Efficacy of *Bacillus thuringiensis* Against *Phyllocnistis citrella* (Lepidoptera: Phyllocnistidae)

CARLA DIAS, PATRÍCIA GARCIA, NELSON SIMÕES, AND LUÍSA OLIVEIRA[1]

Departamento de Biologia and CIRN, Universidade dos Açores, P-9501-801 Ponta Delgada Codex, Açores, Portugal

J. Econ. Entomol. 98(6): 1880–1883 (2005)

ABSTRACT The purpose of this work was to analyze the efficacy of *Bacillus thuringiensis* Berliner on the control of *Phyllocnistis citrella* Stainton (Lepidoptera: Phyllocnistidae) in laboratory and field trials. In the laboratory, four *B. thuringiensis* were used: Dipel (commercial formulation) tested at the concentrations of 25×10^6 and 25×10^8 spores/ml and the isolates K, 6, and 15 (collections of the University of Azores) at the concentration of 25×10^6 spores/ml. A surfactant solution of nonoxinol also was tested with or without the different suspensions of *B. thuringiensis*. Leaves of *Citrus sinensis* (L.) Osbeck with second or third instars of leafminer larvae were used in all tests. Bacterial suspensions were applied topically on the surface of intact leaf mines or by injection inside the mine, near the head of the leafminer. When injecting both concentrations of Dipel into the mines, mortality of the leafminers increased compared with the topical application, although no significant differences were observed. The addition of the nonoxinol to the Dipel suspension, applied topically, increased the effect of *B. thuringiensis*, but differences were not significant. The mortality of the leafminers treated only with the nonoxinol solution increased significantly 48 h after treatment, compared with the control group, suggesting an insecticidal effect of this surfactant when used at a concentration of 0.01%. All the tested *B. thuringiensis* were equally active against the leafminer, either when applied topically or by mine injection. Field trials showed a significant difference between larval mortality of the control group and the results observed at the trees treated with *B. thuringiensis* 48 h after treatments.

KEY WORDS *Phyllocnistis citrella*, *Bacillus thuringiensis*, biological control, larval mortality

THE LEAFMINER *Phyllocnistis citrella* Stainton (Lepidoptera: Phyllocnistidae) has long been a citrus pest of general distribution throughout Asia. In the past 10 yr, it rapidly invaded other areas and is now present in all citrus-producing areas of the world (Heppner 1993, Hoy and Nguyen 1997, Legaspi et al. 1999, Garcia-Marí et al. 2002). In the Azores, it was first detected on Santa Maria Island in 1996 and was found all over the archipelago a short time later (Vieira 1997, Elias and Soares 1998).

Females of the leafminers deposit eggs individually on the adaxial or abaxial sides of young leaves (Amalin et al. 2002, Garcia-Marí et al. 2002). After hatching, the larvae feed the leaf parenchyma, and pupation occurs on the leaf margin, under a slight curl of the leaf (Del Bene et al. 1998, Del Bene and Landi 1999, Chermiti et al. 2001). The epidermis of the citrus leaf provides substantial protection for leafminers and presents a significant barrier to control by using chemical or microbial insecticides (Smith and Hoy 1995, Shapiro et al. 1998, Legaspi et al. 1999).

Bacillus thuringiensis Berliner subsp. *kurstaki* is the bacterial insecticide most widely used for controlling Lepidoptera larval populations (Broderick et al. 2000, Lacey et al. 2001). It is safe for many nontarget insects, and it has a minimal impact on the environment (Jyoti and Brewer 1999). Because of the complexity of *B. thuringiensis,* several biotic and abiotic factors can have pronounced effects on the outcome of a control application (Ali and Young 1993a, b; Ben-Dov et al. 2003). Because *P. citrella* is protected inside the mine, Shapiro et al. (1998) suggested the use of an organosilicone surfactant because this solution can reduce the surface tension and increase the penetration of the *B. thuringiensis* suspension through the epidermis of the citrus leaf.

In this study, we compared the effect of a commercial formulation of *B. thuringiensis* (Dipel) and three isolates of *B. thuringiensis* tested with or without a nonionic surfactant (frequently used in agriculture), applied by two different methods: topical and mine injection. The effect of two different concentrations of the commercial formulation of *B. thuringiensis* was further tested in field trials in a citrus grove.

