Field Evaluation of Transgenic Tobacco Containing Genes Encoding *Bacillus* thuringiensis ∂-Endotoxin or Cowpea Trypsin Inhibitor: Efficacy Against *Helicoverpa xea* (Lepidoptera: Noctuidae)

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ABSTRACT Transgenic tobacco plants expressing ***Bacillus thuringiensis*** var. ***kurstaki*** HD-73 delta-endotoxin or cowpea, ***Vigna Unguiculata,*** trypsin inhibitor (CpTI) were evaluated for efficacy against artificial infestations of ***Helicouerpa zea*** (Boddie) under field conditions. Mortality of ***H. zea*** larvae was significantly higher and leaf damage significantly lower for the genotype containing ***Bacillus thuringiensis*** gene compared with nontransgenic control. Few ***H. zea*** larvae survived beyond first instar on the ***Bacillus thuringiensis*** genotype. Larval mortality was also higher and leaf damage lower on the CpTI genotype than its corresponding control, but the effect was less consistent and less pronounced than that produced by the ***Bacillus thuringiensis*** genotype. Neither transgenic genotype had a significant effect on natural infestations of the other phytophagous or predacious insects that were monitored.

KEY WORDS Transgenic tobacco, ***Helicouerpa zea, Bacillus thuringiensis***

PROTECTION OF FOOD AND FIBER CROPS from insect attack has relied heavily on the use of synthetic insecticides for the past several decades. The disadvantages of this dependence are now evident, and alternative control tactics are actively being sought including the use of transgenic plants which are resistant to insect attack. This alternative offers several advantages, including relatively selective control of target pest species, crop protection throughout the season, and protection that is independent of environmental conditions. Genes targeted for introduction into crop plants include ***Bacillus thuringiensis*** genes coding for delta-endotoxins and a protease inhibitor gene from cowpea, ***Vigna unguiculata*** (L.), that codes for a trypsin inhibitor (CpTI).

When ***Bacillus thuringiensis*** is applied to foliage, several applications may be required for long-term protection because it can be washed off by rain, diluted by plant growth, and inactivated by sunlight (Aronson et al. 1986). The cloning of genes encoding insecticidal proteins and expression in transgenic plants may provide an alternate delivery system for crop protection. ***B. thuringiensis*** genes have been genetically engineered into tobacco (Barton et al. 1987, Vaeck et al. 1987) and tomato (Fischhoff et al. 1987) and found to be efficacious in laboratory testing. More recently, transgenic tomato plants expressing the ***Bacillus thuringiensis*** var. ***kurstaki*** HD-1 *(cryIA(b))* genes (Delannay et al. 1989) and cotton plants expressing the HD-1 and HD-73 *(cryIA(c))* genes (Perlak et al. 1990) have been demonstrated to be efficacious against major insect pest species in the greenhouse or field.

Cowpea trypsin inhibitor is a small polypeptide belonging to the Bowman-Birk type of double-headed serine protease inhibitors. Feeding trials using artificial diets containing CpTI have demonstrated it to be efficacious against several insect species (Gatehouse & Boulter 1983). When expressed in transgenic tobacco, it enhances resistance to *Heliothis virescens* (F.) (Hilder et al. 1987).

The objectives of this trial were to evaluate, under field conditions, the effect of transgenic tobacco containing genes encoding ***Bacillus thuringiensis*** HD-73 or CpTI on survival of larvae of

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Helicoverpa zea (Boddie), feeding damage by ***H. zea*** larvae, and naturally occurring infestations of other insects.

