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Susceptibility of Brazilian populations of *Diatraea saccharalis* to Cry1Ab and response to selection for resistance

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ABSTRACT

The sugarcane borer, *Diatraea saccharalis* (F.), is a key pest of sugarcane and other grasses, which is a current focus of research aiming to develop transgenically resistant genotypes. However, the current susceptibility of Brazilian populations of the sugarcane borer to Bt toxins is unknown. Laboratory assays were carried out to characterize the susceptibility of sugarcane borer to the Bt toxin Cry1Ab and to select a resistant strain for additional studies on Cry1Ab resistance. Susceptibility was characterized by exposing neonates of different Brazilian colonies of sugarcane borer to different concentrations in meridic diet using surface application of Cry1Ab toxin. Selection for Cry1Ab resistance was carried out by exposing neonates to Cry1Ab-expressing maize (MON 810). The resistance of the borer populations to Cry1Ab was variable with LC_{50} and EC_{50} values reaching about 30-fold. The larvae responded positively to Cry1Ab selection exhibiting a 55-fold increase in resistance after four generations. This suggests the suitability of using leaves containing Bt-expressing genes for selection and the existence of variability of Bt-resistance in populations of the sugarcane borer.

1. Introduction

The development of transgenic sugarcane varieties expressing *Bacillus thuringiensis* (Bt) toxin genes against the sugarcane borer, *Diatraea saccharalis* (F.), is a current focus of attention (Arencibia et al., 1997; Braga et al., 2003), and these crops are expected to be commercially available in the next decade. A transgenic maize variety expressing the pro-toxin Cry1Ab was introduced in the US North Corn Belt in 1996 as a preventive measure against the European and the southwestern corn borers, *Ostrinia nubilalis* (Hübner) and *Diatraea grandiosella* Dyar, respectively. Subsequently, in 1999, transgenic maize was planted in the southern US region for managing the sugarcane borers *D. saccharalis* and *D. grandiosella* (Castro et al., 2004a).

Bt-Transgenic maize expressing Cry1Ab was deregulated in Brazil in 2008, and in 2013 over 50% of the maize cultivated in the country was of Bt-transgenic cultivars (ABRASEM, 2014). Maize expressing Cry1Ab is less efficacious against *D. saccharalis* than against the European corn borer (Huang et al., 2007). Hence, the potential development of Cry1Ab resistance in the sugarcane borer may take place more quickly, compromising the effectiveness of the Bt-toxin against the sugarcane borer even more. This correlates with evidence that the sugarcane borer has evolved detectable levels of resistance in the laboratory as well as the initial detection of a major Cry1Ab resistance allele in the state of Louisiana in the US (Huang et al., 2007).

The sugarcane borer appears to have adapted well to maize plants in the southern US, where it is a prevailing pest species (Castro et al., 2004a; Huang et al., 2006, 2007). Curiously, this species is only a secondary maize pest in Brazil, leading to concerns of overlap in maize and sugarcane cultivation. The shift in host preference of *D. saccharalis* observed in the US may also take place in Brazil, but this is unlikely because of the high abundance of sugarcane, the borer's primary host plant in Brazil.

Selection for Bt-toxin resistance in field-collected insects is possible using Bt-expressing plant varieties, various parts of the plant, and Bt-contaminated diet as well as other alternatives (Gould et al., 1995; Ferré and Van Rie, 2002; Pereira et al., 2008). However, the production of purified toxin is expensive in contrast to Bt toxin-expressing plants (González-Cabrera et al., 2013). The objective of our study was to assess the variability in Cry1Ab susceptibility among different populations of the sugarcane borer and to select for resistance in a mixed population in the laboratory.

