Pedosphere **28**(1): 94–102, 2018
doi:10.1016/S1002-0160(18)60006-2
ISSN 1002-0160/CN 32-1315/P

Published by Elsevier B.V. and Science Press

PEDOSPHERE

www.elsevier.com/locate/pedosphere

Adsorption and Desorption of Cry1Ab Proteins on Differently Textured Paddy Soils

Ling LIU, Stefan KNAUTH and Thilo EICKHORST*

Soil Microbial Ecology, University of Bremen, 28359 Bremen (Germany)

(Received July 19, 2017; revised January 19, 2018)

ABSTRACT

In recent years, selected *cry* genes from *Bacillus thuringiensis* (*Bt*) encoding the production of Cry proteins (*Bt* toxins) have been engineered into crop plants (*Bt*-crops). Through the cultivation of *Bt* crops and the application of *Bt* pesticides, Cry proteins could be introduced into arable soils. The interaction between the proteins and soils was analyzed in this study to investigate the affinity of Cry proteins in paddy soil ecosystems. Four Paddy soils were selected to represent different soil textures. Cry proteins were spiked in soils, and the amount of protein adsorbed was measured over 24 h. Desorption of Cry1Ab proteins from paddy soils was performed by washing with sterile Milli-Q water (H_2O_{MQ}), and subsequently extracted with an extraction buffer. The paddy soils had a strong affinity for Cry1Ab proteins. Most of the Cry1Ab proteins added (> 98%) were rapidly adsorbed on the paddy soils tested. More Cry1Ab proteins were adsorbed on non-sterile soils than on sterile soils. Less than 2% of the adsorbed Cry1Ab proteins were desorbed using H_2O_{MQ}, while a considerable proportion of the adsorbed proteins could be desorbed with the buffer, ranging from 20% to 40%. The amount of proteins desorbed increased with the increases in the initial amount of Cry1Ab proteins added to the paddy soils. The concentration of Cry1Ab proteins desorbed from the paddy soils was higher for sterile soils than non-sterile ones. Our results indicate that *Bt* toxins released via the cultivation of *Bt* crops, the application of *Bt* pesticides can be adsorbed on paddy soils, and soil texture could impose an impact on the adsorption capability.

Key Words: affinity, *Bt* toxin, extraction, protein solution, soil texture, sterile soil

Citation: Liu L, Knauth S, Eickhorst T. 2018. Adsorption and desorption of Cry1Ab proteins on differently textured paddy soils. *Pedosphere*. **28**(1): 94–102.

INTRODUCTION

Bacillus thuringiensis is a gram-positive soil bacterium with the affiliation to the class of *Bacilli* belonging to the phylum Firmicutes, and is commonly known as *Bt* (Parry *et al.*, 1983; Read *et al.*, 2003). It is characterized by its ability to form parasporal crystals during sporulation (Read *et al.*, 2003). The proteins in these crystals, referred to as Cry endotoxins or *Bt* toxins, exhibit insecticidal activity against specific insect groups, many of which are economically important crop pests. After ingestion by a susceptible insect, *Bt* toxins are solubilized in the insect midgut (pH > 10), and proteolytically activated into a toxin core in the insect digestive fluid (Höfte and Whiteley, 1989; Crecchio and Stotzky, 2001). The activated toxins are then bound to midgut receptors and inserted into the apical membrane, where the toxins generate ion channels or pores, thus disturbing the osmotic balance. Consequently, the cells swell and lyse (Harvey *et al.*, 1983; Knowles and Ellar, 1987; Hofmann *et al.*, 1988; Schnepf *et al.*, 1998). To date, *Bt* toxins are classified into four major groups and several subgroups characterized by both structural similarities and the insecticidal spectra of the toxins. The four major groups are Lepidoptera-specific (Cry1), Lepidoptera- and Diptera-specific (Cry2), Coleoptera-specific (Cry3), and Diptera-specific (Cry4) (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998; Mohan and Gujar, 2001).

The highly specific insecticidal effect led to the development of pesticides based on a mixture of cells, spores, and parasporal crystals of *Bt* (Beegle and Yamamoto, 1992). For decades, *Bt* has proven to be a useful alternative or supplement to synthetic chemical insecticides applied in agriculture, forest management, and mosquito control (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998; Crecchio and Stotzky, 2001; Dohrmann *et al.*, 2013). Typical agricultural formulations of *Bt* consist of wettable powders, spray concentrates, liquid concentrates, dusts, baits, and time-release rings. Products of *Bt* used as microbial pest co-

*Corresponding author. E-mail: eickh@uni-bremen.de.

ntrol agents in agriculture and forestry are already commercially available, which are based on formulations of different *Bt* subspecies (Höfte and Whiteley, 1989; Icoz and Stotzky, 2008), *e.g.*, Dipel® (DF, dry flowable *Bt kurstaki*), Xentari® (WG, *Bt aizawai*), and NEUDORFF® (StechmückenFrei, *Bt israelensis*).

The toxins from *Bt* are attractive as applied pesticides, and also for delivery via genetically modified (GM) plants. The *cry* genes coding for insecticidal crystal proteins have been cloned from *Bt*. With the insertion of exogenous *cry* genes in plant chromosomal DNA, *Bt* toxins can be expressed within plants (Schnepf *et al.*, 1998; Crecchio and Stotzky, 2001; Dohrmann *et al.*, 2013). Selected *cry* genes have been introduced into major crops, such as maize (Koziel *et al.*, 1993; Kramarz *et al.*, 2007), rice (Fujimoto *et al.*, 1993), and potato (Adang *et al.*, 1993), to produce their own *Bt* toxins. Transgenic *Bt* crops are considered as a powerful and promising alternative strategy for the protection of crops against insect infection (Höfte and Whiteley, 1989). Commercial cultivation of GM plants began in 1996 and has expanded rapidly, with 20.3 million ha of *Bt* crops representing 37% of all GM crops in 2007 (Helassa *et al.*, 2009). The global area of GM crops continued to increase in recent years, reaching 175.2 million ha in 2013 (James, 2013). Given the rapid increase in the commercial cultivation of transgenic *Bt* crops, their environmental effects can't be completely assessed (Icoz and Stotzky, 2008; Helassa *et al.*, 2009). The *Bt* toxins are continuously produced by the *Bt* plants and apparently persist for some time in plant tissues (Sims and Holden, 1996; Sims and Ream, 1997). Insecticidal proteins from *Bt* plants are released into the soil ecosystem by different pathways, *e.g.*, via root exudates during plant growth (Saxena *et al.*, 1999; Saxena and Stotzky, 2000), via the incorporation of plant residues after the harvest of *Bt* plants (Tapp and Stotzky, 1998), and probably via some input from pollen (Losey *et al.*, 1999; Pagel-Wieder *et al.*, 2007).

