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Fate of insecticidal *Bacillus thuringiensis* Cry protein in soil: differences between purified toxin and biopesticide formulation

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ABSTRACT

BACKGROUND

Bacillus thuringiensis produces insecticidal proteins known as Cry and its efficiency and absence of side-effects make it the most widely used biopesticide. There is little information on the role of soils in the fate of Cry proteins from commercial biopesticide formulations, unlike toxins from genetically modified crops that have been intensively studied in recent years. The persistence of Cry in soil was followed under field and laboratory conditions.

RESULTS

Sunlight accelerated loss of detectable Cry under laboratory conditions but little effect of shade was observed under field conditions. The half-life of biopesticide proteins in soil under natural conditions was about one week. Strong temperature effects were observed, but they differed for biopesticide and purified protein, indicating different limiting steps.

CONCLUSION

For the biopesticide the observed decline in detectable protein was due to biological factors, possibly including the germination of *B. thuringiensis* spores and was favoured by higher temperature. In contrast for purified proteins, the decline in detectable protein was slower at low temperature, probably because the conformational changes of the soil-adsorbed protein, that cause fixation and hence reduced extraction efficiency, are temperature dependent.

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1 INTRODUCTION

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

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

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There have been few studies of the persistence of biopesticide-derived spores and toxins in the environment.[9, 10] Bt has been found to persist in soils and waters for days or months, and in some favourable circumstances spores may germinate.[10, 11] Although there is no mechanism by which *Bt* protoxins or toxins may be harmful to mammals, the persistence has two conflicting consequences. Firstly, the longer the toxin remains intact and in contact with the plant to be protected, the longer is the period of protection. Secondly, the persistence of the protoxin or toxin at sub-lethal levels could increase the probability of acquisition of resistance and possibly the exposure of non-target insects, via soil or crop residues. In contrast to biopesticides Bt, there have been many studies of the environmental fate of Bt toxins derived from genetically modified (GM) crops since their commercialization in 1996.[12, 13] Some of these studies have been conducted in the field monitoring the presence of Cry proteins originating from GM crops, but many were conducted under controlled conditions using purified protein produced by bacteria. This contrast reflects the different perception of risk from a natural and a genetically engineered product. Protoxins and toxins are usually observed to decline rapidly in soil, but may remain detectable for months.

The roles of soil in determining the fate of Cry proteins are potentially very important. Soil acts as an efficient UV-filter, thereby potentially prolonging the conservation of crystals. The microbial activity of soil, including catalytic activity of extracellular proteases, contributes to the decline in insecticidal protein. Although the solubilisation of crystals in soils may be slow, given that soil pH is rarely strongly alkaline, crystals will eventually be solubilized and truncated. As for other proteins, soluble Cry proteins released into soil are rapidly adsorbed on soil organo-mineral surfaces.[14-18] Adsorbed proteins are largely immobilized [19] and adsorption has various consequences. Adsorption is thought to confer both physical and chemical protection against microbial breakdown, although recent studies of fungal phosphatases indicate that this may not always be the case.[20, 21] Conformational

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changes due to electrostatic and hydrophobic interactions may modify the biological properties of the proteins. Conformational changes may change with time and this may cause the extraction efficiency to decrease, a phenomenon known as aging or fixation. No published studies have successfully distinguished between breakdown of Cry protein and fixation as causes of the observed decline in extractable-detectable protein in soil.[15, 22]

The aim of this study was to follow the persistence in soil of detectable Cry toxins applied in a commercial formulation of *B. thuringiensis* var. *kurstaki* crystals and spores. The persistence was followed under field conditions, varying the mode of application (canopy protection or exposure to direct sunlight, application morning or afternoon). The persistence was also monitored in soil with no crop under controlled laboratory conditions, varying temperature and exposure to sunlight and in aqueous solution without soil as a function of temperature and nutrient supply. For comparison, the persistence of purified Cry1Ac toxin applied to the same soil was monitored under controlled conditions. Purified protein was preferred to protein produced by a GM plant since it is available in a large amount and its fate does not depend on the decay kinetics of plant material. Purified Cry proteins are considered to be so nearly identical to GM plant produced proteins that they are used in homologation studies. We were interested in the effect of temperature on persistence since previous studies with purified Cry proteins showed a strong temperature effect, despite no effect on soil microbial activity from which we concluded that protein conformational changes following adsorption were temperature dependent.[15] However for biopesticides there are no data on the temperature effect on persistence. Prewetting soil prior to the application of Bt was another variable chosen to modify microbial activity and so to distinguish between physico-chemical and microbial driving forces.