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2. Materials and methods

2.1. Insects

Field collection of insect populations was difficult because natural sugarcane borer populations were low, so we used laboratory populations from five locations in Brazil. Sugarcane borers were obtained from the Federal University of Alagoas (coded CECA-UFAL), Maceió county, state of Alagoas, and the Federal University of Lavras (UFLA), Lavras county, state of Minas Gerais, as well as from the sugarcane mills in Santa Helena county, state of Goiás, in Conchal county, state of São Paulo, and in Campo Novo de Parecis county, state of Mato Grosso. These locations were chosen as they are within the main sugarcane production regions, and received field-collected *D. saccharalis* every two years to avoid inbreeding depression of the borer populations that are maintained in high numbers for mass rearing of a parasitoid species used for biological control of the pest. Upon arrival in the laboratory, eggs masses were sequentially disinfected in 1% formaldehyde, then in distilled water, and finally in 1% copper sulfate pentahydrate. After air drying, 200–300 eggs were placed in 500-ml glass jars, each with a perforated lid sealed by a metallic net containing 250 ml of artificial diet (King and Hartley, 1985). When larvae were sent instead of eggs, they were directly placed in the glass jars containing the diet, whereas eventual pupae that were collected were contained in polyvinyl chloride (PVC) cages 20 cm wide and 10 cm high until adults emerged and mated. The inner surface of the PVC cages was covered with sulfite paper as an oviposition substrate.

Eggs, larvae, and adults were maintained in a rearing room at 27 °C, 60% RH and a photoperiod of 14:10 h (L:D). 20-d-old larvae were transferred to 15 cm diam Petri dishes containing artificial diet (King and Hartley, 1985) until they pupated. The pupae were sexed and transferred to PVC cages (120 adults per cage with a sex ratio of 2 females to 3 males) for adult emergence. Offspring from the next generation were used for bioassays.

2.2. Cry1Ab concentration-response bioassays

Concentration–response bioassays were established for each insect population obtained (as well as for the mixed population obtained from the original populations) and used for Cry1Ab resistance selection. The purified Cry1Ab toxin was obtained from Prof. Blair Siegfried (Department of Entomology, University of Nebraska–Lincoln, NE). The toxin was prepared by fermentating a recombinant strain of *Escherichia coli* (Migula) that expresses Cry1Ab (strain ECE53) (Lee et al., 1992). Toxin preparations were quantified by densitometry of the 60–65 kDa peptides after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared to a standard curve for bovine serum albumin (BSA) (Crespo et al., 2008). Endotoxin was lyophilized, shipped overseas and stored.

The concentration-response experiments were carried out in a completely randomized design using neonates from different cages to minimize their relatedness, and each assay was replicated 3–4 times for each population depending on availability of larvae. The 128-well bioassay trays (C-D International, Pitman, NJ) received 1 ml of diet per well, which allowed for simultaneous testing of seven toxin concentrations and a control (*i.e.*, no toxin) on each population. The lyophilized toxin was solubilized in 0.1 M sodium carbonate-bicarbonate buffer pH 10.4 to obtain a stock of 0.43 μg/μl of Cry1Ab in the buffer. Dilutions were prepared in 0.1% Triton X-100, and 30 μl of toxin solution were applied to the surface of the diet within the tray wells to ensure uniform application. The range of toxin concentrations tested were 2–128 ng toxin/cm^2, based on the LC_{50} and LC_{90} values estimated by Tan et al., (2011). Control treatments consisted of diet treated with 0.1% Triton-X 100 only. After air drying, each tray well containing both diet and toxin received a single neonate larvae (24 h old) transferred with a fine paint brush, and wells were covered with vented, adhesive lids (C-D International) once each group of 16 larvae had been transferred.

Sixteen larvae were tested at each toxin concentration in a tray, and 3–4 trays (*i.e.*, replicates) were used for each population (48–64 larvae per concentration). The trays were maintained under controlled conditions (27 °C, 60% RH, and 24 h dark) for seven days, after which larval mortality was determined by observing movement when larvae were touched by the tip of a fine paint brush and if they did not molt. As a result, the criterion for mortality used here accounts for both severe growth inhibition and death. The weight of larvae surviving exposure was also recorded. Data for mortality and larval growth inhibition were standardized by using the untreated control to adjust for natural mortality and the percentage of growth inhibition was calculated and analyzed using probit (Marçon et al., 1999; PROC PROBIT; SAS Institute, 2008). The LC_{50} and EC_{50} values obtained were used to estimate the resistance ratios and respective 95% confidence intervals (Robertson et al., 2007).

