Differences in Susceptibility and Physiological Fitness of Mexican Field *Trichoplusia ni* Strains Exposed to *Bacillus thuringiensis*

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ABSTRACT The use of different commercial *Bacillus thuringiensis* (Bt) products in the Bajio guanajuatense area in Mexico began 12 yr ago, and resistance to Bt in this area has been reported for *Plutella xylostella* (L.) The current study provides a baseline response and resistance potential to Bt in field and laboratory strains of Bajio *Trichoplusia ni* (Hübner). Differences in susceptibility to Bt among *T. ni* populations were observed. *T. ni* neonates collected in Romita, Guanajuato, were more susceptible to Bt than those collected in Salvatierra or San Luis de la Paz, Guanajuato. After five generations of exposure to XenTari in the laboratory, decreased susceptibility was found only in the Salvatierra insects, with an LC_{50} that was 2.1-fold greater than that of a Mexican laboratory strain. The XenTari-selected San Luis de la Paz strain was from 16- to 87-fold more resistant to Cry1A protoxins than U.S. (US) and Mexican laboratory strains. Although Cry1Ab is not a component of XenTari, this strain also was significantly less susceptible to Cry1Ab toxin compared with a US strain, with a resistance ratio of 40.4. The larval weights and lengths, pupal lengths, and percentage of pupation were significantly lower for the Salvatierra strain than for all other strains. The relationship of *T. ni* susceptibilities to Bt Cry toxins and protoxins after several generations of exposure to XenTari and its similarity to *P. xylostella* behavior.

KEY WORDS *Trichoplusia ni*, susceptibility, physiological fitness, insect resistance monitoring

The cabbage looper, *Trichoplusia ni* (Hübner), and the diamondback moth, *Plutella xylostella* (L.), are important pests of crucifers and cole crops as well as lettuce, beans, tomato, tobacco, spinach, pea, turnip, potato, and sweet potato (Parker et al. 2001). These crops represent considerable economic value. For example, during 1996 and 1997 in Texas, growers planted >9,308 Ha (23,000 acres) of cole crops of an estimated value of >US$82 million (Liu et al. 1999). Broccoli and cauliflower are attacked primarily by *P. xylostella* and *T. ni* in both Mexico and the United States (Cartwright et al. 1987, Bujanos-M. 1999). Previous reports on thresholds revealed a ratio of 1.0–0.2 for *T. ni*:*P. xylostella* populations, but the threshold for cabbage and cauliflower is 0.25 cabbage looper equivalents during the heading to harvest stage (Bérubé 2002). An economic threshold of 0.75 *T. ni* equivalents per plant (in a survey of 50 or more plants) was estimated for most crops (Bérubé 2002). These data reflect the significant damage of *T. ni* to cole crops. In Mexico, three to five generations of *T. ni* can be present in the same crop each year (Bujanos-M. 1999). Control of pests by synthetic insecticides has become more difficult because of resistance development and associated costs, estimated at US$700/ha for synthetic pesticides and US$450/ha for integrated pest management (IPM) (Tierra Fértil 2005).

To date, the most successful bioinsecticide used in IPM programs is based on *Bacillus thuringiensis* (Bt), a soil bacterium that produces insecticidal proteins (Cry) and spores (Chattopadhyay et al. 2004). Bt production, formulation, and field application are affordable and feasible, making the active ingredient a competitive alternative to synthetic pesticides (Cerón 2001). Generally, the low impact on the environment and low toxicity to mammals make this bioinsecticide an attractive choice. Thus, Bt applications for pest control have increased worldwide.

In Mexico, the use of commercial Bt products began 12 yr ago, and the acceptance of Bt-based insecticides has improved greatly in the last decade. Bt products applied in the Bajio area include XenTari (*B. thuringiensis* subsp. *aizawai*), Lepinox (*B. thuringiensis* subsp. *kurstaki*), and Dipel (*B. thuringiensis* subsp. *kurstaki*) (Table 1). In Guanajuato, crops have been exposed to Bt commercial products (Xentari, Lepinox, and Dipel) for more than 5 years (Salazar-Solís 2002). In the Bajío area in central Mexico, 37 tons of formulated Bt were applied in fields in 2001 (Salazar-Solís 2002). With increasing selection pressure, Bt-resistant

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Table 1. Characteristics of commercial *B. thuringiensis* products

Characteristic	Dipel 2X	XenTari	Lepinox LPW
Company	Valent Bioscience, Corp.	Valent Bioscience, Corp.	Ecogen Inc.
Serotype	*B. thuringienses* subsp. *kurstaki*	*B. thuringienses* subsp. *aizawai*	*B. thuringienses* subsp. *kurstaki*
Genes *cry*	1Aa, 1Ab, 1Ac, 2Aa, 2Ab	1Aa, 1Ab, 1Ca, 1 d	1Aa, 1Ac, 2A, 1F
Type	Wettable powder	Sprayable granules	Sprayable granules
Use	0.5–1 kg/ha	1–1.5 kg/ha	0.5–1 kg/ha
Potency	32,000 IU/mg	16,000 IU/mg	15,000 IU/mg

P. xylostella have been collected frequently in this area (Díaz-Gómez et al. 2000), where farmers usually grow >30,000 ha of broccoli and cauliflower per year, yielding ≈360,000 ton/yr. Although Bt resistance levels in *P. xylostella* are not extremely high (Díaz-Gómez et al. 2000), it is important to collect baseline data for future comparisons and to evaluate the physiological differences in insect populations to devise resistance management strategies. There are no reports of any *T. ni* field populations with resistance to Bt products, but a rapid increase in resistance to Bt among greenhouse populations has been reported (Janmaat and Myers 2003, Kain et al. 2004).

