MON 95379 Bt maize as a new tool to manage sugarcane borer (Diatraea saccharalis) in South America

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Abstract

Background: The sugarcane borer (SCB), Diatraea saccharalis (Fabricius) (Lepidoptera: Crambidae), is a key pest of maize in Argentina, and genetically modified maize, producing Bacillus thuringiensis (Bt) proteins, has revolutionized the management of this insect in South America. However, field-evolved resistance to some Bt technologies has been observed in SCB in Argentina. Here we assessed a new Bt technology, MON 95379, in the laboratory, greenhouse and field for efficacy against SCB.

Results: In a laboratory leaf disc bioassay, both MON 95379 (producing Cry1B.868 and Cry1Da_7) and Cry1B.868_single maize (producing only Cry1B.868) resulted in 100% mortality of SCB. The level of Cry1B.868 in the Cry1B.868_single maize is comparable to that in MON 95379 maize. However, the Cry1Da_7 protein does not have high efficacy against SCB, as evidenced by < 20% mortality on Cry1Da_7_single leaf tissue. Total (100%) mortality of SCB in a Cry1B.868_single tissue dilution bioassay indicated that Cry1B.868_single maize meets the criteria to be classified as a high dose. Similar median lethal concentration (LC50) values were observed for MON 89034-R and susceptible SCB strains exposed to Cry1B.868 protein. MON 95379 also controlled SCB strains resistant to MON 89034 (Cry1A.105/Cry2Ab2) and Cry1Ab. Under field conditions in Brazil and Argentina, MON 95379 maize plants were consistently protected from SCB damage.

Conclusion: MON 95379 maize will bring value to maize growers in South America by effectively managing SCB even in locations where resistance to other Bt-containing maize technologies has been reported.

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

Keywords: SCB; insect resistance management; Bt plant; Cry1B.868

1 INTRODUCTION

The sugarcane borer (SCB), Diatraea saccharalis (Fabricius) (Lepidoptera: Crambidae), is a key lepidopteran maize pest in South America, primarily in Argentina,1,2 where the economic losses due to SCB injury to maize were estimated at approximately US$170 million annually.3 Neonate SCB larvae typically feed on the leaves of maize plants, and after molting to second instar they enter the stem, producing characteristic tunneling of the maize stalks.4 This injury to the stalk ultimately disrupts the flow of water and nutrients, impacting yield potential and increasing the frequency of stalk lodging.5 Because SCB larvae mainly feed within the stalks of maize plants, the efficacy of foliar insecticides to manage this species is limited.4 Bacillus thuringiensis (Bt) maize plants, which produce insecticidal proteins derived from the naturally occurring soil bacterium Bacillus thuringiensis (i.e. Cry1Ab, Cry1F, Cry1A.105 and Cry2Ab2), provided superior management of SCB relative to foliar insecticide sprays and were highly adopted by farmers in Argentina.5–7 However, the evolution of insect resistance...
resistance to Bt crops is considered the main challenge to the sustainable use of these technologies. In fact, SCB resistance to Cry1F and Cry1A.105 proteins, which reduces the field efficacy of TC1507- and MON 89034-based Bt maize technologies targeted against this species, has been documented but is currently confined to the San Luis Province in Argentina. Many of the cases of insect resistance to Bt crops have been associated with a limited ability to kill heterozygous individuals in populations of the target pests (i.e. low dose) and or with poor compliance with refuge planting recommendations. Reducing the fitness of heterozygous individuals through the deployment of Bt plants carrying high concentrations of effective Bt protein(s), is acknowledged to be a key factor for successfully managing insect resistance to Bt crops. Planting a structured refuge with non-Bt maize would sustain a population of susceptible insects, which are expected to mate with the rare resistant insects emerging from a Bt field. Structured refuges can be defined as a deliberately planted non-Bt crop in association with the Bt crop with placement and size recommendations. These matings reduce the likelihood of a resistant insect to pass along the resistance trait to its offspring, thus delaying the evolution of field-evolved resistance in the population of the target pest. Poor compliance with structured refuge recommendations is frequently understood as the main driver of the SCB resistance cases detected in San Luis, Argentina. The SCB resistance case in San Luis has been well managed through an effective partnership among farmers, the biotechnology and seed industries, and regulators in Argentina. Nevertheless, the limited number of effective Bt modes of action for SCB (i.e. Cry1Ab and Vip3A protein; Cry2Ab2) has had a direct impact on the capacity of farmers to effectively manage this pest in Argentina, particularly in the affected region of San Luis. MON 95379, an insect-protected maize event, was developed to produce the insecticidal proteins Cry1B.868 and Cry1Da_7, which protect against feeding damage caused by targeted pest Lepidoptera. Cry1B.868 is a chimeric protein comprising domains I and II from Cry1Be (Bt), domain III from Cry1Ca (Bt subsp. aizawai) and the C-terminal protoxin domain from Cry1Ab (Bt subsp. kurstaki). Cry1Da_7 is a modified Cry1Da protein derived from Bt subsp. aizawai. Results from Wang et al. and Horikoshi et al. indicated that MON 95379 provides an effective tool to manage fall armyworm (Spodoptera frugiperda Lepidoptera: Noctuidae) resistant to Cry1 and likely Cry2Ab2 proteins in South America. Our goals were to (i) evaluate the efficacy of Cry1B.868 and Cry1Da_7 against SCB and (ii) assess the value of MON 95379 for management of SCB resistance in South America.