2.3. Selection for Cry1Ab resistance

Selection for Cry1Ab resistance was performed using surviving neonates fed on MON810 maize leaves. Three seeds of transgenic maize or its non-transgenic isoline were planted in plastic pots fertilized with 20 g/l of "Super Simples" mix of 20% P_2O_5, 28% CaO, 12% S in addition to 20 g/l of ammonium sulfate and 18 g/l of potassium chloride. Four maize leaves from plants at the V3 and V5 stages were harvested and transversally cut into four portions of 5 cm length and placed into 16-cell 5.5 × 3.5 cm plastic trays. One hundred and sixty neonates from each population were placed in the trays and fed for 2 d with 160 g of leaf fragments with transgenic or non-transgenic maize leaves (*i.e.*, 160 larvae per plant type). The surviving larvae were weighted on the third day using an analytical balance (Shimadzu AUW220D, Shimadzu, Kyoto, Japan). Expression of the Bt toxin in the transgenic plants was confirmed using the AgraStrip® Cry1Ab strip test (Romer Labs do Brasil, Campinas, Brazil).

Larvae from each population that survived exposure to transgenic maize were collected on molting to the 2nd instar and transferred to 5 × 3 cm 16-well plastic trays containing 50 ml diet/well and were maintained at 27 °C, RH 60% and 14:10 h L:D, until pupation. The surviving pupae from each population were counted, sexed and pooled into a single population, hereafter referred to as "mixed" population. Twenty males and 20 females from each population (200 pupae in total) were mixed and randomly divided into two groups of 50 male–female pairs, and each group was placed in a mating PVC cage maintained at 27 °C, RH 60%, and 14:10 h L:D. The eggs obtained from these cages were used for the selection process for Cry1Ab resistance. The effect of different portions of Cry1Ab-maize whorl leaves on larval survival and development was subjected to one-way ANOVA (PROC GLM; SAS Institute, 2008).

Eggs from the mixed adult population were placed on 9 cm diam plastic Petri dishes with distilled water-moistened filter paper covering the bottom to stimulate egg hatching. Neonates were fed for 2 d with maize leaves. The procedure was continued for three more consecutive generations. Survival and growth inhibition results, as well as the heritability data, were subjected to regression analysis (PROC GLM; SAS Institute, 2008).

3. Results

3.1. Susceptibility of the sugarcane borer populations to Cry1Ab

The probit model fitted mortality and growth inhibition data as indicated by non-significant Chi-square values ($\chi^2 < 8.5$, $P > 0.05$),

Table 1
Relative susceptibility of populations of the sugarcane borer to the Cry1Ab toxin from Bt.

Population	Generation	No. of insects	Slope ± SE	LC_{50} (95% FL) ng/cm^2	LC_{90} (95% FL) ng/cm^2	χ^2	*P*	RR_{50} (95% CL)
Campo Novo	11	496	1.29 ± 0.14	7.60 (5.48–10.02)	74.54 (50.38–129.98)	7.43	0.19	23.99 (6.06–94.90)
CECA-UFAL	37	378	2.17 ± 0.24	10.32 (8.08–12.82)	40.13 (30.45–58.61)	7.65	0.18	32.56 (8.33–127.13)
Santa Helena	5	432	1.90 ± 0.22	2.84 (2.11–3.56)	13.45 (10.48–19.00)	3.26	0.66	10.09 (5.73–12.47)
UFLA	15	416	2.05 ± 0.20	5.15 (4.30–6.23)	21.73 (17.07–29.91)	7.47	0.19	14.7 (4.17–63.19)
F_1 Mixed population	1	512	1.11 ± 0.13	3.19 (2.01–4.47)	45.97 (31.27–79.99)	2.14	0.83	10.06 (5.85–18.86)
São Paulo	32	512	1.20 ± 0.28	0.32 (0.03–0.81)	3.69 (2.01–5.81)	8.04	0.15	1.00
F_4 Mixed population	4	512	1.27 ± 0.15	17.55 (13.06–23.18)	179.33 (111.47–366.01)	3.81	0.58	55.33 (32.71–93.54)