Through both the application of *Bt* pesticides and the commercial cultivation of transgenic *Bt* plants, *Bt* toxins can be released into the soil ecosystem. Their environmental fate relies on interactions with soil; thus the effects of interactions between *Bt* toxins and soil particles on the persistence and insecticidal activity of the toxins should be evaluated (Crecchio and Stotzky, 2001; Hung *et al.*, 2016). In controlled microcosm experiments and field studies, it has been shown that the toxins could be adsorbed and thus persist in soil for up to 8 months (Tapp and Stotzky, 1998; Saxena and Stotzky, 2002; Zwahlen *et al.*, 2003). When the soil is spiked with *Bt* toxins, the toxins are bound to non-sterile soils and still detectable after 40 d (Tapp and Stotzky, 1995a, b). Moreover, the binding protects toxins against microbial degradation, but the insecticidal activity of the toxins is retained (Tapp and Stotzky, 1995b; Koskella and Stotzky, 1997). It is also essential to understand the reversibility of adsorption, as the ability of desorption influences the mobility and persistence of toxins in soil (Helassa *et al.*, 2009). Desorption of *Bt* toxins has been investigated in a few studies. In general, it has been reported that *Bt* toxins could not be desorbed from soil or soil minerals with either water or buffer (Tapp *et al.*, 1994; Crecchio and Stotzky, 2001; Chevallier *et al.*, 2003). In contrast, a considerable proportion of *Bt* toxins could be desorbed from minerals (*i.e.*, montmorillonite and kaolinite) with water (Saxena and Stotzky, 2000; Helassa *et al.*, 2009).

The adsorption and desorption of *Bt* toxins on soil are a function of soil (mainly clay content but also humic substances), source of the toxins (produced by transgenic *Bt* plants *versus Bt* strains), and the particular insecticidal crystal proteins under investigation (Palm *et al.*, 1996; Clark *et al.*, 2005; Pagel-Wieder *et al.*, 2007; Helassa *et al.*, 2009; Sander *et al.*, 2012; Tomaszewski *et al.*, 2012). With the commercial cultivation of transgenic *Bt* rice (*e.g.*, TT51-1) containing exogenous *cry1Ab* genes from *Bt* subsp. *kurstaki* (Wu *et al.*, 2013; Chen *et al.*, 2014), it is reasonable to investigate the fate and behavior of Cry1Ab proteins (*Bt* toxins) in paddy soils. The objective of this study was to investigate the adsorption of Cry1Ab proteins on paddy soils spiked with pure proteins, and the desorption of the adsorbed proteins. Four differently textured paddy soils were selected to investigate differences in the adsorption and desorption behaviors of the proteins among soils. Sterile treatments for soils were also considered in this study in comparison to non-sterile treatments. Several concentrations of Cry1Ab proteins were introduced to investigate soil adsorption ability over time. Desorption was determined by washing with water and subsequent extraction with a specific extraction buffer.

MATERIALS AND METHODS

Paddy soils and Cry1Ab proteins

Differently textured paddy soils were selected for this study, which had been described before (Eickhorst and Tippkötter, 2009). According to the World Reference Base for Soil Resources (IUSS Working group WRB, 2006) and the USDA texture classification (Soil

Survey Staff, 1996), these soils are classified as: sandy loam (Hydragric Anthrosol; LC), loam (Anthraquic Cambisol; MC), silt loam (Gleyic Fluvisol; TX), and silty clay (Stagnic Anthrosol; TL). Some physicochemical characteristics of the four selected soils were analyzed by methods at the group of Soil Microbial Ecology in University of Bremen, Germany, shown in Table I. Additional information concerning their soil matrices and particle morphologies can be obtained from thin section microscopy presented by Eickhorst and Tippkötter (2009). Both sterile and non-sterile treatments were applied to each paddy soil. For sterile treatments, the soils were sterilized by autoclaving at 121 °C for 30 min, followed by air-drying on a sterile workbench.

TABLE I

Selected physico-chemical parameters of the four paddy soils used, a Hydragric Anthrosol (LC), an Anthraquic Cambisol (MC), a Gleyic Fluvisol (TX), and a Stagnic Anthrosol (TL)

Soil	Clay	Silt	Sand	Texture[a)]	pH($CaCl_2$)	EC[b)]
		%				$\mu S\ cm^{-1}$
LC	12.5	26.2	61.3	Sandy loam	4.7	34
MC	23.3	44.0	32.7	Loam	4.6	51
TX	9.2	65.0	25.8	Silt loam	6.5	78
TL	41.0	58.1	0.9	Silty clay	4.7	174

[a)] According to Soil Survey Staff (1996).
[b)] Electrical conductivity.

Cry1Ab proteins were obtained from the Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, USA, and had been tested for the quantification of Cry1Ab toxins in *Bt* (Crespo *et al.*, 2008).

Adsorption of Cry1Ab proteins on paddy soils

A stock solution of Cry1Ab proteins (10 μg mL^{-1}) was diluted using sterile H_2O_{MQ} for the preparation of working solutions (500, 1 000, and 2 000 ng mL^{-1}). An aliquot of 1 mL Cry1Ab protein solution was pipetted into 200 mg paddy soil in test tubes (2 mL), generating an initial protein addition of 2.5, 5.0, or 10.0 μg g^{-1} soil. To test the adsorption of Cry1Ab proteins on the walls of tubes, a treatment of pure Cry1Ab protein solutions without soil was included. In addition, sterile H_2O_{MQ} was pipetted into the same amount of paddy soil, set up as a control. The soil suspension was incubated and rotated in test tubes (2 mL) at 25 °C in the dark for 1, 6, and 24 h. After each incubation period, the suspension was centrifuged at 12 000 × *g* for 10 min. The resulting supernatants were stored for further determination of Cry1Ab protein concentrations via enzyme-linked immunosorbent assay (ELISA), which is highly preferred due to its specificity and sensitivity of analysis, allowing it to be applied in soil systems (Zwahlen *et al.*, 2003; Baumgarte and Tebbe, 2005). The difference between the amounts of the proteins added and those detected in the supernatants was used to calculate the amount of proteins adsorbed on paddy soils. Soil pellets obtained after 24 h of incubation were prepared for desorption.

