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Transgenic sugarcane expressing *Bacillus thuringiensis* Cry1Ac protein offers new possibilities for controlling the giant borer, *Telchin licus* (Drury), a pest difficult to control

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ABSTRACT

The giant borer, *Telchin licus* (Drury), is an important insect pest of sugarcane in Central and South America. This pest is difficult to control with chemical or biological control approaches due to the larvae behavior of hiding in the deepest parts of the plants. Manual scavenging and skewer killing have been the main approaches for monitoring and control of *T. licus* in Brazil, the world's largest sugarcane producer. Recently, Brazil launched the first commercial use of transgenic sugarcane expressing *Bacillus thuringiensis* (Bt) proteins to control the sugarcane borer, *Diatraea saccharalis* (F.), a primary insect pest of sugarcane in the Americas. In this study, five independent field/greenhouse tests were conducted to evaluate the larval survival of and plant injury by *T. licus* on five non-Bt and five Bt sugarcane varieties containing a single *cry1Ac* gene. The multiple field/greenhouse tests showed that Cry1Ac sugarcane was highly effective to control *T. licus*; and the high control efficiency was consistent across varieties, from immature to mature plant stages, as well as for F0 generation plants and F1 ratoons. In addition, diet-incorporated bioassays were performed to determine the susceptibility of three field-collected *T. licus* populations in Brazil to diet treated with purified Cry1Ac protein or with a 25-fold dilution of Cry1Ac sugarcane stalk tissue. The diet bioassays exhibited that the field *T. licus* populations were also susceptible to the purified Cry1Ac protein, as well as to diet treated with the 25-fold dilution of stalk tissue. The results of this study provide compelling evidence that the single-protein Cry1Ac sugarcane varieties offer new possibilities for managing the *T. licus*, a pest that is difficult to control with other current available technologies.

1. Introduction

The giant borer, also known as the banana borer, *Telchin licus* (Drury) (Lepidoptera: Castniidae), has been reported causing extensive damage to sugarcane crops in several countries in Central and South America (Silva Júnior et al., 2008; Bustillo, 2013; Lima et al., 2020). In Brazil, its first occurrence was reported in 1927 in a sugarcane mill located in Pernambuco State (Guagliumi, 1972). Since then, *T. licus* has been reported as one of the most important pest species on sugarcane, causing losses of the crop yield up to 25% in the country's north and northeast regions, including Alagoas, Amapá, Amazonas, Bahia, Maranhão, Pará, Paraíba, Rio Grande do Norte, Pernambuco and Sergipe states (Mendonça et al., 1996; Anselmi, 2008). In addition, this pest is also found in the southeastern region, São Paulo, and Minas Gerais state of the country where it is still considered a secondary pest of sugarcane (Fig. 1) (Almeida et al., 2007; CTC unpublished data).

Adults of *T. licus* are moths about 35 mm long and 90 mm in wingspan. Unusually as a moth species, adults of *T. licus* have clavate antennae and diurnal habits (Gallo et al., 2002; Gullan et al., 2007). Besides sugarcane, *T. licus* has several other plant hosts such as Musaceae, Poaceae and Orchidaceae species (Guagliumi, 1972; Mendonça et al., 1996; González and Cock, 2004). In Brazil, a few closely related cryptic but genetically isolated entities of *T. licus* have been reported; and at least three subspecies, *T. l. licus* in southeast and northeast, *T. l. laura* and *T. l. albomaculata* in Mato Grosso and Rondonia state have been identified through mitochondrial gene sequences (Silva-Brandão et al., 2013) (Fig. 1). In addition, other subspecies were identified by genetic variation within the species *T. licus* and *T. atymnius,* affecting

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sugarcane and alternative hosts in Colombia (Aya et al., 2022).

The life cycle of *T. licus* takes about 180 days (Gallo et al., 2002; Gullan et al., 2007). The greater activity of adults and oviposition occurs at the warmest period of the day. Eggs are laid on the ground, close to the base of sugarcane plants. After hatching, larvae start feeding at the base of plants near to the soil, causing galleries towards the rhizomes and stalks (Guagliumi, 1972) (Fig. 2). Later stage larvae can reach up to 8 cm long with milky white color (Fig. 3). The pupae are formed inside pupal chambers prepared with plant fibers by mature larvae. The pupa stage lasts between 30 and 45 days. The adults, when emerging, come out through the holes made by the immature and have an average longevity of 10 days (Guagliumi, 1972; Almeida and Arrigoni, 2009). The large extension of stalk galleries and giant holes for the formation of pupal chambers weaken the plants, harm the sprouting of ratoons, reduce the germination capacity of ratoons, decrease plant masses, and even lead to the death of plant clumps (Fig. 3). In addition, the giant adult emergence holes allow the invasion of microorganisms associated with 'red hot' disease (*Colletotrichum falcatum* and *Fusarium moniliforme*), which causes sucrose inversion, affecting sugar and ethanol production (Guagliumi, 1972; Viveiros, 1989; Gallo et al., 2002).

Brazil plants approximately one third of the world's 26 million hectares of sugarcane annually planted area (FAO, 2018; OECD-FAO, 2019). During the last decades, Brazilian sugarcane encounters increasing infestations of *T. licus*. Traditionally, Brazilian sugarcane growers used fire to remove sugarcane leaves before harvesting (Bernhardt et al., 2000; Arbex et al., 2007; Leal et al., 2013). In recent years, Brazil adopted a 'Green Cane' harvesting system which has greatly changed Brazilian sugarcane ecosystems (Ma et al., 2014; Chagas et al., 2016; Lemos et al., 2019). Studies have shown that the absence of fire in the 'Green Cane' harvesting systems favors the survival and reproduction of *T. licus* and the remaining plant straws in the field with 'Green Cane' harvesting can provide hiding for this pest (Dinardo-Miranda and Fracasso, 2013). More importantly, in sugarcane fields, *T. licus* larvae hide in the deepest parts of plants (Fig. 3), and thus, the access by predators/parasitoids is difficult. Application of chemical and biological insecticides is very inefficient (Figueirêdo et al., 2002; Weng et al., 2011; Santos et al., 2017; Silva Júnior, 2019; Santos, 2021; Pabón-Valverde et al., 2022). To date, manual scavenging and skewer killing have been the main methods for monitoring and control of *T. licus* in Brazil, which are very expensive with high labor costs (Gallo et al., 2002; Silva Júnior et al., 2008)

Adoption of transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins has been an effective biotechnological tool for control of several major pest species in maize, cotton, and soybean (Srikanth et al., 2011; Cristofoletti et al., 2018). In 2017, Brazil launched the world's first commercial use of transgenic sugarcane expressing Cry1Ab protein to control a primary sugarcane pest, the sugarcane borer, *Diatraea saccharalis* (F.) and in the following years, three other varieties were launched, expressing a single gene *cry1Ac* (de Oliveira et al., 2022). In Bt sugarcane planting, we evaluated that the occurrence of *T. licus* in Bt sugarcane fields, especially in the fields planted with Cry1Ac sugarcane, has been rare including the areas where occurrence of *T. licus* on non-Bt sugarcane were common (Table S1 – Supporting information: Field surveys of insect occurrence of and plant injury by *T. licus* on commercial non-Bt and Bt sugarcane varieties during 2022 and 2023 crop seasons at four locations in Tocantins and Bahia, Brazil), indicating that transgenic Bt sugarcane varieties are effective to control *T. licus*. To evidence this observation, multiple greenhouse/field tests and laboratory bioassays were conducted in this study to evaluate if the transgenic sugarcane containing a single *cry1Ac* transgene can be an effective

