ELISA FARIA DE OLIVEIRA

TRITROPHIC INTERACTIONS BETWEEN *Bt* MAIZE, *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) AND *Podisus nigrispinus* (HEMIPTERA: PENTATOMIDAE)

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Entomologia, área de concentração em Entomologia, para a obtenção do título de Doutor.

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INTERAÇÕES TRITRÓFICAS ENTRE O MILHO *Bt*, *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE) AND *Podisus nigrispinus* (HEMIPTERA: PENTATOMIDAE)

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APROVADA em 28 de junho de 2019.

Dr. Bruno Henrique Sardinha de Souza	UFLA
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2019

Ao meu filho João,

Dedico

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RESUMO GERAL

Plantas transgênicas resistentes a insetos são importantes ferramentas no controle de pragas, e estudos de avaliação de riscos são requeridos para determinar efeitos sobre organismos não-alvo, tais como os inimigos naturais. Estudos de interações tritróficas que integrem aspectos toxicológicos e comportamentais podem fornecer informações confiáveis dos possíveis efeitos das plantas transgênicas sobre organismos do terceiro nível trófico. Neste contexto, objetivou-se estudar as interações tritróficas entre plantas de milho *Bt*, a lagarta-do-cartucho *Spodoptera frugiperda* e o predador *Podisus nigrispinus*. Uma população de *S. frugiperda* resistente ao milho *Bt* foi utilizada para avaliar efeitos diretos da toxina Cry1F na biologia do predador, excluindo os efeitos indiretos que pragas suscetíveis de baixa qualidade podem exercer. Foram também avaliados possíveis efeitos da modificação genética do milho *Bt* na emissão de voláteis e no forrageamento do predador, para isso a emissão de voláteis constitutivos e induzidos de plantas de milho *Bt* foi comparada com plantas não-*Bt*. Plantas induzidas foram tratadas com regurgito larval ou foram danificadas por lagartas resistentes de *S. frugiperda* capazes de alimentar-se igualmente em plantas *Bt* e não-*Bt*, evitando possíveis mudanças nos voláteis induzidos pela herbivoria devido ao comportamento alimentar das lagartas. Experimentos de laboratório e casa-de-vegetação mostraram que lagartas resistentes foram capazes de danificar e sobreviver no milho *Bt*, sem efeitos deletérios na sua biologia. Os bioensaios com *P. nigrispinus* mostraram que não houve efeito direto do milho *Bt* na sua biologia, evidenciado pela alta taxa de sobrevivência, tempo de desenvolvimento ninfal e peso médio dos adultos normais. Análises de GC-MS revelaram que a transformação genética nas plantas *Bt* não resultou em alterações relevantes na emissão de voláteis constitutivos nem na emitida por plantas tratadas com regurgito larval. Apesar da população resistente de *S. frugiperda* provocar danos similares nas plantas *Bt* e não-*Bt*, o perfil de voláteis induzidos pelo herbívoro nas plantas *Bt* apresentou uma composição diferente. Plantas não-*Bt* danificadas pelo herbívoro emitiram derivados de ácidos graxos e monoterpenos que estavam ausentes no perfil de voláteis de plantas *Bt*. Os resultados dos testes de olfatometria mostraram que *P. nigrispinus* não distinguiu entre odores de plantas não danificadas e induzidas pelo regurgito larval, independentemente do genótipo da planta. Enquanto o predador orientou-se preferencialmente por voláteis de plantas não-*Bt* danificadas pelo herbívoro, o predador não diferenciou voláteis de plantas não danificadas e danificadas pelo herbívoro em plantas *Bt*. Quando o predador foi exposto aos voláteis de plantas induzidas pela herbivoria de plantas *Bt* e não-*Bt*, preferiu os emitidos por plantas não-*Bt*, sugerindo que as diferenças qualitativas encontradas entre as plantas *Bt* e não-*Bt* danificadas pelo herbívoro podem ser responsáveis pela atratividade diferenciada. Dado ao fato de que foi utilizado um herbívoro resistente, este é o primeiro relato demonstrando que a inserção do *Bt* no genoma do milho altera qualitativamente as emissões de voláteis nessas plantas, de modo a alterar a orientação do predador aos voláteis induzidos pela herbivoria. Deste modo, o milho Cry1F não apresentou efeitos diretos na biologia do predador *P. nigrispinus*, entretanto, pode afetar o seu forrageamento em busca de presas.

Palavras-chave: Milho transgênico. Organismos não-alvo. Predador generalista. Praga resistente. Voláteis de plantas induzidos pela herbivoria.

GENERAL ABSTRACT

Insect resistant transgenic plants are important tools for pest control, and risk assessment studies are required to assess their effects on non-target organisms, such as the natural enemies. Tritrophic interactions studies that integrate toxicological and behavioral aspects can provide reliable information of the possible effects of transgenic plants on higher trophic level organisms. In this context, the objective of this thesis was to study the tritrophic interactions between *Bt* and non-*Bt* maize plants, the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and the predator *Podisus nigrispinus* (Hemiptera: Pentatomidae). A *Bt*-resistant population of *S. frugiperda* to *Bt* maize was used to evaluate direct effects of the Cry1F toxin on the biology of the predator, excluding indirect effects of low-quality susceptible pests on the biology of the predator. It was also evaluated possible effects of the genetic modification of the *Bt* plants on the volatile emissions and on foraging behavior of the predator *P. nigrispinus*, for this, constitutive and induced volatile emissions of *Bt* maize plants were compared with non-*Bt* plants. Induced plants were treated with larval regurgitant or damaged by *Bt*-resistant larvae of *S. frugiperda* that were able to feed on *Bt* and non-*Bt* plants equally, avoiding possible changes in the herbivore-induced volatile profiles due to larval feeding behavior. The laboratory and semi-field greenhouse assays showed that the *Bt*-resistant *S. frugiperda* were able to damage and survive on *Bt* maize, without deleterious effects on its biology. The bioassays with the predator *P. nigrispinus* showed no direct impact on its biology, as evidenced by the high survival rate, normal nymphal development time and average adult weight after exposure to *Bt*-resistant prey fed on *Bt* maize. GC-MS analyses revealed that *Bt* transformation did not result in relevant changes in the constitutive volatile emission nor in that emitted by regurgitant-treated plants. Despite the fact that *Bt*-resistant *S. frugiperda* population inflicted similar damage on *Bt* and non-*Bt* plants, herbivore-induced volatile blend emitted by *Bt* plants had a different composition. Herbivore-damaged non-*Bt* plants emitted fatty acid derivatives and monoterpenes that were absent in the emission of herbivore-damaged *Bt* plants. Results from olfactometer tests showed that *P. nigrispinus* did not distinguish among the odours of undamaged and regurgitant-treated plants regardless of the plant genotypes. While the predator oriented preferentially to volatiles from herbivore-damaged over undamaged plants of the non-*Bt* genotype, the predator did not differentiate blends emitted by undamaged and herbivore-damaged plants of *Bt* maize. When the predator was exposed to herbivore-induced plant volatiles of *Bt* and non-*Bt* plants, *P. nigrispinus* preferred the latter, suggesting that the qualitative differences found between herbivore-damaged *Bt* and non-*Bt* maize plants might be responsible for the differential attractiveness. Given the fact that we used a *Bt*-resistant herbivore, this is the first report demonstrating that the insertion of *Bt* into maize genome changes qualitatively plant volatile emissions, in ways that disrupt predator orientation to herbivore-induced plant volatiles. Therefore, Cry1F maize had no direct effects on the biology of the predator *P. nigrispinus*, however may affect its prey-searching behavior.

Key-words: Transgenic maize. Non-target organisms. Generalist predator. Resistant prey. Herbivore-induced plant volatiles.

SUMÁRIO

1. INTRODUÇÃO GERAL

Plantas transgênicas com resistência a insetos foram desenvolvidas com o objetivo de minimizar perdas nas lavouras devido ao ataque de pragas. No processo de transgenia, genes da bactéria de solo *Bacillus thuringiensis* (*Bt*) (Berliner, 1915) foram introduzidos no genoma da célula vegetal, dando origem às plantas geneticamente modificadas que conferem resistência a algumas espécies de insetos-praga (ARMSTRONG et al., 1995; HARDKE et al., 2011).

As proteínas *Bt* produzidas pela bactéria são altamente tóxicas aos insetos-alvo. A toxicidade ocorre quando o inseto-alvo ingere a toxina e após sua solubilização e ativação, a proteína *Bt* provoca a ruptura da camada epitelial do mesêntero, levando o inseto à morte (BRAVO; GILL; SOBERÓN, 2007). A solubilização das proteínas *Bt* depende do pH intestinal alcalino de lepidópteros, assim a menor efetividade destas proteínas em coleópteros pode ser devida ao pH neutro ou pouco ácido da ordem. Após a solubilização, as proteínas são ativadas por enzimas digestivas que clivam as protoxinas em polipeptídeos tóxicos. Diferenças na atividade proteolítica entre os insetos-alvo podem também ser responsáveis pela especificidade das toxinas; por exemplo, a principal protease digestiva de lepidópteros é a serino-protease, enquanto nos coleópteros ocorre principalmente cisteína e aspartato-proteases (DE MAAGD; BRAVO; CRICKMORE, 2001).

As proteínas *Bt* são altamente específicas, uma vez que após a sua solubilização e ativação também necessitam de receptores específicos no intestino do inseto (GILL; COWLES; PIETRANTONIO, 1992). Todo esse processo pode contribuir na determinação da especificidade da toxina. Entretanto, as plantas transgênicas com genes *Bt* codificam a expressão de proteínas na forma ativa e solúvel, não necessitando de pH apropriado e proteases específicas para solubilizar e ativar a toxina em subunidades tóxicas (STOTZKY, 2000). O único fator de especificidade nas plantas *Bt* é a presença de receptores na membrana do mesêntero onde as toxinas devem se ligar para que ocorra a toxicidade (STOTZKY, 2000). Dessa forma, uma vez que a especificidade da proteína foi reduzida em plantas transgênicas *Bt*, organismos não-alvo de diferentes níveis tróficos podem ser afetados por essas toxinas (BELL et al., 2001; HILBECK et al., 1998; MALONE et al., 2001).

As proteínas *Bt* são expressas continuamente e estão distribuídas em todos os tecidos da planta *Bt*, inclusive no pólen e néctar. A ingestão do pólen de milho *Bt* pela borboleta monarca, *Danaus plexippus* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) provocou a redução na alimentação e no desenvolvimento do inseto, aumentando sua mortalidade

(LOSEY; RAYOR; CARTER, 1999). Outro estudo com larvas do predador *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) evidenciou a alta mortalidade e o prolongamento no tempo de desenvolvimento larval do crisopídeo após alimentar-se de lagartas de *Ostrinia nubilaris* (Hübner) (Lepidoptera: Pyralidae) expostas a plantas de milho *Bt* (HILBECK et al., 1998).

Uma forma de avaliar os efeitos da toxina *Bt* nos organismos não-alvo, tais como os inimigos naturais, é expor essas espécies benéficas à proteína *Bt* por meio da alimentação com hospedeiros e/ou presas previamente alimentados com plantas *Bt*. Entretanto, as plantas *Bt* causam efeitos tóxicos sobre pragas suscetíveis, podendo comprometer a qualidade nutricional dessas pragas e indiretamente afetar parâmetros biológicos e etológicos dos inimigos naturais. Assim, o uso de pragas resistentes às plantas *Bt* evitaria efeitos indiretos da qualidade da presa na biologia e no comportamento dos inimigos naturais, uma vez que pragas resistentes conseguem alimentar-se e desenvolver-se normalmente em plantas *Bt*, pois são insensíveis a essas toxinas. Estudos prévios evidenciaram o uso de populações de insetos-praga resistentes como ferramenta para avaliar efeitos diretos das toxinas *Bt* na biologia de parasitoides e para determinar possíveis riscos toxicológicos para esses organismos (SCHULER et al., 1999a; 2003).

Os mecanismos de defesas das plantas como compostos tóxicos deterrentes de alimentação fornecem à planta uma proteção direta contra os herbívoros (HARTMANN, 2007). Entretanto, as plantas também se defendem indiretamente, sendo este tipo de defesa denominado de defesa induzida indireta, onde plantas quando danificadas liberam substâncias voláteis atrativas, que servem como guia aos inimigos naturais, sinalizando a presença de fitófagos (MAFFEI; MITHOFER; BOLAND, 2007; TAKABAYASHI et al., 1994; TUMLINSON; LEWIS; VET, 1993; VET; DICKE, 1992).

Neste contexto, os inimigos naturais podem ser afetados negativamente pelas plantas *Bt* devido à baixa qualidade da presa e até mesmo pelo efeito direto da toxina *Bt*, reduzindo sua sobrevivência e fecundidade. Também podem ser afetados durante a localização da planta hospedeira e de sua presa, e o subsequente reconhecimento, pois precisam de pistas olfatórias para contribuir nessa localização. Deste modo, mudanças no perfil de voláteis relacionadas à transgenia podem interferir na localização do habitat da presa (SCHULER et al., 1999b). Essa modificação no perfil de voláteis de plantas transgênicas pode ser explicada por efeitos pleiotrópicos, epistáticos e/ou mutacionais resultantes da inserção de um gene externo (SCHULER et al., 1999b; YAN et al., 2004), ou seja, efeitos que não são devidos aos genes inseridos, porém surgiram na planta transgênica por causa da transformação genética de forma

não intencional. Dessa forma, mudanças no perfil de voláteis induzidos pela herbivoria em plantas *Bt* podem afetar o forrageamento de inimigos naturais, que podem apresentar, por exemplo, uma preferência ou uma repelência a tais odores.

Alguns estudos demonstraram que a indução de voláteis está diretamente relacionada com a injúria que o herbívoro causa na planta hospedeira (DEAN; DE MORAES, 2006; HIMANEN et al., 2009). Assim, a magnitude do dano é determinante na indução do perfil de voláteis, podendo afetar o comportamento de insetos herbívoros e inimigos naturais (DEAN; DE MORAES, 2006; PARE et al., 2005; SCHMELZ et al., 2003). Deste modo, a alimentação do herbívoro induz mudanças na emissão de voláteis nas plantas, mas como as proteínas *Bt* afetam o sistema digestivo do inseto-alvo suscetível e consequentemente sua alimentação, isso pode afetar o processo de indução (SCHULER et al., 1999b). Outra questão, é que alguns aleloquímicos são induzidos quando a alimentação do herbívoro atinge um limiar e esse limiar é modificado em plantas *Bt*, podendo alterar o comportamento de herbívoros e inimigos naturais (POWELL et al., 1998).

Uma forma de evitar diferenças na magnitude do dano, e consequentemente variações nas emissões de voláteis por plantas *Bt* e não-*Bt*, é utilizar como agente indutor insetos-praga resistentes às toxinas *Bt* que podem alimentar-se normalmente nessas plantas. Assim, insetos-praga resistentes podem induzir plantas *Bt* e não-*Bt* de maneira similar, excluindo o efeito do comportamento alimentar e da sua magnitude na indução de voláteis por essas plantas. Embora existam alguns estudos mostrando o perfil de voláteis em plantas *Bt* após a herbivoria (DEAN; DE MORAES, 2006; LIU et al., 2015; MORAES et al., 2011; TURLINGS et al., 2005; VOGLER et al., 2009), pouco foi estudado sobre a interação dessas plantas em um sistema tritrófico, detalhando a interação com o herbívoro-alvo e inimigos naturais.