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2 MATERIALS AND METHODS

2.1 *B. thuringiensis* biopesticide spray. A commercial spray, Vi-Bt, produced by Hubei Kangxin Agro-Industry Co. Ltd, was purchased from Vietnam Pesticide Joint Stock Company and used according to the supplier's recommendations, by dilution in water. This spray is commonly used in Vietnam and the potency unit was given as 16000 IU/mg. It was composed of *B. thuringiensis* var. *kurstaki* (isolate HD-1) crystals and spores. The Materials Safety Data Sheet does not list any chemical additives (http://www.btrdc.com/en/msds/msds-1.htm). The HD-1 strain produces various Cry proteins including Cry1Ac.

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

2.3 Soils and study site. The study site for the field experiment and from which soils were sampled for the controlled laboratory experiment was situated in North Vietnam, near Hanoi, in the Plant Protection Research Institute. The climate is subtropical, with most of the annual rainfall of 1700 mm during the rainy season (May-October) and average daily mean temperatures between 16.5°C (January) and 29.5°C (July). The study plot is used for the cultivation of sweet potato (*Ipomoea batatas*). For laboratory studies and soil analysis, triplicate composite soil samples were collected from the top layer of soil (0-5 cm). The soil

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samples were air-dried, sieved < 200 µm, thoroughly mixed and stored until required. The soil was a sandy loam containing 1.1% organic carbon, with a C/N ratio of 13, a cation exchange capacity of 8.2 and a pH of 8.4.

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

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minutes at 19 000 *g* to separate aqueous and solid phases. Supernatant solution was removed, diluted as required and Cry proteins assayed using ELISA kits (Qualiplate Combo Kit for Cry1Ab/1Ac, Envirologix) following manufacturer's instructions. Low binding plastics (Eppendorf tubes and pipette tips) were used to handle solutions containing Cry.

2.5 Persistence of biopesticide Cry proteins under controlled laboratory conditions. Commercial Bt preparation was suspended in distilled water (50 g dm^{-3}). Soil (10 g) was weighed into Petri dishes and Bt suspension was sprayed onto the soils to give a moisture content of 20%, the amount of solution added was determined by weighing the Petri dishes. Moisture content was adjusted to 40% by pipetting distilled water onto the soils. The soils were incubated under the required conditions and weight checked daily and adjusted for moisture loss as required. The incubation variables were temperature (4°C, 25°C) in darkness or direct sunlight. At intervals, soil was sampled in 3 places from each sample to give composite samples of about 1 g soil from which 5 replicates of 0.1 g were weighed into Eppendorf tubes, protein was extracted and assayed as described above. All incubations were carried out in triplicate.

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

2.7 Persistence of purified Cry1Ac protein in soil under controlled laboratory conditions. Purified Cry1Ac was added to soil by pipetting solution onto soil in Eppendorf

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

3 RESULTS

3.1 Persistence of commercial formulation of Bt crystal proteins in field soil under natural conditions. Soil samples collected after field spraying of the commercial preparations of HD-1 Bt formulated biopesticide were assayed. Soil was either collected under leaf canopy, spayed directly and sampled between rows, or was sprayed and sampled in the afternoon, between rows to give contrasting exposure to sunlight in comparison to the previous treatment. Average data for the three rows are shown in Figure 1 (the coefficient of variation was about 10%). The decrease of detection of Bt toxins in soils as given by anti-Cry1 ELISA tests was similar whatever the sample. There was no coherent effect of canopy protection from sunlight. Detectable Cry tended to be greater for afternoon sprayed soil for the first week, but this was not observed for each row and the effect was not significant when the full data set was considered and compared with either of the other treatments ($P > 0.05$). Similarly detectable Cry was lower for the morning-sprayed inter-row samples than for the other treatments during the first week after spraying, but taking the full data set, the effect was not significant ($P>0.05$). The decline in detectable Cry1A followed approximately first order kinetics (although curvature in the log-linear plot of concentration *vs* time indicates that this is at best an approximate mathematical fitting procedure). The half-life of detectable protein

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was about one week by visual appraisal and calculated to be 9-10 days by linear regression after log transformation of data.

