Soil Inactivation of the *Bacillus thuringiensis* Subsp. *kurstaki* CryIIA Insecticidal Protein within Transgenic Cotton Tissue: Laboratory Microcosm and Field Studies

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The environmental fate of *Bacillus thuringiensis* subsp. *kurstaki* CryIIA insecticidal protein expressed within transgenic cotton plant tissue (= Bt-cotton) was evaluated by determining reduction in the biological activity of the protein incubated in soil for 120 days. Studies were conducted in a laboratory microcosm and under field conditions during the fall and winter of 1995−1996 in St. Louis, MO. An insect bioassay, based on growth inhibition of larval *Heliothis virescens* (F.), was used to estimate DT_{50} values (50% dissipation time = "half-life" of bioactivity) of the CryIIA protein. DT_{50} values were 15.5 and 31.7 days for the laboratory and field, respectively. The percentages of initial CryIIA protein bioactivity remaining after 40 days of incubation were similar for the laboratory and field samples. In both environments, <25% of the initial bioactivity remained after 120 days. These results indicate that the biological activity of CryIIA protein, as a component of postharvest Bt-cotton plants, readily dissipates when cultivated into soil and suggest that laboratory soil microcosms can be useful for estimating the rate at which dissipation occurs in the field.

Keywords: *Soil degradation; transgenic cotton; CryIIA insecticidal protein; Heliothis virescens*

INTRODUCTION

Commercial lines of Bollgard, the first transgenic cotton (*Gossypium hirsutum* L.) introduced for lepidopteran insect control, express the CryIA(c) insecticidal protein from *Bacillus thuringiensis* subsp. *kurstaki* (Btk). Use of the functionally distinct CryIIA protein from Btk, expressed in plants already containing CryIA(c) protein (i.e. "gene-pyramiding") might delay the development of insect resistance and increase the effectiveness of insect control (Tabashnik, 1994). CryIIA protein shares only 37% amino acid sequence homology with CryIA proteins (Donovan et al., 1988) and has a unique insect midgut binding mode of action (English et al., 1994) and a host range that includes Diptera as well as Lepidoptera (Yamamoto and McLaughlin, 1981; Donovan et al., 1988; Moar et al., 1994). Postharvest plant residue from transgenic cotton contains active CryIIA protein (G. Rogan, Monsano Co., unpublished results). It is therefore likely that CryIIA protein will be added to soil upon cultivation of cotton stalks, leaf material, and unharvested bolls. Soil degradation studies of CryIA protein within transgenic cotton were summarized by Ream et al. (1992), but the environmental fate of CryIIA protein within Bt-cotton residue has not been studied. A *Heliothis virescens* (F.) larval growth inhibition bioassay has previously been used to assess the degradation rate of the CryIA(b) insecticidal protein in corn tissue (Sims and Holden, 1996). Evaluation of the relationship between Btk insecticidal protein concentration and larval weight allows accurate quantitation of protein levels in transgenic plant tissues alone and in transgenic plant−soil matrices with a sensitivity as low as 0.5 ng of protein/mL of diet (Sims and Berberich, 1996). In this study, a *H. virescens* larval growth inhibition assay was used to assess the expected environmental fate of the CryIIA insecticidal protein within transgenic cotton tissues. The loss of CryIIA protein biological activity was studied in a laboratory microcosm and under natural conditions of temperature and precipitation in the field.

MATERIALS AND METHODS

Plant Material. The Bt-cotton varieties were generated by *Agrobacterium tumefaciens*-mediated transfer of the *cryIIA* gene from Btk encoding the CryIIA protein, into the genome of the parental cotton line, Coker 312 (C 312) (Klee and Rogers, 1989; Donovan et al., 1988; English et al., 1994). Modifications to the DNA coding sequence of the gene were made to increase plant expression of the full-length, nature-identical CryIIA protein (Perlak et al., 1991). C 312 and Bt-cotton plants were grown in a greenhouse at Chesterfield, MO. Leaf tissue was collected during the matchhead square to first bloom growth stages and immediately placed on dry ice before storage at −80 °C. Frozen material was lyophilized and ground using a mortar and pestle. Insect bioassays of the lyophilized Bt-cotton powder indicated that the concentration of active CryIIA protein was approximately 80 μg/g.

