Interaction Between Linear Furanocoumarins Found in Celery and a Commercial *Bacillus thuringiensis* Formulation on *Spodoptera exigua* (Lepidoptera: Noctuidae) Larval Feeding Behavior

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ABSTRACT The effect of linear furanocoumarins and *Bacillus thuringiensis* subsp. *aizawai* toxins on feeding behavior of beet armyworm, *Spodoptera exigua* (Hübner), larvae was quantified by giving larvae a choice between a control diet and a treated diet (diets contained linear furanocoumarins, *B. thuringiensis*, or linear furanocoumarins plus *B. thuringiensis*). Linear furanocoumarins were tested at concentrations found in the outer leaves of celery, *Apium graveolens* L., plants. Diet consumption and diet preference were quantified by recording the position of the larvae twice daily for 4 d and analyzed using analysis of variance and loglinear models of a 2 × 2 factorial with *B. thuringiensis* and linear furanocoumarins as main effects. Both main effects and their interaction were significant for diet consumption and preference data. The interaction between these 2 factors on larval diet preference indicated a mildly antagonistic effect causing increased deterrence and reduced diet consumption by *S. exigua* larvae; however, by not as much as predicted if *B. thuringiensis* and linear furanocoumarins acted independently. These laboratory results document the combined effect of secondary plant compounds and an entomopathogen on larval behavior. Our results indicate the importance of considering the behavioral ecology of insect herbivores in estimating the compatibility of secondary plant compounds and entomopathogens for the development of integrated pest management programs.

KEY WORDS beet armyworm, insect behavior, allelochemicals, psoralen, xanthotoxin, bergapten

CURRENT INTEGRATED PEST management (IPM) programs for many insect pests such as beet armyworm, *Spodoptera exigua* (Hübner), depend heavily on the use of biorational insecticides, mainly *Bacillus thuringiensis* Berliner based compounds (Trumble 1990; Trumble et al. 1990, 1994; Trumble and Alvarado-Rodriguez 1993). *B. thuringiensis* is a gram-positive bacterium characterized by crystalline inclusions produced during sporulation. The digestion of these crystals in the midgut of many insects results in the release of 1 or more insecticidal proteins, which ultimately can cause death. Gene transfer techniques have produced new *B. thuringiensis* delivery systems resulting in transgenic plants containing *B. thuringiensis* toxins, as well as sprayable products with 2 or more complementary *B. thuringiensis* proteins. The potential widespread use of these technologies and the necessity of developing efficient transgenic *B. thuringiensis* plant-based IPM programs create an urgent need for information on the effect of *B. thuringiensis* toxins on the behavioral ecology of insect pests (Gould 1988, Hokkanen and Wearing 1994, Robison et al. 1994).

The efficacy of *B. thuringiensis* products against insects is influenced commonly by host plant characteristics (Hare 1992, Meade and Hare 1993, 1994, Robison et al. 1994). Particular secondary plant compounds have an important though variable role in this association (Felton et al. 1987, Krishchik et al. 1988, Felton and Duffey 1990, Meade et al. 1994). Many studies have demonstrated additive or synergistic effects between secondary plant compounds and *B. thuringiensis* on insect developmental parameters such as growth or survival (Felton and Dahlman 1984, Trumble et al. 1991, Sivamani et al. 1992, Rajendran and Venkatesan 1993, Meade et al. 1994, but see Krishchik et al. 1988, Navon et al. 1993). However, no studies have analyzed specifically the combined effects of secondary plant compounds and entomopathogens on larval feeding behavior.

Spodoptera exigua is a polyphagous insect pest with a highly mobile larval stage (Van Steenwyk and Toscano 1981, Smits et al. 1987, Berdegué and Trumble 1996). It is a pest of several vegetables including celery, *Apium graveolens* L., which con-

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tains linear furanocoumarins that can act as toxins against unadapted insect herbivores (Berenbaum 1990, Trumble et al. 1990). Furanocoumarins are benz-2-pyrone compounds with a furan ring fused at the 6,7 (linear) or 7,8 (angular) positions. They have been reported in at least 8 plant families, but they occur with diversity and regularity only in the Apiaceae (Umbelliferae) and Rutaceae. Within these 2 families they occur as constituents of hundreds of plant species (Berenbaum 1990).

