

**NATÁLIA DE SOUZA RIBAS**

**PARASITOID SEARCHING BEHAVIOR DRIVEN BY VOLATILES INDUCED ON  
TRANSGENIC MAIZE BY *BT*-RESISTANT AND SUSCEPTIBLE *Spodoptera frugiperda***

Thesis submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Eraldo Lima

Co-adviser: Carla Cristina Marques Arce

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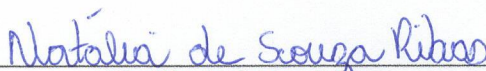
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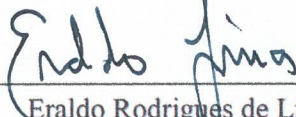
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Eraldo Rodrigues de Lima

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A meus pais (Milton e Rosimar)  
e irmãos (Bruna e Milton), que  
sempre se doaram para a  
realização dos meus sonhos,  
dedico essa conquista!

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Romanos 11:36

## ABSTRACT

RIBAS, Natália de Souza, D.Sc., Universidade Federal de Viçosa, August, 2022. **Parasitoid searching behavior driven by volatiles induced on transgenic maize by Bt-resistant and susceptible *Spodoptera frugiperda*.** Adviser: Eraldo Rodrigues de Lima. Co-adviser: Carla Cristina Marques Arce.

The development and use of toxins from *Bacillus thuringiensis* (*Bt*) to control the main lepidopteran maize pests' populations has been used since 1996. This technology positively impacts agricultural production, causing a reduction in the use of pesticides, minimal effect on non-target organisms, and reduced environmental impact. However, this large-scale cultivation is configured in an environment with a high-pressure selection for resistance. Therefore, there are already many documented cases of resistance in the field, resulting in pest control failures for many *Bt* toxins. Nevertheless, when there is a cost associated with resistance, individuals with resistance alleles could perform less in the absence of *Bt* toxin than the *Bt*-susceptible. This may help slow down the spread of resistance alleles because it can favor susceptible individuals instead the resistant ones. A diversity of fitness costs has been associated with developing and reproducing of resistance in lepidopteran insects. Those are costs affecting the individual directly. However, an related indirect cost, such as being more vulnerable to the attack of natural enemies, has received less attention. It is known that plants under attack with eggs oviposited or larvae feeding can use indirect defenses to attract natural enemies. Moreover, *Bt* and isogenic plants can emit different amounts of volatiles. Therefore, this study aimed to verify possible indirect costs related to changes in the amount of volatiles emitted by *Bt* and non-*Bt* maize plants when oviposited and fed by *Spodoptera frugiperda* and how this affects the eggs parasitoid *Trichogramma pretiosum*. For the egg deposition, our results demonstrated that *T. pretiosum* females preferred volatiles emitted by isogenic maize varieties with eggs of either susceptible or resistant *S. frugiperda* rather than the odors from control plants without eggs. The females did not discriminate between control and plants with *S. frugiperda* eggs on the *Bt* maize varieties. This pattern could be due to the induction of green leaf volatiles ((*E*)-2-hexenal and (*Z*)-3-hexenal) observed in the isogenic plants but not in the *Bt* varieties. For larvae feeding, our results demonstrated that *T. pretiosum* females preferred volatiles emitted by isogenic maize plants treated with the oral secretion of *Bt*-susceptible insects to resistant populations induced plants. On the other hand, in *Bt* plants, they preferred plants treated

with regurgitant from resistant populations than *Bt*-susceptible regurgitant. These results agree with the plants' total volatile; the *T. pretiosum* chose the plants that emitted the highest amounts of volatiles. Thus, these results may help optimize the biological control with *T. pretiosum* in maize fields with *Bt* and isogenic refuge areas to control *S. frugiperda* by promoting the attraction of *T. pretiosum* wasps. Therefore, our study unravels the effects of an ecological disadvantage for the resistant insects in the *Bt* field, which could help delay the evolution of the resistance.

**Keywords:** Tritrophic interaction. Fall armyworm. Induced defense. Maize. *Bt*. Volatiles

## RESUMO

RIBAS, Natália de Souza, D.Sc., Universidade Federal de Viçosa, agosto de 2022. **Comportamento de busca de parasitóides impulsionado por voláteis induzidos em milho transgênico por *Spodoptera frugiperda* suscetível e resistente ao *Bt*.** Orientador: Eraldo Rodrigues de Lima. Coorientadora: Carla Cristina Marques Arce.

O desenvolvimento e uso de toxinas do *Bacillus thuringiensis* (*Bt*) para o controle das principais populações de lepidópteros pragas do milho vem sendo utilizado desde 1996. Essa tecnologia impacta positivamente a produção agrícola, causando redução no uso de agrotóxicos, efeito mínimo sobre organismos não-alvo, e redução do impacto ambiental. No entanto, esse cultivo em larga escala se configura em um ambiente com alta pressão de seleção para resistência. Portanto, já existem muitos casos documentados de resistência no campo, resultando em falhas no controle de pragas para muitas toxinas *Bt*. No entanto, quando há um custo associado à resistência, os indivíduos com alelos de resistência podem ter um desempenho menor na ausência da toxina *Bt* do que os sensíveis ao *Bt*. Isso pode ajudar a retardar a propagação de alelos de resistência, pois pode favorecer indivíduos suscetíveis em vez de resistentes. Uma diversidade de custos de aptidão tem sido associada ao desenvolvimento e reprodução de resistência em insetos lepidópteros. Esses são custos que afetam diretamente o indivíduo. No entanto, um custo indireto relacionado, como ser mais vulnerável ao ataque de inimigos naturais, tem recebido menos atenção. Sabe-se que plantas sob ataque com ovos ou larvas podem utilizar defesas indiretas para atrair inimigos naturais. Além disso, plantas *Bt* e isogênicas podem emitir diferentes quantidades de voláteis. Portanto, este estudo teve como objetivo verificar possíveis custos indiretos relacionados às mudanças na quantidade de voláteis emitidos por plantas de milho *Bt* e não *Bt* quando ovipositadas e alimentadas por *Spodoptera frugiperda* e como isso afeta o parasitóide de ovos *Trichogramma pretiosum*. Para a deposição de ovos, nossos resultados demonstraram que as fêmeas de *T. pretiosum* preferiram voláteis emitidos por variedades isogênicas de milho com ovos de *S. frugiperda* suscetível ou resistente ao invés dos odores de plantas controle sem ovos. As fêmeas não discriminaram entre controle e plantas com ovos de *S. frugiperda* nas variedades de milho *Bt*. Este padrão pode ser devido à indução de voláteis de folhas verdes ((*E*)-2-hexenal e (*Z*)-3-hexenal) observados nas plantas isogênicas, mas não nas variedades *Bt*. Para a alimentação das larvas, nossos resultados

demonstraram que as fêmeas de *T. pretiosum* preferiram voláteis emitidos por plantas de milho isogênicas tratadas com a secreção oral de insetos sensíveis ao *Bt* a plantas induzidas por populações resistentes. Por outro lado, em plantas *Bt*, eles preferiram plantas tratadas com regurgitantes de populações resistentes do que regurgitante de populações suscetíveis ao *Bt*. Esses resultados concordam com a quantidade de volátil total das plantas; o *T. pretiosum* escolheu as plantas que emitiram as maiores quantidades de voláteis. Assim, esses resultados podem ajudar a otimizar o controle biológico com *T. pretiosum* em lavouras de milho com *Bt* e áreas de refúgio isogênico para controle da *S. frugiperda* promovendo a atração de vespas *T. pretiosum*. Portanto, nosso estudo desvenda os efeitos de uma desvantagem ecológica para os insetos resistentes no campo *Bt*, o que poderia ajudar a retardar a evolução da resistência.

Palavras-chave: Interação tritrófica. Lagarta do cartucho do milho. Defesa induzida. Milho. *Bt*. Voláteis

## SUMMARY

GENERAL INTRODUCTION .....	13
REFERENCES .....	15
CHAPTER 1 .....	20
<b><i>Bt</i>-protein expression impairs early <i>Spodoptera frugiperda</i> oviposition induced volatiles and egg parasitoid attraction in maize plants .....</b>	<b>20</b>
ABSTRACT .....	21
1. INTRODUCTION .....	22
2. MATERIAL AND METHODS .....	24
2.1. Insect Rearing .....	25
2.2. Plant Growing Conditions .....	25
2.3. Oviposition treatment .....	26
2.4. Behavior bioassays .....	26
2.5. Volatile collection and analysis .....	27
2.6. Statistical Analysis .....	27
3. RESULTS .....	28
3.1. <i>Parasitoid Response to Maize Volatiles</i> .....	28
3.2. <i>Volatiles sampling and identification</i> .....	29
4. DISCUSSION .....	30
5. CONCLUSION .....	32
6. ACKNOWLEDGMENTS .....	33
7. FUNDING .....	33
8. REFERENCES .....	33
9. FIGURES FIRST CHAPTER .....	46
10. TABLES FIRST CHAPTER .....	50
CHAPTER 2 .....	52
<b>Resistance to <i>Bt</i>-expressing protein of <i>S. frugiperda</i> caterpillars has an indirect cost by increasing the attraction of parasitoids in <i>Bt</i> maize plants .....</b>	<b>52</b>
ABSTRACT .....	53
1. INTRODUCTION .....	54
2. MATERIAL AND METHODS .....	55
2.1. Insects Rearing .....	55

<b>2.2. Plant Growing Conditions</b> .....	56
<b>2.3. Herbivory and simulated-herbivory induction of volatiles in maize plants</b> .....	57
<b>2.4. Volatile collection and analysis</b> .....	57
<b>2.5. Behavior bioassays</b> .....	58
<b>2.6. Regurgitant collection and analysis</b> .....	59
<b>2.7. Statistical Analysis</b> .....	60
<b>3. RESULTS</b> .....	60
<b>3.1. Herbivore-induced plant volatiles by caterpillar and oral secretion</b> .....	60
<b>3.2. Parasitoid attraction depends on the Bt susceptibility of <i>S frugiperda</i> on Bt plants but not in their isogenic line</b> .....	61
<b>3.3. <i>S. frugiperda</i> resistant to Bt proteins impairs the amount of FACs in the regurgitant</b> .....	62
<b>4. DISCUSSION</b> .....	62
<b>5. CONCLUSION</b> .....	65
<b>6. ACKNOWLEDGMENTS</b> .....	65
<b>7. FUNDING</b> .....	66
<b>8. REFERENCES</b> .....	66
<b>9. FIGURES SECOND CHAPTER</b> .....	74
<b>10. TABLES SECOND CHAPTER</b> .....	78
<b>11. GENERAL CONCLUSIONS</b> .....	80

## GENERAL INTRODUCTION

The development and use of toxins from *Bacillus thuringiensis* (*Bt*) to control lepidopteran pest populations of maize were introduced in 1996 in maize plants expressing Cry1Ab toxin (Koziel *et al.*, 1993; Siegfried *et al.*, 2007). This technology represents one of the most significant changes in pest management practices, providing positive impacts on agricultural production, causing a reduction in the use of pesticides and the application cost, minimizing effects on non-target organisms, and reducing environmental impact (MacIntosh *et al.*, 1990; Naranjo *et al.*, 2005; Romeis *et al.*, 2006). Therefore, the positive effects of using this technology had increased the cultivation area worldwide since 1996, when farmers used it for the first time in the US (Siegfried *et al.*, 2007).

In Brazil, transgenic crops were adopted for the first time in 2007, with transgenic plants expressing only one protein with insecticidal action, Cry1Ab toxin (CTNBIO, 2007). These events released in Brazil were called: MON810 and BT11. In 2009, they began to use the transgenic plants expressing the toxin Cry1F, which was more effective in controlling the fall armyworm (FAW) *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (J. E. Smith, 1797) a polyphagous species that attacks many economically essential crops in several countries (Sparks, 1979; CTNBIO, 2009; Blanco *et al.*, 2016). However, large-scale cultivation is configured in an environment with high-pressure selection for resistance (Monnerat *et al.*, 2015; James, 2014; Leite *et al.*, 2016; Faretto *et al.*, 2017).

The accumulation of resistance in the field against *Bt* toxins in FAW populations, in fact, reduces this approach's effectiveness as a control strategy (Omoto *et al.*, 2016; Huang *et al.*, 2014). One method to mitigate that resistance development is the “high dose refuge” (Gassmann *et al.*, 2009). That strategy aims to guarantee a sufficient number of susceptible adults with isogenic plants (refuge area) to mate with resistant adults. The high dose should kill the offspring in *Bt* fields (Carrière & Tabashnik., 2001; Tabashnik *et al.*, 2013). In addition, resistant individuals must present reduced fitness on *Bt* and non-*Bt* plants (Tabashnik *et al.*, 1994; Carrière e Tabashnik, 2001; Bourguet *et al.*, 2003).

Cry1F toxin was a great success, although there are documented cases of resistance in the field resulting in pest control failures (Storer *et al.*, 2010; Tabashnik *et al.*, 2013; Farias *et al.*, 2014;

Huang *et al.*, 2014, de Souza *et al.*, 2022). Thus, people began using *Bt* events, called pyramided events, intending to maximize pest control and delay resistance (Tabashnik *et al.*, 2013). This new technology expressed two or more *Bt* toxins, with different modes of action, to control the same group of target insects and could reduce the resistance because resistance to a single toxin can be faster than to multiple toxins (Zhao *et al.*, 2009; Tabashnik *et al.*, 2013).

Resistance studies are essential as an evolutionary model of the physiological and genetic changes associated and the existence of cost associated with resistance (Raymond *et al.*, 2001). *Bt*-resistant insects can show developmental costs, and individuals who have resistance alleles could have lower performance in the absence of *Bt* toxin than the *Bt*-susceptible individuals (Coustau *et al.*, 2000; Delisle & Vincent, 2002; Carrière *et al.*, 2006; Zhao *et al.*, 2009; Zhang *et al.*, 2014). The cost associated with resistance may help slow down the spread of resistance alleles when refuges are present because they can favor susceptible individuals instead of the resistant ones (Carrière & Tabashnik, 2001; Carrière *et al.*, 2010).

A variety of fitness costs has been associated with the development of resistance in lepidopteran insects. These costs include longer developmental time and reduced mass as well as costs in reproduction as lower pheromone production, lower fecundity, and lower fertility (Delisle & Vincent, 2002; Carrière *et al.*, 2006; Zhao *et al.*, 2009; Zhang *et al.*, 2014; Zhu *et al.*, 2019). Those costs directly affect the individual. However, an related indirect cost, such as being more vulnerable to the attack of natural enemies, has received less attention (Gassmann *et al.*, 2009; Hiltbold & Hibbard, 2018). It is known that plants under attack with eggs oviposited or larvae feeding can use indirect defenses to attract natural enemies (Turlings *et al.*, 1995; Peñaflores *et al.*, 2011; Turlings and Erb, 2018; Nascimento *et al.*, 2021). Moreover, the plant expressing *Bt* and their isogenic relatives may emit different amounts of volatiles (Smith *et al.*, 1996; Dean & De Moraes, 2006; Nascimento *et al.*, 2021). However, it is unknown whether resistant or susceptible insect populations can trigger the same plant defense responses when ovipositing or feeding on the plant and how this affects the third trophic level. Therefore, this study aimed to detect possible indirect costs related to changes in the amount of volatiles emitted by *Bt* and non-*Bt* maize plants (expressing Cry2Ab2 and Cry1A.105 toxins) when oviposited and fed by *S. frugiperda* and how this affect the eggs parasitoid *Trichogramma pretiosum*.

