Fitness cost of resistance to *Bacillus thuringiensis* in velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera, Noctuidae)

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ABSTRACT. Fitness cost of resistance to *Bacillus thuringiensis* in velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera, Noctuidae). Selection pressure to obtain resistant genotypes can result in fitness cost. In this study, we report the effects of the selection pressure of a commercial formulation of *Bacillus thuringiensis* on biological aspects of a Dipel-resistant strain of velvetbean caterpillar, *Anticarsia gemmatalis* Hübner. Comparisons of Dipel-resistant and susceptible individuals revealed significant differences in pupal weight and larval development time. Both strains (Dipel-resistant and susceptible) were susceptible to Cry1Ac toxin expressed in foliar cotton tissues. Resistant and susceptible strains showed low survival rates of 22.5% and 51.2%, respectively, when fed with Greene diet containing Bt-cotton. Larvae bioassayed after three laboratory generations presented lower survival and less instar numbers than individuals maintained in the laboratory for more than 144 generations. Pupal weight was 9.4% lower and larval development time was 1.9 days longer in the resistant population than in the susceptible strain. Other parameters, such as duration of pupal stage, adult longevity, number of eggs per female, oviposition period, and egg fertility, remained unaffected.

KEYWORDS. *Anticarsia gemmatalis*; toxin; Cry1Ac; Dipel-resistant; strain.

RESUMO. Custo adaptativo da resistência a *Bacillus thuringiensis* em lagarta-da-soja, *Anticarsia gemmatalis* Hübner (Lepidoptera, Noctuidae). A pressão de seleção para obter genótipos resistentes pode resultar em custo adaptativo. Neste estudo, relatamos os efeitos da pressão de seleção de uma formulação comercial de *Bacillus thuringiensis* sobre aspectos biológicos de uma raça Dipel-resistente da lagarta-da-soja, *Anticarsia gemmatalis* Hübner. Comparações de indivíduos resistentes e suscetíveis a Dipel revelaram diferenças significativas em peso de pupas e tempo de desenvolvimento larval. Ambas as raças (suscetível e Dipel-resistente) foram suscetíveis à toxina Cry1Ac expressa em tecidos de folhas do algodoeiro. Linhagens resistentes e suscetíveis apresentaram baixas taxas de sobrevivência de 22,5% e 51,2%, respectivamente, quando alimentadas com dieta Greene contendo algodão Bt. As larvas utilizadas no bioensaio após três gerações de laboratório apresentaram menores taxas de sobrevivência e menores números de ínstares do que os indivíduos mantidos no laboratório por mais de 144 gerações. O peso das pupas foi 9,4% menor e o tempo de desenvolvimento foi 1,9 dias mais longo na população resistente do que na raça suscetível. Outros parâmetros, como duração do estágio pupal, longevidade de adultos, número de ovos por fêmea, período de oviposição e fertilidade de ovos, não foram afetados.

PALAVRAS-CHAVE. *Anticarsia gemmatalis*; Cry1Ac; resistente a Dipel; raça; toxina.

Researches in some Brazilian states indicate that the insecticide sprayed area of soybean crops has increased in the last ten years due to the more frequent occurrence of soybean loopers, high stink bug populations, and tetranychid mite resurgences (Sosa-Gómez & Silva 2010; Roggia *et al.* 2008). The imminent introduction of transgenic soybean with the Cry1Ac synthetic *Bacillus thuringiensis* gene in the Brazilian market in 2012 is an interesting alternative to conventional insecticide applications. Potential benefits of Bt-soybean are reduction in insecticide application, increase in natural enemies, and reduction in environmental pollution. However, the availability of Bt toxins in large areas brings a major concern that is the selection of insect genotypes resistant to these toxins (Gould 1998).

The main targets of Bt-soybean technology are the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera, Noctuidae), and the soybean looper, *Pseudoplusia includens* (Walker), species that are widely distributed in Brazilian soybean fields. In the Northeastern region and in Central Brazil, soybean and cotton share the same production system.