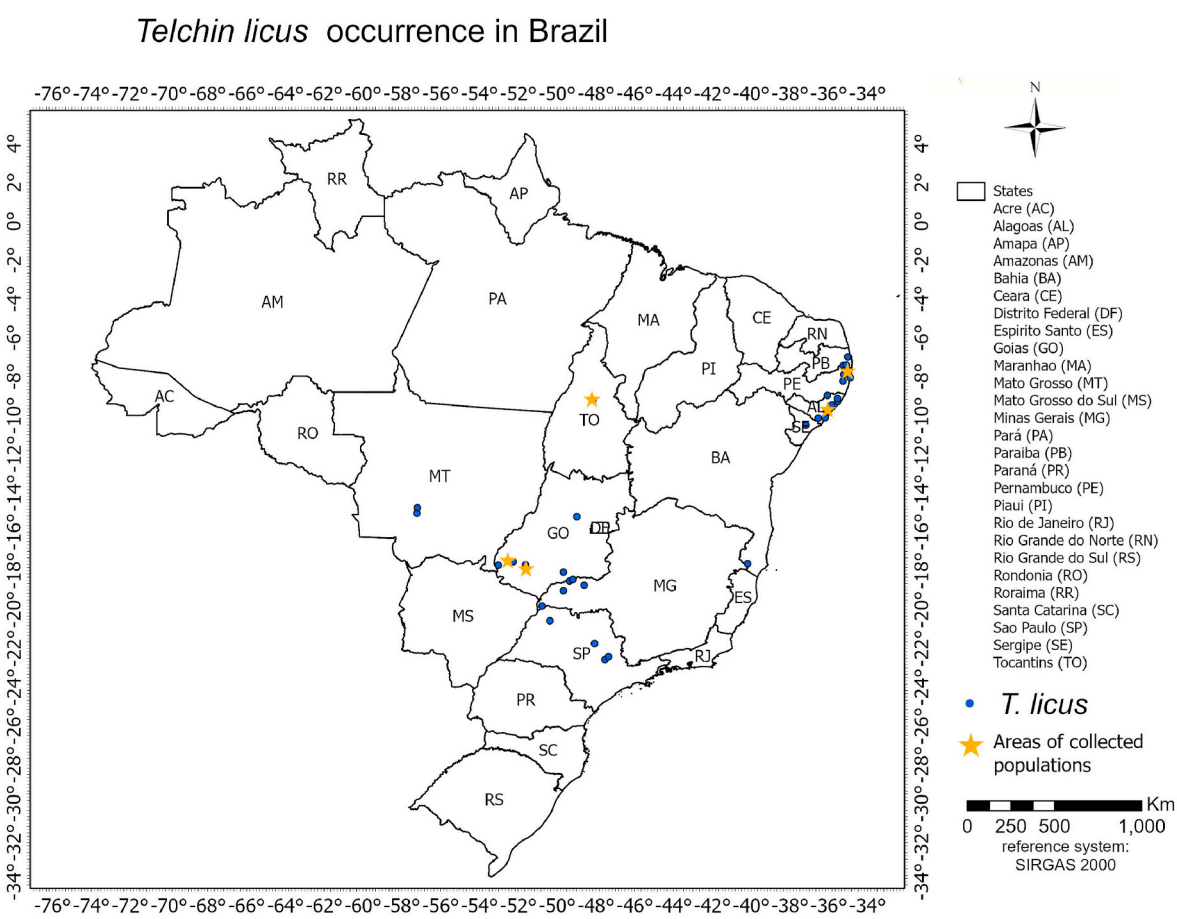

Fig. 1. Map indicating *T. licus* occurrence in Brazil (blue dots) and localities of collected *T. licus* populations used in the study.

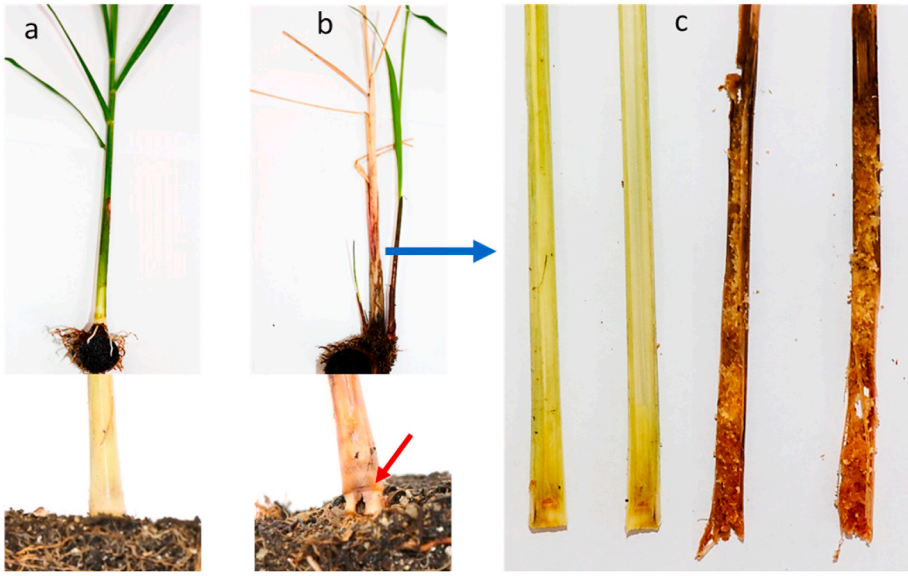

Fig. 2. Sugarcane seedlings developed from vegetative buds on stalk internodes: a: no injury; b: injured by *T. licus* as pointed by the red arrow; c: longitudinal sections of a damaged plant and its first layer of leaves.