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## CHAPTER 1

***Bt*-protein expression impairs early *Spodoptera frugiperda* oviposition induced volatiles and egg parasitoid attraction in maize plants**

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## ABSTRACT

Plants often induce volatile organic compounds in response to herbivory. However, feeding is not necessarily the first contact between plants and herbivores, as females mainly lay their eggs, which can induce volatiles. The use of transgenic plants expressing *Bacillus thuringiensis* (*Bt*) proteins is the most successful strategy for controlling the fall armyworm (FAW) *Spodoptera frugiperda*, the primary pest of maize in South American countries. FAW has shown resistance to most *Bt* proteins; therefore, their management seeks more sustainable tools. In this study, we evaluated the volatiles induced after oviposition over time of resistant and susceptible FAW on *Bt* and isogenic maize varieties and how this induction affected the searching behavior of the egg parasitoid *Trichogramma pretiosum*. Our results demonstrated that *T. pretiosum* females preferred volatiles emitted by isogenic maize varieties (var. 7280 and var. 709) with eggs of either susceptible or resistant *S. frugiperda* rather than the odors from control plants without eggs. The females did not discriminate between control and plants with FAW eggs on the *Bt* maize varieties. This pattern could be due to the induction of green leaf volatiles ((*E*)-2-hexenal and (*Z*)-3-hexenal) observed in the isogenic plants but not in the *Bt* varieties. Our study unravels the effects of *Bt* mediated resistance on crucial biological control agents. Thus, these results may help optimize the biological control with *T. pretiosum* in maize fields with *Bt* and isogenic refuge areas to control FAW by promoting the attraction of *T. pretiosum* wasps.

Keywords: FAW. Natural enemies. Oviposition. Maize. *Bt*. Volatiles

## 1. INTRODUCTION

The fall armyworm (FAW), *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is considered the primary maize pest in South America (Sparks, 1979; Blanco *et al.*, 2016). Maize (*Zea mays* L.) is among the most important cultivated plants worldwide. The management of FAW in this crop is carried out mainly by the widespread cultivation of transgenic maize cultivars expressing the *Bacillus thuringiensis* (*Bt*) protein toxins (Gould, 1998; ISAAA, 2017; Okumura *et al.*, 2013, McCormick, 2020). This strategy can benefit the environment due to the decrease of pesticides application and increase in grain production by at least 30% for controlling defoliators pest, mainly larvae of some lepidopterans (Biotecnología, 2015, Mandal *et al.*, 2020, Rani *et al.*, 2021). However, the accumulation resistance in the field against *Bt* toxins in FAW populations is a real threat to agricultural practices because this could reduce the effectiveness of this tactic as an effective control strategy (Omoto *et al.*, 2016; Huang *et al.*, 2014). For instance, in Brazil, FAW has developed resistance to most maize transgenic *Bt* hybrids in a period of only three years (Monnerat *et al.*, 2015, Farias *et al.*, 2014; 2016, Leite *et al.*, 2016, Fatoretto *et al.*, 2017). Moreover, this technology can negatively impact several groups of non-target organisms such as Hemiptera, Hymenoptera, Neuroptera by affecting the duration of the larval stage, weight of adults, fecundity and survivorship (Faria *et al.*, 2007; Chen *et al.*, 2008; Mason *et al.*, 2008; Schmidt *et al.*, 2009; Lövei *et al.*, 2009; Hilbeck *et al.*, 2012; Resende *et al.*, 2016). For instance, adverse effects were reported by Curis and Bertolaccini (2013), showing that the predator *Eriopis connexa* (Coleoptera: Coccinellidae) when preying FAW feeding on *Bt* maize had a longer duration of the larval stage as well as the adult's weight and fecundity were lower. According to, Rolim *et al.* (2020) the parasitoid *Palmistichus elaeisis* (Hymenoptera: Eulophidae) has lower survivorship, altered host searching, and poorer reproductive performance when *Bt*-treated FAW larvae were used as a host. However, several studies have also reported positive and neutral effects of these toxins on natural enemies (Salama & Zaki, 1983; Van, 1991; Baur & Boethel, 2003; Yang *et al.*, 2005; Mohan *et al.*, 2008, Zenner-de-Polanía, 2021). For example, a neutral effect was observed by Spagnol *et al.* (2020) on *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) a parasitoid of FAW after feeding on *Bt* maize plants. Moreover, a positive effect of *Bt* maize at field condition on the predator *Nusalala tessellate* (Neuroptera: Hemerobiidae) abundance was observed by Frizzas *et al.* (2017). Therefore, the direct effects of *Bt* toxins on non-target insects are well

documented, especially in relation to parasitoids and predators. Nevertheless, the impact of *Bt* plants on the long-range cues used by parasitoids to locate hosts, such as volatiles is not well documented.

Responses to environmental stimuli are crucial for plants survival and they have evolved several defense strategies to cope for instance to herbivore attack. These strategies can be heritable constitutive and induced defenses produced only upon herbivore attack (McCall *et al.*, 1994; Karban & Baldwin, 1997; Dicke & Van Poecke, 2002; Howe & Jander, 2008). Plants under attack can use indirect defenses to attract natural enemies by inducing long-range cues, the so-called "herbivore-induced plant volatiles" (HIPVs). Used by parasitoid wasps to find their hosts were reported by many authors (Turlings *et al.*, 1990; Turlings *et al.*, 1995; De Moraes *et al.*, 1998; Paré & Tumlinson, 1999; Turlings & Wäckers, 2004; Turlings & Erb, 2018; Aljibory & Chen, 2018). It is known that different inbred lines of maize induce a different emission rate of HIPVs (Degen *et al.*, 2004; Gouinguéné *et al.*, 2001). In addition, the pattern of HIPVs emitted by inbred maize lines attacked by FAW can recruit the parasitic wasps *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) and *Cotesia marginiventris* (Hymenoptera: Braconidae) in the field (Degen *et al.*, 2012) and under laboratory conditions (Peñaflor *et al.*, 2011b; De Lange *et al.*, 2020). Regarding responses to HIPVs in *Bt* plants, little is known. For instance, Smith *et al.* (1996) showed that volatiles produced by *Bt* and non-*Bt* undamaged tomatoes plants have a different blend composition. On the other hand, Dean & De Moraes (2006) showed that *Helicoverpa zea* (Lepidoptera: Noctuidae) larvae fed on *Bt* and non-*Bt* maize, the volatile emissions were significantly lower in the transgenic plants than their isogenic maize lines. Moreover, Nascimento *et al.* (2021a) showed that the volatiles of non-*Bt* maize were more attractive to the adult females of *T. pretiosum* than to the volatiles from a *Bt* maize. Therefore, the changes in the volatiles profile could change the parasitoids' searching behavior.

Most of the studies measured volatiles in plants attacked by chewing caterpillars, making the "oviposition-induced plant volatiles" (OIPV) less known, especially for *Bt* and non-*Bt* plants. For instance, Téllez-Rodríguez *et al.* (2014) showed that FAW number of egg masses per plant was significantly higher on *Bt* than on non-*Bt* plants at fields condition.

Many insects, especially the Lepidoptera species, lay their eggs directly on the host plant; thus, the oviposition could be considered the first encounter with herbivores. Several studies have

shown that egg laying itself can induce plant defenses, e.g., volatiles (Hilker & Fatouros, 2015; Hilker & Fatouros, 2016; Meiners & Hilker, 1997; Hilker & Meiners, 2002a; Tamiru *et al.*, 2011; Fatouros *et al.*, 2012; Anastasaki *et al.*, 2015). The advantage of this strategy could be that plant defenses would be activated before the attack of neonate larvae (Geiselhardt *et al.*, 2013; Bandoly *et al.*, 2015). According to, Pashalidou *et al.* (2020) showed that *Brassica oleracea* and *Brassica nigra* induced plant volatiles in response to oviposition of *Pieris brassicae* (Lepidoptera: Pieridae). Mutyambai *et al.* (2015) showed that maize landraces respond to stemborer egg deposition by releasing HIPVs that attract *Trichogramma bournieri* (Hymenoptera: Trichogrammatidae) and *Cotesia sesamiae* (Hymenoptera: Braconidae), an egg and larval parasitoids, respectively. In addition, Piesik *et al.* (2013) reported that *Meligethes aeneus* F. (Coleoptera: Nitidulidae) damage, progressively induced:  $\beta$ -caryophyllene, (*E*)- $\beta$ -farnesene, and (*Z*)- $\beta$ -ocimene after oviposition in *Brassica napus*. Wegener *et al.* (2001) showed that egg deposition of the elm leaf beetle, *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae) in *Ulmus minor* induced:  $\beta$ -caryophyllene and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) as major compounds. However, it is known that oviposition can lead to the suppression of plant defenses, although the mechanism remains unknown. For instance, Bruce *et al.* (2010) showed that the stemborer, *Chilo partellus*, oviposition reduces the emission of (*Z*)-3-hexenyl acetate in an African grass (*Brachiaria brizantha*). Moreover, Peñaflor *et al.* (2011a) showed that egg laying by the FAW reduces the constitutive emission of Linalool in maize plants.

This study was carried out to investigate the oviposition-induced volatile emissions over time and the attraction of a generalist egg parasitoid *Trichogramma pretiosum* to *Bt*- and its isogenic maize plants upon oviposition by *Bt*-resistant (population RR) and *Bt*-susceptible (population SS) of *Spodoptera frugiperda* moths. Thus, we hypothesize that females of FAW *Bt*-resistant population will induce less volatiles on *Bt* maize plants after oviposition, which will impact an egg parasitoid host location. Our study unravels the effects of *Bt* mediated resistance on important biological control agent. These results may help optimize the biological control of FAW with *T. pretiosum* in maize fields with *Bt* and refuge areas with isogenic plants.

## 2. MATERIAL AND METHODS

## 2.1. Insect Rearing

Two populations of *Spodoptera frugiperda* were used in this study: one resistant (hereafter refer to as *S. frugiperda* RR) and one susceptible (hereafter refer to as *S. frugiperda* SS) to plants expressing Cry1A.105 and Cry2Ab *Bt* toxins. Both populations are genetically very similar but differ in their resistance to *Bt* toxins (Santos-Amaya *et al.*, 2015). To evaluate FAW mortality *Bt*-susceptibility was used for feeding on the insects with *Bt*-expressing plants or with their isogenic, non-*Bt* expressing counterparts (Santos-Amaya *et al.*, 2015).

Insects were reared under laboratory conditions as described (de Souza Ribas *et al.*, 2022). Briefly, the adults were maintained in PVC cages (20 cm diameter x 30 cm height) covered with paper on the inner walls for oviposition. A piece of cotton soaked in a 10% sugar and 1% ascorbic acid solution was offered every two days *ad libitum* as the insects food source. Eggs were collected every two days and stored in 500 ml plastic bags until hatching. We kept groups of neonates in plastic cups until they reached the 2<sup>nd</sup> instar. After that, the caterpillars were individually place in 16-cell PVC trays (Advento do Brasil Ind. e Comércio de Plásticos Ltda, São Paulo, Brazil) until pupation. All caterpillar stages were fed on an artificial diet (Greene *et al.*, 1976). The insects were reared in controlled conditions ( $27 \pm 3^\circ \text{C}$ ,  $70 \pm 15\%$  R.H., and 14:10 h L/D photoperiod).

The generalist egg parasitoid *Trichogramma pretiosum* was purchased from Ecotrix Biodefensivos (Viçosa, MG, Brazil), and fed on pure honey and maintained under controlled conditions ( $27 \pm 3^\circ \text{C}$ ,  $70 \pm 15\%$  R.H., and 14:10 h L/D photoperiod). The adults were sexed using the antennal morphology dimorphism (Querino & Zucchi, 2003).

## 2.2. Plant Growing Conditions

Four maize varieties were used: two expressing Cry1A.105 and Cry2Ab toxins (BM 709 PRO2 and GNZ 7280 PRO2) and their respective non-*Bt* expressing isogenic varieties (BM 709, GNZ 7280), respectively. Seeds were purchased in Biomatrix and Geneze (Brazil). Plants were grown in 180 ml plastic pots filled with soil (MecPlant, Brazil) under greenhouse conditions at  $27 \pm 3^\circ \text{C}$ , 70% R.H. and 14:10 h L/D photoperiod. Plants was watered every day and when reashing 10 – 15 days old or three to four fully developed leaves, the experiments started.

### 2.3. Oviposition treatment

To evaluate the preference of *T. pretiosum* parasitoid females for the volatiles emitted by the different maize varieties upon oviposition by the two *S. frugiperda* populations (SS and RR), we offered *Bt* and isogenic maize plants to 12 virgin females and males of three to four day-old, overnight, in acrylic cages for oviposition under controlled conditions (24°C, 70% R.H. and 14:10 h L/D photoperiod). The next morning, plants containing between five to seven egg masses were selected for experiments. The plants were from susceptible (SS) and resistant (RR) population offered separately.

### 2.4. Behavior bioassays

To test the response of the egg parasitoid to maize plants volatiles induced by oviposition of *S. frugiperda* (SS and RR), we evaluated the *T. pretiosum* female's preference to maize volatiles of *Bt* and isogenic plants. The plants were used after 15 hours of the oviposition treatment. For this, two-choice bioassays were performed in a y-tube olfactometer as described by a Janssen, (1999). Briefly, the olfactometer consisted of a Y-shaped glass tube (27 cm in length × 3.5 cm in diameter) which is connected to a vacuum pump that pushes the air at 0.3 m/s. The airflow in negative flow was used for pushing the air from the glass containers (50 x 36 x 43) where the odor sources were placed (plants with or without eggs), into the olfactometers arm to the base. A black Y-shaped metal wire in the middle of the Y-tube was used to guide the parasitoid during walking. The airflow was measured by using a hot-wire anemometer (Velocicalc 9545-A, São Paulo, Brazil) before starting of the experiments.

Two to three day-old mated females parasitoid were tested one by one, and each 6 females tested in a row consisted one replicate in the choice bioassay. The females were released on the black Y-shaped metal wire at the base of the Y-tube and observed until they reached the end of one of the arms, at maximum of 5 minutes, when we introduced the next female. Females that did not make any choice within 5 min were scored as having made no choice and excluded from the analysis. After performing each replicate being performed the Y glass tube was replaced by a cleaned one. In addition, volatile sources were switched to the opposite arm of the olfactometer

after each replicate to avoid bias. Six replicates were carried out per combination of volatile sources contrasted. Each parasitoid was used only once to prevent odor learning.

The following choices treatments were tested: maize plants of *Bt* var. 709 PRO2 and 7280 PRO2 with eggs mass of either *S. frugiperda* (SS and RR) vs. control plants (without eggs); and maize plants of isogenic var. 709 and 7280 with eggs mass of either *S. frugiperda* (SS and RR) vs. control plants (without eggs).