O efeito da proteína *Bt* deve ser avaliado de maneira direta por meio de estudos biológicos e avaliado também de maneira indireta por meio de estudos etológicos dos insetos de interesse. Estudos que integrem os aspectos toxicológicos e comportamentais podem fornecer informações reais dos possíveis efeitos das plantas transgênicas sobre organismos do terceiro nível trófico (SCHULER et al., 1999a).

Neste contexto, o objetivo foi estudar as interações tritróficas entre plantas de milho *Bt* e não-*Bt*; a sua principal praga-alvo, a lagarta-do-cartucho do milho *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) e o predador *Podisus nigrispinus* (Dallas, 1851) (Hemiptera: Pentatomidae). Uma população de *S. frugiperda* resistente ao milho *Bt* Cry1F foi utilizada para avaliar efeitos diretos da toxina Cry1F na biologia do predador *P. nigrispinus* e

estudar possíveis efeitos da modificação genética das plantas *Bt* na emissão de voláteis e no forrageamento do predador *P. nigrispinus*.

2. REFERENCIAL TEÓRICO

2.1. Milho geneticamente modificado resistente a insetos

O milho (*Zea mays* L.) está presente em todas as regiões produtoras do Brasil e por ser cultivado em diferentes condições de clima e de solo está amplamente difundido no país. Nos últimos anos, avanços tecnológicos foram introduzidos ao sistema de produção, como por exemplo, a bioengenharia genética com o uso de toxinas *Bt* que permitiu desenvolver plantas geneticamente modificadas resistentes a insetos (CAROZZI; KOZIEL, 1997).

A bactéria entomopatogênica *Bt* é cosmopolita, sendo encontrada naturalmente no solo em diversos ecossistemas do planeta (BOBROWSKI et al., 2003). Foi utilizada inicialmente como bioinseticida na forma de pulverização, sendo os primeiros testes de sua utilização no controle de pragas realizados na Europa na década de 1930 (POLANCZYK; ALVES, 2003).

O desenvolvimento de plantas de milho que expressam proteínas *Bt* é uma alternativa eficiente no controle de insetos-praga, visto que apresenta grande potencial para emprego em programas de manejo integrado de pragas (MIP). As principais vantagens do uso do milho *Bt* são a redução no uso de inseticidas químicos sintéticos, aumento na produtividade das plantas, segurança na utilização e eficiência no controle de pragas (BOBROWSKI et al., 2003).

As lagartas ao se alimentarem dos tecidos das plantas *Bt* ingerem a proteína, que atua nas células epiteliais do intestino médio (BRAVO; GILL; SOBERÓN, 2007). A proteína *Bt* solubilizada e ativada se liga a receptores específicos na membrana do mesêntero e promove a ruptura das células, causando a morte dos insetos antes que os mesmos consigam causar danos significativos à cultura (PIETRANTONIO; FEDERICI; GILL, 1993; GILL, 1995; STOTZKY, 2000).

Estão disponíveis comercialmente no Brasil híbridos de milho *Bt* com dois tipos de proteínas *Bt*, as proteínas Cry (Insecticidal Crystal Protein) e as proteínas Vip (Vegetative Insecticidal Protein) (CTNBio, 2019). A toxicidade das proteínas Cry é atribuída às inclusões proteicas sintetizadas durante o processo de esporulação, as quais são formadas por polipeptídeos que se acumulam na periferia dos esporos na forma de cristais, e tornam-se tóxicos após a ingestão por lagartas suscetíveis (CAROZZI; KOZIEL, 1997). Alguns isolados

de *Bt* são capazes de sintetizar outras proteínas tóxicas durante sua fase de crescimento vegetativo, as quais não formam cristais e não têm homologia com as proteínas Cry, sendo denominadas de proteínas Vip (ESTRUCH et al., 1996).

No Brasil, estão liberados para comercialização na cultura do milho dois eventos expressando a toxina Cry1Ab (eventos MON810 e Bt11), um evento expressando a toxina Cry1F (evento TC1507), um evento expressando a toxina Vip3A (evento MIR162) e um evento expressando duas toxinas Cry1A105 e Cry2Ab2 (evento MON89034), sendo todos os eventos com atividade sobre lepidópteros-praga. Eventos piramidados expressando combinações das proteínas acima também estão disponíveis comercialmente (CTNBio, 2019). Estes eventos estão registrados para três espécies de pragas-alvo: a lagarta-do-cartucho do milho, *S. frugiperda*; a lagarta-da-espiga do milho, *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) e a broca-da-cana, *Diatraea saccharalis* (Fabricius, 1794) (Lepidoptera: Crambidae). Portanto, os eventos atualmente disponíveis no Brasil oferecem proteção contra as principais espécies de lepidópteros-praga na cultura do milho.

Entretanto, a expressão contínua de toxinas *Bt* pelas plantas transgênicas pode exercer forte pressão de seleção nos insetos-alvo, e como consequência, uma rápida seleção de insetos-praga resistentes. No Brasil, a proteína Cry1F, liberada em 2008 no evento TC1507, apresentou problemas de resistência de *S. frugiperda* em algumas regiões produtoras a partir de 2012, poucos anos após sua liberação comercial (FARIAS et al., 2014). A resistência foi justificada pelos plantios sequenciais, associados ao clima tropical e a elevada população da praga (FARIAS et al., 2014), além de técnicas culturais inadequadas, como por exemplo, a falta de refúgios estruturados formados por milho não-*Bt* (MARTINELLI et al., 2017) e o uso intensivo de inseticidas nessas áreas.

2.2. Aspectos bioecológicos de *Spodoptera frugiperda*

Dentre os insetos-praga mais importantes da cultura do milho, destaca-se a lagarta-do-cartucho do milho, *S. frugiperda*, cujos danos estendem-se por todos os estádios de desenvolvimento da planta, principalmente após a formação do cartucho, onde o inseto se aloja, diminuindo a eficácia do controle químico. Estima-se que a redução na produção causada pela praga seja de até 34% (CRUZ, 1995; CRUZ; TURPIN, 1983).

É uma espécie polífaga com ocorrência em mais de 100 espécies de plantas hospedeiras; possui distribuição geográfica generalizada e encontra-se distribuída em todas as regiões produtoras de milho no Brasil (WAQUIL et al., 2004).

A lagarta-do-cartucho é um inseto holometábolo, ou seja, possui metamorfose completa, passando pelas fases de ovo, larva, pupa e adulto. As larvas recém-eclodidas iniciam a alimentação nas folhas da planta, provocando o sintoma de raspagem. À medida que as larvas crescem, começam a fazer orifícios nas folhas e também penetram no colmo de plantas jovens, o que prejudica o desenvolvimento da planta (GRÜTZMACHER et al., 2000). Essa espécie também ataca a base das espigas e os grãos leitosos, resultando em má formação ou até mesmo a não formação dos grãos (CRUZ, 1995). Ocorrem também danos indiretos causados pelo seu ataque, pois através da sua penetração deixam orifícios que são portas de entrada para fungos e bactérias, agentes causadores de várias doenças nas plantas e que afetam a produção e a qualidade dos grãos (CRUZ, 2003).

A duração da fase larval varia de 12 a 30 dias, sendo que após este período as larvas migram para o solo onde passarão à fase de pupa, com duração de até 25 dias (GALLO et al., 2002; LUGINBILL, 1928). O inseto adulto é uma mariposa de coloração pardo-escura nas asas anteriores e branco-acinzentada nas posteriores, com longevidade de aproximadamente 15 dias (SANTOS et al., 2004). As fêmeas adultas têm hábitos noturnos e podem ovipositar 200 ovos em média, formando uma massa de ovos sobrepostos. O período embrionário é de aproximadamente três dias (SANTOS et al., 2004).

O ciclo biológico de *S. frugiperda* em plantas de milho completa-se em 25 dias à temperatura de 25ºC, podendo obter-se até 11 gerações do inseto por ano (BUSATO; GRUTZMACHER; GARCIA, 2005). Entretanto, todas as fases da vida do inseto são influenciadas por fatores climáticos que podem diminuir ou prolongar a duração de determinadas fases.

O controle de *S. frugiperda* é realizado por meio de inseticidas pertencentes aos grupos químicos dos piretroides e organofosforados, para os quais já apresenta resistência (CARVALHO et al., 2013). O plantio de plantas transgênicas que expressam toxinas *Bt* também vem sendo utilizado no controle dessa praga. Porém, tanto o uso intensivo de inseticidas químicos quanto à exposição contínua às plantas *Bt* contribuíram para a seleção de indivíduos resistentes de *S. frugiperda* (CARVALHO et al., 2013; FARIAS et al., 2014). Portanto, tornam-se necessárias pesquisas por métodos alternativos de controle, no intuito de promover um manejo eficaz e sustentável desse inseto-praga.

O manejo integrado da tecnologia *Bt* com o controle biológico, realizado por inimigos naturais, pode contribuir para suprimir a evolução dos genes de resistência às toxinas *Bt* (SCHULER et al., 1999a).

2.3. Aspectos bioecológicos de *Podisus nigrispinus*

O predador *P. nigrispinus* é uma espécie nativa do Brasil; possui hábito alimentar generalista e é encontrado em diferentes agroecossistemas alimentando-se principalmente de larvas de lepidópteros, incluindo lagartas de *S. frugiperda* (ZANUNCIO et al., 1996; 2008). Ninfas e adultos de *P. nigrispinus* podem alimentar-se de diferentes tipos de presas e na ausência destas, podem alimentar-se de substratos vegetais, o que possibilita ao predador se estabelecer e manter sua população, mesmo quando a densidade da praga é baixa (COLL; GUERSHON, 2002).

O desenvolvimento ninfal e as características reprodutivas de *P. nigrispinus* foram avaliadas por Oliveira et al. (2004). Os autores observaram alta viabilidade ninfal do predador quando alimentado com larvas de *S. frugiperda*, com variação de 80 a 95%. A presa *S. frugiperda* proporcionou ao predador maior produção e viabilidade de ovos do que larvas de *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), inseto utilizado na criação massal de muitos predadores. Dessa forma, foi destacada a alta qualidade nutricional de larvas de *S. frugiperda* para o desenvolvimento do predador *P. nigrispinus*.

O potencial de predação de fêmeas de *P. nigrispinus* sobre larvas de *S. frugiperda* foi avaliado por Zanuncio et al. (2008). Os autores constataram uma resposta funcional dependente da densidade da presa, com maior consumo de presas em altas concentrações, evidenciando a eficiência do predador no controle de *S. frugiperda*.

Alguns estudos indicaram integração positiva entre plantas *Bt* e o predador *P. nigrispinus* no controle de *S. frugiperda* (LEITE et al., 2014; MALAQUIAS et al., 2014). Leite et al. (2014) demonstraram que plantas de milho *Bt* foram menos danificadas por larvas de *S. frugiperda* quando o predador *P. nigrispinus* estava presente, auxiliando no controle do inseto-praga. Malaquias et al. (2014) verificaram que o tipo de resposta funcional do predador *P. nigrispinus* não foi afetado negativamente pelo genótipo da planta, no caso algodão *Bt* e não-*Bt*.

Percevejos predadores do gênero *Podisus* têm recebido destaque como agentes de controle biológico, tais como *P. nigrispinus* para o controle de pragas florestais no Brasil (ZANUNCIO et al., 1992) e *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) no controle de lagartas desfolhadoras em cultivos protegidos na Europa (DE CLERCQ, 2000).

Estudos que integrem informações biológicas com a capacidade predatória de inimigos naturais sobre presas potenciais permitem avaliar o impacto desses organismos no controle

biológico de insetos-praga e, dessa forma, táticas de manejo integrado de pragas podem ser adotadas corretamente.

2.4. Plantas *Bt* e organismos não-alvo

A interação entre plantas *Bt* e organismos não-alvo é amplamente discutida na comunidade científica em busca de informações referentes aos efeitos das proteínas *Bt* sobre insetos polinizadores (DUAN et al., 2008; MALONE et al., 2001), inimigos naturais (LÖVEI; ANDOW; ARPAIA, 2009; ROMEIS; MEISSLE; BIGLER, 2006), pragas secundárias (LIU et al., 2005; FERNANDES et al., 2012), e até mesmo sobre vertebrados (SIEGEL, 2001).

Organismos não-alvo são espécies que podem estar expostas às proteínas *Bt*, de forma direta ou indireta, mas que não são alvos específicos dessas toxinas (ANDOW; HILBECK, 2004). A forma direta ou bitrófica é quando o organismo entra em contato direto com a proteína *Bt* por meio do consumo da planta ou de seus produtos, como néctar e pólen. A forma indireta ou tritrófica é aquela na qual os insetos podem adquirir a proteína *Bt* via alimentação de herbívoros ou pelo consumo do "honeydew" de insetos que se alimentaram da planta *Bt* (GROOT; DICKE, 2002).

Em um estudo de exposição tritrófica, Schuler et al. (1999a) avaliaram o desenvolvimento do parasitoide *Cotesia plutellae* (Kurdjumov, 1912) (Hymenoptera: Braconidae) em lagartas de *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) alimentadas em folhas de canola *Bt* ou canola convencional. Os autores não constataram efeito do alimento no parasitismo das larvas; no entanto, os parasitoides não completaram o ciclo biológico nas lagartas alimentadas na canola *Bt*, pois as mesmas morreram poucos dias após a ingestão dos tecidos da planta. A taxa de emergência dos parasitoides foi de 63% nas lagartas alimentadas com plantas não-*Bt*. Em outro ensaio, utilizaram lagartas de *P. xylostella* resistentes à proteína *Bt* e sob as mesmas condições experimentais não encontraram diferenças significativas quanto à sobrevivência do parasitoide em lagartas alimentadas em canola *Bt* e canola convencional, ocorrendo 54 e 56% de parasitismo, respectivamente.

Entretanto, o comportamento de parasitismo envolve estímulos complexos que estão relacionados à localização do habitat do hospedeiro, o seu reconhecimento e a aceitação pelo parasitoide. Dessa forma, os autores compararam a resposta de *C. plutellae* aos voláteis de plantas de canola *Bt* e convencional, danificadas pelo herbívoro ou artificialmente. As plantas *Bt* foram menos danificadas quando atacadas por lagartas provenientes de uma população suscetível, comparativamente à planta convencional, e os parasitoides apresentaram maior

preferência às plantas não-*Bt*. Porém, quando as folhas foram danificadas artificialmente, em proporções similares, não houve diferença na preferência entre voláteis de plantas *Bt* e não-*Bt*. O mesmo ocorreu quando o dano foi realizado por lagartas resistentes à proteína *Bt*, mostrando que a atratividade das plantas *Bt* ou não-*Bt* aos parasitoides foi similar independente da forma de dano. Porém, a magnitude do dano resultou em uma mudança de comportamento do parasitoide que preferiu voláteis de plantas com maior intensidade de dano.

Dessa forma, organismos benéficos, como os inimigos naturais, podem ser afetados pelas plantas *Bt* tanto em seus parâmetros biológicos como também em suas características comportamentais, como nas etapas de forrageamento em busca de hospedeiro/presa (HAN et al., 2016). Portanto, estudos que integrem aspectos toxicológicos e comportamentais da espécie não-alvo são necessários para o estudo de um cenário mais realista do efeito das plantas transgênicas sobre esses organismos.

2.4. Plantas *Bt* e compostos voláteis

As plantas possuem um complexo sistema de defesa contra doenças e insetos-praga (KARBAN; BALDWIN, 1997). As defesas pré-formadas ou constitutivas são aquelas naturalmente presentes nas plantas, enquanto que as defesas pós-formadas ou induzidas são ausentes ou de pouca expressividade em plantas sadias (AGRAWAL, 2000; 2007). Porém, as defesas induzidas tornam-se evidentes e são ativadas somente após o dano ou ataque do herbívoro (KARBAN; BALDWIN, 1997).