3.2 Persistence of commercial formulation of Bt crystal proteins in field soil under laboratory conditions. Figure 2 shows the decline in detectable Cry1A toxins (average of three repetitions) after application of biopesticides spray to replicate soil samples under laboratory conditions. At 25°C in the dark there was a slow, gradual decline with about 70% of the initially detectable protein remaining after one week. In some cases an increase in Cry was initially observed before a net decline. In contrast, at 4°C there was a fast initial decrease of detectable toxins, reaching less than 20% of the initial load after only one day followed by a slower decline. Sunlight accelerated the rate of decrease of detectable Cry1A toxins with respect to the soil maintained at 25°C in the dark. However, this effect was less than that of low temperature. Time dependence of detectable Cry was significantly different for each of the three treatments ($P<0.05$).

3.3 Effect of soil solution (SS) and nutrient solution (NS) on the persistence of Bt crystal proteins from the commercial formulation. Figure 3 shows the time dependence of detectable Cry incubated in various aqueous solutions at either 25°C or 4°C. At 4°C, the amount of detectable toxin remained fairly constant throughout the 14 days of the experiment, for all the solution compositions, although a small increase after day 3 was observed in presence of soil extract and nutrient solutions. Similarly at 25°C in water, there was no change in the amount of detectable Cry protein. However, when maintained at 25°C in the presence of either nutrient solution or soil solution, the amount of detectable Cry1A toxins increased. In both 100% nutrient solution and soil solution the maximum Cry was detected after 1 week and then decreased by about 20% in the following week. The level of maximum detectable Cry was smaller and the time taken to reach this maximum was greater for Cry incubated in diluted nutrient solution. After 7 days, when the contrast between nutrient

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solutions was greatest, the concentration of Cry in 100% NS was 1.3 times that in 10% NS and 1.6 times that in 1% NS. The average rate of increase in detectable Cry was thus 30% less in 10% NS and 40% less in 1% NS than in 100% NS.

3.4 Persistence of purified Cry1Ac toxin in soil under laboratory conditions. Figure 4 shows the results of the control experiment that monitored purified Cry1Ac toxin incubated with soil under similar conditions as the Bt biopesticides. The amount of detectable protein decreased rapidly at 25°C in contact with soil. However, prewetting the samples to induce a microbial flush prior to addition of Cry protein limited the decrease so that about twice the amount of Cry remained detectable in the prewet sample in comparison to the soil wet directly with Cry solution. The decline in the amount of purified Cry1Ac toxin was markedly less when incubated at 4°C, in comparison to 25°C. At 4°C, Cry only fell to about 60% of the initial value, in comparison to about 15% at 25°C. At low temperature, soil prewetting had no significant effect on the subsequent rate of decline of detectable Cry. The decline of purified Cry at either 4°C or 25°C was significantly different to the decline of biopesticide Cry at each temperature (Fig.2).

4 DISCUSSION

There are very few studies of the persistence of Bt spores and proteins in the environment. This is largely due to the assumption that being a natural product, there is no danger associated with its use. Field observations indicate that insecticidal properties persist for a few days[23, 24], and this information is sufficient for users to time applications with respect to the presence of insects. Industrial research has aimed to optimize efficiency by protecting crystals from UV-light, improving the adhesion of the product to plant parts to minimize run-off and optimizing spray storage and utilization.[5, 24] Spores and the bacterium may survive in soil and water for weeks or even longer.[9-11, 23, 25] One study has shown that the Cry1Ab

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protein from the commercial product Dipel ® remains detectable in soil for a few days.[26] The paucity of data contrasts with the number of studies of the toxin produced by genetically modified (GM) crops in field studies and the purified protein in soil microcosms.[12] These studies are prompted by the fears of exposure of non-target insects and the risk of acquisition of resistance by the exposure of target insects to non-lethal levels of the toxin. The longer the protein remains in the environment, the greater will be the probability of both undesired effects.