Materials and Methods

Plant Materials. Four genotypes of *Nicotiana tabacum* were used in this experiment: *N. tabacum* var. Samsun NN, *N. tabacum* var. Samsun NN transformed with the T-DNA of plasmid pROK/CpTI+5 (herein referred to as Samsun+CpTI), *N. tabacum* var. Xanthi nc and *N. tabacum* var. Xanthi nc transformed with the T-DNA of plasmid pWB139 (herein referred to as Xanthi+*Bt*). The tobacco transformant and T-DNA CpTI+5 has been previously described (Hilder et al. 1987). The T-DNA of plasmid pWB139 contains a single gene which codes for a translational fusion of the first half of the insecticidal crystal protein of *Bacillus thuringiensis* HD-73, fused to kanamycin resistance gene NPTII from Tn5. The gene is under control of the CaMV 35S promoter and the **3'** region of the tomato protease inhibitor I gene. It is flanked by the nopaline promoter and the nopaline 3' end. PWB139 has a structure identical to plasmid pWB146 (Barnes 1990), except that *Bacillus thuringiensis* codons 1 thru 612 of *Bacillus thuringiensis* strain HD-73 replace the firefly luciferase codons of pWB146, and codons 3, 4, and 5 of the *Bacillus thuringiensis* gene have been altered to form a HindIII restriction site. Tobacco plants transgenic for pWB139 T-DNA were produced as described by Barnes (1990) and Horsch et al. (1985), with selection of shoots resistant to kanamycin as coded for by the *Bacillus thuringiensis* Btlkan gene. Line WP35 was selected for this trial based on earlier laboratory efficacy tests against lepidopteran larvae. Homozygous seed of this line were used in the experiments described here.

Field Site and Experimental Design. Tobacco seedlings were started in greenhouses located in Davis, CA, and when 5 wk old (about four true leaves), they were transplanted to 1.5-m-wide (furrow to furrow) preshaped seed beds typically used for processing tomatoes. Field experiments were conducted under permission of USDA–APHIS. The experimental field site was located near Woodland, CA. Transplanting occurred on 23 August, 1989, and the field was immediately sprinkle-irrigated. No additional irrigations were applied. The field was treated with carbaryl (24 August) and diazinon (29 August) for control of flea beetles.

A randomized complete-block design was used with four replicates. A replicate consisted of a row of 12 plants spaced 55 cm apart. At least 1 m of row was left unplanted between plots to act as a buffer, and the outermost row of the tobacco planting, which was not used in the trial, acted as a border. The treatments consisted of the four tobacco genotypes described above, which were subjected to three types of infestation at four distinct times. This resulted in 48 (6 plots per row by eight rows) experimental plots per block.

Infestations. The three types of infestation were (1) plots artificially infested (one time) with *H. zea* eggs followed by up to four samples taken at about weekly intervals (referred to herein as "seasonal" plots), (2) plots artificially infested (one time) with eggs and left undisturbed until harvest (referred to as "harvest" plots), and (3) plots not artificially infested. Plots not artificially infested were intended to provide information on naturally occurring populations of ***H. zea*** and other insects.

Eggs of *H. zea* were obtained from the Southern Field Crops Insect Management Laboratory, USDA–ARS, Stoneville, MI. This colony had been reared for ~12 generations on a soybean flour–wheat germ diet. Eggs were shipped to Davis, CA, where the organdy cloth, upon which the eggs had been layed, was cut into strips each containing ~100 eggs. Eggs were held at variable temperatures ranging from 10 to 27°C so that eggs would hatch shortly after being placed in the field.

Seasonal and harvest plots were infested on 15 September, 22 September, 2 October, and 9 October by placing the organdy strips within the young leaves at the apex of plants. On 15 September, the rate of infestation was ≈100 eggs (one strip) per plant. On subsequent dates, the rate of infestation was ≈200 eggs (two strips) per plant. Eight strips of eggs per infestation date were held in the laboratory to determine egg hatch in the absence of field-mortality factors. Preliminary infestations indicated that very few eggs or early instars were surviving because of heavy predation by *Nabis* spp. To establish *H. zea* successfully, each plant was carefully searched and predators eliminated from the uppermost portion of the plant at the time of infestation. Once predators were removed, egg strips were placed on the plant, and the uppermost leaves were enclosed in a semiporous plastic bag (25 by 30 cm) (Delnet; AET, Middletown, DE). After 7–10 d, the bags were removed. Bags were not placed on uninfested plants.