Because of the concern of increased resistance to Bt Cry proteins in *T. ni* populations exposed to Bt formulations, the current study focused on monitoring the baseline susceptibilities to individual Cry proteins of field populations of *T. ni* in different geographical locations in Mexico. In addition, the potential Bt resistance development of field strains and their susceptibilities to Cry proteins have been evaluated after selection with a Bt commercial product, XenTari, and physical parameters of survivors have been evaluated. The present information can be used for future comparison studies of the effects of Bt sprays on *T. ni* populations.

Materials and Methods

Insects. *T. ni* strains included a laboratory strain (NL) obtained from Dr. Howard T. Dulmage (USDA–ARS, Weslaco, TX), reared since 1982 in our facilities and crossed with wild Mexican populations every 5–7 yr to avoid homocygamy-related problems; a laboratory strain from United States (US), kindly provided by Dr. Behle from the National Center for Agriculture Utilization Research, USDA–ARS, Peoria, IL; and three field strains, SAL from Salvatierra, ROM from Romita, and GTO from San Luis de la Paz, all located in Guanajuato State, east central Mexico. Larvae of various instars were collected from broccoli, cabbage, and cauliflower fields from November 2001 to May 2003, but only SAL and GTO were established as *T. ni* laboratory colonies. All insect populations were reared on artificial diet (Tamez-Guerra et al. 1998) at 25 ± 2°C, 55 ± 10% RH, and a photoperiod of 16:8 (L:D) h. When larvae reached the pupal stage, ≈30 females and 25 males were pooled to initiate the colony. The SAL strain had low survival and was crossed with NL in the fifth generation (<50% of the population remained).

After two to three generations, *T. ni* neonates from selected field strains were separated into two colonies, and one colony was exposed to XenTari (SAL-X and GTO-X). Neonates of the US laboratory strain also were separated, and one colony was selected with XenTari (US-X). A cross of XenTari-selected field-exposed insects (SAL-X) and laboratory insects (NL) was exposed to XenTari over several generations (SAL-X+NL-X). Survivors from each generation of Bt-selected field strains were tested for susceptibility to XenTari or individual Cry toxins in a six-dose droplet feeding bioassay and compared with the susceptibility of SAL-X+NL-X or US-X.

Bt Formulations and Cry Toxins. To test for Bt resistance, we evaluated two commercial Bt products: Lepinox LPW (Ecogen, Inc., imported and distributed in Mexico by Agroquímica de México S. de R.L. de C.V., Zapopan, Jal.) and XenTari (Valent Biosciences Corporation, Libertyville, IL., imported and distributed in Mexico by DuPont México, S.A. de C.V.) (Table 1). Bt Cry proteins were produced from recombinant *Escherichia coli* strains 4101 (Cry1Aa), 4301 (Cry1Ab), and 4201 (Cry1Ac), kindly provided by Dr. Donald Dean (*Bacillus* Genetic Stock Center, The Ohio State University, Columbus, OH). Recombinant strains were grown in 250-ml flasks with 50 ml of YT 2× broth (20 g/liter yeast extract, 32 g/liter tryptone, 10 g/liter NaCl, and 100 μg/ml ampicillin). Inoculated flasks were incubated for 20 h at 37°C and 150 rpm. The culture was used to inoculate 1-liter flasks with 200 ml of YT broth (2% volume of inocula) and incubated at 37°C and 150 rpm for 72 h. The cell culture was then centrifuged at 8,000 × *g*, and the pellet was homogenized in 50 ml of phosphate-buffered saline (PBS) and frozen at −20°C. Cells were resuspended in PBS, sonicated (Microson XL, Misonix Inc., Farmingdale, NY) at 20 W for 10 cycles of alternating 1 min on and 1 min off, and centrifuged at 8,000 × *g* for 20 min at 4°C. Protoxin was present in occluded bodies, which were released after sonication, and were solubilized in 50 mM $NaCO_3$ (Jaquet et al. 1987). Protein was determined by the Bradford technique (Bradford 1976). Solubilized protoxin was treated with trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone treated, EC 3.4.21.4, Sigma-Aldrich Química S.A. de C.V., Toluca, Mexico) at a toxin: trypsin ratio of 20:1. Proteins present in Bt products or recombinant strains were confirmed in 8% polyacrylamide gels stained with Coomassie blue (Sigma-Aldrich, St. Louis, MO) (data not shown).