## 2.5. Volatile collection and analysis

To test whether *Bt* and isogenic maize plants respond differently to oviposition by *S. frugiperda* (SS and RR) moths, volatiles were collected 15 and 30 hours after the oviposition (n= 5-8). Control plants were kept without eggs. Details of number of replicates of each treatment and time point are on Table 1. The plants were individually placed in cylindrical glass chambers (37 cm high × 14 cm diameter) and the plant pots were covered with aluminum foil. Humidified and charcoal-filtered air (1 L/min) were introduced into the chambers and the volatiles were pulled (0.5 L/min) and trapped into 20 mg of Haye Sep Q (80–100 mesh) (Analytical research systems, Florida, USA). The volatiles were collected during the photophase for 3 hours. The filters were eluted with 150 µl of hexane (double-distilled HPLC-grade, Sigma-Aldrich, São Paulo, Brazil). Ten µl of nonyl-acetate (20 ng/µl) were added as internal standard (I.S.). The sample was stored at -80 °C until further analysis.

The volatile samples were analyzed using a gas chromatograph coupled with a mass spectrometer (Shimadzu GCMS – QP2010 SE). One microliter of each sample was injected into a pulsed splitless, Rtx-5MS (Restek chromatography products), mode onto a DB-5 column (30 m × 0.25 mm; df = 0.25 µm; J&W Scientific, Folsom, CA, USA), using helium as carrier gas (170 kPA). The temperature was maintained at 40 °C for 3 min, then increased to 100 °C at 8 °C/min and subsequently to 230 °C at 5 °C/min, followed by a post-run of 3 min at 250 °C. Helium was kept at a constant flow of 1.1mL/min<sup>-1</sup>. The identification and quantification of compounds were performed using comparisons to the mass spectra of NIST 17 library spectra and confirmed with the spectra of commercial standards.

## 2.6. Statistical Analysis

Statistical analyses were performed in R (v. 4.0.0; R Development Core Team, 2020) using Analysis of Variance (ANOVA), followed by residual analysis to confirm the suitability of distributions of the tested models. Generalized Linear Models (GLM) with a Gaussian distribution were used to test the differences in volatile emission rates emitted by maize plants with and without eggs of *Bt* and isogenic plants over time and the time spent by the parasitoid to choose between the treatments in the olfactometer bioassay. Least Squares Means (*emmeans*) were used compare significant differences among treatments. The choice of the parasitoid between maize plants with eggs or control plants and maize plants with eggs or only the egg masses were accessed by using a Generalized Linear Models (GLM) with a Binomial distribution.

### 3. RESULTS

#### 3.1. Parasitoid Response to Maize Volatiles

*Bt* expression impaired *T. pretiosum*'s ability to discriminate between control maize plants and FAW-oviposited plants. On the other hand, *T. pretiosum* females preferred the isogenic maize varieties that had eggs laid rather than plants without eggs. Both isogenic lines showed the same pattern of preference, but *Bt* ones revealed some slight differences in the parasitoid attraction among varieties.

The parasitoid females preferred volatiles emitted by isogenic plants *var.* 7280 with eggs of both *S. frugiperda* (SS and RR) rather than those from control plants without eggs (Fig. 1A). The same pattern was observed when we offered the odors of plants with eggs and the volatiles only from egg masses (laid on paper); they prefer the plants with eggs (Fig. 1C). Interestingly, when we offered the volatiles from eggs laid in *Bt* plants *var.* 7280, the females wasps showed no preference for plants with eggs laid by both *S. frugiperda* (SS and RR) than for plants without eggs (Fig. 1B). However, the females preferred the odors of plants with eggs of *S. frugiperda* SS than only egg masses (Fig. 1D), which was not the case when the odors offered were from *S. frugiperda* RR and only the eggs.

Isogenic maize *var.* 709 with eggs laid by both *S. frugiperda* population (SS and RR) was more attractive to the parasitoid females than plants without eggs (Fig. 2A). Moreover, they

preferred the odors from plants with eggs of *S. frugiperda* SS instead odors elicited by egg masses (Fig. 2C) and the same tendency was observed for odors of plant with *S. frugiperda* RR ( $p=0.06$ , Fig. 2C). Furthermore, the parasitoids released from *Bt* maize plants *var.* 709 with eggs of *S. frugiperda* SS were more attractive than plants without eggs, but interestingly they do not distinguish between the odors of plants with eggs of *S. frugiperda* RR (Fig. 2B). The same pattern was observed when we contrasted odors from plants with eggs of *S. frugiperda* (SS and RR) and only the egg masses on *Bt* maize plants (Fig. 2D).

### 3.2. Volatiles sampling and identification

The two varieties (*var.* 7280 and 709) expressing *Bt* gene and isogenic maize plants without gene expression constitutively emitted volatiles, however the isogenic varieties were more responsive to oviposition than *Bt* varieties. We detected the constitutive and oviposition-induced volatiles of the green leaf volatiles (GLVs): (*E*)-2-hexenal and (*Z*)-3-hexenal in the *Bt* and isogenic maize plants of both varieties. Moreover, *Bt* maize plants showed to have a late and smaller response than isogenic maize plants. *Spodoptera frugiperda* RR populations seemed to induce more volatiles than *S. frugiperda* SS population (Fig. 3 and 4).

The isogenic maize *var.* 7280 emitted more volatiles after early *S. frugiperda* RR oviposition (15 h, Fig. 3A, Table 2), whereas only after 30 hours this variety increased its emission after oviposition by *S. frugiperda* SS (Fig. 3C, Table 2). The two GLVs were induced after early oviposition, but after 30 hours of oviposition by both *S. frugiperda* (SS and RR) population induced the emission rates of only the (*Z*)-3-hexenal (Table 2). Surprisingly, the *Bt* plants of this variety did not respond to early oviposition of both *S. frugiperda* SS and RR populations (Fig. 3B, Table 3), although a late response was observed (Fig. 3D, Table 3). Both (*Z*)-3-hexenal and (*E*)-2-hexenal were induced after 30 hours by *S. frugiperda* RR and SS oviposition in *Bt* plants (Table 3).

The isogenic maize *var.* 709 emitted more volatiles after *S. frugiperda* RR early oviposition (15 h, Fig. 4A, Table 4), whereas after 30 hours this variety did not respond to oviposition of both *S. frugiperda* SS and RR (Fig. 4C, Table 4). Surprisingly, the *Bt* plants of the *var.* 709 did not respond to oviposition of both *S. frugiperda* SS and RR for both 15 and 30 hours after oviposition (Fig. 4B and D, Table 5). The (*Z*)-2-hexenal was induced in the isogenic *var.* 709 after 15 hours of

oviposition by *S. frugiperda* RR and after 30 hours it was induced by both *S. frugiperda* (SS and RR) populations (Table 5). The other GLV ((Z)-3-hexenal) were not induced at all (Table 5).

#### 4. DISCUSSION

Plants often release volatiles after oviposition; the emitted volatiles can be used by egg parasitoids as cues to enhance host location success (Hilker *et al.*, 2002b; Bruce *et al.*, 2010; Tamiru *et al.*, 2011, Fatouros *et al.*, 2012). In general, we provided evidence supporting this statement. Thereby, oviposition by *S. frugiperda* (RR and SS) populations on different varieties of maize plants emitted a different rate of GLVs, which improved the egg parasitoid host location behavior. This pattern was sounder in isogenic maize plants but not on *Bt* expressing protein maize plants.

In contrast what already has been reported, a suppression of constitutive volatile in maize after *S. frugiperda* oviposition (Peñaflor *et al.*, 2011a), our study found that FAW oviposition (SS and RR populations) increased the emission rate of constitutive GLVs (*E*)-2-hexenal and (*Z*)-3-hexenal in isogenic maize plants of two varieties after 15h and 30h of oviposition. However, the *Bt* expressing varieties did not show an early induction volatile after oviposition *S. frugiperda*. Accurately, the increase in GLV's were observed after 30h of oviposition and only in the variety 7280. It is known that maize plants from different varieties can emit a completely differently volatile profile when under herbivore attack (Degen, 2004, Michereff *et al.*, 2011, Moraes *et al.*, 2011), especially conventional maize hybrids which differences can reach up to 70 times among the extreme varieties (Degen *et al.*, 2004). Few studies relate that volatile emission have been impaired by genetic engineering (Dean & De Moraes, 2006; Sun *et al.*, 2013; Liu *et al.*, 2015). Therefore, *Bt* expressing protein plants can cause major impact in volatile responses to herbivory, especially after caterpillars feeding (Schuler *et al.*, 2003; Turlings *et al.*, 2005; Dean & De Moraes, 2006; Tamiru *et al.*, 2011; Liu *et al.*, 2015; Wang *et al.*, 2018). For instance, Xu *et al.*, (2019) reported that maize *Bt* lines emitted higher HIPV amounts when treated with simulated herbivory by applying regurgitant of *S. littoralis*, in comparison to its isogenic line. On the other hand, *Bt* maize lines, after 24 hours attacked by *S. frugiperda* larvae emitted less HIPV than its non-*Bt* isoline (Nascimento *et al.*, 2021a). Additionally, Turlings *et al.*, (2005) showed that *Bt* maize plants after *S. littoralis* regurgitant application emitted less HIPVs than its isogenic line. The literature related to HIPV induction in *Bt* expressing plants by chewing herbivores are extensive, only very

little is known regarding volatiles induced by insect oviposition (OIPV). To the best of our knowledge, only study done by Nascimento *et al.*, (2021b) observed that after FAW oviposition of *Bt* expressing maize hybrids released some specific volatile compounds in high amounts and the TPS 10 and LOX 10 genes expression compared to their isogenic line were higher. It is known that overexpression of TPS10 genes in maize plants increases the emission of (*E*)- $\alpha$ -bergamotene and (*E*)- $\beta$ -farnesene. Random phenotypic changes in plant defense systems due to pleiotropic effects or introduction of exogenous genes could result in losses in the ability to respond through HIPV emission at early phenological stages (Schuler *et al.*, 1999). Therefore, the lack of response by our *Bt* maize plants after oviposition by FAW could be due to no induction of the respective genes. Thence, further investigations of genes related to volatile induction such as GLVs should be performed to explain better this pattern.

Differences in volatile emissions by plants in response to herbivory can impact the natural enemies host foraging behavior, especially parasitoid wasps as *T. pretiosum* and *Cotesia plutellae* (Smith *et al.*, 1996; Dean & De Moraes., 2006; Téllez-Rodríguez *et al.*, 2014; Nascimento *et al.*, 2021a, 2021b; Schuler *et al.* 1999; 2004). According to Da Silva *et al* (2021) and Sousa *et al* (2021) after 48 hours of FAW oviposition maize plants were more attractive to *T. pretiosum* than non-oviposited plants. Peñaflor *et al* (2011b) showed that the GLVs (*E*)-2-hexenal, (*Z*)-3-hexenal, (*E*)-3-hexen-1-ol and (*Z*)-3-hexenyl are the main candidates for of *T. pretiosum* attraction, agreeing with our results. In addition, Sen *et al* (2005), relates that *T. chilonis*, were reactive to GLVs (olfactory responses of antennal chemoreceptors). Nascimento *et al* (2021a) showed that the emission of HIPVs induced by FAW larvae feeding isogenic maize, were more attracted to *T. pretiosum* than to the ones from a *Bt* maize hybrids. Indeed, the most literature related to the attraction of a parasitoid to GLVs are due to a larval feeding. It is possible that elicitors present in the oviposition secretions can be used as a signal to trigger defense responses in plants (Hilker *et al.*, 2002a; Colazza *et al.*, 2004; Reymond *et al.*, 2013, Sousa *et al.*, 2021). However, different plant varieties might respond differently to these elicitors that could translate in different HIPVs emission (Hilker & Fatouros, 2016). For instance, Hilker *et al* (2002b) showed that egg laid by *Diprion pini* induces local and systemic emission of attractive volatiles to the egg parasitoid *Chrysonotomyia ruforum*. Paralelly, other cues must be used by parasitoids to find their host such as insect scales that are used as kairomones (Colazza *et al.*, 1997; Boo & Yang, 2000; Schöller & Prozell, 2002; Milonas *et al.*, 2009). Additionally, Vargas *et al.*, (2021) showed that *T. pretiosum*

used as chemical clues insect scales of FAW *S. frugiperda* to search eggs. Thus, the egg parasitoid finds their hosts using a combination of several olfactory clues and the volatiles from the eggs itself it is only one of them.

Evolution of insects resistance to *Bt* toxins has required the consideration of alternative pest management and improved insect resistance management. In addition, the natural selection of resistance by insects may also lead to a fitness cost (Gassmann *et al.*, 2009). For instance, it is known that *S. frugiperda* resistant to the protein Cry1A.105 and Cry2Ab *Bt* toxins has a strong fitness costs related to its development (Santos-Amaya *et al.*, 2022). In addition, Hiltpold & Hibbard (2018) showed that *Bt* maize hybrids emitted higher amount of volatiles when attacked by *Bt*-resistant *Diabrotica virgifera virgifera* larvae than by *Bt*-susceptible larvae, which do not lead to an induced fitness cost to the herbivore since they do not affected natural enemies below ground. In our study we did observe this induced cost, since FAW resistant population did not induced any volatiles in *Bt* maize plants after oviposition. However, isogenic lines resistant *S. frugiperda* population induced more GLV than the susceptible *S. frugiperda* population.

In summary, we herein showed that oviposition itself increased the OIPV emission in one isogenic variety of maize but not in another one, by increasing volatiles related to attractiveness an egg parasitoid. We also showed that this volatile induction changed between *Bt* and non-*BT* plants also affected parasitoids search. Currently, it is known that HIPVs are used as tool in the integrated pest management to attract and retain natural enemies in field (El-Shafie & Faleiro, 2017; Sharma *et al.*, 2019; Conboy *et al.*, 2020). Thus, these results may help to optimize the biological control by *T. pretiosum* in maize fields with *Bt* and isogenic refuge areas to control FAW, by promoting an increase in parasitism rates and a greater viability of biological control programs. In maize to manage *S. frugiperda*, other clues should be studied in combination with volatiles released from the plants after moth oviposition to better understand aiming the best integrated pest management.

## 5. CONCLUSION

Our results showed that isogenic maize plants with FAW eggs are more attractive to female parasitoids than control maize plants (without eggs). Overall, oviposition by resistant and susceptible FAW populations in isogenic maize plants induced the emission rates of green leaf volatiles (GLVs): (*E*)-2-hexenal and (*Z*)-3-hexenal and these volatiles improved the egg parasitoid

searching behavior. Interestingly, the *Bt* expressing protein plants impaired the emission of GLV after oviposition by FAW, which led the parasitoid females to not discriminate between plant with and without eggs on *Bt* plants.