Materials and Methods

B. thuringiensis. One commercial formulation of *B. thuringiensis* subsp. *kurstaki* (Dipel, [serotype 3A 3B], 16,000 U.I./mg, wettable powder [WP], Bayer, Car-

[1] Corresponding author, e-mail: ziza@notes.uac.pt.

naxide, Portugal), one *B. thuringiensis* subsp. *kurstaki* (K), and the isolates 6 and 15 collected from soils in Azores-Portugal. The Azorean collections are maintained under culture in the Department of Biology of the University of Azores. Bacteria were grown on UG medium according to Thiery and Frachon (1997).

Laboratory Experiments. Leaves containing actively feeding second or third instars of *P. citrella* were completely excised with petioles from *Citrus sinensis* (L.) Osbeck trees. To keep the leaves turgescent during the bioassay, each petiole was covered by wet cotton and placed into a 9-cm-diameter petri dish.

Dipel was tested at the concentrations of 25×10^6 and 25×10^8 spores/ml and the isolates K, 6, and 15 were tested only at the concentration of 25×10^6 spores/ml. The lower concentration (25×10^6 spores/ml) corresponds to that recommended for Dipel by the manufacturer for the control of Lepidoptera. Suspensions were made with or without the nonionic surfactant nonoxinol (Molhante Aderente CUF, 250 g [AI]/liter, emulsifiable concentrate [EC], Agroquisa, Lisboa, Portugal). The nonionic surfactant when tested alone was used at the concentration recommended by the manufacturer (0.01%); when tested with *B. thuringiensis*, we added 1 volume of nonoxinol solution (0.01%) to 99 volumes of each tested concentration of *B. thuringiensis.* All bacterial suspensions and control tests were done with sterile water.

A control group was performed using sterile water. Toxicities of *B. thuringiensis* and of the agricultural surfactant solution against *P. citrella* were tested using two methods: topical application that tests the penetrability and resulting mortality when suspensions and/or solutions were applied to the surface of intact leaf mines (with the epidermal barrier intact); and mine injection that tests the mortality when suspensions and/or solutions are directly applied within the mines, circumventing the epidermal barrier. For the topical bioassay, 4 μl of the tested suspension or solution was placed on the mine surface, directly above the head of an actively feeding larva, without puncturing the mine. For the mine injection bioassay, 4 μl of tested suspension or solution was introduced into the mine, by puncturing the leaf epidermis surrounding the head of the leafminer. All suspensions or solutions were applied with a microliter syringe (10-μl Hamilton). For each test, 28–41 larvae were treated. The petri dishes containing the *C. sinensis* leaf with the treated larvae were placed at 21 ± 1°C under natural daylight, corresponding to a photoperiod of 13:11 (L:D) h (Beck 1968). Larval mortality was recorded 24, 48, 72, and 96 h after treatments. Mortality of the larvae was confirmed when individuals exhibited a total lack of external or peristaltic movements when probed.

Preliminary Field Experiments. These preliminary experiments were carried out to confirm if the commercial formulation of *B. thuringiensis* is able to control *P. citrella* under field conditions, by using the concentrations tested in the laboratory experiments. Field experiments were done at Ginetes, south coast of São Miguel Island, in a *C. sinensis* plantation. All trees had been pruned 2 yr earlier and were no >1.80 m in height. Trees were in four rows, with four trees each, a total of 16 trees within the plot. Distance between rows and between trees was 5 m. The first row was used for the control (treated with sterile water), the second row was not treated, the third row was treated with the lower concentration of Dipel (25×10^6 spores/ml) and, the fourth row was treated with the higher concentration of Dipel (25×10^8 spores/ml). Treatments were performed only once using a handheld atomizing spray gun. Each tree was sprayed with 1 liter of the test solution. Forty-eight hours after spraying, 30 *C. sinensis* leaves containing at least one leafminer larva were collected and immediately brought to the laboratory for observation under a stereomicroscope. The number of alive and dead larvae was record.

Data Analysis. Mortality data were tested by analysis of variance (ANOVA) for qualitative values. When ANOVA showed significant differences ($P <$ 0.05) among data sets, paired comparisons of each mean were made using Sheffé tests (Zar 1996). When analyzing differences between two data sets, *t*-tests were used. All analyses were performed using SPSS 10.0 for Windows (SPSS Inc. 1999).