Considering that selection of resistant genotypes of *A. gemmatalis* to Bt in laboratory is fast and that *B. thuringiensis* based products (Bactospeine, Dipel, and Thuricide) have been commercialized in Brazil since the early 1980s, knowledge related to the biology of resistant strains to *B. thuringiensis* is important. The fitness cost is especially useful for delaying pest resistance (Gassmann *et al.* 2009). Also, the detection of fitness cost involving Bt-resistant strains is crucial to understand possible interactions between Bt (Dipel®) use to control pests in refuge areas and the toxin available through engineered plants. Dipel WP® is a commercial formulation of HD-1 strain of *B. thuringiensis* subsp. *kurstaki* that contains Cry1A(a), Cry1A(b), and Cry1A(c) endotoxins (Masson *et al.* 1990). Therefore, the consequences of a resistant colony selection with this bacterial strain, which possesses a toxin complex, are unpredictable.

During the course of the bioassays carried out with Bt-cotton tissues in our laboratory, we observed differences in susceptibility between colonies with different numbers of generations. Similar responses to Cry toxins have been obser-

ved in *Heliothis virescens* (Fabricius) (Lepidoptera, Noctuidae) laboratory colonies (Albernaz 2011).

This work aims to clarify if populations of *A. gemmatalis* resistant to *B. thuringiensis* express fitness cost in parameters such as life span, number of instars, fertility, and fecundity, to verify their response to Cry1Ac toxin present in cotton tissues from commercial transgenic plants, and to compare the susceptibility of *A. gemmatalis* colonies with different numbers of laboratory generations.

MATERIAL AND METHODS

Insect colonies. An *A. gemmatalis* susceptible strain was initiated in 1998 from field-collected insects in the municipality of Sertanópolis, in the state of Paraná. Approximately 800 larvae were collected in wooden screen cages containing soybean leaves and transported to the laboratory. Adults obtained from these larvae were maintained in oviposition cages. Insects were reared on artificial diet, following the procedures of Hoffmann-Campo *et al.* (1985), at 26 ± 2°C, 70 ± 10% RH, under 14:10 (L:D) photoperiod.

After 67 generations the colony was splitted into two. One of them was challenged with Dipel WP® (16,000 IU.mg^{-1}, 32 g.kg^{-1}, and inert 968 g.kg^{-1}, Abbott Laboratories, North Chicago, IL, US) for 144 generations. A selection pressure was applied to obtain approximately 80% mortality based on the probit analysis of the previous generation and select a resistant strain, named Sertanópolis resistant strain (SRS). No selection pressure was applied to the other population, named Sertanópolis susceptible strain (SSS), which was kept in rearing for 194 generations, under the same conditions used for other colonies.

Individuals from the susceptible and resistant colonies were bioassayed to verify the resistance ratio status. In the bioassays we also used a velvetbean caterpillar field population collected in Cruz Alta, in the state of Rio Grande do Sul, which was reared for three generations, under the same conditions used for the other colonies, and was named Cruz Alta susceptible strain (CASS).

Bioassays. Dipel WP® was incorporated into the artificial Greene diet (Greene *et al.* 1976), after being cooled to 50°C, without formalin and antibiotics. Dilutions were prepared to obtain six concentrations for each population. The susceptible population was treated with 5.8, 9.9, 17.0, 28.8, 49.3, and 83.5 μg of active ingredient (a.i.) per mL of diet, whereas the Dipel-resistant population was treated with 704, 1184, 2016, 3424, 5824, and 9920 μg of a.i. per mL of diet. Control larvae were fed on the diet plus sterile distilled water in the same volumes used in the treatments containing Dipel WR®. Aliquots of 10 mL of treated and untreated diets were dispensed into 50 mL plastic cups. After reaching room temperature, groups of three third-instar larvae were placed in each cup, totaling 60–64 larvae per concentration, and fed on the diet for 24 h. After this period, the larvae were transferred to cups with no Bt-diet. Regression lines between log-dose and probit were estimated with POLO-PC statistical software (LeOra Software 1987).

Lethal concentration (LC$_{50}$) values and their respective fiducial limits (FL$_{95}$) were used to compare differences between susceptible and resistant populations.