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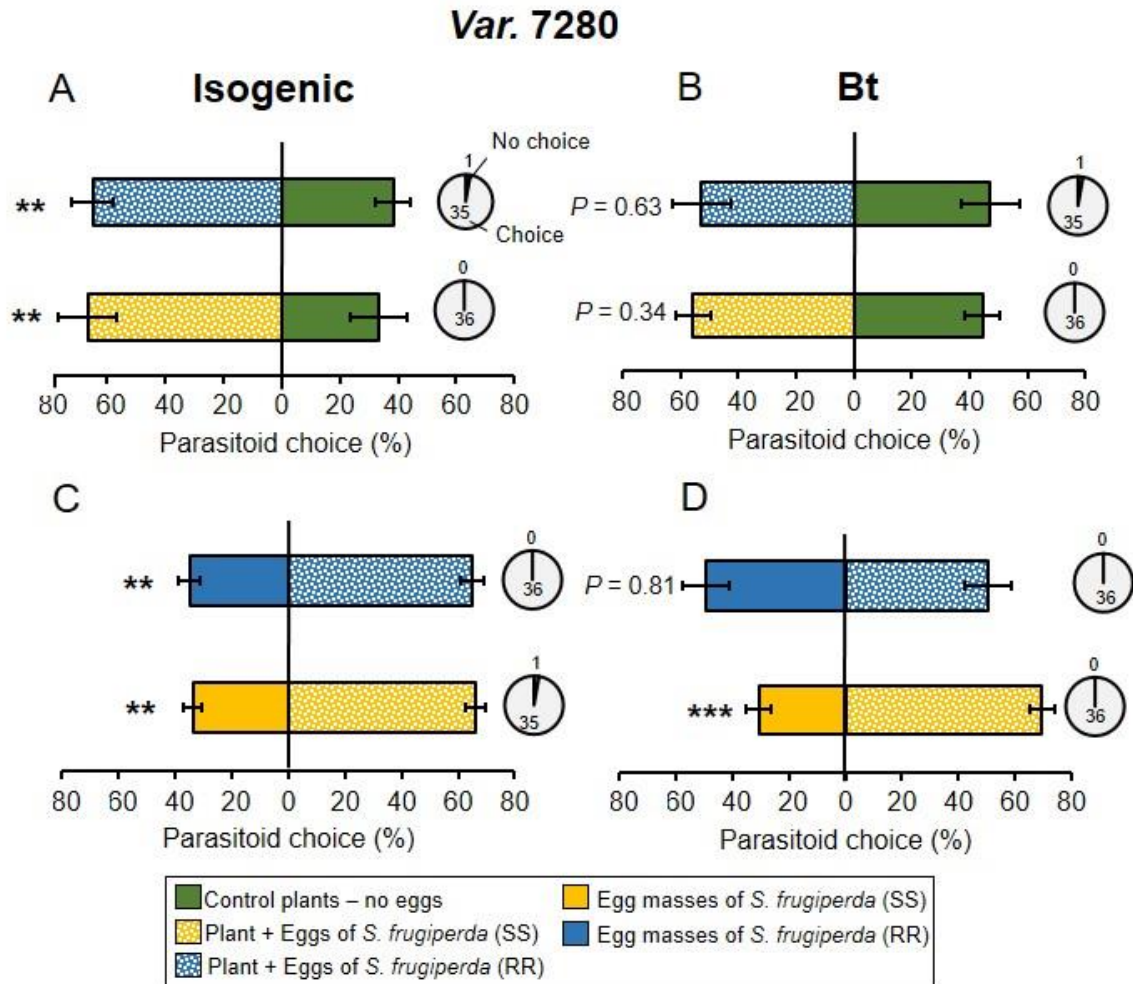
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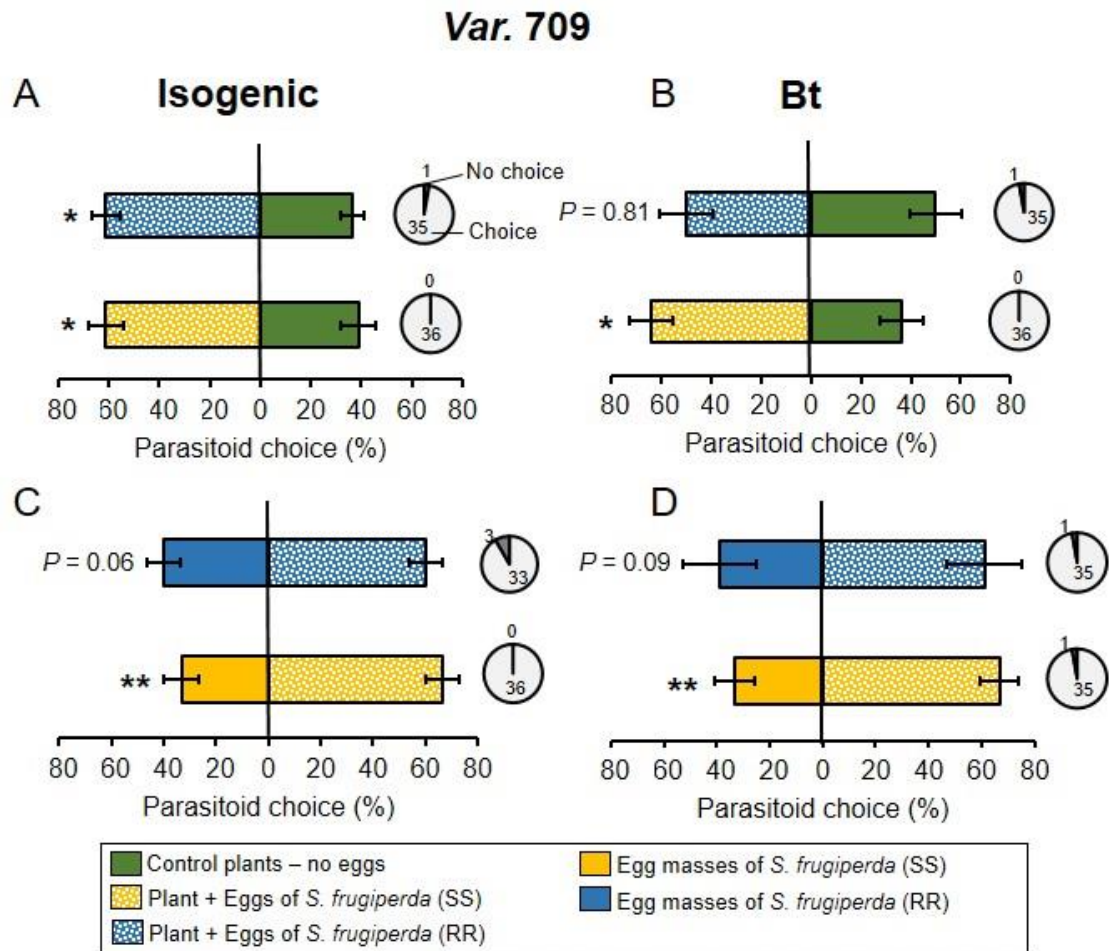
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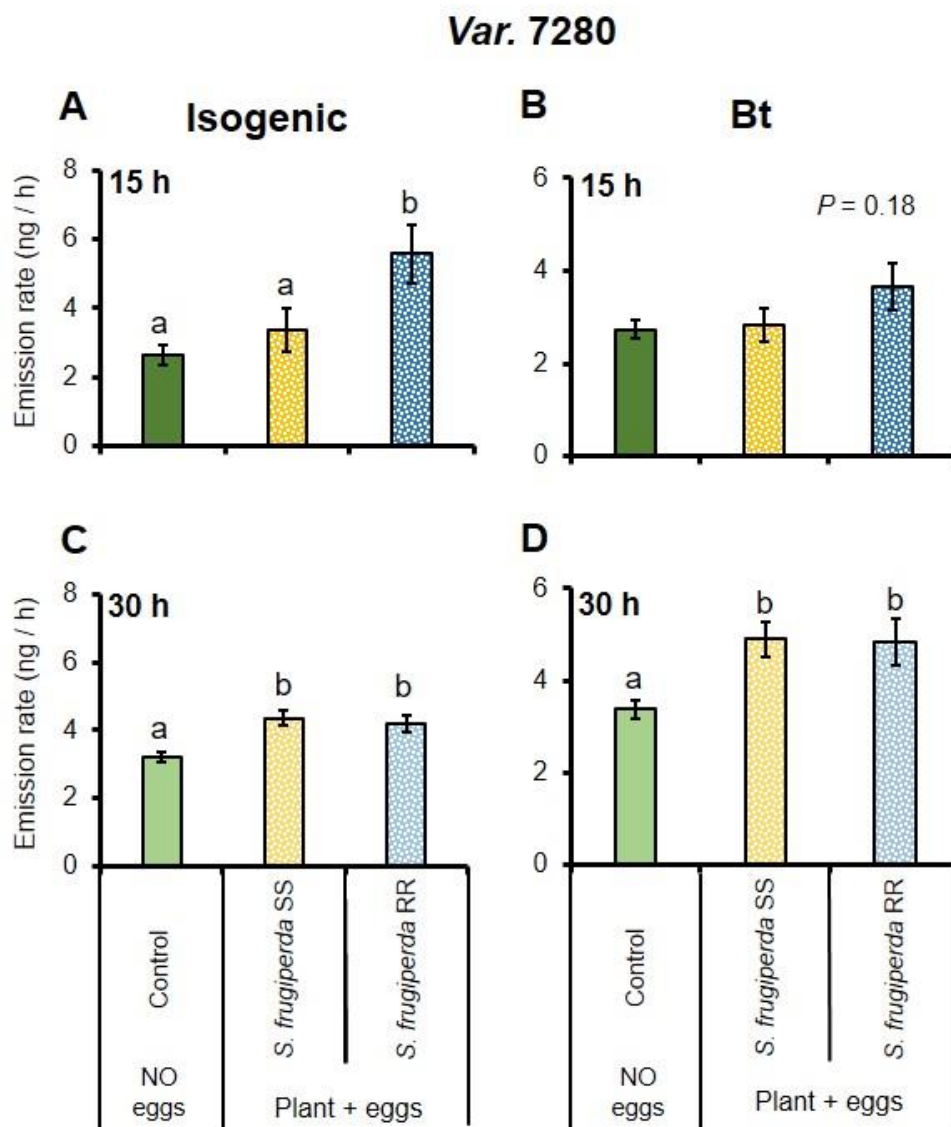
## 9. FIGURES FIRST CHAPTER



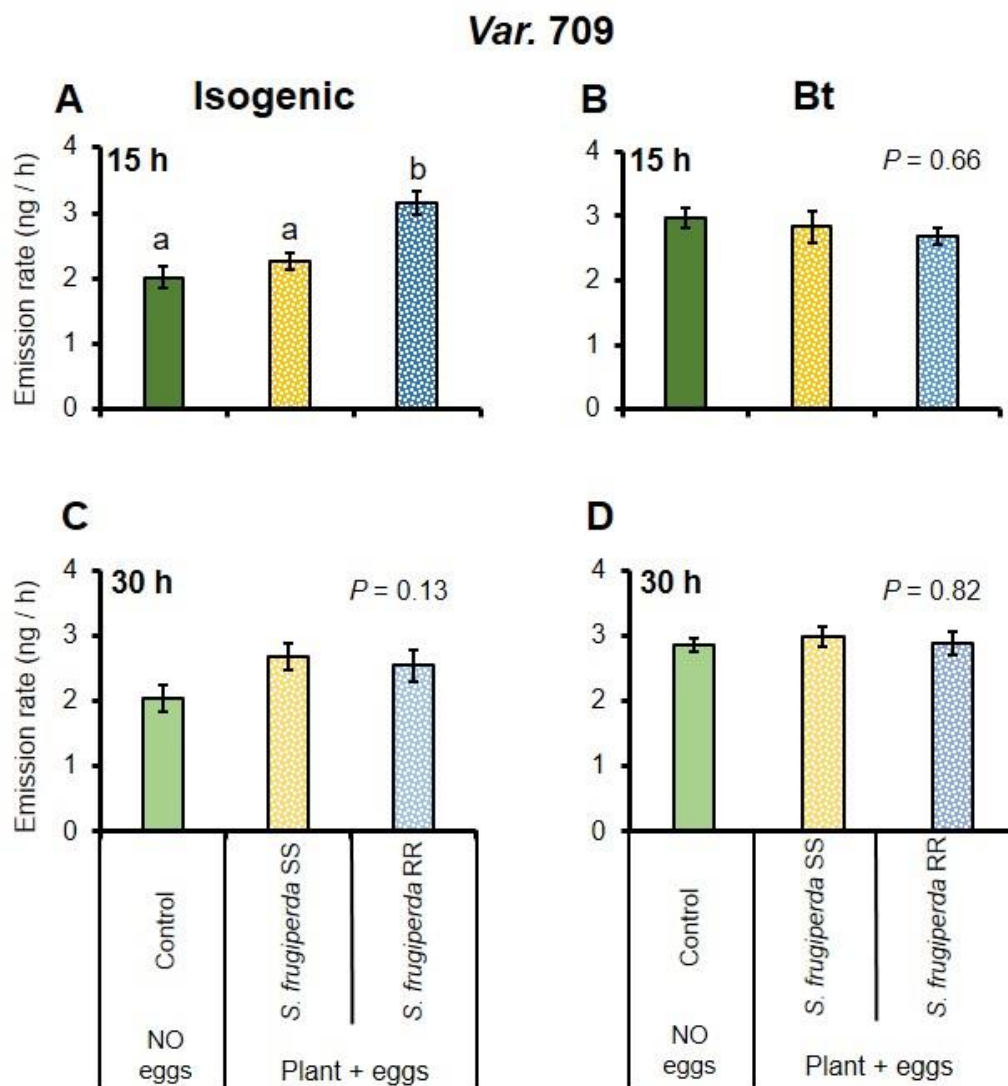
**Figure 1. GLVs of isogenic maize plants induced by oviposition are more attractive to a generalist parasitoid wasp.** Preference (%) of females of *T. pretiosum* parasitoids to HIPVs of var. 7280 isogenic (A, C, n=6) and Bt (B, D, n=6) maize plants induced by oviposition of *S. frugiperda* (SS and RR) after 15 hours in Y-olfactometer. Bars represent the percentages of females that chose the treatments ( $\pm$  S.E.). Pie charts indicate the number of females that chose or not. FDR-corrected  $P$  values are given for treatment comparisons [generalized linear model (family, binomial)], followed by pairwise comparisons of Least Squares Means (LSM). \*\*\*  $P < 0.001$ , \*\*  $P < 0.05$ .



**Figure 2. GLVs of Isogenic maize plants induced by oviposition attract more the generalist parasitoid wasps.** Preference (%) of females of *T. pretiosum* parasitoids to HIPVs of var. 709 Isogenic (A, C, n=6) and Bt (B, D, n=6) maize plants induced by oviposition of *S. frugiperda* (SS and RR) after 15 hours in Y-olfactometer. Bars represent the percentages of females that chose the treatments ( $\pm$  S.E.). Pie charts indicate the number of females that chose or not. FDR-corrected *P* values are given for treatment comparisons [generalized linear model (family, binomial)], followed by pairwise comparisons of Least Squares Means (LSM). \*\* *P* < 0.05, \* *P* < 0.05.