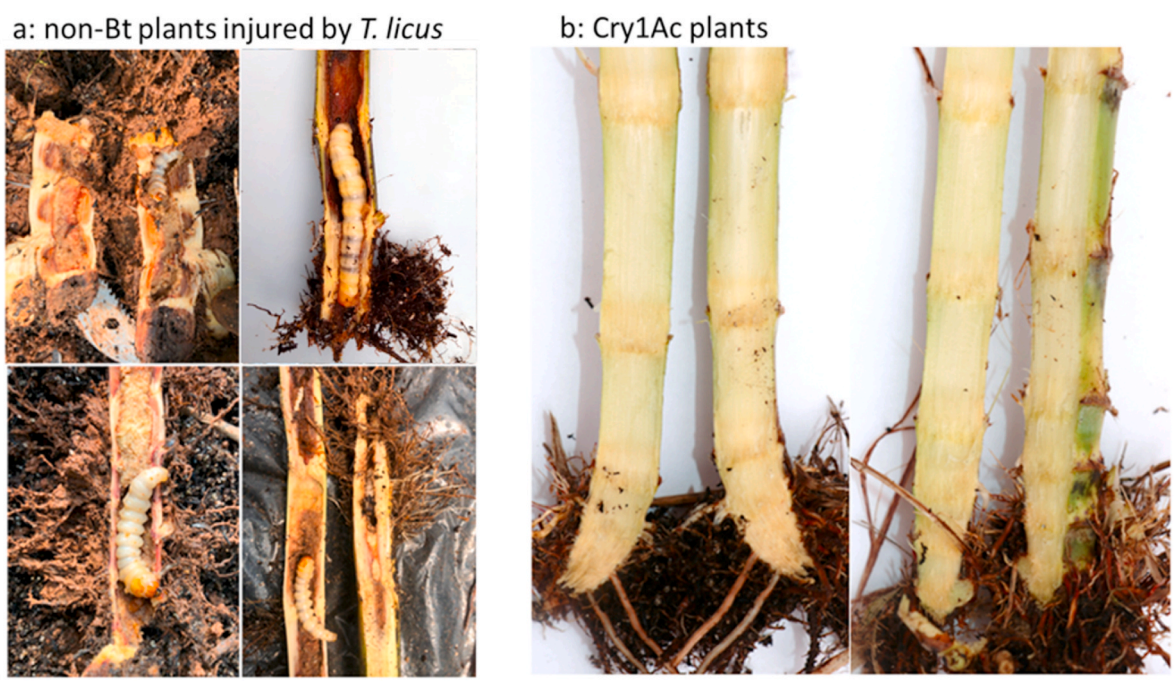

Fig. 3. Longitudinal sections of non-Bt greenhouse-grown mature plants (a) damaged by *T. licus* and Cry1Ac plants (b). Larvae of *T. licus* tunneling inside the deepest part of non-Bt stalks; the large extension of stalk galleries and giant holes for formation of pupal chambers weaken the plants, harm sprouting of ratoons, reduce germination capacity of ratoons, decrease plant masses, and even, lead to death of plant clumps.

approach for managing *T. licus* in Brazil. Documenting if Bt sugarcane varieties are effective against *T. licus* should open new possibilities for controlling this hard-to-control pest.

2. Materials and methods

2.1. Source of T. licus populations used in the study

During 2020–2022, six field collections of *T. licus* adults were performed in commercial non-Bt sugarcane fields at five locations in four states of Brazil (Table S2 - Supporting information: Field collections of *T. licus* adult moths used in this study; Fig. 1). In each collection, 40 to 309 adult moths were collected using standard insect nets. The moths from each collection were placed in a bug dorm (100 cm length x 45 cm width and 55 cm high). A cup containing distilled water was put inside the center base of the dorm to provide water for the moths. The adult dorm was placed in an insect rearing room (28 ± 4 °C, 60 ± 10% RH, and 12:12h, light:dark photoperiod) for egg-laying for 5 days. Eggs produced from the field-collected adults were harvested and placed in Petri dishes (6.5 cm diameter x 1.5 cm height) with moistened filter paper and kept in room conditions (28 ± 2 °C, 60 ± 10% RH, and a 14:10 h, light: dark photoperiod) until hatching. Newly hatched F1 neonates (<24 h old) produced from each of the six field collections were used for one or two of the seven tests described below (Table S2 -

Supporting information: Field collections of *T. licus* adult moths used in this study).

2.2. Sources of sugarcane varieties and Cry1Ac proteins

A total of seven independent tests (thereafter named Test-I to Test-VII) were conducted in the study. Test-I to Test-V evaluated larval survival of and plant injury by *T. licus* on five non-Bt and five Bt sugarcane varieties in greenhouse/field conditions. The five Bt sugarcane varieties were CTC7515BT, CTC9001BT, CTC9003BT, BT79 and BT95; all five varieties contain a same single transgene *cry1Ac*. Among these, CTC7515BT, CTC9001BT and CTC9003BT and have been commercially planted in Brazil. The five non-Bt varieties (RB867515, CTC9001, CTC9003, NBT79 and NBT95) were non-Bt isolines corresponding to each of the five Bt varieties, respectively (Table 1). Each of Test-I to Test-V consisted of two (one Bt and one non-Bt) to ten (five Bt and five non-Bt) varieties listed in Table 1. All non-Bt and Bt sugarcane varieties used in these tests were provided by the Centro de Tecnologia Canavieira (CTC: Piracicaba, SP, Brazil).

Test-VI investigated the performance of *T. licus* on diet treated with a 25-fold dilution of plant tissues of three non-Bt and three Cry1Ac sugarcane varieties (Table S2 - Supporting information: Field collections of *T. licus* adult moths used in this study). Test-VII assessed the susceptibility of three *T. licus* populations to Cry1Ac proteins in a diet-incorporated bioassay. The Cry1Ac used in the bioassays was lyophilized trypsin-activated protein with a purity of 99.9% (Wu et al., 2009). The purified Cry1Ac protein was obtained from Dr. Marianne Puztai-Carey, Department of Biochemistry, Case Western Reserve University Cleveland, OH, USA.

2.3. Larval survival of and plant injury by T. licus on greenhouse-grown immature F0 generation plants of non-Bt and Cry1Ac sugarcane (Test-I to Test-III)

Three tests (Test-I, –II, and –III) were performed to evaluate the larval survival and plant injury of *T. licus* on greenhouse-grown immature F0 sugarcane plants. Test-I consisted of four Cry1Ac sugarcane varieties (CTC7515BT, BT79, CTC9001BT and BT95) and their corresponding four isoline non-Bt varieties (RB867515, CTC9001, NBT79 and NBT95). Each of Test-II and –III also included eight varieties, among which six varieties were the same as evaluated in Test-I (excluding BT95 and NBT95), plus two new ones: CTC9003BT and CTC9003 (Table 1). In each test, vegetative buds with approximately 3-cm long stalks were individually cultured in each cell of 50-cell trays (1 plant/cell) containing commercial soil substrates (Carolina Soil, Pardinho, São Paulo, Brazil) in a CTC's greenhouse room (Piracicaba, São Paulo, Brazil) maintained at 30 ± 4 °C and 70 ± 20% RH. Each cultural cell had a size of 5 cm long, 5 cm wide and 10 cm in height. Test-I was conducted in 2020, while Test-II and Test-III were performed during 2022. Normal irrigation regimes were applied for all three plantings in the greenhouse cultures.

In Test-I, after 45 days of planting, when the plants reached the end of germination stage with *circa* 5–6 leaves, plants were transplanted individually in each cylinder-shape well of 24-well culturing trays (each well with 6 cm diameter x 10 cm height). At the same time, one F1 neonate (<24 h old) produced from field-collected adults (F0) of *T. licus* (Table S2 - Supporting information: Field collections of *T. licus* adult moths used in this study) was inoculated at the base of each plant stalk in each well in the greenhouse. In Test-II and –III, 30 days after culturing in the cells (at germination stage with 4–5 leave), plants were transferred individually to 12.5 L plastic pot with a mixture of soil and commercial substrate in a ratio 1:1 and continued to grow in the greenhouse conditions as described in Test-I. After 150 days of transplanting, when the plants reached the grand growth phase with actual cone formation and elongated, one F1 neonate was infested at the plant base in each pot.

The plant germination trays or pots in the three tests were arranged in a randomized block design with 3–4 replications for each sugarcane variety in Test-I, or 4 replications in Test-II and Test-III. Each replication consisted of 12 plants (wells) in Test-I or 6 plants (pots) in Test-II and III. Number of live larvae and plant injury status (yes/no) for each plant/tiller were checked after 14 days (Test-I), 28 days (Test-II), or 60 days (Test-III) after the neonate inoculations. Plant injury status by *T. licus* larvae was categorized into two levels: 'no injury' representing no feeding, or plant just having scraping but not exceeding the second layer of the leaves; and 'injured' for injuries passing through the second layer of the leaves, or observation of cavities, galleries and necrotic tissue, or dead heart symptoms (Figs. 2 and 3).

