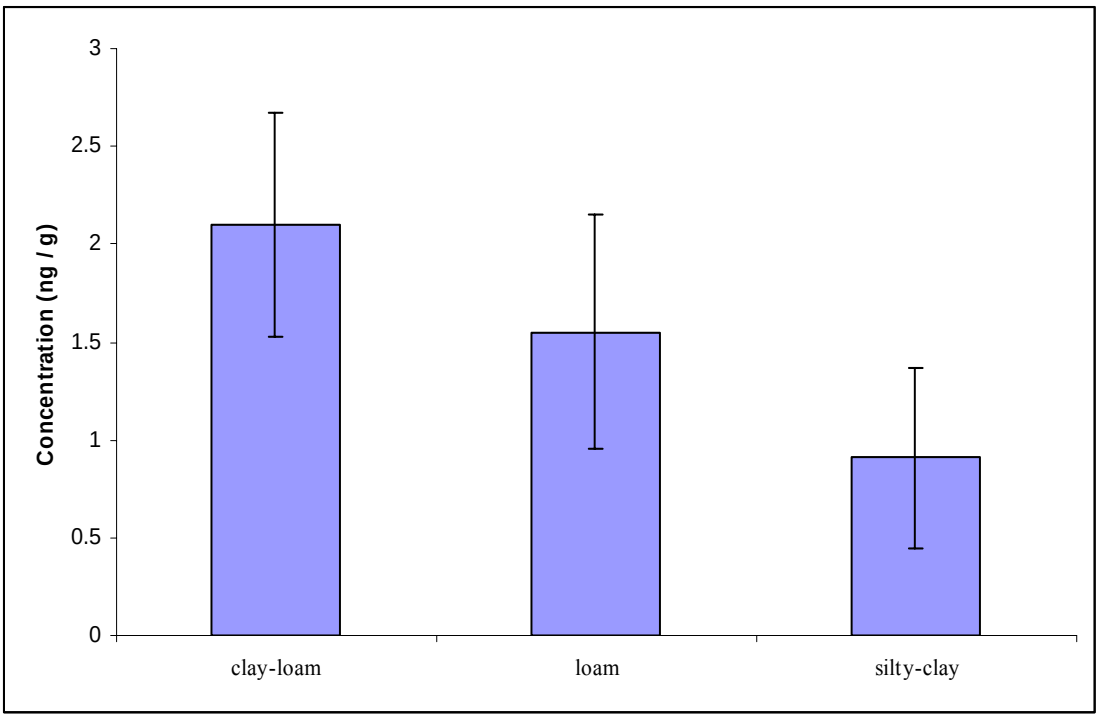

Figure 3.10 Cry1Ab concentrations in rhizosphere soil at mid-season 2006 for different soil textural classifications (ng / Cry1Ab protein g^{-1} soil).

Cry3Bb1 and Cry1Ab protein concentrations in rhizosphere soils from the various sites were examined. It appeared from the averages of the Cry protein concentrations that Cry3Bb1 protein had a higher concentration (5.43 ng g^{-1} soil) in these soils compared to Cry1Ab (1.59 ng g^{-1} soil) (Figure 3.11). There was a significant difference between the Cry proteins ($F=6.00$; $df=1$; $\rho=0.0153$).

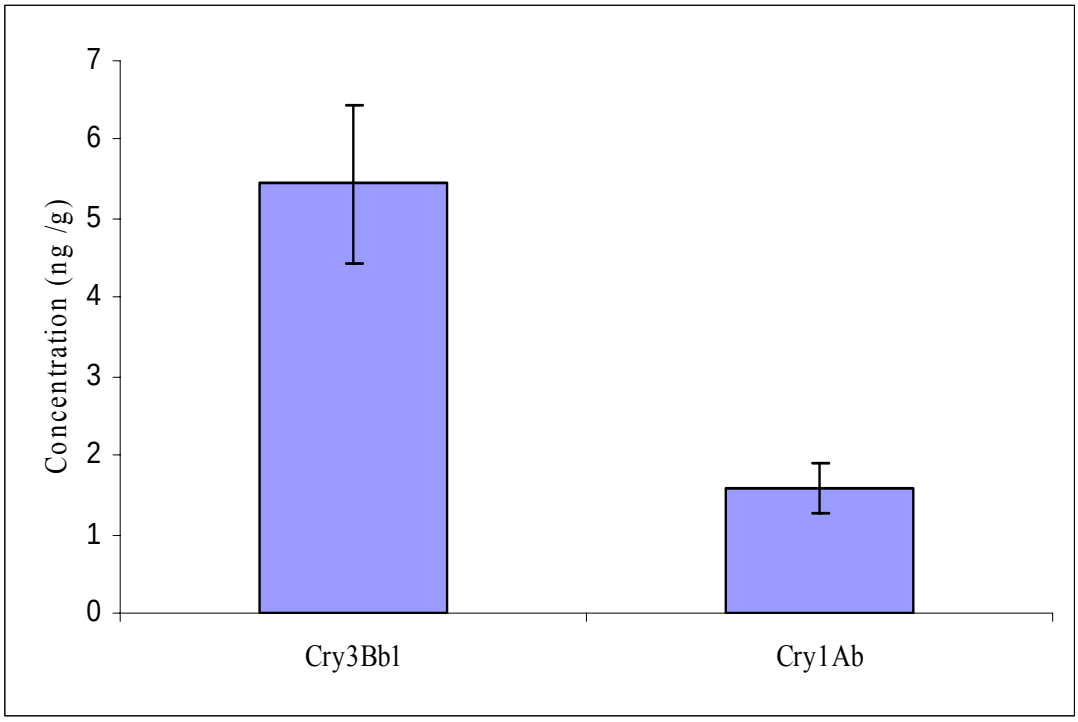

Figure 3.11 Comparison between Cry3Bb1 and Cry1Ab concentrations in rhizosphere soils at mid-season 2006 (ng Cry1Ab protein g^{-1} soil).

3.5 Discussion

The concentrations of Cry3Bb1 protein in corn tissue from the Aurora, NY, field site was estimated to be 5.54 ± 1.70 ng g^{-1} in leaves, 1.36 ± 0.68 ng g^{-1} in stalks, 1.10 ± 0.59 ng g^{-1} in cobs and 0 ng g^{-1} in roots by ELISA. Low concentrations of Cry3Bb1 in plant tissues (1.6 ± 0.20 µg g^{-1}) were also reported by Icoz et al. (2007) and by Wander and Gunapala (2004), where they reported Cry3Bb1 concentrations at 1.7 to 2.5 µg g^{-1} in leaves, 0.4 to 1.0 µg g^{-1} in stems and 0.1 to 0.4 µg g^{-1} in roots. However, this is not in agreement with other studies. Prihoda and Coats (2008) reported 24.6 ± 5.6 and 96.6 ± 7.6 µg g^{-1} for leaf and root concentrations; EcoStrat (2002) reported 81 and 41 µg $g^{-1}$for leaf and root samples; the USEPA (2007) reported 13 to 54 µg g^{-1} above-ground plant material and Vaughn et al. (2000) reported 3.2 to 66 µg g^{-1} in roots. The reason for these discrepancies is not clear but may be a result of different environmental conditions where these crops were grown, the source and age of seeds, and the method of extraction and analysis (Icoz et al., 2007).

Many studies propose that there is a need to investigate the presence of Bt proteins in agricultural field soils on a case-by-case basis, where Bt corn is repeatedly planted to assist in evaluating the accumulation and persistence of Cry proteins in soils (Ahmad et al., 2005; Icoz et al., 2007; Icoz and Stotzky, 2008). Examining the actual concentrations in soil will also assist in estimating the potential exposure of soil organisms to these proteins (Jehle, 2007). The modified and optimized method, based on the protein extraction protocol described by Palm et al. (1994), worked effectively to detect and quantify Cry proteins in field soils. Ahmad et al. (2005) found that Cry3Bb1 was rapidly degraded and. thus, was not detected in field soil. Furthermore, Icoz et al. (2007) reported a rapid decrease in Cry3Bb1 concentrations in Kitchawan

soil and determined that no protein was detected after 50 days. Icoz et al. (2008) did not detect Cry3Bb1 protein in their field soil study.

I found in this investigation that Cry3Bb1 (and Cry1Ab) protein(s) were released in root exudates of transgenic Bt corn events MON 863 and MON 810 as determined by ELISA and were present mid-season at various concentrations in rhizosphere soils gathered from six field locations where transgenic Bt corn was grown in NY State. These results are similar to Cry1Ab (in Bt corn and rice), Cry 3A (in Bt potato) and other Cry3Bb1 protein detection studies (Icoz et al., 2007), but in low concentrations.

The persistence of Cry3Bb1 protein was first evaluated at the Musgrave Research Farm in Aurora, NY, in 2006, where it was determined that Cry3Bb1 protein does not persist from mid-season to after harvest in the same growing season in this agricultural field soil (pH=7.71; 32% clay composition). From these results, it was determined that mid-season rhizosphere soils were needed for the remainder of the study to evaluate the release of Cry proteins into soils.