Helicouerpa zea Abundance and Feeding Damage. At approximately weekly intervals following infestations, three plants were randomly sampled from each seasonal or uninfested plot or both. Each seasonal plot was sampled four times following infestation. Uninfested plots were sampled fewer times as described below. The three plants were cut at soil level, placed together in a large plastic bag, and transported to the laboratory where they were shaken vigorously in a large metal can. Insects were collected, identified, and counted. Plants sampled on the first date following infestation still had the

plastic bags attached. Insects contained in the bags were recorded separately but were later pooled with insect data from the entire plant. Insects recorded per three plants included *H. zea* larvae by instar, Nabidae, Aphididae, and Chrysomelidae (flea beetles). *H. zea* instars were estimated by comparing the head capsule size of larvae in samples from the field with known instar head capsules (glued to a glass microscope slide). These comparison were made using a dissecting microscope. Other insects were present but in low numbers. For the first two observations following infestations, the numbers of insects other than *H. zea* were recorded from both seasonal and uninfested plots ($n = 8$).

On 15 November, eight plants from the harvest plots infested on 2 and 9 October were cut off at soil level and shaken into a large metal container in the field, and the number of *H. zea* larvae per plot was recorded.

On 31 October, the leaf closest to the apex of the plant that was at least 15 cm long and the two leaves immediately below it were removed from each of four plants in the harvest plots (total of 48 leaves per genotype) infested 22 September and 2 October. Additionally, 36 to 48 leaves were sampled from uninfested Samsun and Xanthi plots. Feeding damage (proportion leaf area removed) by *H. zea* larvae was estimated by measuring the leaf area of each leaf and cut-out tracings of the same leaf using a LI-COR 3000 leaf area meter (Lincoln, NE).

Data Summary and Statistical Analyses. The mean numbers of *H. zea* larvae and miscellaneous insects were analyzed using repeated-measures analysis of variance (ANOVA) (Abacus Concepts 1989). The ANOVA model included genotype, date of infestation, block, sample week (a repeated measure), and interactions. Data were transformed as the natural log before analysis. Because of significant interactions between genotype and sample week and genotype and date of infestation, *H. zea* data were subsequently analyzed within sample week (averaged across infestation date) and by each combination of sample week and infestation date (Milliken & Johnson 1984). Insect data other than for *H. zea* were analyzed using data from seasonal or uninfested plots or both, as previously described. Such data were available from only the first two sample weeks. The experiments were designed for planned comparisons of the density of *H. zea* larvae and of miscellaneous insect species on tobacco genotypes. The following single degree of freedom contrasts were used: Xanthi versus Xanthi+*Bt* (X versus X+), Samsun versus Samsun+CpTI (S versus S+), Xanthi+*Bt* versus Samsun+CpTI (X+ versus S+), and Xanthi with and without *Bacillus thuringiensis* versus Samsun with and without CpTI.

The mean number of *H. zea* larvae in harvest plots from the 2 and 9 October infestations and mean proportion leaf area removed by larval feeding among genotypes were also subjected to ANOVA and compared using contrasts. Before analyses, the number of *H. zea* larvae per three plants was transformed as described above.

The effect of genotype on age-specific survivorship of *H. zea* larvae was assessed by constructing a life table using the larval records from seasonal plots. The number of *H. zea* eggs applied and the number of surviving larvae by instar were summed across all infestation and sample dates. The total number of *H. zea* eggs applied to each genotype was 33,600. However, after adjustment for the proportion of eggs which did not hatch in the control test in the laboratory, the total number of eggs that hatched in the field was estimated to be 16,286 per genotype. The percentages of eggs that hatched in the laboratory for the four dates of infestation were 48.5, 83.0, 39.5, and 22.9, respectively. Age-specific survivorship (lx) was calculated by dividing the number of larvae recorded for each instar by the number of eggs in the initial cohort.

Potato Y virus infected a portion of the tobacco in the field trial. The infection was apparent throughout the field but was most common in one block. Infected plants appeared chlorotic and stunted. It was assumed that the indirect effect (if any) of the virus on insect abundance would be accounted for by the blocked experimental design.

Maximum and minimum temperatures were obtained from a local weather station and used to generate accumulated degree-day (DD) values for the time periods starting with the day of each infestation through the last seasonal or harvest sample of each infestation. Lower and upper thresholds of 13.9 and 34.5°C for *H. zea* development were used for degree-day calculations (Wilson & Barnett 1983).