A forma direta de defesa induzida na planta em resposta ao dano está baseada no aumento de compostos de defesas, como os inibidores de proteases presentes em várias espécies de plantas (KANT et al., 2004). Já a forma indireta de defesa induzida constitui a produção de compostos voláteis que servem como indicadores da presença de herbívoros na planta (AGRAWAL et al., 2002; DICKE et al., 2003). Os compostos voláteis induzidos pela herbivoria são liberados pelas plantas no ar (BALDWIN, 2010) e as tornam atrativas aos inimigos naturais que usam essas pistas químicas para localizar o seu hospedeiro e/ou presa (TURLINGS et al., 2005; VET; DICKE, 1992).

As plantas *Bt* podem diferir quanto ao perfil de voláteis liberado como resultado do processo de transgenia, o qual pode alterar o mecanismo de defesa da planta devido a um efeito pleiotrópico ou mutacional quando um gene externo é inserido no genoma da célula vegetal (SCHULER et al., 1999b). Outro ponto a ser considerado, é que a constante produção

de proteínas *Bt* pelas plantas transgênicas requer aumento na alocação de recursos (HIMANEN et al., 2009), podendo afetar os metabolismos primário e secundário das plantas *Bt*; e dessa forma, comprometer a emissão de compostos voláteis por essas plantas. A potencial mudança no perfil de voláteis em plantas *Bt* pode impactar negativamente as interações tritróficas nos cultivos transgênicos e comprometer a resposta dos inimigos naturais aos odores emitidos por essas plantas (HIMANEN et al., 2009; YAN et al., 2004).

Estudos com plantas *Bt* mostraram que as mudanças no perfil de voláteis induzidos por essas plantas podem ser também consequência direta do grau de herbivoria (DEAN; DE MORAES, 2006; HIMANEN et al., 2009), ou seja, o consumo da planta *Bt* por um inseto-alvo suscetível inibirá a sua alimentação, e consequentemente, a indução de voláteis por essas plantas também será inibida. Porém, existem métodos para evitar essa diferença na magnitude de dano pelo herbívoro entre plantas *Bt* e não *Bt*, como por exemplo, a indução artificial das plantas (TURLINGS et al., 1998; 2005) e o uso de insetos-praga resistentes às plantas *Bt* (SCHULER et al., 1999a; 2003).

Em um trabalho com o parasitoide especialista *C. plutellae* foi demonstrada maior atração do parasitoide por plantas *Bt* danificadas por indivíduos resistentes do que por indivíduos suscetíveis (SCHULER et al., 1999a). A maior atração ocorreu provavelmente devido à grande extensão do dano provocado pelas lagartas resistentes que resultou em uma maior produção de voláteis atrativos ao parasitoide. A hipótese de que diferenças no comportamento alimentar, dentro de uma mesma espécie de praga, esteja influenciando a preferência de inimigos naturais, tem grande relevância em estudos de manejo da resistência, uma vez que pragas *Bt*-resistentes não são mais controladas pelas plantas transgênicas, porém os inimigos naturais podem exercer controle efetivo dessas populações resistentes, limitando a propagação dos genes de resistência (SCHULER et al., 1999a).

Em outro estudo com o parasitoide *C. plutellae* foi observado que o parasitoide não distinguiu entre plantas *Bt* e não-*Bt* danificadas na mesma magnitude, artificialmente ou por lagartas resistentes, indicando que possivelmente o processo de transformação genética não estaria causando mudanças no perfil de voláteis das plantas *Bt* (SCHULER et al., 2003). Porém, em ambos os estudos não foram realizadas análises quantitativas e qualitativas dos compostos voláteis liberados pelas plantas.

Dessa forma, estudos etológicos são importantes para o entendimento das interações multitróficas que ocorrem entre organismos em uma teia alimentar. O conhecimento dos compostos químicos que medeiam à localização de plantas infestadas com herbívoros pelos inimigos naturais também é importante para a melhor compreensão da interação inseto-planta

influenciando o terceiro nível trófico (BLEEKER et al., 2009; PROFFIT et al., 2011; VAN WIJK; BRUIJN; SABELIS, 2011).

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ARTIGO 1

RESISTANCE OF *Spodoptera frugiperda* TO *Bt* MAIZE TO ASSESS DIRECT EFFECTS ON THE PREDATOR *Podisus nigrispinus*

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Abstract

Genetically modified plants are important tools for pest control, and risk assessment studies are required to assess their effects on non-target organisms. Tritrophic interaction studies of the effect of *Bt* plants on the third trophic level through the consumption of *Bt*-susceptible prey were unable to separate the effects of feeding on low-quality prey from the direct effects of the *Bt* plant. The use of *Bt*-resistant pests could avoid the potential indirect effects of susceptible pests with high toxicity and poor nutritional quality on the biology of natural enemies. In this context, the objective of our study was to evaluate the direct effects of Cry1F maize on the predator *Podisus nigrispinus* (Hemiptera: Pentatomidae) after feeding on a *Bt*-resistant population of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Our study showed that *Bt*-resistant *S. frugiperda* were able to damage and survive on *Bt* maize in laboratory and semi-field greenhouse assays without deleterious effects on its biology, avoiding potential indirect effects on the biology of the predator. The bioassays with the predator showed no direct impact on its biology, as evidenced by the high survival rate, normal nymphal development time and average adult weight after exposure to *Bt*-resistant prey fed on *Bt* maize. In this case, our study provides reliable information to ensure that Cry1F maize does not harm the generalist predator *P. nigrispinus*.

Keywords: Cry1F, fall armyworm, generalist predator, non-target effects, resistant prey, transgenic maize

1. Introduction

Transgenic insect-resistant plants were developed with the objective of reducing crop losses due to insect damage. In the transgenic process, genes of the soil bacterium *Bacillus thuringiensis* Berliner (*Bt*) were introduced into the plants, resulting in genetically modified plants with high resistance to some insect pests (Tabashnik et al. 2013). The *Bt* proteins present in *Bt* plants are highly toxic to target insects. The toxicity occurs when the target insect feeds on the *Bt* plant and the active toxin binds to specific receptors on the midgut, promoting pore formation in the epithelial membrane and leading to insect death (Gill et al. 1992, Groot & Dicke 2002).

Although a high specificity is attributed to these proteins, some non-target organisms may also be affected (Hilbeck et al. 1998, Malone et al. 2001). A way to evaluate these effects on non-target beneficial organisms, such as predators, is to offer them prey previously fed on *Bt* plants and evaluate the effects on their biology after this exposure.

However, a common methodological problem is that predators feed on *Bt*- susceptible prey whose biology is affected by the *Bt* toxin. Consequently, *Bt*-susceptible prey usually present low nutritional quality that could result in indirect effects on predator biology. Therefore, the direct effect of *Bt* plants on non-target organisms can be masked or unclear because these organisms are feeding on poisoned prey with compromised nutritional quality due to exposure to *Bt* toxins (Lawo et al. 2010, Tian et al. 2012).

A more accurate way to assess the risks of *Bt* plants to non-target organisms is by using resistant prey (Schuler et al. 1999). *Bt*-resistant prey are able to develop normally on *Bt* plants without deleterious effects because these prey are insensitive to *Bt* toxins, and after exposure to *Bt* plants, the prey can be offered to natural enemies to determine the real risk to these non-target insects (Schuler et al. 2003, Romeis et al. 2011, Tian et al. 2012).

Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) is an important pest of maize, soybean, cotton, rice and sorghum (Sparks 1986). The fall armyworm was the first lepidopteran pest to be reported as resistant to *Bt* plants in the field (Tian et al. 2012). Resistance to *Bt* maize (Herculex, Cry1F, TC1507 event) was first documented in Puerto Rico in 2006 (Storer et al. 2010) and in Brazil in 2012 (Farias et al. 2014), a few years after its commercial release in these countries. This pest resistance gives us an opportunity to study tritrophic interactions with a *Bt*-resistant herbivore, a *Bt* plant and a non-target predator.

Podisus nigrispinus (Dallas) (Hemiptera: Pentatomidae) is a generalist predator and promising biocontrol agent of lepidopteran pests (Mohaghegh et al. 2001). The predator is

native to Brazil, where it often occurs in maize crops, and nymphal and adult stage prey on more than 30 different pests of economic importance (Torres et al. 2006), including *S. frugiperda*. Thus, *P. nigrispinus* may be exposed to *Bt* proteins present in *Bt* maize crops by feeding on lepidopteran prey.

Previous studies have shown negative effects of *Bt* maize on *P. nigrispinus* after exposure to *Bt*-fed prey (Cunha et al. 2012, De Jesus et al. 2014, Leite et al. 2014), but none of these studies were conducted with a *Bt*-resistant prey, excluding indirect prey-mediated effects. Although, Cunha et al. (2012) have reported ultrastructural changes in digestive cells of the midgut of *P. nigrispinus* after the predator fed with susceptible-larvae of *S. frugiperda* reared on *Bt* cotton, the authors did not determine whether these changes affected the biology and the behavior of the predator.

Given the potential risk of *Bt* plants to non-target beneficial organisms and the opportunity to explore a tritrophic interaction, the aims of this study were to (1) characterize the biological parameters of a *Bt*-resistant *S. frugiperda* population and compare them with those of a *Bt*-susceptible population; and (2) assess the direct effects of Cry1F maize on *P. nigrispinus* through the consumption of *Bt*-resistant *S. frugiperda*.

2. Materials and methods

2.1. Plants

Maize hybrids seeds of 30F53H (Herculex, Cry1F, TC1507 event) and its non-*Bt* isogenic equivalent 30F53 were provided by DuPont do Brasil, "Divisão Pioneer Sementes" (Santa Cruz do Sul, RS, Brazil). Seeds were sown in a commercial plant substrate (Bioplant®) in plastic pots (300 mL) weekly and kept in a greenhouse with an anti-aphid screen to avoid herbivore infestation. Plants were grown until they reached the V4 to V8 stage. After the V4 stage the plants were transferred to 2 L plastic pots. Subsequently, plants were used in experiments or for insect rearing. After the trials, to confirm *Bt* gene expression by the maize plants, AgraStrip® Cry1F test strips (Romer Labs®, Getzersdorf, Austria) were used according to the manufacturer's instructions. All tests for the presence of the Cry1F protein (*Bt* plants) or its absence (non-*Bt* isogenic plants) were as expected.

2.2. Insects

A *Bt*-susceptible population of *S. frugiperda* (LAB) was obtained from the rearing laboratory at the Department of Entomology, Federal University of Lavras (Lavras, MG, Brazil). This population was never exposed to *Bt* maize or any insecticide, and it was used as a susceptible reference strain in all bioassays. A *Bt*-resistant population of *S. frugiperda* (LEM) was obtained from the insect rearing laboratory at DuPont do Brasil, "Divisão Pioneer Sementes" (Panaltina, GO, Brazil). This population was collected by DuPont Pioneer technicians from *Bt* maize fields in Luis Eduardo Magalhães (BA, Brazil), and it was able to damage transgenic maize because it was resistant to Cry1F protein. Caterpillars were reared at room temperature at 25 ± 2 ºC, with a relative humidity of 70 ± 10% and photoperiod of 12:12 (L:D) hours. Adults were maintained in PVC tubes lined with paper to support oviposition and were fed on a honey and distilled water (10%) solution. Eggs were collected every day and placed in Petri dishes. Larvae were fed on artificial diet (Greene et al. 1976) and transferred to a plastic box until pupation.

Podisus nigrispinus were obtained from the rearing laboratory at the Department of Entomology, Federal University of Lavras (Lavras, MG, Brazil). Nymphs in the 1st instar were fed only with a honey and distilled water (10%) solution. Nymphs in the 2nd instar were fed on third or fourth instar *S. frugiperda* larvae *ad libitum*, and water was provided on a wet cotton ball and refreshed every 24 h. Predator rearing was also maintained under controlled conditions at a temperature of 25 ± 2 ºC, a relative humidity of 70 ± 10% and a photoperiod of 12:12 (L:D) hours.

2.3. Biology of Spodoptera frugiperda populations

The experiments were conducted in the Ecotoxicology and IPM Laboratory of the Federal University of Lavras (Lavras, MG, Brazil) under controlled conditions, with a temperature of 25 ± 2 ºC, relative humidity of 70 ± 10% and photoperiod of 12:12 (L:D) hours. For the bioassays, neonates of *Bt*-susceptible or *Bt*-resistant *S. frugiperda* were individually kept in a 50 mL plastic cup. In each cup, 10 mL of agar and a filter paper dish were added to prevent foliar dryness. A 4 cm² foliar dish from leaves of transgenic maize or leaves of isogenic maize was offered daily. The leaves used in the bioassay were excised from V4 to V8 plants. In each treatment, 50 larvae were used. The survival of the larvae was evaluated over 120 hours, and the weight and length of the live larvae were measured.

2.4. Maize plants efficacy trials

The efficacy of maize plants was evaluated with a greenhouse experiment under natural light and temperature conditions (February–March 2017, Lavras, Minas Gerais, Brazil). V4 maize plants (*Bt* or non-*Bt*) were infested with 5 neonates of *S. frugiperda* (*Bt*-resistant or *Bt*-susceptible), and they were maintained in voile cages to prevent larval escape. The injury was evaluated 7 days after infestation. Foliar damage was scored using a 1-9 rating scale, also known as the Davis scale (Davis et al. 1992), whereby a score of 1 indicates no damage and a score of 9 indicates total destruction of the whorl by insect feeding. The experiment was conducted in a randomized complete block design with 5 replications.

2.5. Podisus nigrispinus biology

The experiments were conducted in the Ecotoxicology and IPM Laboratory of Federal University of Lavras (Lavras, MG, Brazil) in controlled conditions, with a temperature of 25 ± 2 ºC, relative humidity of 70 ± 10% and photoperiod of 12:12 (L:D) h. For the bioassays, 2nd instar nymphs of *P. nigrispinus* were used because at this stage and beyond, the nymphs assume a predatory habit (Zanuncio et al. 1996). Only distillated water was offered to 1st instar nymphs through a wet cotton ball. For the 2nd instar nymphs, distillated water (wet cotton ball) and *S. frugiperda* larvae were offered. Larvae of the *Bt*-resistant population were fed transgenic maize leaves or leaves of the isogenic non-*Bt* maize for 48 hours before being offered to the predators. This time was sufficient for *Bt* toxin ingestion by larvae (Lawo et al. 2010). Maize leaves of both genotypes, between vegetative stages V4 to V8, were used. The leaves were offered in a quantity of approximately 4 cm² in 50 mL plastic cups. In each cup, 10 mL of agar and a filter paper dish were added to prevent foliar dryness.

For each treatment, 50 nymphs of *P. nigrispinus* were individually placed in Petri dishes (4 cm diameter); a group of 50 nymphs were fed with resistant larvae that fed on transgenic maize, and a second group of 50 nymphs were fed with resistant larvae that fed on isogenic maize. Nymphs were fed *ad libitum* with 3rd to 4th instar larvae of *S. frugiperda*, according to the treatments above, and distilled water was added daily. The predators were monitored daily until the adult stage, and the following data were recorded: duration and survival of each nymphal instar, duration of the total nymphal stage and the weight of the newly emerged adults.

