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Neotropical maize genotypes with different levels of benzoxazinoids affect fall armyworm development

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Abstract

Plants are equipped with various defensive attributes against herbivores, including volatile and nonvolatile compounds. In maize plants, benzoxazinoids mediate resistance against some herbivores, with the most abundant being (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2H-1,4-benzoxazin−3(4H)-one (DIMBOA-Glc), and its corresponding aglucone 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA). Both compounds have been shown to interfere in the larval development of generalist herbivores but are less effective on specialist, that is, grass-feeding, herbivores. Using a Brazilian population of *Spodoptera frugiperda*, we investigated (i) the level of constitutive benzoxazinoids in Neotropical maize genotypes, that is, Zapalote Chico, Mirt 2A, Sintético Spodoptera, L3, BRS 4103 and BRS 1040 (ii) the effect of *S. frugiperda* herbivory on benzoxazinoid levels in these genotypes and (iii) the impact of the genotypes on the development of *S. frugiperda* larvae. The results showed that the six maize genotypes produce different levels of benzoxazinoids, with Mirt 2A and BRS 1040 producing constitutively higher levels of HDMBOA-Glc and DIMBOA-Glc respectively compared to the other genotypes. When feeding on BRS 1040 and Mirt 2A, *S. frugiperda* larvae took an additional week to pupate, but this effect does not affect larval survival, what was the same and high on all the genotypes (>70%). Furthermore, production of DIMBOA-Glc and HDMBOA-Glc in these genotypes was suppressed, suggesting that *S. frugiperda* larvae can alter maize defence plant responses. In summary, our results demonstrate that Neotropical maize genotypes produce varying levels of benzoxazinoids, genotypes respond differently to *S. frugiperda* herbivory and *S. frugiperda* is able to cope with secondary metabolite-based defence in Neotropical maize.

KEYWORDS
benzoxazinoids, direct defence, fall armyworm, maize genotypes

INTRODUCTION

Brazil is the third highest global producer of maize with a planting area of 17.495 million hectares (CONAB, 2019), and although it is the fourth highest exporter, most of the production is destined for internal markets. Crops are attacked by several pests causing severe losses in crop yield, predominantly the fall armyworm, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), which is present in the Americas, Africa and Asia (Cruz et al., 2010; Midega et al., 2018). Despite the intensive use of insecticides to manage this pest, populations in maize production have increased, resulting in the use of additional applications and the development of insecticide resistance. Furthermore, the

introduction of Bt maize in Brazil has contributed to a significant reduction of pest populations. Maize is a particularly important commodity crop for smallholder and family-run farms in Brazil, with 30% of the growers producing maize in areas less than 1 hectare, but due to the prohibitive cost of insecticide and Bt maize deployment, alternative interventions for *S. frugiperda* management on these farms are urgently required.

Plants are equipped with various defensive attributes against insect herbivores, including volatile and nonvolatile secondary metabolites. Resistance of plants to herbivores is generally classified as (i) antixenosis: when herbivores show a nonpreference due to a plant trait has a negative effect on herbivore behaviour (ii) antibiosis: a plant has characteristics that negatively affect herbivore development and (iii) tolerance: a plant is able to cope with herbivory without significant decline in yield (Mitchell et al., 2016; Painter, 1951). In general, plants maintain defence secondary metabolite production at low levels because of the high metabolic cost, with production being induced when triggered by herbivore feeding or by signalling from herbivore-damaged neighbouring plants. Plants that are under high herbivore pressure are more likely to display high levels of constitutive defence than inducible defences (Wittstock & Gershenzon, 2002; Zangerl & Rutledge, 1996). For maize, benzoxazinoids provide resistance against herbivores (Erb et al., 2015; Oikawa et al., 2004), with the most abundant benzoxazinoids in young maize plants being (2*R*)-2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA-Glc), and its corresponding aglucone 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA). Both DIMBOA-Glc and DIMBOA have been shown to interfere in the larval development of generalist herbivores such as *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) (Rostás, 2007) and *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) (Glauser et al., 2011). However, insects that preferentially feed on grasses appear to be less affected by benzoxazinoids; this is the case for *S. frugiperda*, which is able to detoxify these compounds (Glauser et al., 2011; Wouters et al., 2014, 2016), *Mythimna separata* Walker larvae (Lepidoptera: Noctuidae) (Sasai et al., 2009) and *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Alouw & Miller, 2014). Benzoxazinoids are produced in the cytoplasm, after which they are transported and stored in the extravacuolar space of the mesophyll parenchyma protoplasts as glucosides (Massardo et al., 1994; Wouters et al., 2016). When the destruction of plant tissues by herbivory occurs, the glucosides come into contact with β-glucosidases that have been compartmentalized in the vacuole, and are hydrolysed to aglucones, which are toxic both to herbivores and to plants. This compartmentalization avoids the formation of aglucones, protecting the plants against autotoxicity.

Understanding how plant defences are activated by herbivores and the influence of volatile and nonvolatile secondary metabolites against herbivore development is important for the selection of cultivars and genotypes with herbivore resistance (Dicke & Hilker, 2003; Erb et al., 2015; Gatehouse, 2002; Turlings & Ton, 2006). Although, the production of benzoxazinoids in maize plants and their role against insects is well documented to different insects (Glauser et al., 2011; Köhler et al., 2015; Rostás, 2007) there is no information about the effect of *Spodoptera frugiperda* Brazilian population inducing bezoxazinoids in Neotropical maize cultivars. Studies have shown that benzoxazinoids can change with the age of the plants (Cambier et al., 2000), and also the amount of these compounds in young and old leaves within plants at same age is different (Köhler et al., 2015). In this study, we investigated the impact of *S. frugiperda* herbivory on (i) benzoxazinoid production in six Neotropical maize genotypes, that is, Zapalote Chico, Mirt 2A, Sintético Spodoptera, L3, BRS 4103 and BRS 1040; (ii) the impact of the genotypes on the development of *S. frugiperda* larvae. We asked the following questions: (i) Are there differences in benzoxazinoids levels among Neotropical maize genotypes? (ii) Do *S. frugiperda* herbivory affect the benzoxazinoids levels among the maize genotypes in L3 phase? (iii) How do differences in benzoxazinoid content affect *S. frugiperda* development?