Results

Seasonal and Harvest Samples. Analysis of variance of mean *H. zea* density per genotype within each sample week showed a significant ($P < 0.05$) interaction between genotype and infestation for weeks 2 and 3. Therefore, genotypes were compared for each week and infestation date combination (Fig. 1). Because this interaction was not significant for weeks 1 and 4, contrasts were also used to compare genotype means averaged across infestation dates for these two weeks (Table 1).

The ANOVA for weeks 1 ($F = 4.22$; df = 3, 45; $P = 0.01$) and 4 ($F = 13.29$; df = 3, 45; $P = 0.0001$) indicated a significant genotype effect. Contrasts showed the density of *H. zea* larvae recorded on Xanthi+*Bt* was significantly lower than on Xanthi or Samsun+CpTI (Table 1). During week 4, but not week 1, significantly fewer *H. zea* larvae

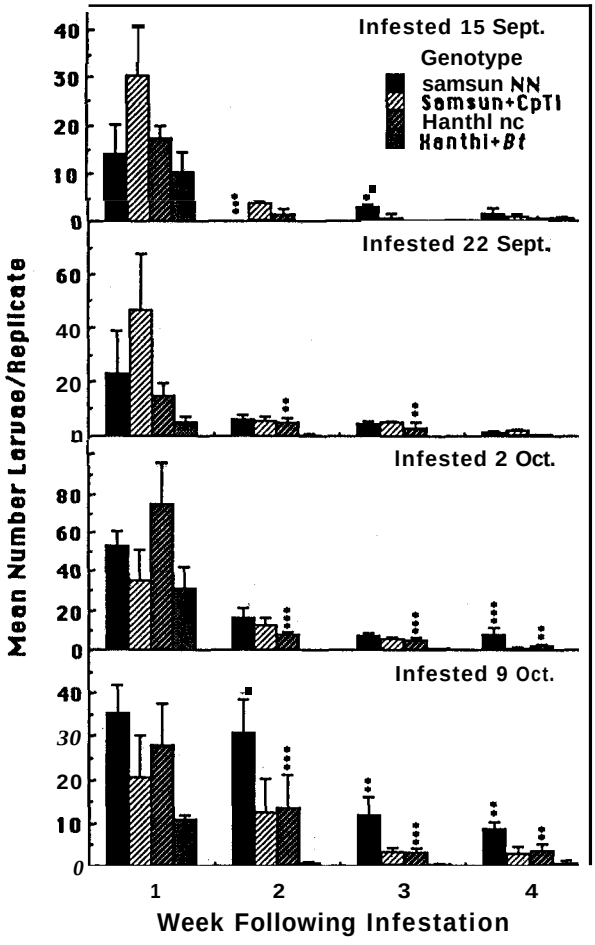

Fig. 1. Mean number (±SEM) of H. *zea* larvae per replicate (three plants) for genotypes of tobacco evaluated at weekly intervals following artificial infestations. Genotypes within each date of observation (week following infestation) and date of infestation compared separately using single degree of freedom contrasts. Levels of significance between transgenic and respective control genotype indicated with asterisks (**, $P < 0.01$; ***, $P < 0.001$). Asterisks always associated with control genotype. Lack of asterisk indicates no significant difference.

were recorded on Samsun+CpTI than on Samsun.

When each sample week and infestation date combination was analyzed separately (Fig. 1), the mean number of *H.* zea larvae recorded on Xanthi+*Bt* was, with few exceptions, the lowest of all genotypes and frequently significantly lower than Xanthi. Apparently, Xanthi+*Bt* was having an affect on larval survival within the first week following infestations, because it consistently had the lowest number of larvae. The efficacy of Xanthi+*Bt* became more apparent with time. In the second and third observation week, significantly lower numbers of *H. **zea*** were associated with the Xanthi+*Bt* genotype for three dates of infestation. The efficacy of the ***Bacillus*** thuringiensis toxin no doubt becomes more apparent with time because of the time required for larvae to feed and the toxin to cause mortality.