Bioassays. *Droplet Feeding Bioassay.* To evaluate the susceptibility of field-collected *T. ni* to Bt, the insecticidal activity of XenTari and Lepinox on second or third generation neonates was tested, following the technique described by Behle et al. (2000). In this bioassay, the percentage of mortality was obtained from a large number of larvae (>200), and surviving larvae were monitored through various generations to the adult stage. Bt doses were mixed with a feeding solution of 2% (wt:vol) sucrose and 0.4% (wt:vol) of an edible blue dye (ASIS, Monterrey, Nuevo León, Mexico). The same solution without Bt was tested as a control. For each sample, small drops (≈1 μm each) of the blue solution per dose were pipetted with a short-neck Pasteur pipette at regular intervals in a 50- by 9-mm plastic petri dish. Fifty *T. ni* neonates were placed in the central part of each dish to feed from the colored drops of solution. Twenty-five fed neonates (selected by the blue color) were transferred to individual diet cups with 5 ml of modified artificial wheat germ diet (McGuire et al. 1997). Larvae for each treatment were incubated in the dark at 28°C for 7 d. Larvae that may have died because of handling were identified by a blue color and were nonfeeding (as judged by an intact diet surface), and they were excluded from data analysis. Each bioassay was repeated three times. After incubation, the numbers of live and dead larvae were counted to calculate the insecticidal activity of each Bt product.

Overlay Bioassay. This assay was used to test susceptibility to individual Cry toxins and solubilized and activated (trypsin-digested) toxins. Because this assay does not provide larvae for future generations, it was performed only for mortality determination. Protoxins and toxins were tested, because proteolytic activity is involved in dissolving and activating Bt Cry toxins and has been associated with a Bt resistance mechanism (Oppert et al. 1996, 1997). Cry1Aa, Cry1Ab, and Cry1Ac were selected as test toxins, because some strains of *T. ni* have increased susceptibility to these toxins (Estada and Ferré 1994). Cry1Aa is a component of both XenTari and Lepinox, whereas XenTari has Cry1Ab but not Cry1Ac, and Lepinox has Cry1Ac but not Cry1Ab. For this assay, serial doses of toxin in PBS, selected for each *T. ni* strain and toxin from preliminary susceptibility tests (data not shown), were made in 50 μl of PBS and applied to each well of 12-well tray (Corning Life Sciences, Acton, MA) containing 2 cm^2 of artificial diet with 0.1% bovine serum albumin in PBS as a control (Iracheta et al. 2000). Doses were air-dried for 2 h and infested with one *T. ni* neonate per well. The same procedure was used to compare with *T. ni* fed solubilized but nonpurified, nontrypsin-activated Cry protoxins (similar doses, diet, and bioassay technique). Mortality was recorded after 120 h, and the LC_{50} was calculated using probit analysis. Each bioassay was performed in triplicates on different days.

Selection for Bt Resistance Assays. Bt resistance assays were designed to detect resistance development among field-collected strains versus laboratory strains. To select larvae that survived Bt commercial products, *T. ni* neonates from each strain were exposed to a dose corresponding to the LC_{50} for each Bt product, by using a droplet bioassay as described above. Surviving larvae were allowed to complete their life cycle as adults and placed in a reproductive chamber for oviposition. Progeny larvae and subsequent generations were reexposed to Bt to select for resistance and labeled with "-X" followed by the number of the generation exposed to Bt (except for the first generation). Larvae from each generation were tested in a single-dose bioassay in triplicate, by using the LC_{50} dose given by probit analysis. Data from each replicate detected decreased susceptibility and the need to use higher XenTari doses in the next generation. If the mortality value from a single dose test was lower than 30%, the next generation larvae were exposed to a five-dose bioassay with higher international units (IU) of Bt to determine the LC_{50}. For comparison, the LC_{50} for Lepinox also was determined for Bt-selected larvae. Bt susceptibility of each *T. ni* strain was considered significantly different when 95% confidence intervals (CI_{95}) of the resistance ratios (RRs) for the LC_{50} data (using droplet or overlay bioassays) did not overlap. In addition, we compared the life cycle duration and fertility of Bt exposed strains as indicators of fitness.

Fitness Parameters. Physical characteristics of larvae from each generation of *T. ni* strains exposed to Bt also were used to evaluate fitness parameters, by using the same conditions as for colony rearing. Containers were 30-ml plastic cups, with 15 ml of artificial diet each, kept at the same temperature, humidity, and photoperiod as for rearing. Differences were evaluated by recording the 5-, 7-, 9-, and 12-d larval weight and larval and pupal length of 30 randomly selected insects. Larvae were incubated until the pupal stage, and percentage of pupation and pupal length were recorded. The size of larvae and pupae was measured as body length by using a digital Vernier. Pupae were monitored for adult eclosion in a 50-cm-high and 40-cm-diameter container, reared under the same conditions. Pupae were placed in an open paper bag, which was removed after adult eclosion to count the empty cocoons as an adult-hatching indicator. Egg fertility was evaluated by removing the cloth sheet where eggs were laid and counting the total of nonhatched as a larval eclosion failure indicator. Each test was performed in triplicate on different dates. Cycle delay was evaluated by recording the days needed to reach each instar.

Statistical Analysis. Dose response was evaluated by probit analysis (POLO-PC, LeOra Software 1987), based on Finney 1971), to provide LC_{50} values with CI_{95} and slopes of the dose–mortality curves. The resistance ratio was calculated by POLO-PC and was considered significant when the CI_{95} did not include the value 1 (Robertson and Preisler 1992). Statistical analysis of the fitness parameters was performed using analysis of variance (ANOVA) with repeated measures and one-sample Kolmogorov–Smirnov test ANOVA with SPSS computational program (SPSS Inc. 2004).