Desorption of Cry1Ab proteins from paddy soils

After the adsorption procedures, sterile H_2O_{MQ} (1 mL) was added to the soil pellets. The suspensions were equilibrated by end-over-end shaking at 25 °C in the dark for 24 h before centrifugation and analysis of Cry1Ab proteins in the supernatants.

After first desorption with H_2O_{MQ}, the resulting pellets were then resuspended in 400 μL extraction buffer, containing 0.1 mol L^{-1} Na_2CO_3, 0.1 mol L^{-1} $NaHCO_3$, 5 mmol L^{-1} ethylenediaminetetraacetic acid, 10 mmol L^{-1} dithiothreitol, 50 mmol L^{-1} $Na_4P_2O_7{\cdot}10H_2O$, and 0.1% Triton X-100 (pH 10). Based on the results of a preliminary test, the extraction buffer (as described above and reported by Wang *et al.* (2006)) had a higher extraction efficiency than other two buffers which were prepared according to the protocol in a commercial ELISA kit (AP 003, Envirologix, Portland, USA) and the recipe described in Palm et *al.* (1994) (data not shown). The suspensions were then rotated at 25 °C in the dark for 1 h. After centrifugation (12 000 × *g* for 10 min), Cry1Ab proteins in the supernatants were determined via ELISA.

Quantification of Cry1Ab proteins

The amount of Cry1Ab proteins in sample solutions was analyzed by ELISA using a commercial QualiPlate™ kit (sandwich assay: primary antibody followed by secondary anti horseradish peroxidase; AP 003, Envirologix, Portland, USA). The main procedure was the incubation of an enzyme conjugate and sample solution in a well coated with primary antibodies. After washing, a substrate was added and reacted with the enzyme to produce a stained product, indicating the presence of Cry1Ab proteins. The final solution was measured for absorbance at a wavelength of 450 nm (EMax® Endpoint ELISA Microplate Reader, Molecular Devices, Sunnyvale, USA). Inverse and linear relationships can be observed between the protein concentrations and the absorbance. A standard curve of purified Cry1Ab proteins was included in each individual analysis and thus used for calculating concentrations of Cry1Ab proteins in the samples. The sample solutions were diluted so that they were within the de-

tection range of the ELISA test. The concentration of Cry1Ab proteins was referred to 1 g soil.

Statistical analysis

The adsorption and desorption experiments were conducted in triplicate. The data are presented as means and standard errors (SEs). Statistical analyses were performed the software SPSS Statistics 20 (International Business Machines Corp., Armonk, USA) using one-way analysis of variance (ANOVA) and Duncan's test.

RESULTS AND DISCUSSION

Adsorption of Cry1Ab proteins on the silt loam soil

The Cry1Ab proteins were rapidly adsorbed on the silt loam soil when the soil was spiked with Cry1Ab proteins at three different concentrations (Fig. 1). Adsorption of Cry1Ab proteins on the silt loam soil tended to increase over time. More than 98% of the proteins were adsorbed on the soil after 1 h; thereafter, only a small amount of proteins was adsorbed. When the Cry1Ab protein concentration increased from 2.5 to 10.0 g g^{-1} soil, the amount of proteins adsorbed on the soil increased. The amount of Cry1Ab proteins adsorbed on the silt loam soil were close to 2.50, 4.98, and 9.98 μg g^{-1} soil when the initially added proteins were 2.5, 5.0, and 10.0 μg g^{-1} soil, respectively. Concentrations of proteins adsorbed on the non-sterile soils were slightly higher than those adsorbed on the sterile soil. The protein concentrations in the control tubes containing Cry1Ab protein solutions without soil did not change during the experiment, which were 500, 1 000, and 2 000 ng mL^{-1} (data not shown), indicating that there was no adsorption of Cry1Ab proteins on the walls of the tubes.

The adsorption of *Bt* toxins on paddy soils is important for assessing the environmental risk associated with the commercial cultivation of *Bt* crops (Pagel-Wieder *et al.*, 2007). Thus, the effect of contact time on the adsorption of Cry1Ab proteins on paddy soil was investigated in this study. Most of the Cry1Ab proteins added were adsorbed on the silt loam soil after 1 h (Fig. 1). Previous studies have also shown that Cry1Ab proteins are adsorbed on clay minerals within 1 h, and the adsorption is completed after 4 h (Tapp *et al.*, 1994; Pagel-Wieder *et al.*, 2007). Sundaram (1996) investigated the adsorption of *Bt* toxins on the sandy loam and clay loam soils, and found that the amount of *Bt* toxins adsorbed on the sandy loam soil reached a maximum after 3 h, in contrast to the maximum on the clay loam soil after 4 h. In addition, a longer contact time does not result in an increase in *Bt* toxin adsorption (Crecchio and Stotzky, 2001). The adsorption isotherms of *Bt* toxins on the clay soil are the L-type (Giles *et al.*, 1974), suggesting a low affinity interaction as reported in several studies (Crecchio and Stotzky, 2001; Fu *et al.*, 2007; Helassa *et al.*, 2009). The adsorption of Cry1Aa proteins on kaolinite and montmorillonite could be fitted to the Langmuir isotherm (Helassa *et al.*, 2009). Adsorption of *Bt* toxins on a constant amount of humic acids increases with increasing toxin concentrations, until a plateau is reached (Crecchio and Stotzky, 1998).