Bt corn varieties with variations in constructs containing Cry3Bb1 and Cry1Ab were grown at different sites in NY State to investigate the release of Cry protein into the rhizosphere. Overall, results of this study showed differences in Cry3Bb1 and Cry1Ab concentrations at different sites, in different constructs, and in soils with different textures. The highest concentration of Cry3Bb1 was reported for Avon, NY, at 78.64 ng g^{-1}, while the highest concentration of Cry1Ab was reported for Albion, NY, at 7.88 ng g^{-1}. These two locations represent soils which contain clay contents ranging from 32-55%. This agrees with others studies which found that the binding of Cry1Ab protein on clays reduced its bioavailability to microorganisms and may be responsible for its persistence in soil (Ahmad et al., 2005; Fiorito et al., 2007; Icoz et al., 2008). Therefore, this study supports other findings that soil texture affects the

efficiency of Cry protein extraction, but most importantly, soil texture leads to differences in Cry protein residence time in soils. Thus, heavy-textured soils are problematic for estimating rhizosphere loads to soil. Consequently, soil recovery conversions were calculated to account for adsorption to the soil matrix during protein extraction from field soils.

Different constructs also resulted in different protein concentrations in soil. Results from the quantification of Cry3Bb1 protein in soil suggest that the construct for Roundup resistance (RR) causes a decrease in Cry3Bb1 protein release into rhizosphere soil compared to soils where Bt corn plants were grown that do not contain the RR construct. However, the opposite was the case for the Cry1Ab protein rhizosphere soil concentrations reported in this study. The addition of the RR construct in these Bt corn plants resulted in higher concentrations of Cry1Ab compared to Bt corn plants without the RR construct. Furthermore, soils examined for the Cry3Bb1 protein also had higher concentrations in soils compared to the Cry1Ab protein. This finding supports the results of Ecostrat (2002) and the USEPA (2000), where they reported higher Cry3Bb1 protein expression in corn tissues (leaves, and roots) compared to the Cry1Ab protein. Lastly, it was found that the CB + RW "stacked" construct released more Cry3Bb1 protein compared to the other Bt corn varieties. Thus, it appeared that the addition of the CB construct affected the expression of *cry*3Bb1 gene in these varieties.

The results from this study show that Cry3Bb1 protein in root exudates and/or decaying plant residues is available for rapid decomposition and does not persist in these soils. These results support the findings of Ahmad (2005) and Icoz et al. (2007) that Cry3Bb1 protein in soil is rapidly biodegraded and does not persist or accumulate in soil. Furthermore, this study has shown that soil where Bt corn plants containing

stacked constructs expressing both Cry3Bb1 and Cry1Ab protein are grown, control of insect pests is improved, but the proteins do not persist in field soil. Therefore, although the production and release of Cry3Bb1 proteins in root exudates is common, Cry3Bb1 does not appear to persist or accumulate in soil in concentrations that may constitute a hazard to beneficial soil-dwelling organisms.

Protein adsorption in soils is directly related to soil texture, surface-charge and surface area. Protein loads tended to follow the order: clay > silt > sand. Persistence and accumulation may occur where the rate of the addition of Cry protein to soil exceeds the inactivation and/or degradation by both abiotic and biotic factors (Tapp and Stotzky, 1995, Crecchio and Stotzky, 1998; Ahmad, 2005).

This is the first field study reporting the presence of Cry3Bb1 protein in rhizosphere soils at mid-season at six NY field soils. More importantly, Cry3Bb1 was quantified in these soils. Another major result of this study was that, despite detectable levels of Cry3Bb1 protein measured in plant biomass and in mid-season rhizosphere soil, Cry3Bb1 protein does not persist or accumulate under NY State conditions from mid-season to after harvest in the same growing year. In conclusion, all the measurements performed in this study led to the conclusion that Cry3Bb1 proteins do not persist or accumulate in the environment under field conditions in NY State at different localities under different climatic conditions.

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CHAPTER 4 Conclusion

4.1 Key Findings

First, the modification of the Palm extraction buffer (Palm et al., 1994) was explored to overcome extraction difficulties and poor protein recoveries and to optimize and develop an accurate, effective and reliable means of extracting Bt protein from NY State field soils. The results indicated that Cry3Bb1 protein rapidly adsorbs well to clay and humic particles in field soil with binding being more pronounced in soils with higher clay contents. This study confirmed that soil texture affects the efficiency of Cry protein extraction from field soil. Thus, soils containing higher % clay showed reduced extraction efficiencies (Table 2.11; Figure 2.4). In addition, modification of pH, incubation time, and the incorporation of glass beads has given way to optimal Cry3Bb1 protein extraction from soil (10.50 ± 8.46 to 96.67 ± 3.08%, see Table 2.13). Thus, one major result of this study is that it is feasible to conduct efficient quantitative assays to evaluate the persistence of Cry3Bb1 and Cry1Ab proteins in field soils. The sensitivity of the method was <1.0 ng protein g^{-1} of soil and it could quantify Cry protein levels as low as 0.5 ng g^{-1} soil.

Another major result of this study established that Bt corn expressing the *cry*3Bb1 gene releases Cry3Bb1 protein into soil from roots and residues of transgenic Bt corn under NY State environmental conditions. Cry protein concentration in rhizosphere soil was quantified. Cry3Bb1 and Cry1Ab proteins were detected and quantified in all the mid-season 2006 field soils sampled from six sites. However, this study showed that Cry3Bb1 did not accumulate or persist from one growing season (after-harvest) to the next (pre-planting 2006: long-term), nor from mid-season to after

harvest within the same growing season (short-term) at the Aurora field site in 2006. This supports the findings of Icoz et al. (2007) and Icoz et al. (2008) that Cry3Bb1 protein is rapidly biodegraded and does not accumulate or persist in soil. Furthermore, statistical tests of field data supported my hypothesis that corn hybrids vary in the amount of protein released by roots into the rhizosphere and that Cry3Bb1 expression is influenced by the insertion of additional constructs and other environmental factors (Figure 3.5). Another finding of this study supported my hypothesis that soil texture affects the efficiency of Cry protein extraction and leads to differences in Cry protein residence time in soils. Data shown in Figures 3.6 and 3.9 support this conclusion and show that soils with higher clay composition do influence Cry protein adsorption and as a result show differences in residence times in various field soils. However, soils with higher clay contents do not necessarily enhance soil accumulation nor result in persistence from one growing season to the next.

The last major finding of this study supported the hypothesis that Cry3Bb1 and Cry1Ab proteins differ in their persistence in different soils. Results of this study showed that *cry*3Bb1 gene expression varied in different corn varieties and at different sites and that Cry3Bb1 protein released in rhizosphere deposits into soil likely resulted from these factors; however, these differences were not significant (Figure 3.11). Since Cry3Bb1 protein concentrations in above- and below- ground corn tissue were low (0.02 to 12.39 ng Cry3Bb1 protein g^{-1} plant residue), the release of Cry3Bb1 protein in root exudates was also likely low, thus, the potential for Cry3Bb1 protein to persist beyond the growing season under NY State conditions is also likely low.

Bt protected crops have demonstrated significant benefits since their introduction in 1996. These products provide a level of pest protection that is generally superior to that of conventional chemical pesticides. As a result, Bt corn requires fewer

applications of externally applied pesticides. Thus, the adoption of Bt corn provides higher crop yields and economic value to growers (Betz et al., 2000). Therefore, the concern over the rapid adoption and widespread use of TC's, particularly Bt corn has directed the focus of this study, which concluded that the potential for exposure to Cry3Bb1 protein from Bt corn residues and root exudates in soil is likely to be very low. Cry3Bb1 protein, therefore, appears not to pose any ecological risks to soil organisms. These products are fully suitable for introduction into widespread commercial agriculture. The importance of pH and other physicochemical and biological characteristics of soil need to be determined to get a better understanding of the persistence of Cry3Bb1 in diverse field soils. In addition, the effect of "stacked" traits (Cry1Ab and Cry3Bb1) on binding to (to determine if it is rapid and also binds strongly) and accumulation in soil needs further study as does developing optimal extraction methods for these proteins from field soil to better assess their persistence and potential hazard to non-target organisms.