Table 2. Relative susceptibility of *T. ni* neonates to *B. thuringiensis* commercial products in a feeding droplet bioassay

Strain	Bt product	LC_{50} (CI_{95})[a]	Slope ± SE	Resistance ratio (CI_{95})[b]
NL[c]	XenTari	1.17 (0.88–1.46)	2.35 ± 0.14	1
	Lepinox	1.01 (0.25–2.1)	1.63 ± 0.18	1
US	XenTari	1.54 (0.86–2.04)	2.80 ± 0.14	1
ROM	XenTari	0.19 (0.11–0.32)	1.03 ± 0.15	0.16 (0.09–0.21)
SAL	XenTari	1.26 (0.74–1.93)	1.73 ± 0.16	NSD
	Lepinox	1.49 (0.79–2.37)	1.62 ± 0.18	NSD
GTO	XenTari	1.03 (0.90–1.27)	3.71 ± 0.14	NSD
	Lepinox	1.92 (1.04–2.73)	1.71 ± 0.18	NSD

[a] LC_{50} is the concentration (IU of active ingredient $\times 10^3$/ml) resulting in 50% mortality; CI_{95} = 95% CL.
[b] Resistance ratios of field strains were calculated using POLO-PC software based on the LC_{50} versus that of the laboratory strain, NL. NSD, not significantly different compared with NL, based on CI_{95} of RR value.
[c] Neonates of second generation of laboratory reared insects. GTO, San Luis de la Paz field-collected insects; NL, Mexican laboratory strain; ROM, Romita field-collected insects; SAL, Salvatierra field-collected insects; US, U.S. laboratory strain.

Results

Susceptibility to XenTari. *T. ni* larvae were collected from the Bajio region in Guanajuato during 2001 and 2002. *T. ni* larvae collected from Irapuato were highly parasitized and were not healthy for laboratory colony establishment. Additionally, eggs obtained from insects collected in Villagrán were not fertile. Therefore, only results from larvae from the Romita (ROM), Salvatierra (SAL), and San Luis de la Paz (GTO) areas are reported. In a droplet feeding bioassay, neonates from the second generation of ROM larvae were significantly more susceptible (6-fold susceptibility increase) to XenTari than a laboratory strain (NL), demonstrating more than a six-fold increase in susceptibility (Table 2). NL, US, SAL, and GTO strains had similar susceptibility to either XenTari or Lepinox.

Percentage of mortality and pupation of neonates from each generation of the SAL strain exposed to XenTari in single-dose bioassays are provided in Table 3. For comparison, the mortality of NL not exposed to XenTari was lower than 10% (data not shown). Pupation percentages of SAL neonates exposed to Bt always were lower compared with those of the NL strain (average of 44 versus 81%, respectively). Furthermore, although the dose of XenTari was slightly increased, fourth generation neonates had the lowest mortality rate (27%), and there was a significant difference in the LC_{50} values of SAL-X5 exposed to Bt formulations (Xentari: 3.2 IU $\times 10^3$/ml, CI_{95} = 2.2–5.2, slope ± SE = 1.35 ± 0.17; Lepinox: 3.2 IU $\times 10^3$/ml, CI_{95} = 2.6–4.1, slope ± SE = 2.18 ± 0.18) because of nonoverlapping CI_{95} with NL. Although the percentage of pupation of the fifth generation was similar to that of previous generations of Bt-surviving insects, the viability of pupae was very low (only nine adults emerged from pupae); thus, the surviving adults were crossed with NL adults (SAL-X-NL).

Table 3. Response of different generations of *T. ni* Salvatierra field-collected strain neonates to XenTari in a single-dose droplet feeding bioassay

Generation	No. of larvae	Dose (IU $\times 10^3$/ml)	% mortality	% pupation
1	200	1.0	55.0	40.0
2	160	1.0	50.0	50.5
3	119	1.0	58.9	48.7
4	116	1.5	26.7	40.2
5	226	2.5	58.8	41.9

The susceptibility of almost all the strains to XenTari, as tested in a droplet bioassay, was similar (data not shown). The LC_{50} of NL and US strains were 1.6 and 1.5 IU $\times 10^3$/ml, respectively. Only the strain SAL-X5 (after five generations of exposure to XenTari in the laboratory) was significantly different, with a LC_{50} of 3.1 IU $\times 10^3$/ml (2.1–5.2) and an RR value of 2.1 (1.2–4.2) with the unselected NL strain. Because differences were found in the enzymatic activity profile (unpublished data) and susceptibility to Bt Cry toxin among laboratory strains and the SAL strain exposed to Bt, SAL-X was crossed with NL and US strains. The resulting progeny from crosses of SAL-X+US and SAL-X+NL were not significantly different in XenTari susceptibility compared with the unselected laboratory strains (data not shown).

When the susceptibilities of NL and US strains to XenTari were compared in an overlay bioassay, we found no significant differences in toxin susceptibility among the strains (data not shown), similar to droplet bioassay results. However, the first generation US strain exposed to XenTari in the laboratory (US-X) was more susceptible to XenTari than the unselected NL (data not shown). No further differences in susceptibility were observed in US-X after three generations of exposure to XenTari.

Susceptibility to Cry1Aa, Cry1Ab, and Cry1Ac Protoxins and Toxins. The protoxin and toxin of Cry1Aa, Cry1Ab, and Cry1Ac were tested with strains of *T. ni.* Overall, the field strain GTO-X was less susceptible than laboratory strains to Cry1Aa, Cry1Ab, and Cry1Ac protoxins and toxins (Tables 4–6). All unselected laboratory strains had similar susceptibility to Cry protoxins and toxins, with the exception of a significantly decreased susceptibility of NL larvae to Cry1Aa protoxin when compared with US larvae, >91-fold (Table 4). In addition, the US strain was more susceptible to Cry1Aa protoxin compared with the NL strain.

