

Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil

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Abstract

BACKGROUND: The first *Bt* maize in Brazil was launched in 2008 and contained the MON 810 event, which expresses Cry1Ab protein. Although the Cry1Ab dose in MON 810 is not high against fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), MON 810 provided commercial levels of control. To support insect resistance management in Brazil, the baseline and ongoing susceptibility of FAW was examined using protein bioassays, and the level of control and life history parameters of FAW were evaluated on MON 810 maize.

RESULTS: Baseline diet overlay assays with Cry1Ab ($16 \mu\text{g cm}^{-2}$) caused 76.3% mortality to field FAW populations sampled in 2009. Moderate mortality (48.8%) and significant growth inhibition (88.4%) were verified in leaf-disc bioassays. In greenhouse trials, MON 810 had significantly less damage than non-*Bt* maize. The surviving FAW larvae on MON 810 (22.4%) had a 5.5 day increase in life cycle time and a 24% reduction in population growth rate. Resistance monitoring (2010–2015) showed a significant reduction in Cry1Ab susceptibility of FAW over time. Additionally, a significant reduction in the field efficacy of MON 810 maize against FAW was observed in different regions from crop season 2009 to 2013.

CONCLUSIONS: The decrease in susceptibility to Cry1Ab was expected, but the specific contributions to this resistance by MON 810 maize cannot be distinguished from cross-resistance to Cry1Ab caused by exposure to Cry1F maize. Technologies combining multiple novel insecticidal traits with no cross-resistance to the current Cry1 proteins and high activity against the same target pests should be pursued in Brazil and similar environments.

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Keywords: fall armyworm; Cry1Ab protein; *Bt* maize; insect resistance management

1 INTRODUCTION

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is historically one of the most destructive and economically important insect pests of maize (*Zea mays* L.) in Brazil.^{1–3} FAW has a highly polyphagous feeding behaviour that includes the consumption of different cultivated host plants in Brazil, such as rice⁴ and cotton in the Brazilian Cerrados.⁵ The considerable economic losses caused by FAW to maize plants led growers to rely on rigorous management based on the use of chemical insecticides, contributing to the evolution of resistance to most of those chemicals.^{6,7} In 2008, MON 810 maize expressing the Cry1Ab protein from *Bacillus thuringiensis* (*Bt*) was the first *Bt* maize technology deployed in Brazil and provided significant levels of control against FAW.^{8–10} Thereafter, different *Bt* maize technologies were launched, targeting FAW in Brazil, reaching up to 80% penetration in the maize-growing areas during cropping season 2014.¹¹

The evolution of resistance in target insect pests remains the main challenge to the sustainable deployment of crops expressing *Bt* proteins.^{12,13} Proactive insect resistance management (IRM) strategies were proposed to delay the evolution of resistance to *Bt* proteins^{14–16} and consequently prolong the

benefits of *Bt* crops.^{17–19} The most successful strategy adopted for single-mode-of-action *Bt* crops has been the high-dose/refuge strategy, which is based on the following assumptions: *Bt* plants control nearly all of the heterozygous insects (causing resistance to be functionally recessive); the resistant alleles are rare; refuge areas formed by non-*Bt* plants are available to provide susceptible insects to contribute to dilution of the resistance alleles.^{12–14} Although most insect pest populations have remained susceptible to *Bt* crops worldwide, resistance cases typically occur in situations where these assumptions have not been met.¹³

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Field-evolved resistance to *Bt* crops has been documented in several species, including *Busseola fusca* (Fuller) resistant to Cry1Ab maize in South Africa,²⁰ FAW resistant to Cry1F maize in Brazil and in Puerto Rico,^{21–23} and also on the US mainland,²⁴ *Pectinophora gossypiella* (Saunders) resistant to Cry1Ac cotton in India²⁵ and *Diabrotica virgifera virgifera* LeConte resistant to Cry3Bb1 maize in the United States.²⁶

Even though MON 810 maize has contributed to integrated pest management (IPM) in Brazil,¹⁰ it is not a high-dose product for FAW primarily owing to the moderate activity of Cry1Ab against this species.^{27,28} The relatively moderate activity of Cry1Ab against FAW, in combination with a tropical/subtropical environment that allows multiple maize-growing seasons²⁹ and low compliance with non-*Bt* refuge recommendations,²³ poses a risk of resistance evolution to MON 810 in FAW. Moreover, recent reports of field-evolved Cry1F resistance in FAW in Brazil^{22,23} and the likely cross-resistance between current Cry1 proteins^{30–35} bring more complexity to IRM programs, ultimately challenging the durability of MON 810 in Brazil. In this paper, we present data indicating a shift in Cry1Ab susceptibility in FAW in Brazil over time, along with a shift in the performance of Cry1Ab-containing MON 810 hybrids. The IRM implications of MON 810 being a moderate-dose product against FAW are discussed.

2 MATERIALS AND METHODS

2.1 Baseline susceptibility and resistance monitoring

FAW populations collected in crop season 2001 were previously used for evaluating the susceptibility and natural variation of this species to Cry1Ab using a diet-incorporated bioassay. Although diet incorporation is a more robust assay type, diet overlay methodology is simpler and requires less protein, which is useful when screening insects that are less sensitive to the *Bt* protein in question.³⁶ After the 2001 collection, a diet overlay bioassay was implemented, and field populations collected in crop season 2009 (supporting information Table S1) were used to bridge the two bioassay methodologies and to establish a diagnostic concentration for monitoring resistance. FAW populations were then sampled for resistance monitoring during each maize-growing season from 2010 to 2015 and from multiple regions (supporting information Table S1). Each crop season consisted of two maize-growing seasons, designated as first crop (spring/summer) and second crop (autumn/winter). The first crop of a given year is actually planted late in the previous year. Approximately 1000 FAW larvae were collected on non-*Bt* maize at each sampling location. Larvae were reared on artificial diet adapted from Kasten et al.³⁷ For the diet-incorporated bioassay,³⁸ 7–8 concentrations ranging from 0.1 to 100 µg Cry1Ab mL⁻¹ artificial diet were used (16 neonates × 8 replicates), while for the diet overlay assay,³⁹ 7–8 concentrations ranging from 0.001 to 28.6 µg Cry1Ab cm⁻² were used. An FAW laboratory strain obtained from EMBRAPA Maize and Sorghum (Sete Lagoas, MG, Brazil) in 1996, and maintained since then without selection pressure from insecticides or *Bt*, was used as a susceptible reference (SUS). Cry1Ab protein was provided by Monsanto Company (St Louis, MO) at a concentration of 1.65 mg purified Cry1Ab mL⁻¹ 100 mM sodium carbonate buffer (pH ~10) and stored in an ultra-low freezer at $-80 \pm 5^\circ\text{C}$. Monitoring bioassays were conducted as described in Bernardi et al.⁴⁰ Functional mortality (i.e. larvae did not moult to second instar) was assessed after 7 days. The weight of survivors was also recorded. The moulting inhibitory concentration (MIC₅₀) was estimated by probit