Frequently, the mean number of *H. **zea*** larvae recorded on the Samsun+CpTI genotype was numerically lower than on Samsun. However, on only five occasions was this difference significant (Fig. 1).The greater time for CpTI to show any significant effect may reflect the difference in the mode of action between an antimetabolite and a toxin.

Plots infested on 15 September had the lowest number of *H.* zea larvae, apparently because these plants were infested with 100 eggs per plant versus 200 on subsequent dates of infestation (Fig. 1).The decline in larval densities with later samples following infestation apparently reflected attrition because of natural mortality.

This is the first reported example of tobacco containing Bacillus thuringiensis HD-73 or CpTI showing efficacy against an insect pest under field conditions. Neither the Xanthi+*Bt* nor Samsun+CpTI had an apparent effect on the number of naturally occurring Nabidae ($F = 1.27$; df = 3,105; $P = 0.29$), Aphididae ($F = 0.40$; df = 3, 105; $P = 0.75$), or Chrysomelidae ($F = 1.48$; df = 3, 105; $P = 0.22$) recorded (Table 2).

Analysis of variance indicated a significant genotype effect among harvest samples taken from plots infested 2 or 9 October ($F = 28.12$; df = 3, 9; $P = 0.0001$ and $F = 6.28$; df = 3, 9; $P = 0.014$, respectively). Contrasts of mean number of *H.* zea larvae present in harvest samples examined on 15 November (infested 2 or 9 October) indicated that Xanthi+Et had significantly fewer larvae than Xanthi (Fig. 2). Only one larva (fourth instar) was found associated with Xanthi+*Bt*. Significantly more larvae were recorded on Samsun+CpTI than on Samsun on

Table 1. Mean numbers of *H. zea* larvae recorded on transgenic and control genotypes of tobacco

Genotypes	Mean no. H. *zea* (±SEM)		Contrasts	Level of significance[a]	
	Week 1	Week 4		Week 1	Week 4
Samsun NN (S)	**31.44 (5.81)**	**4.81 (1.26)**	S vs. S+	ns	**0.002**
Samsun+CpTI (S+)	**33.25 (7.06)**	**1.56 (0.45)**	X vs. X+	0.004	**0.002**
Xanthi nc (X)	**33.63 (7.98)**	**1.73 (0.42)**	S+ vs. X+	**0.006**	**0.005**
Xanthi+*Bt* (X+)	**14.38 (3.69)**	**0.29 (0.15)**	S, S+ vs. X, X+	**0.048**	<0.001

[a] Level of significance for single degree of freedom contrasts, df = 1, 45; ns, not significant.

Table 2. Mean numbers of naturally occurring Nabidae, Aphididae, and Chrysomelidae recorded on transgenic and control genotypes of tobacco

Genotypes	Mean no. insects (± SEM)[a]		
	Nabidae	Aphididae	Chrysomelidae (flea beetles)
Samsun NN	23.43 (3.77)	6.07 (0.99)	10.10 (1.41)
Samsun+CpTI	26.59 (3.64)	7.56 (1.18)	6.97 (0.85)
Xanthi nc	20.68 (2.76)	6.71 (1.26)	5.74 (0.69)
Xanthi+*Bt*	26.77 (4.30)	7.24 (1.19)	7.41 (0.87)

[a] No significant differences between transgenic and their respective control genotypes.

plants infested 2 October. This may be a reflection of the relatively low number of larvae present on the plants. Differences were not significant for plants infested 9 October. Xanthi+*Bt* had significantly fewer larvae than did Samsun+CpTI on both dates of infestation ($P < 0.01$). Differences between Xanthi and Samsun were not significant.