**Figure 3. Bt maize plants var. 7280 have a late HIPV emission response to FAW oviposition than their isogenic line.** Total emission rate of volatiles emitted by isogenic (A, C, n=5-8) and Bt (B, D, n=5-8) var.7280 maize plants with egg masses of: *S. frugiperda* (SS and RR) and plants without eggs (controls), after 15 and 30 hours of oviposition. Bars represent the average ( $\pm$ S.E.) of total emission of (Z)-3-hexenal and (E)-2-hexenal volatiles. *P* values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Different letters indicate significant differences among treatments ( $P < 0.05$ ).



**Figure 4. Bt maize plants var. 709 do not respond to FAW oviposition.** Total emission rate of volatiles emitted by isogenic (A, C, n=5-8) and Bt (B, D, n=5-8) var.709 maize plants with egg masses of: *S. frugiperda* (SS and RR) and plants without eggs (controls), after 15 and 30 hours of oviposition. Bars represent the average ( $\pm$ S.E.) of total emission of (Z)-3-hexenal and (E)-2-hexenal volatiles. *P* values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (LSMeans). Different letters indicate significant differences among treatments ( $P < 0.05$ ).

## 10. TABLES FIRST CHAPTER

**Table 1.** Number of replicates used in each treatment of maize plants.

Treatments	Var. 709				Var. 7280			
	Isogenic		Bt		Isogenic		Bt	
	15h	30h	15h	30h	15h	30h	15h	30h
Control	6	6	5	6	6	6	8	6
<i>S. frugiperda</i> SS	5	8	7	8	7	8	7	8
<i>S. frugiperda</i> RR	5	6	6	6	6	6	6	6

**Table 2.** Emission rate of volatiles (mean  $\pm$ SE) emitted (ng/hour) by isogenic *Var. 7280* of maize plants ( $n = 5-8$ ) oviposited by either *S. frugiperda* (SS and RR) and plants without eggs (control). Volatiles were collected after 15 and 30 of oviposition. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

Var. 7280	Isogenic			
	15 h		30 h	
	(Z)-3-hexenal	(E)-2-hexenal	(Z)-3-hexenal	(E)-2-hexenal
Control	1.32 $\pm$ 0.10 a	1.33 $\pm$ 0.17 a	1.48 $\pm$ 0.13 a	1.74 $\pm$ 0.06 a
<i>S. frugiperda</i> SS	1.52 $\pm$ 0.25 a	1.85 $\pm$ 0.39 a	2.47 $\pm$ 0.13 b	1.89 $\pm$ 0.11 a
<i>S. frugiperda</i> RR	2.31 $\pm$ 0.34 b	3.26 $\pm$ 0.49 b	2.38 $\pm$ 0.15 b	1.81 $\pm$ 0.12 a

**Table 3.** Emission rate of volatiles (mean  $\pm$ SE) emitted (ng/hour) by Bt *Var. 7280* of maize plants ( $n = 5-8$ ) oviposited by either *S. frugiperda* (SS and RR) and plants without eggs (control). Volatiles were collected after 15 and 30 of oviposition. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

Var. 7280	Bt			
	15 h		30 h	
	(Z)-3-hexenal	(E)-2-hexenal	(Z)-3-hexenal	(E)-2-hexenal
Control	1.25 $\pm$ 0.07 a	1.49 $\pm$ 0.17 a	1.88 $\pm$ 0.17 a	1.50 $\pm$ 0.09 a
<i>S. frugiperda</i> SS	1.43 $\pm$ 0.14 a	1.40 $\pm$ 0.25 a	2.66 $\pm$ 0.22 b	2.24 $\pm$ 0.21 b
<i>S. frugiperda</i> RR	1.51 $\pm$ 0.24 a	2.14 $\pm$ 0.27 a	2.65 $\pm$ 0.29 b	2.19 $\pm$ 0.25 b

**Table 4.** Emission rate of volatiles (mean  $\pm$ SE) emitted (ng/hour) by isogenic *Var. 709* of maize plants ( $n = 5-8$ ) oviposited by either *S. frugiperda* (SS and RR) and plants without eggs (control). Volatiles were collected after 15 and 30 of oviposition. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

<i>Var. 709</i>	Isogenic			
	15 h		30 h	
	(Z)-3-hexenal	(E)-2-hexenal	(Z)-3-hexenal	(E)-2-hexenal
<b>Control</b>	1.03 $\pm$ 0.11 a	0.98 $\pm$ 0.06 a	1.08 $\pm$ 0.13 a	0.96 $\pm$ 0.10 a
<b><i>S. frugiperda</i> SS</b>	1.18 $\pm$ 0.07 a	1.08 $\pm$ 0.08 a	1.34 $\pm$ 0.12 a	1.32 $\pm$ 0.09 c
<b><i>S. frugiperda</i> RR</b>	1.28 $\pm$ 0.07 a	1.88 $\pm$ 0.13 b	1.28 $\pm$ 0.11 a	1.26 $\pm$ 0.11 c

tests,  $P < 0.05$ ).

**Table 5.** Emission rate of volatiles (mean  $\pm$ SE) emitted (ng/hour) by Bt *Var. 709* of maize plants ( $n = 5-8$ ) oviposited by either *S. frugiperda* (SS and RR) and plants without eggs (control). Volatiles were collected after 15 and 30 of oviposition. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

<i>Var. 709</i>	Bt			
	15 h		30 h	
	(Z)-3-hexenal	(E)-2-hexenal	(Z)-3-hexenal	(E)-2-hexenal
<b>Control</b>	1.29 $\pm$ 0.05 a	1.67 $\pm$ 0.14 a	1.45 $\pm$ 0.13 a	1.4 $\pm$ 0.09 a
<b><i>S. frugiperda</i> SS</b>	1.5 $\pm$ 0.14 a	1.34 $\pm$ 0.11 a	1.53 $\pm$ 0.07 a	1.44 $\pm$ 0.09 a
<b><i>S. frugiperda</i> RR</b>	1.1 $\pm$ 0.07 a	1.6 $\pm$ 0.07 a	1.49 $\pm$ 0.08 a	1.39 $\pm$ 0.11 a

## CHAPTER 2

**Resistance to *Bt*-expressing protein of *S. frugiperda* caterpillars has an indirect cost by increasing the attraction of parasitoids in *Bt* maize plants**

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## ABSTRACT

Plants often release volatiles after herbivory and natural enemies can use these volatiles to find hosts. The fall armyworm (FAW) *Spodoptera frugiperda*, the primary pest of maize in South American countries, is mostly controlled by transgenic plants expressing *Bacillus thuringiensis* (*Bt*) proteins. However, this pest has shown resistance to most *Bt* proteins, and to delay the event in the field is very challenging. Therefore, a cost related to fitness could reduce the evolution of the insect resistance but their fact has not received much attention. In this study, we evaluated the volatiles induced after herbivory and caterpillar's oral secretion application resistant and susceptible FAW populations were applied on *Bt* and isogenic maize plants as well as how this induction affected the searching behavior of the egg parasitoid *Trichogramma pretiosum*. In addition, we measured the elicitor volicitin and volicitin-like compounds in the regurgitant of both *S. frugiperda* populations. Our results demonstrated that *T. pretiosum* females preferred volatiles emitted by isogenic maize plants treated with oral secretion of *Bt*-susceptible than resistant insects populations induced plants. On the other hand, in *Bt* plants, parasitoids preferred plants treated with regurgitant from resistant populations than *Bt*-susceptible ones. These results are in agreement with the total volatiles emitted by the plants, the plants that emitted the highest amounts of volatiles were preferred by the *T. pretiosum*. Furthermore, the *Bt*-susceptible insects produced the biggest abundance of volicitin and volicitin-like rather than the resistant populations. Therefore, our study unravels the effects of an ecological disadvantage for the resistant insects in *Bt* field and this could help delay the evolution of the resistance.

Keywords: Natural enemies. Tritrophic interaction. Fall armyworm. Induced defense. *Bt*

## 1. INTRODUCTION

Plants often release volatiles after herbivory so-called HIPVs (herbivore-induced plant volatiles), and natural enemies can use these volatiles to find their host (Peñaflor *et al.*, 2011; Nascimento *et al.*, 2021a, 2021b). The emission of volatiles can be triggered after the damage itself, but only the damage is not enough to induce a complete plant defense response (Heil, 2009; Heil & Land., 2014; Choi & Klessig., 2016). When feeding on the plants, herbivores release their oral secretion that can contain elicitors, which plants can recognize and use to activate a downstream signaling defense responses (Mattiacci *et al.*, 1995; Alborn *et al.*, 1997; Turlings *et al.*, 2000; Louis *et al.*, 2013). Various elicitors have been described as activators of the plant immune system (Turlings *et al.*, 1993; Mattiacci *et al.*, 1995; Schmelz *et al.*, 2006; Louis *et al.*, 2013). As volicitin and inceptin elicitors detected in the oral secretion of many caterpillars of *Spodoptera* species (Alborn *et al.*, 1997; Turlings *et al.*, 2000; Schmelz *et al.*, 2006). Moreover, volicitin is an example of elicitor detected in the most important maize pest in South America, the fall armyworm (FAW), *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) (Sparks, 1979; Blanco *et al.*, 2016).

The use of insecticides for many years was the only option to control FAW an important pest, until the registration of a field release of transgenic maize cultivars expressing the *Bacillus thuringiensis* (*Bt*) protein toxins (James & Krattiger, 1996; Okumura *et al.*, 2013; Biotecnología, 2015; McCormick, 2020). This technology was used for many years in our country without any problems to control FAW until these insects began to present resistance to the *Bt* toxins (Monnerat *et al.*, 2015, Farias *et al.*, 2014, Faretto *et al.*, 2017; Santos-Amaya *et al.*, 2022). The resistant insects can develop and reproduce in *Bt* plants, and consequently, resistance reduces the effectiveness of strategy approach as a control tactic (Omoto *et al.*, 2016; Huang *et al.*, 2014). One approach used to delay the resistance in the field is the refuge strategy and the *Bt* high dose (Gassmann *et al.*, 2009). The refuge area strategy aims at ensuring a sufficient number of susceptible adults in non-*Bt* plants (non *Bt* variety area surroundings *Bt* crop) to mate with resistant adults, this way the offspring should be killed by a high dose encountered at *Bt* fields (Carrière & Tabashnik., 2001; Tabashnik *et al.*, 2013). Alternatively to delay the resistance could be a fitness cost and if there is a fitness cost associated with resistance, susceptible individuals should do better in non-*Bt* areas (refuges), which may reduce the allelic frequency of resistant insects (Gassmann *et*

*al.*, 2009). Therefore, when fitness cost happens, the evolution of the resistance can be reduced or delayed.

A diversity of fitness costs has been associated with the development of resistance in lepidoptera insects, including longer developmental time, reduced mass, lower pheromone production, lower fecundity, as well as, lower fertility (Delisle & Vincent, 2002; Carrière *et al.*, 2006; Zhao *et al.*, 2009; Zhang *et al.*, 2014; Zhu *et al.*, 2019). Santos-Amaya *et al.*, (2022) related delay in the developmental time, as well as, reduction in survival rate and pupal weight in *S. frugiperda* individuals resistant to a Cry1A.105+Cry2Ab2 expressing *Bt* maize plants compared to susceptible insects. Furthermore, de Souza *et al* (2022) showed that the spermatophore mass, time since the onset of mating and fertility for *S. frugiperda* resistant to a Cry1Fa expressing *Bt* maize plants were negatively affected. Those are costs related to the development and reproduction affecting directly the individual. The indirect cost of turning more vulnerable to natural enemies attack has received less attention (Gassmann *et al.*, 2009; Hiltpold & Hibbard, 2018).

There is a reasonable acknowledgment that plants under attack can use indirect defenses to attract natural enemies (Turlings *et al.*, 1995; Turlings & Wäckers, 2004; Peñaflores *et al.*, 2011; Turlings & Erb, 2018; Nascimento *et al.*, 2021a), as well as, that *Bt* and isogenic plants can emit different amounts of volatiles (Smith *et al.*, 1996; Dean & De Moraes, 2006; Nascimento *et al.* 2021a). However, how resistant and susceptible insect populations can trigger the same plant defense responses and how this affects the third level trophic is unknown. Thus, for a better understanding of FAW resistant and susceptible populations specificity of the herbivory inducing plant volatiles, detailed analyses of HIPV in *Bt* and isogenic plants, as well as, of regurgitant of these populations are necessary. Additionally, the behavioral responses of natural enemies to these volatiles could be very important to evaluate the indirect cost of *Bt* resistance. For this, we hypothesize that FAW *Bt*-resistant population will induce less volatiles on *Bt* maize plants after caterpillar feed, which will impact an egg parasitoid host location.

## **2. MATERIAL AND METHODS**

### **2.1. Insects Rearing**

Three resistant populations of *Spodoptera frugiperda* to an event expressing Cry1A.105 + Cry2Ab *Bt* toxins were used: *S. frugiperda* Bahia RR, *S. frugiperda* Canapolis RR, and *S. frugiperda* Cajuri RR, and one susceptible isoline (SS) *S. frugiperda* Bahia SS. The laboratory Bahia RR and SS colonies have the same genetic background (Santos-Amaya *et al.*, 2015). The four populations were obtained from the Laboratory of Insect-Plant Interaction at the Universidade Federal de Viçosa. After seven generations, we checked the population's susceptibility and the mortality of caterpillars by offering *Bt* plants expressing Cry1A.105 + Cry2Ab *Bt* toxins are able to cause 50% mortality (LC50) or 50% growth inhibition (Santos-Amaya *et al.*, 2015). Briefly, the adults were maintained in PVC cages (20 cm diameter x 30 cm height) covered with paper on the inner walls for oviposition, eggs were collected every two days and kept in 500 ml plastic bags until hatching. A piece of cotton soaked in a 10% sugar and 1% ascorbic acid solution was offered every two days *ad libitum* as adults food source. We kept groups of neonates in plastic cups until they reached the 2<sup>nd</sup> instar. After that, the caterpillars were individually placed in 16-cell PVC trays (Advento do Brasil Ind. e Comércio de Plásticos Ltda, São Paulo, Brazil) until pupation. All caterpillar stages were fed on an artificial diet (Greene *et al.*, 1976). The insects were reared in controlled conditions ( $27 \pm 3^\circ \text{C}$ ,  $70 \pm 15\%$  R.H., and 14:10 h L/D photoperiod).

The generalist egg parasitoid *Trichogramma pretiosum* was purchased from Ecotrix Biodefensivos (Viçosa, MG, Brazil). They were fed on pure honey and maintained under controlled conditions ( $27 \pm 3^\circ \text{C}$ ,  $70 \pm 15\%$  R.H., and 14:10 h L/D photoperiod). The adults were sexed according to antennal morphology dimorphism (Querino & Zucchi, 2003).