In the present study, detectable Cry1A proteins from biopesticide decreased gradually with time under field conditions. The half-life was about one week and the protein remained detectable after one month, the maximum period of the trial. We are not aware of any similar field data with which these findings can be compared. However the kinetics of decline are markedly different to those usually reported for purified protein in soil microcosms or resulting from GM crops.[15, 27-29] GM or purified Bt toxins in soil usually decline rapidly in the first few days and then more slowly over the following weeks, with half-lives of between less than one day and up to one week. This was the pattern observed for the present soil contaminated with purified Cry1Ac protein, incubated at 25°C. The difference did not therefore arise because of any inherent difference between the soils studied in this and other studies.

The comparison between the field study and the application of biopesticide under laboratory conditions (25°C in darkness) shows marked difference in the fate of Cry proteins. Under laboratory conditions in the dark, the level of detectable Cry decreased more slowly than in the field. It should be noted that the extraction method used would not only desorb protein from soil but would also solubilize any protein remaining in crystal form. Douville et al.[26] compared the extraction of Cry1Ab from soil using extraction solutions at pH 7.4 and 10.5 and assumed that the former extracted only truncated protein whereas the later

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solubilized the protoxin. In fact, at alkaline pH both proteins would be solubilized/desorbed and the extraction yield of truncated Cry protein would be more efficient at pH 10.4 than at pH 7.4. In a previous study we observed poor extraction yields at neutral pH, particularly in the absence of surfactants.[30] This is in accordance with the very low extraction yields reported at pH 7.4 or by water.[26, 31] The differences between laboratory and field conditions include temperature, sunlight and application conditions. In the field, suspension containing the Cry crystals could percolate in depth, allowing some dilution, whereas the layer of soil in the Petri dishes used in the laboratory trial was only about 5 mm thick. However this is unlikely to be the major cause of the observed difference since there was no rainfall in the first week of the field sampling. Preliminary studies showed that there was no effect of moisture content on the dynamics of either biopesticide or purified Cry under laboratory conditions suggesting that the difference was not due to the surface layer of the soil in the field being drier than for the laboratory study. Feng et al.[32] also report no effect of moisture content on the release of Cry1Ab from transgenic straw and its subsequent decline in soil.

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

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prevented the sunlight effect to be established. Other effects were stronger and dominated. The larger effect of sunlight in the laboratory conditions may be in part due to the shallow soil layer, 5 mm, affording less protection to Cry.

4.2 Effect of temperature on persistence in soil. Previous studies have shown that low incubation temperature, 4°C, of purified Cry1Aa toxin with four contrasting soils conserved protein more than incubation at 25°C.[15] Two mechanisms for this temperature effect were considered to explain the observation. The first was that lower microbial activity at low temperature slowed microbial breakdown of the protein. However this was discounted because stimulation of microbial activity or inhibition by chemical methods or sterilization did not have marked effects. The other, preferred, hypothesis was that conformational changes of the adsorbed protein led to increasing fixation on the soil surface and hence decreasing extraction yield. We postulated that the fixation was dominated by hydrophobic interactions since they are known to decrease with decreasing temperature. Feng et al.[32] reported an increase in the rate of decline of Cry1B in soil with increasing temperature, but it is impossible to distinguish between the temperature effect on release of protein from straw and its subsequent degradation or fixation on soil.