Data on number of surviving larvae were transformed with log (x+1), while percentage plant injury data were converted to arcsine ($x^{1/2}$) for normalization; and the transformed data were analyzed with one-way analysis of variance (ANOVA) (PROC GLM, SAS Institute, 2010). In addition, to increase the degree of freedom in the statistical analyses, data on the number of larvae and plant injury were combined across the three tests and the combined data were analyzed with a mixed model using one-way ANOVA with 'test' as a random factor (PROC MIXED, SAS Institute, 2010; Kaur et al., 2019). Treatment means within a test or in the combined analysis were separated using Tukey's HSD tests at $\alpha = 0.05$.

2.4. Larval survival of and plant injury by T. licus on field/greenhouse-grown mature F0 generation plants of non-Bt and Cry1Ac sugarcane (Test-IV)

Pre-sprouted seedlings of one Cry1Ac sugarcane variety (CTC9001BT) and its non-Bt isoline, CTC9001 (Table 1) were planted in open fields in a CTC's farm located in Fazenda Santo Antônio, Piracicaba, São Paulo, Brazil. After 120 DAP (Days After Planting), plants together with all their stalks and tillers were transplanted in 100 L plastic pots (one clump per pot) containing a mixture of soil and commercial substrate in a ratio 1:1 in a CTC's greenhouse room at the same location in Test-I. At the same time, seven F1 neonates produced from field-collected *T. licus* moths were infested in the base of plants/tillers in each pot. The planting pots in greenhouse were arranged in five blocks and each block contained a pot with Bt plants and another with non-Bt plants. The numbers of live larvae, and plant/tiller injury status in each pot were checked after 31 days of larval infestation. Data on the number of larvae per pot and percentages of plants/tillers injured were analyzed using paired Student t-test (PROC TTEST, SAS Institute, 2010) and the two pots in a same block were treated as a pair.

Table 1
Sugarcane varieties evaluated in the study.

Sugarcane variety	Abbr. in figures	Transgene	Used in tests
RB867515	NBT75	Non-Bt isoline of CTC7515BT	Test-I, Test-II, Test-III, Test-III, Test-V, Test-VI
CTC7515BT	BT75	Cry1Ac	Test-I, Test-II Test-III, Test-V, Test-VI
CTC9001	NBT91	Non-Bt isoline of CTC9001BT	Test-I, Test-II, Test-III, Test-IV
CTC9001BT	BT91	Cry1Ac	Test-I, Test-II, Test-III, Test-IV
CTC9003	NBT93	Non-Bt isoline of CTC9003T	Test-II, Test-III, Test-VI
CTC9003BT	BT93	Cry1Ac	Test-II, Test-III, Test-VI
NBT79	NBT79	Non-Bt isoline of BT79	Test-I, Test-II, Test-III, Test-V
BT79	BT79	Cry1Ac	Test-I, Test-II, Test-III, Test-V
NBT95	NBT95	Non-Bt isoline of BT95	Test-I, Test-V
BT95	BT95	Cry1Ac	Test-I, Test-V

2.5. Larval survival of and plant injury by T. licus on mature F1 ratoons of non-Bt and Cry1Ac sugarcane (Test-V)

Three Cry1Ac sugarcane varieties (CTC7515BT, BT79 and BT95) and their non-Bt isolines (RB867515, NBT79 and NBT95) were planted in an open field at a CTC's farm in the same location as described above. After 150 days of harvesting of the F0 generation plants, stalks/tillers of mature ratoons of the Bt and non-Bt varieties were collected and transplanted in 100 L plastic pots (one clump per pot) containing a mixture of soil and commercial substrate in a ratio 1:1 in a CTC's greenhouse room at the same location described above. Right after the transplanting in the greenhouse, seven F1 neonates of *T. licus* were infested in the base of plants/tillers in each pot. The planting pots in greenhouse were arranged in a randomized complete block design with five replications and one pot per replication. The number of live larvae per pot and percentage of plants/tillers injured in each pot were checked after 90 days of larval infestation. Data on number of larvae per pot and percentage of plants/tillers injured were transformed and the transformed data were analyzed in the same ways as described in Test-I to Test-III.

2.6. Diet-incorporated bioassays with 25-fold dilution of sugarcane plant tissue (Test-VI)

Stalks of three Cry1Ac sugarcane varieties (CTC7515BT, CTC9003BT, and CTC9001BT) and their respective non-Bt isolines (RB867515, CTC9003, and CTC9001) were planted in an open field at a CTC's farm as described above. After 150 days when the plants reached the grand growth phenological stage, stalks containing three basal internodes were collected and used as plant materials for the bioassays. Field-collected stalks were cleaned with distilled water and neutral detergent, and carefully sliced into small pieces. Stalk tissues from each variety were individually lyophilized and then grounded into powder. Lyophilized plant tissue powder was stored in −80 °C until use.

A diet-incorporated method was used in the bioassays. To prepare diet for the bioassays, an amount of 1 g of lyophilized sugarcane tissue powder was placed in a 200-mL plastic cup (COPAZA Descartáveis Plásticos Ltda, Içara, Santa Catarina, Brazil) containing 24 g of artificial diet (Southland Products Inc., Lake Village, AZ, USA) and mixed with a spatula for 1 min. In the bioassay, approximately 1 mL of the final diet mixture was placed in each well of 128-well trays (CD-International, Pitman, NJ, USA) using a 20 mL syringe (Becton Dickison Ind. Cirúrgica Ltda, Curitiba, Paraná, Brazil). After diet in the wells were cooled to room temperature. One F1 neonate (<24 h old) of *T. licus* produced from field-collected adult moths (Table S2- Supporting information: Field collections of *T. licus* adult moths used in this study) was placed on the diet surface in each well using a paint brush. Bioassay trays were placed in an environmental chamber maintained at 27 ± 2 °C, 60 ± 10% RH and a 14:10 h, light:dark photoperiod.

Each Bt and non-Bt sugarcane variety in a bioassay was replicated four times with 16 or 32 larvae in each replication. The number of dead larvae plus living larvae that were stunted and still in 1st and 2nd instars, as well as the body weight of surviving larvae were recorded after 10 days. Larval mortality was calculated as 'practical mortality': larval mortality (%) = 100 * total number of (dead larvae + living larvae that were stunted at the 1st or 2nd instar stage after 10 days)/total number of larvae assayed (Huang et al., 2007). Larval growth inhibition at a Bt concentration was calculated as: growth inhibition (%) = 100* (larval body weight on control diet – body weight on Bt diet)/body weight on control diet (Huang et al., 2007). Data on larval mortality and growth inhibition were transformed using arcsine ($x^{1/2}$) for normalization and the transformed data were analyzed using one-way ANOVA (PROC GLM, SAS Institute, 2010). Treatment differences were separated using Tukey's HSD tests at $\alpha = 0.05$.