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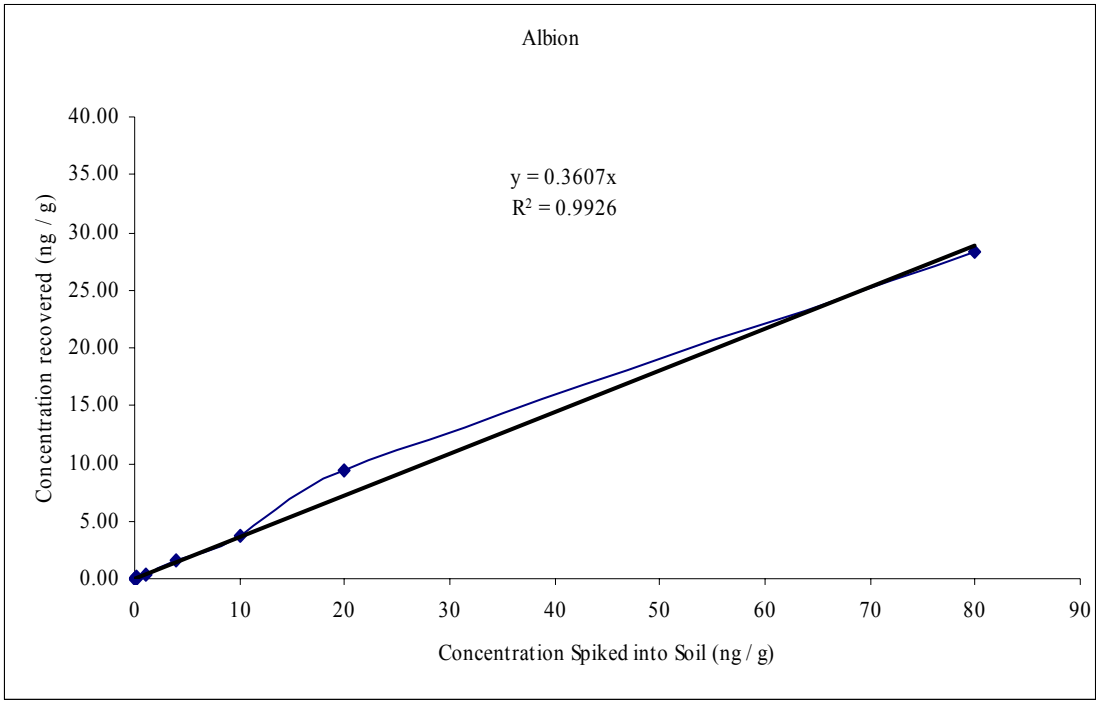
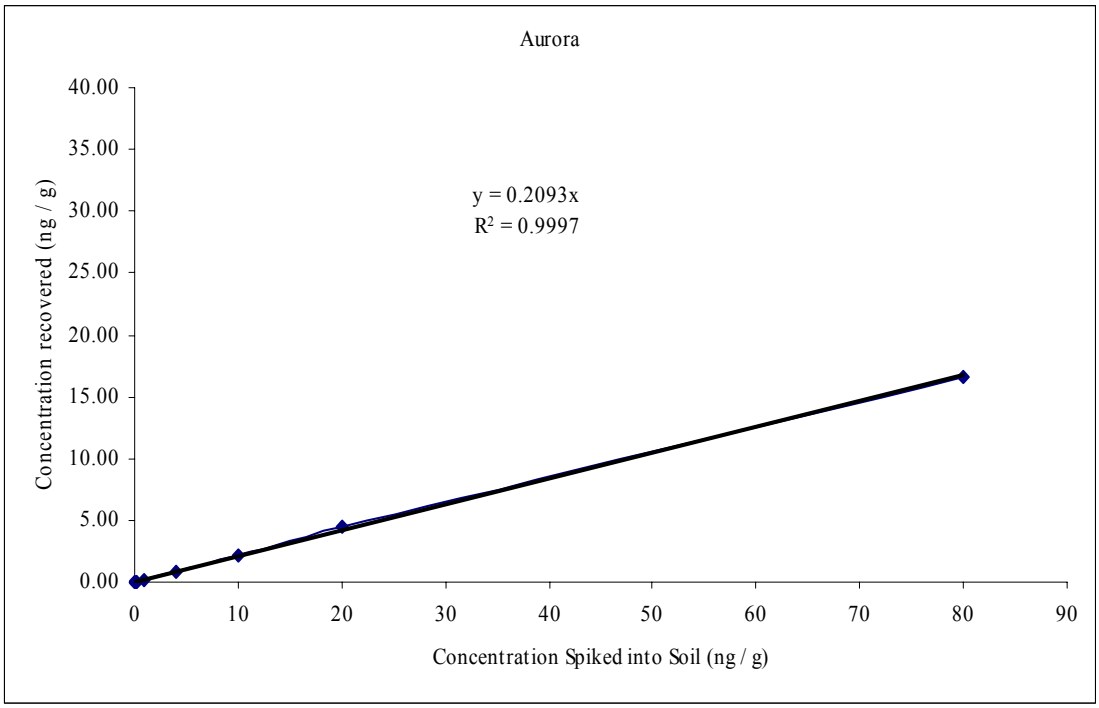
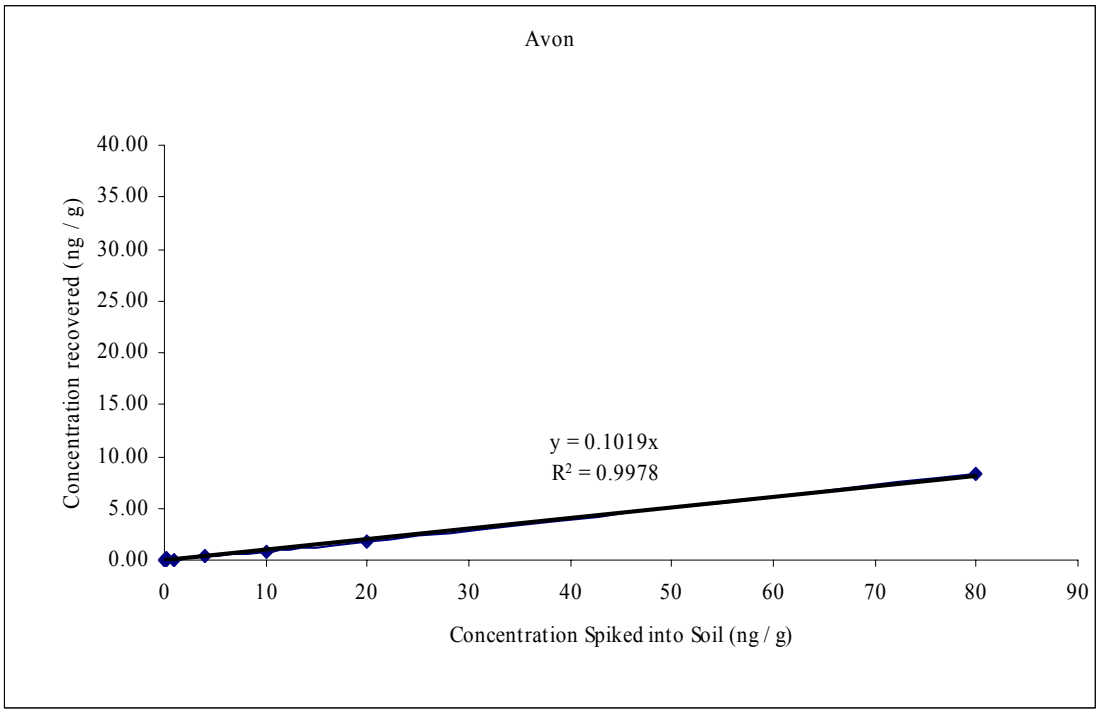
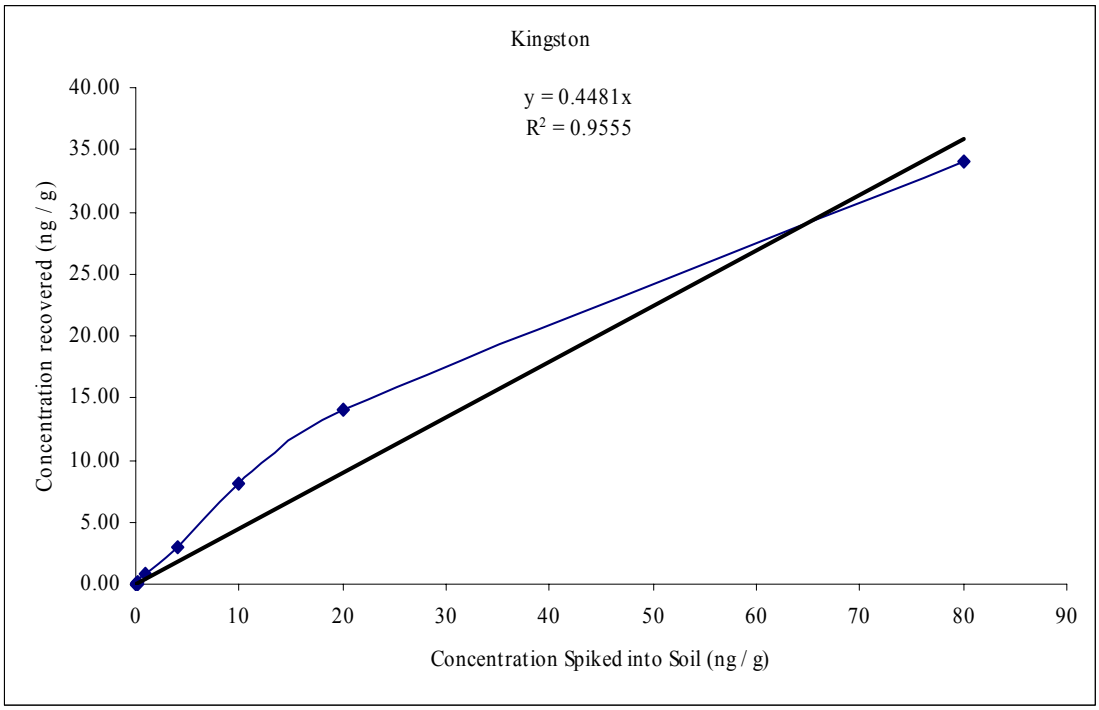
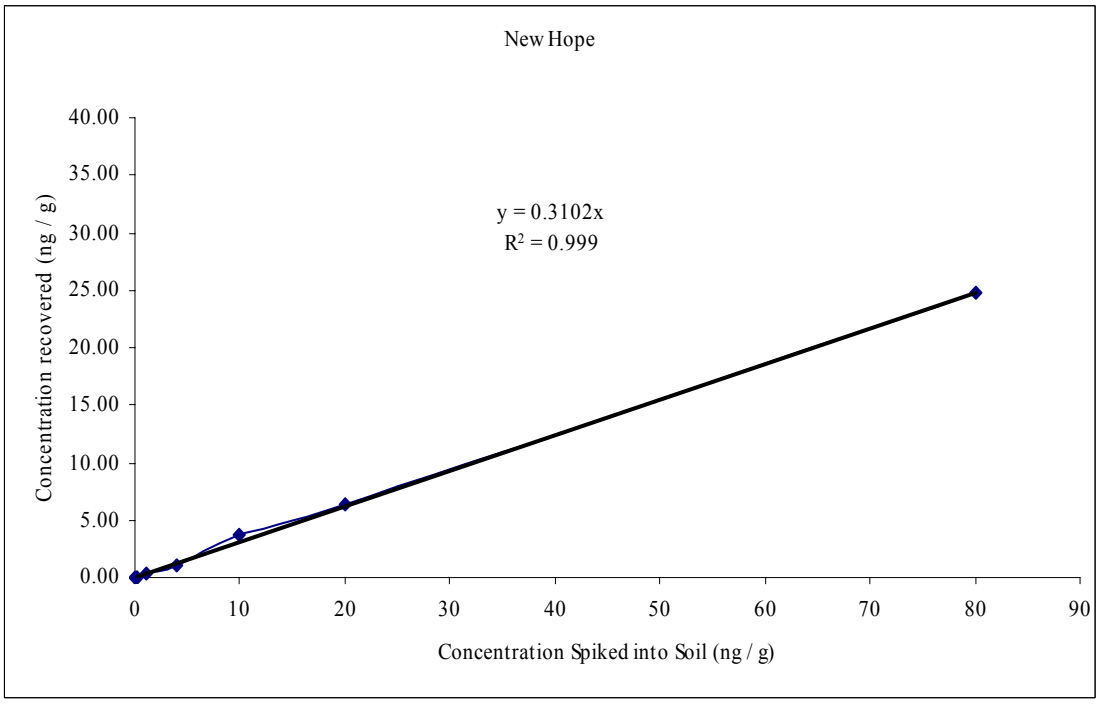
APPENDIX A