analysis.⁴¹ The effective concentration (EC₅₀) was estimated according to Sims et al.⁴² using non-linear regression analysis with JMP software.⁴³ Insect susceptibility to Cry1Ab was monitored through bioassays carried out using 16 µg Cry1Ab cm⁻² as a diagnostic concentration (nine replicates of 112 larvae). A total of 66 FAW field populations were tested from crop season 2009 to 2015 in Brazil.

2.2 Leaf-disc bioassays

Completely expanded leaves were removed from the whorl region of MON 810 and non-*Bt* maize plants cultivated in a greenhouse at the V4, V6 and V8 phenological stages. Leaf discs 2.4 cm in diameter were cut using a metallic cutter and placed on a non-gelled mixture (before solidification) of water–agar at 2.5% (1 mL well⁻¹) in acrylic plates with 12 wells (Costar®; Corning, Tewksbury, MA). Leaf discs were separated from the water–agar layer by a filter paper disc. One FAW neonate larva (0–24 h old) was placed on each leaf disc using a fine brush. Plates were sealed with plastic film and placed in a climatic chamber (temperature $27 \pm 1^\circ\text{C}$; relative humidity $60 \pm 10\%$; photoperiod 14:10 h light:dark). The experimental design was completely randomised, with ten replicates per treatment; each replicate consisted of 12 neonate larvae, for a total of 120 neonate larvae tested for each phenological stage. Functional mortality, weight and instars of survivors were assessed after 5 days. Mortality obtained on MON 810 maize was corrected on the basis of mortality on non-*Bt* maize. To evaluate larval growth inhibition, the weight of survivors was compared with that from non-*Bt* maize. Mortality data and growth inhibition (x) were transformed using the formula $\sqrt{x + 0.5}$, the data were subjected to analysis of variance and the means were compared by *t*-test ($P \leq 0.05$) (PROC TTEST⁴⁴).

2.3 Whole-plant assays

MON 810 and non-*Bt* maize were sown in a greenhouse at a density of 5 seeds per linear metre following a completely randomised experimental design. Within each block, the rows of maize (3.0 m length × 0.5 m between rows) represented the experimental replicates (plots). At phenological stage V6, each plant was infested with 100 FAW neonates. After 7 days, ten consecutive plants were evaluated in each plot. Foliar damage was evaluated using the Davis scale (0 = no damage, 9 = severe damage),⁴⁵ and the number of living caterpillars in the whorl was determined. Both measurements (x) were transformed using the formula $\sqrt{x + 0.5}$, because raw data were not normally distributed as determined by the Shapiro–Wilk test. The transformed data were subjected to analysis of variance, and the means were compared by *t*-test ($P \leq 0.05$) (PROC TTEST⁴⁴).

2.4 Life history traits of FAW fed on MON 810 maize

MON 810 and non-*Bt* maize were sown in the greenhouse into 12 L plastic pots (two seeds per pot). Completely expanded leaves were removed from the whorl region of the plants when they reached V6 and cut into sections of ~ 40 cm². Thereafter, leaves were placed into sterilised glass tubes (8.5 cm length × 2.5 cm diameter) containing a wad of hydrophobic cotton. One FAW neonate larva (0–24 h old) was placed into each glass tube using a fine brush. Leaves were changed every 48 h over the larval development period. Tubes were sealed with plastic film and placed in a climatic chamber (temperature $27 \pm 1^\circ\text{C}$; relative humidity $60 \pm 10\%$; photoperiod 14:10 h light:dark). The experimental design was completely randomised with 20 replicates, each one consisting of ten

tubes. For each treatment, the following biological parameters were evaluated: length and survival of egg, larval and pupal stages; total cycle (egg to adult); larval weight at 10 days after infestation; pupal weight; sex ratio; length of pre-oviposition, oviposition and post-oviposition periods; adult longevity, fecundity and fertility.

Length and survival of egg, larval and pupal stages and total cycle (egg to adult) were determined in daily observations. Adult longevity, fecundity and fertility were determined from 15 couples (raised on either MON 810 or non-Bt leaf tissue) kept in PVC cages (23 cm height \times 10 cm diameter) internally coated with a paper towel (oviposition substrate) and closed at the top with a voile-type fabric. Adults were fed with a 10% honey aqueous solution provided on cotton balls. The number of eggs and the mortality of adults were assessed daily. To determine the embryonic period and survival, 50 eggs were obtained from each couple. Eggs were placed into flat-bottomed glass tubes (8.5 \times 2.5 cm). A piece of paper (2 \times 1 cm) was placed inside the tube and moistened daily with distilled water. The tube was sealed at the top with plastic film. The eggs were observed daily, and the number of hatched larvae was counted. The larval stage length, larval and pupal weights and the length of the pre-oviposition, oviposition and post-oviposition periods (x) were transformed using the formula $\sqrt{x + 0.5}$, because raw data were not normally distributed as determined by the Shapiro–Wilk test. Each biological parameter was then subjected to analysis of variance, and the means were compared by t -test ($P \leq 0.05$) (PROC TTEST⁴²). Putative deviations in the sex ratio were assessed using a chi-square test (χ^2) ($P \leq 0.05$) (PROC FREQ⁴²). Life tables were calculated by estimating the mean interval between generations (T), net reproductive rate (R_0), intrinsic growth rate (r_m) and finite growth rate (λ). The parameters of the life table were estimated using the lifetable.sas⁴⁶ procedure with SAS software.⁴⁴