2.7. Susceptibility of T. licus field populations to purified Cry1Ac protein (Test-VII)

Three *T. licus* populations were collected from two locations in Maceió, AL and Pedro Afonso, TO, Brazil during 2022 (Table S2 - Supporting information: Field collections of *T. licus* adult moths used in this study). A diet-incorporated method similarly as described in the bioassays with the 25-fold dilution of plant tissue was used to determine the susceptibility of field-collected *T. licus* populations to purified Cry1Ac protein. Each bioassay consisted of a non-Bt control and six Bt concentrations: 0.01, 0.0316, 0.1, 0.316, 1.0, and 3.16 μg/g. To prepare the appropriated Bt concentrations, purified Cry1Ac protein was dissolved in distilled water, and the Bt solutions were fully mixed with the artificial diet mentioned above. Control diet was added with the same amount of distilled water. Similarly, as described in the bioassays with diluted plant tissue, 1 mL of the Cry1Ac-treated or control diet was dispended into each well of the 128-well trays (CD-International, Pitman, NJ, USA). After the diet was cooled to room temperature, one F1 neonate (<24h after hatching) was placed on the diet surface in each well, and wells were then sealed with ventilated self-adhesive lids. For each concentration in a bioassay, there were 3–6 replications with 16 larvae in each replication. The bioassay trays were held in a growth chamber maintaining 27 ± 2 °C, 60 ± 10% RH and 12:12h, light:dark photoperiod).

The number of dead larvae and living larvae stunted at 1st instar was assessed 6 days after neonate inoculation. Larval mortality was calculated as 'practical mortality': larval mortality (%) = 100 * total number of (dead larvae + living larvae that were stunted at the 1st instar stage after the 6-day bioassay)/total number of larvae assayed. Percentage of growth inhibition of live larvae at each Cry1Ac concentration was computed using the same formula as described above. Data on the larval mortality and growth inhibition were transformed and analyzed in the same ways as described in the above bioassays with diet containing plant tissue. In addition, the observed practical mortality for each replication in a bioassay was adjusted for control mortality (Abbott, 1925). The corrected mortality data were also analyzed using the probit model (Finney, 1971) to calculate the median lethal concentration (LC_{50}) that resulted in 50% 'practical mortality' and the associated 95% confidence limits (95% CLs) for each of the three insect populations.

3. Results

3.1. Greenhouse-grown immature plants of Cry1Ac sugarcane were highly effective against T. licus

F0 generation plants of the five Bt sugarcane varieties containing a single *cry1Ac* gene at the various immature stages examined in Test-I to –III consistently showed highly effective against *T. licus*. The effect of sugarcane variety on larval survival was significant across the three tests ($F_{7,15\text{-}20} = \geq 10.22$; $P < 0.0001$) and in the combined analysis ($F_{9,67} = 32.70$; $P < 0.0001$). Across the five non-Bt varieties and three tests, 0.38 to 0.94 larvae per plant/tiller survived at the terminations of the tests; and the survivorship rates were generally not significantly different among varieties in each test and in the combined analysis (Fig. 4). In contrast, on Cry1Ac sugarcane, an average of 0.003–0.11 larvae per plant/tiller were recovered in Test-I; none living larvae were found in Test-II; and 0.0 to 0.06 larvae were detected in Test-III across all Bt sugarcane varieties examined (Fig. 4). The differences in the larval survival between non-Bt and Bt varieties were significant across virtually all treatments and all three tests, as well as for the combined analysis.

Similarly, F0 generation plants of the five Cry1Ac sugarcane varieties were highly effective in protecting plants from injury by *T. licus*. The effect of sugarcane variety on plant injury was significant across all three tests ($F_{7,15\text{-}20} = \geq 8.13$; $P < 0.0001$) and in the combined analysis ($F_{9,67} = 26.10$; $P < 0.0001$). In Test-I across the four non-Bt varieties,

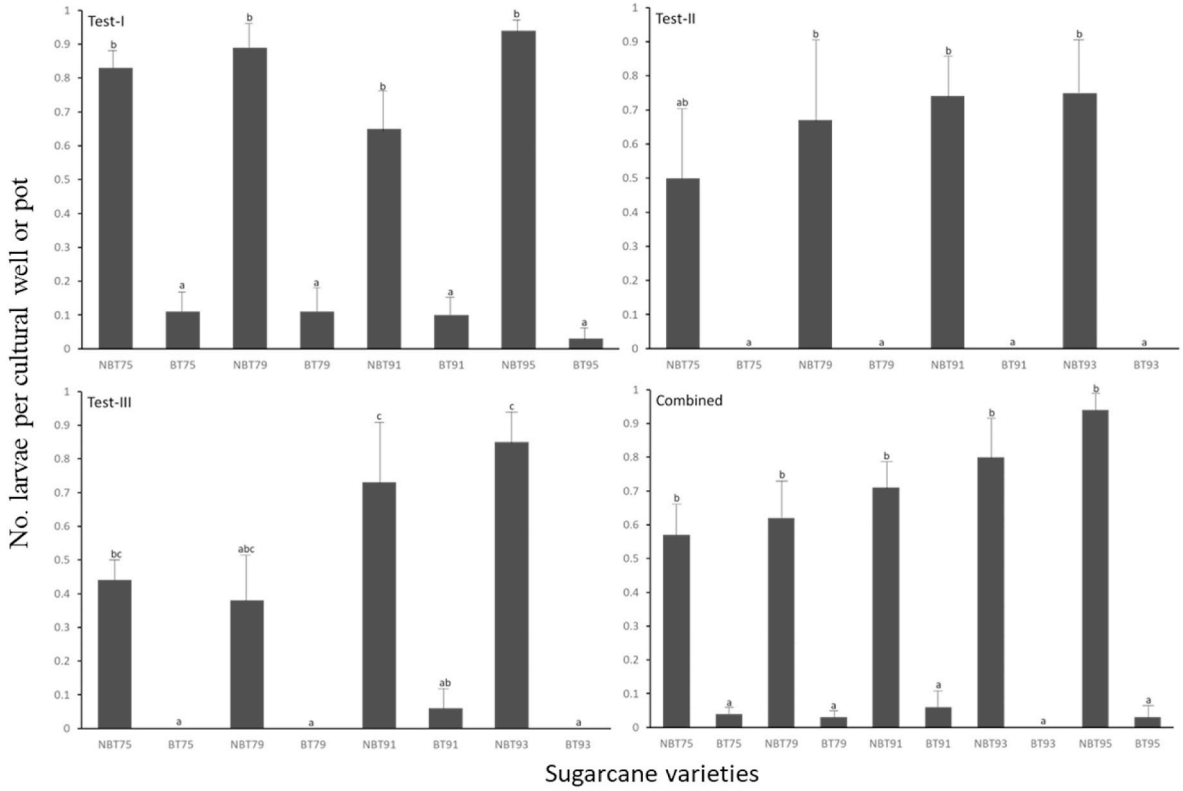

Fig. 4. Number (mean ± sem) of *T. licus* larvae per planting well (Test-I) or pot (Tests-II and –III) on 10 greenhouse-grown non-Bt and Cry1Ac sugarcane varieties in immature F0 generation plants. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