Albion
y = 0.3607x
R² = 0.9926
Concentration recovered (ng / g)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
10
20
30
40
50
60
70
80
90
Concentration Spiked into Soil (ng / g)


Aurora
y = 0.2093x
R² = 0.9997
Concentration recovered (ng / g)
Concentration Spiked into Soil (ng / g)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
0
10
20
30
40
50
60
70
80
90


Avon
y = 0.1019x
R² = 0.9978
Concentration recovered (ng / g)
Concentration Spiked into Soil (ng / g)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
0
10
20
30
40
50
60
70
80
90


Kingston
y = 0.4481x
R2 = 0.9555
Concentration recovered (ng / g)
Concentration Spiked into Soil (ng / g)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
0
10
20
30
40
50
60
70
80
90


New Hope
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R2 = 0.999
Concentration recovered (ng / g)
Concentration Spiked into Soil (ng / g)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
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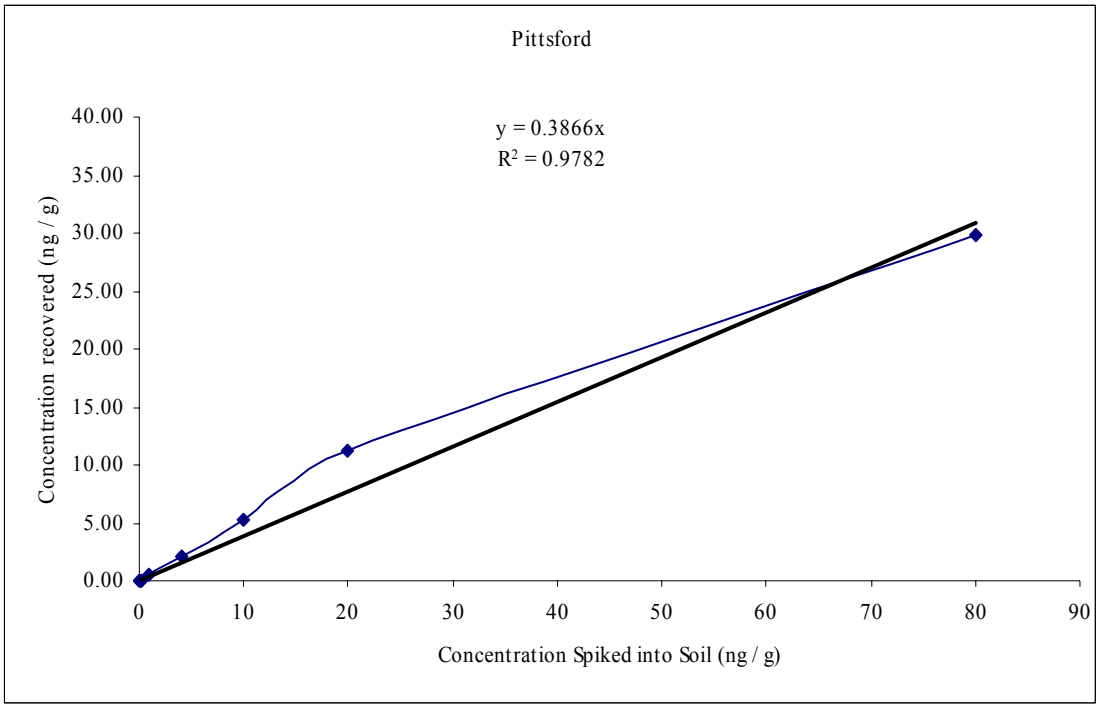
0
10
20
30
40
50
60
70
80
90


Pittsford
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R2 = 0.9782
Concentration recovered (ng / g)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
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0
10
20
30
40
50
60
70
80
90
Concentration Spiked into Soil (ng / g)