2.5 Monitoring of MON 810 field efficacy

Leaf damage caused by FAW (Davis scale)⁴³ and the number of large larvae (≥ 1.5 cm in length) were evaluated on a weekly basis in six commercial MON 810 and non-Bt maize fields in Brazil from crop season 2009 to 2013: Passo Fundo (RS), Rolândia (PR), Araguari (MG) and Luis Eduardo Magalhães (BA) during the first crop season, and Campo Verde (MT) and Dourados (MS) during the second crop season. At all locations, a randomised complete block design with three replicates was established using commercial maize hybrids adapted to each location. Each replicate consisted of at least 3 ha. Twenty sampling spots were referenced in each replicate to be the reference for pest scouting and damage evaluation. For each weekly evaluation, ten sampling spots with five plants each were evaluated in each of three replicates, totalling 150 plants per treatment (total of 300 plants per location). In summary, considering the six locations evaluated for the first and second crop-growing seasons, two entries (MON 810 and non-Bt maize), three replicates and ten sampling spots per replicate, a total of 360 sampling spots with five plants each (total of 1800 plants) were evaluated, representing each week of crop growth. The number of larvae ≥ 1.5 cm in the whorl and the percentage of plants with damage rated ≥ 3 for each plot (Bt and non-Bt) were calculated on the basis of the damage to each plant evaluated in each plot (50 plants before tasselling and 30 plants after) in each location for every weekly sampling. The average was then calculated for the location in a specific weekly evaluation during the 5 years of monitoring. To evaluate the impact of MON 810 on larval counts and plant damage, and to assess whether that impact

had declined over time, total larval counts and average percentage of plants with damage rated ≥ 3 were computed for each plot (MON 810 and non-Bt maize) over all plant growth stages. Then, linear models were used to relate the square root of larval counts or square root of percentage of plants damaged to each crop season, treatment and crop season \times treatment interaction. Linear model analysis was conducted using R statistical software, v.3.0.2 (<http://www.r-project.org>).

3 RESULTS

3.1 Baseline susceptibility to Cry1Ab

Baseline susceptibility curves using a diet-incorporated bioassay for FAW sampled in crop season 2001 indicated moderate susceptibility to Cry1Ab and large natural variation across the populations. EC_{50} values (i.e. concentrations that caused 50% growth inhibition of FAW larvae) ranged from 0.30 (0.22–0.45) $\mu\text{g Cry1Ab mL}^{-1}$ diet (population RS) to 3.67 (0.23–6.24) $\mu\text{g Cry1Ab mL}^{-1}$ diet (population GO) (Table 1). The difference between the most susceptible and most tolerant populations was approximately 12-fold. The susceptible reference strain (SUS) had an EC_{50} similar to that of the most susceptible field population, 0.33 (0.22–1.13) $\mu\text{g mL}^{-1}$ diet.

Owing to the moderate activity of Cry1Ab against FAW and the large amount of protein that would be required for the subsequent resistance monitoring programme, a diet overlay bioassay was implemented, and field populations sampled in crop season 2009 were used to bridge the methodologies and establish a diagnostic concentration. Using the new methodology, both mortality and growth inhibition responses could be used to identify shifts in susceptibility. For the susceptible reference strain, 16 $\mu\text{g cm}^{-2}$ caused 98% mortality, while tenfold less (1.6 $\mu\text{g cm}^{-2}$) was sufficient to cause near-complete growth inhibition (Fig. 1). In the case of field populations (BA-16 and MT-10), mortality and growth inhibition using 16 $\mu\text{g cm}^{-2}$ were approximately 80.2 and 71.7% (BA-16) and 99.7 and 94.4% (MT-10) respectively (Fig. 1). Therefore, the diagnostic concentration selected for resistance monitoring based on both mortality and growth inhibition was 16 $\mu\text{g cm}^{-2}$.

3.2 Leaf-disc and whole-plant assays

FAW neonates were moderately susceptible to the Cry1Ab expressed in MON 810 maize (Table 2). At 5 days after infestation,

Table 1. Susceptibility of *S. frugiperda* larvae sampled from multiple locations in crop season 2000/01 and exposed to the Cry1Ab protein in a diet-incorporated bioassay

Population code	City, state	Sampling date	EC_{50} (95% FL) ^a
SUS ^b	Sete Lagoas, MG	–	0.33 (0.22–1.13)
RS	Santa Maria, RS	January 2001	0.30 (0.22–0.45)
BA	Barreiras, BA	November 2000	0.36 (0.21–0.83)
MT	Rondonópolis, MT	December 2000	0.37 (0.22–1.24)
MG	Uberlândia, MG	January 2001	0.43 (0.28–1.14)
SP	Guaira, SP	December 2000	0.97 (0.45–6.40)
PR	Rolândia, PR	November 2000	1.49 (0.59–5.76)
GO	Montividiu, GO	November 2000	3.67 (0.23–6.24)

^a EC_{50} : effective concentration of protein ($\mu\text{g mL}^{-1}$ diet) required to cause 50% growth inhibition relative to untreated controls at 7 days. FL: fiducial limit.

^b SUS: susceptible reference strain.

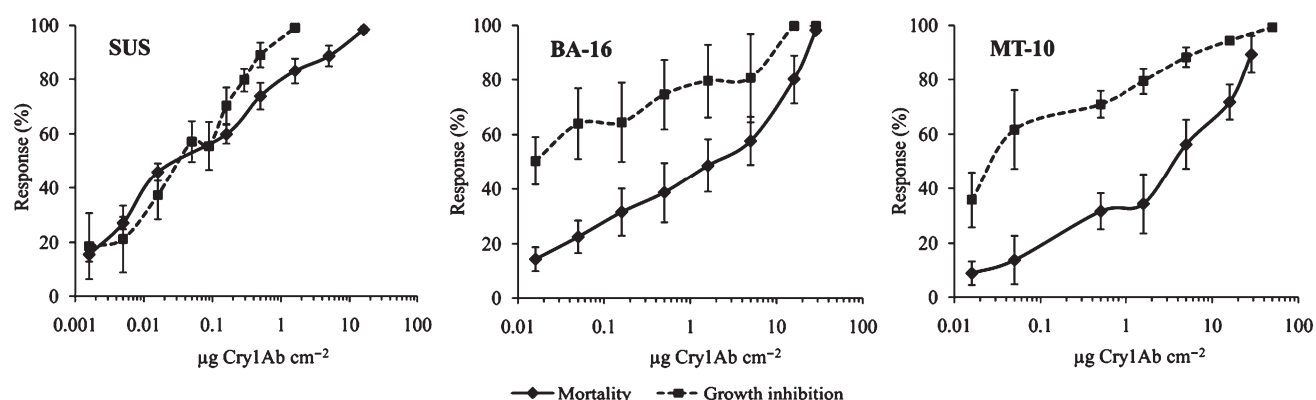


Figure 1. Comparisons of dose–response curves for mortality and growth inhibition of *S. frugiperda* neonate larvae exposed to increasing concentrations of the Cry1Ab protein in a diet overlay bioassay. SUS is the susceptible reference strain; BA-16 and MT-10 are field-collected populations. Each point represents the mean response from the replicates tested. Error bars represent the standard errors (SEs).

