JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Rapid Degradation of the Cry1F Insecticidal Crystal Protein in Soil

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The gene for the core Cry1F insecticidal crystal protein (ICP) from *Bacillus thuringiensis* Berliner (Bt) has been incorporated into the genome of maize plants, *Zea mays* L. Plants expressing this ICP are protected from attack by various Lepidopteran pests including the European corn borer, *Ostrinia nubilalis* (Hübner). The stability of the Cry1F ICP in soil was assessed in a laboratory study designed to determine the persistence of the active protein residue in soil over time, using insect bioassay as the analytical quantification method. The GI_{50} (concentration estimated to inhibit growth by 50%) rose at each consecutive incubation interval, indicating a consistent decline in Cry1F activity over time. The residue data were poorly described by a first-order model when fit to either the full data or a truncated data set where the last interval (28 days) was excluded. Data were well described by a shift-log model, and this model predicted DT_{50} (time until 50% decay) and DT_{90} (time until 90% decay) values of 0.6 and 6.9 days, respectively. This rapid degradation rate was consistent with other Bt proteins evaluated in our laboratory.

KEYWORDS: ***Bacillus thuringiensis*; soil degradation; Cry1F; transgenic plants**

INTRODUCTION

Plants expressing insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* Berliner (Bt) are integral features of modern crop production systems. The fate and toxicity of the Bt ICPs have been well documented (*1*) and are substantiated by a history of safe use for both sprayable formulations and plant-expressed ICPs.

Numerous reports document the fate of Bt ICPs in agricultural soils and amended soil mixtures (*2*−*8*). These studies differ significantly in design and analysis. Some studies show highly variable ICP activity in soil over time (*6*, *7*), perhaps due to analytical difficulties. Others indicate rapid to moderate initial rates of Bt ICP degradation in soil, but with some fraction of residual activity remaining (*2*−*4*, *8*). Multiphasic decline patterns of this sort are commonly observed for organic compound degradation in soil and may reflect competitive processes of sorption and degradation governing soil environmental fate, or they may reflect difficulties in long-term maintenance of microbially robust soils in laboratory settings (*9*). Empirical models that adequately represent nonlinear decline in time are important to fully understand organic chemical degradation that does not obey simple first-order kinetics (*3*). This is especially true for the plant-expressed ICPs, where the potential for season-long release to the soil environment may have implications to ICP fate in the field environment (*10*).

The gene for the core Cry1F ICP from Bt has been incorporated into maize plants, *Zea mays* L. Plants expressing this ICP are protected from attack by various Lepidopteran pests including the European corn borer, *Ostrinia nubilalis* (Hübner). This plant-incorporated protectant has been registered with the Environmental Protection Agency and is being commercialized under the Herculex I trademark (*11*). The stability of the Cry1F ICP in soil was assessed in a laboratory study designed to determine the persistence of the active protein residue in soil over time, using insect bioassay. Insect bioassay is a common approach for determining the fate of Bt proteins in soil, because the insecticidal activity of the ICPs is maintained for residues that are not readily extractable and/or quantifiable by other methods (*5*). Thus, tracking the reduction in the activity of the proteins over time provides a more realistic measure of potential ecological effects than does a chemical assay for incompletely extracted ICPs.

MATERIALS AND METHODS

The Cry1F protein used in this study was isolated from a recombinant *Pseudomonas fluorescens* strain as an 11.4% active-ingredient (AI) powder. The recombinant protein was used because isolating sufficient quantities of the ICP from transgenic plants was not feasible. Furthermore, it appears that purified Bt proteins degrade more slowly than those contained within plant tissue (*12*), making the use of this test material a more conservative approach to evaluating environmental persistence. A field-moist Ap horizon soil sample of Drummer silt loam (fine-silty, mixed, superactive, mesic Typic Endoaquolls) was obtained from Illinois (**Table 1**). Cry1F was incorporated at 18.1 μg of AI/g of soil (dry weight equivalent) into soil adjusted to approximately the 100

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10.1021/jf025630u CCC: $22.00
Published on Web 10/15/2002

Table 1. Soil Characterization Results

characteristic	value
pH	7.0
CEC[a] (mequiv/100 g)	28.7
organic matter (%)	6.1
WHC[b](%) at 100 kPa	32.4
bulk density (g/cm^3)	1.07
textural classification[c]	silt loam
particle size distribution[c]	
sand (%)	21
silt (%)	58
clay (%)	21