MATERIAL AND METHODS

Insects

Spodoptera frugiperda larvae were obtained from a laboratory colony maintained at Embrapa Genetic Resources and Biotechnology in Brasília, DF, Brazil (15° 46′ 46″ S, 47° 55′ 46 W). They were reared in plastic containers on an artificial diet based on beans (*Phaseolus vulgaris* L.) (Schmidt et al., 2001), in environmental chambers, with a 25 ± 2°C temperature, 65 ± 10% relative humidity and a 14 L:10 D photoperiod. Larvae were used in the experiments when they had reached the 2nd instar. Prior to all experiments, they were starved for 24 h.

Plants

Maize seeds were obtained from Embrapa Maize and Sorghum in Sete Lagoas, Minas Gerais state, Brazil (19° 27′ 57″ S, 44° 14′ 48″ W), and germinated on damp paper. After 3 days, they were transplanted into pots filled with a mixture of soil and organic substrate (at a ratio of 1:1 w/w) and maintained in a greenhouse (14 L:10 D photoperiod). The plants used in all experiments had three fully expanded leaves (∼12 days). Two groups of genotypes were assessed with differing levels of resistance to *S. frugiperda*: resistant genotypes (the landrace Zapalote Chico, Mirt 2A and Sintético Spodoptera), and moderately resistant (L3 and commercial genotypes [BRS 4103 and BRS 1040]) (Costa et al., 2006; Viana & Potenza, 2000).

EXPERIMENT 1: BENZOXAZINOID PRODUCTION IN SIX NEOTROPICAL MAIZE GENOTYPES

Maize plants subjected to *S. frugiperda* herbivory

To evaluate the effects of *Spodoptera frugiperda* larval herbivory on benzoxazinoid production in maize, the plants of genotypes Zapalote

Chico, Mirt 2A, Sintético Spodoptera, L3, BRS 4103 and BRS 1040, were placed in cylindrical glass chambers (internal volume of 10 L) with five 2nd instar *S. frugiperda* larvae, that were starved for 24 h, or with no larvae to obtain undamaged plants ($N = 6$ plants/treatment). After 22 h, the larvae were removed, and plants were immediately subjected to benzoxazinoid extraction.

Benzoxazinoid extraction

All leaves from maizeplants of each genotype and treatments described previously were harvested, immediately frozen in liquid nitrogen and then macerated in a porcelain mortar and pestle to a fine powder. The samples were weighed (100 mg), added to methanol: acetic acid (99:1, 5 ml) and sonicated for 10 min at room temperature in an ultrasonic bath. The organic phase was then transferred to another vial, and the extraction solution was added twice more to the pellet and again sonicated for 10 min at room temperature. The organic phases were combined and concentrated to 1 ml using a rotary evaporator. Afterwards, the combined organic phase was centrifuged at 6300 rpm for 20 min; the obtained supernatants were collected, filtered through a syringe filter with a hydrophilic PTFE membrane (25 mm diameter × 0.45 μm pore; Millex, Millipore) and analysed via high-performance liquid chromatography (HPLC).

Benzoxazinoid chemical analysis

An aliquot of each extract (10 ul) was analysed by HPLC (Flexar, Perkin Elmer, USA) equipped with a quaternary pump and photodiode array detector. The HPLC analyses were conducted using a reversed-phase C18 analytical column (4.6 mm diameter × 150 mm in length, with a 3 μm film). Analyses were conducted using a gradient composed of two solvents: (A) deionized water + acetic acid 0.025% and (B) methanol: isopropanol:acetic acid (95:5:0.025). The initial gradient consisted of 90% solvent A and 10% B for 1 min, followed by 50% solvent A for 11 min, and then the initial conditions for another 20 min. The solvent flow rate was 0.65 ml min^{-1}, the oven temperature was maintained at 24°C, and the total run time was 32 min. All solvents used were HPLC grade. For the detection of benzoxazinoids, a spectrophotometric detector with a tungsten lamp with a photodiode array (Waters, model 996) was used. The wavelengths of 262 and 282 nm were monitored during the analysis. For quantification, the external standard curve method was used, and the curve was constructed with different methanolic synthetic solutions of 6-methoxybenzoxazolin-2(3H)-one (MBOA) and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one DIMBOA (0.75, 1.0, 3.0, 7.5, 15, 30 and 45 μg ml^{-1}). DIMBOA was obtained following the same procedure as described by Chandra et al. (2013). The area corresponding to the compound was calculated and applied to the equation of the calibration standards to estimate the concentration of all compounds in the sample.

To confirm the chemical identification of the benzoxazinoids using liquid chromatography coupled to mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR), 90 plant extracts containing benzoxazinoids were combined and fractionated using the following method adapted from Baumeler et al. (2000). An ACE C-18 10 μm column (dimensions: 250 × 4.6 mm) was used in a DGU-20A5 Prominence Degasser (Shimadzu Corporation, Kyoto, Japan), and the mobile phase involved deionized water plus 0.025% acetic acid (solvent A) and methanol: isopropanol (95:5) plus 0.025% acetic acid (solvent B) in gradient mode. The program was as follows: 0–15 min, 10% B; 15–45 min, 40% B; 45–55 min, 75% B; 55–62 min, 95% B; and 62–65 min, 10% B. The injection volume was 1 μl, with a flow rate of 1 ml min^{-1} and run time of 65 min. The semipreparative analysis was conducted using the same conditions of the analytical analysis, but with an ACE C-18 10 μm column (dimensions: 150 mm × 12.8 mm), the injection volume was 50 μl, with a flow rate of 4 ml min^{-1}. The absorbance was monitored at 260 and 280 nm. The analysis resulted in six peaks whose maximum wavelengths were typical of benzoxazinoid compounds (Figure S1).