provided estimates of the toxicological parameters LC_{50} and EC_{50} with their respective 95% fiducial limits and resistance ratios with 95% confidence intervals (Tables 1 and 2). The LC_{50} values ranged from 0.32 ng/cm^2 (São Paulo) to 10.32 ng/cm^2 (CECA-UFAL), which corresponds to a 32-fold variation in the resistance ratios at LC_{50} (RR_{50}) (Table 1). There was substantial variation in the Cry1Ab susceptibility among populations of the sugarcane borer and relatively low variation of the intra-population heterogeneity among populations, with slopes from the concentration–mortality curves ranging from 1.11 to 2.17 (Table 1). Similar results were also obtained on larval growth, with little variability in intra-population heterogeneity and resistance ratios reaching 12-fold for CECA-UFAL population (Table 2). The mixed population in its F1 generation showed resistance ratios of 10 and 30 fold based on mortality and larval growth inhibition, respectively (Tables 1 and 2).

3.2. Selection for Cry1Ab resistance

Larval survival and growth after ingestion of different parts of the whorl leaves were similar, indicating similar levels of expression of Cry1Ab throughout these leaves. Cry1Ab toxin expression was confirmed by the immunological strip test. The mixed population of the sugarcane borer that was subjected to selection showed 55-fold increase of Cry1Ab resistance in the fourth generation (Table 1). Larval survival for the Cry1Ab-selected strain reached levels similar to the non-selected larvae maintained on the non-transgenic isoline after four generations of selection (Fig. 1A). The weight gain of the surviving larvae also differed between the Cry1Ab-selected and non-selected insects, without exhibiting significant variation along successive generations of Cry1Ab selection (Fig. 1B). Offspring-parent regression for survivorship data indicated a significant gain of survivorship on Cry1Ab maize upon selection for resistance, while no significant offspring-parent regression coefficient was observed for insects maintained on non-transgenic maize as expected (Fig. 2).

4. Discussion

The concentration–response susceptibility estimates of the Brazilian sugarcane borer populations indicate up to 30-fold variability in the range of the LC_{50} and EC_{50} estimates, which overlap with those reported by Tan et al., (2011). Such variability is frequent and likely due to many factors, including geographic origin, host, previous selection pressure and mating patterns (Roush and McKenzie, 1987; Marçon et al., 1999; Huang, 2006; Huang et al., 2008; Carrière et al., 2010; Crespo et al., 2010). The knowledge of the existing population variability to Cry1Ab susceptibility is important to allow proper management of this control tool in minimizing the risk of selection and evolution of Cry1Ab resistance (Roush and McKenzie, 1987; Gassmann et al., 2009; Carrière et al., 2010; Crespo et al., 2010).

The toxin expression in the different parts of the plant potentially affects selection for resistance (Castro et al., 2004b; McAllister et al., 2004; Wu et al., 2007). Such variation has been reported in maize leaves during the reproductive stage (Székács et al., 2010; Bakhsh et al., 2011); however, our data indicate no detectable variation in Cry1Ab expression in maize leaves during the vegetative stage of the plant. The use of the Cry1Ab-expressing leaves from transgenic maize allowed for the successful selection of resistance to this toxin in a mixed population of the sugarcane borer. Larval survival under Cry1Ab exposure reached levels similar to those of larvae maintained in non-transgenic isoline after four generations of selection. Therefore, the pre-existence of Cry1Ab resistant alleles does not appear to be particularly rare in Brazil, and the use of a mixed population for Cry1Ab resistance selection allowed for relatively quick selection, as would be expected in such cases (Tabashnik, 1992). The selection for Cry1Ab resistance using maize leaves expressing the toxin was also successfully achieved in the armyworm *Mythimna unipuncta* Haworth (1809) (Lepidoptera: Noctuidae) (González-Cabrera et al., 2013), but this technique is still uncommon in the laboratory despite its potential, as demonstrated in our study with the sugarcane borer.