Table 1. Mortality (%) of *P. citrella* larvae at different times after topical treatment of leaf mine or leaf mine injection (*) with Dipel (25×10^6 spores/ml), Dipell (25×10^8 spores/ml), sterile water (control), and the nonoxinol solution (surfactant)

Treatment	*n*	24 h	48 h	72 h	96 h
Dipel	32	15.6a	56.3a	71.9a	93.8a
Dipel*	41	14.6a	75.6a	87.8a	97.6a
Dipell	30	33.3a	86.7a	96.7a	100.0a
Dipell*	32	48.4a	96.8a	100.0a	100.0a
Control	34	0.0a	0.0a	0.0a	0.0a
Control*	32	0.0a	0.0a	0.0a	3.1a
Surfactant	32	0.0a	21.9a	59.4a	75.0b
Surfactant*	32	3.1a	34.4a	75.0a	93.8a

In each group, means in each column followed by a different letter are significantly different ($P < 0.05$, *t*-test).

Results

Laboratory Trials. For both concentrations, when injecting the leaf mines with Dipel, mortality increased compared with the topical application; however, no significant differences were observed (Table 1). When the mines were injected with the nonoxinol solution, an increase in the mortality was observed; however, significant difference between treatments was detected only 96 h after treatment (Table 1). For the control group, no significant differences were observed (Table 1).

The addition of the nonoxinol to the Dipel solution, applied topically, seems to increase the effect of *B. thuringiensis;* nevertheless, no significant differences were observed between treatments (Table 2). When comparing the effect of the nonoxinol solution (alone) with the control group, we observed a significant increase in the larval mortality just 48 h after

Table 2. Mortality (%) of *P. citrella* larvae at different times after topical treatment with Dipel at 25 × 10⁶ spores/ml with (D+) or without (D) the nonoxinol, sterile water (control), and the nonoxinol solution (surfactant)

Treatment	*n*	24 h	48 h	72 h	96 h
D	32	15.6a	56.3a	71.9a	93.8a
D+	28	28.6a	78.8a	89.3a	100.0a
Control	34	0.0a	0.0b	0.0b	0.0b
Adherent	32	0.0a	21.9a	59.4a	75.0a

In each group, means in each column followed by a different letter are significantly different ($P < 0.05$, *t*-test).

treatment, suggesting an insecticidal effect of this agricultural surfactant when used at a concentration of 0.01% (Table 2).

No significant differences were observed between leafminer mortality either caused by the tested solutions of *B. thuringiensis* or by the interaction between *B. thuringiensis* and the nonoxinol solution, when solutions were applied topically (Table 3). Similar results were observed when applying the treatments by direct injection, with the exception of the mortalities caused by Dipel and isolate 15 that were significantly higher than the observed with nonoxinol (Table 3). Mortality caused by Dipel and by the isolates of *B. thuringiensis*, 48 h after treatments (either by topical or direct injection), was significantly higher than the mortality observed in the control (Table 3).

Preliminary Field Experiments. A significant difference was observed between the mortality of the leafminers from the control group and those at the trees treated with Dipel. Leafminer mortality was significantly higher when trees were sprayed with a Dipel solution at 25×10^8 spores/ml compared with trees treated at the lower concentration (25×10^6 spores/ml) (Table 4).

Results show that the leafminer's mortality obtained in field was significantly less than that observed in the laboratory, when using the same concentrations of Dipel ($t = -3.806$, df = 63.743, $P < 0.001$ for 25×10^8 spores/ml; and $t = -2.926$, df = 48.742, $P = 0.005$ for

Table 3. Mortality (%) of *P. citrella* larvae 24 and 48 h after topical and direct injection treatments, with Dipel (D) and isolates K, 6, 15 (all at 25 × 10⁶ spores/ml) with nonoxinol solution (surfactant) and with sterile water (control)

Treatment	Topical treatment			Mine injection		
	n	24 h	48 h	*n*	24 h	48 h
D	32	15.6a	56.3a	41	14.6a	75.6a
K	32	9.4a	50.0a	32	9.4a	59.4ac
6	33	3.0a	54.6a	32	9.4a	65.6ac
15	32	0.0a	56.3a	32	6.3a	75.0a
Surfactant	32	0.0a	21.9ab	32	3.1a	34.4bc
Control	34	0.0a	0.0b	32	0.0a	0.0b
	F	2.603	9.539		1.389	15.704
	df	5, 189	5, 189		5, 195	5, 195
	P	0.027	<0.001		0.230	<0.001

ANOVA *F*, df, and *P* values.
Means in each column followed by a different letter are significantly different ($P < 0.05$, Scheffé test).

Table 4. Percentage of dead larvae of *P. citrella* 48 h after treatment with Dipel at 25 × 10⁸ (Dipell) and 25 × 10⁶ spores/ml (Dipel) in field experiments

Treatment	*n*	Dead larvae (%)
Dipell	125	56.90a
Dipel	101	26.88b
Control	135	5.08c
ANOVA		
F		50.08
df		2, 86
P		<0.05

ANOVA *F*, df, and *P* values.
Means in each column followed by a different letter are significantly different ($P < 0.05$ Scheffé test).