Independently, both the linear furanocoumarins and *B. thuringiensis* proteins affect *S. exigua* larval diet choice. The CryIC protoxin/spore mixture present in commercial *B. thuringiensis* formulations, purified CryIC protoxin, and the CryIC activated toxin all act as feeding deterrents (Berdegué et al. 1996). Similarly, the 3 primary linear furanocoumarins found in *A. graveolens* have proven to function as feeding deterrents for *S. exigua* larvae, even in the relatively low concentrations found in healthy plants (Berdegué et al. 1997). Collectively, linear furanocoumarins have proven compatible with *B. thuringiensis* in their negative effect on survival and developmental time of *S. exigua* (Trumble et al. 1991). However, we could not find any reports on the combined effect of linear furanocoumarins and *B. thuringiensis* on larval diet consumption or choice. Hence, the objective of this study was to examine the nature of the interaction (i.e., additive, synergistic, or antagonistic) between linear furanocoumarins and a commercial *B. thuringiensis* formulation on larval feeding behavior of *S. exigua*.

Materials and Methods

Insects for this study were obtained from a *S. exigua* colony at the University of California, Riverside. *S. exigua* were collected originally in 1982 from Orange County, California, and have been maintained on artificial diet (modified from Patana 1969) in incubators at 28 ± 2°C and a photoperiod of 14:10 (L:D) h. New genetic material has been added from the same area annually since 1983 with the last addition made 8 mo before our study. Previous studies have documented the independent effect of *B. thuringiensis* toxins and linear furanocoumarins on 3 distinct feeding behaviors (Berdegué et al. 1996, 1997) of 3rd instars. Furthermore, this stage is characterized by a significant increase in larval activity (unpublished data). Therefore, cohorts of 2- to 12-h-old 3rd instars were used in all tests.

To quantify the effect of linear furanocoumarins and *B. thuringiensis* on larval feeding behavior, larvae were given a choice between a control diet and a treated diet (treated diet containing linear furanocoumarins, *B. thuringiensis*, or linear furanocoumarins plus *B. thuringiensis*) following a methodology adapted from Gould et al. (1991) and described by Berdegué et al. (1996). The test arenas were circular 150-ml plastic cups with 4% agar (wt:vol) in the bottom; 4 holes were arranged in a cardinal design just above the agar. Microcentrifuge tubes (1.5 ml), 2 with control diet and 2 with treated diet, were arranged alternately in the holes so that tubes with the same type of diet were in opposite sides of the cup.

The linear furanocoumarin diet incorporated 3 different linear furanocoumarins; psoralen, bergapten (5-methoxypsoralen), and xanthotoxin (8-methoxypsoralen) (Sigma, St. Louis, MO), all of which occur in *A. graveolens* (Trumble et al. 1990). We tested the combined effects of these linear furanocoumarins at a concentration of [28.00 μg of bergapten + 18.00 μg of xanthotoxin + 4.00 μg of psoralen]/g diet. These particular concentrations were chosen because these levels were reported by Diawara et al. (1995) for healthy outer *A. graveolens* ('Tall Utah 5270-R') leaves and because previous studies have shown deterrence of 3rd and 4th instar *S. exigua* at these levels (Berdegué et al. 1997). We incorporated linear furanocoumarins into artificial diet by dissolving the compounds in acetone and mixing the solution with 3 g of a nonnutritive fiber (Alphacel, ICN, Costa Mesa, CA). The acetone was evaporated and the nonnutritive fiber with linear furanocoumarins was resuspended in 27 ml of distilled water and warm artificial diet was added to this mixture (Chan et al. 1978).

The *B. thuringiensis* commercial formulation used was XenTari, a *B. thuringiensis* subsp. *aizawai*-based product containing *Cry*IA(a), *Cry*IA(b), *Cry*IC, *Cry*ID, and *Cry*IIB toxins (Abbott, 1992 *B. thuringiensis* Products manual, North Chicago, IL). This formulation is >7 times more toxic to *S. exigua* than *B. thuringiensis* subsp. *kurstaki* products (e.g., Dipel 2X, Abbott) (Moar and Breed 1994). We incorporated 50 μg of XenTari per gram of diet by dissolving the formulation in Tween surfactant at 0.10% (vol:vol) (Fisher, Pittsburgh, PA), and mixing this solution into warm artificial diet according to the methodology of Moar and Trumble (1990). This XenTari concentration caused <10% mortality in choice tests between diet containing *B. thuringiensis* and control diet with 3rd instars (M.B., unpublished data). Also, this concentration caused deterrence of *S. exigua* larvae in previous studies (Berdegué et al. 1996).