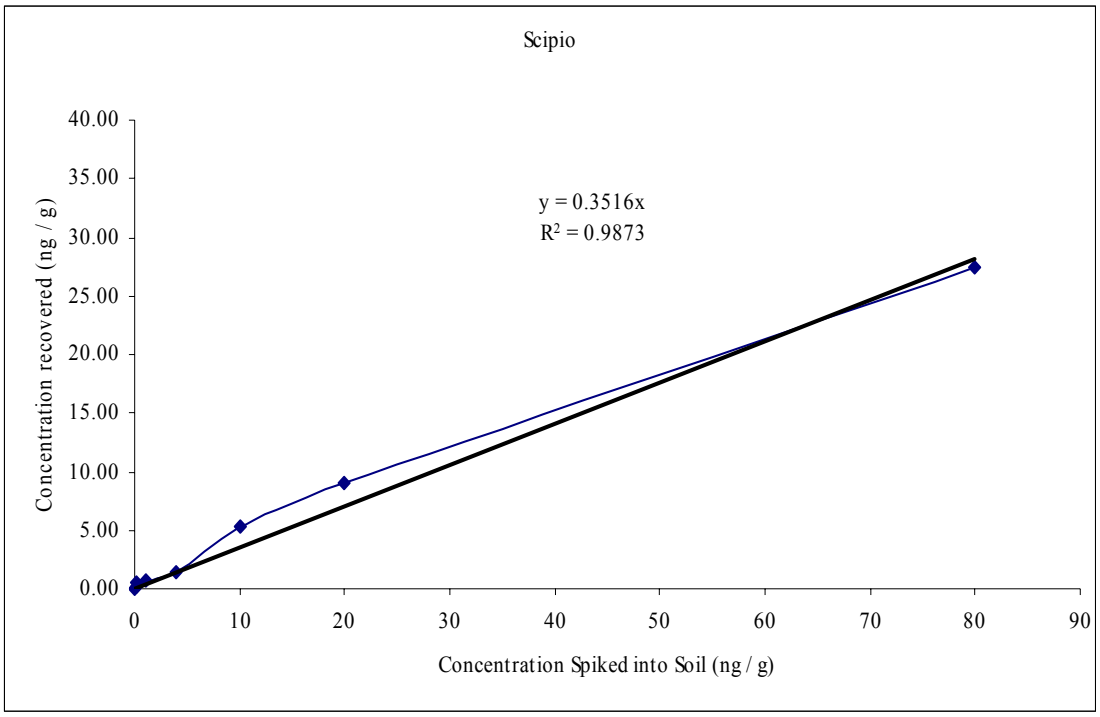
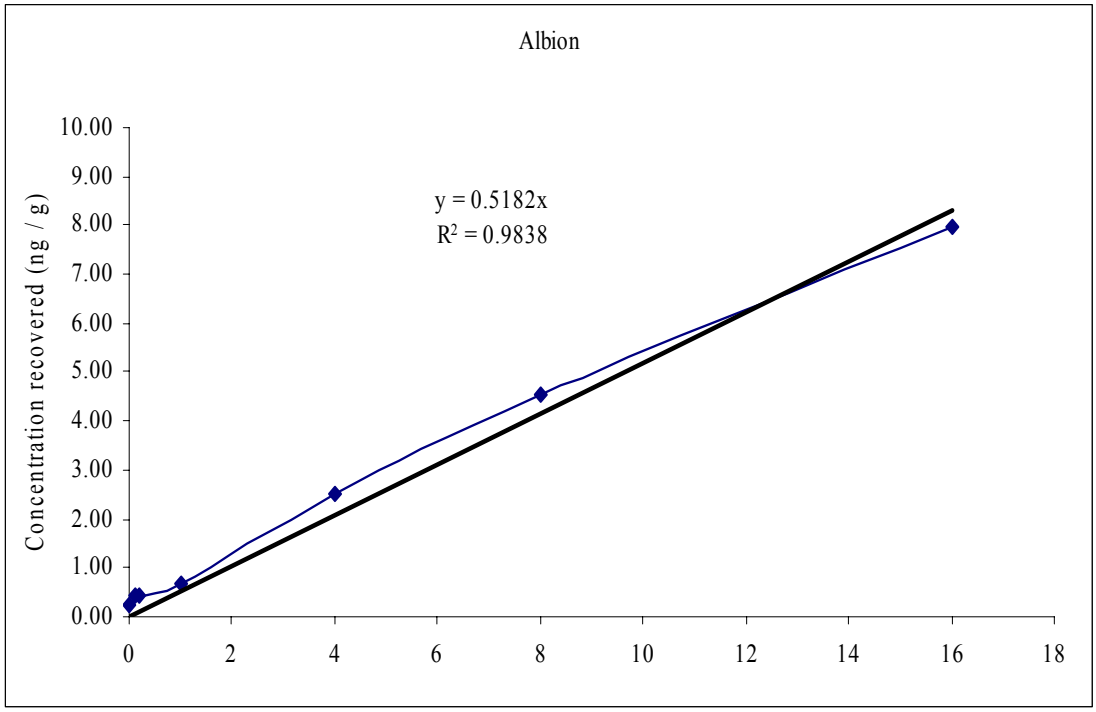
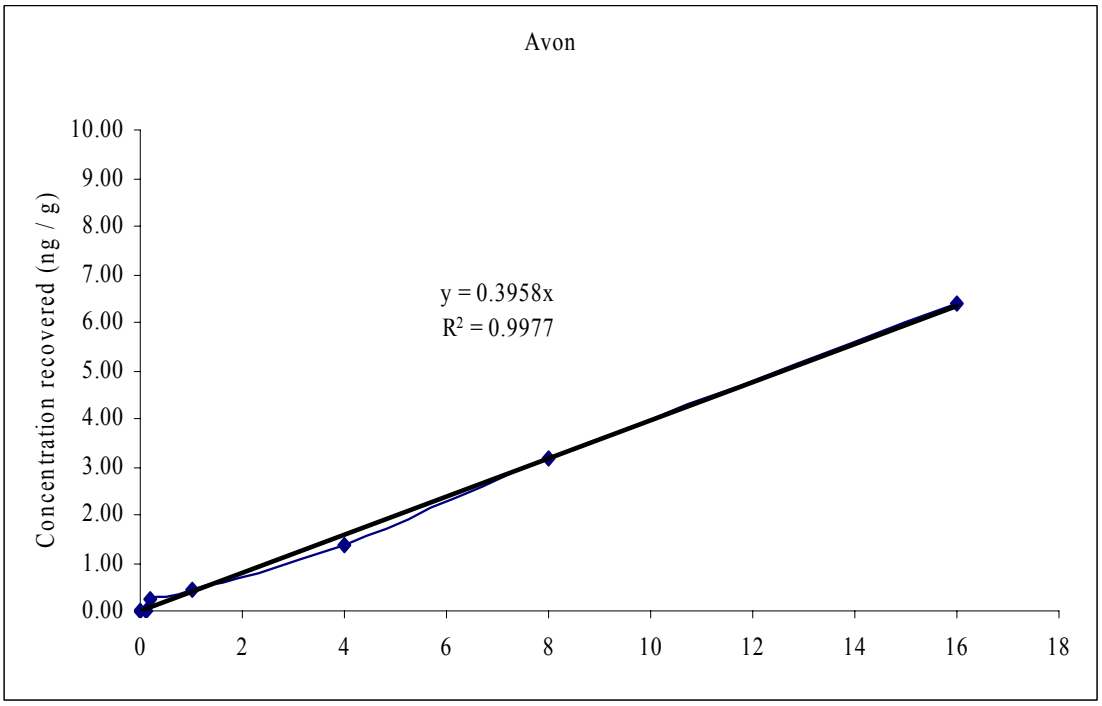
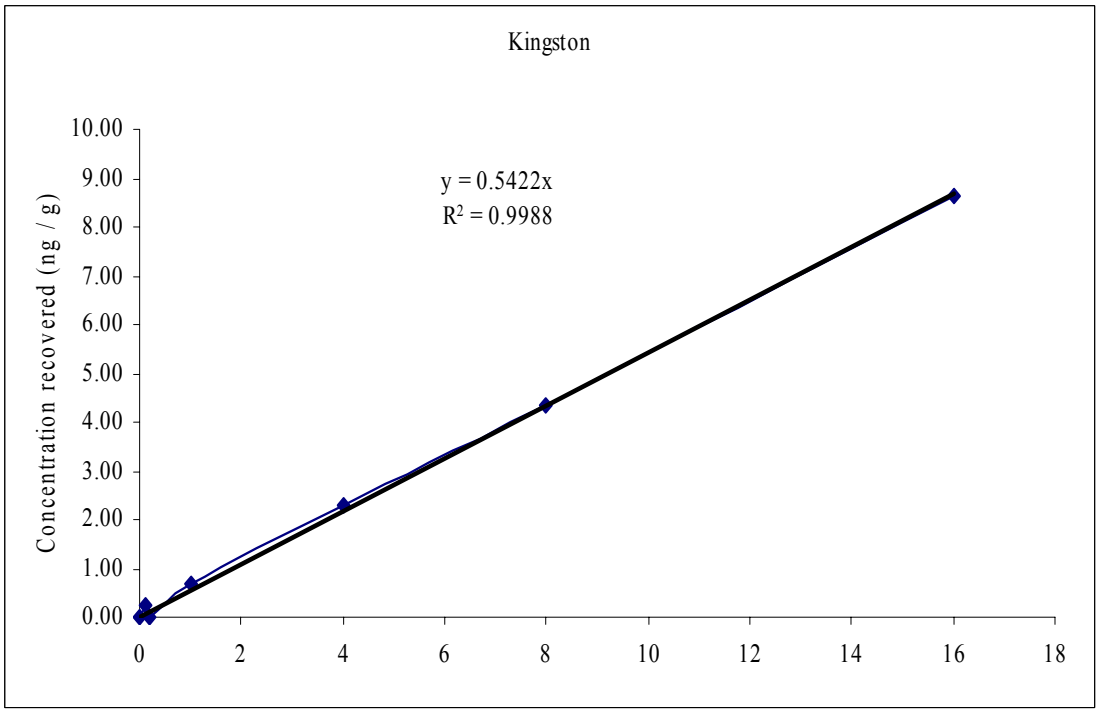
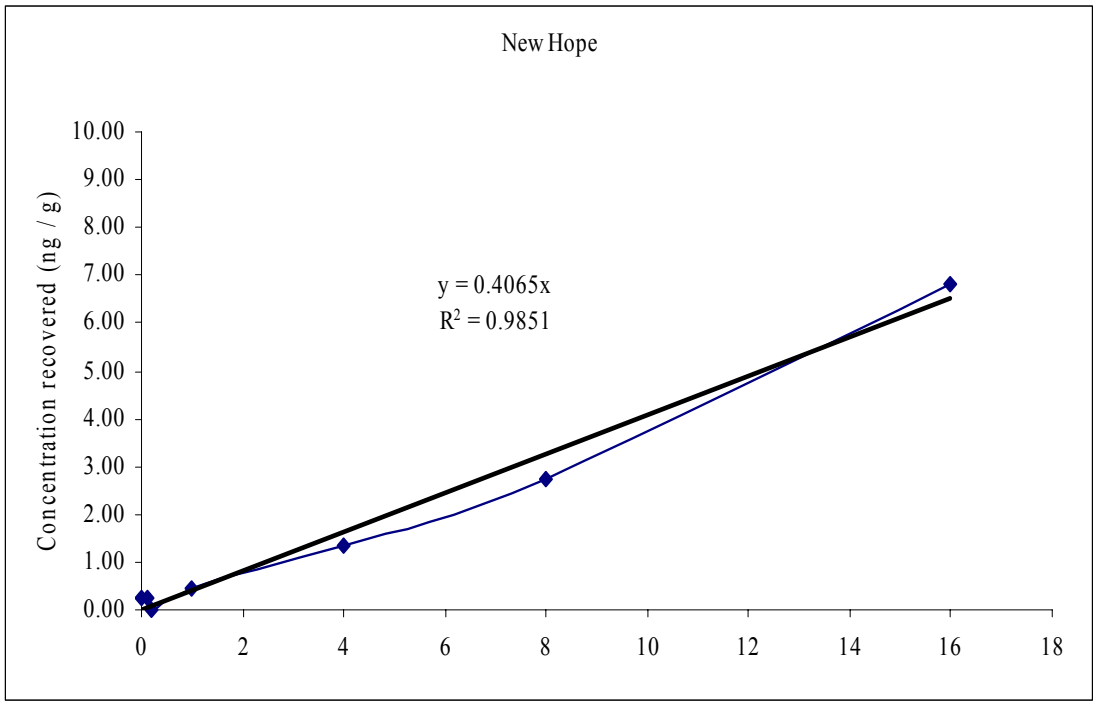