25×10^6 spores/ml). In laboratory, leafminer mortality was 86.7 and 56.3% for the Dipel concentrations of 25×10^8 and 25×10^6 spores/ml, whereas in the field the observed values were of 56.9 and 26.9%, respectively.

Discussion

Laboratory experiments demonstrated that Dipel, *B. thuringiensis kurstaki* (K) and the isolates 6 and 15 were effective against *P. citrella* larvae, indicating that all are valuable candidates to control this pest. All the tested bacterial suspensions caused larval mortality >50% within 48 h after treatment. Our results showed that the tested *B. thuringiensis* were similarly active against the leafmines, either when applied topically or by injection, demonstrating that *B. thuringiensis* penetrates into leaf mines killing the larvae, as observed by Shapiro et al. (1998). Polanczyk et al. (2000), in a study with different strains of *B. thuringiensis* against the second instars of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) observed a similar mortality between *B. thuringiensis aizawai* and *B. thuringiensis thuringiensis* and between *B. thuringiensis dendrolimus* and *B. thuringiensis darmstadiensis*, suggesting that these results could be related to the composition of crystals and their toxic potential. Follas (1995) observed the same in a study with two different strains of *B. thuringiensis* against brassica lepidopteran pests.

When comparing the activity of the commercial formulation (Dipel) against the leafminer, we observed an increase (although not significant) in the mortality of the leafminers treated by direct injection, compared with the topical application, regardless of the tested concentrations. This finding can be explained by the protection given by the epidermis of the citrus leaf (Smith and Hoy 1995, Shapiro et al. 1998, Legaspi et al. 1999).

The agricultural surfactant (nonoxinol solution) tested in this study had two different effects on leafminer mortality. First, it had insecticidal activity when applied alone against the leafminer at the recommended concentration. Other surfactants, such as the organosilicone products, are also recognized by their direct or indirect toxicity to soft-bodied insects and mites (Shapiro et al. 1998, Cowles et al. 2000). Second, it seems to increase the efficacy of the commercial formulation of *B. thuringiensis.* That nonoxinol is a nonionic surfactant can explain its use with pesticides to help them to penetrate the plant cuticles (O'Brien 2004). Other agricultural adjuvants are recommended to control or to help the control of *P. citrella,* such as the organosilicone surfactants (Shapiro et al. 1998) and white mineral oil (Del Bene et al. 1998).

The tested Azorean isolates (6 and 15) were effective against *P. citrella* under laboratory conditions, indicating that when formulated in an adequate manner both can have a potential in the control of the leafminer in Azores, avoiding the introduction of exotic bacteria in the Islands ecosystems.

The significantly higher mortality of the leafminers from the trees treated with *B. thuringiensis* in field trials suggests that the commercial formulation might possibly be a solution for the control of *P. citrella* in leaves of *C. sinensis.* In Cuba, *B. thuringiensis* is used mainly in field programs for the control of different pests, such as *P. citrella,* as discussed by Vega (1999). Nevertheless, more field studies will need to be performed to understand the effect of *B. thuringiensis* against *P. citrella* and to determine the optimum timing of multiple applications.

References Cited

Ali, A., and S. Y. Young. 1993a. Effects of rate and spray volume of *Bacillus thuringiensis* var. *kurstaki* on activity against *Heliothis virescens* (Lepidoptera: Noctuidae) and persistence in cotton terminals. J. Econ. Entomol. 86: 735–738.

Ali, A., and S. Y. Young. 1993b. *Bacillus thuringiensis* var. *kurstaki* activity against larvae of *Heliothis zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) on cotton. J. Econ. Entomol. 86: 1064–1068.

Amalin, D. M., J. E. Peña, R. E. Duncan, H. W. Browning, and R. Mcsorley. 2002. Natural mortality factors acting on citrus leafminer, *Phyllocnistis citrella,* in line orchards in south Florida. BioControl 47: 327–347.

Beck, S. D. 1968. Insect photoperiodism. Academic, New York.

Ben-Dov, E., D. Saxena, Q. Wang, R. Manasherob, S. Boussiba, and A. Zaritsky. 2003. Ingested particles reduce susceptibility of insect larvae to *Bacillus thuringiensis.* J. Appl. Entomol. 127: 146–152.