In general, temperatures were relatively cool during the field trial. Degree days (C°) accumulated between the date of each of the four infestations and the last seasonal or harvest sample for each infestation were 157.1, 163.0, 125.6, and

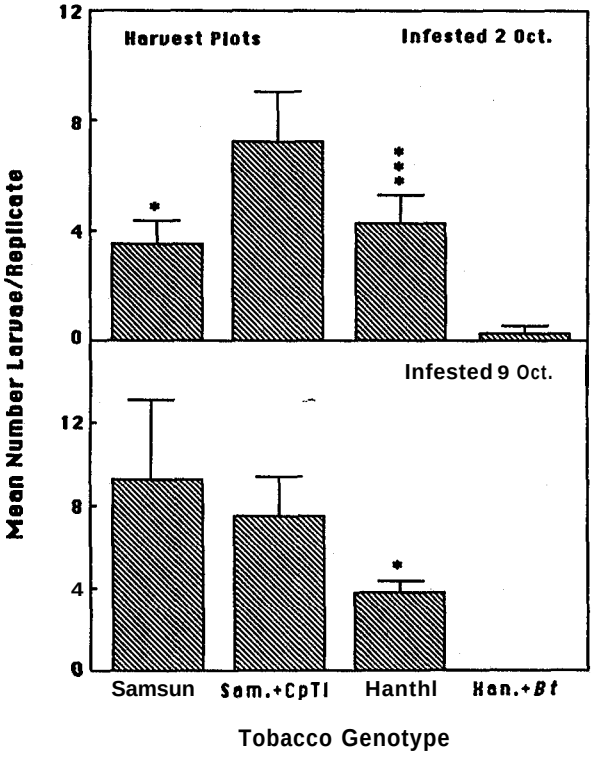

Fig. 2. Mean number (±SEM) of H. *zea* larvae per replicate (eight plants) recorded on four genotypes of tobacco examined on 15 November. Levels of significance between transgenic and respective control genotypes indicated with asterisks (*, $P < 0.05$; ***, $P < 0.001$). Asterisks always associated with control genotype. Lack of asterisk indicates no significant difference.

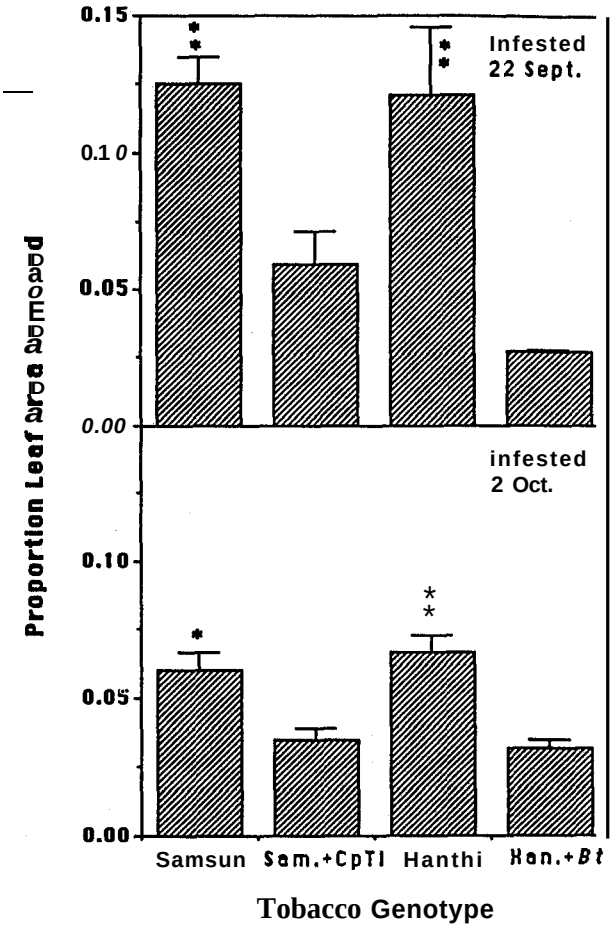

Fig. 3. Mean proportion (±SEM) of leaf area removed from leaves (four replicates of 12 plants) sampled from four genotypes of tobacco infested with H. *zea* and evaluated on 31 October. Levels of significance between transgenic and respective control genotypes indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$). Asterisks always associated with control genotype. Lack of asterisk indicates no significant difference.

107.7, respectively. These are all less then the 182.8 DD required for completion of *H. zea* larval development (Wilson & Barnett 1983) and indicates that larvae would still have been present on plants during the entire sampling period of each infestation.