2 MATERIALS AND METHODS

2.1 Efficacy of MON 95379, Cry1B.868 and Cry1Da_7 against SCB in leaf disc bioassays

Leaf disc bioassays were performed in 2020 with a susceptible strain of SCB susceptible to MON 89034 maize (MON 89034-SCB BRZ, Table 1) maintained in the laboratory for > 50 generations without exposure to Bt proteins or infusion of wild genes in Brazil. MON 95379 (producing Cry1B.868 and Cry1Da_7), Cry1B.868_single, Cry1Da_7_single and non-Bt, all in the same genetic background, were planted in a greenhouse at Santa Cruz das Palmeiras, SP, Brazil. For bioassays, the newest completely expanded leaves were excised at the V4–V5-growth stage of maize plants and kept in a refrigerator until use on the same day. Leaf discs of 1.8 cm diameter were cut using a metallic cutter and placed into 128-well bioassay trays (BIO-BA-128; CD International Inc., Pitman, NJ, USA) containing a 2% agar–water solution (0.5 mL per well). One neonate SCB larva (< 24 h old) was placed in each well of the tray using a fine paint brush, and the tray was sealed with plastic adhesive (BIO-CV-16; CD International Inc.) that allowed air exchange. Trays were placed in a climatic chamber at 25 ± 2 °C, 70 ± 10% relative humidity (RH) and 14:10 h (light/dark) photoperiod. For each maize line, eight replicates of 16 larvae were tested, for a total of 128 larvae per line. After 5 days, leaves were replaced every 2 days with the corresponding treatment. Larval mortality was recorded at two time points, 7 and 14 days; larval instar and weight were recorded at 14 days. The total number of tested and dead insects were used to estimate the 95% confidence interval (CI) for the probability of mortality, according to a binomial distribution. Data analysis was performed using the function binom.probit from the package binom in R statistical software (R version 4.0.2). Percent mortality scores were considered significantly different when their 95% CI did not overlap. Larval instar and weight from the survivors were compared using a t-test (P ≤ 0.05), using JMP software.

2.2 Tissue dilution to assess dose of Cry1B.868 necessary to control SCB

The susceptible strain of SCB used in this study was received from Louisiana State University, Baton Rouge, LA, USA (SCB-SS-2009, Table 1), which was susceptible to MON 89034 and MON 810 maize. Bioassays were performed with lyophilized and powdered maize leaf tissue producing Cry1B.868. The bioassay was performed in 2018 in Chesterfield, MO, USA. The amount of lyophilized tissue was weighed in 50-ml centrifuge tubes using a fresh/dry correlation of each sample to estimate the amount that, when incorporated into artificial diet, would result in 8x, 16x, 25x, 32x and 64x dilutions relative to fresh tissue (125, 62.5, 40, 31.25 and 15.63 mg of lyophilized tissue per milliliter of each 50-ml centrifuge tube, respectively). For the dilution calculation, it was assumed that 1 mL of fresh leaf tissue is 1 g and the lyophilization process results in 80% weight loss, resulting in a fresh: dry correlation of 5:1. An 8x dilution relative to fresh tissue of negative isoline control was also prepared to control for potential maize tissue effects. Artificial diet (Southland Products Inc., Lake Village, AR, USA) was prepared according to the manufacturer’s instructions except that the amount of water was reduced by 20%. The diet was placed in 500-ml squeeze bottles that were kept in a hot-water bath set at 48–56 °C. The diet was incorporated at a 4:1 diet/sample ratio and mixed using a vortex. The final dilutions in the diet were 25, 12.5, 8, 6.25 and 3.13 mg of lyophilized maize tissue per milliliter of artificial diet, respectively. Diet (1 mL per well, 16 wells per treatment replicate) was pipetted into a 128-well C-D International tray using a repeater pipette. The diet was allowed to cool before infesting. One SCB neonate was placed in each well with a fine-tip paint brush and then sealed with a ventilated self-adhesive lid. The assay was incubated at 27 °C, 60% RH, in total darkness. Assays were evaluated 7 days post-infestation, when mortality and the instars of any living larvae were determined. The bioassays were replicated three times with 16 larvae per replicate except for the 8x dilution, which had two replicates of 16 individuals. Molt inhibition (dead + first instar) data were analyzed using the function binom.probit from the package binom in R statistical software (R version 4.0.2), as previously described.
2.3 Cross-resistance evaluation of a MON 89034-R SCB strain to Cry1B.868 in diet-overlay bioassay and leaf disc bioassay