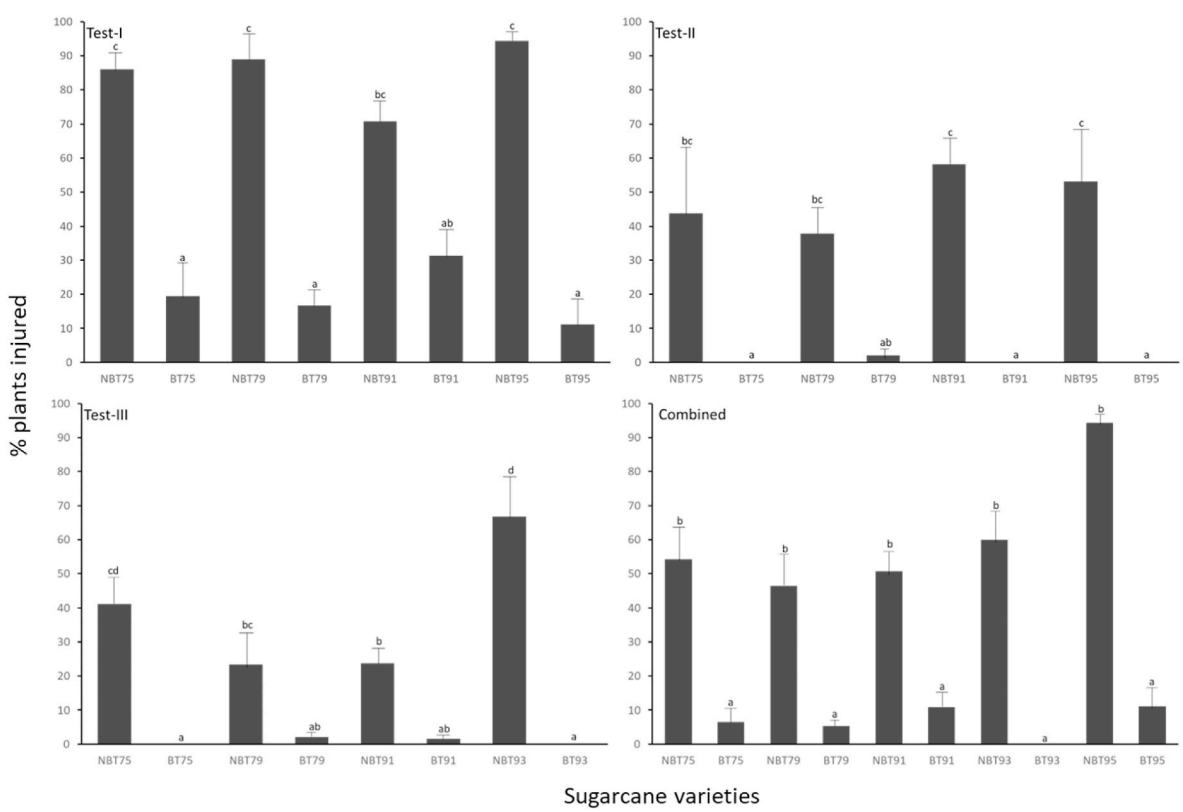

Fig. 5. Percentages (mean ± sem) of plants injured by *T. licus* larvae on immature F0 generation plants of 10 greenhouse-grown non-Bt and Cry1Ac sugarcane varieties. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

70.8–94.4% (with an average of 85.1%) plant/tillers were injured (Fig. 5). The overall plant injury rates of non-Bt sugarcane in Test-II and –III appeared to be somewhat less than that in Test-I with an average of 48.3% in Test-II and 38.6% in Test-III. In contrast, the plant injury rates on Bt plants in Test-I ranged from 11.1 to 31.3% with an average of 19.6%; and the values were only 0.0–2.1% with an average of 0.5% for Tests-II and –III, respectively (Fig. 5). The plant injury rates on Bt plants were significantly less than those of non-Bt plants across all varieties and the three tests, as well as in the combined analysis with few exceptions.

3.2. Field/greenhouse-grown mature F0 plants of Cry1Ac sugarcane were also highly effective against T. licus

Mature F0 BT91 plants expressing Cry1Ac protein in Test-IV were also highly effective against *T. licus*. After 31 days of infestation with 7 neonates per pot containing mature plants that were transplanted from open fields to the greenhouse conditions, an average of 4.6 larvae per pot were recovered on the non-Bt sugarcane variety NBT91. In contrast, no live larvae were found from BT91 variety (Fig. 6). Correspondingly, the plant injury rate of the non-Bt variety was 41.6%, while none of the Bt plants were injured by the insect. Paired *t*-test showed that the differences between the non-Bt and Bt varieties were significant for larval survival ($t = 9.2$; df = 4; $P = 0.0008$) and for plant injury ($t = 6.33$; df = 4; $P = 0.0032$).

3.3. Field/greenhouse grown mature F1 ratoons of Cry1Ac sugarcane were also highly effective against T. licus as the F0 generation plants

Again, Test-V showed that F1 mature ratoons of sugarcane plants expressing Cry1Ac protein were also highly effective in *T. licus* control. As observed in Test-IV on mature F0 plants, the effects of sugarcane variety on larval occurrence and plant injury in Test-V were all significant ($F_{5,20} = 10.88$; $P < 0.0001$ for larval survival and $F_{5,20} = 40.25$; $P < 0.0001$ for plant injury). On the three non-Bt varieties, an average of 0.8–1.2 larvae per pot were recovered; and 13.5–46.2% plants/tillers were injured by the insect, while no live larvae or injured plants were found across all three Bt varieties (Fig. 7). As shown in the F0 generation plants, the differences in larval survival and plant injury between the three non-Bt varieties in the F1 plants were not significant.

3.4. Diet treated with 25-fold diluted Cry1Ac sugarcane stalk tissue was effective against T. licus

Diet treated with 25-fold diluted Cry1Ac sugarcane stalk tissue in Test-VI was very effective against *T. licus*. The effect of sugarcane varieties on larval mortality was significant ($F_{5,18} = 200.35$; $P < 0.0001$). Larval 'practical' mortalities on diet containing non-Bt stalk tissue after 10 days were not significant among the three varieties, ranging from 25.0 to 30.0%, while the value on diet containing Cry1Ac stalk tissue was 100% for all three Bt varieties (Fig. 8). In addition, compared to larvae on the control diet, growth of all living larvae (either still in the 1st or 2nd instars) on diet treated with Bt sugarcane stalk tissue were severely inhibited with average reductions of larval body weight by 68.1–93.5% after 10 days (Fig. 8). The larval growth inhibition rates were not significantly ($P > 0.05$) different among the three Bt varieties.

3.5. Field T. licus populations in Brazil were susceptible to purified Cry1Ac protein

In the diet-incorporated bioassays with purified Cry1Ac protein, larval mortalities increased as Cry1Ac concentrations increased, and the overall trends of increase for the three insect populations were similar (Fig. 9). ANOVA showed that effect of *T. licus* populations on larval mortality was significant at 0.0316 μg/g ($F_{2,10} = 5.29$; $P = 0.0271$), but not significant at other concentrations ($F_{2,10} \leq 2.25$; $P \geq 0.1302$) (Fig. 9). At 0.0316 μg/g, the mortality (35.4%) of AL-TL was greater ($P < 0.05$) than the mortalities of TO-TL-1 (7.2%) and TO-TL-2 (10.4%) *T. licus* population. Probit analysis with the dose-mortality data showed an LC_{50} of 0.14 μg/g with 95% CLs of 0.07–0.27 for AL-TL, 0.34 with 95% CLs of 0.20–0.63 for TO-TL-1, and 0.24 with 95% CLs of 0.10–0.53 for TO-TL-2. The differences in the LC_{50} values were not significant among the three populations based on their overlapped 95% CIs.

Effect of *T. licus* populations on larval growth inhibition was significant at each of the three higher concentrations ($F_{2,10} \geq 5.03$; $P \leq 0.0308$), but not significant at the three lower concentrations ($F_{2,9\text{-}10} \leq 1.11$; $P \geq 0.3705$). In general, growth inhibitions of living larvae were also increased as Bt concentrations increased, but the slops of the increase at 0.01 μg/g and 0.0316 μg/g were greater than those observed at the four higher Cry1Ac concentrations (Fig. 9). Larval growth inhibitions reached 60.3–74.7% at 0.0316 μg/g, and 85.2–95.6% at ≥ 0.1 μg/g across insect populations (Fig. 9). The differences in larval growth inhibitions among the three insect populations at each of the three higher concentrations were significant, but the differences were small, ≤5.1% (Fig. 9).