2.6. Statistical analysis

All statistical analyses were carried out in R v 3.3.1 (R Development Core Team 2013). Data of biology of *S. frugiperda* populations and *P. nigrispinus* biology were analysed with a generalized linear model (GLM) with a Gaussian error distribution. Survival curves of *S. frugiperda* populations were estimated applying the Weibull model using the Survival package. Data of weight and length larval of *S. frugiperda* and weight of *P. nigrispinus* adults were subjected to Shapiro-Wilk and Bartlett tests to confirm normal distribution and homoscedasticity. Subsequently, the data were analysed using ANOVA and Tukey's test. When assumptions of normality or homoscedasticity were violated, a generalized linear model (GLM) with a Gaussian error distribution was utilized. The efficacy trial was analysed by analysis of variance (ANOVA), with means separation using the Scott-Knott test.

3. Results

3.1. Biology of Spodoptera frugiperda populations

The survival rate of *S. frugiperda* was affected by the plant genotype (GLM, $F_{1, 22}$ = 12.86, $P < 0.01$) and by the type of population ($F_{1, 21}$ = 76.83, $P < 0.001$). Furthermore, a significant interaction ($F_{1, 20}$ = 15.87, $P < 0.001$) was observed between genotype and population. The survival of the susceptible larvae was significantly higher in those fed on non-*Bt* maize leaves compared with those fed on *Bt* maize leaves ($F_{1, 10}$ = 44.02, $P < 0.001$), while resistant larvae fed on *Bt* or non-*Bt* maize leaves survived equally ($F_{1, 10}$ = 0.06, P = 0.813). The survival of susceptible larvae was significantly lower in those fed on non-*Bt* and *Bt* leaves compared with resistant larvae ($F_{1, 10}$ = 6.10, $P < 0.05$ and $F_{1, 10}$ = 640, $P < 0.001$, respectively) (Figure 1).

The survival curves of susceptible larvae were different when the larvae were exposed to *Bt* maize leaves and non-*Bt* maize leaves ($P < 0.001$) (Figure 2A), while the survival curves of resistant larvae fed on *Bt* and non-*Bt* maize leaves were similar ($P > 0.05$) (Figure 2B).

The weight ($F_{1, 69}$ = 9.03, $P < 0.01$) and length ($F_{1, 76}$ = 8.50, $P < 0.01$) of *S. frugiperda* larvae were affected by the plant genotype (Figure 3). Both the weight and length of the susceptible larvae were significantly greater in those fed on non-*Bt* plants ($F_{1, 69}$ = 9.69, $P <$ 0.01 and $F_{1, 76}$ = 32.57, $P < 0.01$, respectively), whereas resistant larvae had a similar weight and length for both plant genotypes ($F_{1, 69}$ = 3.71, $P > 0.05$ and $F_{1, 76}$ = 0.25, $P > 0.05$, respectively). Resistant larvae on *Bt* maize plants were heavier and larger than susceptible

larvae ($F_{1, 69}$ = 5.57, P < 0.05 and $F_{1, 76}$ = 24.51, P < 0.01, respectively), but no significant difference was found in larval weight and length between the populations on non-*Bt* plants ($F_{1, 69}$ = 0.79, P > 0.05 and $F_{1, 76}$ = 3.17, P > 0.05, respectively).

3.2. Maize plants efficacy trials

Bt maize plants infested with susceptible larvae showed little injury and no live larvae 7 days after infestation, indicating that the population was unable to survive on *Bt* maize. This *S. frugiperda* population caused intermediate injury to non-*Bt* maize plants. The *Bt*-resistant *S. frugiperda* population caused great injury to *Bt* and non-*Bt* maize plants, indicating extensive whorl damage. The injury caused by *Bt*-resistant population to *Bt* maize plants was not significantly different from that of non-*Bt* maize plants (P > 0.05) (Figure 4).

3.3. Podisus nigrispinus biology

The exposure of *P. nigrispinus* nymphs to *Bt*-resistant *S. frugiperda* previously fed transgenic or isogenic maize did not significantly affect the survival of the predator ($F_{1, 8}$ = 0.067, P = 0.803). The survival percentage of predators was 84 ± 6.78% when exposed to resistant larvae fed on non-*Bt* maize and 82 ± 3.74% when exposed to resistant larvae fed on *Bt* maize (Figure 5).

Total nymphal development time ($F_{1, 8}$ = 0.545, P = 0.482) and adult weight ($F_{1, 8}$ = 0.603, P = 0.460) of *P. nigrispinus* were statistically similar when the predator was fed with resistant larvae fed on non-*Bt* maize or *Bt* maize (Table 1 and Figure 6). The duration of total nymphal development was 15.06 ± 0.09 days when the predator was exposed to resistant larvae fed on non-*Bt* maize and 14.97 ± 0.09 days when exposed to resistant larvae fed on *Bt* maize. The adult weight of *P. nigrispinus* exposed to resistant larvae fed on non-*Bt* maize was 50.13 ± 2.56 mg and 47.70 ± 1.79 mg when exposed to resistant larvae fed on *Bt* maize.

4. Discussion

The evaluation of potential effects of *Bt* plants on non-target organisms, such as predators and parasitoids, is generally performed by offering prey that were previously fed on *Bt* plants to these organisms. However, the great majority of studies use prey with susceptibility to *Bt* plants (Romeis et al. 2006, Naranjo 2009).

The use of *Bt*-resistant herbivores is a new tool to explore the direct effects of *Bt* plants on tritrophic non-target studies, without the methodological problem of indirect effects of susceptible herbivores (Shelton et al. 2016). The potential prey-mediated effects can be mistakenly measured as a direct effect of *Bt* toxicity (Romeis et al. 2011), and the use of *Bt*-resistant prey eliminates the possible effects of prey quality on natural enemy's biology.

We showed in laboratory and greenhouse assays that a resistant population of *S. frugiperda* was able to damage and survive on *Bt* maize plants, demonstrating that this population was insensitive to the Cry1F protein produced by the transgenic maize. Tian et al. (2012) also observed a *S. frugiperda* strain that developed resistance to Cry1F maize and was able to complete its life cycle on *Bt* maize in laboratory conditions without the deleterious effects of the Cry1F protein. These data are consistent with Storer et al. (2010) and Farias et al. (2014) who showed that resistant populations of *S. frugiperda* are highly insensitive to Cry1F toxin expressed in event TC1507 maize. These initial trials were necessary to confirm the resistance of the *S. frugiperda* population in laboratory and semi-field conditions, and to posteriorly explore the direct effect of *Bt* plants on non-target organisms overcoming host quality effects.

Our tritrophic bioassays with *P. nigrispinus* showed that there was no direct effect of feeding on *Bt* maize by *Bt*-resistant *S. frugiperda* on *P. nigrispinus* biology. The survival and the nymphal stage development time of the predator *P. nigrispinus* were not significantly different when fed resistant larvae previously fed *Bt* or non-*Bt* maize. The weight gain of the adults was also not significantly affected by this exposure. In contrast, Leite et al. (2014) showed a delay in the nymphal development time and a biomass reduction in the fifth instar of *P. nigrispinus* when predators fed *Bt*-susceptible *S. frugiperda* that were exposed to *Bt* maize. The authors attributed this delay in development to the poor quality of the *Bt*-fed prey due to the susceptibility of *S. frugiperda* larvae to *Bt* toxin. However, in our study, the prey that we used was resistant to *Bt* maize, and the *Bt* exposure had no effect on its biology; therefore, these indirect effects were avoided.

The simple presence of the *Bt* protein in the midgut of the biocontrol agent is not an assurance of toxicity, because the receptors in the midgut membrane need to bind to the *Bt* protein for the disruption of the midgut cells, a pre-requisite for direct toxicity (Gill et al. 1992, Bravo et al. 2007). In a histological study, Cunha et al. (2012) verified that *P. nigrispinus* showed ultrastructural changes in the digestive cells after feeding on susceptible *S. frugiperda* larvae exposed to *Bt* cotton, indicating that the predator acquired the *Bt* protein from the prey. The midgut of the predator was affected by induced disorganization in the

midgut cells, and according to the authors, these changes could disturb the predation ability; however, no predation or biological assay was performed to confirm this.

From our results, no negative effect was observed on biological parameters of *P. nigrispinus* after ingesting *Bt*-fed prey. Leite et al. (2014) indicated that *P. nigrispinus* reduced the larval survival and damage of *S. frugiperda* on *Bt* maize plants, under semi-field conditions, indicating a positive integration between *Bt* plants and the predator for successful *S. frugiperda* control. A similar result was found by Malaquias et al. (2014), who that showed a functional response by *P. nigrispinus* on *S. frugiperda* control was not affected by the cotton genotype (*Bt* or non-*Bt*) used in the trials. Thus, there is no evidence that *Bt* plants affect the biology and predatory ability of the predator *P. nigrispinus*.

A 'worst-case' toxicological study demonstrated that the predator *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) exhibited no direct effect of Cry1Ab toxin when presented in an artificial diet, and the predator was possibly able to digest or degrade the toxin into a non-toxic form (Romeis et al. 2004). In the bioassays, the authors feed the predator with high concentrations of the *Bt* toxin; however, the use of purified *Bt* proteins in artificial diets may not reproduce a real ecological scenario, such as the predation or parasitism of natural enemies of herbivores that feed *Bt* plants (Arpaia et al. 2017).

Schuler et al. (2004) showed that the parasitism rates by *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae) on *Bt*-resistant *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) larvae was not significantly different when the larvae were fed on *Bt* and non-*Bt* plants. The study also showed that the parasitoid was able to control the resistant host and that the presence of *Bt* toxin in the resistant host had no direct effect on the parasitoid biology. Ferry et al. (2006) demonstrated that biological responses did not differ between beetles (*Pterostichus madidus*) that fed on *Bt*-resistant larvae of *P. xylostella* reared on *Bt* plants and those reared on non-*Bt* plants, even with *Bt*-resistant larvae delivering high levels of toxin to the predator.

Tritrophic studies with *Bt*-resistant *S. frugiperda*, *Bt* maize and the predators *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) (Tian et al. 2012), *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae) (Tian et al. 2013), *Geocoris punctipes* (Say) (Hemiptera: Geocoridae) and *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) (Tian et al. 2014a), and the parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) (Tian et al. 2014b) showed no direct effects on the life history of these natural enemies over more than one generation when they were exposed to *Bt* proteins indirectly through the prey/host. The studies also showed that the *Bt* protein was transferred to the natural enemies

after prey/host feeding, but the *Bt* protein was diluted and did not bioaccumulate, resulting in its degradation to non-active fragments.

Similar to other studies presented here, our study suggest that the Cry1F protein presents in *Bt* maize had no direct impact on the predator *P. nigrispinus*, as evidenced by the high survival and normal development rates after exposure to *Bt*-resistant prey fed on *Bt* maize. The tritrophic studies with *Bt*-resistant herbivores utilized non-target organisms from different orders and families of natural enemies (see Shelton et al. 2016 for a review), and all studies showed that the Cry proteins currently used in *Bt* crops for the control of Lepidoptera are not harmful to these beneficial organisms.

Although there are data for some *Bt* plants and some natural enemy species, the database is still far from sufficient to assure the effects of *Bt* proteins. First, there are recent *Bt* proteins, such as Vip3A, with few studies done thus far, and second, all existing studies can not be generalized to other organisms (Lövei et al. 2009). Therefore, risk assessment studies are important to identify potential effects of *Bt* plants on a case-by-case basis and studies are increasingly necessary due to our vast biodiversity and the complexity of tritrophic interactions.

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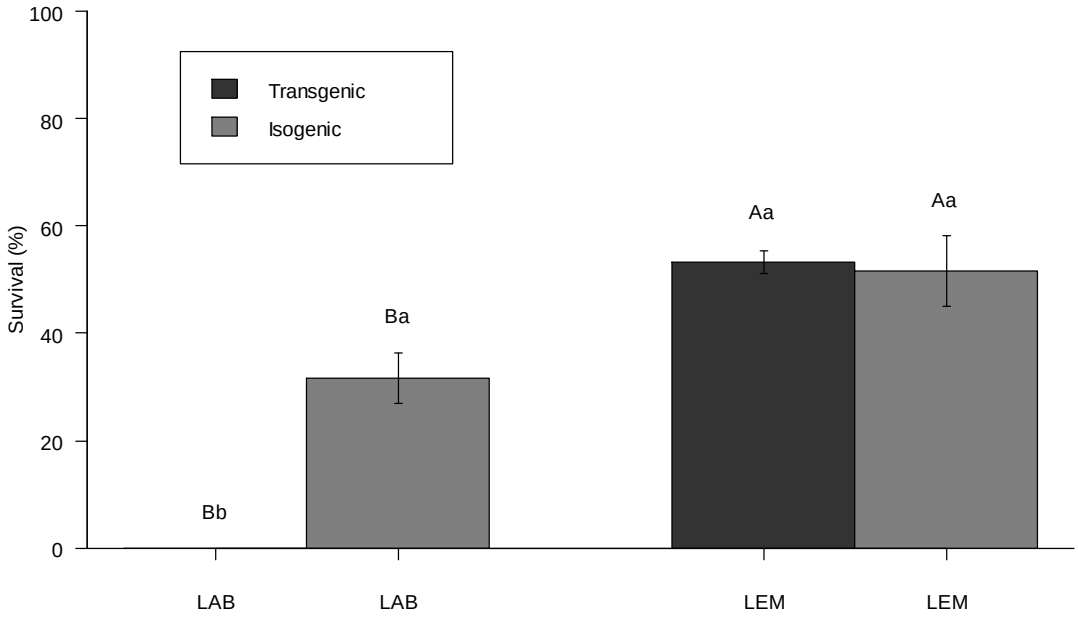

Figure 1: Survival of susceptible larvae (LAB) and resistant larvae (LEM) of *Spodoptera frugiperda* fed on *Bt* (transgenic) and non-*Bt* maize leaves (isogenic). Uppercase letters compare the populations in each treatment, and lowercase letters compare the treatments in each population. Means (± SE) followed by different letters are significantly different (contrast among populations/treatments after a GLM).