## **2.2. Plant Growing Conditions**

Maize plants (*Zea mays* L.) of *Bt* (var. GNZ 7280 PRO2, Geneze seeds) and isogenic lines were used (GNZ 7280, Geneze Seeds). These *Bt* varieties were chosen since *S. frugiperda* RR populations are resistant to these toxins expressed (Cry1A.105 + Cry2Ab) and the isogenic plants without the toxin expression. Plants were grown in 180 ml plastic pots filled with soil (MecPlant, Brazil) under greenhouse conditions at  $27 \pm 3^\circ \text{C}$ , 70% R.H. and 14:10 h L/D photoperiod and watered every other day. Plants with 10-15 days-old and three to four fully developed leaves were used in all experiments.

### 2.3. Herbivory and simulated-herbivory induction of volatiles in maize plants

To assess whether there is an adaptive cost related to the HIPV emission by isogenic and *Bt* maize plants under *S. frugiperda* caterpillar and simulation of caterpillar attack, we sampled HIPVs from plants attacked by caterpillars of *S. frugiperda* either resistant or susceptible populations to *Bt* proteins. The HIPVs were collected for three hours from isogenic maize plant after 12 hours under attack of five caterpillars on third instar per plant and control from plants without herbivory (n=6). To standardize the amount of damage, we simulated caterpillars attack by applying regurgitant on mechanically wounded leaves (Figure 1) (Turlings *et al.*, 1993; Arce *et al.*, 2021). Briefly, wounding was inflicted by scratching the leaf (~2 cm<sup>2</sup>) with a serrated forceps and 10 µl of pure regurgitant collected from *S. frugiperda* populations caterpillars (RRs and SS) was applied on the wound site either on *Bt* and isogenic plants. Control plants for the wounding was performed by adding 10 µl of water. For details of regurgitant collection see below section. Isogenic and *Bt* maize plants (n = 6) were treated two times and two leaves were induced (3<sup>rd</sup> and 4<sup>th</sup> leaves), after two hours of the first induction the plants were induced once more. Then, the HIPVs were collected during the three following hours.

### 2.4. Volatile collection and analysis

To sample the HIPVs emitted by isogenic maize plants induced either by herbivory damage or regurgitant application with the four populations of *S. frugiperda* (SS and RR), the plants were individually placed in cylindrical glass chambers (37 cm high × 14 cm diameter) and the plant pots were covered with aluminum foil. Briefly, humidified and charcoal-filtered air (1 L/min) were introduced into the chambers and the volatiles were pulled (0.5 L/min) and trapped into 20 mg of Haye Sep Q (80–100 mesh) (Analytical research systems, Florida, USA). After 3 hours of sampling, the filters were eluted with 150 µl of hexane (double-distilled HPLC-grade, Sigma-Aldrich, São Paulo, Brazil) and 10 µl of nonyl-acetate (20 ng/µl) was added as internal standard (I.S.). The sample was stored at -80 °C until further analysis.

The volatile samples were analyzed in a gas chromatograph coupled with a mass spectrometer (Shimadzu GCMS – QP2010 SE). One microliter of each sample was injected into a pulsed splitless mode onto a Rtx-5MS (Restek chromatography products) column, DB-5 (30 m ×

0.25 mm;  $df = 0.25 \mu\text{m}$ ; J&W Scientific, Folsom, CA, USA) using helium as carrier gas (170 kPa), at a constant flow of  $1.1\text{mL}/\text{min}^{-1}$ . The temperature was maintained at  $40 \text{ }^\circ\text{C}$  for 3 min, then increase to  $100 \text{ }^\circ\text{C}$  at  $8 \text{ }^\circ\text{C}/\text{min}$  and subsequently to  $230 \text{ }^\circ\text{C}$  at  $5 \text{ }^\circ\text{C}/\text{min}$ , followed by a post-run of 3 min at  $250 \text{ }^\circ\text{C}$ . The identification and quantification of compounds were performed using comparisons to the mass spectra of commercial standards and NIST 17 library spectra.

## 2.5. Behavior bioassays

To evaluate whether there is a female's parasitoid preference among HIPV emitted by isogenic maize plants under simulating herbivory by regurgitant application of *S. frugiperda* either resistant or susceptible to *Bt* proteins (SS and RR), we evaluated *T. pretiosum* female's response to HIPVs in a choice bioassay in a y-olfactometer. The plants were treated the same manner as described above for HIPV collection and the experiments were performed during the following three hours after the last regurgitant application. Two-choice bioassays were performed in a y-tube olfactometer according to Janssen, (1999). Briefly, the olfactometer consists of a Y-shaped glass tube (27 cm in length  $\times$  3.5 cm in diameter) connected to a vacuum pump that pushes the air at 0.3 m/s. The airflow had a negative pressure flow, for pushing the air from the glass containers (50 x 36 x 43), where the odor sources are placed into the olfactometers arm to the base. A black Y-shaped metal wire in the middle of the Y-tube is used to guide the parasitoid during walking. Before starting the experiments the airflow was measured by using a hot-wire anemometer (Velocicalc 9545-A, São Paulo, Brazil).

The parasitoids were offered to HIPV's release by plants-treatments as follow plants treated: *Bt* plants with regurgitant from larvae of Bahia SS vs Bahia RR; Bahia SS vs Mechanical Damage; Bahia SS vs Cajuri RR and Bahia SS vs Control Plants, the same were offered to isogenic plants. We also compared between *Bt* and isogenic plants in the following choices: *Bt* plants with regurgitant from Bahia SS vs isogenic plants with regurgitant from Bahia SS and *Bt* plants with regurgitant from Bahia RR vs isogenic plants with regurgitant from Bahia RR.

The parasitoid mated females of two to three days-old were released on the black Y-shaped metal wire at the base of the Y-tube until reached the end of one of the arms for a maximum of 5 minutes, then the next female was introduced. We tested one by one, and every eight females in a row consisted of one replicate in the choice bioassay. Six to eight replicates were carried per

combination of volatile sources contrast. Each parasitoid was used only once to prevent odor learning. Females that did not make a choice within 5 min were scored as having made no choice and excluded from the analysis. After each replicate being performed the Y glass tube was replaced by a cleaned one. In addition, volatile sources were switched to the opposite arm of the olfactometer after each replicate to avoid bias.

## 2.6. Regurgitant collection and analysis

To evaluate whether there is an adaptive cost related to resistance, we measured the amount of fatty acid-derived elicitors, specially volicitin N-(17-hydroxylinolenoyl)-L-glutamine, an elicitor in the caterpillar from the *Spodoptera* genus (Alborn *et al.*, 2000; Turlings *et al.*, 2000). Therefore, we collected the regurgitant of *S. frugiperda* of all resistant and susceptible populations fed on the isogenic maize variety. For this, third to fourth instar caterpillars were squeezed close to the head, which forced them to regurgitate (Turlings *et al.*, 1993). Then, 10  $\mu$ l of regurgitant from two caterpillars were collected per replicate (N=5 per treatment). The caterpillars were previously fed on maize leaves (isogenic line) for 24 h. The regurgitant samples were stored at  $-80^{\circ}\text{C}$  until further use. Volicitin and volicitin-like compounds were extracted by adding 990 ml of MeOH:H<sub>2</sub>O 50% on each sample (Arce *et al.*, 2021). Then, the samples were vortexed and centrifuged at 10000 rpm for 15 minutes. Finally, we collected the supernatant and used for volicitin analysis.

The analyses of volicitin were performed by Agilent, the method was optimized for an Ultra-High-Performance Liquid Chromatography (UHPLC) coupled online to a mass spectrometer QQQ (triple quadrupole). The solvent flow rate is  $0.25\text{ml min}^{-1}$  in a column at  $30^{\circ}\text{C}$ . The ionization method used in the mass spectrometry was an ESI (Electrospray Ionization) under the conditions: gas temperature of  $300^{\circ}\text{C}$ , nitrogen flow rate of  $10\text{L min}^{-1}$ , nebulizer pressure of 35psi and capillary voltage of 4000V (Vital *et al.*, 2019). Chromatographic separation was performed by reverse phase columns, such as an analytical Zorbax Eclipse Plus C18 ( $1.8\ \mu\text{m}$ ,  $2.1 \times 150\text{mm}$ ) and a guard column Zorbax SB-C18,  $1.8\ \mu\text{m}$  (Agilent Technologies, Santa Clara, California, USA). The mass spectrometer was operated by negative mode. The samples were injected using the MRM method, which was used to generate the standard curves. The data were analyzed using the Skyline software and, after obtaining peak area values, and related molecules based on the determination of the most

probable molecular formula as well as fragmentation pattern (typical fragment at  $m/z$  145.0615 corresponding to a glutamine).

## 2.7. Statistical Analysis

Statistical analyses were performed in R (v. 4.0.0; R Development Core Team, 2020) using Analysis of Variance (ANOVA), followed by residual analysis to verify the suitability of distributions of the tested models. Generalized Linear Models (GLM) with a Gaussian distribution were used to verify the differences in volatile emission rates emitted by maize plants induced by real caterpillars and with regurgitant application. Least Squares Means (*emmeans*) were carried out for pairwise comparison. To check the preference of the parasitoid a Generalized Linear Models (GLM) with a Binomial distribution were used. A hierarchical cluster heatmap were carried using MetaboAnalyst 5.0.

## 3. RESULTS

### 3.1. Herbivore-induced plant volatiles by caterpillar and oral secretion

Our results showed that perhaps a cost related to the resistance to *Bt* toxins on the emission of volatiles in maize plants seems to happen (Fig. 2). The isogenic maize plants emitted higher amounts of HIPV 12 hours after being attacked by susceptible *S. frugiperda* caterpillar's population than the resistant populations (Fig. 2A-B). For instance, we found ten compounds induced by *S. frugiperda* caterpillars in isogenic plants: 3-(Z)-Hexenal, 2-(E)-Hexenal, (Z)-3-Hexen-1-ol, (Z)-3-Hexen-1-ol, acetate,  $\beta$ -Linalool, DMNT ((E)-4,8-dimethyl-1,3,7-nonatriene), (Z)- $\alpha$ -Bergamotene, (E)- $\beta$ -Farnesene,  $\beta$ -Sesquiphellendrene, TMTT (4,8,12-trimethyltrideca-1,3,7,11-tetraene) (Figure 2A-E, Table 1). Furthermore, the susceptible Bahia SS caterpillars induced more emission of Linalool, DMNT, (Z)- $\alpha$ -Bergamotene, (E)- $\beta$ -Farnesene and TMTT than resistant populations and undamaged plants (Figure 2C-E, Table 1). In addition, isogenic maize plants induced higher amounts of 3-(Z)-Hexenal under attack of resistant Canapolis RR population (Figure 2E, Table 1).

The cost related to the *Bt* resistance on *S. frugiperda* caterpillars on the emission of volatiles in maize plants was strong when the induction of the plants were done by simulating herbivory and

regurgitant treatment both in isogenic and *Bt* plants (Fig. 3). Therefore, the isogenic maize plants emitted higher amounts of HIPV after mechanical injury and application of regurgitant collected from *S. frugiperda* Bahia SS susceptible population than from any resistant populations (Fig. 3A, 3c and 3E). Although the *Bt*-resistant population from Canapolis showed a similar induction as the susceptible caterpillar regurgitant (Fig. 3A). We detected eight compounds induced by *S. frugiperda* caterpillars in isogenic plants followed by mechanical injury followed by regurgitant application: 3-(Z)-Hexenal, 2-(E)-Hexenal, (Z)-3-Hexen-1-ol, (Z)-3-Hexen-1-ol, acetate, Linalool, DMNT ((E)-4,8-dimethyl-1,3,7-nonatriene), Indole and (E)- $\beta$ -farnesene (Fig. 3E and 3G, Table 2). The overall emission of HIPV after regurgitant application showed to be responsible for this pattern at 66 % of the time (Fig. 3C). The susceptible Bahia SS regurgitant induced higher emissions of Indole and 3-(Z)-Hexenal when treated on isogenic maize plants HIPVs emission of indole than resistant populations, mechanically damaged and undamaged plants (Fig. 3E and 3G, Table 2). Regarding the response of *Bt* maize to *S. frugiperda* regurgitant, we observed that these plants strongly respond to regurgitant of resistant Bahia RR population followed by Canapolis RR population (Fig. 3B, 3D, 3F), and we found the same eight compounds detected on the isogenic plants (Fig. 3F and Table 3). The overall emission of HIPV after resistant regurgitant application were responsible for this pattern, at very low rate reaching up to 33 % of the times (Fig. 3D). Moreover, all the resistant populations of *S. frugiperda* induced higher emissions of DMNT, but only the resistant Bahia RR induced higher rates of (Z)-3-Hexen-1-ol, acetate when treated on *Bt* maize plants (Fig. 3F, 3H, Table 3).

### **3.2. Parasitoid attraction depends on the *Bt* susceptibility of *S. frugiperda* on *Bt* plants but not in their isogenic line**

The *S. frugiperda* *Bt* protein resistant impaired *T. pretiosum* wasp's ability in discriminating between maize plants volatiles emitted after simulating herbivory by applying regurgitant of caterpillars (Fig. 4A-C). The parasitoid wasp *T. pretiosum* were strongly attracted to the HIPVs emitted by the isogenic maize plants treated with regurgitant of *S. frugiperda* susceptible population rather than undamaged plants, mechanical damage only, regurgitant application of the resistant populations Bahia RR and Cajuri RR (Figure 4A). Moreover, the *Bt* expressing maize plants induced with regurgitant of the susceptible Bahia SS population were also more attractive to the female's wasps than undamaged and mechanically damaged plants (Fig. 4B). Interestingly, the

regurgitant of the resistant population Bahia RR were more attractive to the parasitoid than the HIPV emitted by the susceptible *S. frugiperda* regurgitant (Fig. 4B). Surprisingly, Cajuri RR regurgitant provoked a different pattern of parasitoid choice (Fig. 4B). Yet, the parasitoid females did not discriminate between *Bt* plants treated with either resistant or susceptible regurgitant (Figure 4B). additionally, when contrasting the HIPVs emitted from isogenic and *Bt* maize plants induced by Bahia SS *S. frugiperda* regurgitant the females of *T. pretiosum* preferred the odors from isogenic maize plants then from the *Bt* plants (Fig. 4C). In the other hand, when the plants were treated with the regurgitant of the resistant Bahia RR population the parasitoid did not show any preference (Fig. 4C).

### 3.3. *S. frugiperda* resistant to *Bt* proteins impairs the amount of FACs in the regurgitant

The chemical analyses of the regurgitant of the four populations of *S. frugiperda* used Bahia RR, *S. Canapolis* RR, Cajuri RR and Bahia SS fed on isogenic maize plants revealed the presence of four different fatty acid amino acids conjugates (FACs): 18:2-glutamine-1, 18:3-glutamine-1, 18:2-OH-glutamine-1 and 18:3-OH-glutamine-1 (volicitin) (Fig. 5, Table 4). Interestingly, the resistance to *Bt* toxins constrains the amount of FACs present in the regurgitant of *S. frugiperda* Bahia, Canapolis and Cajuri resistant populations which was not observed in the susceptible population Bahia (Fig. 5, Table 4). The main FAC, volicitin is the one found in highest amounts in all treatments and the population Bahia SS is the one that showed the more abundance of this elicitor rather than the other three RR populations (Fig. 5, Table 4).