2.3.1 Diet-overlay bioassay
The SCB-susceptible laboratory strain used in this bioassay was maintained on artificial diet for 46 generations without exposure to Bt proteins or infusion of wild genes in Argentina (Table 1). The MON 89034-resistant SCB strain (MON 89034-R SCB) was sampled in Quines, SL, Argentina and maintained in the laboratory for 37 generations until use in the bioassay (Table 1). For every generation, the MON 89034-R strain was reared on MON 89034 maize tissue for the first 14 days and then transferred to artificial diet without Bt protein until the pupal stage. In preliminary 4-day leaf disc assays, the mortality of insects from this resistant strain was 15.8 ± 4.5% on MON 89034 × MON 88017 and 0.0 ± 0.0% on non-Bt maize. A laboratory strain of SCB susceptible to MON 89034 was used as reference (MON 89034-SS SCB ARG). MON 89034 maize produces two Bt proteins with activity against Lepidoptera, Cry1A.105 and Cry2Ab2, and MON 88017 maize produces a modified cry3Bb1 gene from Bt subsp. kumamotoensis. This gene encodes the protein Cry3Bb1, which protects maize plants against feeding injury by corn rootworm larvae (Diabrotica spp.) but has no activity against SCB.

Diet-overlay bioassays were performed in 2020 in Argentina with purified Cry1B.868 protein (1 mg mL⁻¹) produced by Bayer Crop Science US (Chesterfield, MO, USA). The efficacy of Cry1Da_7 maize against SCB was low (see Section 3), so this experiment was done only for Cry1B.868. The Cry1B.868 protein was stored at −80 °C until use. Cry1B.868 protein was diluted in buffer [25 mmol L⁻¹ sodium chloride (NaCl), 25 mmol L⁻¹ carbonate, pH 10] to produce concentrations in the final overlay ranging from 1.56 to 100 ng cm⁻². The protein solutions were pipetted at a rate of 30 μL per well into a 128-well tray containing 1 mL of artificial diet per well. Buffer without protein was used for the control. Each well was infested with one neonate SCB larva, for a total of 64 larvae tested per concentration of Cry1B.868. Trays were incubated at 27 ± 2 °C, 70 ± 10% RH and 14 h:10 h (light/dark) photoperiod. Larval mortality was recorded after 14 days. Larvae that did not respond to a slight touch with a paint brush were considered dead. The results from this concentration–response bioassay was used to estimate the median lethal concentration (LC₅₀) and 95% CI using probit analysis in SAS 9.19

2.3.2 Leaf disc bioassay
For the leaf disc bioassay to evaluate cross-resistance, the same SCB strains described in Section 2.3.1 were used. MON 95379 (Cry1B.868/Cry1Da_7), Cry1B.868_single and non-Bt, all in the same laboratory colony, were used. MON 89034-SS SCB BRZ colony was used as a reference. MON 95379 maize produces Cry1B.868/Cry1Da_7, Cry1B.868_single and non-Bt. This experiment was conducted in 2020 in Argentina. (MON 95379 Cry1B.868/Cry1Da_7), Cry1B.868_single and non-Bt, all in the same laboratory colony, were used. MON 95379 maize produces Cry1B.868/Cry1Da_7, Cry1B.868_single and non-Bt. This experiment was conducted in 2020 in Argentina.