However, Sundaram (1996) investigated the adsorption pattern of *Bt* toxins on forest soils using the Freundlich adsorption isotherm and a linear isotherm equation instead of the Langmuir equation, because of the heterogeneity of the soil surface. Moreover, at higher concentrations, the linear model showed deviations. It has been reported that the adsorption of *Bt* toxins on the sandy and clay loam soils can be up to

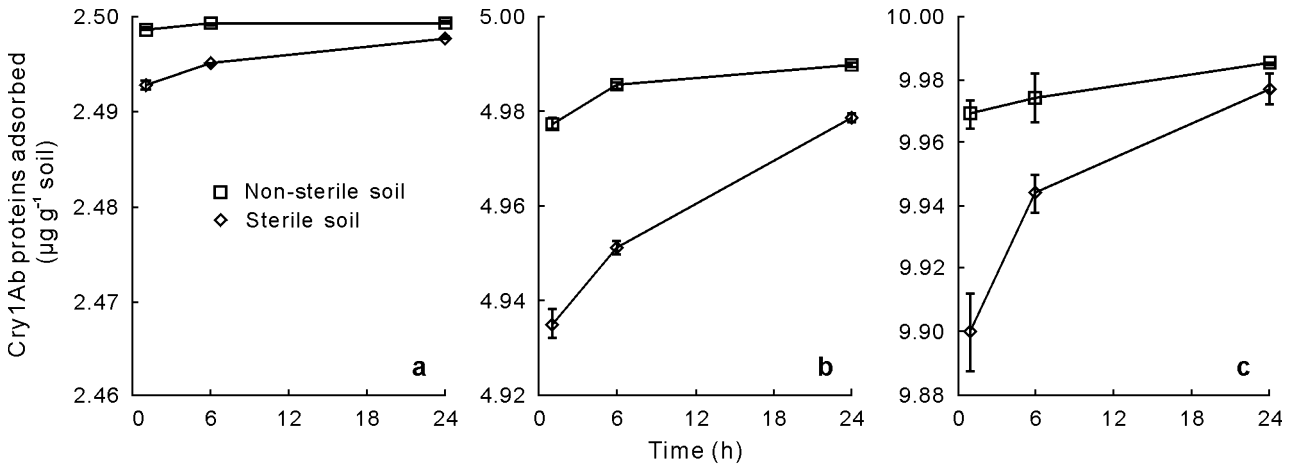

Fig. 1 Adsorption of Cry1Ab proteins on a silt loam paddy soil with an initial Cry1Ab protein addition of 2.5 (a), 5.0 (b), or 10.0 (c) μg g^{-1} soil. Vertical bars indicate standard errors of the means ($n = 3$).

301 and 474 μg g^{-1} soil, respectively (Sundaram, 1996). In this study, the high (> 98%) and rapid adsorption of Cry1Ab proteins on the silt loam paddy soil could be explained by the low concentrations of Cry1Ab proteins used, which resulted in large numbers of unoccupied surface sites on the adsorbent, that were easily accessible for molecules of Cry1Ab proteins (Pagel-Wieder *et al.*, 2007).

Autoclaving was conducted in this study to inhibit microbial activity. We detected lower concentrations of Cry1Ab proteins in supernatants from the non-sterile treatment than the sterile treatment, indicating higher adsorption of Cry1Ab proteins on the non-sterile soils than the sterile soils. Douville *et al.* (2005) suggested that sterilization could significantly inhibit the degradation of *Bt* toxins in soil and water. Our study indicated that the inactivation of indigenous microorganisms in the silt loam soil could lead to less degradation and thus higher amounts of free Cry1Ab proteins in the supernatants, which resulted in a decreasing adsorption of Cry1Ab/Ac proteins on the sterile soil.

Adsorption of Cry1Ab proteins on differently textured paddy soils

The adsorption of Cry1Ab proteins on differently textured paddy soils was measured using two initial concentrations of proteins added to the soil, *i.e.*, 2.5 and 5.0 μg g^{-1}. When the paddy soils were spiked with the Cry1Ab proteins, more than 98% of the proteins were adsorbed (Table II, Fig. 2). The adsorption was almost completed within 1 h, despite a slight increase in the amounts of proteins adsorbed afterwards. The amount of adsorbed Cry1A proteins on the sandy loam soil was more for the non-sterile treatments than the sterile treatments. Similar trend for the differences between the sterile and non-sterile treatments were found

TABLE II

Concentrations of Cry1Ab proteins adsorbed on four differently textured paddy soils[a)] with an initial Cry1Ab protein addition of 2.5 or 5.0 μg g^{-1} soil

Cry1Ab proteins added	Paddy soil	Cry1Ab protein concentration in sterile soil		Cry1Ab protein concentration in non-sterile soil	
		1 h	24 h	1 h	24 h
μg g^{-1} soil		μg g^{-1} soil			
2.5	Sandy loam	2.490 7 ± 0.0008[a)]	2.495 5 ± 0.000 3	2.494 2 ± 0.000 9	2.495 7 ± 0.000 2
	Loam	2.493 8 ± 0.000 7	2.496 4 ± 0.000 1	2.497 4 ± 0.000 2	2.498 7 ± 0.000 1
	Silt loam	2.492 7 ± 0.000 4	2.497 7 ± 0.000 2	2.498 6 ± 0.000 1	2.499 4 ± 0.000 1
	Silty clay	2.496 4 ± 0.000 7	2.497 4 ± 0.000 8	2.496 6 ± 0.000 1	2.498 7 ± 0.000 0
5.0	Sandy loam	4.940 6 ± 0.001 5	4.9765 ± 0.000 5	4.961 4 ± 0.007 2	4.982 2 ± 0.001 7
	Loam	4.958 1 ± 0.007 3	4.995 5 ± 0.000 1	4.986 5 ± 0.003 4	4.998 6 ± 0.000 0
	Silt loam	4.943 9 ± 0.003 2	4.987 6 ± 0.000 8	4.977 4 ± 0.001 3	4.989 6 ± 0.000 4
	Silty clay	4.995 3 ± 0.000 4	4.997 8 ± 0.000 1	4.996 1 ± 0.000 9	4.998 5 ± 0.000 2

[a)] Means ± standard errors ($n = 3$).