Test System. Four treatment conditions were examined, each corresponding to combinations of location (laboratory microcosm or field) and tissue type (Bt-cotton or control cotton). We used samples of a Dupo loam soil [30% sand, 62% silt, 26% clay, 4.7% organic matter, pH 7.0, and field capacity (% moisture at −33 kPa) = 19.0%] that was originally obtained in 1987 from St. Charles, MO, and maintained since that time within an exposed field storage bin at Chesterfield, MO. Dupo loam was chosen because it has physical characteristics (texture, pH, and organic matter) representative of many soils used to grow cotton. Soil collected for this study in 1995 was temporarily stored (24 h) in continuous darkness at 22−23 °C prior to being sifted through a 1.4-mm sieve (U.S. Standard Sieve No. 14) to remove extraneous debris. Moisture was then adjusted to obtain 75% field capacity. Three replicate 50-mL-capacity polypropylene centrifuge incubation tubes were prepared per sampling time for each treatment. Tubes containing Bt-cotton plus soil and C 312 cotton plus soil treatments for the laboratory microcosm study contained a uniform mixture

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of approximately 3.5 g of soil (dry wt) and 0.175 g of Bt-cotton tissue. The estimated preincubation concentration of active CryIIA protein per gram of soil was therefore [(80 μg/g) (0.175 g)] ÷ 3.5 g = 4.0 μg/g. For field-incubated samples, the bottoms of the sample centrifuge tubes were perforated with five or six holes to permit drainage, and the tubes were partially filled with tight-fitting cylinders of polyurethane sponge to prevent sample material from shifting to the bottom. A column of sample material approximately 5 cm long was then created by adding 16−17 g of cotton tissue plus soil to fill the tubes within 1−1.5 cm of the top opening. The field soil plus cotton tissue samples had the same proportions used for the laboratory study [i.e. 3.5 g of soil (dry wt)/0.175 g of cotton tissue].

Sample Incubation. Laboratory centrifuge tubes had one 2−3-mm-diameter ventilation hole punched into the cap to allow for air exchange. Samples, excluding the $t = 0$ tubes, were capped and placed inside a 48 × 48 × 38 cm Plexiglas chamber. Air flow and high humidity were maintained by a pan of water at the bottom of the chamber through which air was bubbled using an aquarium air pump (Whisper 800, Secondnature, Oakland, NJ). The chamber was covered with a cardboard box to provide continuous darkness. Temperatures during the study ranged from 24 to 27 °C. The relative humidity inside the chamber was estimated as >80% due to the surface area and aeration of the water reservoir. Previous studies determined that these incubation conditions maintained the initial soil moisture level for at least 43 days (Sims and Holden, 1996). Replicate tubes (three) for each treatment were sampled six times: 0, 10, 20, 50, 70, and 120 days. Incubations were terminated by taping the cap hole and moving the tubes to −80 °C, a temperature at which the CryIIA protein within cotton plant tissue is stable. Field sample tubes were held upright within test tube racks. Three racks and tubes were separated by approximately 30 m, in a triangular arrangement, and buried with the tops of the tubes slightly above ground level. Field incubation was started on September 7, 1995, at Chesterfield, MO. Tubes were sampled at 0, 7, 21, 42, 70, and 120 days and stored at −80 °C until evaluated.

Bioassay. After completion of the incubations, the replicate samples were thawed and the entire contents (laboratory samples) or approximately 3.675 g dry wt (field samples) was suspended in a volume of 20 mL of a 0.17% agar solution. Five doses of each 20-mL suspension were transferred to separate labeled test tubes. The doses were 6.0, 2.0, 0.75, 0.25, and 0.10 mL of the test suspension. Negative control material for each incubation time consisted of 3.5 g of soil (dry wt) and 0.175 g of C 312 cotton tissue, incubated for the same duration as the respective Bt-cotton treatment, mixed into 20 mL of a 0.17% agar solution. For the 2.0−0.10-mL treatment doses, control material was added to adjust the final volume to 6.0 mL. Negative controls contained 6.0 mL of the C 312 + soil + agar mix. Each dose of treatment or control sample was mixed into 30-mL total volume of artificial insect diet (6.0 mL of sample plus 24 mL of insect diet) (King and Hartley, 1992) and presented to larvae of *H. virescens*. Larvae tested were first instar, 24−48 h old (posteclosion), and weighed <1 mg. For each of the three replicates, 24 test larvae were incubated for 7 days at approximately 28 °C. Survivors were weighed in groups of 15−24, and the mean larval weight for each replication was calculated and used in the statistical analysis. Sublethal doses of CryIIA and other Btk proteins stunt *H. virescens* growth (MacIntosh et al., 1990; Sims and Berberich, 1996). The magnitude of weight reduction was used as an index of the level of CryIIA protein bioactivity in each sample.

Data Analysis. We calculated the mean larval weight of surviving larvae exposed to dilutions made from replicate tubes of sample incubation dates. Nonlinear regression analysis was then used to fit the three-parameter logistic function to mean larval weight for the doses (including control) for each sample date. The logistic model is

$$W_x = W_0/[(1 + (x/EC_{50})^B] \quad (1)$$

In this model W_x is a dependent weight variable, W_0 is the expected control weight, x is the amount (mL) of the test sample (20-mL dilution) added to insect diet (30 mL total), EC_{50} is the EC_{50} (effective concentration reducing larval growth by 50% compared to control) expected for the test sample, and B is the logistic function "slope" parameter.