The effect of prewetting the soil on the amount of detectable purified Cry toxin at 25°C is probably related to the release of microbial organic matter induced by the plasmoptysis of microbial cells due to the rapid water potential increase of the soil.[33] This organic matter which is released before the addition of Cry will adsorb on soil mineral surfaces and thus compete with the toxin adsorption sites and decrease the fixation process. A similar competition effect has been observed for the adsorption of extracellular enzymes on soil organo-mineral complexes.[34]

A strong effect of temperature on biopesticide Cry in laboratory conditions was also observed, but it was the reverse of what happened for purified Cry, namely a positive effect of higher

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temperature on detectability of the toxin. One of the important differences between biopesticide and purified protein is the presence of spores as well as crystal protein in the former. We can thus postulate that the temperature effect for biopesticide in the laboratory is predominantly biological, with the possibility that spores could produce more protein, thus counteracting the decline that dominates the trend in the field. This hypothesis is strengthened by the fate of biopesticide in aqueous solutions which was designed to test the effect of temperature without the effect of adsorbing surfaces. Detectable biopesticide protein increased with time when incubation conditions favoured microbial development, namely at 25°C rather than at 4°C and in solutions containing nutrients, either aqueous soil extract or nutrient solution, rather than pure water. The temperature effect of soil and nutrient solutions on the increase in Cry concentration could be interpreted as the solubilisation of crystals, but this would not explain the effect of nutrient solution concentration and the absence of an effect in water. Spore germination with protein production occurring at 25°C in the presence of nutrients seems a more likely explanation. The nutrient solution contained bactopeptone which supplies L-alanine which is a very efficient germinant for *Bacillus* spore germination.[35] Dilution of nutrient solution caused the increase in protein to be slower and the maximum value lower, which is consistent with bacterial growth. In the presence of the soil solid phase the increase would be tempered by protein adsorption, the activity of soil proteases and competition with other bacteria and so the net effect is a constant level of protein or a slow decline. When low temperature inhibited bacterial growth in the laboratory the decline in protein was rapid, as observed in the field. The fact that protein dynamics at low temperature are different for biopesticide and purified protein shows that the two processes do not have the same rate limiting factors. We postulate that the limiting factor for purified protein is the on-going fixation of protein on soil surfaces leading to increasing irreversibility of adsorption. For the biopesticide formulation, protein fixation is counteracted by an induction of the spore

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germination. The fact that the soil solution is as effective for this process as nutritive solution means that some soil solution compounds can act as germinants.

5. CONCLUSIONS

The persistence of Cry proteins in the field results from average conditions of sunlight and temperature. The rate of decline of detectable protein from biopesticide differs from that of purified protein. For purified protein, ongoing fixation of the adsorbed protein leading to decreasing extractability dominates the observed time trends. Biopesticide protein trends depend on the additional processes of spore germination and the protective effects of commercial additives.

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FIGURE LEGENDS

Figure 1.

Persistence of Cry1A toxins from a commercial *Bt* formulated biopesticide under field conditions as a function of period after spraying, for three conditions of spraying-sampling. Average of three repetitions of spraying (Coefficient of variation about 15% between subsamples within each area sprayed and between areas sprayed). Error bars (not always visible) show variation between the three areas sprayed.

Figure 2.

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

Figure 3.

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

Figure 4.

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

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References

1. Sansinenea E. Discovery and description of *Bacillus thuringiensis*. In *Bacillus thuringiensis Biotechnology*, ed. by Sansinenea E. Springer Science: Dordrecht Heidelberg London New York, pp. 3-18 (2012).
2. Ongena M and Jacques P, Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology*; **16** 115–125 (2008).
3. Lacey LA, Frutos R, Kaya HK and Vail P, Insect pathogens as biological control agents: Do they have a future? *Biological Control*; **21** 230-248 (2001).
4. de Maagd RA, Bravo A and Crickmore N, How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics*; **17** 193-199 (2001).
5. Sanchis V, From microbial sprays to insect-resistant transgenic plants: history of the biopesticide *Bacillus thuringiensis.* A review. *Agronomy for Sustainable Development*; **31** 217-231 (2011).
6. Crickmore N, Using worms to better understand how *Bacillus thuringiensis* kills insects. *Trends in Microbiology*; **13** 347-350 (2005).
7. Crickmore N, Beyond the spore - past and future developments of *Bacillus thuringiensis* as a biopesticide. *Journal of Applied Microbiology*; **101** 616-619 (2006).
8. Pérez-García A, Romero D and de Vicente A, Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Current Opinions in Biotechnology*; **22** 187-193 (2011).
9. Vilas-Bôas LA, Vilas-Bôas GFLT, Saridakis HO, Lemos MVF, Lereclus D and Arantes OMN, Survival and conjugation of *Bacillus thuringiensis* in a soil microcosm. *FEMS Microbiology Ecology*; **31** 255-259 (2000).