Table 2. Mortality and growth inhibition of *S. frugiperda* larvae after 5 days of feeding on leaf discs of MON 810 and non-Bt maize sampled at various phenological stages

Treatment	Phenological stage		
	V4	V6	V8
<i>Mortality (%)^a</i>			
MON 810	49.2 ± 1.1	50.4 ± 4.1	46.9 ± 6.2
Non-Bt maize	8.3 ± 3.4	4.2 ± 2.1	5.8 ± 1.9
<i>Growth inhibition (%)^a</i>			
MON 810	87.0 ± 0.8	91.5 ± 1.0	86.7 ± 1.2
Non-Bt maize	–	–	–

^a Values represent means ± SE after correction for non-Bt maize (control). Growth inhibition on MON 810 was calculated relative to larval growth on non-Bt maize. There was a statistically significant difference (t -test at $P \leq 0.05$) between MON 810 and non-Bt maize for all variables analysed. Mortality (%) at phenological stages V4 ($t = 4.87$; $df = 1, 18$; $P < 0.0001$), V6 ($t = 4.90$; $df = 1, 18$; $P < 0.0001$) and V8 ($t = 4.94$; $df = 1, 18$; $P < 0.0001$) and for variable growth inhibition (%) at phenological stages V4 ($t = 5.56$; $df = 1, 18$; $P < 0.0001$), V6 ($t = 6.02$; $df = 1, 18$; $P < 0.0001$) and V8 ($t = 5.49$; $df = 1, 18$; $P < 0.0001$).

Table 3. Average foliar damage, percentage of plants with damage ≥ 3 and number of living *S. frugiperda* larvae per plant after 7 days of feeding on MON 810 and non-Bt maize (V6 stage) in greenhouse trials

Treatment	Average damage ^{a, b}	Plants with damage ≥ 3 (%) ^b	Living larvae per plant ^b
MON 810	1.75 ± 0.08	15.00 ± 1.83	1.30 ± 0.45
Non-Bt maize	5.17 ± 0.97	90.00 ± 4.47	5.97 ± 2.06

^a Values represent means ± SE of leaf damage scores on the Davis scale (0 = no damage; 9 = severe damage).⁴³

^b There was a statistically significant difference (t -test at $P \leq 0.05$) between MON 810 and non-Bt maize for all variables analysed. Average damage ($t = 6.56$; $df = 1, 6$; $P = 0.0003$), plants with damage ≥ 3 (%) ($t = 10.49$; $df = 1, 6$; $P < 0.0001$) and living larvae per plant ($t = 2.57$; $df = 1, 6$; $P < 0.0210$).

FAW mortality on MON 810 maize was 48.8%, significantly higher than on non-Bt maize (6.1%). This result was consistent across all phenological stages evaluated (V4, V6 and V8). Although FAW mortality was not high on MON 810 maize leaves, the surviving larvae were severely affected. At 5 days after infestation, significant larval growth inhibition (88.4%) was observed at all phenological stages (Table 2).

These results were consistent with greenhouse trials in which MON 810 maize plants showed low levels of damage caused by FAW. At 7 days after infestation, MON 810 maize showed foliar damage significantly lower than that of non-Bt maize (Table 3). Average damage on MON 810 maize was 1.75 ± 0.08 on the Davis scale (i.e. small circular lesions), with only 15% of plants showing damage ≥ 3 . This level and frequency of damage is below the economic threshold adopted by growers in Brazil (20% of plants with damage ≥ 3). Conversely, the average damage on non-Bt maize was 5.17 ± 0.97 , with 90% of plants presenting damage ≥ 3 . The lower damage on MON 810 maize plants was a consequence of reduced feeding and moderate control of FAW, which resulted in fewer (1.30 ± 0.45) living larvae per plant in the whorl than on the non-Bt plants (5.97 ± 2.06) (Table 3).

3.3 Life history traits on MON 810

There was no significant difference in the length of the egg ($t = 0.59$; $df = 1, 25$; $P = 0.4491$) and pupal ($t = 3.50$; $df = 1, 38$; $P = 0.0692$) stages for FAW fed on MON 810 and non-Bt maize. However, a significant (5 day) increase in the length of the larval stage ($t = 36.73$; $df = 1, 38$; $P < 0.0001$) was observed, resulting in a difference in the egg–adult period ($t = 73.37$; $df = 1, 38$; $P < 0.0001$) and an increase of 12.1% in the time to complete the life cycle on MON 810 plants (Fig. 2A). Egg ($t = 1.22$; $df = 1, 38$; $P = 0.0558$) and pupal ($t = 0.12$; $df = 1, 38$; $P = 0.7322$) survival were not significantly different on MON 810 and non-Bt plants; however, larval survival on MON 810 was significantly lower than on non-Bt plants ($t = 14.40$; $df = 1, 38$; $P < 0.0001$) (Fig. 2B). This lower survival directly affected the number of insects completing the life cycle and consequently the egg–adult survival, which was reduced from 42.2% (non-Bt) to 22.4% for MON 810 ($t = 12.55$; $df = 1, 38$; $P < 0.0001$) (Fig. 2B). Larval weight was also significantly lower for insects feeding on Bt maize ($t = 34.98$; $df = 1, 38$; $P < 0.0001$). At 10 days after infestation, larvae fed on MON 810 plants had a mean larval weight of 35.4 ± 4.8 mg, about one-fifth that of larvae fed on non-Bt plants (174.3 ± 8.5 mg) ($t = 8.64$; $df = 1, 38$; $P < 0.0001$), which led to reduced pupal weight on MON 810 (150.8 ± 4.6 mg on MON 810 versus 203.6 ± 3.3 mg on non-Bt maize) ($t = 34.98$; $df = 1, 38$; $P < 0.0001$). Sex ratio (0.48 versus 0.51) was not affected ($\chi^2 = 12.92$; $df = 13$; $P = 0.4538$). Larval growth inhibition on MON 810 plants did not affect FAW adult longevity ($t = 1.81$; $df = 1, 51$;