The six fractions collected from HPLC semipreparative analysis were initially evaporated using a rotary evaporator until 1 ml, after the fraction was dried under a nitrogen stream. These fractions were analysed by LC–MS and NMR to elucidate the structure of each compound.

LC–MS/MS analyses were performed on a Prominence system (Shimadzu Corporation, Kyoto, Japan) coupled to a tandem spectrometer (Applied Biosystems, DS Sciex, Concord, Canada) equipped with an electrospray ion source operating in negative ionization mode. The ion spray voltage was maintained at 4500 V. The turbo gas temperature was 500°C, the nebulizing gas pressure was 60 psi, the curtain gas pressure was 25 psi, the heating gas pressure was 60 psi and the collision gas pressure was 5 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion-to-product ion conversion, with MRM parameters for the benzoxazinoid ions obtained from the literature (except for the quadrupoles Q1 and Q3 *m/z* values of 418 and 372) and MRM parameters for DIMBOA optimized from infusion experiments with standards (Q1 *m/z* 210; Q3 *m/z* 149; and using the following potential declustering potential 15 V; entrance potential 8 V; cell entrance potential 12 V; collision energy 16 V; cell exist potential 4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems, Darmstadt, Germany) was used for data acquisition and processing.

^{1}H NMR spectra were acquired on a spectrometer operating at 500 MHz (Bruker Avance, Billerica, USA) equipped with a 2.5 mm selective inverse probe (SEI) and with deuterated methanol (CD_3OD) used as a solvent (~1.0 mg of sample was dissolved in ~200 μl of solvent, which was then transferred into a 2.5 mm micro-NMR tube). The ^{1}H spectra were collected via free induction decay (FID). The two-dimensional (2D) experiments were performed, and the data were processed with the software provided by Bruker. Standard pulse sequences were used for 1H-1H COSY 90°. Two-dimensional inverse hydrogen-detected heteronuclear long-range correlation ^{1}H- HMBC experiments were carried out using the software provided by Bruker. Six peaks were collected, and the NMR and LC–MS analysis confirmed these peaks, the data of which are shown as Data S1.

Chemicals

Methanol and HPLC grade water were purchased from Fisher Scientific (Leic., UK), and isopropanol (≥99.8%) and acetic acid (≥99%) were purchased from Sigma-Aldrich, (St Louis, MO, USA). Deuterated methanol (CD_3OD, 99.9% D) solvent for nuclear magnetic resonance (NMR) was purchased from Goss Scientific, (UK) and stored over activated 4 Å molecular sieves. Standards of BOA and MBOA were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and acetic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

EXPERIMENT 2: INFLUENCE OF NEOTROPICAL MAIZE GENOTYPES ON *SPODOPTERA FRUGIPERDA* DEVELOPMENT

To evaluate the influence of the six genotypes on the development of *Spodoptera frugiperda* larvae, each maize plant of the six genotypes selected received one 2nd instar *S. frugiperda* larva (with a 0.51 mm cephalic capsule) starved for 24 h. Every 3 days, the development of the larvae was monitored by measuring the cephalic capsule, (under a micrometric ocular stereoscopic microscope (Zeiss Stemi SV 11, Germany at 10× magnification) until the insects reached the pupal stage. Plants were replaced for new one each tree days. Pupal weight was measured 24 h after moulting and cuticular hardening using an analytical balance (Shimadzu ATX, Japan). The insects were reared in environmental chambers at 25 ± 2°C, 65 ± 10% relative humidity and a 14 L:10 D photoperiod. Forty replicates were conducted for each genotype.

Statistical analysis

The total amount of benzoxazinoids and individual compounds in each treatment were analysed using GLM in conjunction with a gamma distribution and an inverse link function. To evaluate the influence of all compounds in separating the maize volatile chemical profiles among treatments (genotypes), principal component analysis (PCA) was applied to the multivariate data. The PCA was performed with a variance–covariance matrix and disregarded comparison using palaeontological statistics software (PAST v.3.10). *Spodoptera frugiperda* cephalic capsule width was analysed via GLM with repeated measures, and the weight of pupae was analysed via GLM with Gaussian distribution and identity link function. To evaluate the effect of different maize genotypes on *S. frugiperda* larval survival, survival was represented by Kaplan–Meier survival curves and analysed by a log-rank test using the survival functions of the "Survival" package (April 26, 2021, version n 3.2-11, Survival R package, n.d.). All analyses were performed using the statistical program R 3.6.0.