Table 1. Description of Diatraea saccharalis strains used in the experiments

| Strain            | Location | Characteristic          | Year of collection | Generation | Additional information                                      |
|-------------------|----------|-------------------------|--------------------|------------|------------------------------------------------------------|
| MON 89034-SS      | Brazil   | Laboratory susceptible  | 2009               | >50        | Maintained in artificial diet without selection            |
| SCB BRZ           |          |                         |                    |            |                                                            |
| SCB-SS-2009       | USA      | Laboratory susceptible  | 2009               | >50        | Maintained in artificial diet without selection           |
| Cry1Ab-R SCB      | USA      | Cry1Ab resistant        | 2009               | >50        | Colony from Louisiana State University and maintained under selection with Cry1Ab corn leaf¹⁶ |
| MON 89034-SS      | Argentina| Laboratory susceptible  | 2017               | 46         | Maintained in artificial diet without selection           |
| SCB ARG           |          |                         |                    |            |                                                            |
| MON 89034-R       | Argentina| Cry1A.105 resistant      | 2019               | 33         | Maintained on MON 89034 tissue for 14 days and then artificial diet until pupation |
| SCB               |          |                         |                    |            |                                                            |

Table 2. Efficacy of MON 95379 (producing Cry1B.868/Cry1Da_7) maize against Diatraea saccharalis (MON 89034-SS SCB BRZ colony) in laboratory leaf disc bioassays

| Material          | Protein                     | Percent mortality 7 days | Percent mortality 14 days | Number of larvae by instar (14 days) | Larval weight (mg) |
|-------------------|-----------------------------|--------------------------|---------------------------|--------------------------------------|--------------------|
|                   |                             | L2  | L3  | L4  | L5  |                           |                    |
| MON 95379         | Cry1Da_7/                    | 100 | 100 | 100 | 100 |                           |                    |
|                   | Cry1B.868                   |     |     |     |     |                           |                    |
| Cry1B.868_single  | Cry1B.868                   | 100 | 100 | 100 | 100 |                           |                    |
| Cry1Da_7_single   | Cry1Da_7                    | 10.9| 18.7| 18.7| 18.7|                           |                    |
| Non-Bt            | Non-Bt                      | 0.8 | 1.5 | 1.5 | 1.5 |                           |                    |
| Test              |                             | 0.0 | 0.0 | 0.0 | 0.0 |                           |                    |
| t-Ratio           |                             | 1.07| 4.35| 7.11| 10.1363|                         |                    |
| P                 |                             | 0.30| 0.0007| <0.0001| <0.0001|                         |                    |

Within a column, values marked with the same letter are not significantly different according to the indicated test.
same genetic background, and MON 89034 × MON 88017 were planted in a greenhouse at San Pedro, BA, Argentina in 2020. As Cry1Da_7 efficacy for SCB is low (see Section 3), this experiment was done only for Cry1B.868 single. The bioassay followed the same methodology described in Section 2.1. Mortality data were analyzed using the function `binom.probit` from the package `binom` in R statistical software (R version 4.0.2)\(^\text{17}\), as previously described.

### 2.4 Cross-resistance evaluation of Cry1Ab-R SCB strain to MON 95379 in leaf disc bioassay and whole-plant assay

Plants for the leaf disc bioassays (MON 95379, MON 89034 × MIR162 and non-Bt) were grown in a Conviron set to the following conditions: 16.8 h light/dark, 28 °C day/23 °C night (± 2 °C), 650 μmol m\(^{-2}\) s\(^{-1}\), 50 ± 15% RH. One kernel was sown per pot, in 4.5”-square pots with Berger BM6 soil (Hummert International, Earth City, MO, USA). The pots were hand-watered without fertilizer until germination, then hand-watered with fertilizer (15-5-15 + 40 g; Jack’s Professional LX Water-Soluble Fertilizer, Allentown, PA, USA) as needed throughout the remainder of the experiment. Both leaf disc bioassays and whole-plant assays were conducted in 2020 in Chesterfield, MO, USA.

#### 2.4.1 Leaf disc bioassay

The first fully expanded leaves from V4 plants were used for bioassays. Each harvested leaf was cut into eight pieces (1” × 1” each) and placed (one piece per well) into 32-well trays. Immediately after setting up the leaf pieces, the plates were infested with neonates (<16-h old) from two SCB strains (Cry1Ab-R SCB [SCB-RR-43A] and SCB-SS-2009, a related Cry1Ab-S strain), both supplied by Louisiana State University (Table 1). The Cry1Ab-R SCB strain was established from field collections in 2009 and was estimated to have a resistance ratio to Cry1Ab of >526 in a 2013 study.\(^\text{16}\) A total of 32 insects were tested for each plant material and insect genotype. The 32-well trays were covered with stick-on, breathable lids and incubated at 27 ± 2 °C, 16:8 h (light/dark) photoperiod, 45 ± 5% RH. Larval mortality was evaluated at 6 days after infestation. Mortality data were analyzed using the function `binom.probit` from the package `binom` in R statistical software (R version 4.0.2)\(^\text{17}\), as previously described.