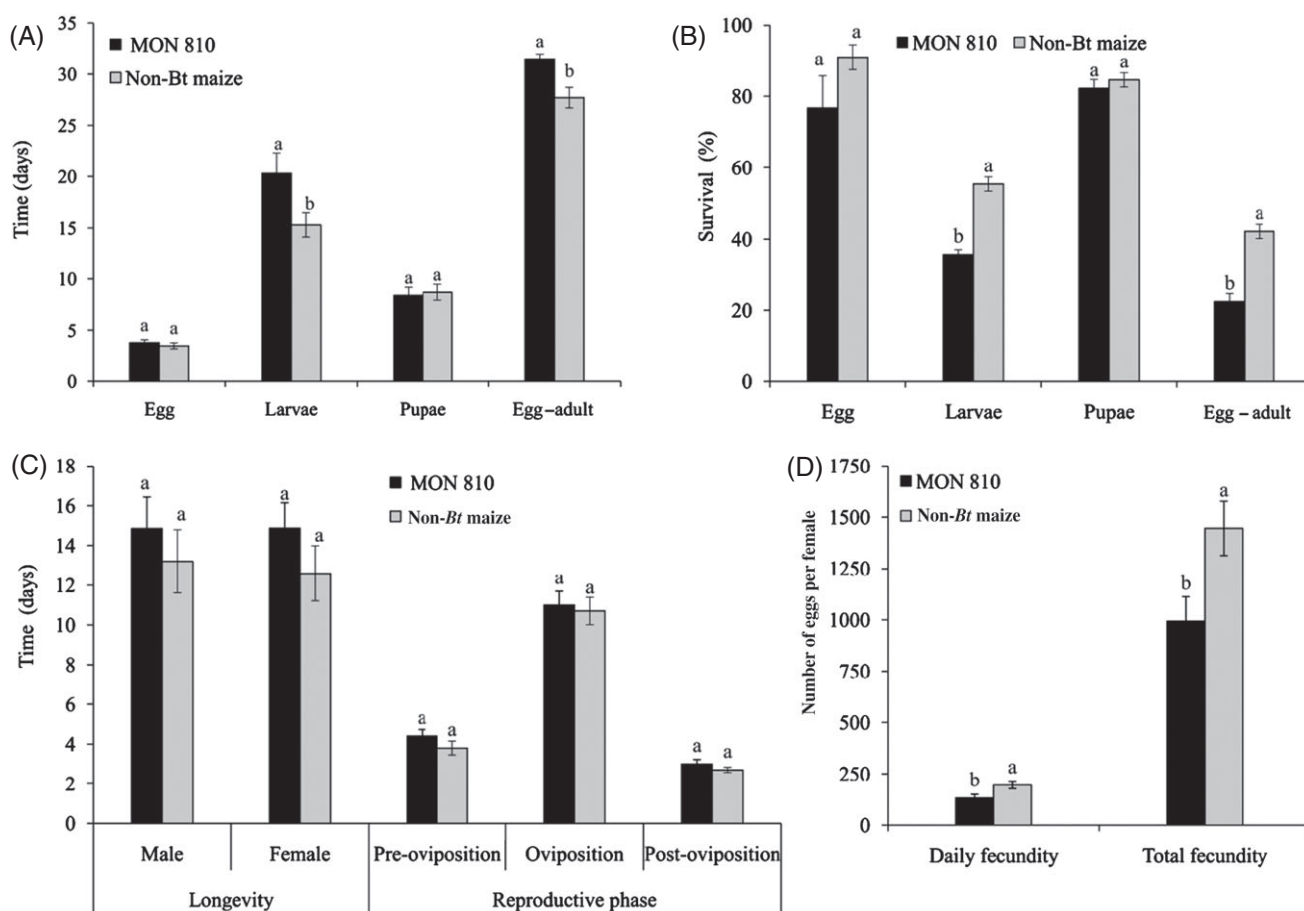


Figure 2. Life history traits of *S. frugiperda* fed on MON 810 and non-Bt maize: (A) length of each life stage; (B) survival percentage at each life stage; (C) male and female longevity and length of each reproductive phase; (D) daily and total fecundity. Within each life history trait, bars marked with the same letter are not significantly different (t -test at $P \leq 0.05$). Error bars represent the standard errors (SEs).

$P = 0.1853$) or the lengths of the pre-oviposition ($t = 1.83$; $df = 1, 25$; $P = 0.1885$), oviposition ($t = 0.54$; $df = 1, 25$; $P = 0.4693$) and post-oviposition ($t = 1.08$; $df = 1, 25$; $P = 0.5298$) periods (Fig. 2C). However, daily ($t = 11.67$; $df = 1, 25$; $P = 0.0033$) and total fecundity ($t = 12.43$; $df = 1, 25$; $P < 0.0001$) were significantly reduced by MON 810 maize (Fig. 2D). The effects of MON 810 maize on larval development and net reproductive rate affected the parameters in the fertility life table (Table 4). There was a 5.5 day increase in the mean length of a generation (T) when FAW fed on MON 810 maize, resulting in fewer generations expected per year. In addition, net reproductive rate (R_0 , i.e. the capacity to generate female descendants) was reduced by 54%. Based on these results, after 42.1 days (T), 139.9 females are expected from each FAW female on MON 810 maize, whereas after 36.6 days (T), 316.9 females are expected on non-Bt maize. The number of females expected after time T is calculated by multiplying the net reproductive rate (R_0) by the finite rate of population increase (λ). The intrinsic rate of population increase (r_m) was positive for both treatments, indicating population growth, although it was 24% lower on MON 810 plants; likewise, the finite growth rate (λ) was 3.8% lower.