Some physiological insect traits may be affected by the selection for either insecticide or toxin resistance (Cousteau et al., 2000; Guedes et al., 2006; Araújo et al., 2008a,b; Gassmann et al., 2009). The sugarcane borer exhibited a decrease in weight when maintained with an artificial diet without selection for Cry1Ab resistance, which led to smaller adults with reduced egg production and thus reduced fecundity (data not shown). In contrast, the insects selected for Cry1Ab resistance did not exhibit a significant decline in weight gain with selection, indicating an apparent lack of adaptive costs associated with Cry1Ab resistance at least for the resistance levels obtained, which was unexpected based on previous studies (Carrière et al., 2001; Tabashnik et al., 2000, 2003).

Table 2
Relative growth inhibition for populations of the sugarcane borer fed on diet containing the Cry1Ab toxin from Bt.

Population	Generation	No. of insects	Slope ± SE	EC_{50} (95% FL) ng/cm^2	EC_{90} (95% FL) g/cm^2	χ^2	*P*	RR_{50} (95% CL)
Campo Novo	11	496	1.16 ± 0.13	2.43 (1.46–3.47)	30.84 (21.76–50.18)	8.47	0.13	7.67 (1.91–30.69)
CECA-UFAL	37	378	1.76 ± 0.20	3.86 (2.84–4.92)	20.66 (15.41–31.17)	3.48	0.63	12.18 (3.09–47.91)
Santa Helena	5	432	1.32 ± 0.23	0.82 (0.26–1.50)	7.68 (5.41–11.58)	6.57	0.25	2.59 (0.63–13.56)
UFLA	15	416	1.48 ± 0.27	0.77 (0.26–1.33)	5.64 (4.08–8.37)	3.49	0.63	2.43 (0.53–11.11)
F_1 Mixed population	1	512	1.41 ± 0.14	4.20 (3.09–5.38)	33.10 (24.43–49.87)	2.11	0.83	29.58 (20.09–43.18)
São Paulo	32	512	1.31 ± 0.29	0.14 (0.01–0.70)	4.15 (1.046–8.25)	1.01	0.96	1.00
F_4 Mixed population	4	512	1.21 ± 0.14	2.86 (1.76–4.04)	32.96 (23.37–53.50)	3.09	0.69	20.14 (12.38–32.45)