## 4. DISCUSSION

It is well known that resistance can lead to a fitness cost related to the development and some reproductive features for the individual (Gassmann *et al.*, 2009; Zhao *et al.*, 2009; Zhang *et al.*, 2014 de Souza *et al.*, 2022; Santos-Amaya *et al.*, 2022). Those are direct cost, because they affected directly the performance of the insects. Although, in other cases an indirect fitness cost might happen when the resistance affects indirectly an individual, resulting in biggest induction of volatiles on the plants and attracting more natural enemies (Gassmann *et al.*, 2009). Here, *S. frugiperda* population resistant to *Bt*- expressing protein suffered an indirect cost related to this feature, since the parasitoid females of *T. pretiosum* were more attracted by HIPV from *Bt* maize

plants induced by regurgitant from the same population. Beside that the stronger induction is partially explained by volicitin, which was higher in the regurgitant from Bahia-SS FAW populations. However, the *Bt*-resistant FAW ones produced lower amount of the elicitor, therefore we can not exclude that this population also have other factors triggering the plant responses.

Recently, Hiltbold & Hibbard (2018) related indirect cost inflicted in *Bt* hybrids in maize, which *Bt* plants only induced the emission of volatiles when attacked by resistant *Diabrotica virgifera virgifera*, otherwise maize plants were not elicited when attacked by *Bt*-susceptible *D. virgifera virgifera* population. Interestingly, this difference in volatiles emission did not affect the entomopathogenic nematodes (*Steinernema carposcopsae* and *Heterorhabditis megidis*) host searching. In our study, we provide evidence that supports an indirect fitness cost. Higher emission of volatiles was observed when the resistant *S. frugiperda* population fed on *Bt* maize rather than when *Bt*-susceptible *S. frugiperda* and a clear preference of the parasitoid *T. pretiosum* females towards plants that emitted the highest amount of volatiles. Thus, the indirect cost related to the genotype of *S. frugiperda* resistant and susceptible affected the attraction of an important eggs parasitoid *T. pretiosum*. *Bt* plants attracted more when parasitoids fed by resistant insects. Therefore, it could be postulated that an ecological disadvantage in the resistant insects attacking *Bt* maize plants, since they could have more chances to be found by natural enemies and this could help slow down the evolution of the resistance.

It is known that plants under attack by chewing herbivores trigger their induction of defense through an elicitor derived from the attackers. *Spodoptera* spp (Turlings *et al.*, 1993; Turlings *et al.*, 2000; Schmelz *et al.*, 2006; Blanco *et al.*, 2016., Arce *et al.*, 2021) are known to have volicitin and volicitin-like compounds in their regurgitant, used by plants as downstream signaling (Mattiacci *et al.*, 1995; Turlings *et al.*, 2000; Louis *et al.*, 2013, Arce *et al.*, 2021). We found a higher amount of these elicitors in the regurgitant of *Bt*-susceptible *S. frugiperda* caterpillars fed on isogenic maize plants rather than in *Bt*-resistant populations that were fed on isogenic maize plants. This could explain the higher volatile emission rates emitted by isogenic plants either attacked by caterpillars or induced with regurgitant of *Bt*-susceptible *S. frugiperda* population. Regurgitant of *S. littoralis* and *S. frugiperda*, induced different amount of volatiles depending if the maize genotype *Bt* or isogenic (Turlings *et al.*, 2005; Xu *et al.*, 2019; Nascimento *et al.*, 2021a). The lack of response by *Bt* maize plants after larval feeding or regurgitant application could be due to no induction of the

*Bt* genes. Thus, further investigations of genes related to volatile induction should be performed to explain better this pattern.

However, we did not observe the same pattern when *Bt* plants were induced with regurgitant whereas the *Bt*-resistant regurgitant from Bahia and Canapolis populations induced the most the *Bt* plants. Therefore, it is presumable that other factors beside the elicitor amount is playing a role in this process. It is known that different species of caterpillars can induce different volatiles (Geervliet *et al.*, 1997; De Moraes *et al.*, 1998; Turlings *et al.*, 1998; Cai *et al.*, 2014) and that different species of caterpillars produce different elicitors or amount of elicitors (Pohnert *et al.*, 1999; Mori *et al.*, 2001; Arce *et al.*, 2021). However, to our best knowledge, this is the first time that the difference between the same specie resistant's populations from different places has been reported. Schmelz *et al* (2006) showed that inceptin was another elicitor found in the oral secretion of *S. frugiperda* after feeding on beans that functions as signal initiating specific plant responses to the caterpillar attack, what means that elicitor production can change, depending on the host. In addition, we know that the same species from different locations can produce different amounts of important compounds related to the communication between females and males (Hansson *et al.*, 1990; Miller *et al.*, 1997; Andrade *et al.*, 2000). Furthermore, Arce *et al* (2021) showed that *Spodoptera exigua* fed on maize plants produced the same quantity of the elicitor volicitin as on cotton, but the plants induced different amount of volatiles depending on the host. Therefore, we hypothesize that can exist another factor that is triggering induction of volatiles on *Bt* plants by resistant population of FAW that remains to be tested, as genes related to volatile induction, the possible existence of another elicitor and the degree of the insect resistance.

In summary, our findings revealed an indirect fitness cost to *Bt*-resistant *S. frugiperda* population since greater amount of volatiles is induced and therefore attracted more parasitoid in *Bt* maize plants. Currently, HIPVs are used as tool in the integrated pest management to attract natural enemies in field (James, 2003; James & Grasswitz, 2005; Sharma *et al.*, 2019; Conboy *et al.*, 2020). Therefore, these results may help to optimize the biological control with *T. pretiosum* in maize fields with *Bt* and isogenic refuge areas to control FAW. For instance, HIPVs: m-cymene and 4'-ethylacetophenone from damaged *Ricinus communis* plants are already being used to attract the braconid wasp *Peristenus spretus* a native endoparasitoid of the mirid bug *Apolygus lucorum* (Xiu *et al.*, 2019). Furthermore, an egg parasitoid *Anaphes iole* have its attraction augmented

following applications of HIPVs: (Z)-3-hexenyl acetate or  $\alpha$ -farnesene (Williams *et al.*, 2008). In addition, we showed that *Bt*-resistant insects fed on *Bt* plants produced a biggest induction of DMNT in relation to *Bt*-susceptible ones fed on isogenic plants. Contrarily, a biggest induction of Indole when *Bt*-susceptible insects fed rather than *Bt*-resistant ones. Thus, other clues should be studied in combination with volatiles released from the *Bt* and isogenic plants to find best-integrated pest management and retard the evolution of resistance.

## 5. CONCLUSION

Therefore, in our study, we identified the amount of elicitors of caterpillars from resistant and susceptible FAW populations and the behavioral responses of natural enemies to the volatiles changes by the resistant and susceptible herbivore induced plant volatiles. These results unravel the effects of *Bt* mediated resistance on important biological control agent and may help optimize the biological control of FAW with *T. pretiosum* in maize fields with *Bt* and refuge areas. Our results showed that susceptible populations produced a greater amount of the elicitor volicitin and volicitin-like rather than in resistant individuals. This may have affected the induction of volatiles, because when resistant insects fed on *Bt* plants there was a bigger induction of volatiles comparing with susceptible fed on and the opposite happens with isogenic plants. In addition, parasitoid host location was affected by the amount of volatiles and they preferred plants that induced larger amount of volatiles either on *Bt* or isogenic or between the same plant genotype. Therefore, resistant insect populations of FAW were a disadvantage in *Bt* maize plants even producing less volicitin they were able to elicit a stronger response in *Bt* plants. This shows that more than one elicitor is required to trigger a response in *Bt* maize plants.

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## 7. FOUNDING

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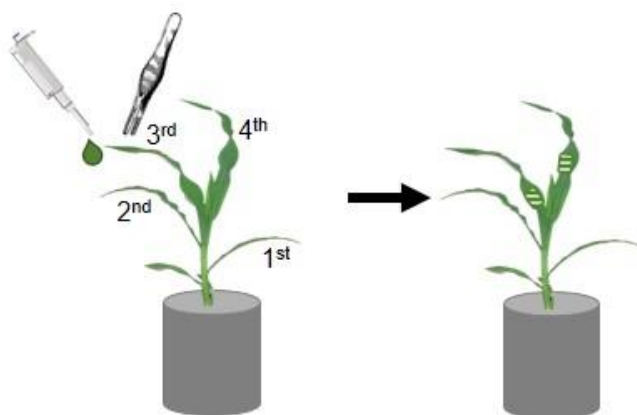
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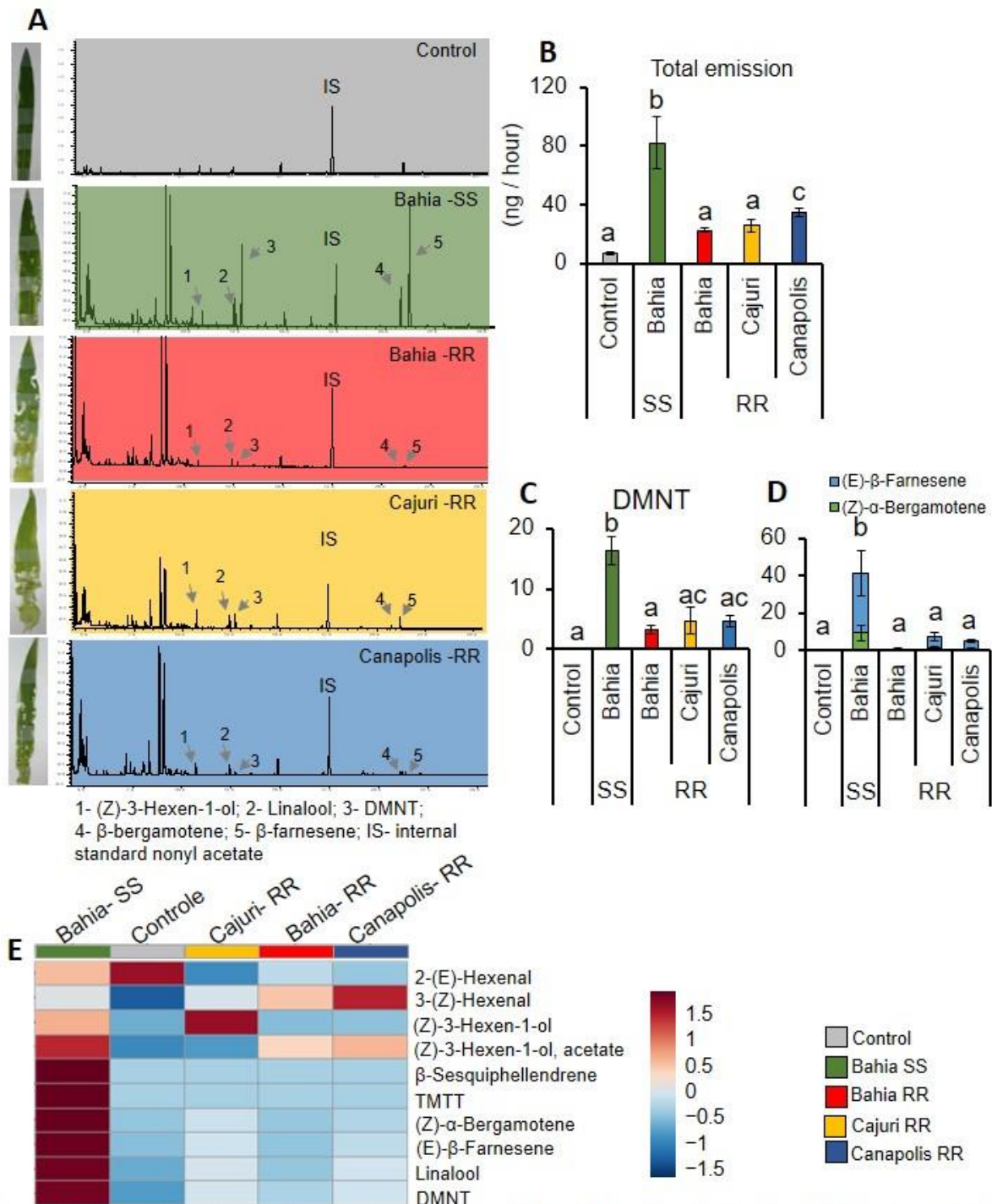
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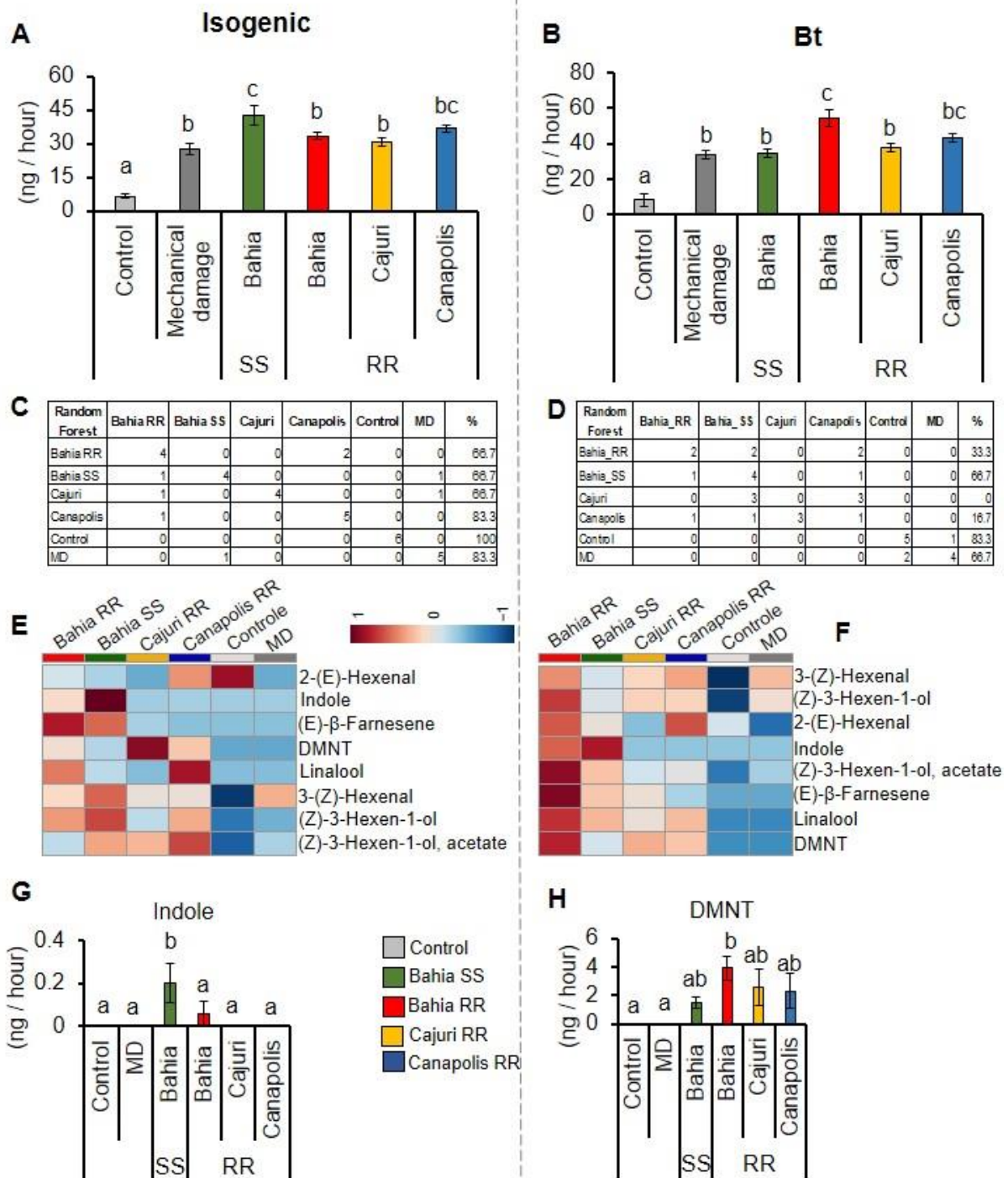
## 9. FIGURES SECOND CHAPTER