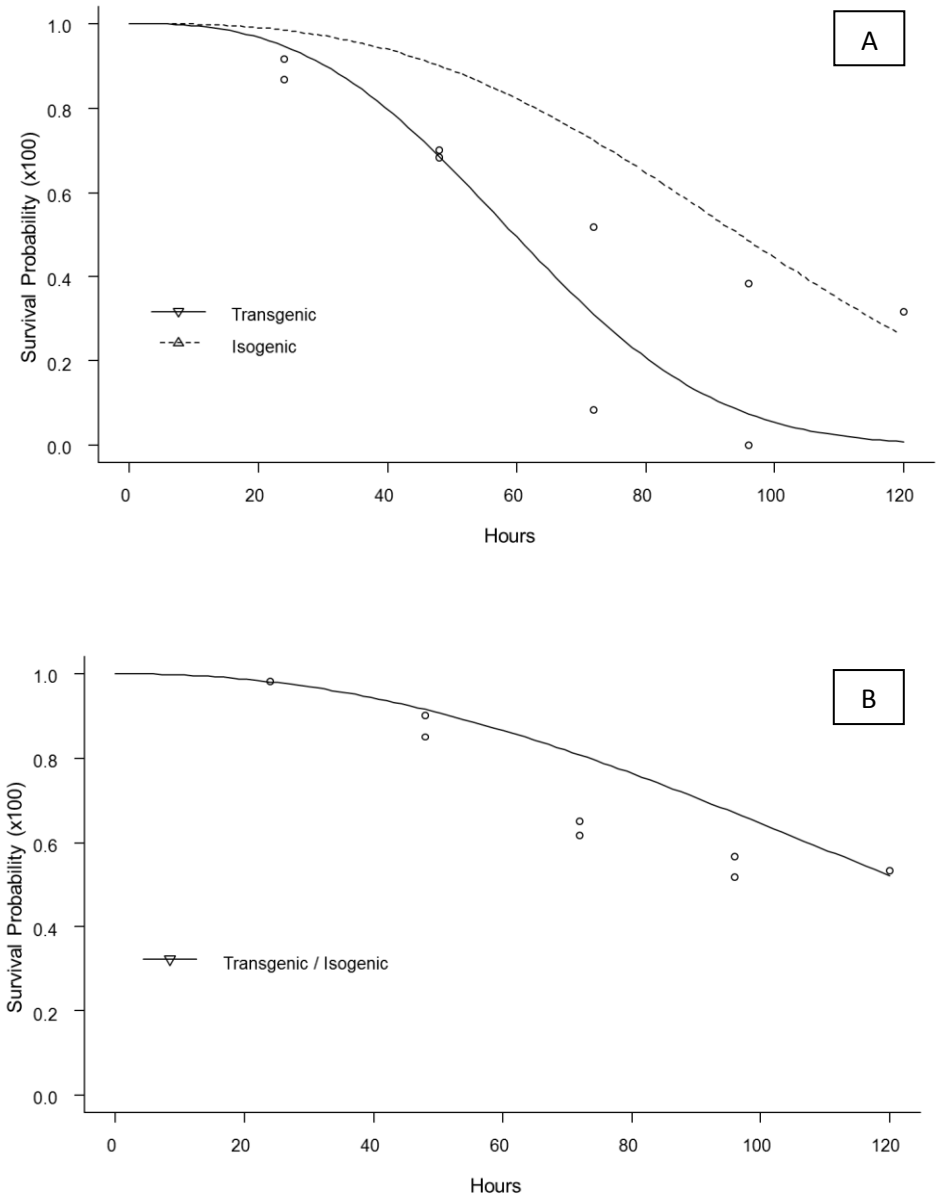

Figure 2: Survival curves of *Spodoptera frugiperda* populations exposed to *Bt* Cry1F maize (transgenic) and non-*Bt* maize (isogenic) throughout larval development. (A) *Bt*-susceptible population, (B) *Bt*-resistant population.

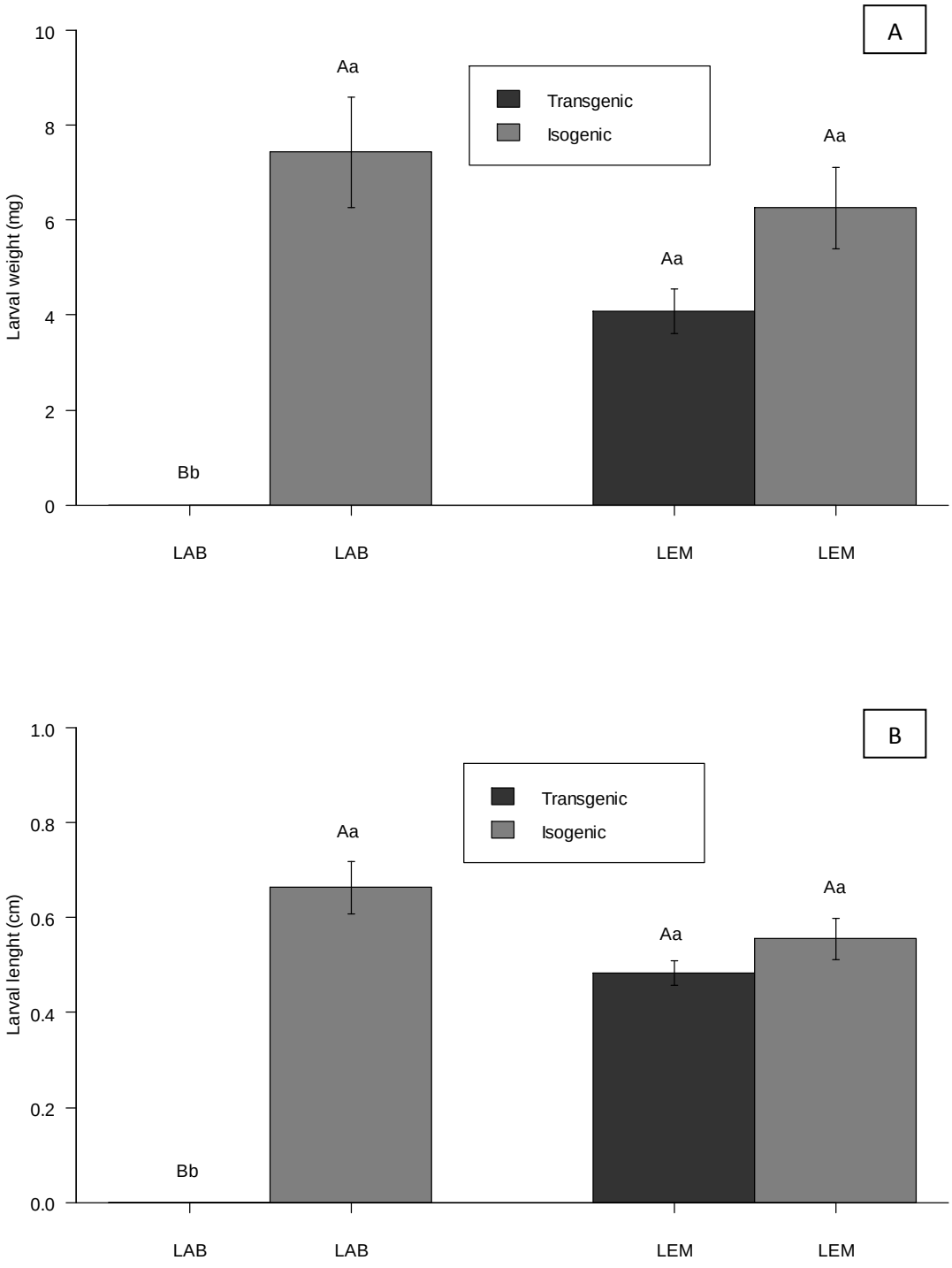

Figure 3: Larval weight (A) and larval length (B) of *Spodoptera frugiperda* populations (LAB: *Bt*-susceptible population, LEM: *Bt*-resistant population) exposed to *Bt* Cry1F maize (transgenic) and non-*Bt* maize (isogenic). Means (± SE) followed by different letters were significantly different (ANOVA, $P \leq 0.05$). Uppercase letters compare the populations in each treatment, and lowercase letters compare the treatments in each population.

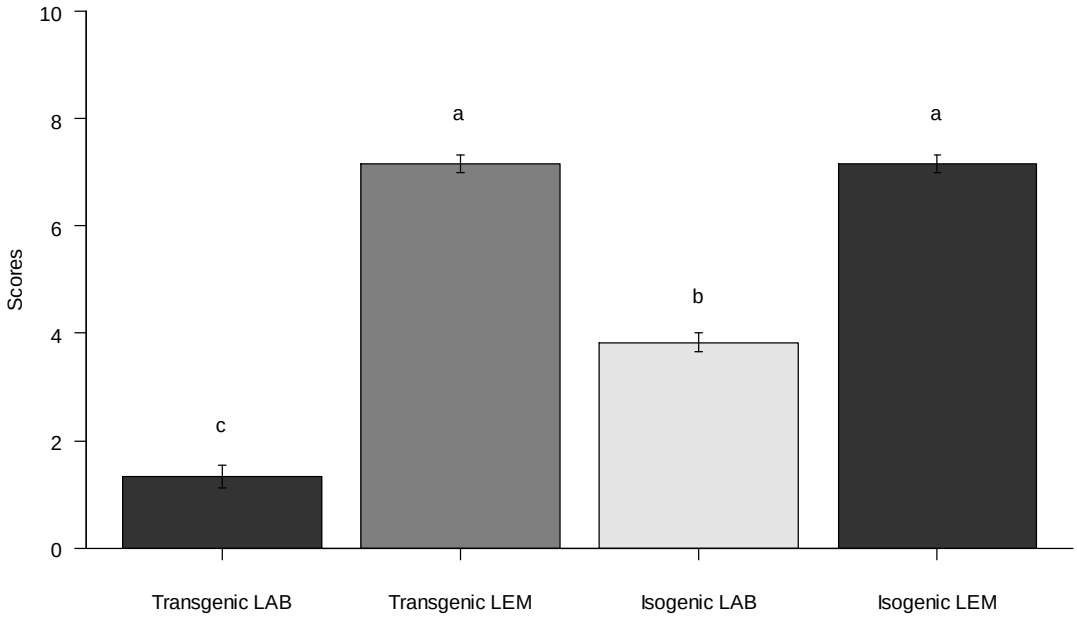

Figure 4: Damage scores of *Bt* maize (transgenic) and non-*Bt* maize (isogenic) infested by a *Bt*-susceptible population (LAB) or *Bt*-resistant population (LEM) of *Spodoptera frugiperda*. Means (± SE) followed by different letters are significantly different (ANOVA, $P \leq 0.05$).

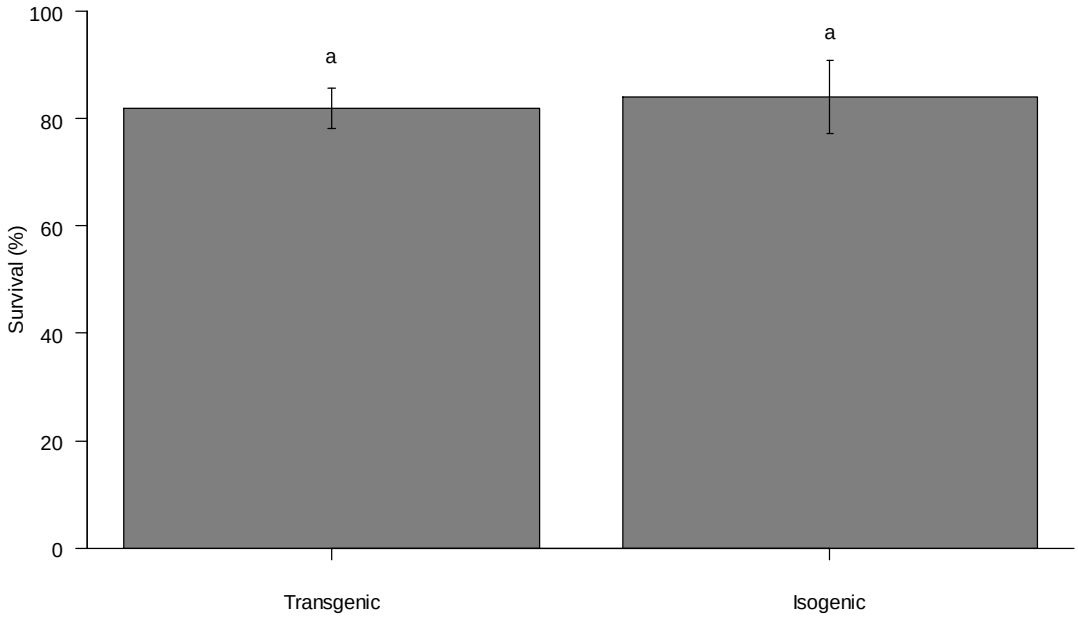

Figure 5: Survival of *Podisus nigrispinus* nymphs exposed to *Bt*-resistant larvae of *Spodoptera frugiperda* previously fed on *Bt* maize (transgenic) and non-*Bt* maize (isogenic). Means (± SE) followed by the same letter are not significantly different (GLM, $P \leq 0.05$).

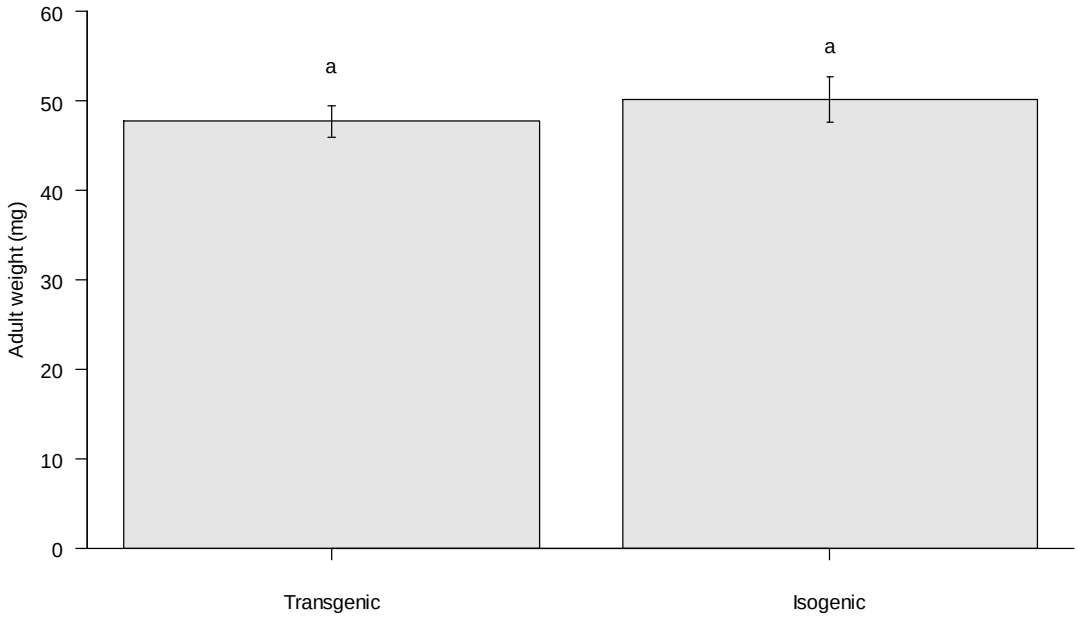

Figure 6: Adult weight of *P. nigrispinus* exposed to *Bt*-resistant larvae of *S. frugiperda* previously fed on *Bt* maize (transgenic) and non-*Bt* maize (isogenic). Means (± SE) followed by the same letter are not significantly different (ANOVA, $P \leq 0.05$).

Table 1. Nymphal stage development of *Podisus nigrispinus* fed with *Bt*-resistant larvae of *Spodoptera frugiperda* previously fed on *Bt* and non-*Bt* maize.

Plant genotype	*S. frugiperda* population	Nymphal stage development duration (days)				Total nymphal development (days)
		Second	Third	Fourth	Fifth	
Non *Bt* maize	Resistant	3.38 ± 0.08a (n = 42)	2.94 ± 0.05a (n = 42)	3.07 ± 0.11a (n = 42)	5.68 ± 0.05a (n = 42)	15.06 ± 0.09a (n = 42)
Bt maize	Resistant	3.29 ± 0.12a (n = 41)	2.95 ± 0.10a (n = 41)	2.91 ± 0.06a (n = 41)	5.82 ± 0.07a (n = 41)	14.97 ± 0.09a (n = 41)

Mean ± SE followed by the same letter in the column are not significantly different by ANOVA ($P \leq 0.05$). The n indicates the number of individuals of each nymphal stage that were used in the ANOVA from the total of 50 nymphs at the start of the experiment.