Broderick, N. A., R. M. Goodman, K. F. Raffa, and J. Handelsman. 2000. Synergy between Zwittermicin A and *Bacillus thuringiensis* subsp. *kurstaki* against gypsy moth (Lepidoptera: Lymantriidae). Environ. Entomol. 29: 101–107.

Chermiti, B., M. Braham, M. Znaidi, H. Gahbiche, B. Messelmani, M. Dali, and M. Messelmani. 2001. Premiers résultats sur l'acclimatation d'*Ageniaspis citricola* Logvinovskaya (Hym., Encyrtidae), parasitoide spécifique de *Phyllocnistis citrella* Stainton (Lep., Gracillariidae). J. Appl. Entomol. 125: 45–52.

Cowles, R. S., E. A. Cowles, A. M. McDermott, and D. Ramoutar. 2000. "Inert" formulation ingredients with activity: toxicity of trisiloxane surfactant solution to twospotted spider mites (Acari: Tetranychidae). J. Econ. Entomol. 93: 180–188.

Del Bene, G., and S. Landi. 1999. Observations on the citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) in Florentine historic gardens. Adv. Hortic. Sci. 13: 12–19.

Del Bene, G. D., S. Landi, and R. Russo. 1998. Chemical control of *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) on ornamental citrus. J. Agric. Environ. Int. Dev. 92: 83–102.

Elias, R. B., and A. O. Soares. 1998. Novos insectos fitófagos em pomares de citrinos da Ilha de S. Miguel (Açores). Açoreana 8: 525–529.

Follas, G. 1995. The efficacy of *Bacillus thuringiensis* and lufenuron against Lepidoptera pest in brassicas. N.Z. Plant Prot. Conf. 47: 341–345.

Garcia-Marí, F., C. Granda, S. Zaragoza, and M. Agustí. 2002. Impact of *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) on leaf area development and yield of mature citrus trees in the Mediterranean Area. J. Econ. Entomol. 95: 966–974.

Heppner, J. B. 1993. Citrus leafminer *Phyllocnistis citrella* in Florida (Lepidoptera: Gracillariidae: Phyllecnistinae). Trop. Lepid. 4: 49–64.

Hoy, M. A., and R. Nguyen. 1997. Classical biological control of the citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae): theory, practice, art and science. Trop. Lepid. 8: 1–19.

Jyoti, J. L., and G. J. Brewer. 1999. Median lethal concentration and efficacy of *Bacillus thuringiensis* against banded sunflower moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 92: 1289–1291.

Lacey, L. A., R. Frutos, H. K. Kaya, and P. Vail. 2001. Insect pathogens as biological control agents: do they have a future?. Biol. Control 21: 230–248.

Legaspi, J. C., J. V. French, M. E. Schauff, and J. B. Woolley. 1999. The citrus leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in south Texas: incidence and parasitism. Fla. Entomol. 82: 305–316.

O'Brien, J. 2004. Adding a surfactant to your tank mix. Albion–Metalosate Plant Nutrition News 5: 2.

Polanczyk, R. A., R.F.P. Silva, and L. M. Fiuza. 2000. Effectiveness of *Bacillus thuringiensis* strains against *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Braz. J. Microbiol. 31: 1–8.

Shapiro, J. P., W. J. Schroeder, and P. A. Stansly. 1998. Bioassay and efficacy of *Bacillus thuringiensis* and an organosilicone surfactant against the citrus leafminer (Lepidoptera: Phyllocnistidae). Fla. Entomol. 81: 201–210.

Smith, J. M., and M. A. Hoy. 1995. Rearing methods for *Ageniaspis citricola* (Hymenoptera: Encyrtidae) and *Cirrospilus quadristriatus* (Hymenoptera: Eulophidae) released in a classical biological control program for the citrus leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae). Fla. Entomol. 74: 600–608.

SPSS Inc. 1999. SPSS Base 10.0 applications guide. SPSS Inc., Chicago, IL.

Thiery, I., and E. Frachon. 1997. Identification isolation, culture and preservation of entomopathogenic bacteria, pp. 57–77. *In* L. Lacey [ed.], Manual of techniques in insect pathology. Academic, San Diego, CA.

Vega, O.F.L. 1999. A review of *Bacillus thuringiensis* (Bt) production and use in Cuba. Biocontrol News Info. 20: 47N–48N.

Vieira, V. 1997. Lepidoptera of the Azores Islands. Bul. Mun. Funchal. 49: 5–76.

Zar, J. H. 1996. Biostatistical analysis. Prentice-Hall International Editions, London, United Kingdom.

Received 26 April 2005; accepted 13 July 2005.