[a] Cation exchange capacity. [b] Water holding capacity. [c] Hydrometer method. (Soil characterization by Agvise, Northwood, ND.)

kPa moisture capacity. This was accomplished by mixing 500 μg of Cry1F powder (11.4% AI) into 4.05 g of soil (29% moisture). This concentration was chosen to achieve sufficient biological activity to allow degradation to be tracked through time. The treated soil samples were placed in 50 mL plastic centrifuge tubes, each containing a <2 mm diameter hole in the cap for gas exchange. Tubes containing the soil samples were held in an incubator within a dark chamber at 24 °C and 60−65% RH. A total of 28 tubes containing the Cry1F−soil mixture were prepared. Four tubes were removed from the chamber after 0, 3, 5, 7, 10, 14, and 28 days of incubation and immediately frozen at −70 °C.

Bioassays were conducted with tobacco budworm, *Heliothis virescens* (F.), neonates (French Agricultural Research, Lamberton, MN), because this insect is very sensitive to the Cry1F ICP. On each of four bioassay dates (replicates), a single vial from each incubation interval was brought to a volume of 20 mL with a 0.2% aqueous agar solution. These soil−agar suspensions were diluted 0-, 3-, 6-, 12-, and 60-fold with 0.2% agar. Liquefied Stoneville insect diet (*13*) was cooled to 50 °C in a water bath, and 6 mL aliquots of the aforementioned soil suspensions were added to 24 g of diet to produce theoretical concentrations of 475, 158, 79, 40, and 8 ng of AI/mL of diet (based on initial fortification). Diet from each treatment was added to 30 wells of a bioassay tray (C-D International, Pitman, NJ) on each bioassay date. Control treatments including nontreated soil or diet alone were included in each experiment (208−294 wells/bioassay). A positive control consisting of soil fortified with Cry1F on the date of the bioassay was included in two of the experiments. This fortified control was applied at the same concentrations as the incubated samples, and with the same sample size within each of two of the four bioassays (30 wells per treatment). The fortified control was used to determine if the Cry1F protein degraded during storage in the freezer.

After the diet cooled and solidified, a single neonate tobacco budworm was placed in each well of the bioassay tray, and the trays were sealed with vented lids. Bioassays were held in the dark for 6 days at 24 °C and 60−75% RH, after which time mortality and the total weight of surviving larvae was recorded.

Percent growth inhibition was calculated for each treatment within each bioassay by comparing the total weight of insects in each treatment with those in the treatments containing nontreated soil and nontreated diet, since these controls appeared to be comparable. The weights were adjusted for the total number of insects in each treatment, and dead insects were considered to weigh nothing. This measure of efficacy incorporated the effects of mortality and growth retardation and helped to avoid artifacts that can occur when mortality is ignored. The concentration providing 50% growth inhibition (GI_{50}) and the 95% confidence interval for each incubation interval and control treatment were estimated by regressing the probit of average growth inhibition against the logarithm of concentration.

The fraction of residue remaining at each incubation interval was estimated by dividing the initial GI_{50} by the GI_{50} determined for each incubation interval. The fit of the data to a first-order decay pattern was evaluated graphically by plotting the natural logarithm of the percent remaining residue against time and looking for a linear relationship. The data were also evaluated by fitting a shift-log model (*3*, *14*). Briefly, the shift-log model is a nonmechanistic linearization procedure that involves adding a single constant to all of the time values and regressing the logarithm of the residue against the logarithm of the transformed time. The constant and the other two model parameters (slope and intercept) are estimated using nonlinear regression (PROC NLIN, *15*). Times until 50% decay (DT_{50}) and 90% decay (DT_{90}) were calculated on the basis of the shift-log model.