Equation 1 provided a single curve for each series of CryIIA test substance dilutions (within a soil and incubation time) and corresponding *H. virescens* larval weight responses. The SAS nonlinear regression procedure NLIN (SAS Institute, 1992) was used to fit eq 1 to the mean total larval weight data. Expected values for W_0 were estimated using mean weights of control larvae. Expected EC_{50} values were interpolated from doses bracketing the larval weight that was 50% of the control weight. The primary logistic curve parameter of interest was the EC_{50} or the concentration of test suspension that reduced larval growth by 50%. The nonlinear regression estimates of the EC_{50} and the corresponding 95% confidence intervals are presented in this paper for the laboratory microcosm and field samples.

Estimation of the DT_{50} Values. The DT_{50} is the time required for a 50% reduction in the concentration of bioactive material. Because the EC_{50} is inversely proportional to the CryIIA protein concentration, as the concentration of CryIIA protein dissipates, the EC_{50} increases. Thus, at DT_{50}, when 50% of the initial material remains, the EC_{50} will have doubled. DT_{50} estimates were obtained by first calculating

$$(EC_{50,\, t=0}) \quad (2)$$

Linear interpolation was then used to compute the DT_{50} located between the two sample times with known EC_{50} values bracketing eq 2.

Table 1. Reduction in the Biological Activity of CryIIA Protein within Transgenic Cotton Tissue Incorporated into Soil

incubation day	EC_{50}[a] (95% CI)[b]	
	laboratory microcosm	field
0	0.24 (0.20−0.28)	0.24 (0.20−0.28)
7		0.16 (0.13−0.18)
10	0.43 (0.35−0.51)	
(15.5)[c]	(0.48)[c]	
20	0.52 (0.37−0.66)	
21		0.21 (0.17−0.26)
(31.7)[c]		(0.48)[c]
42		0.74 (0.42−1.06)
50	0.81 (0.57−1.04)	
70	0.81 (0.64−0.98)	0.89 (0.41−1.38)
120	1.02 (0.65−1.38)	1.40 (0.92−1.89)

[a] EC_{50} = concentration (μg/mL) of test material reducing tobacco budworm larval weight to 50% of control larval weight. [b] 95% confidence interval of the EC_{50} estimate. [c] Estimated DT_{50} and EC_{50} where DT_{50} = time to 50% degradation of test material (see text for methods).

RESULTS

Persistence of CryIIA Protein Bioactivity. The initial ($t = 0$) bioactivity of the CryIIA protein rapidly decreased upon soil exposure as indicated by EC_{50} values that increased with incubation duration (Table 1; Figure 1). The estimated DT_{50} values of the CryIIA protein incubated under laboratory and field conditions were 15.5 and 31.7 days, respectively (Table 1). During the first 21 days of incubation, the rate of CryIIA protein dissipation appeared to be more rapid under laboratory conditions but the 50-day laboratory samples and 42-day field samples had statistically similar bioactivities (Table 1). The percentages of $t = 0$ bioactivity remaining in the 120-day laboratory (23.5%) and field samples (17.1%) were not significantly different (Table 1).

Bioassay Suitability. Recovery of CryIIA protein bioactivity from soil was determined by comparing the

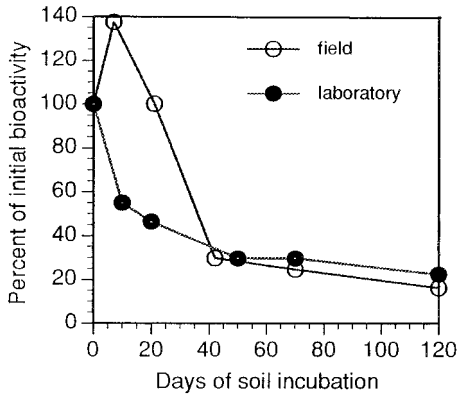

Figure 1. Reduction in the biological activity of CryIIA insecticidal protein within cotton plant tissue incorporated into soil.

EC_{50} values obtained for the Bt-cotton plus soil and Bt-cotton alone at $t = 0$. The bioactivity of Bt cotton plus soil (EC_{50} = 0.24 mL; 95% CI = 0.20−0.28) was not statistically different from that of Bt-cotton alone (EC_{50} = 0.27 mL; 95% CI = 0.14−0.41).