Leaf Damage. Transgenic genotypes had significantly less leaf damage than their respective controls (Fig. 3). Approximately 3% of the leaf area was removed from the Xanthi+*Bt* versus >12% for the control Xanthi. Uninfested nontransgenic plants averaged ≈3% leaf area removal. There was no significant difference in the amount of leaf area removed between Xanthi+*Bt* and Samsun+CpTI on either date, nor were there differences between control genotypes ($P > 0.05$). Had the leaf area measurements been taken on a later date than the plots infested on 2 October, the differences between the genotypes may have been greater and similar

Table 3. Age-specific survivorship of *H. zea* on four tobacco genotypes, 1989

Instar	Tobacco genotype							
	Samsun NN		Samsun+CpTI		Xanthi nc		Xanthi+*Bt*	
	Total no. survivors	l_x (%)	Total no. survivors	l_x (%)	Total no. survivors	l_x (%)	Total no. survivors	l_x (%)
1	602	3.70	522	3.21	556	3.41	231	1.42
2	142	0.87	145	0.89	95	0.58	4	0.02
3	101	0.62	47	0.29	38	0.23	5	0.03
4	39	0.24	28	0.17	19	0.12	2	0.01
5	15	0.09	8	0.05	7	0.04	0	0.00

l_x, total number of survivors per initial cohort of eggs; 16,286 eggs per genotype; 48 plants (16 replicates of three plants each) per genotype.

to that observed for the 22 September infestation; i.e., larvae would have had more time to damage control genotypes.

Life-Table Analysis. Only five H. zea larvae were recorded in uninfested plots, indicating that native *H. zea* were probably not supplementing the artificially infested plots. These infestations may have also been minimized by heavy predation by Nabis spp. In all genotypes, the greatest mortality occurred early, with <4% surviving to first instar (Table 3). There is no doubt some error is associated with a life table for first instars when samples are taken only at weekly intervals. However, these results provide adequate relative estimates of survival. The efficacy of Xanthi+*Bt* was greatest against early instars. This genotype had the lowest number of first instars, and very few larger larvae were recorded. Overall, fewer larvae were recorded on Samsun+CpTI than on Samsun.

Discussion

Results from our field study demonstrated that transgenic tobacco was efficacious against artificial infestations of *H. zea*. The level of control obtained with Xanthi+*Bt* was excellent; very few larvae survived beyond the first instar. In addition, leaf damage on this genotype was minimal and similar to that on uninfested plants. These results are especially encouraging because H. zea has been reported to be less sensitive to Bacillus thuringiensis than some other lepidopteran pests (MacIntosh et al. 1990). Results with Samsun+CpTI were less dramatic but demonstrated that this protease inhibitor has the potential to increase mortality to *H. zea* and reduce leaf-feeding damage.

Tobacco was selected as the model plant for this experiment. Although Samsun NN and Xanthi nc are not commercial varieties, the level of control observed here, especially with Xanthi+*Bt*, would be acceptable in a wide range of commercial crops. These results and those of Barton et al. (1987), Fischhoff et al. (1987), Vaeck et al. (1987), and others indicate the potential for the use of transgenic crop plants in agriculture. Many crops are amendable to genetic engineering including tomatoes, cotton, potato, crucifers, and soybeans. The range of insect species controlled may be extended by using combinations of Bacillus thuringiensis genes. The insecticidal activity of Bacillus thuringiensis may also be enhanced when in combination with proteinase inhibitors (MacIntosh et al. 1990), which could also expand the range of activity to less sensitive insect targets or allow effective use of lower expression levels of Bacillus thuringiensis protein in the plant tissue.

The advantages of transgenic crop plants are obvious; however, one potential disadvantage is the development of resistance by target insects. This possibility should be carefully considered in the development and use of transgenic crop plants (Gould 1988). There is a wealth of evidence showing insect resistance to conventional insecticides. Recently, resistance in field populations of diamondback moth, Plutella *xylostella* (L.), to foliar applications of Bacillus thuringiensis has been demonstrated (Tabashnik et al. 1990). The probability of resistance to protease inhibitors is less likely because they effect the active site of a major digestive enzyme. An examination and consideration of the ecological significance that transgenic crop plants have to agroecosystems is essential to assure that this rapidly developing pest-control tactic will become an integral and long-term component of crop production.

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