Fitness components. The following lifetime parameters of Dipel-resistant and susceptible populations were evaluated: larval stage, larval viability, number of instars, pupal weight, sex ratio, adult lifespan, and egg number per female per day. All studies were performed at 26 ± 1.5°C and 70 ± 10% RH, under a 14:12 (L:D) photoperiod. Neonate larvae were individualized and confined in 50 mL plastic cups containing artificial diet; pupal weight was determined 48 h after pupation; the number of abnormal pupae was observed; and the weight of 700 neonates from each strain was determined in 14 groups of 50 larvae each.

The heads of 145 neonate larvae from each population were measured using a Motic Images Plus 2.0 ML software and a stereoscopic microscope equipped with a Moticam 2300 3.0M Pixel digital camera (Motic China Group Co. Ltda.). The measurements of the distance between the frontal setae were performed from the second to the fifth instars for a more accurate determination (Pérez *et al.* 2005).

One day before adult emergence, 20 couples were placed in PVC tube cages (12 cm diameter x 22 cm height), fed on a solution containing 0.6% honey bee, 6% sugar, 0.06% sorbic acid, 0.06% methylparaben, and 40% pilsen beer, and kept in environmental chambers at 25 ± 1°C, with a 14-h photophase. Sheets of paper were placed on the inner wall of the PVC cages to collect the eggs. The number of eggs per female was recorded daily to estimate fecundity and egg hatch. The mean viability and mean incubation time with the respective standard error of mean were calculated. Comparisons between Dipel-resistant and susceptible strains were performed using Student's t-test or nonparametric analysis if assumptions of normality and equal variance f or parametric analysis were not fulfilled (Systat Software Inc. 2008).

Bioassay with Bt-cotton and non-Bt-cotton tissues. Since Cry1Ac toxin was unavailable, non-transgenic cotton and cotton-Bt leaves from 30-day old cotton plants [Delta Opal and Nu Opal (Bollgard I)] were lyophilized, grounded, and blended with the artificial Greene diet in the proportion of 2 g of dry weight in 40 mL of the diet. Dipel-resistant *A. gemmatalis* neonates (SRS) and two susceptible populations (SSS and CASS) were fed on Greene diet with and without transgenic and non-transgenic cotton tissues. The bioassay was set up after 144 generations of the resistant strain. Survival after 7 days, larval weight, and instar changes were the response variables statistically analyzed. Each variable was analyzed by ANOVA and means compared by Tukey's multiple comparison test at 5% probability. Statistical analyses were performed using Sigmaplot for Windows version 11.0 (Systat Software Inc. 2008).

RESULTS AND DISCUSSION

Biological parameters. The results of the probit analysis and the associated parameters for the resistant popula-

tions were: LC_{50} = 6,642.1 μg of a.i. per mL of diet; n = 248 larvae; FL_{95} = 4,312.4–15,589.9; b = 0.97; SE = 0.33; χ^2 = 0.79. The same parameters for the susceptible populations were: LC_{50} = 77.2 μg of a.i. per mL of diet; n = 309 larvae, FL_{95} = 66.2–89.2; b = 3.4; SE = 0.4; χ^2 = 2.5 (Fig. 1). Dipel-resistant population presented resistance ratio 86 times higher than the population that did not receive selection pressure. Average weight of susceptible larvae (n = 700) was 0.078 ± 0.006 mg and of Dipel-resistant larvae was 0.083 ± 0.005 mg, results that did not present significant statistical difference using Student's t-test (t = 0.58; P = 0.569).
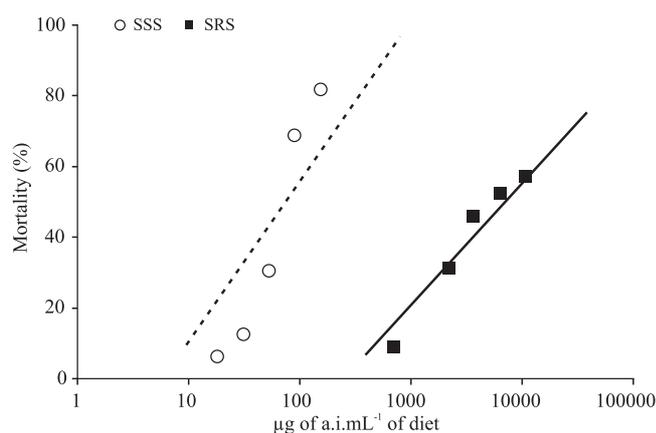

Fig. 1. Probit analysis of mortality of susceptible (SSS) and Bt-resistant (SRS) 3rd instar larvae of velvetbean caterpillar exposed to Cry toxins from a commercial formulation of *Bacillus thuringiensis*, HD-1 strain.