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10. Tetreau G, Alessi MV, S., Perigon S, David JPR, S. and Despres L, Fate of *Bacillus thuringiensis* subsp *israelensis* in the Field: Evidence for Spore Recycling and Differential Persistence of Toxins in Leaf Litter. *Applied and Environmental Microbiology*; **78** 8362-8367 (2012).

11. Tilquin M, Paris M, Reynaud S, Despres L, Ravenel P, Geremia RA and Gury J, Long lasting persistence of bacillus thuringiensis Subsp. israelensis (Bti) in mosquito natural habitats. *Plos One*; **3** 1-10 (2008).

12. Icoz I and Stotzky G, Fate and effects of insect-resistant Bt crops in soil ecosystems. *Soil Biology & Biochemistry*; **40** 559-586 (2008).

13. Clark BW, Phillips TA and Coats JR, Environmental fate and effects of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *Journal of Agricultural and Food Chemistry*; **53** 4643-4653 (2005).

14. Quiquampoix H and Burns RG, Interactions between proteins and soil mineral surfaces: Environmental and health consequences. *Elements*; **3** 401-406. (2007).

15. Helassa N, A. MC, Quiquampoix H, Noinville S, Déjardin P, Frutos R and Staunton S, Effects of physicochemical interactions and microbial activity on the persistence of Cry1Aa Bt (*Bacillus thuringiensis*) toxin in soil. *Soil Biology & Biochemistry*; **43** 1089-1097 (2011).

16. Fu Q, Hu H, Chen S, Huang Q and Liang W, Adsorption of insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* by some Chinese soils: effects of organic acid ligands addition. *Plant and Soil*; **296** 35-41 (2007).

17. Muchaonyerwa P, Chevallier T, Pantani OL, Nyamugafata P, Mpepereki S and Chenu C, Adsorption of the pesticidal toxin from *Bacillus thuringiensis* subsp *tenebrionis* on tropical soils and their particle-size fractions. *Geoderma*; **133** 244-257 (2006).

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Accepted Article
Version postprint

18. Hung TP, Truong LV, Binh ND, Frutos R, Quiquampoix H and Staunton S, Comparison of the affinity and extraction yield of trace amounts of three Cry proteins from *Bacillus thuringiensis* in contrasting types of soils. *European Journal of Soil Science*; **67** 90-98 (2016).

19. Helassa N, Daudin G, Noinville S, Janot JM, Dejardin P, Staunton S and Quiquampoix H, Mobility of adsorbed Cry1Aa insecticidal toxin from *Bacillus thuringiensis* (Bt) on montmorillonite measured by fluorescence recovery after photobleaching (FRAP). *Philosophical Magazine*; **90** 2365-2371 (2010).

20. Kedi B, Sei J, Quiquampoix H and Staunton S, Persistence of catalytic activity of fungal phosphatases incubated in tropical soils. *Soil Biology & Biochemistry*; **56** 69-74. (2013).

21. Nannipieri P, Sequi P and Fusi P. Humus and Enzyme Activity. In *Humic Substances in Terrestrial Ecosystems*, ed. by Alessandro P. Elsevier Science B.V.: Amsterdam, pp. 293-328 (1996).

22. Hung TP, Truong LV, Binh ND, Frutos R, Quiquampoix H and Staunton S, Persistence of detectable insecticidal proteins from Bacillus thuringiensis (Cry) and toxicity after adsorption on contrasting soils. *Environmental Pollution*; **208** 318-325 (2016).

23. Konecka E, Baranek J, Bielinska I, Tadeja A and Kaznowski A, Persistence of the spores of B. thuringiensis subsp kurstaki from Foray bioinsecticide in gleysol soil and on leaves. *Science of the Total Environment*; **472** 296-301 (2014).