XenTari selected *T. ni* strains US-X, SAL-X, and SAL-X+NL-X were significantly more susceptible to Cry1Aa toxin than NL insects (Table 4). However,

Table 4. Response of *T. ni* neonates to Cry1Aa toxin or protoxin in an overlay bioassay

Strain[a]	Cry1Aa protein	LC_{50} (CI_{95})[b]	Slope ± SE	Resistance ratio (CI_{95})[c]	
				NL	US
NL	Toxin	6.90 (2.6–32.3)	0.88 ± 0.17	1	NSD
	Protoxin	54.7 (13.5–4,920)	0.70 ± 0.26	1	91.1 (58.0–1,730)
US	Toxin	4.15 (1.2–43.1)	0.38 ± 0.25	NSD	1
	Protoxin	0.60 (0.30–0.89)	1.10 ± 0.14	<1	1
US-X	Toxin	0.07 (0.02–0.21)	0.80 ± 0.26	<1	<1
	Protoxin	2.40 (0.83–64.8)	0.60 ± 0.22	NSD	NSD
US-X3	Toxin	2.40 (0.88–5.58)	0.97 ± 0.25	NSD	NSD
	Protoxin	2.70 (0.94–41.5)	0.60 ± 0.14	NSD	4.50 (2.60–11.9)
GTO-X3	Toxin	5.30 (3.97–36.2)	0.86 ± 0.23	NSD	NSD
	Protoxin	9.30 (4.35–44.1)	0.60 ± 0.21	NSD	15.5 (8.30–34.9)
SAL-X	Toxin	0.50 (0.15–1.28)	0.70 ± 0.13	<1	NSD
SAL-X+NL-X	Toxin	2.00 (0.70–4.6)	0.96 ± 0.26	NSD	NSD
SAL-X+NL-X	Protoxin	1.62 (0.49–3.54)	1.10 ± 0.23	<1	NSD

[a] GTO, San Luis de la Paz field-collected strain; NL, Mexican laboratory strain; SAL, Salvatierra field-collected strain; SAL-X+NL, progeny from SAL-X and NL cross, with X indicating exposure to XenTari under laboratory conditions and the number indicating the selection generation; US, U.S. laboratory strain.

[b] LC_{50} is the concentration (nanograms × $10^2/cm^2$) resulting in 50% mortality; CI_{95} = 95% CIs. Average from four replications, testing 12 neonates per dose.

[c] Resistance ratios were calculated using POLO-PC software based on the LC_{50} of the laboratory strains NL or US. NSD, not significantly different, based on CI_{95} of RR value.

US-X3 and GTO-X3 were less susceptible to Cry1Aa protoxin than US insects after three generations of laboratory exposure to XenTari, with resistance ratios of 4.50 and 15.5, respectively.

There were no significant differences in Cry1Ab protoxin or toxin susceptibility in unselected laboratory strains (Table 5). SAL-X3 and GTO-X3 were significantly less susceptible to Cry1Ab toxin than US, with RR values of 6.20 and 40.4, respectively, whereas GTO-X3 was 49.8-fold more resistant to Cry1Ab protoxin compared with the NL strain. Therefore, GTO-X3 was more resistant to Cry1Ab protoxin than NL and more resistant to Cry1Ab toxin than US.

US-X3 was more susceptible to Cry1Ac toxin compared with the unselected US strain, but this strain was also less susceptible to Cry1Ac protoxin compared with the NL strain (RR = 21; Table 6). GTO-X3 was less susceptible to Cry1Ac protoxin compared with NL and US laboratory strains (RR = 87 and 22, respectively; Table 6). SAL-X+NL-X was significantly less susceptible to Cry1Ac protoxin than NL, with RR = 7 (Table 6).

Fitness Parameters. Significant differences in weights and lengths of larvae and pupae and pupation percentages were observed in comparisons of *T. ni* strains. Weight averages of 30 randomly selected larvae from each of the *T. ni* strains demonstrated significant differences ($P < 0.0001$) (Fig. 1). The repeated measures ANOVA revealed a significant effect of days [$F(3, 396) = 2420.7$; $P < 0.0001$], treatments [$F(5, 396) = 41.3$; $P < 0.0001$], and days versus treatments [$F(15, 396) = 16.05$; $P < 0.0001$] in the weights data (Fig. 1). In general, differences in larval weight were observed at each instar, but after 12 d incubation,

Table 5. Response of *T. ni* neonates to Cry1Ab toxin or protoxins in an overlay bioassay