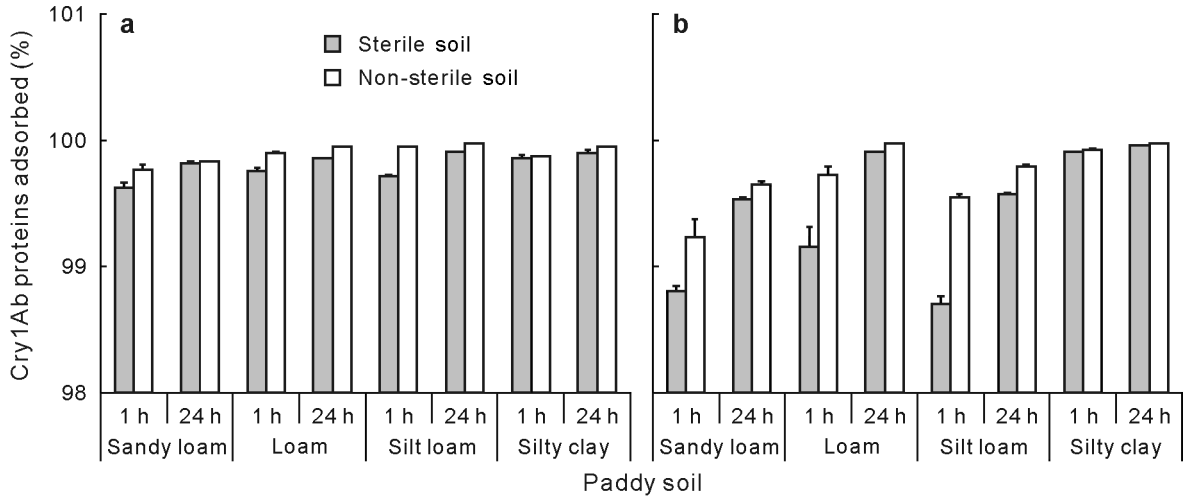

Fig. 2 Adsorption of Cry1Ab proteins on differently textured (sandy loam, loam, silt loam, and silty clay) paddy soils with an initial Cry1Ab protein addition of 2.5 (a) or 5.0 (b) μg g^{-1} soil after 1 and 24 h. Vertical bars indicate standard errors of the means ($n = 3$).

on the other three paddy soils.

The proportion (> 98%) and rapidity (within 1 h) of adsorption observed indicated that paddy soils have a strong affinity for Cry1Ab proteins at a low Cry1Ab protein concentration (2.5 μg g^{-1} soil). When the initial concentration of Cry1Ab proteins was increased to 5.0 μg g^{-1} soil, there was a slight difference in protein adsorption among the four paddy soils: adsorption was lower on the sandy loam soil than the silty clay soil. Doerr *et al.* (2000) pointed out that sandy soils are particularly prone to hydrophobicity as expressed as soil water repellency, due to their smaller surface area in comparison to clayey soils. Therefore, sandy soils are more easily saturated with hydrophobic organic molecules (Helassa *et al.*, 2011). This is consistent with the greater amount of Cry1Ab proteins adsorbed on the silty clay soil than the sandy loam soil (Table II).

Ideally, sterilization should inhibit microbial activity and have no effect on soil physicochemical properties, but such a sterilization method does not exist (McNamara *et al.*, 2003). The heat from autoclaving may release labile carbon and carbon dioxide from soil organic matter and also induces mineralogical changes (Egli *et al.*, 2006). Shaw *et al.* (1999) stated that abiotic changes to the soil during the sterilization process may complicate the interpretation of comparisons between sterile and non-sterile soils. Therefore, differences in the adsorption of Cry1Ab proteins between sterile and non-sterile paddy soils could be caused by the inactivation of microorganisms, and consequently less degradation (as discussed above), as well as by other abiotic changes during autoclaving.

Desorption of Cry1Ab proteins adsorbed on paddy soils

After adsorption, the desorption of Cry1Ab proteins from paddy soils was performed by washing with sterile H_2O_{MQ}, and subsequent extraction with extraction buffer. The desorption efficiency of the water was much lower than that of the extraction buffer. No more than 2% of the adsorbed Cry1Ab proteins were desorbed from the four paddy soils by washing with sterile H_2O_{MQ} (Fig. 3).

A considerable proportion (20%–40%) of the adsorbed proteins was desorbed from the silt loam soil with the extraction buffer (Table III). Comparing desorption of Cry1Ab proteins from the silt loam soil, the amount of the desorbed proteins differed due to different initial concentrations of the proteins that were spiked into the soil ($P < 0.05$). The more the Cry1Ab proteins that were initially added, the more that proteins were desorbed. The concentrations of proteins desorbed from the non-sterile silt loam soil were *ca.* 0.7, 2.0, and 3.0 μg g^{-1} soil for the initial protein concentrations of 2.5, 5.0, and 10.0 μg g^{-1} soil, respectively (data not shown). However, when expressed as the percentage of adsorbed proteins desorbed, the desorption did not increase with increasing initial protein concentration (Table III). When the concentrations of proteins added to the soil increased from 5.0 to 10.0 μg g^{-1} soil, the relative desorption decreased from 40.3% to 31.5%, respectively. Desorption of Cry1Ab proteins was significantly higher from the sterile silt loam soil than the non-sterile silt loam soil ($P < 0.05$).

Studies on the desorption of *Bt* toxins using water have reported contrasting findings. Crecchio and Sto-

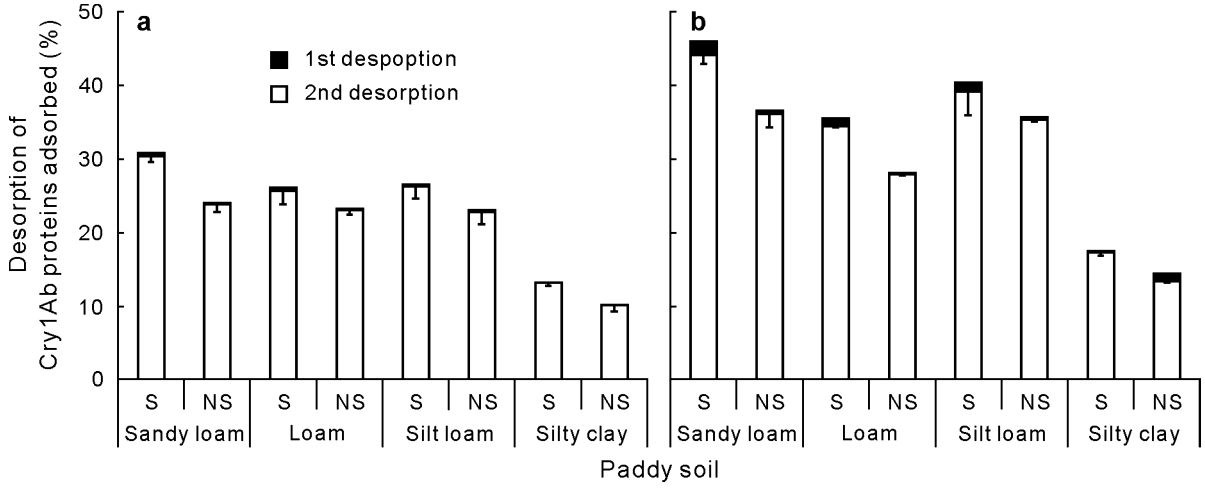

Fig. 3 Desorption of the Cry1Ab proteins adsorbed on differently textured (sandy loam, loam, silt loam, and silty clay) sterile (S) and non-sterile (NS) paddy soils with an initial Cry1Ab protein addition of 2.5 (a) or 5.0 (b) μg g^{-1} soil. Cry1Ab protein desorption from the paddy soils was evaluated based on washing with sterile Milli-Q® water (1st desorption) and subsequent extraction with a specific extraction buffer (2nd desorption). Vertical bars indicate standard errors of the means ($n = 3$).