RESULTS

Experiment 1: Benzoxazinoid production in six maize genotypes

The benzoxazinoids HMBOA-Glc, DIMBOA-Glc, HMBOA, DIMBOA, HDMBOA-Glc and MBOA were identified in all six maize genotypes evaluated in this study (Figure S1). There were significant differences when considering the mean total amount of the six benzoxazinoids identified in this study from undamaged maize plants (UDP) (ANODEV, $\chi^2 = 36.688$, df = 5, $p < 0.001$) (Figure 1), with the genotypes Mirt 2A and BRS 1040 producing higher levels of benzoxaxinoids compared to other genotypes (Figure 1; Table S1). There was no significant difference between Mirt 2A and BRS 1040 (Figure 1; Table S1). In contrast, when comparing the total amount of the six benzoxazinoids identified in this study produced by *S. frugiperda* - damaged maize plants (*Sf*-DP), there was no difference among genotypes (ANODEV, $\chi^2 = 7.221$, df = 5, $p = 0.204$) (Figure 1; Table S2). Mirt 2A and BRS 1040 produced lower amount of benzoxazinoids when submitted to 22 h of *S. frugiperda* herbivory compared to UDP of the same genotype (Mirt 2A: ANODEV, $\chi^2 = 18.689$, df = 1, $p < 0.001$; BRS1040: ANODEV, $\chi^2 = 5.929$, df = 1, $p = 0.014$) (Figure 1). For all the other genotypes, there were no differences in the total amount of benzoxazinoids produced when comparing plants that were subjected to 22 h of herbivory by *S. frugiperda* larvae and UDP of the same genotype (Sintético Spodoptera: ANODEV, $\chi^2 = 0.378$, df = 1, $p = 0.538$; L3: ANODEV, $\chi^2 = 0.106$, df = 1, $p = 0.744$; BRS4103: ANODEV, $\chi^2 = 1.657$, df = 1, $p = 0.198$ and Zapalote Chico: ANODEV, $\chi^2 = 2.373$, df = 1, $p = 0.123$) (Figure 1).

When the individual benzoxazinoids were analysed by comparing the production between UDP and *Sf*-DP-22h, there were no

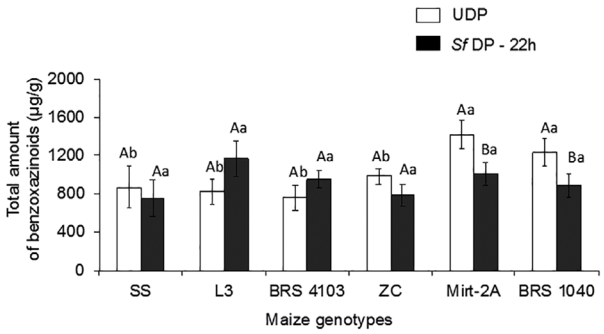

FIGURE 1 Mean total amount (µg g^{-1} plant material ± SEM) of benzoxazinoids present in two-week old undamaged maize plants (UDP) and 2nd instar *Spodoptera frugiperda*-damaged maize plants (22 h larval feeding) (*Sf* DP - 22h). Neotropical maize genotypes SS, ZC, L3, Mirt 2A, BRS 4103 and BRS 1040 were used in this study. Different capital letters indicate significant differences in the level of benzoxazinoids between undamaged and damaged plants within each genotype ($p < 0.05$). Different lowercase letters indicate significant differences in the level of benzoxazinoids between genotypes submitted to the same treatment ($p < 0.05$). Statistically significant effects were determined by GLM with Gamma distribution, deviance and contrast analysis.

differences in benzoxazinoid production for genotypes Sintético Spodoptera, Zapalote Chico, L3 and BRS 4103 (Figure 2a,b,e,f; Table S3). BRS 1040 produced higher levels of DIMBOA-Glc and DIMBOA (Figure 2c; Table S3), and Mirt 2A produced higher levels of HMBOA-Glc, DIMBOA-Glc and HDMBOA-Glc (Figure 2d; Table S3).

When the production of individual compounds on UDP were compared among the different genotypes, the results showed that the production was different for HMBOA-Glc (ANODEV, $\chi^2 = 13.857$, df = 5, $p = 0.016$), DIMBOA-Glc (ANODEV, $\chi^2 = 42.062$, df = 5, $p < 0.0001$), DIMBOA (ANODEV, $\chi^2 = 51.658$, df = 5, $p < 0.0001$), HDMBOA-Glc (ANODEV, $\chi^2 = 31.069$, df = 5, $p < 0.0001$) and MBOA (ANODEV, $\chi^2 = 18.902$, df = 5, $p = 0.002$) (Figures 2a–f). Mirt 2A produced constitutively higher levels of HMBOA-Glc and HDMBOA-Glc (Figure 2; Table S4 and Figure S2a), BRS 1040 produced constitutively higher levels of DIMBOA-Glc compared to other genotypes (Figure 2; Table S4 and Figure S2a), and BRS 4103 produced lower levels of DIMBOA-Glc and DIMBOA compared to other genotypes (Figure 2; Table S4 and Figure S2a).

When the production of individual compounds on *Sf*-DP 22h from different genotypes were analysed, differences were observed for DIMBOA-Glc (ANODEV, $\chi^2 = 229.43$, df = 5, $p < 0.0001$), HMBOA (ANODEV, $\chi^2 = 13.099$, df = 5, $p = 0.002$), DIMBOA (ANODEV, $\chi^2 = 32.259$, df = 5, $p < 0.0001$), HDMBOA-Glc (ANODEV, $\chi^2 = 12.516$, df = 5, $p = 0.023$) and MBOA (ANODEV, $\chi^2 = 72.253$, df = 5, $p < 0.0001$) (Figures 2a–f; Table S5 and Figure S2b).

To evaluate whether the production of the six identified benzoxazinoids could be specific for each maize genotype evaluated,