Table 2. Tobacco Budworm GI_{50} Results with Cry1F Incubated with Soil

treatment	GI_{50} (95% confidence interval) (ng of AI Cry1F/mL of diet)
fortified control	7.7 (5.8–10.3)
0-day incubation	12.3 (10.2–14.8)
3-day incubation	60.4 (44.3–82.2)
5-day incubation	98.9 (65.7–148.9)
7-day incubation	109.4 (50.1–239.1)
10-day incubation	186.0 (137.2–252.0)
14-day incubation	268.8 (143.8–502.4)
28-day incubation	316.9 (175.7–571.8)

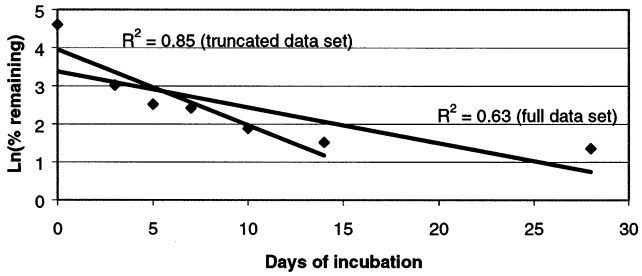

Figure 1. Plot of Cry1F decay in soil, demonstrating fit of first-order kinetic model.

RESULTS AND DISCUSSION

Growth inhibition was used to index the activity of the Cry1F in soil because mortality was <7% by the second sampling time of 3 days, even at the highest concentration tested. The GI_{50} for the fortified control was not significantly different than the GI_{50} of the frozen 0-day treatment (based on overlap of the 95% confidence intervals), but it was numerically more active (**Table 2**). If this was a meaningful effect, it may have been caused by breakdown of the Cry1F in the freezer or by degradation of the Cry1F in the soil samples during the short time prior to freezing. In either case, this would make the estimates of degradation made here conservative (slower), since the initial activity (residue) would actually be higher than that assumed here. A factor that may have affected the reliability of the fortified-control result is its lower level of replication (only included in two of the four bioassays) compared to the other treatments.

The GI_{50} rose at each consecutive incubation interval, indicating a consistent decline in Cry1F activity over time. The residue data were poorly described by a first-order model when fit either to the full data or a truncated data set where the last interval (28 days) was excluded (**Figure 1**). Data were well described by the shift-log model (**Figure 2**), and this model predicted DT_{50} and DT_{90} values of 0.6 and 6.9 days, respectively. Irrespective of the method for describing the fit of the data, <4% of the initial activity remained after 28 days, indicating a rapid decline in activity over this time interval.

These results are consistent with previous studies conducted with a full-length (containing a leaving group) preparation of the Cry1F ICP determined in a soil from the mid-south cotton-growing area of the United States (DT_{50} less than 1 day) (*2*).

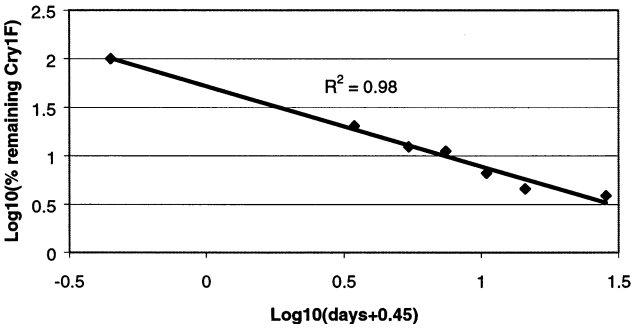

Figure 2. Plot of Cry1F decay in soil, demonstrating fit of shift-log model.

The short residual seen for these two Cry1F ICPs in field-collected soils is also consistent with results seen in our laboratory for another Bt ICP, Cry34Ab1/Cry35Ab1, where the results are well fit by the shift-log model ($R^2 = 0.98$), and where the DT_{50} was estimated at less than 2 days on the basis of this model (*3*).

Recent field-accumulation studies with Bt cotton also confirmed that Bt ICPs degrade rapidly in the soil environment (*16*). These studies, which monitored fields where Bt cotton was cropped for up to 6 years in succession, found no detectable soil residues of Cry1Ac at the limits of detection (15−20 μg of AI/kg of soil) as determined by both immunoassay of extractable residues and insect bioassay. The environmental fate of Cry1F protein, as expressed in Herculex I hybrids, is similarly being evaluated in field accumulation studies to verify the prediction of rapid soil degradation made from this laboratory study.

ACKNOWLEDGMENT

We thank Mark Krieger, Ian van Wesenbeeck, Jim Knuteson, Penny Hunst, Katherine Armstrong, Laura Tagliani, John Cuffe, and Donald Stuart of Dow AgroSciences, and Dan Moellenbeck and Larry Zeph of Pioneer Hi-Bred International for reviewing the manuscript. We also thank Gary Misich, Ricerca Inc., for technical assistance.

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Received for review April 26, 2002. Revised manuscript received September 16, 2002. Accepted September 16, 2002.

JF025630U