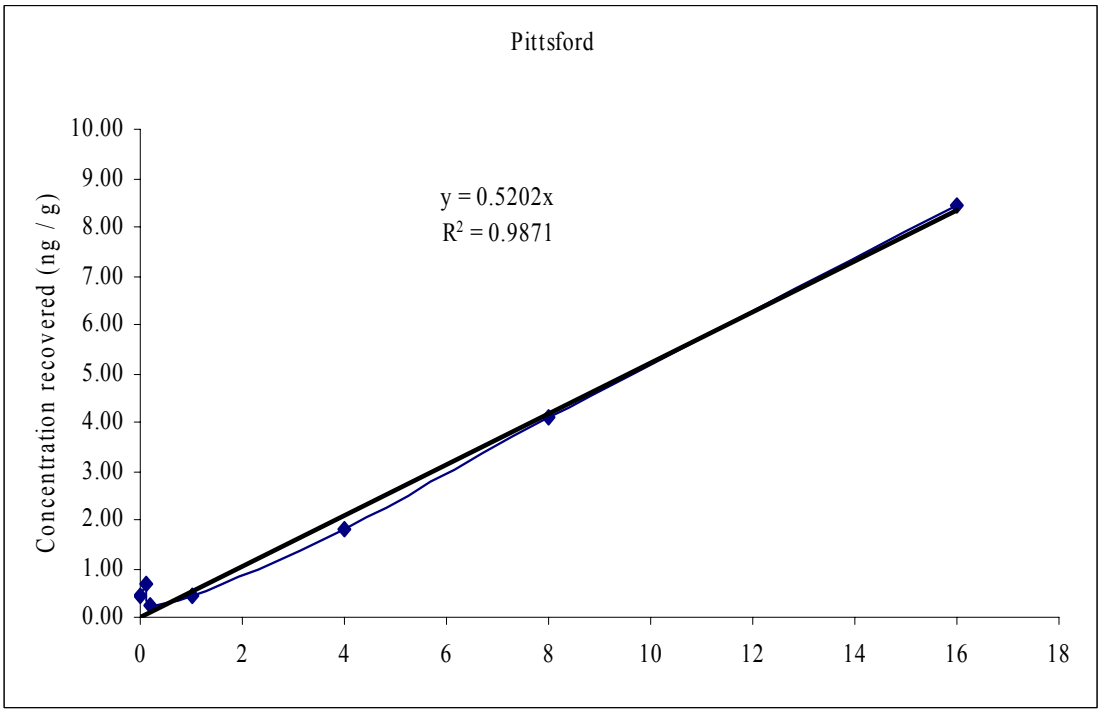
Figure A.1 Standard curves for determining corrected amounts of Cry3Bb1 protein in Albion, Aurora, Avon, Kingston, New Hope, Pittsford, and Scipio soils from NY State based on extraction efficiency of the protein.


Albion
Concentration recovered (ng / g)
y = 0.5182x
R² = 0.9838
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
2
4
6
8
10
12
14
16
18


Avon
Concentration recovered (ng / g)
y = 0.3958x
R² = 0.9977
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
2
4
6
8
10
12
14
16
18


Kingston
Concentration recovered (ng / g)
y = 0.5422x
R² = 0.9988
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
2
4
6
8
10
12
14
16
18


New Hope
Concentration recovered (ng / g)
y = 0.4065x
R2 = 0.9851
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
2
4
6
8
10
12
14
16
18


Pittsford
Concentration recovered (ng / g)
y = 0.5202x
R2 = 0.9871
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
2
4
6
8
10
12
14
16
18

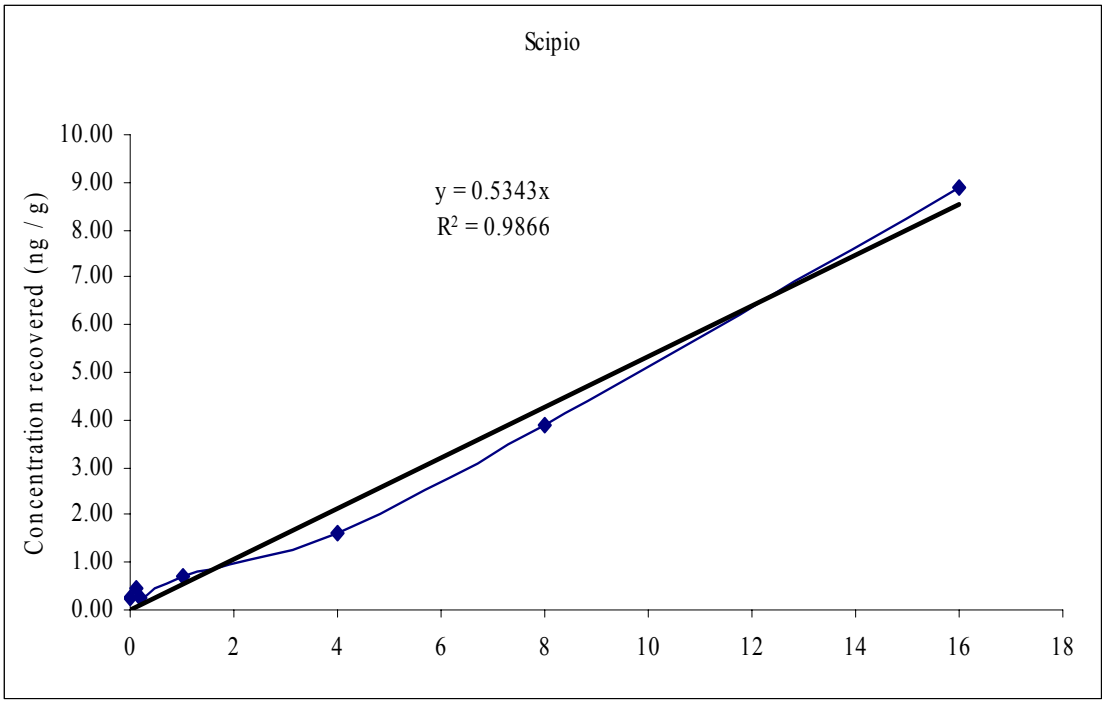


Figure A.2 Standard curves for the determination of corrected amounts of Cry1Ab protein in Albion, Avon, Kingston, New Hope, Pittsford, and Scipio soils from New York State based on its extraction efficiency of the protein.

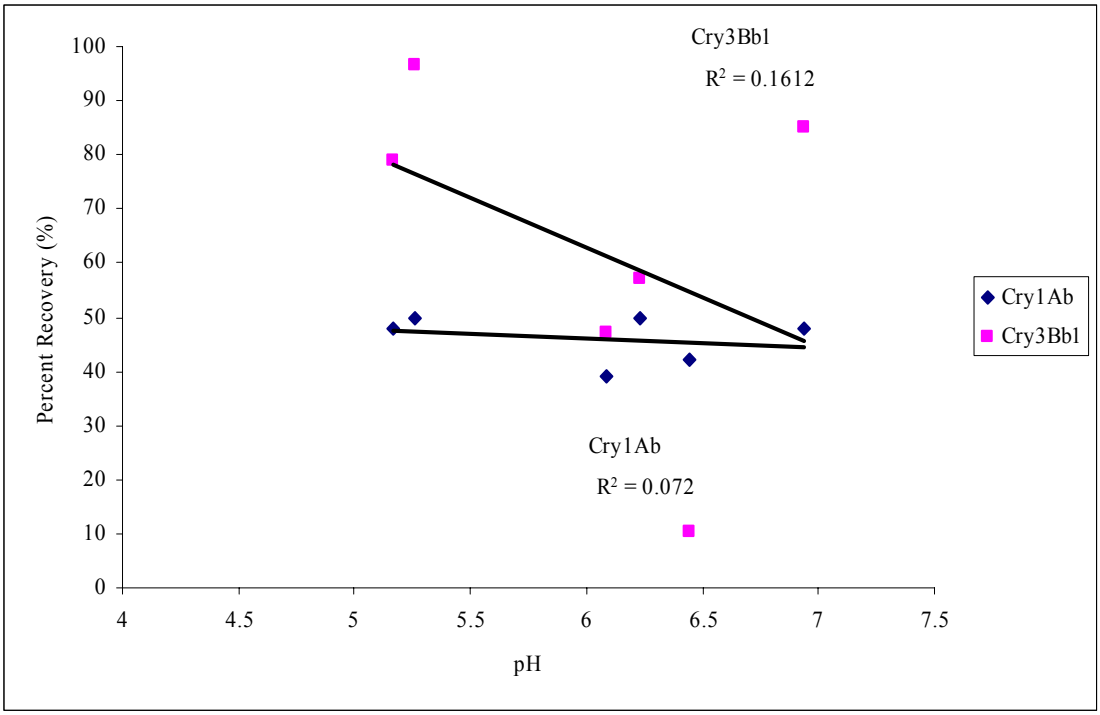


Figure A.3 Comparison of effects of pH on percent recovery (%) between Cry3Bb1 and Cry1Ab.