3.4 Monitoring of Cry1Ab susceptibility using a diagnostic concentration

After establishing a diagnostic concentration for Cry1Ab, a resistance monitoring programme was initiated in Brazil. From 2010 to 2015, 64 FAW field populations were sampled across the most

Table 4. Fertility life table of *S. frugiperda* larvae fed on MON 810 maize and non-Bt maize

Biological parameter ^{a, b}	MON 810	Non-Bt maize	<i>P</i> -value
T	42.1 ± 0.28 b	36.6 ± 0.36 a	<0.0001
R_0	124.8 ± 17.23 b	272.0 ± 29.94 a	<0.0001
r_m	0.115 ± 0.003 b	0.152 ± 0.003 a	<0.0001
λ	1.121 ± 0.004 b	1.165 ± 0.004 a	<0.0001

^a T = mean length of a generation (days); R_0 = net reproductive rate (females per female per generation); r_m = intrinsic rate of population increase (per day); λ = finite rate of population increase (per day).

^b Means in the same row followed by the same letter are not significantly different for two-tailed t -tests for pairwise group comparisons (at $P \leq 0.05$).

relevant maize-growing areas in both crop seasons (first and second crop) and subjected to bioassays with Cry1Ab protein at the diagnostic concentration of $16 \mu\text{g cm}^{-2}$. In spite of the large variation in FAW response to Cry1Ab observed among the field populations tested, an increase in the mean survivorship was observed over the years (Fig. 3A). To test whether survivorship had increased over time, the observed survivorship in collected populations was regressed against season sampled using logistic regression (a generalised linear model with a 'quasi-binomial' link) weighted by

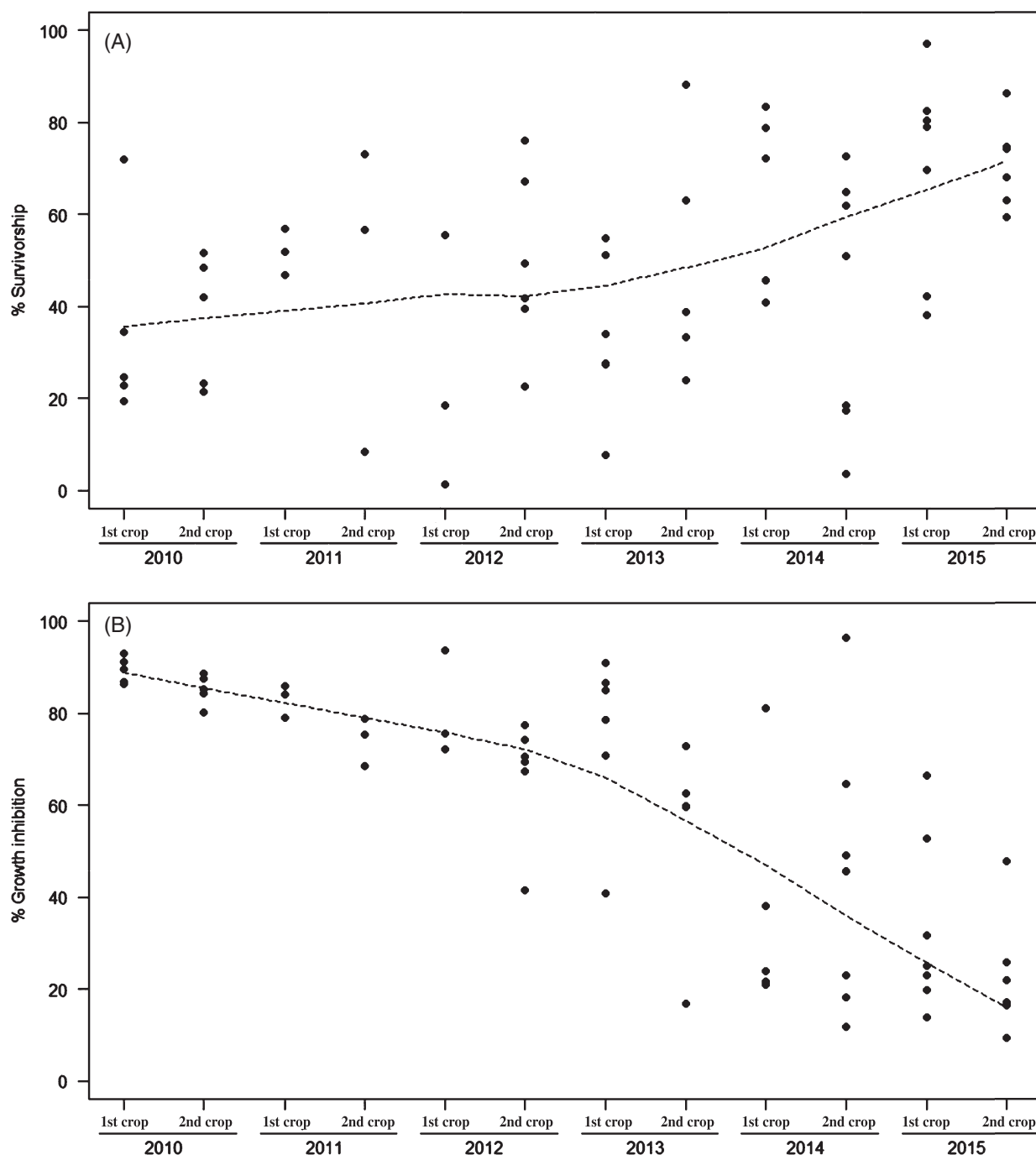


Figure 3. Mean survivorship (A) and growth inhibition (B) of *S. frugiperda* from different locations and crop seasons in Brazil in a diet overlay bioassay using $16 \mu\text{g cm}^{-2}$ of Cry1Ab protein. Within a crop season, mean values for different locations are separated to improve visibility. Dashed trend lines are locally weighted scatterplot smoothers computed using the lowess function in R.

number of insects tested at the diagnostic dose. The slope of the regression is positive (suggesting increasing survivorship over time) and statistically significant ($P < 0.01$).