TABLE III

Desorption of the Cry1Ab proteins adsorbed on a silt loam paddy soil[a)] with an initial Cry1Ab protein addition of 2.5, 5.0, or 10.0 μg g^{-1} soil by washing with sterile Milli-Q® water (1st desorption) and subsequent extraction with a specific extraction buffer (2nd desorption)

Cry1Ab proteins added	Cry1Ab proteins desorbed			
	Sterile soil		Non-sterile soil	
	1st desorption	2nd desorption	1st desorption	2nd desorption
μg g^{-1} soil	%			
2.5	0.14 ± 0.02[a)]a[b)]	26.2 ± 1.6a	0.06 ± 0.01a	22.7 ± 1.6a
5.0	1.16 ± 0.07b	39.1 ± 3.3c	0.27 ± 0.01b	35.3 ± 0.2b
10.0	1.12 ± 0.13b	30.4 ± 1.9b	0.30 ± 0.08b	20.2 ± 2.4a

[a)]Means ± standard errors ($n = 3$).
[b)]Means followed by different letters within each column are significantly different at $P < 0.05$.

tzky (1998) used water to wash *Bt* toxins adsorbed on humic acids, and found that water desorbed a significant amount of *Bt* toxins from the humic acids, ranging from 20% to 55%. Helassa *et al.* (2009) reported that up to 14% of *Bt* toxins can be desorbed from both montmorillonite and kaolinite. Lee *et al.* (2003) found that water desorbed only 2% to 12% of the adsorbed proteins. In this study, only minimal desorption of Cry1Ab proteins by sterile H_2O_{MQ} was observed, indicating paddy soils served as adsorbent were different from pure clay minerals due to the heterogeneity of soil. The *Bt* toxins can be adsorbed on soils by hydrogen bonding, ligand exchange, and hydrophobic, electrostatic, and van der Waal interactions (Quiquampoix and Burns, 2007). Helassa *et al.* (2009) found an important electrostatic effect of pH on adsorption, as well as other effects, which is in agreement with the results obtained in this study. Desorption using sterile H_2O_{MQ} was rather inefficient, whereas the use of a specific extraction buffer containing Triton X-100 (a nonionic surfactant) with the pH adjusted to 10 extracted nearly 40% of the adsorbed toxins.

At the three concentrations of Cry1Ab proteins added to the silt loam soil, most of the proteins (> 98%) could be absorbed in the soil; however, through desorption with sterile H_2O_{MQ} and the specific extraction buffer, more than 50% of the Cry1Ab proteins were tightly bound on the soil. The toxins can remain in the soil and retain their insecticidal properties for up to 8 months (Tapp and Stotzky, 1995b, 1998; Palm *et al.*, 1996). This could elicit and enhance the selection of target organisms, constituting a risk to the soil environment (Heckel, 1994; Alstad and Andow, 1995; Crecchio and Stotzky, 2001).

The paddy soil-Cry1Ab protein interaction was partly reversible, as the sterile H_2O_{MQ} and the specific extraction buffer desorbed a considerable amount of Cry1Ab proteins. Desorption of adsorbed Cry1Ab proteins from the other three paddy soils was far lower with H_2O_{MQ} than that with the specific extraction buffer ($P < 0.05$), which is consistent with the data obtained for the silt loam soil, as described above.

Different amounts of the *Bt* toxins (Cry1Ab proteins) were desorbed from the differently textured paddy soils. Desorption of the adsorbed proteins was lower from the silty clay soil than from the sandy loam soil. When the concentration of added Cry1Ab proteins was maintained at 2.5 μg g^{-1} soil, more Cry1Ab proteins were desorbed from the sandy loam soil than from the silty clay soil (Fig. 3a). When the Cry1Ab proteins were added at a concentration of 5.0 μg g^{-1} soil, the amount of Cry1Ab proteins desorbed from the sandy loam soil was 2.5 times as much as that from the silty clay soil (Fig. 3b). Desorption of Cry1Ab proteins from each paddy soil increased with increasing amounts of added proteins. When the initial concentration of Cry1Ab proteins increased from 2.5 to 5.0 μg g^{-1} soil, the amount of proteins desorbed from the sterile sandy loam soil increased from 0.76 to 2.28 μg g^{-1} soil (data not shown), indicating an increase in relative desorption from 30.7% to 45.9%, respectively (Fig. 3). In addition, desorption with sterile H_2O_{MQ} was more at higher concentrations of Cry1Ab proteins in comparison to lower ones. Similar trends of the relative desorption were obtained for the other three paddy soils, *i.e.*, the loam, silt loam, and silty clay soils.

The data indicated that the Cry1Ab proteins were more strongly bound on the silty clay soil than on the sandy loam soil, indicating a greater affinity for the silty clay soil. It is known that *Bt* toxins react with the organomineral surfaces of soils, and clay minerals play a dominant role due to their large specific surface areas and surface charges (Quiquampoix, 1987a; b; Quiquampoix and Burns, 2007; Helassa *et al.*, 2011). The

intensity of adsorption differed in the affinity for paddy soils with different textures. Once Cry1Ab proteins were adsorbed on the silty clay soil, the interaction between the Cry1Ab proteins and the silty clay soil became stronger with increasing time, and the proteins adsorbed were less desorbed in comparison to the sandy loam soil.

CONCLUSIONS

The affinity of Cry proteins has important implications for soil monitoring. The persistence of proteins adsorbed, which cannot be desorbed by either the sterile H_2O_{MQ} or extraction buffer suggested that Cry proteins released from the cultivation of GM crops would accumulate in soil ecosystems. Soil properties, due to their complexity, should not be considered a simple factor affecting the fate of Cry proteins in paddy soil ecosystems.

REFERENCES

Adang M J, Brody M S, Cardineau G, Eagan N, Roush R T, Shewmaker C K, Jones A, Oakes J V, McBride K E. 1993. The reconstruction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. *Plant Mol Biol.* **21**: 1131–1145.