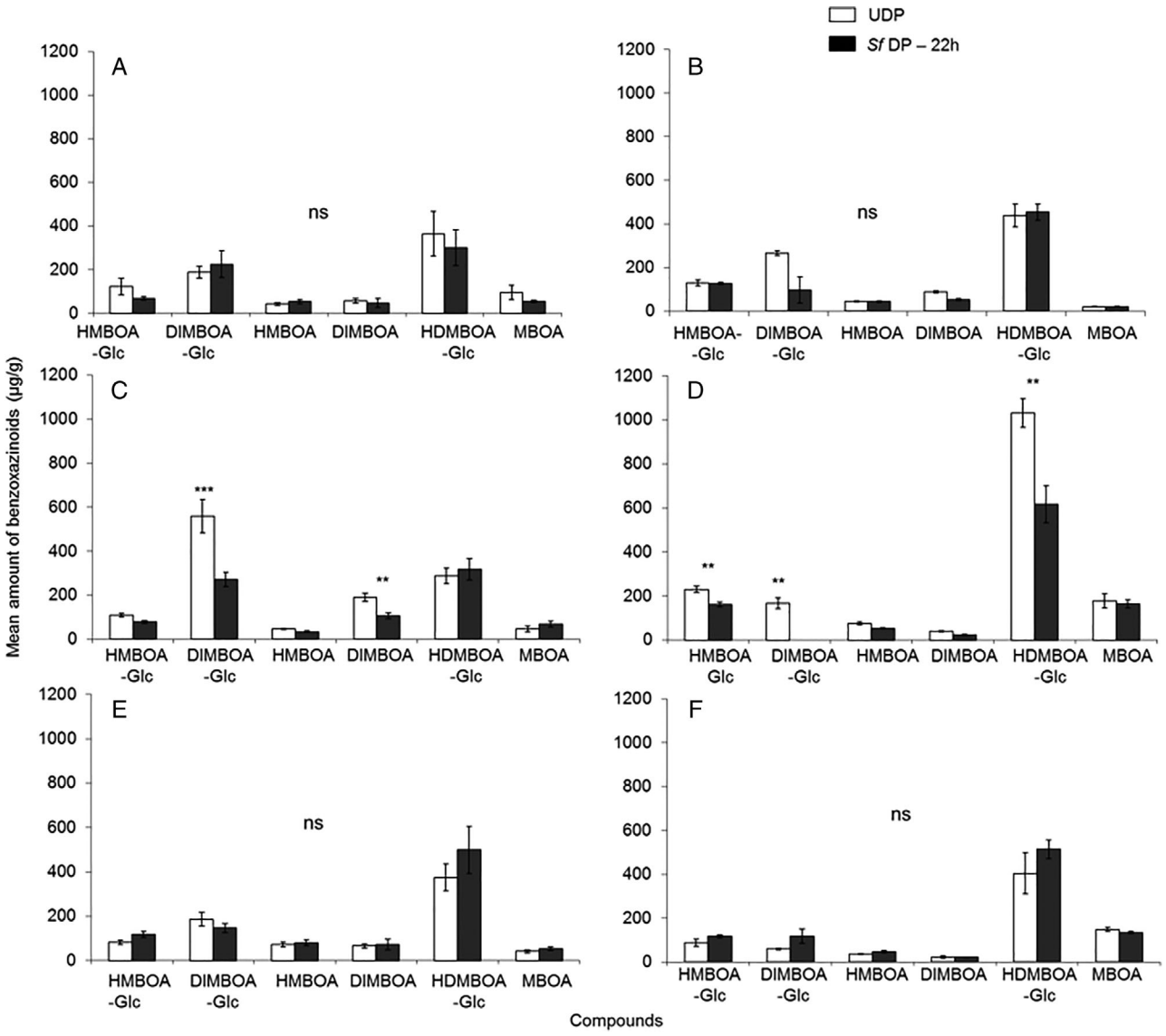

FIGURE 2 Mean amount (µg g^{-1} plant material ± SEM) of individual benzoxazinoids present in two-week old undamaged maize plants (UDP) and 2nd instar *Spodoptera frugiperda*-damaged maize plants (22 h larval feeding) (*Sf* DP - 22h). Neotropical maize genotypes were used in this study: (a) SS, (b) ZC, (c) BRS 1040, (d) Mirt 2A, (e) L3 and (f) BRS 4103. Asterisks indicate differences between undamaged plants and *S. frugiperda*-damaged plants per genotype. Statistically significant effects were determined by GLM with Gamma distribution and deviance analysis, $\alpha = 0.05$, $^{**}p \leq 0.01$ and ≥0.001, $^{***}p \leq 0.001$; ns: not significant).

multivariate analysis was applied; principal component analysis (PCA) of undamaged plants (UDP) and *S. frugiperda* - damaged plants (*Sf*-DP-22h). For all genotypes showed a clear separation of Mirt 2A and BRS 1040 in undamaged plants (Figure 3a). Whereas, when evaluating the *Sf*-DP-22h treatment is possible to observe that this separation is not so clear, indicating that the levels of the compounds get closer among the genotypes (Figure 3b). In this analysis al genotypes present the same benzoxazinoids, therefore the separation of the genotypes in PCA is based on the amounts of benzoxazinoids extracted from each genotype. The genotype BRS 1040 and Mirt 2A were separated due to a relatively high production level of DIMBOA-Glc and HMBOA-GLc, respectively (Figure 3a). No other genotypes were separated from each other.

Experiment 2: Influence of six maize genotypes on *Spodoptera frugiperda* development

S. frugiperda larval development varied when reared on six different genotypes of Neotropical maize ie Zapalote Chico, Mirt 2A, Sintético Spodoptera, L3, BRS 4103 and BRS 1040. Larvae took one extra week to pupate when reared on Mirt 2A and BRS 1040 compared to the other genotypes (Table 1). The cephalic capsule width was different between the treatments (ANODEV, $\chi^2 = 54.63$, df = 5, $p < 0.001$); larvae that fed on Mirt 2A and BRS 1040 possessed smaller cephalic widths compared to larvae fed on the other genotypes (Table 1) whilst larvae that fed on genotypes Sintético Spodoptera and L3 possessed larger cephalic capsule widths compared with larvae that fed on Mirt 2A, BRS 4103 and BRS 1040 (Table 1). In addition, larvae that fed on the Zapalote Chico genotype possessed higher cephalic capsule widths than larvae that fed on the Mirt 2A and BRS 1040 (Table 1). When comparing the cephalic capsule widths of larvae fed on Mirt 2A and BRS 1040, there was no significant difference in width (Table 1). *Spodoptera frugiperda* larval survival was high on all cultivars (more than 70%) (Figure 4), and there was no influence of maize genotypes on larval survival (Log-rank test: $\chi^2 = 9.1$, df = 5, $p = 0.1$) (Figure 4). There was no significant difference between the weight of female and male pupae fed on different maize genotypes (females: ANODEV, $\chi^2 = 2.903$, df = 5, $p = 0.406$; males: ANODEV, $\chi^2 = 8.654$, df = 5, $p = 0.123$) (Table S6).