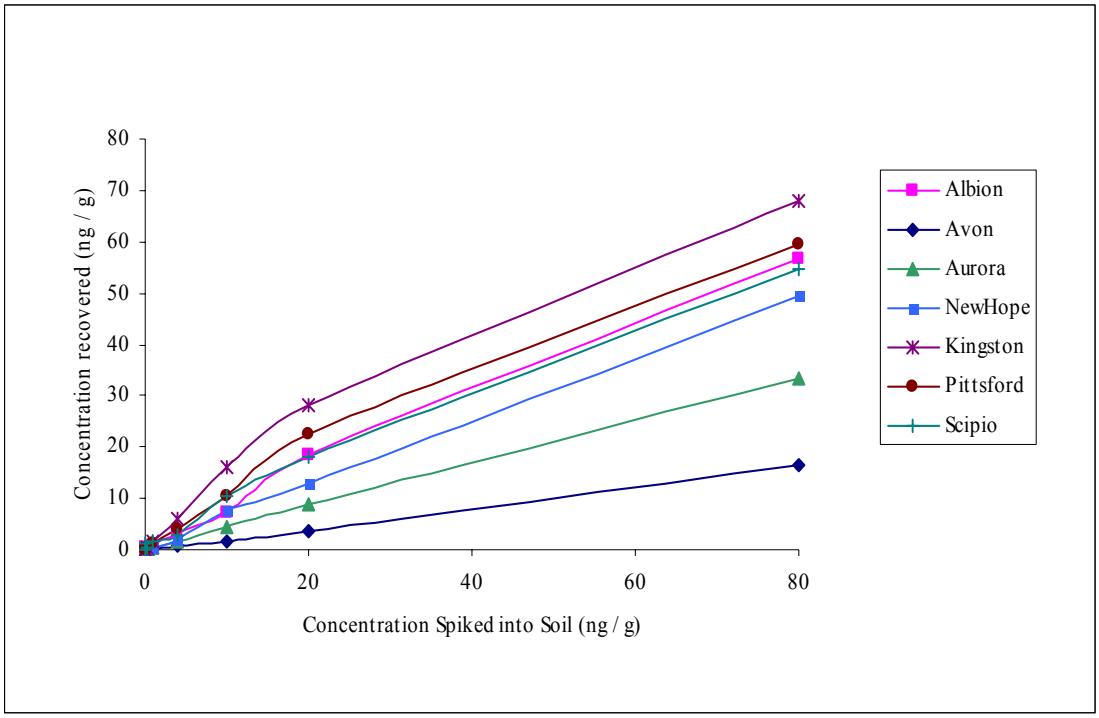


Figure A.4 Concentration of Cry3Bb1 recovered as a function of percent clay (%).

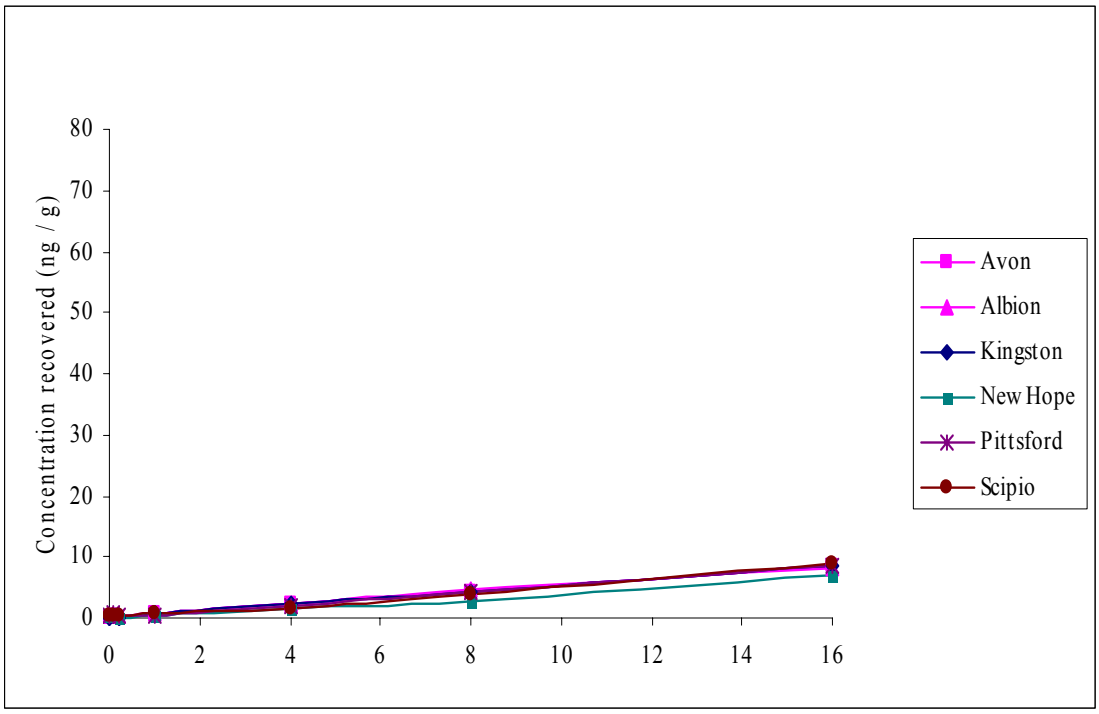


Figure A.5 Concentration of Cry1Ab recovered as a function of percent clay (%).

APPENDIX B

Table B.1 Quantification of Cry3Bb1 Protein in corn residues.

	(ng Cry3Bb1 protein g^{-1} plant residue)			
Plot	**Cob**	**Leaves**	**Stalk**	**Root**
1	0.03	11.33	6.83	0.00
3	0.00	3.23	0.12	0.00
6	4.01	11.37	0.41	0.00
7	0.00	0.06	0.02	0.00
13	0.36	12.39	2.17	0.00
15	0.07	2.00	0.06	0.00
16	0.02	0.20	0.00	0.00
18	1.51	10.72	1.76	0.00
19	0.03	0.78	0.02	0.00
21	4.94	3.27	2.25	0.00

Table B.2 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Aurora field plots in 2006 as determined by ELISA.

	Sampling time		
Plot	**Pre-planting**	**Mid-season**	**After Harvest**
1	0.00	3.33	0.05
3 / 11	0.00	3.95	0.00
6	0.00	0.43	0.00
13	0.00	2.10	0.00
18	0.00	4.86	0.00
21	0.00	35.86	0.00
24	0.00	0	0.00

Table B.3 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Kingston field sites mid-season 2006 (6551) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
K5 101	1	A1	TA5859	CB+RW	2.67
K5 201	2	A2	TA5859	CB+RW	24.31
K5 304	3	A3	TA5859	CB+RW	21.09
K5 103	1	B1	TA675-13	RR+CB+RW	1.87
K5 203	2	B2	TA675-13	RR+CB+RW	0.00
K5 302	3	B3	TA675-13	RR+CB+RW	6.96
K5 105	1	C1	TA67657	RR+RW	0.53
K5 202	2	C2	TA67657	RR+RW	16.29
K5 301	3	C3	TA67657	RR+RW	6.96
K5 205	1	D1	DKC 6168	RR+RW	0.00
K5 303	2	D2	DKC 6168	RR+RW	1.07
K5 104	1	E1	TA6704	RW	5.89
K5 204	2	E2	TA6704	RW	2.13
K5 305	3	E3	TA6704	RW	2.42
K5 307			TA6750	-	0.00