Strain[a]	Cry1Ab protein	LC_{50} (CI_{95})[b]	Slope ± SE	Resistance ratio (CI_{95})[c]	
				NL	US
NL	Toxin	1.60 (0.60–22.1)	0.90 ± 0.32	1	NSD
	Protoxin	0.70 (0.29–8.89)	1.00 ± 0.13	1	NSD
US	Toxin	0.50 (0.24–0.97)	1.00 ± 0.11	NSD	1
	Protoxin	2.00 (0.80–22.1)	0.90 ± 0.37	NSD	1
US-X3	Toxin	1.00 (0.41–20.3)	1.10 ± 0.17	NSD	NSD
	Protoxin	2.40 (1.41–7.99)	1.10 ± 0.12	NSD	NSD
SAL-X3	Toxin	3.10 (1.46–6.60)	1.30 ± 0.16	NSD	6.20 (4.10–17.8)
	Protoxin	6.30 (3.80–9.57)	1.00 ± 0.22	NSD	NSD
GTO-X3	Toxin	20.2 (8.40–43.8)	0.70 ± 0.25	NSD	40.4 (16.0–83.8)
	Protoxin	34.9 (18.0–70.1)	0.70 ± 0.15	49.8 (31.0–92.3)	NSD
SAL-X+NL-X	Toxin	0.50 (0.27–1.00)	0.80 ± 0.17	NSD	NSD
	Protoxin	2.20 (0.70–8.78)	0.80 ± 0.20	NSD	NSD

[a] GTO, San Luis de la Paz field-collected strain; NL, Mexican laboratory strain; SAL, Salvatierra field-collected strain; SAL-X+NL, progeny from SAL-X and NL cross, with X indicating exposure to XenTari under laboratory conditions and the number indicating the selection generation; US, U.S. laboratory strain.

[b] LC_{50} is the concentration (nanograms × $10^2/cm^2$) resulting in 50% mortality; CI_{95} = 95% confidence intervals. Average from four replications, testing 12 neonates per dose.

[c] Resistance ratios were calculated using POLO-PC software based on LC_{50} versus the laboratory strains NL or US. NSD, not significantly different based on CI_{95} of RR value.

Table 6. Response of *T. ni* neonates exposed to Cry1Ac toxin or protoxin in an overlay bioassay

Strain[a]	Cry1Ac protein	LC_{50} (CI_{95})[b]	Slope ± SE	Resistance ratio (CI_{95})[c]	
				NL	US
NL	Toxin	0.40 (0.13–4.76)	0.60 ± 0.20	1	NSD
	Protoxin	0.10 (0.048–0.18)	0.90 ± 0.18	1	NSD
US	Toxin	0.70 (0.24–3.54)	0.90 ± 0.19	NSD	1
	Protoxin	0.40 (0.14–1.01)	1.00 ± 0.12	NSD	1
US-X3	Toxin	0.07 (0.02–0.21)	0.80 ± 0.26	NSD	<1
	Protoxin	2.10 (0.62–27.7)	0.80 ± 0.20	21.0 (8.20–54.9)	NSD
GTO-X3	Toxin	0.80 (0.3–2.4)	0.80 ± 0.22)	NSD	NSD
	Protoxin	8.70 (5.18–50.1)	0.80 ± 0.17	87.0 (46.0–181.3)	21.8 (9.50–67.2)
SAL-X+NL-X	Toxin	2.10 (0.62–27.7)	0.70 ± 0.27	NSD	NSD
	Protoxin	0.70 (0.29–1.88)	0.90 ± 0.21	7.00 (3.20–13.7)	NSD
SAL-X+NL-X4	Toxin	1.60 (0.48–4.17)	0.80 ± 0.21	NSD	NSD
	Protoxin	0.20 (0.13–0.43)	1.00 ± 0.25	NSD	NSD

GTO, San Luis de la Paz field-collected strain; NL, Mexican laboratory strain; SAL, Salvatierra field-collected strain; SAL-X+NL, progeny from SAL-X and NL cross, with X indicating exposure to XenTari under laboratory conditions and the number indicating the selection generation; US, U.S. laboratory strain.

[b] LC_{50} is the concentration (nanograms per $10^2/cm^2$) resulting in 50% mortality; CI_{95} = 95% confidence intervals. Average from four replications, testing 12 neonates per dose.

[c] Resistance ratios were calculated using POLO-PC software based on LC_{50} versus the laboratory strains NL or US. NSD, not significantly different based on CI_{95} of RR value.

GTO and GTO-X weighed significantly less than all other strains. Using Tukey's multiple comparisons, the GTO-X strain was significantly different ($P < 0.0001$) than any other treatment; SAL-X strain was significantly different ($P < 0.005$) than GTO-X, US, and GTO strains; NL was significantly different ($P < 0.0001$) than GTO-X and US strains; US-X was significantly different ($P < 0.0001$) than GTO-X and US strains; US was significantly different ($P < 0.001$) than any other strain; and GTO was significantly different ($P < 0.005$) than GTO-X, SAL-X, and US strains.

Similarly, larvae in the GTO-X strain were significantly smaller than those in all other strains (data not shown). The repeated measures ANOVA revealed significant effect of days [$F(3, 383) = 15{,}606.1$; $P < 0.0001$], treatments [$F(5, 383) = 160.85$; $P < 0.0001$], and days versus treatments [$F(15, 383) = 41.3$; $P < 0.0001$] in larval and pupal lengths (data not shown). Using Tukey's multiple comparisons, the larval length of GTO-X was significantly different ($P < 0.0001$) than any other treatment; NL, SAL-X+NL, US-X, US, and GTO were significantly different ($P < 0.001$) than any other treatment. Additionally, the one-sample Kolmogorov–Smirnov test ANOVA indicated a significant effect of treatments [$F(5, 95) = 43.5$, $P < 0.0001$] in pupal lengths. The length of pupae from the laboratory strains (NL and US) and the XenTari-exposed US strain (US-X) was significantly more than those from