ARTIGO 2

HERBIVORY BY *Bt*-RESISTANT FALL ARMYWORM ON *Bt* MAIZE DOES NOT INDUCE ATTRACTIVE VOLATILES TO THE THIRD TROPHIC LEVEL

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ABSTRACT

Genetic transformation that occurs on transgenic plants can result in unintended effects on plant traits, such as alter the emission of volatiles in response to herbivory. These herbivore-induced plant volatiles are important to the natural enemies that use them to find their prey or host and any change in the volatile profile can negatively impact the tritrophic interactions. We determined whether the genetic transformation of maize with Cry1F gene affects the constitutive and induced plant volatiles in ways that alter foraging behavior of the predator *Podisus nigrispinus* (Hemiptera: Pentatomidae). GC-MS analyses revealed that *Bt* transformation did not result in relevant changes in the constitutive volatile emission nor in that emitted by regurgitant-treated plants. Despite the fact that *Bt*-resistant *Spodoptera frugiperda* (Lepidoptera: Noctuidae) population inflicted similar damage on *Bt* and non-*Bt* plants, herbivore-induced volatile blend emitted by *Bt* plants had a different composition. Herbivore-damaged non-*Bt* plants emitted fatty acid derivatives and monoterpenes that were absent in the emission of herbivore-damaged *Bt* plants. Results from olfactometer tests showed that *P. nigrispinus* did not distinguish among the odours of undamaged and regurgitant-treated plants regardless of the plant genotypes. While the predator oriented preferentially to volatiles from herbivore-damaged over undamaged plants of the non-*Bt* genotype, the predator did not differentiate blends emitted by undamaged and herbivore-damaged plants of *Bt* maize. When the predator was exposed to herbivore-induced plant volatiles of *B*t and non-*Bt* plants, *P. nigrispinus* preferred the latter, suggesting that the qualitative differences found between herbivore-damaged *B*t and non-*Bt* maize plants might be responsible for the differential attractiveness. Given the fact that we used a *Bt*-resistant herbivore, this is the first report demonstrating that the insertion of *Bt* into maize genome changes qualitatively plant volatile emissions, in ways that disrupt predator orientation to herbivore-induced plant volatiles.

Keywords: induced plant defenses, *Podisus nigrispinus*, *Spodoptera frugiperda*, transgenic maize, tritrophic interaction, volatiles

1. INTRODUCTION

Transgenic plants with insect resistance were developed with the objective of reducing crop losses due to feeding damage of pests. In the genetic transformation, genes of the soil bacterium *Bacillus thuringiensis* Berliner (*Bt*) were introduced into the plant genome to express toxic proteins to lepidopteran and/or coleopteran pests (Sanahuja et al. 2011, Tabashnik et al. 2013).

Transgenic maize expressing *Bt* has been commercialized since 1996 and its benefits include reduced insecticide application, increased yields, and efficient pest control (Christou et al. 2006, Sanahuja et al. 2011). Additionally, *Bt* proteins exert effect on a narrow taxonomic group of insects, unlike chemical insecticides, and their use also may contribute to conservation of natural enemies (Sanahuja et al. 2011).

The *Bt* maize with expression of Cry1F protein (Herculex, event TC1507) was proposed to protect from attack by lepidopteran pests, such as *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), an important pest of maize (Sparks 1979). However, field-evolved *S. frugiperda* resistance to Cry1F maize was documented in Puerto Rico in 2006 (Storer et al. 2010), and in Brazil in 2012 (Farias et al. 2014), a few years after its commercial release in these countries.

The genetic transformation with the insertion of an external gene into a plant may result in physiological alterations on the plant (Haslberger 2003, Cellini et al. 2004), such as increased production of lignin in *Bt* maize plants (Saxena and Stotzky 2001) and changes on isoenzymes activity in *Bt* cotton plants (Ding et al. 2001). Thus, physiological alterations in the plants due to breeding process may result in modifications in the primary and secondary metabolism, and alter the tritrophic interactions between plants, herbivores and natural enemies (Yan et al. 2004, Himanen et al. 2009, Chen et al. 2015).

It is well-known that plants under attack of arthropod herbivores release herbivore-induced plant volatiles (HIPVs) that serve as cues to the third trophic level, such as predators and parasitoids, in prey/host searching (Agrawal et al. 2002, Dicke et al. 2003). Thus, HIPVs emission supports the recruitment of natural enemies that indirectly help the plants on the pest control (Dicke et al. 1990). Therefore, foraging behavior of natural enemies may have considerable effects on pest control, and may add a biocontrol service on *Bt* crops. Currently, this integration is relevant due to cases of pest resistance where *Bt* plants do not have more effect on the *Bt*-resistant pest, and thus, the biological control provided by the natural enemies could help to suppress the evolution of pest resistance (Schuler et al. 1999a, 2003).

Some studies have been conducted to evaluate the effect of the genetic transformation on the volatile profiles of *Bt* plants and its influence on natural enemy behavior. In general, the studies showed that parasitoids do not discriminate foraging on *Bt* and non-*Bt* plants (Turlings et al. 2005, Himanen et al. 2009, Moraes et al. 2011, Liu et al. 2015). This behavior may be attributed to similar blend volatiles between *Bt* and non-*Bt* plants (Moraes et al. 2011, Liu et al. 2015); however, the indiscrimination also was observed where the blend of volatiles were qualitative similar, but distinct in quantitative terms (Turlings et al. 2005).

Turlings et al. (2005) showed that regurgitant-treated *Bt* and non-*Bt* maize plants emitted the same number of volatile compounds that were quantitatively more emitted by non-*B*t plants, but even emitting larger amounts of volatiles, two parasitoids did not distinguished between the odours of the *Bt* and the non-*Bt* plants. However, differences in volatile emissions in either concentration and/or composition may influence the response of beneficial organisms (Yan et al. 2004).

Dean and De Moraes (2006) also showed that herbivore-damaged non-*Bt* maize plants induced larger amounts of volatiles than *Bt* plants due to reduced feeding damage by *Bt*-susceptible herbivores on *Bt* plants and not precisely the result of genetic transformation. A way to avoid induction variability between *Bt* and non-*Bt* plants, and consequently differences on volatile emissions would be ensure equal treatment on both plant genotypes. Like for example, the artificial damage of the leaves and posterior application of larval regurgitant, that mimic the real herbivory (Turlings et al. 1990, 2005), and through the use of a *Bt*-resistant pest that are insensitive to *Bt* toxins and can normally feed on *Bt* plants (Schuler et al. 1999a, 2003). The use of these methods of plant induction excludes the effects of reduced target herbivory, and only the effects of the genetic transformation would be evaluated.

Here we investigated whether volatile emissions varied between *Bt* maize plants, with Cry1F gene inserted, and non-*Bt* maize plants. First, we compared the volatile profiles of undamaged plants to determine whether *Bt* and non-*Bt* plants differed in constitutive emissions, and compared induced volatile emissions from regurgitant-treated plants and herbivore-damaged plants that were damaged by a *Bt*-resistant population of *S. frugiperda*. Then, we addressed if any alteration of the volatile profiles can modify the prey-searching behavior of the predator *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae), a natural enemy of *S. frugiperda* found in maize crops in Brazil. To our knowledge, this is the first study to address differences on volatile profile between *Bt* and non-*Bt* maize plants, induced by a *Bt*-resistant pest, and its impact on the third trophic level.

2. MATERIALS AND METHODS

2.1. Plants

Maize hybrids seeds of 30F53H (Herculex, event TC1507, Cry1F) and its non-*Bt* isogenic equivalent, 30F53, were provided by DuPont do Brasil, "Divisão Pioneer Sementes" (Santa Cruz do Sul, RS, Brazil). Seeds were sown in a commercial plant substrate (Bioplant®) in plastic pots (300 mL) weekly and kept in a greenhouse made of anti-aphid screen and inside cages of fine-mesh fabric to prevent herbivore infestation. Plants at the V4 stage, approximately 4 weeks after sowing, were used in the behavioral trials and volatile collection assays. After the trials, to confirm *Bt* gene expression by the maize plants, AgraStrip® Cry1F test strips (Romer Labs®, Getzersdorf, Austria) were used according to the manufacturer's instructions. All tests for the presence of the Cry1F protein (*Bt* plants) or its absence (non-*Bt* isogenic plants) were as expected.

2.2. Insects

A *Bt*-susceptible population of *S. frugiperda* (LAB) was obtained from the rearing laboratory at the Department of Entomology, Federal University of Lavras (Lavras, MG, Brazil). This population was never exposed to *Bt* maize or any insecticide, and it was used as a susceptible reference strain. A *Bt*-resistant population of *S. frugiperda* (LEM) was obtained from the insect rearing laboratory at DuPont do Brasil, "Divisão Pioneer Sementes" (Planaltina, GO, Brazil). This population was collected from *Bt* maize fields in the municipality of Luis Eduardo Magalhães (BA, Brazil), and it was considered resistant to Cry1F protein because it was able to damage transgenic maize plants. Larvae were reared in a acclimatized room at temperature of 25 ± 2 ºC, relative humidity of 70 ± 10% and photoperiod of 12:12 (L:D) hours. Adults were maintained in PVC tubes lined with paper to support oviposition and were fed on a honey and distilled water (10%) solution. Eggs were collected daily and placed in Petri dishes, where larvae hatched and were transferred to plastic boxes containing artificial diet (Greene et al. 1976) to feed on until pupation.

Podisus nigrispinus were obtained from the rearing laboratory at the Department of Entomology, Federal University of Lavras (Lavras, MG, Brazil). Nymphs in the 1st instar were fed only with a honey and distilled water solution (10%). Nymphs in the 2nd instar were fed on third- or fourth-instar *S. frugiperda* larvae *ad libitum*. A cotton ball soaked with water

served as water source for insects and it was replaced daily. Predator rearing was also maintained under controlled conditions at a temperature of 25 ± 2 ºC, a relative humidity of 70 ± 10% and a photoperiod of 12:12 (L:D) hours.

2.3. Foliar consumption of susceptible and resistant S. frugiperda

To measure the foliar consumption of *S. frugiperda* populations, a single neonate of *S. frugiperda* (*Bt*-susceptible or *Bt*-resistant) was enclosed in a clip cage (1 cm diameter) attached to the third fully-extended leaf of a V4-stage *Bt* maize or non-*Bt* maize plant. In this way, the same leaf area, limited by the clip cage, was provided for neonates to feed. After 48 h, the foliar consumption by the neonate was measured using the software ImageJ (Rasband 2016). Parameters recorded also included larval length. Six replicates were performed in this assay.

2.4. Volatile Collection and Analysis

We collected volatile emissions from V4-stage maize plant of the transgenic and the isogenic hybrid: undamaged, herbivore-damaged or regurgitant-treated plants.

Herbivore-damaged plants were infested with two third-instar *S. frugiperda* larvae for 24 h. The larvae were left feeding on the plant during the experiments. We used a resistant population of *S. frugiperda* that was able to damage the transgenic maize, as confirmed in previous experiment (see Figure 1).

To obtain larval regurgitant, fourth-instar *S. frugiperda* larvae were fed on transgenic or isogenic plants for 24 h, after this feeding time, the larval regurgitant was manually collected with a pipette tip by pressing the oral cavity of the caterpillars and catching the fluid released. This regurgitant was stored in glass vials at -70ºC until its use. To obtain regurgitant-treated plants, two leaves (third and fourth fully-extended leaf) were scratched with a razor blade about 1 cm² and applied 10 µL of larval regurgitant to each site. Plants were treated with larval regurgitant at 08:00 AM and the experiments started 6 h later.

Prior to volatile collection, the pots with the plants were carefully wrapped in aluminum foil to reduce the emission of volatiles from below ground parts or the substrate. Maize plants were placed individually in sealed glass chambers (10 cm diameter and 25 cm high) connected to the ARS Volatile Collection System (ARS, Gainesville, FL, USA) coupled to charcoal filters, humidifiers and flow-meters. Filtered and humidified air was pumped

through PTFE (polytetrafluoroethylene) hoses into sealed glass chambers containing the plants as odor sources. The glass chambers were connected to filters with the adsorbent polymer Haysep® (30 mg, 80/100 mesh, Alltech Assoc.) where the air was pulled out by a vacuum pump. The air flow was adjusted to 0.6 L min^{-1}. The polymer filters were washed with 150 µL of hexane and the extracts were placed in sealed glass vials, which were stored in a freezer at - 30 °C until analysis. We added 4 µL of a 10 ng μL^{-1} nonyl acetate solution as internal standard to each sample.

Samples were analysed in a Shimadzu 2010 gas chromatograph (GC) equipped with a flame ionization detector (FID) and using an HP-1MS column (30 m × 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). We adopted Electron Impact (EI) in full scan as ionization method. Detector was calibrated to mass range analysis from 35 to 250 m z^{-1} and 25 ms of maximum ion time. Two microliters of each sample were injected into a splitless injector, with helium as carrier gas. The column temperature was kept at 40°C for 5 min, and then was increased at 5°C min^{-1} until it reached 150°C, held for 1 min, and then increased at 20°C min^{-1} until it reached 250°C and held for 20 min. Each compound was quantified based on the compound peak area relative to nonyl acetate in the GC-FID.

To characterize the compounds, samples were injected into a Shimadzu QP 2010 Ultra gas chromatograph coupled with a quadrupole mass spectrometer (GC-MS Quadrupole), adopting the same settings used for the GC-FID. Compounds were identified by calculating the Kovats index, by comparing their mass spectra to the NIST 08 Mass Spectral Library (Sigma-Aldrich, St. Louis, MO, USA) and, when available, to the mass spectra of synthetic standards (Sigma-Aldrich, St. Louis, MO, USA, purity 98%).

Volatile collection were made from six plants of each treatment for 4 h and were split into two blocks during the photoperiod, from 9:00-13:00 h and from 14:00-18:00 h using supplemental light for plants (Philips Green Power tubular LED, 12L:12D, 120 µmol m^{-1} s^{-1}, 400–700 nm). Herbivore-damaged plants were sampled on the morning (9:00-13:00 h) after 24 h of larval feeding and regurgitant-treated plants were sampled on the afternoon (14:00-18:00 h) after 6 h of induction.

2.5. Olfactometer Bioassay

A glass Y-tube olfactometer (main arm: 9 cm long; side arms: 9 cm long; 1 cm internal diameter; arms angle 90º) was used to investigate the responses of *P. nigrispinus*. A humidified charcoal filtered airflow of 1.2 L/min was equally split into two parts, and pushed

into glass chambers (12 cm diameter and 23 cm high) containing the odor sources. Each odor source consisted of a V4-stage maize plant of either the transgenic or the isogenic hybrid: undamaged, herbivore-damaged or regurgitant-treated plants, as described above.

A single second-to-third instar *P. nigrispinus* nymph, starved for 24 h, was introduced into the olfactometer main arm and its choice was recorded. Insect choice was considered when it crossed a threshold line, located in the middle of the side arm, and stayed in the top half of the arm for at least 5 s. Nymphs that did not choose within 10 min period were excluded from the data analysis.

After each trial, the olfactometer was inverted to prevent directional bias. After every five nymphs, Y-tube olfactometer and the glass chambers were cleaned with water and acetone and sterilized (100ºC) and a new pair of plants was tested. The experiments were conducted in the laboratory under controlled conditions at 25 ± 2ºC, 70 ± 10% RH and during the day (09:00-18:00 h), when the predator is more active (Torres et al. 2002).

The following trials were conducted: (1) undamaged isogenic plants (UI) *vs.* herbivore-damaged isogenic plants (HI); (2) undamaged transgenic plants (UT) *vs.* herbivore-damaged transgenic plants (HT); (3) herbivore-damaged isogenic plants (HI) *vs.* herbivore-damaged transgenic plants (HT); (4) undamaged isogenic plants (UI) *vs.* regurgitant-treated isogenic plants (RI); (5) undamaged transgenic plants (UT) *vs.* regurgitant-treated transgenic plants (RT), and (6) regurgitant-treated isogenic plants (RI) *vs.* regurgitant-treated transgenic plants (RT).