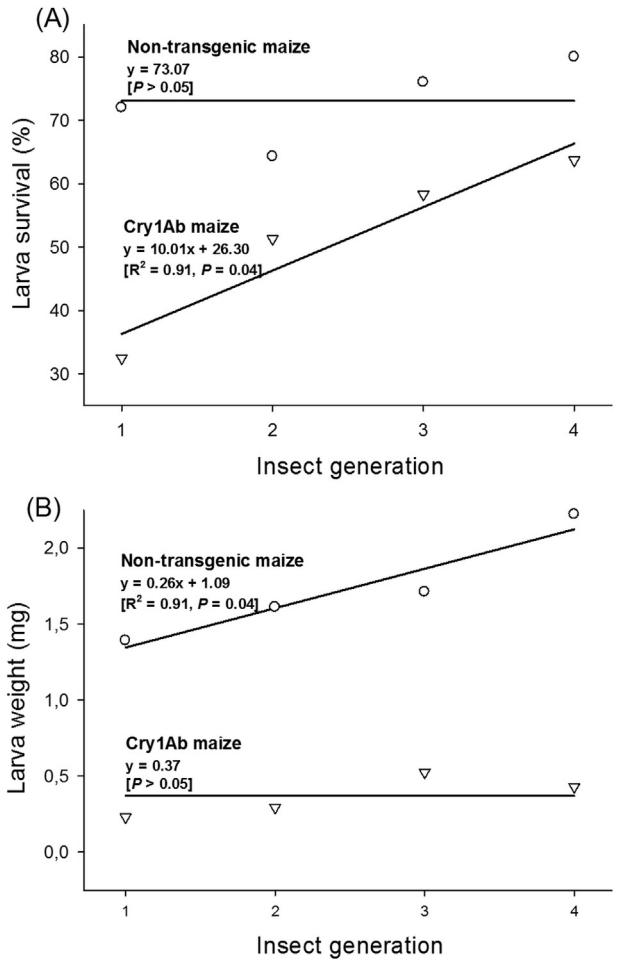

Fig. 1. Larval survival (A) and larval weight (B) from a mixed population of the sugarcane borer exposed to maize leaves expressing the pro-toxin Cry1Ab or maize leaves from a non-transgenic isoline.

Such non-detectable expression of adaptive costs is sometimes observed for insecticide resistance (e.g., Guedes et al., 2006) and has even been minimized in the sugarcane borer by the transfer of the larvae feeding on maize leaves to an artificial diet for the completion of development, a possibility that has already been reported in other species (Cousteau et al., 2000; Gassmann et al., 2009). This absence of cost may occur because an artificial diet tends to favor the development and survival of the insects because of its superior nutritional quality (Schoonhoven et al., 2005). Therefore, the transfer of the resistant insects from the host leaves to an artificial diet may have minimized the effects of Cry1Ab resistance costs while potentially relaxing the adverse effects of the host plant on the larval development by preventing a longer exposure to the host defensive phytochemicals, which favor improved larval development (Carriére et al., 2001; Janmmat and Myers, 2003).

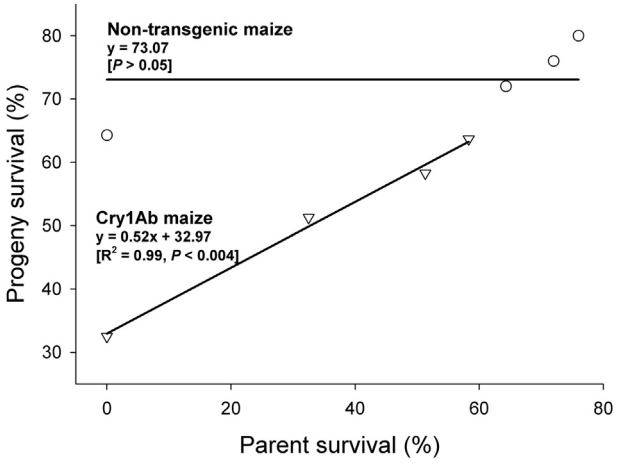

Fig. 2. Offspring-parent regression for survivorship data showing the gain by selection after each generation of selection in a mixed population of the sugarcane borer exposed to maize leaves expressing the pro-toxin Cry1Ab or maize leaves from a non-transgenic isoline.

In conclusion, we observed significant variations in the susceptibility of Brazilian populations of the sugarcane borer to Cry1Ab, which reached levels as high as 30-fold when larval mortality and growth inhibition were assessed. These findings spark concerns regarding the future use of transgenic maize and sugarcane expressing Cry1Ab toxins in Brazil because the evolution of sugarcane borer resistance to this toxin could take place upon their large-scale field use, which would require resistance management tactics to maximize the sustainable use of these transgenic crops. The mixing of the gathered populations allowed for sufficient variability for quick (four-generation) laboratory selection for Cry1Ab resistance in larvae of the sugarcane borer, although based on these results, we cannot predict whether resistance will develop in the field because the conditions of exposure and intensity of selection will differ under field settings. Cry1Ab resistance in the sugarcane borer does not appear to be associated with a fitness cost, thus making the management of resistant populations more difficult.

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