#### 2.4.2 Whole-plant assay

In addition to the leaf disc bioassay, eight R2-stage plants per entry were infested with Cry1Ab-R SCB neonate larvae to evaluate whole-plant efficacy. Neonates (<16-h old) were suspended in corn cob grit, the mixture was calibrated so that the infestation rate was consistently six neonates per plant, and a bazooka was used to infest one shot per plant into the leaf axil of the primary ear.\(^\text{20}\) At 21 days after infestation, the maize stalks were split open along the entire length and stalk tunneling was measured. Results reported are the average of total stalk tunneling length per plant, in centimeters. Data were submitted to analysis of variance (ANOVA), and the treatment averages were compared by the Tukey test (\(P ≤ 0.05\)), using JMP software.\(^\text{18}\)

### 2.5 Field efficacy of MON 95379 maize for SCB in Argentina and Brazil under natural infestation

Argentina field trials were conducted during the 2019 (2018/2019) season at six sites: América, BA; Tacuari, BA; Urdinarrain, Er; Margarita, SF; Candelaria, SL; Cañete, TM. The Candelaria site was in San Luis, a region with reported field-evolved resistance of SCB to Cry1F and Cry1A.105 proteins. Therefore, commercial references were added to the experimental design of the trial carried out in Candelaria, where a total of five treatments (MON 95379, MON 89034 × MON 88017, MON 810, MIR162 and non-Bt maize) were planted. At the remaining Argentinian sites, the trials had only two treatments: MON 95379 and non-Bt maize. The study was established at each site with a randomized complete block design with four replications. The plot size of each treatment consisted of four rows with 10 m length, with row spacing of 0.52 or 0.70 m depending on the location. The plots were separated by two rows of conventional maize along their length. Fertilizer, irrigation, agricultural chemicals and other management practices were applied as necessary according to local agronomic practices and the purpose of the study. Damage by SCB was evaluated in the two central rows of each plot within 10 days prior to harvest (R6 stage). The number of tunnels, tunnel length (in centimeters) and live larvae (numbers of larvae ≤ 1.5 cm and > 1.5 cm in length) were evaluated by examining ten plants (five consecutive plants per row). Data from Candelaria, SL, were submitted to ANOVA, and the treatment averages were compared by the Tukey test (\(P ≤ 0.05\)), using JMP software.\(^\text{18}\) For the

![Figure 1](image1.png) **Figure 1.** Molt inhibition (dead + first instar) (95% CI) of *Diatraea saccharalis* (SCB-SS-2009 colony) in tissue dilution bioassay to assess dose of Cry1B.868. Values marked with the same letter are not significantly different due to overlap of 95% CIs. Error bars represent 95% CI.

![Figure 2](image2.png) **Figure 2.** Concentration–response curves of MON 89034-resistant (MON 89034-R SCB) and susceptible (MON 89034-SS SCB ARG) *Diatraea saccharalis* strains exposed to purified Cry1B.868 protein.
remaining sites, treatment averages were compared with a t-test 
\((P \leq 0.05)\), using JMP software.  

Brazil field trials were conducted in 2019 (2018/2019) and 2020 
(2019/2020) at six Bayer Crop Science research sites: Náo-Me-
Toque, RS; Rolândia, PR; Santa Cruz das Palmeiras, SP; Cachoeira 
Dourada, MG; Sorriso, MT; Luís Eduardo Magalhães, BA. Part of 
the same study evaluating *Spodoptera frugiperda* is available in 
Horikoshi et al.  

A total of six and four treatments were in the 
original experimental designs in the 2019 and 2020 seasons, 
respectively, which were planted in a randomized block with four 
replicates per treatment. Here we present only results compari-
sions among MON 95379, MON 89034 × MON 88017 and non-Bt 
maize. The plot size of each treatment in 2019 consisted of eight 
rows with 8-m length and a row spacing of 0.5 m. In 2020, the plot 
size was six rows with 5-m length and a row spacing of 0.5 m. The 
SCB damage was evaluated once during the R3–R5 stage. A total 
of 15 consecutive plants within one row of plants was evaluated. 