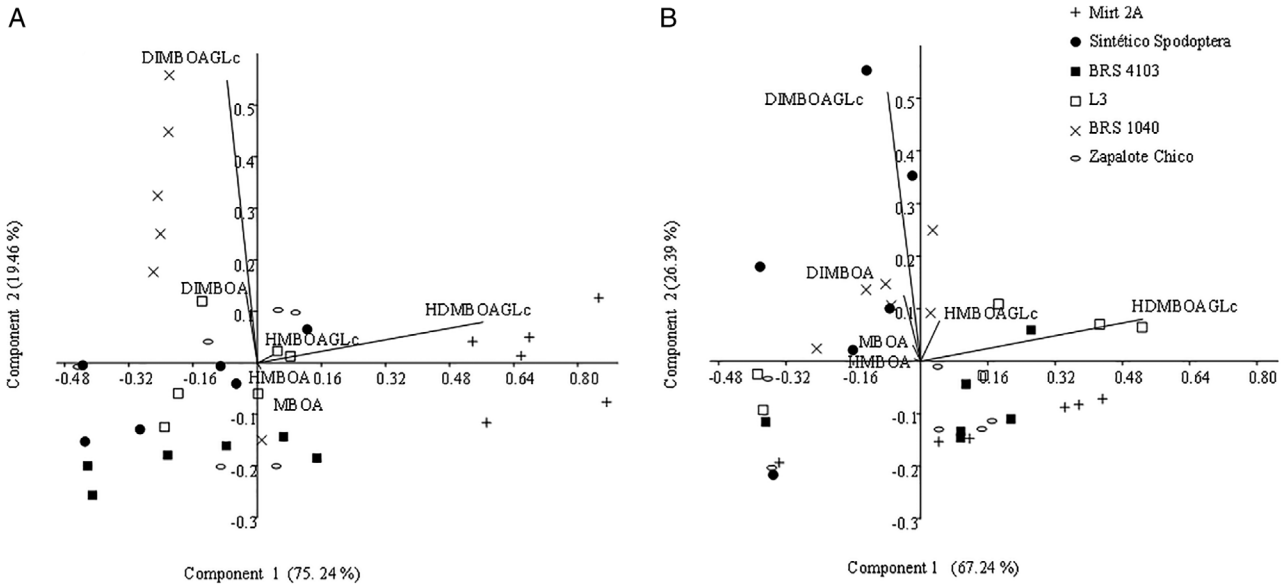

FIGURE 3 Principal component analysis (PCA) of components 1 and 2 of the benzoxazinoids emitted by (a) undamaged maize plants and (b) 2nd instar *Spodoptera frugiperda*-damaged maize plants (22 h larval feeding). Neotropical maize genotypes used in this study were: Mirt 2A, Sintetico spodoptera, Zapalote Chico, BRS 1040, BRS 4103 and L3.

TABLE 1 Mean width (mm ± SEM) of cephalic capsules of fall armyworm, *Spodoptera frugiperda*, larvae feeding on Neotropical maize genotypes (25 ± 2°C, 65 ± 10% RH and photoperiod of 14 h)

Evaluation (day)	SS	L3	BRS 4103	ZC	Mirt 2A	BRS 1040
3rd	1.55 ± 0.35^{a}	1.52 ± 0.35^{a}	1.34 ± 0.25^{bc}	1.50 ± 0.35^{ab}	1.26 ± 0.21^{c}	1.27 ± 0.21^{c}
6th	2.26 ± 0.41^{a}	2.10 ± 0.38^{a}	1.95 ± 0.30^{bc}	2.14 ± 0.40^{ab}	1.83 ± 0.28^{c}	1.85 ± 0.21^{c}
9th	2.41 ± 0.47^{a}	2.39 ± 0.41^{a}	2.69 ± 0.30^{b}	2.69 ± 0.32^{b}	2.49 ± 0.28^{ab}	2.46 ± 0.33^{ab}
12nd	—	—	—	—	2.75 ± 0.29^{a}	2.82 ± 0.36^{a}

Note: Evaluations were performed every three days until the larvae reached the pupal stage. '—' represent no data for genotypes SS, L3, BRS and ZC. The means followed by the same letter in a line are not significantly different according to the GLM test at the 5% level.