2.6. Statistical Analysis

Data of foliar consumption and larval length of *S. frugiperda* populations were analyzed with a generalized linear model (GLM) with a Gaussian error distribution. The preference of the predator in the olfactometer bioassays was analyzed with binomial tests. Data for plant volatile composition were analyzed by MANOVA and principal components analysis (PCA). The total amount of plant volatiles were analyzed with a generalized linear model (GLM) followed by F test. Statistical analyses were conducted in R software version 3.3.1 (www.R-project.org), except for the MANOVA that was performed in Minitab® Release 14 (Minitab, State College, PA, USA).

3. RESULTS

3.1. Foliar consumption of susceptible and resistant S. frugiperda

Susceptible *S. frugiperda* larvae consumed less tissue of *Bt* maize plants (mean, 0.02 ± 0.005 cm²) than non-*Bt* maize plants (mean, 0.09 ± 0.021 cm²) (GLM, $F_{1, 10} = 10.09$, $P < 0.01$). No significant difference was found in leaf consumption by resistant *S. frugiperda* larvae on *Bt* (mean, 0.17 ± 0.047 cm²) and non-*Bt* maize plants (mean, 0.15 ± 0.038 cm²) ($F_{1, 10} = 0.18$, $P = 0.680$). Susceptible larvae consumed less foliar tissue than resistant larvae on *Bt* maize plants ($F_{1, 10} = 10.93$, $P < 0.01$), but there was no difference in leaf consumed between the populations on non-*Bt* maize plants ($F_{1, 10} = 2.02$, $P = 0.185$) (Figure 1).

Size of the susceptible and the resistant larvae was not affected by the plant genotype ($F_{1, 5} = 2.40$, $P = 0.196$ and $F_{1, 8} = 3.20$, $P = 0.111$, respectively). While no significant difference was found in larvae length comparing the populations on non-*Bt* plants ($F_{1, 7} = 0.18$, $P = 0.685$), resistant larvae on *Bt* maize plants were larger than susceptible larvae ($F_{1, 5} = 6.25$, $P < 0.05$) (Figure 2).

3.2. Volatile Collection and Analysis

Multivariate analysis revealed that the composition of chemical groups of volatiles differed among treatments (MANOVA, Wilk's Criterion, $P < 0.001$).

We observed that undamaged non-*Bt* plants released six volatile compounds: the fatty acid derivatives 3-hexenal and (2*Z*)-2-hexen-1-ol, the benzenoid indole and the monoterpenes β-myrcene, β-linalool and camphor. The volatile profile of undamaged *Bt* plants closely approximated that of undamaged non-*Bt* plants, also released six volatile compounds: the fatty acid derivatives 3-hexenal, the aromatic compound indole and the monoterpenes β-myrcene, β-linalool, camphor and limonene (Table 1). The total amount of constitutive volatiles of *Bt* and non-*Bt* plants was similar (GLM, $P = 0.935$) (Table 2), however, each plant genotype emitted one different compound.

Regurgitant-treated non-*Bt* plants emitted nine volatile compounds: the fatty acid derivatives 3-hexenal and 2-hexenyl acetate, the benzenoid indole, the monoterpenes β-myrcene, β-linalool and camphor, the homoterpenes (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and the sesquiterpene (*E*)-β-farnesene. Regurgitant-treated *Bt* plants emitted the same volatile compounds that regurgitant-treated non-*Bt* plants, except by the fatty acid derivatives that only the compound (2Z)-2-hexen-1-ol was released (Table 1). The total quantity of volatiles

induced by regurgitant-treated *Bt* and non-*Bt* plants was similar ($P = 0.472$). Although there was an increase in the total concentration of volatiles compared to undamaged plants, this increase was not significant ($P = 0.469$) (Table 2).

Herbivore-damaged non-*Bt* plants released a volatile blend of 15 compounds: the fatty acid derivatives 3-hexenal, 2-hexanol, (*Z*)-3-hexen-1-ol, (2*Z*)-2-hexen-1-ol and 2-hexenyl acetate, the benzenoids phenethyl acetate and indole, the monoterpenes β-myrcene, β-ocimene, β-linalool and neryl acetate, the homoterpenes DMNT and TMTT and the sesquiterpenes (*E*)-β-farnesene and α-bergamotene. The composition of volatiles emitted by herbivore-damaged *Bt* plants consisted in the fatty acid derivatives 3-hexenal and (*Z*)-2-hexen-1-al, the benzenoids phenethyl acetate and indole, the monoterpenes β-myrcene, β-linalool and camphor, the homoterpenes DMNT and TMTT and the sesquiterpenes (*E*)-β-farnesene and α-bergamotene, totalizing 11 compounds (Table 1). The total amount of volatiles emitted by herbivore-damaged *Bt* and non-*Bt* plants was similar ($P = 0.939$). There was a significant increase in the total concentration of volatiles compared to undamaged plants and regurgitant-treated plants of isogenic genotype ($P = 0.018$) and transgenic genotype ($P < 0.001$) (Table 2).

Analyses of the volatile composition using PCA showed the separation of herbivore-damaged plants (*Bt* and non-*Bt*) from the other treatments along PC 1, which accounted for 47.8% of the variance (Figure 3). The fatty acid derivatives (2-hexanol, (*Z*)-3-hexen-1-ol and 2-hexenyl acetate) and monoterpenes (β-ocimene and neryl acetate) were positively correlated to the blend emitted by herbivore-damaged non-*Bt* plants, while the volatile emissions of herbivore-damaged *Bt* plants was positively influenced by the fatty acid derivative (*Z*)-2-hexen-1-al and the monoterpenes β-myrcene and camphor.

3.3. Olfactometer Bioassay

The predator *P. nigrispinus* did not discriminate odours from herbivore-damaged *Bt* plants and undamaged *Bt* plants (Figure 4A, Binomial test, $P = 1.000$). However, nymphs of the predator preferred volatiles emitted from herbivore-damaged non-*Bt* plants over undamaged non-*Bt* plants (Figure 4A, $P = 0.028$). When *P. nigrispinus* was given a choice between herbivore-damaged *Bt* plants and herbivore-damaged non-*Bt* plants, the nymphs oriented preferentially to volatiles from non-*Bt* plants (Figure 4B, $P = 0.029$).

The nymphs of *P. nigrispinus* did not show any preference for volatiles of regurgitant-treated *Bt* plants and undamaged *Bt* plants (Figure 4C, $P = 0.405$), as also volatiles of

regurgitant-treated non-*Bt* plants and undamaged non-*Bt* plants (Figure 4C, $P = 0.728$). Similarly, nymphs did not discriminate between regurgitant-treated *Bt* plants and regurgitant-treated non-*Bt* plants (Figure 4D, $P = 0.080$).

4. DISCUSSION

The genetic transformation that occurs on transgenic plants can result in alteration of volatile emissions compared to isogenic plants (Yan et al. 2004, Turlings et al. 2005, Dean and De Moraes 2006, Himanen et al. 2009), and can negatively impact the tritrophic interactions, since that natural enemies use volatile cues to find their host or prey (Agrawal et al. 2002, Dicke et al. 2003).

Here we demonstrated that the total quantity of constitutive volatiles of undamaged plants (*Bt* and non-*Bt*) was similar, and the plants emitted a similar volatile composition according to PCA, despite that blends had a single exclusive component. Moreover, the herbivory by *S. frugiperda* larvae dramatically increased the volatile emission of transgenic and isogenic maize plants compared with undamaged plants. Herbivore-damaged plants (*Bt* and non-*Bt*) emitted almost 7-fold more volatiles than undamaged plants, demonstrating that the concentration of some constitutive volatiles and the blend composition were altered after herbivory on both plant genotypes.

Our results of the olfactometer bioassays showed that nymphs of *P. nigrispinus* prefer the odours of herbivore-damaged isogenic plants over those from undamaged isogenic plants, and this finding is consistent with our GC-MS analyses: herbivore-damaged plants (*Bt* and non-*Bt*) released several new volatiles in higher concentrations. However, the nymphs of the predator did not show preference between herbivore-damaged plants and undamaged plants from transgenic maize, even these herbivore-damaged plants increasing the volatile emissions.

Additionally, when odours from herbivore-damaged isogenic plants and herbivore-damaged transgenic plants were evaluated, another time, the nymphs orientated towards the odours from isogenic plants. The total amount of volatiles on herbivore-damaged plants (*Bt* and non-*Bt*) was similar, however, there were qualitative differences in the volatile blend emitted and five compounds constituted a major proportion of this difference: the fatty acid derivatives 2-hexanol, (*Z*)-3-hexen-1-ol, 2-hexenyl acetate, and the monoterpenes β-ocimene and neryl acetate, emitted only by herbivore-damaged isogenic plants. Thus, these qualitative

differences may explain the attractiveness of the predator *P. nigrispinus* to volatile blends emitted from these herbivore-damaged plants from non-*Bt* maize.

It is well-known that plants under attack by herbivores often induce volatiles that may orient natural enemies to these plants and protect them from herbivore damage (Agrawal et al. 2002, Dicke et al. 2003) and thus, HIPVs are highly attractive to natural enemies because indirectly indicate the presence of the herbivore. However, in our study, the predator *P. nigrispinus* was able to distinguish these herbivore-induced volatiles only from non-*Bt* plants, suggesting a possible effect of genetic transformation of the *Bt* maize on volatile emissions.

A previous study indicated that non-*Bt* maize plants emitted significantly more volatiles than *Bt* plants as a result of intensive larval damage on non-*Bt* plants and reduction of larval feeding on *Bt* plants due to *Bt* protein toxicity to *Bt*-susceptible herbivore (Dean and De Moraes 2006). A way to avoid this methodological problem, in our experiments, was control the leaf damage using a *Bt*-resistant population of *S. frugiperda* that was insensitive to *Bt* toxin and could normally feed on *Bt* maize plants. We showed that the laboratory population of *S. frugiperda* fed on artificial diet, and thus without selection pressure exerted by *Bt* maize, consumed about 5-fold less foliar tissue of *Bt* plants than non-*Bt* plants. While the foliar consumption of the *Bt*-resistant *S. frugiperda* was equal on *Bt* and non-*Bt* plants, avoiding possible changes in the herbivore-induced volatile profiles due to larval feeding behavior. Similar results were reported by Schuler et al. (1999a, 2003), who found a similar feeding damage by *Bt*-resistant larvae of *Plutella xylostella* (Lepidoptera: Plutellidae) on *Bt* and non-*Bt* leaves of oilseed rape.

Another way to standardize the leaf damage is to use artificial damage of the leaves and posterior application of larval regurgitant (Turlings et al. 1998, Turlings et al. 2005). The volatile profiles induced by regurgitant-treated plants are generally similar to those from herbivore-damaged plants and the method is considered efficient to induce plants (Degen et al. 2004, Turlings et al. 2005).

In our experiments, the volatile emissions of regurgitant-treated plants differ qualitatively but not quantitatively from undamaged plants on both plant genotypes; few new volatiles at low to moderate concentrations were induced by regurgitant-treated plants. The results from olfactometer bioassays showed that nymphs of *P. nigrispinus* did not distinguish among the odours of undamaged and regurgitant-treated plants of transgenic and isogenic plants. Moreover, the volatile profiles of regurgitant-treated plants (*Bt* and non-*Bt*) differed quantitatively and qualitatively from herbivore-damaged plants; more volatiles in greater quantities were emitted to those herbivore-damaged. A possible explanation for this

difference is that the treatment with larval regurgitant was inefficient to reproduce the real herbivory represented by herbivore-damaged plants. A lack of attractiveness of the predator by induced isogenic plants may be explained because many volatile compounds, such as some fatty acid derivatives and terpenes compounds were not released by regurgitant-treated isogenic plants. Others studies also showed a weak induction of fatty acid derivatives and terpenes by regurgitant-treated plants when compared with herbivore-damaged plants (Turlings et al. 1998, Dean and De Moraes 2006), and these compounds appear to be determinant in the attraction of the predator *P. nigrispinus* to herbivore-damaged isogenic plants.

In contrast to our results, previous studies showed that when the foliar damage was manipulated to produce a same damage level, *Bt* maize plants and its non-*Bt* lines emitted a similar volatile profiles (Dean and De Moraes 2006) or presented only quantitative differences (Turlings et al. 2005). However, in our study, qualitative differences appear to be responsible for the response of the predator to herbivore-damaged isogenic plants.

Specific plant volatile may be responsible for attracting natural enemies, and the identification of bioactive compounds in the volatile blends requires a combination of behavioral and chemical studies (D'Alessandro and Turlings 2006). For example, it is known that fatty acid derivatives are important volatiles in the orientation of generalist natural enemies (Maeda et al. 2015, Naranjo-Guevara et al. 2017). Electroantennograms studies showed that the predator *P. nigrispinus* is sensitive to fatty acid derivatives and other plant volatiles that have the same or closely related compounds of the aggregation pheromone of the predator (Sant'ana and Dickens 1998). Thus, these compounds may be used as cues for locate potential prey and for reproductive behaviors (Sant'ana et al. 1999).

The alteration of volatile profiles on *Bt* plants may result in lack of attractiveness by natural enemies to these plants, as occurred in our behavioral trials, and negatively impact on biological control by beneficial organisms. Turlings et al. (2005) showed that *Bt* and non-*Bt* maize plants emitted the same 11 volatiles after induction with larval regurgitant, seven compounds were quantitatively more emitted by non-*B*t plants. However, the reduced volatile emissions by *Bt* plants did not affect the recruitment of two beneficial parasitoids. In our study, even herbivore-damaged *Bt* plants emitting greater quantities of volatiles compared with undamaged *Bt* plants, the predator *P. nigrispinus* was unable to discriminate between these plants. However, the predator showed a clear preference for herbivore-damaged non-*Bt* plants that presented higher amounts of volatiles compared with undamaged non-*Bt* plants and some exclusive fatty acid derivatives and monoterpenes compared with herbivore-damaged *Bt*

plants, and likely this preference is mediated by a specific mixture of volatiles or by individual compounds (Ishiwari et al. 2007, Maeda et al. 2015). Future studies may unravel the role of the exclusive compounds of herbivore-damaged non-*Bt* maize blend on the predator's recruitment.

Importantly, our study show that genetic modification with Cry1F gene can result in variation of volatile emissions between *Bt* and non-*Bt* maize plants, and qualitative differences in the odours emitted by *Bt* and non-*Bt* plants appear to be reflected in the responses of the predator. This variation can be explained by the changes in resource allocation and investment for *Bt* production that can occur on transgenic plants (Turlings et al. 2005, Himanen et al. 2009) and/or due to unintended pleotropic effects of genetic modification of plants (Schuler et al. 1999b, Haslberger 2003, Cellini et al. 2004). To the best of our knowledge, this is the first example of qualitative differences on volatile profiles between *Bt* and non-*Bt* maize plants with effects on the foraging behavior of a natural enemy.

To conclude, our findings suggest that the use of *Bt* maize may incur in problems in integrated pest management, such as negative impacts on the efficiency of biological control agents in finding prey or host, because of the reduced or lack of attractiveness of HIPVs emitted by *Bt* plants. Although the main purpose of *Bt* crops is not to be damaged by pests, *Bt* plants are subject to be damaged by resistant pests. Therefore, the recruitment of natural enemies through of volatiles emission by *Bt* plants can be compromised, translating into consequences to pest control by these beneficial organisms that could suppress the evolution of pest resistance and ensure the life-spam of *Bt* technology.