The parameters analyzed were number of tunnels, tunnel length 
(in centimeters) and live SCB larvae (numbers of larvae ≤ 1.5 cm 
and > 1.5 cm in length). Data were submitted to ANOVA and in 
case of significance \((P \leq 0.05)\) treatment averages were compared 
by least squares (LS) means contrasts。

3 RESULTS

3.1 MON 95379, Cry1B.868 and Cry1Da_7 efficacy 
against SCB in leaf disc bioassay

As described in Horikoshi et al., the leaf production of Cry1B.868 
protein in greenhouse-grown MON 95379 (producing Cry1B.868/
Cry1Da_7) and in the experimental line Cry1B.868_single was 
86 and 81 \(\mu\)g g\(^{-1}\) fresh weight (FW), respectively, whereas production of 
Cry1Da_7 in MON 95379 and in the Cry1Da_7_single was 
17 and 4.7 \(\mu\)g g\(^{-1}\) FW, respectively. SCB showed 100% mortality 
on MON 95379 and Cry1B.868_single maize tissue at 7 days

![Figure 3.](image-url)

**Figure 3.** (A) Percent mortality (95% CI) of MON 89034-R (MON 89034-R SCB) and -susceptible (MON 89034-SS SCB ARG) strains of *Diatraea saccharalis* on MON 95379 (producing Cry1Da_7/Cry1B.868), Cry1B.868_single and non-Bt leaf tissue. (B) Percent mortality (95% CI) of Cry1Ab-R (Cry1Ab-R SCB) and -susceptible (SCB-SS-2009) strains of *Diatraea saccharalis* on MON 95379, MON 89034 (Cry1A.105/Cry2Ab2) × MIR162 (Vip3Aa20) and non-Bt leaf tissue. Values marked with the same letter within each SCB strain type are not significantly different due to overlap of 95% CIs. Error bars represent 95% CI.
Mortality on Cry1Da_7_single was higher than on non-Bt, with 10.9% and 18.7% mortality at 7 and 14 days, respectively (Table 2).

Instar evaluation was made only for survivors on Cry1Da_7_single and non-Bt maize at 14 days. No difference was observed in the number of third-instar larvae (t-ratio = 1.07; df = 1, 14; P = 0.30) (Table 2). Cry1Da_7_single maize resulted in significantly more fourth-instar larvae than the non-Bt control (t-ratio = 4.35; df = 1, 14; P = 0.0007), while non-Bt maize resulted in more fifth-instar larvae (t-ratio = 7.11; df = 1, 14; P < 0.0001) (Table 2). The surviving larvae on Cry1Da_7_single also showed reduced larval weight compared to those on non-Bt maize (t-ratio = 10.14; df = 1, 14; P < 0.0001) (Table 2).

### 3.2 Tissue dilution to assess dose of Cry1B.868 necessary to control SCB

Bioassays with lyophilized Cry1B.868 tissue showed 100% molt inhibition of SCB at all dilutions (8×, 16×, 25×, 32×, 64×), differing significantly from the non-Bt control (Fig. 1). When exposed to artificial diet containing tissue of non-Bt maize, approximately 98% of the tested SCB larvae developed either to second (10.41%) or third instar (87.50%).

### 3.3 Cross-resistance evaluation of MON 89034-R SCB strain to Cry1B.868 in diet-overlay bioassay and leaf disc bioassay

The MON 89034-R and susceptible SCB strains both showed increasing mortality with increasing Cry1B.868 protein concentration, with 100% mortality of larvae at 100 ng cm\(^{-2}\) (Fig. 2). The estimated LC\(_{50}\) for the MON 89034-R strain was 7.81 (5.41–10.30), similar to that estimated for the susceptible strain 6.08 (4.71–7.50), with overlapping 95% CIs (Table 3). The resistance ratio (RR), calculated by dividing the LC\(_{50}\) of MON89034-R by that of the susceptible strain, was 1.28 (Table 3).

The MON 89034-R strain showed 100% mortality at 7 days when exposed to MON 95379 and Cry1B.868_single maize (Fig. 3(A)). Low mortality (23.75%) of MON 89034-R on MON 89034 × MON 88017 was observed, significantly lower than the mortality observed on MON 95379 and Cry1B.868_single maize and significantly higher than that on non-Bt maize (Fig. 3(A)). The susceptible SCB strain showed 100% mortality on the MON 95379,
Cry1B.868 single and MON 89034 × MON 88017 leaf tissue, significantly higher than that found on non-Bt maize (Fig. 3(A)).