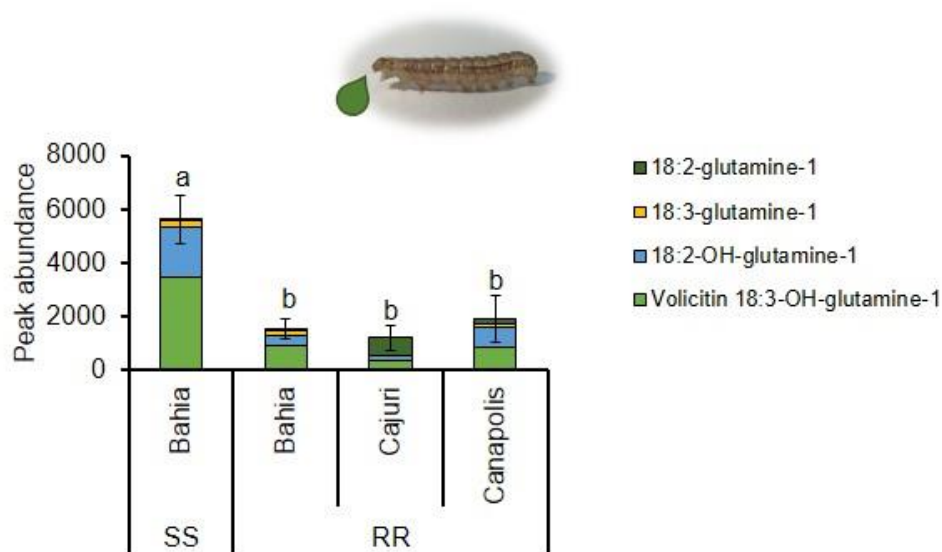
**Figure 1: Illustration of simulated herbivory on maize plants.** Four-leaves stage maize plants used to measure herbivore-induced plant volatile (HIPV) emissions. The third and fourth leaf were wounded every two hours with a serrated forceps and immediately afterward 10  $\mu$ l of regurgitant were applied on the wounded sites. The HIPVs were collect after the second time of induction.



**Figure 2: Isogenic maize plants are strongly induced after *S. frugiperda* attack and this pattern is Bt susceptibility dependent.** (A) Typical chromatograms of maize plants (n=6) undamaged (control) and attacked by five second instar larvae of *S. frugiperda* Bahia SS, Bahia RR, Cajuri RR and Canapolis RR populations after 12 hours under damage. (B) Average ( $\pm$ S.E.) of total amount of HIPV emitted by maize plants 12 hours after *S. frugiperda* RR and SS populations attack. (C-D) Average ( $\pm$ S.E.) of the most representative compounds emitted by maize plants 12 hours after damage. (E) Hierarchical clustering heatmaps of the HIPVs by maize after 12 hours damage by different *S. frugiperda* RR and SS populations. Different letters indicate significant differences between treatments ( $P < 0.05$ ). ( $P$  values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (*emmeans*)).



**Figure 3: Isogenic and Bt maize plants are strongly induced after *S. frugiperda* regurgitant and this pattern is Bt susceptibility dependent. (A-B)** Average ( $\pm$ S.E.) of total amount of HIPV emitted by isogenic and Bt maize plants ( $n=6$ ) after regurgitant application of *S. frugiperda* RR and SS populations. **(C-D)** Contingency tables of the performance of each treatment in percentages based on random forest analysis. **(E-F)** Hierarchical clustering heatmaps of the HIPVs by isogenic and Bt maize after regurgitant application of *S. frugiperda* RR and SS populations. **(G-H)** Average ( $\pm$ S.E.) of the most representative compounds emitted by maize plants 12 hours after damage. Different letters indicate significant differences between treatments ( $P < 0.05$ ). ( $P$  values are given for treatments [generalized linear model (family, Gaussian)] followed by pairwise comparisons of Least Squares Means (*emmeans*)).



**Figure 5. Resistance to Bt maize plants drives the amount of volicitin and volicitin-like compounds in the regurgitant of *S. frugiperda* caterpillars fed on isogenic maize plants ( $n = 5$ ).** Bars represent average ( $\pm$  SE). Different letters indicate significant differences between treatments ( $P < 0.05$ ).  $P$  values are given for treatment comparisons [generalized linear model (family, Gaussian)], followed by pairwise comparisons of Least Squares Means (LSM).

## 10. TABLES SECOND CHAPTER

**Table 1:** Emission rate of HIPV (mean  $\pm$ SE) emitted (ng/hour) from isogenic maize plants (n=6) attacked by *Spodoptera frugiperda* populations: Bahia SS, Bahia RR, Cajuri RR and Canapolis RR caterpillars and undamaged plants. Volatiles were collected after 12 of herbivory for 3 hours. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

Compounds	Undamaged	Bahia SS	Bahia RR	Cajuri RR	Canapolis RR	P value
3-(Z)-Hexenal	2.62 $\pm$ 0.28 a	10.49 $\pm$ 2.16 b	13.17 $\pm$ 1.38 b	10.21 $\pm$ 2.13 b	18.94 $\pm$ 0.87 c	< 0.001
2-(E)-Hexenal	3.97 $\pm$ 0.55 a	2.41 $\pm$ 0.83 b	1.37 $\pm$ 0.3 bc	0.27 $\pm$ 0.17 c	1.04 $\pm$ 0.28 d	< 0.001
(Z)-3-Hexen-1-ol	0 $\pm$ 0 a	1.29 $\pm$ 0.48 ab	0.12 $\pm$ 0.12 a	2.2 $\pm$ 1.17 b	0.17 $\pm$ 0.17 a	0.04
(Z)-3-Hexen-1-ol, acetate	0 $\pm$ 0 a	5.85 $\pm$ 1.40 b	3.29 $\pm$ 0.94 b	0.4 $\pm$ 0.4 a	3.9 $\pm$ 1.23 b	< 0.001
$\beta$ -Linalool	0 $\pm$ 0 a	3.43 $\pm$ 1.07 b	0.32 $\pm$ 0.14 a	0.89 $\pm$ 0.41 a	0.84 $\pm$ 0.51 a	< 0.001
DMNT	0 $\pm$ 0 a	16.32 $\pm$ 2.35 b	3.26 $\pm$ 0.62 a	4.72 $\pm$ 2.19 ac	4.65 $\pm$ 0.94 ac	< 0.001
(Z)- $\alpha$ -Bergamotene	0 $\pm$ 0 a	9.4 $\pm$ 4.14 b	0.09 $\pm$ 0.09 a	1.47 $\pm$ 0.78 a	0.81 $\pm$ 0.17 a	< 0.001
(E)- $\beta$ -Farnesene	0 $\pm$ 0 a	32.01 $\pm$ 12.16 b	0.67 $\pm$ 0.28 a	5.71 $\pm$ 2.44 b	4.35 $\pm$ 0.75 b	< 0.005
$\beta$ -Sesquiphellendrene	0 $\pm$ 0	0.24 $\pm$ 0.21	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.29
TMTT	0 $\pm$ 0 a	0.73 $\pm$ 0.17 b	0 $\pm$ 0 a	0 $\pm$ 0 a	0 $\pm$ 0 a	< 0.001

**Table 2:** Emission rates (ng/h) of HIPV from isogenic maize plants (n=6) induced by regurgitant application of *Spodoptera frugiperda* caterpillars resistant and susceptible to Bt toxins. The caterpillars were fed on isogenic plant prior the regurgitant collection. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

Compounds	Undamaged	MD	Bahia SS	Bahia RR	Cajuri	Canapolis	P values
3-(Z)-Hexenal	2.62 $\pm$ 0.28 a	23.17 $\pm$ 2.77 b	27.37 $\pm$ 1.8 bc	20.19 $\pm$ 1.11 b	18.62 $\pm$ 0.65 b	18.92 $\pm$ 1.26 b	< 0.0001
2-(E)-Hexenal	3.97 $\pm$ 0.55 a	0 $\pm$ 0 b	0.66 $\pm$ 0.34 b	1.12 $\pm$ 0.32 b	0 $\pm$ 0 b	2.59 $\pm$ 0.19 bc	< 0.0001
(Z)-3-Hexen-1-ol	0 $\pm$ 0 a	2.11 $\pm$ 0.32 ab	9.03 $\pm$ 2.12cd	7.27 $\pm$ 0.52c	3.74 $\pm$ 0.65 bc	6.88 $\pm$ 0.33c	< 0.0001
(Z)-3-Hexen-1-ol, acetate	0 $\pm$ 0 a	2.11 $\pm$ 1.46 ac	4.45 $\pm$ 1.77 ac	2.69 $\pm$ 0.9 ac	4.33 $\pm$ 1.22 ac	5.77 $\pm$ 0.62 bc	< 0.05
$\beta$ -Linalool	0 $\pm$ 0 a	0 $\pm$ 0 a	0.12 $\pm$ 0.08 a	0.5 $\pm$ 0.25 ab	0 $\pm$ 0 a	0.73 $\pm$ 0.21 b	< 0.005
DMNT	0 $\pm$ 0 a	0 $\pm$ 0 a	0.8 $\pm$ 0.37 a	1.58 $\pm$ 0.42 ac	4.01 $\pm$ 1.37 bc	2.01 $\pm$ 0.91 ac	< 0.005
Indole	0 $\pm$ 0 a	0 $\pm$ 0 a	0.2 $\pm$ 0.09 b	0.06 $\pm$ 0.06 ab	0 $\pm$ 0 a	0 $\pm$ 0 a	< 0.05
(E)- $\beta$ -Farnesene	0 $\pm$ 0 a	0 $\pm$ 0 a	0.02 $\pm$ 0.01 a	0.03 $\pm$ 0.02 a	0 $\pm$ 0 a	0 $\pm$ 0 a	0.17

**Table 3:** Emission rates (ng/h) of HIPV from Bt maize plants (n=6) induced by regurgitant application of *Spodoptera frugiperda* caterpillars resistant and susceptible to Bt toxins. The caterpillars were fed on isogenic plant prior the regurgitant collection. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

Compounds	Undamaged	MD	Bahia SS	Bahia RR	Cajuri RR	Canapolis RR	P values
3-(Z)-Hexenal	4.91 ± 2.99 a	23.71 ± 1.3 b	18.71 ± 1.45 b	26.26 ± 3.82 b	22 ± 1.33 b	25.23 ± 1.48 b	< 0.0001
2-(E)-Hexenal	1.94 ± 0.52 ab	0.28 ± 0.18 a	02.22 ± 0.44 ab	3.63 ± 1.05 b	1.3 ± 0.13 ab	3.68 ± 1.5 b	< 0.05
(Z)-3-Hexen-1-ol	1.36 ± 0.70 a	26.95 ± 1 bcd	6.26 ± 0.84 c	10.44 ± 1.15 d	7.48 ± 0.63 bcd	7.41 ± 0.75 bcd	< 0.0001
(Z)-3-Hexen-1-ol, acetate	0 ± 0 a	2.67 ± 0.86 a	5.46 ± 0.64 b	9.29 ± 1.36 c	3.67 ± 0.67 b	4.11 ± 0.95 b	< 0.0001
β-Linalool	0 ± 0 a	0 ± 0 a	0.61 ± 0.04 bc	0.89 ± 0.21 b	0.46 ± 0.06 c	0.60 ± 0.12 bc	< 0.0001
DMNT	0 ± 0 a	0 ± 0 a	1.47 ± 0.41 ab	3.93 ± 0.82 b	2.59 ± 1.27 ab	2.31 ± 1.24 ab	< 0.05
Indole	0 ± 0 a	0 ± 0 a	0.01 ± 0.01 a	0.01 ± 0.01 a	0 ± 0 a	0 ± 0 a	0.39
(E)-β-Farnesene	0 ± 0 a	0 ± 0 a	0.05 ± 0.02 a	0.10 ± 0.06 a	0.04 ± 0.02 a	0.02 ± 0.01 a	0.08

**Table 4:** Fatty acid conjugates volicitin and volicitin-like compounds present in the regurgitant of *Spodoptera frugiperda* caterpillars (n=5) resistant and susceptible to Bt toxins after feeding on isogenic maize plants. Statistically differences among treatments are indicated by different letters for each compound (Anova followed by FDR-corrected post-hoc tests,  $P < 0.05$ ).

FAC	SS		RR		P value
	Bahia	Bahia	Cajuri	Canapolis	
18:3-OH-glutamine-1	3485.6 ± 654.01 a	900.4 ± 221.34 b	356.2 ± 141.2 b	841 ± 434.98 b	< 0.001
18:2-OH-glutamine-1	1842 ± 238.02 a	385.6 ± 84 b	190 ± 70.55 b	775 ± 362.4 b	< 0.001
18:3-glutamine-1	250.2 ± 13.15 a	216.8 ± 71.95 ab	36 ± 14.17 c	110.6 ± 48.2 bc	< 0.05
18:2-glutamine-1	50.8 ± 6.76 a	64.4 ± 4.57 a	647.6 ± 229.17 bc	183.2 ± 31.09 ab	< 0.001

## 11. GENERAL CONCLUSIONS

- Different hybrids of maize plants induce different volatiles amount and their profile.
- Isogenic and *Bt* maize plants respond different by oviposition and larval feeding induces different amounts of volatiles.
- Resistant populations to an event expressing Cry1A.105 + Cry2Ab *Bt* toxins showed a fitness cost related to the production of the elicitor volicitin and volicitin-like comparing to the *Bt*- susceptible population.
- The insect genotype (resistant or susceptible) impairs the amount of volatiles induced by maize plants.
- The amount of volatiles induced by *Bt* and its isogenic maize plants causes effect on host search of an egg parasitoid, *T. pretiosum*.
- The biggest the amount of volatiles induction, the biggest is the parasitoid attraction.
- The presence of a cost to the FAW population resistant to Cry1A.105 + Cry2Ab *Bt* toxins can contribute to slow down the selection for *Bt*-resistance.
- The degree of cost changed depending on the insects location
- For a better understanding of changes in eggs parasitoid behaviour and how this can influence the *S. frugiperda* resistance management, studies on the field are required considering the fall armyworm genotype and the differences between isogenic and *Bt* maize plants.