Table B.4 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Kingston field sites mid-season 2006 (6451) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
K4 106	1	A1	PIO34H39	CB+RW	0.00
K4 205	2	A2	PIO34H39	CB+RW	4.02
K4 304	3	A3	PIO34H39	CB+RW	2.42
K4 107	1	B1	TA552-13	RR+CB+RW	0.00
K4 204	2	B2	TA552-13	RR+CB+RW	4.02
K4 301	3	B3	TA552-13	RR+CB+RW	2.67
K4 109	1	C1	PIO36N73	RR+CB+RW	0.27
K4 203	2	C2	PIO36N73	RR+CB+RW	2.42
K4 309	3	C3	PIO36N73	RR+CB+RW	1.07
K4 108	1	D1	TA5524	RW	0.00
K4 209	2	D2	TA5524	RW	0.00
K4 302	3	D3	TA5524	RW	0.00
K4 101	4	D4	TA5854	RW	10.93
K4 207	5	D5	TA5854	RW	0.00
K4 306	6	D6	TA5854	RW	7.22
K4 104			TA5510	-	0.82

Table B.5 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Pittsford field sites mid-season 2006 (6551) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
P5 704	1	A1	TA5859	CB+RW	16.05
P5 806	2	A2	TA5859	CB+RW	6.51
P5 902	3	A3	TA5859	CB+RW	61.56
P5 705	1	B1	TA675-13	RR+CB+RW	0.00
P5 801	2	B2	TA675-13	RR+CB+RW	10.33
P5 903	3	B3	TA675-13	RR+CB+RW	5.74
P5 702	1	C1	TA67657	RR+RW	1.15
P5 703	2	C2	DKC61-68	RR+RW	0.38
P5 701	1	D1	TA6704	RW	25.23
P5 807	2	D2	TA6704	RW	0.00
P5 707			TA6750	-	0.00

Table B.6 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Albion field sites mid-season 2006 (6351) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
AL3 105	1	A1	DKC46-22	RR+CB+RW	1.19
AL3 204	2	A2	DKC46-22	RR+CB+RW	2.00
AL3 308	3	A3	DKC46-22	RR+CB+RW	0.00
AL3 101	4	A4	DKC51-39	RR+CB+RW	1.19
AL3 208	5	A5	DKC51-39	RR+CB+RW	0.97
AL3 307	6	A6	DKC51-39	RR+CB+RW	0.36
AL3 106	7	A7	PI38P-10	RR+CB+RW	0.78
AL3 207	8	A8	PI38P-10	RR+CB+RW	1.36
AL3 303	9	A9	PI38P-10	RR+CB+RW	0.56
AL3 108	1	D1	DKC46-24	RR+RW	0.00
AL3 201	2	D2	DKC46-24	RR+RW	3.94
AL3 206			PI38P-05	-	0.00

Table B.7 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at New Hope field sites mid-season 2006 (6351) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
NH3 408	1	A1	DKC46-22	RR+CB+RW	0.48
NH3 501	2	A2	DKC46-22	RR+CB+RW	0.00
NH3 601	3	A3	DKC46-22	RR+CB+RW	0.32
NH3 405	4	B1	DKC51-39	RR+CB+RW	1.29
NH3 507	5	B2	DKC51-39	RR+CB+RW	0.00
NH3 605	6	B3	DKC51-39	RR+CB+RW	0.81
NH3 607	1	C1	PI38P-10	RR+CB+RW	2.39
NH3 503	2	C2	PI38P-10	RR+CB+RW	0.32
NH3 403	3	C3	PI38P-10	RR+CB+RW	3.13
NH3 407	1	D1	DKC46-24	RR+RW	1.74
NH3 502	2	D2	DKC46-24	RR+RW	0.00
NH3 608	3	D3	DKC46-24	RR+RW	0.00
NH3 508			PI38P-05	-	0.00

Table B.8 Concentration (ng g^{-1}) of Cry3Bb1 protein in rhizosphere soil for Cry3Bb1 at Pittsford field sites mid-season 2006 (6351) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
SC3 706	1	A1	DKC46-22	RR+CB+RW	0.09
SC3 707	2	A2	DKC46-22	RR+CB+RW	0.25
SC3 802	3	A3	DKC46-22	RR+CB+RW	0.55
SC3 901	4	A4	DKC46-22	RR+CB+RW	0.09
SC3 705	5	B1	DKC51-39	RR+CB+RW	0.00
SC3 805	6	B2	DKC51-39	RR+CB+RW	0.09
SC3 902	1	B3	DKC51-39	RR+CB+RW	0.00
SC3 801	2	C1	PI38P-10	RR+CB+RW	0.09
SC3 905	3	C2	PI38P-10	RR+CB+RW	0.09
SC3 708	1	D1	DKC46-24	RR+RW	0.51
SC3 806	2	D2	DKC46-24	RR+RW	0.09
SC3 907	3	D3	DKC46-24	RR+RW	0.09
SC3 702			PI38P-05	-	0.00

Table B.9 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Albion field sites mid-season 2006 (6351) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
AL3 101	1	A1	RR+CB+RW	DKC51-39	7.88
AL3 208	2	A2	RR+CB+RW	DKC51-39	1.77
AL3 103	3	A3	RR+CB	DKC50-20	0.38
AL3 205	1	B1	RR+CB	DKC50-20	0.65
AL3 105	2	B2	RR+CB+RW	DKC46-42	0.19
AL3 204	3	B3	RR+CB+RW	DKC46-42	0.79

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Table B.10 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Avon field sites mid-season 2006 (6551) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
AV5 401	1	A1	RR+CB+RW	TA675-13	0.60
AV5 402	2	A2	RR+CB+RW	TA552-13	0.00
AV5 502	3	A3	RR+CB+RW	TA675-13	1.48
AV5 602	4	A4	RR+CB+RW	TA675-13	6.73
AV5 404	1	B1	CB+RW	TA5859	0.00
AV5 504	2	B2	CB+RW	TA5859	0.00
AV5 607	3	B3	CB+RW	TA5859	0.18
AV5 402			-	TA6750	0.00

Table B.11 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Avon field sites mid-season 2006 (6451) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
AV4 405	1	A1	RR+CB+RW	Pio36N73	0.00
AV4 501	2	A2	RR+CB+RW	Pio36N73	0.00
AV4 603	3	A3	RR+CB+RW	Pio36N73	0.35
AV4 406	1	B1	CB+RW	Pio34H39	0.28
AV4 509	2	B2	CB+RW	Pio34H39	0.25
AV4 604	3	B3	CB+RW	Pio34H39	0.55
AV4 506	1	C1	RR+CB+RW	TA552-13	1.10
AV4 609	2	C2	RR+CB+RW	TA552-13	2.20
AV4 407			-	Pio36N70	0.00

Table B.12 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Kingston field sites mid-season 2006 (6551) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
K5 101	1	A1	CB+RW	TA5859	0.07
K5 201	2	A2	CB+RW	TA5859	5.98
K5 304	3	A3	CB+RW	TA5859	3
K5 103	1	B1	RR+CB+RW	TA675-13	0.2
K5 302	2	B2	RR+CB+RW	TA675-13	1.35
K5 307			-	TA6750	0

Table B.13 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Kingston field sites mid-season 2006 (6451) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
K4 106	1	A1	CB+RW	Pio34H39	0.31
K4 205	2	A2	CB+RW	Pio34H39	0.13
K4304	3	A3	CB+RW	Pio34H39	0.02
K4 107	1	B1	RR+CB+RW	TA552-13	0.02
K4 204	2	B2	RR+CB+RW	TA552-13	2.19
K4 301	3	B3	RR+CB+RW	TA552-13	0.09
K4 109	1	C1	RR+CB+RW	Pio36N73	6.74
K4 203	2	C2	RR+CB+RW	Pio36N73	0.28
K4 309	3	C3	RR+CB+RW	Pio36N73	1.37
K4 103			-	Pio36N70	0.00

Table B.14 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Pittsford field sites mid-season 2006 (6551) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
P5 702	1	A1	RR+CB+RW	TA552-13	4.19
P5 801	2	A2	RR+CB+RW	TA552-13	0.04
P5 904	3	A3	RR+CB+RW	TA552-13	3.17
P5 703	4	A4	RR+CB+RW	Pio36N73	0.37
P5 809	5	A5	RR+CB+RW	Pio36N73	0.12
P5 907	6	A6	RR+CB+RW	Pio36N73	4.33
P5 704	1	B1	CB+RW	Pio34H39	0.35
P5 803	2	B2	CB+RW	Pio34H39	0.88
P5 901	3	B3	CB+RW	Pio34H39	0.12
P5 706			-	Pio36N70	0.00

Table B.15 Concentration (ng g^{-1}) of Cry1Ab protein in rhizosphere soil for Cry1Ab at Pittsford field sites mid-season 2006 (6451) as determined by ELISA.

Soil Site	Plot	Replication	Variety	Construct	Concentration
P4 704	1	A1	CB+RW	TA5859	3.92
P4 806	2	A2	CB+RW	TA5859	0.71
P4 902	3	A3	CB+RW	TA5859	8.00
P4 705	1	B1	RR+CB+RW	TA675-13	0.37
P4 801	2	B2	RR+CB+RW	TA675-13	5.46
P4 903	3	B3	RR+CB+RW	TA675-13	0.50
P4 707			-	TA6750	0.00