3.4 Cross-resistance evaluation of Cry1Ab-R SCB strain to MON 95379 in leaf disc bioassay and whole-plant assay

The Cry1Ab-R and susceptible SCB strains showed 100% mortality on MON 95379 and MON 89034 × MIR162 leaf tissue at 6 days after infestation, significantly higher than that on non-Bt maize (Fig. 3(B)).

In the whole-plant assay, the Cry1Ab-R strain did not cause tunneling in MON 95379 and MON 89034 × MIR162 maize plants, differing significantly from the observed tunneling in non-Bt maize, 5.1 ± 1.3 cm (df = 3.20; F = 10.98; P = 0.0002).

3.5 Field efficacy of MON 95379 maize against SCB in Argentina

Among the six field trial locations in Argentina, three (Tacuarí, BA; Urdinarain, ER; Margarita, SF) did not show natural SCB infestation high enough to enable the detection of differences in damage between MON 95379 and non-Bt (Fig. 4). The number of tunnels and tunnel length were significantly higher in non-Bt than in MON 95379 in América, BA, and Cañete, TM (Fig. 4). The number of SCB larvae found per plant was not different between non-Bt and MON 95379 across these locations, except for the results of larvae > 1.5 cm in América, BA, which were significantly more prevalent on non-Bt maize (Fig. 4).

A higher SCB infestation level was observed in the trial located in Candelaria, SL, a region where resistance to Cry1F and Cry1A.105 proteins in SCB was previously detected. Significantly lower number of tunnels, tunnel length and number of live larvae ≤ 1.5 cm were observed for MON 95379 maize relative to non-Bt maize (Fig. 5). The number of tunnels and live larvae ≤ 1.5 cm was similar between MON 89034 × MON 88017 and non-Bt maize, while tunnel length and number of live larvae > 1.5 cm were higher on non-Bt plants (Fig. 5).

No significant differences were observed among MON 95379, MIR162 and MON 810 maize for any of the parameters evaluated (Fig. 5). Details on the results of statistical analysis are available in Supporting Information Tables S1 and S2.
3.6 Field efficacy of MON 95379 maize against SCB in Brazil

The field trials in Brazil were conducted in two consecutive seasons across six locations (Figs 6 and 7). In both years, the SCB infestations were not as high as those observed in Argentina. The maximum average tunnel length observed was 6.05 cm for non-Bt maize in Sorriso, MT, in 2020 (Fig. 7). In both 2019 and 2020, MON 95379 and MON 89034 × MON 88017 plants consistently showed less SCB damage (number of tunnels and tunnel length) than non-Bt maize plants under conditions when non-Bt plants showed measurable damage (Figs 6 and 7). No significant differences in SCB injury were observed between MON 95379 and MON 89034 × MON 88017 plants (Figs 6 and 7). The numbers of live SCB larvae were not significantly different among treatments at any of the locations evaluated in 2019 and 2020, likely due to low pest pressure (Figs 6 and 7). Details on the results of statistical analysis are available in Table S3.

4 DISCUSSION

The Cry1B.868_single maize plant tissue provided complete mortality of SCB at 7 days, whereas the Cry1Da_7_single tissue produced <20% mortality at 14 days. The results also showed complete larval mortality of SCB neonates on MON 95379 (producing Cry1B.868/Cry1Da_7) tissue at 7 days, demonstrating that the efficacy of MON 95379 against SCB was largely due to activity of the Cry1B.868 protein. According to mathematical models, in an effective Bt pyramid, each protein should kill at least 95% of
susceptible individuals with absence of cross-resistance among them.\textsuperscript{12,21} Therefore, MON 95379 by itself does not meet the criteria of an effective Bt pyramid for managing resistance in SCB. However, the tissue dilution bioassay indicated that the mortality from Cry1B.868 in MON 95379 meets the criteria of a high-dose Bt plant. The US Environmental Protection Agency (EPA) defines a high-dose plant as one that produces 25-fold the dose necessary to kill 99\% of susceptible individuals, while Caprio et al. recommended a criterion of 50-fold the necessary dose.\textsuperscript{22,23} According to both definitions, the Cry1B.868 protein produced in maize plants at a level like that in MON 95379 (i.e. in Cry1B.868\_single maize) could meet the high-dose criteria in our study. MON 95379 can be considered functionally a high-dose single-mode-of-action Bt plant to manage SCB, and it will be extremely valuable to farmers in South America, primarily in Argentina, where resistance to current commercial proteins (e.g. Cry1F and Cry1A.105) has been documented.\textsuperscript{5,6}