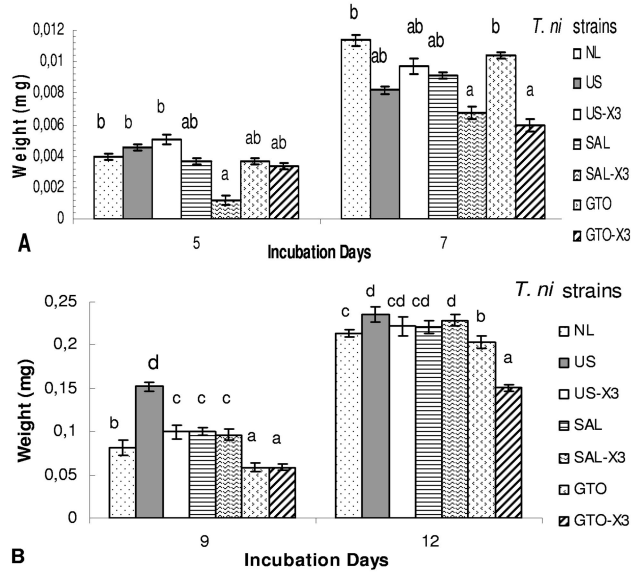

Fig. 1. Weight of *T. ni* larvae. Average weights (± SE) of 30 randomly collected larvae were measured at 5- and 7- (A) and 9- and 12-d (B) incubation.

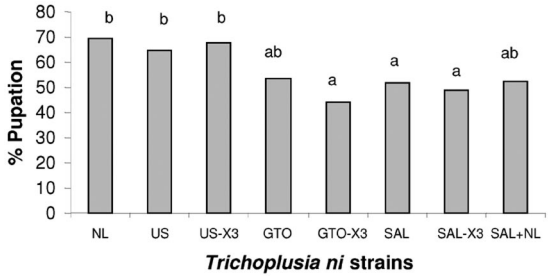

Fig. 2. Percentage of pupation of *T. ni* strains. Data represent percentages of 30 randomly selected pupae. Columns with different letters are significantly different based on multiple comparisons dependent variable, Tukey's honestly significant difference ($\alpha = 0.05$).

SAL-X+NL-X and nonexposed or XenTari exposed field strains GTO and GTO-X, with $F(5, 95) = 43.489$, $P < 0.0001$. Pupal weight of *T. ni* strains was not significantly different ($P > 0.05$, data not shown). The pupation percentage of GTO-X3 was significantly lower than NL ($P < 0.02$) (Fig. 2). There were no significant differences in egg fertility (larval eclosion) and time needed to complete each cycle among the tested strains ($P > 0.05$, data not shown).

Discussion

Although field resistance to bioinsecticides is less common, at least 12 insect species have developed resistance to Bt under laboratory conditions (Ferré and van Rie 2002). In Mexico, resistance to specific Bt protoxins and toxins has been observed among *P. xylostella* field strains (Díaz-Gómez et al. 2000). In the current study, we reported differences in susceptibility to Bt among laboratory and field-collected *T. ni* strains. Field-collected *T. ni* strains were from the geographical area where Bt-resistant *P. xylostella* strains were obtained. In Guanajuato, susceptible crops have been exposed to Bt commercial products (Dipel, Xentari, or Lepinox) for more than five years (Salazar-Solís 2002). We found significant differences in Bt susceptibility among *T. ni* larvae collected from three Guanajuato towns (Romita larvae were less susceptible to Bt than those from Salvatierra and San Luis de la Paz). Similarly, differences in susceptibility to Bt field-collected *P. xylostella* larvae among monitored areas in Bajio Guanajuatense also were reported (Díaz-Gómez et al. 2000).

In addition to Bt use, the Mexican IPM programs for *T. ni* control recommend the applications of azaradictine, nucleopolyhedroviruses, or spinosad, and release of parasitoids [*Trichogramma* spp. and *Diadegma insulare* (Cresson)] (Bujanos-M. 2000). Naturally, nucleopolyhedroviruses can kill 20–45% of the population, and *D. insulare* can parasitize 10–30% of the pest population (Bujanos-M. 2000). In general, when we tested either XenTari or Lepinox, we observed similar susceptibility values among strains. Although differences in susceptibility to XenTari were not detected among selected *T. ni* strains, differences in susceptibility to individual Bt Cry toxins were observed. The selection formulation, XenTari, lacks of Cry1Ab. However, there were higher levels of resistance to Cry1Ab toxin (40.4-fold increase) and protoxin (49.8-fold increase) in GTO-X3 compared with US and NL, respectively, even though Cry1Ab was not present in the selection formulation. These differences can be explained in part by the increased susceptibility of the US strain to the toxin form of Cry1Ab, whereas the NL strain was more susceptible to the protoxin.

A previous report on the susceptibility of *T. ni* to Cry toxins revealed that a strain originally from Quebec, Canada, had a similar susceptibility to Cry1Aa toxin compared with the NL and US strains (Estada and Ferré 1994). Decreased susceptibility of the NL strain to Cry1Aa was previously reported by Iracheta et al. (2000). However, Mexican *T. ni* strains were more susceptible to Cry1Ac than the Canadian strain, similar to a previous report by Moar et al. (1990). Estada and Ferré (1994) also reported a 31-fold increase of resistance to Cry1Ab after seven generations of selection in a *T. ni* strain. When SAL larvae were exposed to XenTari, we observed a significant decrease in Bt susceptibility after five generations, whereas the GTO strain demonstrated decreased susceptibility after three generations of exposure. SAL-X resistance reverted after adults were crossed with adults from a laboratory strain (NL), similar to reports in *P. xylostella* (Tabashnik et al. 1994a,b). The reversion may have resulted from the loss of recessive genes associated with Bt resistance in the low numbers of SAL-X survivors.