During the last six maize-growing seasons of monitoring (from first crop 2013 to second crop 2015), a significant increase was observed. Growth inhibition of survivors relative to untreated controls also showed an apparent decrease in FAW susceptibility to Cry1Ab over the maize-growing seasons. The spread in percentage growth inhibition among locations increased dramatically over time (Fig. 3B). Overall, a large natural variation in FAW response

to Cry1Ab was observed. Nevertheless a significant increase in mean survivorship was observed from first crop 2010 to second crop 2015, increasing from 33.0 to 70.9%, while mean growth inhibition of survivors decreased from 89.4 to 23.1% in the same timeframe. Both results indicated a reduction in FAW susceptibility to Cry1Ab protein over time. To test whether growth inhibition had decreased over time, the observed growth inhibitions in collected populations were regressed against season sampled using a logistic generalised linear model with a 'quasi-binomial' link, weighted by number of insects tested at the discriminating dose. The slope

Table 5. Average percentage of plants with foliar damage caused by *S. frugiperda* rated ≥ 3 (Davis scale) observed over all sampling periods on MON 810 and non-Bt maize fields, by state and crop season. Each number reported is the average of three replicates and represents a total of 150 plants per treatment per location per crop season. Sampling periods with no reported damage on either plant type were excluded from the average. Numbers in parentheses are the number of insecticide sprays applied in each treatment. 'Reduction' is the proportion of reduction in damage on MON 810 maize plants relative to non-Bt plants

State ^a	Treatment	Crop season				
		2009	2010	2011	2012	2013
BA	Non-Bt	28.33 (4)	28 (5)	25.33 (4)	54.93 (5)	12.48 (4)
BA	MON 810	1.33 (0)	13.33 (0)	4.27 (1)	37.33 (2)	3.81 (4)
BA	Reduction	0.95 (4)	0.52 (5)	0.83 (3)	0.32 (3)	0.69 (0)
MG	Non-Bt	30.44 (2)	20.13 (2)	45.67 (3)	38.8 (2)	63.11 (1)
MG	MON 810	1.56 (1)	5.47 (0)	16.33 (1)	7.47 (2)	16.56 (1)
MG	Reduction	0.95 (1)	0.73 (2)	0.64 (2)	0.81 (0)	0.74 (0)
MS ^b	Non-Bt	49.56 (1)	32.95 (2)	29.05 (1)	38 (0)	27.56 (1)
MS	MON 810	3.89 (0)	1.43 (0)	0 (1)	3.6 (0)	4.67 (1)
MS	Reduction	0.92 (1)	0.96 (2)	1 (0)	0.91 (0)	0.83 (0)
MT	Non-Bt	16.67 (2)	52.42 (2)	24.76 (1)	29.07 (0)	28.22 (1)
MT	MON 810	0 (1)	18.42 (0)	3.14 (0)	4 (0)	10 (1)
MT	Reduction	1 (1)	0.65 (2)	0.87 (1)	0.86 (0)	0.65 (0)
PR ^b	Non-Bt	56.1 (3)	23.07 (3)	78.83 (4)	55.52 (3)	28.1 (2)
PR	MON 810	1.81 (1)	2.27 (1)	24.83 (0)	38.76 (1)	8.48 (1)
PR	Reduction	0.97 (2)	0.9 (2)	0.68 (4)	0.3 (2)	0.7 (1)
RS	Non-Bt	5.93 (0)	12.86 (0)	36.17 (1)	0.33 (0)	NA ^c
RS	MON 810	0.2 (0)	1.24 (0)	1.08 (0)	0 (0)	NA
RS	Reduction	0.97 (0)	0.9 (0)	0.97 (1)	1 (0)	NA

^a The effect of MON 810 was statistically significant for each state ($P \leq 0.05$) in all cases, with less damage on MON 810 plants.

^b There was a statistically significant crop season \times treatment interaction (evidence of a linear decrease in MON 810 control over time) at this site.

^c NA: data were not collected. Maize plants in the plots were severely affected by an early frost.

of the regression is negative (suggesting decreasing growth inhibition over time) and statistically significant ($P < 0.01$).

3.5 Monitoring of MON 810 field efficacy

Counts of large (≥ 1.5 cm) FAW larvae and the average percentage of plants with damage rated ≥ 3 on MON 810 and non-Bt maize were analysed for six states across 5 years. Generally, more insecticide sprays were applied to non-Bt maize than to MON 810, although in the last few years an increase in the number of sprays could be observed on MON 810 maize. In spite of the use of sprays on both MON 810 and non-Bt plants, in a statistical analysis of percentage of plants damaged, the effect of MON 810 was statistically significant for each state ($P \leq 0.05$) in all cases, with less damage on MON 810 maize plants (Table 5). Results from two of the six states – MS and PR – indicated a statistically significant crop season \times treatment interaction, i.e. evidence of a decrease in MON 810 control over time. The same pattern was observed for the number of large larvae. The effect of MON 810 was statistically significant for each state ($P \leq 0.05$); in all cases, fewer large larvae were found on MON 810 maize plants (Table 6). In one state (MS), there was a statistically significant crop season \times treatment interaction, suggesting a decrease in MON 810 control over time. The increase in sprays on MON 810 may have contributed to controlling FAW and thus reducing damage and incidence of large larvae on MON 810 plants. For instance, the number of insecticide sprays on MON 810 increased from 0 to 4 in BA (Tables 5 and 6).

4 DISCUSSION

Baseline susceptibility results indicated that FAW larvae sampled on maize-producing areas in Brazil were moderately susceptible

to the Cry1Ab protein expressed in MON 810 maize. Many reports have confirmed that FAW is more tolerant to Cry1Ab than other lepidopteran species.^{27,28} Protein binding assays have shown Cry1Ab to have low affinity for midgut tissue sections and isolated brush border membrane vesicles of FAW,⁴⁷ which may explain the tolerance of FAW to Cry1Ab. Another hypothesis is that tolerance is related to faster degradation of the protein in the larval midgut of FAW than in the midgut of more susceptible insects.⁴⁸

The results presented herein also indicate moderate susceptibility of FAW to Cry1Ab protein in leaf-disc and whole-plant assays performed with MON 810 maize. In spite of the moderate levels of larval mortality found on MON 810 maize leaf tissue, significant larval growth inhibition was observed throughout the maize phenological stages tested. Reduction in larval survivorship and mass gain (larvae and pupae) were found by Lynch *et al.*⁴⁹ when FAW neonates were fed with excised maize whorl leaves containing Cry1Ab. Regardless of the moderate activity of Cry1Ab against FAW larvae, MON 810 made an important contribution to maize IPM in Brazil.^{8,50} However, under conditions highly conducive to FAW in Brazil, MON 810 fields can reach the action threshold (20% of plants with damage rated ≥ 3 on the Davis scale) and therefore may require a certain amount of spraying, as observed in the field evaluations in this study. In such cases, MON 810 has been effectively used in association with other IPM tools.^{9,10}