Alstad D N, Andow D A. 1995. Managing the evolution of insect resistance to transgenic plants. *Science.* **268**: 1894–1896.

Baumgarte S, Tebbe C C. 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Mol Ecol.* **14**: 2539–2551.

Beegle C C, Yamamoto T. 1992. History of *Bacillus thuringiensis* Berliner research and development. *Can Entomol.* **124**: 587–616.

Chen R, Wang Y, Zhu Z, Lan Q K, Zhao X, Wang H Y, Guo Y Z, Wang Q Y. 2014. Development of the one-step visual loop-mediated isothermal amplification assay for genetically modified rice event TT51-1. *Food Sci Technol Res.* **20**: 71–77.

Chevallier T, Muchaonyerwa P, Chenu C. 2003. Microbial utilisation of two proteins adsorbed to a vertisol clay fraction: Toxin from *Bacillus thuringiensis* subsp. *tenebrionis* and bovine serum albumin. *Soil Biol Biochem.* **35**: 1211–1218.

Clark B W, Phillips T A, Coats J R. 2005. Environmental fate and effects of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *J Agr Food Chem.* **53**: 4643–4653.

Crecchio C, Stotzky G. 1998. Insecticidal activity and biodegradation of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound to humic acids from soil. *Soil Biol Biochem.* **30**: 463–470.

Crecchio C, Stotzky G. 2001. Biodegradation and insecticidal activity of the toxin from *Bacillus thuringiensis* subsp. *kurstaki* bound on complexes of montmorillonite-humic acids-Al hydroxypolymers. *Soil Biol Biochem.* **33**: 573–581.

Crespo A L B, Spencer T A, Nekl E, Pusztai-Carey M, Moar W J, Siegfried B D. 2008. Comparison and validation of methods to quantify Cry1Ab toxin from *Bacillus thuringiensis* for standardization of insect bioassays. *Appl Environ Microb.* **74**: 130–135.

Doerr S H, Shakesby R A, Walsh R P D. 2000. Soil water repellency: Its causes, characteristics and hydro-geomorphological significance. *Earth-Sci Rev.* **51**: 33–65.

Dohrmann A B, Küting M, Jünemann S, Jaenicke S, Schlüter A, Tebbe C C. 2013. Importance of rare taxa for bacterial diversity in the rhizosphere of *Bt*-and conventional maize varieties. *ISME J.* **7**: 37–49.

Douville M, Gagné F, Masson L, McKay J, Blaise C. 2005. Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. *Biochem Syst Ecol.* **33**: 219–232.

Egli M, Mirabella A, Kägi B, Tomasone R, Colorio G. 2006. Influence of steam sterilisation on soil chemical characteristics, trace metals and clay mineralogy. *Geoderma.* **131**: 123–142.

Eickhorst T, Tippkötter R. 2009. Management-induced structural dynamics in paddy soils of south east China simulated in microcosms. *Soil Till Res.* **102**: 168–178.

Fu Q L, Hu H Q, Chen S W, Huang Q Y, Liang W. 2007. Adsorption of insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* by some Chinese soils: Effects of organic acid ligands addition. *Plant Soil.* **296**: 35–41.

Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K. 1993. Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. *Nat Biotechnol.* **11**: 1151–1155.

Giles C H, D'Silva A P, Easton I A. 1974. A general treatment and classification of the solute adsorption isotherm part. II. Experimental interpretation. *J Colloid Interf Sci.* **47**: 766–778.

Harvey W R, Cioffi M, Dow J A T, Wolfersberger M G. 1983. Potassium ion transport ATPase in insect Epithelia. *J Exp Biol.* **106**: 91–117.

Heckel D G. 1994. The complex genetic basis of resistance to *Bacillus thuringiensis* toxin in insects. *Biocontrol Sci Techn.* **4**: 405–417.

Helassa N, M'Charek A, Quiquampoix H, Noinville S, Déjardin P, Frutos R, Staunton S. 2011. Effects of physicochemical interactions and microbial activity on the persistence of Cry1Aa Bt (*Bacillus thuringiensis*) toxin in soil. *Soil Biol Biochem.* **43**: 1089–1097.

Helassa N, Quiquampoix H, Noinville S, Szponarski W, Staunton S. 2009. Adsorption and desorption of monomeric Bt (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite. *Soil Biol Biochem.* **41**: 498–504.

Hofmann C, Lüthy P, Hütter R, Pliska V. 1988. Binding of the delta endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (*Pieris Brassicae*). *Eur J Biochem.* **173**: 85–91.

Höfte H, Whiteley H R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev.* **53**: 242–255.

Hung T P, Truong L V, Binh N D, Frutos R, Quiquampoix H, Staunton S. 2016. Persistence of detectable insecticidal proteins from *Bacillus thuringiensis* (Cry) and toxicity after adsorption on contrasting soils. *Environ Pollut.* **208**: 318–325.

Icoz I, Stotzky G. 2008. Fate and effects of insect-resistant *Bt* crops in soil ecosystems. *Soil Biol Biochem.* **40**: 559–586.

IUSS Working Group WRB. 2006. World Reference Base for Soil Resources. World Soil Resources Reports No. 103. FAO, Rome.

James C. 2013. Global Status of Commercialized Biotech/GM Crops: 2013. International Service for the Acquisition of Agri-Biotech Applications, Ithaca.

Knowles B H, Ellar D J. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochim Biophys Acta.* **924**: 509–518.

Koskella J, Stotzky G. 1997. Microbial utilization of free and clay-bound insecticidal toxins from *Bacillus thuringiensis* and their retention of insecticidal activity after incubation with microbes. *Appl Environ Microbiol.* **63**: 3561–3568.

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

Kramarz P E, De Vaufleury A, Carey M. 2007. Studying the effect of exposure of the snail *Helix aspersa* to the purified *Bt* toxin, Cry1Ab. *Appl Soil Ecol.* **37**: 169–172.

Lee L, Saxena D, Stotzky G. 2003. Activity of free and clay-bound insecticidal proteins from *Bacillus thuringiensis* subsp. *israelensis* against the mosquito *Culex pipiens*. *Appl Environ Microb.* **69**: 4111–4115.

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

McNamara N P, Black H I J, Beresford N A, Parekh N R. 2003. Effects of acute gamma irradiation on chemical, physical and biological properties of soils. *Appl Soil Ecol.* **24**: 117–132.