DISCUSSION

CryIIA protein within Bt-cotton tissue was added to test soil at an ecologically meaningful concentration. Immediately prior to harvest, Bt-cotton contained approximately 34 μg of CryIIA protein/g of fresh weight of tissue, on the basis of enzyme-linked immunosorbent assay (ELISA) analysis of three field-grown transgenic lines, and the mean fresh weight per plant was 238 g (G. Rogan, Monsanto Co., St. Louis, unpublished data). Using these results, the estimated total amount of CryIIA protein added per acre as a component of Bt-cotton plants would be 60 000 plants/acre × 238 g/plant × 34 μg/g = 486 g. If the cotton plant matrix containing the CryIIA protein is uniformly incorporated into the top 7.6 cm of soil and 1 cm^3 soil = 1 g, then the maximum "field load" expected is 486 g ÷ (10890 ft^3 × 28317 cm^3) or 1.6 μg/cm^3. Therefore, the level of CryIIA protein added to soil in the present study (4.0 μg/g dry wt of soil) is reasonably consistent with the maximum anticipated concentration of CryIIA protein from post-harvest cultivation of Bt-cotton plant residue. This field estimate also represents a maximum exposure of nontarget organisms to CryIIA protein. In situations of deeper tillage, concentration of the CryIIA protein would be proportionally lower and nontarget exposure would consequently be reduced.

Accurate determination of the residual biological activity of insecticidal proteins in soil is critical in assessing environmental fate and exposure risks to nontarget species. The *H. virescens* bioassay allowed direct monitoring of this bioactivity. The data demonstrate that incorporation of cotton plant material into a soil matrix did not reduce the bioavailability of the CryIIA protein. Similarly, CryIA proteins bound tightly to components of soil such as clay (Venkateswerlu and Stotzky, 1992; Tapp et al., 1994) retained bioavailability (Tapp and Stotzky, 1995a). The biological fate of these soil-bound insecticidal proteins is difficult to accurately quantify using immunoassay procedures such as ELISA because soil-bound proteins resist extraction procedures (Palm et al., 1994; Stotzky, 1986) or provide only semiquantitative data (Tapp and Stotzky, 1995b).

The dissipation rate of CryIIA protein from Bt-cotton was relatively rapid during the first 40 days of incubation but slower during the next 80 days. Abundant fungal growth observed in the laboratory Bt plus soil treatment tubes suggests that microbial activity contributed to the loss of bioactivity (West, 1984). Sunlight irradiation can reduce the toxicity of crystals from *B. thuringiensis* strain HD-1, which produces both CryIA and CryIIA proteins (Pozsgay et al., 1987; Pusztai et al., 1991; Höfte and Whiteley, 1989). It is therefore possible that sunlight exposure may have assisted in the inactivation of CryIIA protein in field samples located on, or near, the soil surface. Despite exposure to sunlight, the rate of CryIIA protein dissipation in the field was initially slower than that for indoor samples. This result is correlated with a lack of precipitation for the first 30 days of field incubation. However, after 42 days, the concentrations of active CryIIA protein remaining in the field and laboratory, based on overlapping 95% confidence intervals of EC_{50} estimates from days 42−50, 70, and 120, were statistically similar (Table 1). The reduced rate of degradation in the final 80 days of the study may have been a consequence of lowered microbial activity following depletion of available nutrients in the plant samples. In addition, lower temperatures in fall/winter would have attenuated microbial activity in the field samples. Overall, our results are consistent with the findings of Donegan et al. (1995), who demonstrated the stimulatory, but short-term, effects of transgenic CryIA(c) protein cotton plants on bacterial and fungal populations.

Recent studies have provided data indicating that *B. thuringiensis* insecticidal proteins, as components of plant tissue, will rapidly lose bioactivity when incorporated into agricultural soils. Sims and Holden (1996) demonstrated that CryIA(b) protein, added to soil within transgenic corn tissue, had an estimated DT_{50} of 1.6 days and a DT_{90} of 15 days. Palm et al. (1994) reported on soil dissipation of CryIA(b) and CryIA(c) proteins alone or in a cotton plant tissue matrix using an ELISA detection procedure. Using a different soil type and lower soil moisture and temperature conditions than in the present study, they found that CryIA(b) and CryIA(c) proteins in cotton tissue decreased rapidly, with DT_{50} estimates of approximately 4 and 7 days, respectively. Ream et al. (1992) used a *H. virescens* bioassay to study soil (Dupo) degradation of CryIA(c) protein in transgenic cotton tissue. They determined DT_{50} (95% CI) = 41 (31−62) days and DT_{90} = 136 (101−205) days values, which are consistent with the results presented here for the CryIIA protein. These data indicate that environmental conditions (soil type, microbial composition, weather) and transgenic plant species may have a greater effect on degradation than the specific *B. thuringiensis* protein expressed in the plant tissues.

In summary, our data suggest that studies conducted within laboratory soil microcosms can be useful for predicting the environmental fate of *B. thuringiensis* insecticidal proteins under field conditions. The biological activity of CryIIA protein, within transgenic cotton tissue, is likely to rapidly degrade under conventional cultivation in which cotton plant residue is plowed into the soil after harvest.

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