The linear furanocoumarin plus *B. thuringiensis* diet contained 50 μg/g of XenTari plus 50 μg/g of linear furanocoumarins ([28.00 μg of bergapten + 18.00 μg of xanthotoxin + 4.00 μg of psoralen]/g diet). This diet was prepared by adsorbing the linear furanocoumarins on 3 g of nonnutritive fiber. The fiber was then resuspended in 24.5 ml of distilled water. The desired XenTari concentration was added in 2.5 ml of Tween (0.10 %). This mixture was then incorporated into warm artificial diet. The control diet was obtained by suspending 3 g of nonnutritive fiber in acetone. The acetone was evaporated and the nonnutritive fiber was suspended in 24.5 ml of distilled water and this suspension was mixed with 2.5 ml of Tween (0.10 %).

This mixture was then incorporated into warm artificial diet.

Two larvae were transferred into each arena and the arenas were placed in a walk-in chamber maintained at 27 ± 3°C and a photoperiod of 14:10 (L:D) h. An uncorrected diet consumption estimate was obtained for each microcentrifuge tube with diet by subtracting the final weights from the initial microcentrifuge tube weights. The average water loss for each type of diet was calculated by averaging the differences between initial and final weights of tubes in 3 arenas without larvae, which were maintained under the same experimental conditions. Consumption was estimated by subtracting the average water loss for each type of diet from the uncorrected diet consumption estimate for each tube. The consumption estimates for the 2 tubes with control diet per arena were added to obtain the total amount of consumed control diet per arena. The total amount of consumed, treated diet per arena was obtained by adding the consumption estimates for the 2 treated diet tubes.

Preference data were obtained by recording the position of the larvae twice daily at 0900 and 1600 hours for 4 d. The test lasted 4 d because, under these environmental conditions, the larvae stop feeding and start forming the pupal cell 5 d after the 3rd instar is reached (unpublished data). Preference was calculated by subtracting the number of larvae on treated diet from the number of larvae on control diet. Each arena was considered a replicate and each treatment was replicated 30 times. All choice tests (120 arenas) were run concurrently. For test 1 (see the section below), 2 opposing control tubes were assigned as treated to compare with other tests.

Experimental Design and Statistical Analyses. Larvae were exposed to the following 4 types of choice tests (treatments): (1) control diet versus control diet, (2) control diet versus linear furanocoumarin-containing diet, (3) control diet versus *B. thuringiensis*-containing diet, and (4) control diet versus diet containing linear furanocoumarins plus *B. thuringiensis*.

The consumed control and treated diet data were analyzed separately. Each data set was analyzed using a logarithmic transformation and an analysis of variance (ANOVA) of the 2 × 2 factorial design. Each of the factors (*B. thuringiensis* and linear furanocoumarins) had 2 levels (presence or absence).

Preference data were analyzed using a loglinear model with the CATMOD procedure (SAS Institute 1990). The experiment had 3 main effects: time (2 observations per day for 4 d = 8 levels), *B. thuringiensis*, and linear furanocoumarins with 2 levels each (presence or absence).

A statistically nonsignificant interaction would indicate that *B. thuringiensis* and linear furanocoumarins acted independently and additively. A significant interaction would indicate that these factors acted synergistically (if the resulting combined effect was superior than what would be predicted if *B. thuringiensis* and linear furanocoumarins acted independently) or antagonistically if the resulting combined effect was inferior to what would be predicted if *B. thuringiensis* and linear furanocoumarins acted independently.