24. Bryant JE, Application strategies for *Bacillus thuringiensis*. *Agriculture Ecosystems & Environment*; **49** 65-75 (1994).

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25. Dupont C and Boisvert J, Persistence of bacillus thuringiensis servar. Israelensis toxic activity in the environment and interaction with natural substrates. *Water, Air and Soil Pollution*; **29** 425-438 (1986).

26. Douville M, Gagne F, Masson L, McKay J and Blaise C, Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. *Biochemical Systematics and Ecology*; **33** 219-232 (2005).

27. Herman RA, Wolt JD and Halliday WR, Rapid degradation of the Cry1F insecticidal crystal protein in soil. *Journal of Agricultural and Food Chemistry*; **50** 7076-7078 (2002).

28. Marchetti E, Accinelli C, Talame V and Epifani R, Persistence of Cry toxins and *cry* genes from genetically modified plants in two agricultural soils. *Agronomy for Sustainable Development*; **27** 231-236 (2007).

29. Wang HY, Ye QF, Gan J and Wu LC, Biodegradation of Cry1Ab protein from Bt transgenic rice in aerobic and flooded paddy soils. *Journal of Agricultural and Food Chemistry*; **55** 1900-1904 (2007).

30. Helassa N, Quiquampoix H, Noinville S, Szponarski W and Staunton S, Adsorption and desorption of monomeric Bt (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite. *Soil Biology & Biochemistry*; **41** 498-504 (2009).

31. Wang HY, Ye QF, Gan J and Wu JM, Adsorption of Cry1Ab protein isolated from Bt transgenic rice on bentone, kaolin, humic acids, and soils. *Journal of Agricultural and Food Chemistry*; **56** 4659-4664 (2008).

32. Feng YJ, Ling L, Fan HZ, Liu YH, Tan FX, Shu YH and Wang JW, Effects of temperature, water content and pH on degradation of Cry1Ab protein released from Bt corn straw in soil. *Soil Biology & Biochemistry*; **43** 1600-1606. (2011).

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33. Kieft TL, Soroker E and Firestone MK, Microbial biomass response to a rapid increase in water potential when dry soil is wetted. *Soil Biology & Biochemistry*; **19** 119-126 (1987).

34. Quiquampoix H, A stepwise approach to the understanding of extracellular enzyme activity in soil. II. Competitive effects on the adsorption of a b-D-glucosidase on different mineral surfaces. *Biochimie*; **69** 765-771 (1987).

35. Vary JC and Halvorson OH, Kinetics of germination of Bacillus spores. *Journal of Bacteriology*; **89** 1340-1347 (1965).

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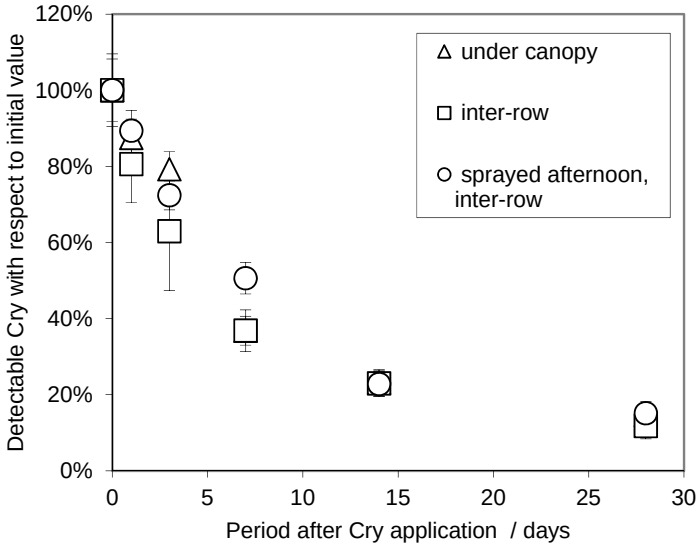