The number of instars measured by distance between the frontal setae, duration of pupal stage, adult longevity, survival, number of eggs per female, and egg fertility recorded for the resistant population did not statistically differ from the results found for the susceptible population (Table I).

Dipel-resistant *A. gemmatalis* strain exhibited significant difference regarding the parameter days at the larval stage, which was 1.9 days longer than in the susceptible strain. Most of this variation was due to differences between the 3rd and 5th instars of the susceptible and resistant strains. However, the pupal weight was significantly lower in the Dipel-resistant strain compared to the susceptible one (Table I). No abnormal pupae were recorded in these two populations.

Bioassays using a diet amended with cotton tissues. Survival of the susceptible population from Cruz Alta was affected by the cotton tissue in the diet, and it was also significantly affected by the Cry1Ac toxin present in the cotton tissues of the transgenic plant (Table II). The susceptible population from Sertanópolis and Dipel-resistant populations with longer time in rearing conditions were not significantly affected by the presence of cotton tissues (Delta Opal) in the diet. In all populations, including the Bt-resistant population, larval weight was affected by the presence of conventional cotton in the diet and severely affected by the presence of Cry1Ac toxin (Table II). The mean number of instars

reached by the larvae was affected by Delta Opal tissues and strongly affected by the presence of Bollgard tissues (Table II). Dipel-resistant *A. gemmatalis* strain was susceptible to the Cry1Ac toxin present in the cotton leaves.

Table I. Means ± SEM of biological parameters of Dipel-resistant strain (Sertanópolis resistant strain – SRS) and susceptible (Sertanópolis susceptible strain – SSS) of *Anticarsia gemmatalis* when the resistant strain presented a resistance ratio of 86 x.

Parameter	SSS[1]	SRS[1]
Larval stage (days)	13.88 ± 0.06 a	15.76 ± 0.07b
1st instar (days)	2.0 ± 0.0 a	2.1 ± 0.03 a
2nd instar (days)	1.96 ± 0.03a	1.94 ± 0.03a
3rd instar (days)	1.38 ± 0.07a	2.02 ± 0.05b
4th instar (days)	2.26 ± 0.41a	2.04 ± 0.06a
5th instar (days)	2.84 ± 0.07a	3.10 ± 0.17b
Pupal stage (days)	8.50 ± 0.05 a	8.45 ± 0.76a
Pupal weight (mg)	266 ± 1.97 a	241 ± 1.89b
Survival at pupal stage (%)	92.00 ± 2.26 a	87.33 ± 2.67a
Adult longevity (days)	13.70 ± 0.80 a	14.1 ± 0.79a
Eggs/female (no.)	755.06 ± 89.69 a	673.39 ± 70.88a
Oviposition phase (days)	11.44 ± 1.04 a	10.56 ± 0.90a
Egg hatch (%)	84.53 ± 2.93 a	78.88 ± 5.33a

[1]Data followed by the same letter within a line are not significantly different using the Mann-Whitney Rank Sum Test (α = 0.05).

Table II. Means ± SEM of larval weight, survival rate, and instars reached by three populations of *Anticarsia gemmatalis* larvae (Sertanópolis susceptible strain – SSS; Cruz Alta susceptible strain – CASS; Sertanópolis resistant strain – SRS) fed on Greene diet alone and Greene diet containing non-Bt (Delta Opal) and Bt-cotton (Bollgard) for 7 days. Number of laboratory generations between parentheses.