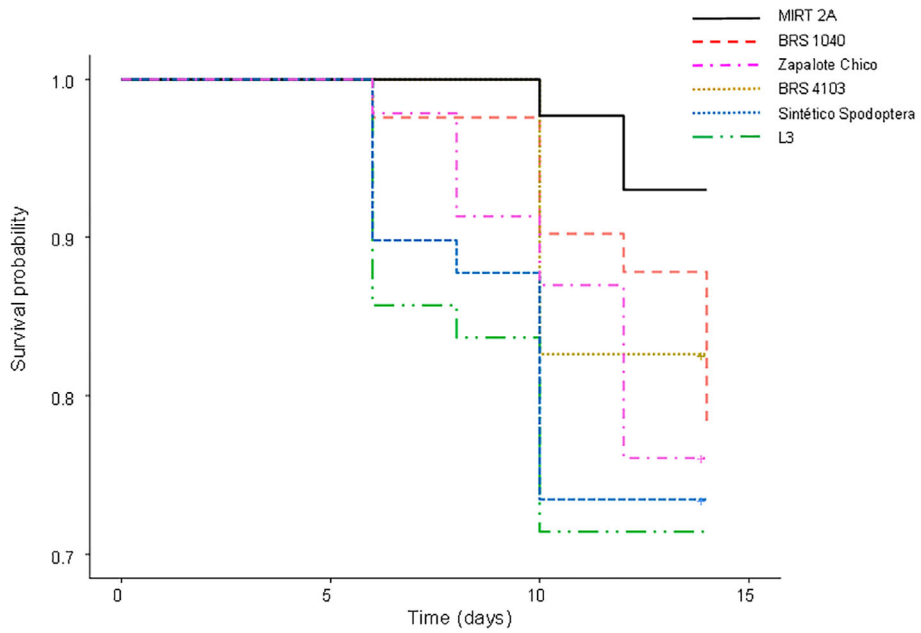

FIGURE 4 Kaplan–Meier survival plots for fall armyworm, *Spodoptera frugiperda*, 2nd instar larvae after feeding on six neotropical maize, *Zea mays*, genotypes. Two-week old Sintetico Spodoptera (SS), Zapalote Chico (ZC), L3, Mirt 2A, BRS 1040 and BRS 4103 were used in experiments and were replaced every 3 days. Larvae were added and were monitored from the 2nd instar stage until either mortality or pupation was reached (Log-rank test, $\chi^2 = 9.1$, df $= 5$, $p = 0.1$).

DISCUSSION

In this study we asked the following questions: (i) Are there differences in benzoxazinoids levels among Neotropical maize genotypes? (ii) Do *S. frugiperda* herbivory affect the benzoxazinoids levels among the maize genotypes? (iii) How do differences in benzoxazinoid content affect *S. frugiperda* development? Our results showed that the six Neotropical maize genotypes studied ie. Zapalote Chico, Mirt 2A, Sintético Spodoptera, L3, BRS 4103 and BRS 1040, had different levels of benzoxazinoids. HMBOA-Glc, DIMBOA-Glc, HMBOA, DIMBOA, HDMBOA-Glc and MBOA were all identified in the six Neotropical maize genotypes, and the amount and variability of the six benzoxazinoids quantified from aerial parts of maize seedlings were similar to those reported elsewhere (Cambier et al., 2000). Analysis of 18 commercial maize hybrids grown in Canada showed significant variation in the total amount of benzoxazinoids; the highest production was close to 1027 μg g^{-1} fresh weight, and the lowest was 42 μg g^{-1} fresh weight. A relatively high level of benzoxazinoids was detected within the first 2 days after germination; it then decreased rapidly, and DIMBOA-Glc remained the major benzoxazinoid quantified during the first 20 days after germination (Cambier et al., 2000). In our study, the major compound quantified in five of the genotypes at 15 days was HDMBOA-Glc, with the highest production in Mirt 2A (1070 μg g^{-1} fresh weight), and similar levels for all other genotypes (500 μg g^{-1} fresh weight). Compared with temperate maize varieties, tropical maize varieties have been reported to have higher levels of HDMBOA-Glc (Meihls et al., 2013), and it was suggested that this could be an adaptation of the maize varieties to the herbivore community, enhancing the resistance of the tropical maize varieties to Lepidoptera larvae (Zhou et al., 2018).

Differences in the levels of benzoxazinoids were observed among genotypes, but as reported in other studies, genotypes with higher levels of benzoxazinoids did not affect survival of *S. frugiperda* larvae (Glauser et al., 2011; Wouters et al., 2014, 2016). Several studies have shown that *S. frugiperda* can detoxify bezoxazinoids produced by maize plants (Glauser et al., 2011; Wouters et al., 2014, 2016). In all the genotypes studied here, more than 70% of the larvae survived, and damage by *S. frugiperda* did not increase the levels of benzoxazinoids. In fact, damage by *S. frugiperda* larvae decreased the levels of DIMBOA-Glc (BRS 1040) or HDMBOA-Glc (Mirt 2A). A previous study using a *S. frugiperda* population, without a geographic location informed, showed that the *S. frugiperda* larvae prefer to feed on young leaves, which in general, contains higher levels of benzoxazinoids, compared to old leaves, whereas *S. littoralis* prefer to feed on old leaves (Köhler et al., 2015). *S. littoralis* feeding on young leaves induce higher levels of benzoxaxinoids, such as HDMBOA-Glc and HDM2BOA-Glc, but when larvae of *S. litorallis* feed on old leaves, there was a negative effect on the bezoxazinoids production. (Köhler et al., 2015). In our study, the larvae of *S. frugiperda* was allowed to feed in all leaves, old and young leaves, and all leaves were used to quantify the benzoxazinoids. Therefore, we cannot infer if *S. frugiperda* will induce a similar response on old and young leaves of maize plants as observed for *S. littoralis* (Köhler et al., 2015). However, even if the feeding of *S. frugiperda* can induce higher production of benzoxazinoids in young leaves, this effect was supressed by the negative effect observed here on the DIMBOA-Glc and HDMBOA-Glc production in the varieties BRS 1040 and Mirt 2A, respectively.