Figure 1

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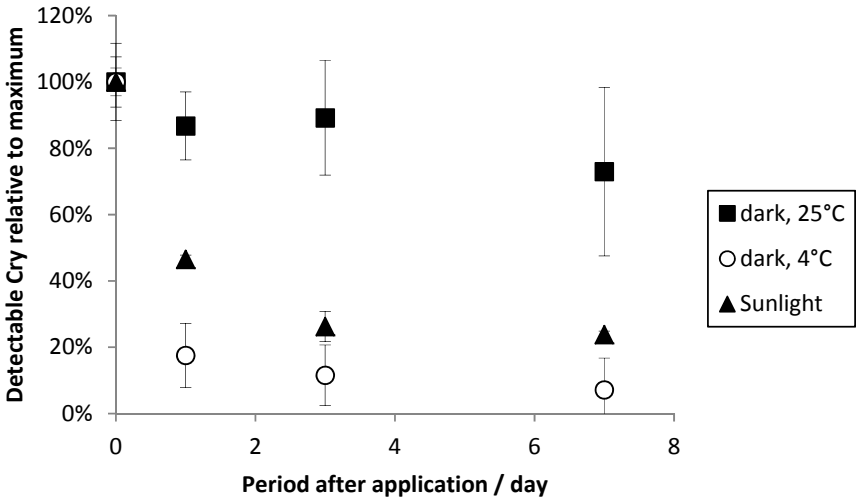

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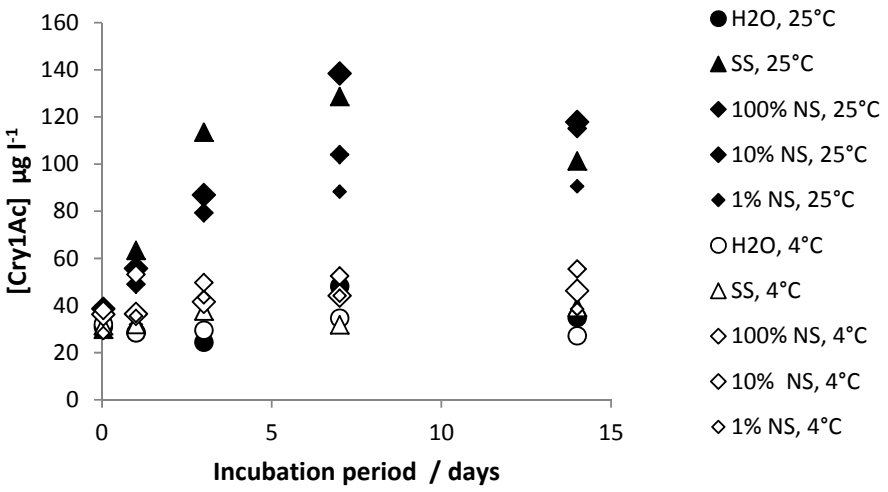

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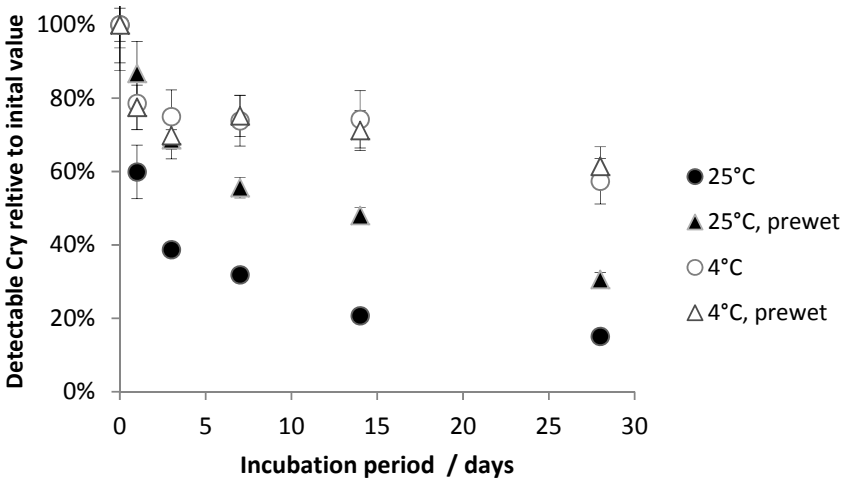

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