In this study, the biological parameters of FAW fed on MON 810 maize plants were found to be different from those on non-Bt maize. Delayed development was observed in FAW larvae reared on MON 810. This effect is likely to be intensified under field conditions, leading to an increase in the probability of exposure to pathogens, natural enemies and other environmental factors.⁵¹ Conversely, the asynchronous emergence of FAW adults that were

Table 6. Total number of *S. frugiperda* large larvae (≥ 1.5 cm) observed over all sampling periods on MON 810 and non-Bt maize fields, by state and crop season. Each number reported is the average across three replicates and represents a total of 150 plants per treatment per location per crop season. Numbers in parentheses are the number of insecticide sprays applied in each treatment. 'Reduction' is the proportion of reduction in large larval counts in MON 810 fields relative to non-Bt fields

State ^a	Treatment	Crop season				
		2009	2010	2011	2012	2013
BA	Non-Bt	48.67 (4)	49.67 (5)	36.67 (4)	85.33 (5)	62 (4)
BA	MON 810	11 (0)	23.33 (0)	9.33 (1)	28.67 (2)	23.33 (4)
BA	Reduction	0.77 (4)	0.53 (5)	0.75 (3)	0.66 (3)	0.62 (0)
MG	Non-Bt	17 (2)	23.67 (2)	40.33 (3)	79.67 (2)	126.67 (1)
MG	MON 810	2.67 (1)	4 (0)	8 (1)	2.67 (2)	12.67 (1)
MG	Reduction	0.84 (1)	0.83 (2)	0.8 (2)	0.97 (0)	0.9 (0)
MS ^b	Non-Bt	152.33 (1)	207.67 (2)	83.33 (1)	56.67 (0)	65 (1)
MS	MON 810	19 (0)	27.33 (0)	1.33 (1)	11.67 (0)	18.33 (1)
MS	Reduction	0.88 (1)	0.87 (2)	0.98 (0)	0.79 (0)	0.72 (0)
MT	Non-Bt	10.67 (2)	33 (2)	14.67 (1)	15.33 (0)	103 (1)
MT	MON 810	0.67 (1)	10.67 (0)	1.67 (0)	1.33 (0)	60 (1)
MT	Reduction	0.94 (1)	0.68 (2)	0.89 (1)	0.91 (0)	0.42 (0)
PR	Non-Bt	89 (3)	38 (3)	168 (4)	82 (3)	175 (2)
PR	MON 810	2.33 (1)	6 (1)	101 (0)	42 (1)	52 (1)
PR	Reduction	0.97 (2)	0.84 (2)	0.4 (4)	0.49 (2)	0.7 (1)
RS	Non-Bt	18 (0)	13 (0)	105 (1)	0.33 (0)	NA ^c
RS	MON 810	1 (0)	2.67 (0)	0.67 (0)	0 (0)	NA
RS	Reduction	0.94 (0)	0.79 (0)	0.99 (1)	1 (0)	NA

^a The effect of MON 810 was statistically significant for each state ($P \leq 0.05$) in all cases, with fewer large larvae on MON 810 plants.

^b There was a statistically significant crop season \times treatment interaction (evidence of a linear decrease in MON 810 control over time).

^c NA: data were not collected. Maize plants in the plots were severely affected by an early frost.

reared on MON 810 and non-Bt maize, as observed herein, could undermine the random mating between insects emerging off the refuge plants (susceptible) and insects selected for resistance. This outcome could ultimately lead to an increase in the frequency of resistance alleles in the population.⁵² Nonetheless, the overlapping of FAW generations typically found in tropical environments²¹ would support the presence of susceptible adults throughout the season.

Although MON 810 has contributed to FAW management in Brazil, it is a moderate-dose Bt event for this insect. According to Roush,¹⁴ this characteristic allows the survival of heterozygous individuals, ultimately reducing refuge effectiveness. Moreover, compliance with refuge requirements has been poor in Brazil.^{22,40} An effective IRM strategy requires control of nearly all heterozygous individuals and reasonable compliance with refuge requirements (size and placement) to provide an adequate number of susceptible insects.¹⁶ Likewise, the environmental conditions in tropical regions such as Brazil allow the intensification of agricultural production,⁵³ contributing to a substantial increase in the size of insect pest populations.^{54,55} Because maize is grown in Brazil during two consecutive growing seasons (first crop and second crop),⁵⁶ FAW populations are able to build up considerably under conditions favouring multiple and overlapping generations.^{4,57} Furthermore, FAW is a polyphagous species, and the spatial and temporal availability of food resources plays an important role in the population dynamics and outbreaks of this species.⁵ These bioecological factors favour high levels of selection pressure against technologies used to control FAW in Brazil, including insecticides^{6,7,58} and Bt proteins expressed in genetically modified plants.^{22,23} Given the moderate efficacy of Cry1Ab against FAW, Bt maize technologies expressing the Cry1F protein have gained a

significant market share in Brazil primarily owing to its higher activity against this species.⁵⁹ In spite of the efficacy of Cry1F against FAW, the current single-mode-of-action Cry1F-based Bt maize available in Brazil does not meet the high-dose requirement.⁶⁰ Field-evolved resistance to Cry1F in FAW was reported in Puerto Rico²¹ and in Brazil.²² Additionally, cross-resistance between Cry1 proteins is expected to occur in FAW, based on receptor binding studies and plant bioassays.^{29–35}

Considering the myriad factors described herein, a reduction in the performance of MON 810 in controlling FAW would be expected. However, the widespread Cry1F resistance in FAW documented in Brazil²³ and the likely cross-resistance between current Cry1 proteins^{30–35} did not allow us to distinguish the specific effects of MON 810 on the detected resistance to Cry1Ab. Nevertheless, the deployment of technologies combining two or more novel insecticidal traits with no cross-resistance to the current Cry1 proteins and high activity against the same set of target pests (pyramids) is seen as an efficient and robust contribution to resistance management,^{61,62} and this strategy should be pursued in Brazil and other environments presenting similar characteristics.

ACKNOWLEDGEMENTS

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for granting a research fellowship to CO (Grant No. 312086/2013-0).

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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