Overall, the GTO-X3 strain demonstrated the highest resistance to Bt protoxins and toxins tested in this study. Similar to Mexican *P. xylostella* resistant to Bt (Díaz-Gómez et al. 2000), neonates of a *T. ni* field strain that were less susceptible to a Bt formulation (SAL-X) also were significantly less susceptible to Cry1Ab toxin compared with a U.S. laboratory strain. However, previous reports with *P. xylostella* indicated that variations in susceptibility to Bt toxins exist among Bt nonexposed strains (González-Cabrera et al. 2001). We observed that all strains were similar in susceptibility to Cry1Ac toxin, but resistance to Cry1Ac protoxin was shown for several of the selected strains. The presence of a high concentration of a specific Cry protein in a particular formulation could result in a lower LC_{50} value among susceptible insects, but if the level of the protein is lower, the LC_{50} value may suggest resistance. This problem has been reported previously by Wilcox et al. (1986) with HD-1, a Bt strain commonly used for Bt production in the early development of Bt products (Cerón 2001).

There is a previous report showing that *T. ni* shares a Cry1Ab and Cry1Ac binding site, but resistance development to Cry1Ab did not induce Cry1Ac resistance (Estada and Ferré 1994). Mexican *T. ni* populations had variable susceptibilities to Cry1A toxins. Although Cry1Aa and Cry1Ab toxin resistance has been reported in the same locus in *T. ni*, the LC_{50} for Cry1Aa toxin with SAL-X+NL-X was about 14-fold

higher than for Cry1Ab toxin. These data suggest that there are differences in either toxin binding sites or affinities for Cry1A toxins in Mexican *T. ni* populations.

In a study conducted by Kain et al. (2004), a *T. ni* population collected from commercial greenhouses had a 24-fold resistance level to Dipel (*B. thuringiensis* subsp. *kurstaki*). This population was selected with Cry1Ac, the major Bt Cry toxin in Dipel, and the resulting strain had a resistance ratio of ≈1000-fold, with a monogenetic inheritance of resistance (Janmaat et al. 2004, Kain et al. 2004). In many of the Mexican XenTari-selected strains, the LC_{50} was higher for the protoxin than the toxin, suggesting that selection may favor individuals with enzymes that activate or solubilize Bt protoxins less efficiently (Oppert 1999). Insects have multiple protease-encoding genes (Reeck et al. 1999), and insects can adapt to toxin via proteinase-mediated mechanisms (Oppert et al. 1997). We have found significant differences in the overall enzymatic activity and protease profiles among the *T. ni* strains reported in this work (unpublished data). In addition, studies on antimicrobial peptide expression in the larval gut of *T. ni* strains by using reverse transcription-polymerase chain reaction demonstrated that, in general, the GTO strain had an increased expression of antimicrobial peptides after exposure to 500 IU/ml of XenTari (unpublished data). Whether this expression is related to a loss in susceptibility to XenTari remains to be elucidated.

Reduction of *T. ni* larval growth after Bt Cry toxin exposure has been reported (Kain et al. 2004). Thus, we also recorded fitness parameters (larval length and weight and pupal percentages) in *T. ni* larvae exposed and not exposed to XenTari. In spite of being less susceptible to Bt products, differences were observed in SAL-X4, with a significant reduction in pupation and fertility percentages compared with the NL strain. We observed that *T. ni* field strains exposed to XenTari were less susceptible to Bt (based on LC_{50} confidence intervals), but they had significantly higher natural mortality and a lower pupation rate compared to unexposed controls, probably because of a fitness cost (Oppert et al. 2000). Similar results were observed among *Leptinotarsa decimlineata* (Say) strains previously exposed or not to Bt (Feldman and Stone 1997). We also observed that the larval weight and length were significantly higher in US, US-X, and GTO-X strains exposed to XenTari compared with those nonexposed larvae. In general, all *T. ni* XenTari-exposed strains had significantly smaller pupae than nonexposed strains. Laboratory experiments have demonstrated that *Heliothis virescens* (F.) larvae fed Bt-corn (*Zea mays* L.) were significantly smaller than those fed on nontransgenic corn (Romeis et al. 2004). We observed that laboratory strains (NL and US) were significantly heavier, and larvae and pupae were larger than the field strain GTO-X. Significant differences in *Pectinophora gossypiella* (Saunders) larval weight, exposed or not to Cry1Ac, also have been reported (Tabashnik et al. 2002).

The data reported in the present study provide a baseline to evaluate Bt effects on Mexican populations of *T. ni* in areas where Bt spray pressure may increase. Our results suggest that, although some differences were observed, resistance of *T. ni* to Bt has not yet become a problem. However, differences were observed in the relative susceptibility to individual Cry toxins, depending on the form. Therefore, variations in digestive proteinases in Mexican *T. ni* populations may provide an advantage, particularly when selection pressure is low. As part of the continuous monitoring of insect species in fields exposed to Bt, it is necessary to evaluate the potential resistance and physiological parameters of survivors from commercial products and transgenic plants in Mexico.

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