4. Discussion

Studies have shown that Bt expression levels in plants can vary among varieties and plant growth stages (Greenplate, 1999; Adamczyk et al., 2001; Zhang et al., 2001; Adamczyk and Meredith, 2004; Wan et al., 2005) and the variations can result in different levels of control efficacy of pests in the field (Huang et al., 2002; Li et al., 2007; Wu et al., 2007; Ghimire et al., 2011; Wangila et al., 2013). Multiple field/-greenhouse tests in the current study showed that transgenic sugarcane varieties containing a single *cry1Ac* transgene were very effective against Brazilian field populations of *T. licus* and the high control efficiency was consistent across sugarcane varieties, from immature and mature plant stages, as well as for F0 generation plants and F1 ratoons. The results of the study provide compelling evidence to demonstrate that the transgenic sugarcane varieties expressing Cry1Ac protein offer

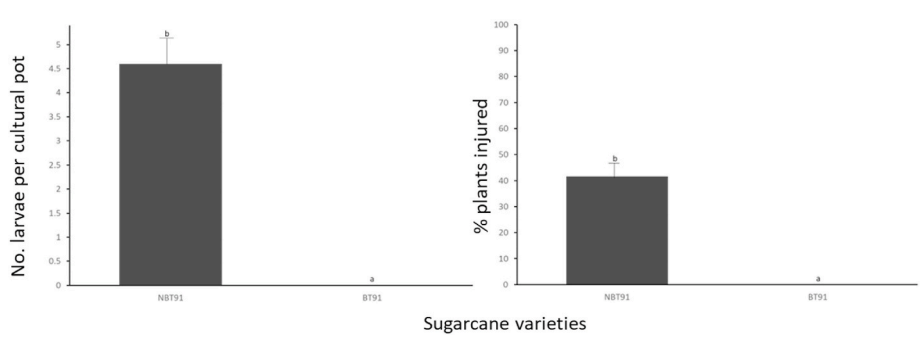

Fig. 6. Larval survival of and plant injury (mean ± sem) by *T. licus* on mature F0 generation plants of two non-Bt and Cry1Ac sugarcane varieties. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

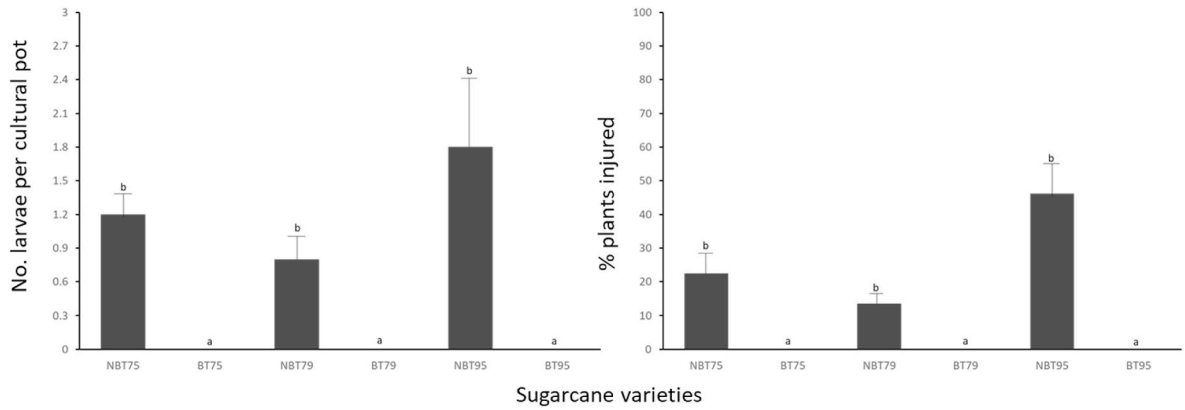

Fig. 7. Larval survival of and plant injury (mean ± sem) by *T. licus* on mature F1 generation plants of six non-Bt and Cry1Ac sugarcane varieties. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

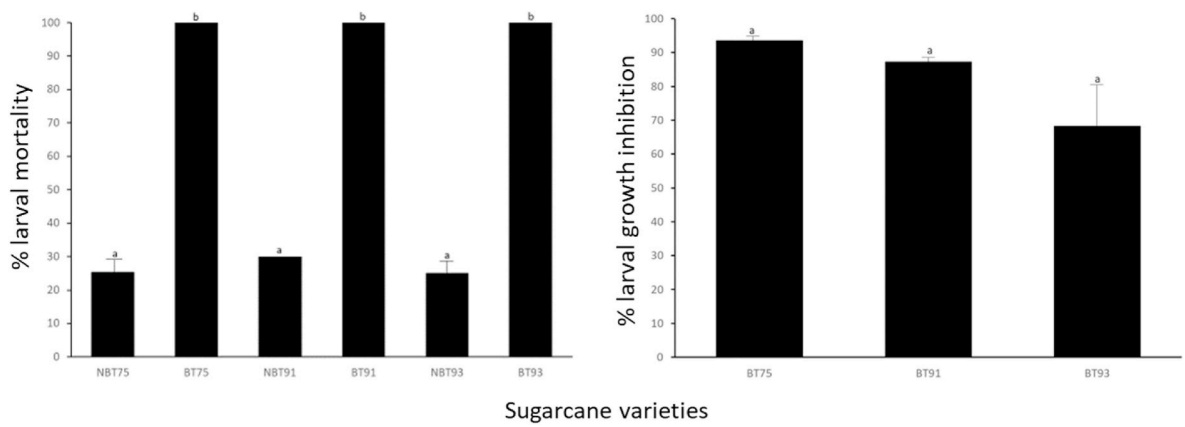

Fig. 8. Larval mortality (%) and growth inhibition (%) (mean ± sem) of *T. licus* on diet treated with 25-fold dilution of non-Bt and Cry1Ac sugarcane tissue. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

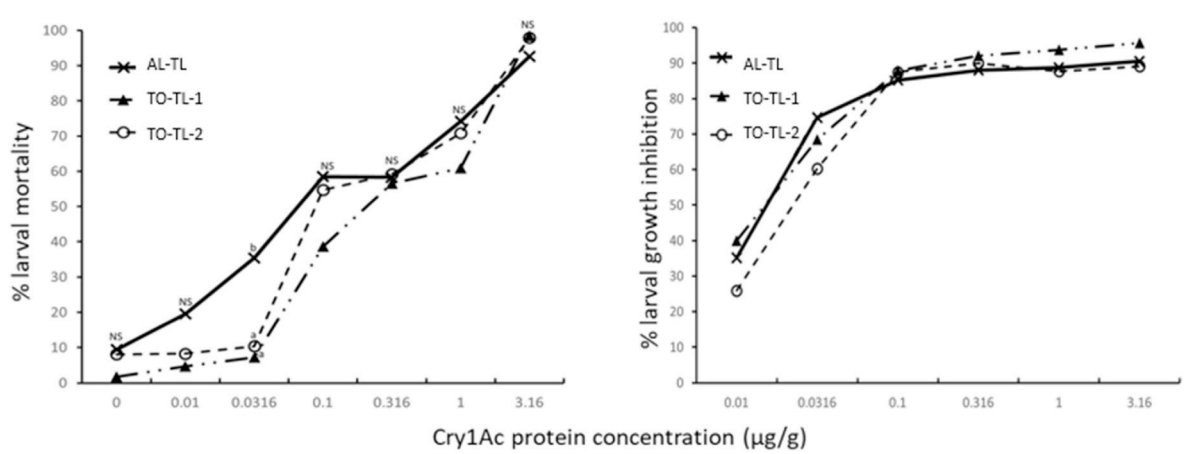

Fig. 9. Larval mortality and growth inhibition of *T. licus* on diet treated with Cry1Ac protein. Mean values followed by a same letter in a figure are not significantly different (Tukey's HSD test, α = 0.05).

new possibilities for controlling *T. licus*, a pest species of sugarcane that is difficult to control in Central and South America.