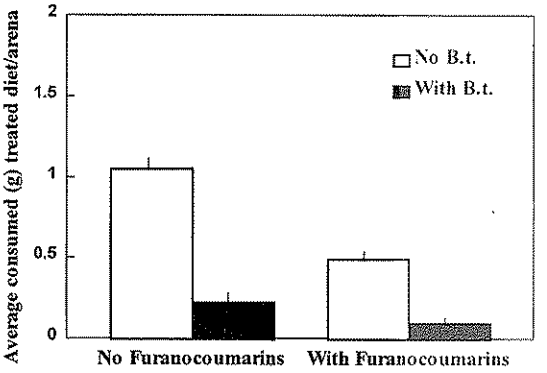

Fig. 1. Interaction plot for the effect of *B. thuringiensis* and linear furanocoumarins on treated diet consumption by *S. exigua* larvae. Two larvae per arena were given the choice between control or treated diet (containing *B. thuringiensis*, linear furanocoumarins, *B. thuringiensis* plus linear furanocoumarins, or control diet). Lines above bars denote standard errors. B.t., *B. thuringiensis*.

Results and Discussion

A decrease in control diet consumption with the addition of *B. thuringiensis* occurred (F = 39.99; df = 1, 29; P = 0.0001), whereas the addition of linear furanocoumarins increased control diet consumption (F = 90.88; df = 1, 29; P = 0.0001) as compared with the control diet versus control diet (test 1). The absence of a significant 2-way interaction between *B. thuringiensis* and linear furanocoumarins indicated that the effect of each factor on consumption of control diet was independent and additive. Feeding inhibition by *B. thuringiensis* has been amply documented in the literature (Gill et al. 1992). Hence, the decrease in control diet consumption with the addition of *B. thuringiensis* was most likely the result of feeding inhibition.

Data for consumption of treated diet also produced significant main effects (*B. thuringiensis*, F = 375.05; df = 1, 29; P = 0.0001; linear furanocoumarins, F = 100.68; df = 1, 29; P = 0.0001), indicating that the addition of either 1 of these 2 factors reduced consumption of treated diet. The 2-way interaction for consumption of treated diet was significant (F = 22.22; df = 1, 29; P = 0.0001), indicating a combined negative effect of *B. thuringiensis* and linear furanocoumarins on consumption of treated diet (Fig. 1). However, the combined negative effect was less than the negative effect if the interaction had been additive. Hence, *B. thuringiensis* and linear furanocoumarins had an

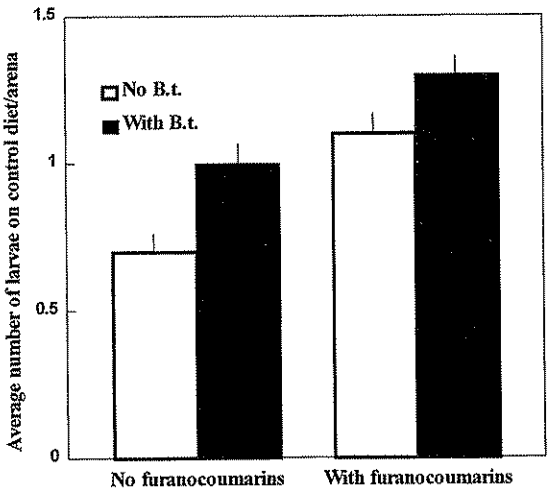

Fig. 2. Interaction plot for the effect of linear furanocoumarins and *B. thuringiensis* on control diet preference by *S. exigua* larvae. Two larvae per arena were given the choice between control or treated diet (containing *B. thuringiensis*, linear furanocoumarins, *B. thuringiensis* plus linear furanocoumarins, or control diet) and their position was recorded twice daily for 4 d. Lines above bars denote standard errors. B.t., *B. thuringiensis*.

antagonistic interaction on consumption of treated diet. This interaction could have been constrained by the fact that treated diet consumption cannot be <0 g; therefore, it should be interpreted with caution. According to Chapman (1974), a feeding deterrent is a compound that deters the insect from further feeding or reduces food intake after initial contact. The interaction between *B. thuringiensis* and linear furanocoumarins on treated diet consumption resulted from an increased deterrence of *S. exigua* larvae because of the combined effect of these 2 factors. However, larval deterrence to diets containing both *B. thuringiensis* and linear furanocoumarins did not result in a proportional increase in control diet ingestion.