Bioassays with the MON 89034-R SCB strain confirmed the absence of cross-resistance between the currently available Cry1A.105 and Cry2Ab2 proteins and Cry1B.868. The dose–response curves were practically overlapping among MON 89034-R and susceptible strains, and complete mortality of MON 89034-R larvae was observed on both MON 95379 and MON 89034\_MIR162 (producing the Vip3Aa20 protein) maize in leaf disc and whole-plant assays. Results presented in Grimi et al.\textsuperscript{5} indicated the absence of cross-resistance between Cry1Ab and Cry1F, Cry1A.105 and Cry2Ab2 in SCB. Plant-tissue-based bioassay studies with fall armyworm also indicated absence of cross-resistance among these proteins.

The figure shows field efficacy evaluation of MON 95379 (producing Cry1B.868/Cry1Da_7) for Diatraea saccharalis management in Brazil in 2020 season. Within each location, values marked with the same letter are not significantly different (LS means contrasts, P ≤ 0.05). ns, no significant difference among the three genotypes. Error bars represent standard error.
including Vip3A. 14,15 Our results from plant-tissue-based/whole-plant bioassays of resistant strains demonstrated lack of cross-resistance between Cry1B.868 and Cry1A.105, Cry2Ab2 and Cry1Ab in SCB. Several studies also demonstrated that Vip3A protein has a distinct structure (and, more importantly, a distinct mode of action) relative to Cry proteins. 24–26

MON 95379 consistently protected maize plants from SCB injury in Argentina and Brazil. The field trials were carried out at multiple locations representing the major corn-growing regions and therefore should have captured the variability in susceptibility to Bt proteins in natural populations of SCB. We examined MON 95379 in a field trial at the Candelaria site in San Luis, Argentina, and this technology showed high efficacy against SCB. San Luis is a specific isolated region with a hot, semi-arid climate and use of irrigation that results in high SCB pressure and promoted field-evolved resistance of SCB to Cry1F and Cry1A.105 proteins. 3,6

Indeed, at this location the non-Bt maize showed the highest injury level observed among all the tested sites in Argentina and Brazil, and SCB injury to MON 89034 × MON 88017 plants were observed. The field result from Candelaria provided further evidence that no cross-resistance is present between Cry1F/Cry1A.105 and Cry1B.868 in SCB natural populations in the region.

MON 95379 will be an important new tool to manage SCB resistance in Argentina. During the 2013 season, unexpected injury due to SCB was detected on TC1507 (Cry1F) and MON 89034 × MON 88017 (Cry1A.105/Cry2Ab2) maize hybrids cultivated in San Luis, Argentina. 5 The affected region covers Ayacucho, a northern county covering the towns of Quines, Balde and Candelaria. Since 2014, through the effort of farmers’ associations and industry, an action plan has been developed to mitigate and contain this situation. To date, SCB resistance to Cry1F/Cry1A.105 seems to be constrained to the areas first affected in San Luis. Nevertheless, monitoring SCB resistance to Bt proteins should continue in the affected and neighboring areas as well the adoption of mitigation plans if necessary. It is important to note that best management practices adopted in the areas affected by resistance in San Luis were successful in maintaining a reasonable low level of damage to Bt maize plants, and consequently the typical yield levels for the region were maintained. 5 The most successful management practices recommended to farmers in the affected area outlined the need for (i) planting of structured refuge, with more than 80% of compliance recently achieved; (ii) proactive monitoring for SCB injury to trigger integrated pest management interventions if necessary and (iii) replacing the affected Bt maize traits with those with effective SCB control, e.g. maize hybrids containing Vip3A and Cry1Ab proteins. 5

Overall, the results from our study indicate that MON 95379 maize will be a valuable new and effective tool to manage SCB in South America.

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AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments; RJH, GF, PMD, JIC, HW, MP and KM collected data; RJH, GF, PMD, KM, SM and RFLO performed the statistical analysis; all authors interpreted the results, discussed and wrote the manuscript.

CONFLICT OF INTEREST

All of the authors are employed by Bayer Crop Science. This research was financed by Bayer Crop Science. The authors declare no additional conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

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

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