Mohan M, Gujar G T. 2001. Toxicity of *Bacillus thuringiensis* strains and commercial formulations to the diamondback moth, *Plutella xylostella* (L.). *Crop Prot.* **20**: 311–316.

Pagel-Wieder S, Niemeyer J, Fischer W R, Gessler F. 2007. Effects of physical and chemical properties of soils on adsorption of the insecticidal protein (Cry1Ab) from *Bacillus thuringiensis* at Cry1Ab protein concentrations relevant for experimental field sites. *Soil Biol Biochem.* **39**: 3034–3042.

Palm C J, Donegan K, Harris D, Seidler R J. 1994. Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin from transgenic plants. *Mol Ecol.* **3**: 145–151.

Palm C J, Seidler R J, Schaller D L, Donegan K K. 1996. Persistence in soil of transgenic plant produced *Bacillus thuringlensis* var. *kurstaki* δ-endotoxin. *Can J Microbiol.* **42**: 1258–1262.

Parry J M, Turnbull P C B, Gibson J R. 1983. A Colour Atlas of Bacillus Species. Wolfe Medical Publications, London.

Quiquampoix H. 1987a. A stepwise approach to the understanding of extracellular enzyme activity in soil I. Effect of electrostatic interactions on the conformation of a β-D-glucosidase adsorbed on different mineral surfaces. *Biochimie.* **69**: 753–763.

Quiquampoix H. 1987b. A stepwise approach to the understanding of extracellular enzyme activity in soil. II. Competitive effects on the adsorption of a β-D-glucosidase in mixed mineral or organo-mineral systems. *Biochimie.* **69**: 765–771.

Quiquampoix H, Burns R G. 2007. Interactions between proteins and soil mineral surfaces: Environmental and health consequences. *Elements.* **3**: 401–406.

Read T D, Peterson S N, Tourasse N, Baillie L W, Paulsen I T, Nelson K E, Tettelin H, Fouts D E, Eisen J A, Gill S R, Holtzapple E K, Økstad O A, Helgason E, Rilstone J, Wu M, Kolonay J F, Beanan M J, Dodson R J, Brinkac L M, Gwinn M, DeBoy R T, Madpu R, Daugherty S C, Durkin A S, Haft D H, Nelson W C, Peterson J D, Pop M, Khouri H M, Radune D, Benton J L, Mahamoud Y, Jiang L X, Hance I R, Weidman J F, Berry K J, Plaut R D, Wolf A M, Watkins K L, Nierman W C, Hazen A, Cline R, Redmond C, Thwaite J E, White O, Salzberg S L, Thomason B, Friedlander A M, Koehler T M, Hanna P C, Kolsto A B, Fraser C M. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature.* **423**: 81–86.

Sander M, Tomaszewski J E, Madliger M, Schwarzenbach R P. 2012. Adsorption of insecticidal Cry1Ab protein to humic substances. 1. Experimental approach and mechanistic aspects. *Environ Sci Tech.* **46**: 9923–9931.

Saxena D, Flores S, Stotzky G. 1999. Insecticidal toxin in root exudates from *Bt* corn. *Nature.* **402**: 480.

Saxena D, Stotzky G. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic *Bt* corn in vitro and in situ. *FEMS Microbiol Ecol.* **33**: 35–39.

Saxena D, Stotzky G. 2002. *Bt* toxin is not taken up from soil or hydroponic culture by corn, carrot, radish, or turnip. *Plant Soil.* **239**: 165–172.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler D R, Dean D H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev.* **62**: 775–806.

Shaw L J, Beaton Y, Glover L A, Killham K, Meharg A A. 1999. Re-inoculation of autoclaved soil as a non-sterile treatment for xenobiotic sorption and biodegradation studies. *Appl Soil Ecol.* **11**: 217–226.

Sims S R, Holden L R. 1996. Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp. *kurstaki* CryIA(b) protein in corn tissue. *Environ Entomol.* **25**: 659–664.

Sims S R, Ream J E. 1997. Soil inactivation of the *Bacillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein within transgenic cotton tissue: Laboratory microcosm and field studies. *J Agr Food Chem.* **45**: 1502–1505.

Soil Survey Staff. 1996. Soil Survey Laboratory Information Manual. Soil Survey Investigations Report No. 42. Version 3.0. United States Department of Agriculture, Portland.

Sundaram K M S. 1996. Sorptive interactions and binding of δ-endotoxin protein from *Bacillus thuringiensis* subsp. *kurstaki* in forest soils. *J Environ Sci Heal B.* **31**: 1321–1340.

Tapp H, Calamai L, Stotzky G. 1994. Adsorption and binding of the insecticidal proteins from *Bacillus thuringiensis* subsp. *kurstaki* and subsp. *tenebrionis* on clay minerals. *Soil Biol Biochem.* **26**: 663–679.

Tapp H, Stotzky G. 1995a. Dot blot enzyme-linked immunosorbent assay for monitoring the fate of insecticidal toxins from *Bacillus thuringiensis* in soil. *Appl Environ Microb.* **61**: 602–609.

Tapp H, Stotzky G. 1995b. Insecticidal activity of the toxins from *Bacillus thuringiensis* subspecies *kurstaki* and *tenebrionis* adsorbed and bound on pure and soil clays. *Appl Environ Microb.* **61**: 1786–1790.

Tapp H, Stotzky G. 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. *Soil Biol Biochem.* **30**: 471–476.

Tomaszewski J E, Madliger M, Pedersen J A, Schwarzenbach R P, Sander M, 2012. Adsorption of insecticidal Cry1Ab protein to humic substances. 2. Influence of humic and fulvic acid charge and polarity characteristics. *Environ Sci Tech.* **46**: 9932–9940.

Wang H Y, Ye Q F, Wang W, Wu L C, Wu W X. 2006. Cry1Ab protein from Bt transgenic rice does not residue in rhizosphere soil. *Environ Pollut.* **143**: 449–455.

Wu Y H, Yang L T, Cao Y L, Song G W, Shen P, Zhang D B, Wu G. 2013. Collaborative validation of an event-specific quantitative real-time PCR method for genetically modified rice event TT51-1 detection. *J Agr Food Chem.* **61**: 5953–5960.

Zwahlen C, Hilbeck A, Gugerli P, Nentwig W. 2003. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Mol Ecol.* **12**: 765–775.