Parameter	SSS (194)	CASS (3)	SRS (144)
Greene diet			
Larval weight (mg)	228 ± 6.1a[1]A[2]	254 ± 3.0aB	170 ± 7.6aA
Survival rate (%)	100aA	93.7 ± 6.2aA	97.9 ± 2.1aA
Instars (mean number)	4.9 ± 0.02aA	3.6 ± 0.19aB	4.8 ± 0.08aA
Greene diet plus Delta Opal			
Larval weight (mg)	16.4 ± 1.5bA	1.5 ± 0.2bA	6.3 ± 0,5bA
Survival rate (%)	85.8 ± 2.5aA	23.7 ± 9.4bB	90.0 ± 10.0aA
Instars (mean number)	3.7 ± 0.08bA	2.1 ± 0.09bB	2.9 ± 0.07bA
Greene diet plus Bollgard			
Larval weight (mg)	1.1 ± 0.1cA	0.7 ± 0.1[3]	1.9 ± 0.2cA
Survival rate (%)	22.5 ± 14.3bA	4.6 ± 2.7bA	51.2 ± 11.6bA
Instars (mean number)	1.8 ± 0.05cA	1.2 ± 0.06cA	1.8 ± 0.06cA

[1]Data followed by the same letter in the columns (lower-case letters) or in the lines are not significantly different using the Tukey's test (P < 0.05).
[2]Data followed by the same letter in the lines (capital letters) are not significantly different using the Tukey's test (P < 0.05).
[3]The number of insects was not enough to carry out statistical analyses.

Although the observed parameters seem to be insignificantly affected or unaffected by the selection pressure with Dipel WP®, the biological meaning of the life history parameters under field conditions should be determined. However, according to Gould *et al.* (2006), the fitness cost measured

in laboratory can be underestimated. In this study, selection pressure performed with Dipel did not increase resistant rates to Cry1Ac, despite the fact that this endotoxin is present in the crystal of HD-1 strain used in the commercial formulation (Masson *et al.* 1990).

The bioassays performed with the resistant and susceptible populations of *A. gemmatalis* fed on diets containing Bt-cotton tissues with Cry1Ac toxin showed that the Dipel-resistant population was susceptible to the toxin as well as both susceptible populations (Table II). In a hypothetical case of frequent use of commercial Bt formulations to control velvetbean caterpillar in soybeans, it does not seem probable that different selection mechanisms involved when using selection pressure with several toxins, such as *B. thuringiensis* var. *kurstaki*, could contribute to select genotypes of this caterpillar resistant to Bt-soybean plants with Cry1Ac gene.

Considering these results, occasional use of Dipel in refuge areas in resistance management programs could not be discarded. Interestingly, *A. gemmatalis* populations maintained for more than 144 generations in the laboratory had more instars and higher tolerance to Dipel than the more recently introduced population in artificial rear and kept for three generations in the laboratory. Velvetbean caterpillar population from Cruz Alta was in the third laboratory generation, possibly less adapted to laboratory conditions, resulting in lower survival and lower instar number than observed in the laboratory strain or in the resistant strain, maintained for more than 144 generations in artificial rearing. Lower susceptibility to Cry1Ac toxin was also observed in *H. virescens* populations kept for several generations in laboratory compared to populations kept for few generations under the same laboratory conditions (Albernaz 2011).

Additional studies with different *A. gemmatalis* populations selected for resistance to Cry1Ac pure toxins are necessary because populations of diverse origins seem to respond differently to the selection pressure with entomopathogens.

Considering that HD-1 strain of Bt has 54% of Cry1Ab toxin, 32% of Cry1Ac, and 14% of Cry1Aa (Masson *et al.* 1990), it seems that in the selective pressure with Dipel®, the contribution of Cry1Ac to favor selection of resistant genotypes in *A. gemmatalis* is minimal.

The cost associated with resistance to Bt is small, at least regarding the parameters evaluated here. Other features associated to fitness that receive influence of resistance to different toxins should be studied. This information could be useful for deploying resistant management strategies for Bt-soybean. Biological parameters, such as number of instars, duration of pupal stage, adult longevity, survival, number of eggs per female, and egg fertility of velvetbean caterpillar individuals are not affected after selection pressure with *B. thuringiensis* var.

kurstaki. However, parameters such as days at the larval stage and pupal weight are affected. Selection pressure performed with *B. thuringiensis* var. *kurstaki* does not contribute to select velvetbean caterpillar individuals resistant to Cry1Ac toxin.

ACKNOWLEDGMENTS

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