The addition of either *B. thuringiensis* (χ^2 = 54.19, df = 1, $P < 0.0001$) or linear furanocoumarins (χ^2= 88.36, df = 1, $P < 0.0001$) elicited a highly significant preference for control diet in choice tests (Fig. 2). The independent effects of *B. thuringiensis* and linear furanocoumarins on larval preference for control diet were consistent with earlier reports (Berdegué et al. 1996, 1997). The *B. thuringiensis* × linear furanocoumarin interaction on larval preference was also significant (χ^2 = 7.04, df = 1, $P = 0.0080$). The difference in larval preference for control diet between the treatments with and without *B. thuringiensis* in the absence of linear furanocoumarins is greater than the difference in larval preference for control diet between treatments with and without *B. thuringiensis* in the presence of linear furanocoumarins (Fig. 2). These results support previous results from consumption data indicating a mildly antagonistic effect of these 2 factors on preference for control diet by *S. exigua* larvae.

A significant time effect (χ^2 = 32.07, df = 7, $P < 0.0001$) implied differences in preference among the observations. To discern if preference for control diet changed as the experiment progressed we performed a linear regression analysis of the average preference ratio, with observations as the independent variable. A significant time effect [REG: $y = 34.05 - 1.43x$ ($r = 0.53$, $P < 0.05$, $n = 8$)] indicated that preference for control diet, although significant throughout the experiment, was reduced as the experiment progressed. The decrease in preference through time was also expected because *S. exigua* larvae have been shown to become habituated or increasingly able to process toxins, or both, as they develop (Berdegué et al. 1996, 1997).

There was a significant linear furanocoumarin × time interaction (χ^2= 19.21, df = 7, $P = 0.0076$), indicating that the presence of linear furanocoumarins in the diet lessens the rate of reduction in preference for the control diet. The *B. thuringiensis* × time interaction was not significant (χ^2= 7.75, df = 7, $P = 0.3556$).

These results should be interpreted with caution because artificial diet experiments are essential simplifications of complex systems, therefore relative preferences for artificial diet in the laboratory are not necessarily equivalent to relative preferences for plant material. The simplicity of our system, although elucidating the interactions between *B. thuringiensis* and linear furanocoumarins on larval behavior, does not take into account factors such as plant phenology, nitrogen content, and physical and ecological characteristic of the plant that have been shown to affect the behavioral ecology of insects (Scriber and Slansky 1981, White 1984, Bernays and Graham 1988, Roden et al. 1992, Berdegue and Trumble 1996). Also, linear furanocoumarins are not distributed uniformly throughout the plant tissue as they are in the diet (Zobel and Brown 1989, Zobel et al. 1991, Zobel and March 1993), and these compounds cooccur with a multiplicity of other secondary plant compounds than may have additive, synergistic, or antagonistic effects themselves.

A few studies in which insects are forced to eat 1 type of diet (no-choice tests) have shown combined and negative effects between *B. thuringiensis* and secondary plant compounds on diet consumption rate (Sivamani et al. 1992, Navon et al. 1993, Rajendran and Venkatesan 1993). However, our laboratory results demonstrate the combined effect of secondary plant compounds and an entomopathogen on larval behavior.

Trumble et al. (1991) concluded that there were no antagonistic interactions between *B. thuringiensis* and linear furanocoumarins that would reduce the effectiveness of *B. thuringiensis* compounds in the field. This conclusion was based on no-choice tests showing an additive effect of *B. thuringiensis*

(Dipel 2X at 25 μg/ml diet) and linear furanocoumarins (135 μg/ml), which caused reduced survival to the pupal stage and increased developmental time of *S. exigua*. However, we found a mildly antagonistic interaction between *B. thuringiensis* and linear furanocoumarins regarding the behavior (deterrence) of *S. exigua* larvae. If reduced *B. thuringiensis* toxin consumption, caused by deterrence, results in increased survival of the targeted insect (Navon et al. 1993, Farrar and Ridgway 1995, but see Meade and Hare 1993), then our results indicate that linear furanocoumarins could reduce direct mortality caused by *B. thuringiensis* products in the field. However, if increased deterrence resulted in reduced developmental rate, mortality caused by environmental factors or biological control agents could increase. This dilemma illustrates the importance of considering the behavioral ecology of insect herbivores in estimating the compatibility of secondary plant compounds and entomopathogens for the development of IPM programs.

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