The genotypes Mirt 2A and BRS 1040 are considered resistant and moderately resistant genotypes, respectively (Costa et al., 2006;

Viana & Potenza, 2000); and *S. frugiperda* larvae took one extra week to pupa in these two genotypes and larvae presented smaller cephalic widths compared to larvae fed on the other genotypes, suggesting an effect of antibiosis due the extending development period. However, this effect did not reflect in higher mortality of larvae in laboratory conditions; *S. frugiperda* late-instar larvae can compensate for the exposure to stressors in early instars (Israni et al., 2020; Wouters et al., 2014). Our hypothesis is that to detoxify the benzoxazinoids ingested from genotypes with higher levels of these compounds, the insect probably waste energy and therefore need to feed more to obtain sufficient energy to pupate. The undamaged genotype Mirt 2A produced significantly higher levels of HDMBOA-Glc, HMBOA-Glc and DIMBOA-Glc compared to those of the *S. frugiperda* -damaged plants, and when compared to those in the other genotypes. When this genotype was subjected to *S. frugiperda* larval herbivory, the levels of the benzoxazinoids decreased. We did not observe an increase in the levels of their respective aglucones that could explain the hydrolysis by the action of endogenous β-glucosidases; aglucones are very unstable and rapidly degrade to MBOA. Therefore, these results suggest that *S. frugiperda* larval feeding on maize can alter benzoxazinoid production in maize plants; similar results were observed by Glauser et al. (2011) when 4th instars *S. frugiperda* were used to feed on maize.

A genotype that is resistant to herbivores and at the same time attracts natural enemies would be the best choice to be used in the field; however, the results obtained here and in Michereff et al. (2019) indicate that the same maize genotype do not present high levels of direct and indirect defence. Sintético Spodoptera was the genotype that present higher levels of volatiles emission among the six maize genotypes evaluated, and it was the most attractive to natural enemies of *S. frugiperda*, the egg parasitoid *Telenomus remus*; whereas Mirt 2A and BRS 1040 were the genotypes that presented the lowest level of constitutive VOCs and HIPVs emission (Michereff et al., 2019). The larval parasitoid *Campoletis flavicincta* Ashmead (Hymenoptera: Ichneumonidae) was shown to be attracted to volatiles emitted by maize plants (variety SWB551) treated with *S. frugiperda* larvae regurgitate (Signoretti et al., 2012). Further studies could evaluate whether HIPVs emitted from different maize genotypes or cultivars influence the attraction of larval parasitoids as observed to egg parasitoids. Thus, a genotype mixture with different characteristics to improve the crop defence can be a strategy to manage herbivores in crops areas. Recently, Grettenberger and Tooker (2020) showed that cultivar mixtures of soybean, selected based on their phenotypic traits and from different companies, in an area with a low pest pressure did not show a clear effect on herbivore and natural enemy populations. Despite this, the authors suggest that plant genotype diversity in crop fields can influence higher trophic levels. We hypothesize that the use of cultivars mixture in crop areas can have a positive effect in insect populations when selecting cultivars with traits that favours the presence of natural enemies, and with traits that present a negative effect on herbivores. Therefore, a cultivar mixture of maize with Sintetico Spodoptera genotype, which produce HIPVs and attract natural enemies (Michereff et al., 2019) with Mirt 2A or BRS 1040 genotypes, where the insects remain longer on larvae phase can be an alternative to manage *S. frugiperda* in maize crops. The prolonged development of *S. frugiperda* larvae on these genotypes can be disadvantageous because they are exposed to natural enemies for an extended period, which can enhance the mortality due to parasitism or predation, as suggested in the slow-growth-high-mortality hypothesis (Clancy & Price, 1987). This effect was observed in *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) in mountain birch *Betula pubescens* (Kaitaniemi & Ruohomaki, 1999) and *Pieris rapae* L. (Lepidoptera: Pieridae) *Brassica oleracea* crop (Benrey & Denno, 1997; Lund et al., 2020), for example.

Maize plants are under high herbivore pressure, suffering constant attach of *S. frugiperda* larvae, *D. melacanthus*, *Dalbulus maidis* (DeLong & Wolcott) (Hemiptera: Cicadellidae) and *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae), for example. This high herbivore pressure explains the constitutive high level of nonvolatile compounds, whereas the amount of volatile compounds was shown to be constitutively low, and HIPVs high levels was observed only in two genotypes from the six evaluated (Michereff et al., 2019). These results provide information to build strategies using genotypes that can improve the action of natural enemies in field conditions like Sintetico Spodoptera genotype, combined with genotypes, which can offer a direct defence against herbivores, like Mirt 2A and BRS 1040 genotypes. This strategy can be applied to the complex of herbivores attacking maize plants. Further studies could evaluate the effect of benzoxazinoids on other herbivores such as the stink bug *D. melacanthus*.

In summary, our results demonstrate that different genotypes respond differentially to herbivory of *S. frugiperda* larvae; the survival of *S. frugiperda* larvae is not affected by different levels of benzoxazinoids, but when feeding on high level bezoxazinoids maize genotypes there is a delay on its development. Further studies could evaluate if this effect can result in more exposition of S. *frugiperda* larvae to natural enemies resulting in lower population levels in crops areas.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Data S1 Supplementary material II. NMR ^{1}H results.

Figure S1 Typical high-performance liquid chromatography (HPLC) analysis of benzoxazinoids present in 2 weeks old leaves of neotropical maize genotypes SS, ZC, L3, Mirt 2A, BRS 1040 and BRS 4103. The profile below is from the SS genotype. Analysis was performed using a Shimadzu Prominence instrument equipped with a PDA and an ACE - C18 column (250 mm × 4.6 mm, 5 μm). Labelled peaks: 1. HMBOA-Glc, 2. DIMBOA-Glc, 3. HMBOA, 4. DIMBOA, 5. HDMBOA-Glc, 6. MBOA

Figure S2 Amount of main benzoxazinoids identified in different genotypes of undamaged and herbivory damage plants.

Table S1 Statistical analysis of data from Figure 1.

Table S2 Statistical analysis of data from Figure 1.

Table S3 Statistical analysis of data from Figure 2.

Table S4 Statistical analysis of data from Figures 2 and S2a.

Table S5 Statistical analysis of data from Figures 2 and S2b.

Table S6 Pupae weight (grams) (means ± SEMs) of *Spodoptera frugiperda* feeding on different maize genotypes.

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