It is known that an important management strategy for sugarcane crops is adopting different cultivars (genotypes) (Moreira, 2000). This guarantees profitability, productivity and harvestability, even with edaphoclimatic factors and other stresses, in addition to disease loss prevention (Landell et al., 1999; Gagliardi and Camargo, 2009).

Bacillus thuringiensis secreted toxins, differently from chemical insecticides, are highly specific. The spectrum and the selectivity of each protein vary depending on the pest target, in other words, the susceptibility among species is frequently different within the same order, family even within the same genus (Yu et al., 2013; Rule et al., 2014; Li et al., 2021). The results presented in this work demonstrate a technological innovation that provides sugarcane with resistance to sugarcane borer, and it is also effective against the giant borer. In addition, this work makes available a range of variety options with different

characteristics to compose a sugarcane crop. For instance, CTC9003BT manifest high tillering, large number of buds, which allows for greater longevity of sugarcane fields. CTC9001BT shows precocity in restrictive environments and adaptability to mechanized harvesting. Furthermore, CTC7515BT has rusticity, rare flowering, and strait stalks, which ensures high quality in harvesting and transporting (CTC, 2023).

Currently, Bt sugarcane planted in Brazil contains either a single gene *cry1Ac* or *cry1Ab*. Field surveys by CTC have suggested that field performance of Cry1Ac sugarcane varieties in *T. licus* control apparently exceed the varieties expressing Cry1Ab (Table S1 – Supporting information, Field surveys of insect occurrence of and plant injury by *T. licus* on commercial non-Bt and Bt sugarcane varieties during 2022 and 2023 crop seasons at four locations in Tocantins and Bahia, Brazil; Márcio Tavares, personal communication). In addition, our previous F2 screen in laboratory also indicated that sugarcane varieties expressing Cry1Ac against *D. saccharalis* was more effective compared to varieties expressing Cry1Ab (de Oliveira et al., 2022). Thus, additional studies are warranted to demonstrate the possibilities for use of the transgenic sugarcane varieties expressing Cry1Ab to control *T. licus*.

Prior to the current study, there was few information available about the susceptibility of field *T. licus* populations to purified Bt proteins (Fonseca et al., 2023). The overall LC50 values of Cry1Ac, as well as the data on larval mortality and growth inhibitions observed in the diet bioassays with Cry1Ac filled well within the ranges observed for *D. saccharalis* with similar bioassay methods in the United States and Brazil (Wu et al., 2009; Zhang et al., 2013; de Oliveira et al., 2022). Efficacy data of Cry1Ac sugarcane varieties against *D. saccharalis* have been well established in Brazil (Cristofoletti et al., 2018; de Oliveira et al., 2022). Thus, the results of the diet-incorporated bioassays of this study provided additional evidence to support the use of Cry1Ac sugarcane for *T. licus* control according to information reported by Fonseca et al. (2023) which found best toxicity by Cry1Ac among Cry1Aa, Cry1Ab and Cry2Aa. In addition, the susceptibility data generated from this study could be used as baselines for resistance monitoring in the future.

Resistance to Bt crops in insect pests has become a great threat for the sustainable use of Bt crop technology. As of today, field resistance to commercial Bt crops has been documented in >20 cases (Tabashnik et al., 2023). Thus, implementing effective insect resistance management (IRM) programs is essential for the continued success of Bt corps. Effective IRM should be carefully evaluated and implemented not only for major target species, but also for secondary pests (Huang, 2021a). Many past studies have shown that one of the most important requirements for an effective IRM program is to plant 'high dose' Bt crop varieties (Huang et al., 2011, 2021b). A high dose Bt crop variety means that the plants express a high level of Bt proteins to kill >95% heterozygous resistant genotype (RS) of the pest populations in the field so that the rare resistant homozygous individuals (RR) of the pest populations can mate with susceptible populations (SS) from refuge areas (US EPA-SAP, 1998). At this moment, because Bt resistant *T. licus* populations are still not known, the 'high dose' qualification could not be assessed directly using a RS population. Therefore, an indirect criterion of 'high dose' was evaluated in the current study. The US EPA Scientific Advisory Panel (SAP) on Bt Plant-Pesticides and Resistance Management (US EPA-SAP, 1998; US-EPA, 2001) used empirical data generated from early studies to define that 'a high dose' should be 'a dose 25 times the toxin concentration needed to kill Bt-susceptible larvae'. This indirect definition of 'high dose' has been used to evaluate the high-dose status of some Bt crops in the USA and other countries (US-EPA, 2001). In the current study, based on this indirect definition, larval mortalities and growth inhibitions of three field populations of *T. licus* were evaluated in diet-incorporated bioassays with a 25-fold dilution of stalk tissues of three Cry1Ac sugarcane varieties. The bioassays showed a 100% 'practical mortality' on the diet treatment with the 25-fold dilution of Bt sugarcane stalk tissue for all three Bt sugarcane varieties tested, and these 'surviving' larvae on Bt tissue treated diet were all heavily stunted with larval growth inhibitions of ≥68.1% relative to the larvae feeding on diet treated with the same amount of the isoline non-Bt plant tissues. The results of the bioassays suggest that the 25-fold diluted concentration of the Cry1Ac sugarcane plant tissue was effective against the Brazilian *T. licus* populations. Additional studies are certainly warranted to document the high dose qualification of the Bt sugarcane varieties for *T. licus* control, but the highly control efficacies in the multiple whole plant and diet-incorporated tests observed in the current study suggest that the Cry1Ac sugarcane varieties might express a dose at least close to the defined level of 'high dose' for controlling *T. licus*.

In summary, the multiple field/greenhouse tests showed that the Bt sugarcane varieties expressing Cry1Ac were highly effective to control *T. licus*, and the high control efficiency was consistent across Bt sugarcane varieties, from immature and mature plant stages, and for F0 generation plants and F1 ratoons. Diet-incorporated bioassays in laboratory further documented that field populations of *T. licus* from Brazil were susceptible to purified Cry1Ac protein, as well as to diet treated with 25-fold dilution of Cry1Ac sugarcane stalk tissue. Data generated from this study, together with the results of our previous study (de Oliveira et al., 2022) demonstrate that the transgenic sugarcane varieties expressing Cry1Ac are effective in controlling both *D. saccharalis* and *T. licus*. Adoption of Cry1Ac sugarcane varieties to manage the two major sugarcane pests, especially *T. licus,* should be a revolutionary possibility in greatly reduce the difficulties associated in this pest management, by mitigating labor costs, elevate control efficacies, and thus considerably increase profits for the sugarcane growers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cropro.2023.106469.

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