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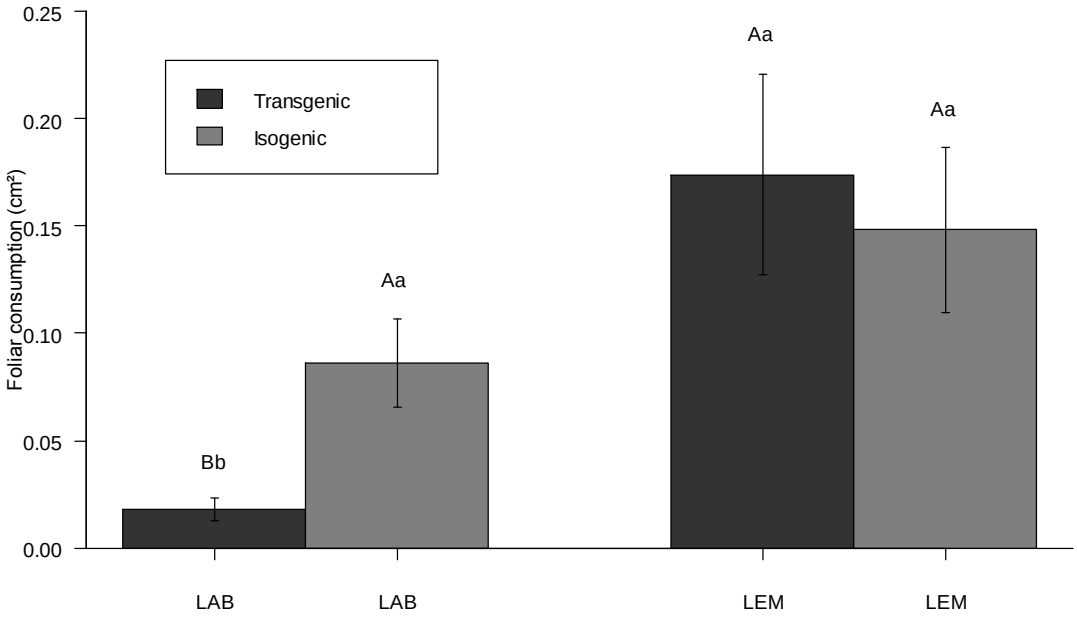

Figure 1: Foliar consumption of *Spodoptera frugiperda* larvae (LAB: *Bt*-susceptible population, LEM: *Bt*-resistant population) exposed to *Bt* Cry1F maize (transgenic) and non-*Bt* maize (isogenic). Means (± SE) followed by different letters are significantly different (contrast among populations/treatments after a GLM). Uppercase letters compare the populations in each treatment and lowercase letters compare the treatments in each population.

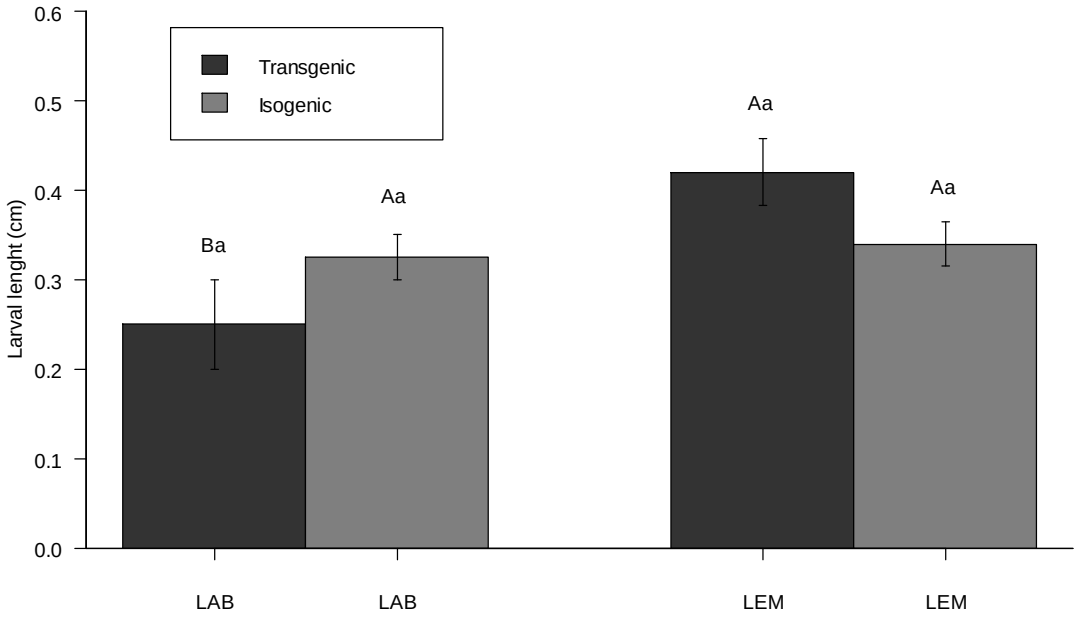

Figure 2: Larval length of *Spodoptera frugiperda* populations (LAB: *Bt*-susceptible population, LEM: *Bt*-resistant population) exposed to *Bt* Cry1F maize (transgenic) and non-*Bt* maize (isogenic). Means (± SE) followed by different letters were significantly different (contrast among populations/treatments after a GLM). Uppercase letters compare the populations in each treatment and lowercase letters compare the treatments in each population.

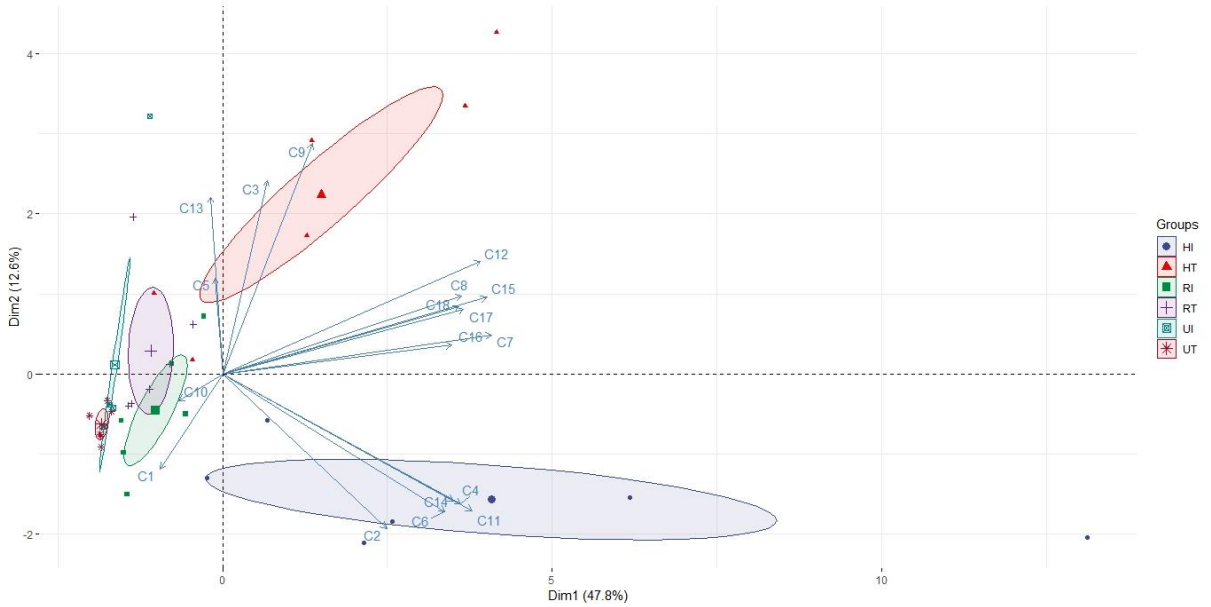

Figure 3: Score plot for principal component analysis (PCA) for the composition of volatiles emitted by HI: isogenic plants damaged by *Bt*-resistant larvae of *Spodoptera frugiperda*, HT: transgenic plants damaged by *Bt*-resistant larvae of *S. frugiperda*, RI: regurgitant-treated isogenic plants, RT: regurgitant-treated transgenic plants, UI: undamaged isogenic plants and UT: undamaged transgenic plants. Volatile compounds are represented by the vectors: C1: 3-hexanal, C2: 2-hexanol, C3: (*Z*)-2-hexen-1-al, C4: (*Z*)-3-hexen-1-ol, C5: (2*Z*)-2-hexen-1-ol, C6: 2-hexenyl acetate, C7: phenethyl acetate, C8: indole, C9: β-myrcene, C10: limonene, C11: β-ocimene, C12: β-linalool, C13: camphor, C14: neryl acetate, C15: (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) , C16: (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), C17: α-bergamotene, and C18: (*E*)-β-farnesene.

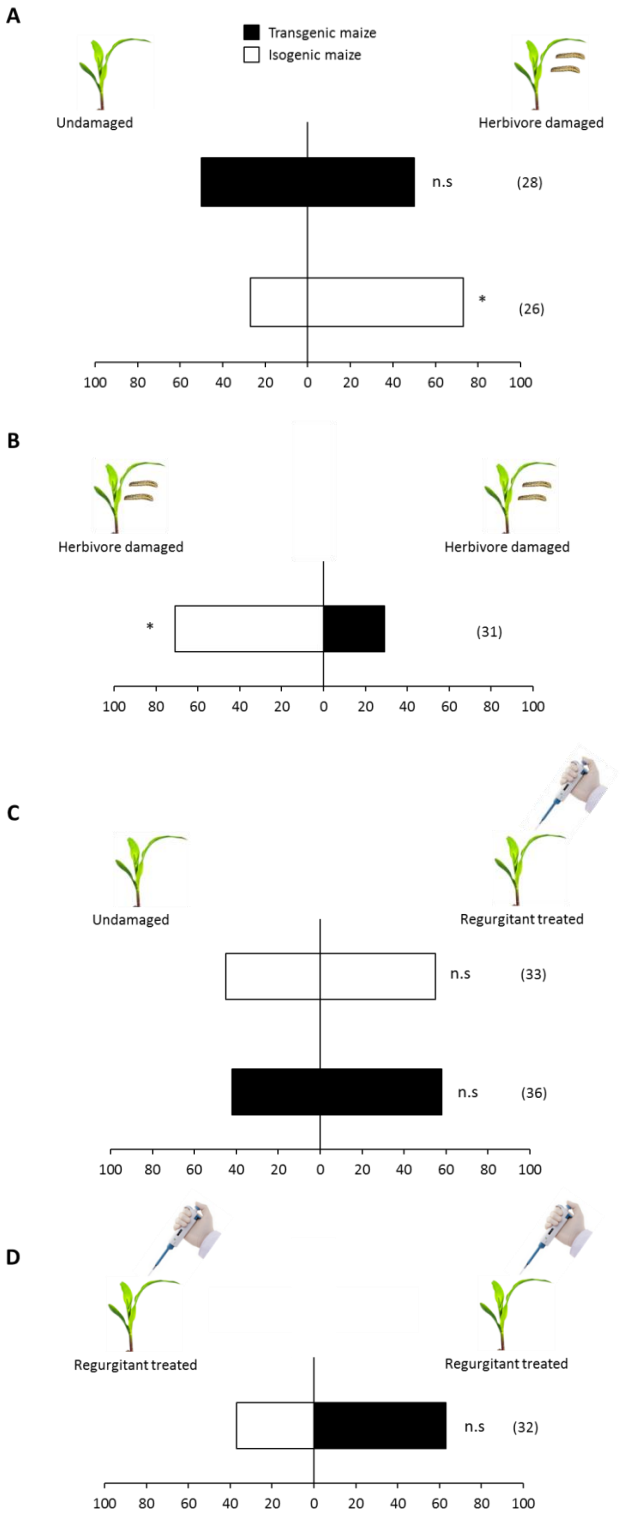

Figure 4: Olfactory preference of *Podisus nigrispinus* nymphs to undamaged plants, plants damaged by *Bt*-resistant larvae of *Spodoptera frugiperda* and regurgitant-treated plants of transgenic maize (dark bars) and isogenic maize (white bars). Forty nymphs were used in each test and the numbers of insects that responded are indicated within parentheses. Asterisks indicate significant difference between responses within a trial (Binomial test, $P \leq 0.05$), n.s = not significant.

Table 1: Individual volatile compounds (ng g^{-1} dry plant tissue) released by undamaged plants, plants damaged by *Bt*-resistant larvae of *Spodoptera frugiperda* and regurgitant-treated plants of transgenic and isogenic maize.

Compounds	Undamaged plants		Herbivore-damaged plants		Regurgitant-treated plants	
	Isogenic	Transgenic	Isogenic	Transgenic	Isogenic	Transgenic
Fatty acid derivatives						
3-hexenal	247.46 ± 73.78	382.50 ± 73.89	170.86 ± 43.75	272.31 ± 115.60	761.26 ± 306.14	-
2-hexanol	-	-	59.06 ± 15.38	-	-	-
(*Z*)-2-hexen-1-al	-	-	-	174.23 ± 54.84	-	-
(*Z*)-3-hexen-1-ol	-	-	403.65 ± 179.48	-	-	-
(2*Z*)-2-hexen-1-ol	162.94 ± 90.87	-	61.83 ± 16.94	-	-	107.08 ± 25.98
2-hexenyl acetate	-	-	266.93 ± 114.23	-	113.42 ± 31.04	-
Total Fatty acid derivatives	410.40 ± 164.65	382.50 ± 73.89	962.34 ± 369.78	446.54 ± 170.44	874.68 ± 337.18	107.08 ± 25.98
Benzenoids						
phenethyl acetate	-	-	391.19 ± 225.45	333.08 ± 108.26	-	-
indole	135.35 ± 47.25	90.16 ± 39.47	1996.53 ± 1386.34	2462.38 ± 1000.31	436.85 ± 235.50	286.03 ± 110.47
Total Benzenoids	135.35 ± 47.25	90.16 ± 39.47	2387.73 ± 1611.79	2795.46 ± 1108.57	436.85 ± 235.50	286.03 ± 110.47
Monoterpenes						
β-myrcene	184.56 ± 111.00	83.07 ± 14.00	130.16 ± 26.42	222.12 ± 52.53	104.16 ± 18.01	102.15 ± 19.98
limonene	-	209.92 ± 141.45	-	-	-	-
β-ocimene	-	-	125.14 ± 47.83	-	-	-
β-linalool	600.77 ± 105.90	502.59 ± 67.74	2586.67 ± 1026.79	2874.13 ± 783.45	1160.72 ± 311.72	946.24 ± 230.19
camphor	214.71 ± 142.30	317.57 ± 120.12	-	712.88 ± 220.49	782.80 ± 484.18	908.09 ± 776.63
neryl acetate	-	-	130.23 ± 46.50	-	-	-
Total Monoterpenes	1000.04 ± 359.20	1113.16 ± 343.31	2972.19 ± 1147.54	3809.13 ± 1056.47	2047.68 ± 795.90	1956.48 ± 1006.82
Homoterpenes						
DMNT	-	-	1958.24 ± 668.09	1906.38 ± 502.66	353.24 ± 157.48	294.51 ± 163.30
TMTT	-	-	484.42 ± 181.04	409.96 ± 129.09	309.38 ± 50.56	399.01 ± 84.21
Total Homoterpenes	-	-	2442.66 ± 849.13	2316.34 ± 631.75	662.62 ± 208.04	693.52 ± 247.51
Sesquiterpenes						
α-bergamotene	-	-	495.13 ± 129.70	416.99 ± 128.27	-	-
(*E*)-β-farnesene	-	-	1335.32 ± 387.51	1197.25 ± 337.84	86.93 ± 18.88	86.03 ± 18.74

Total Sesquiterpenes	-	-	1830.46 ± 517.21	1614.24 ± 466.11	86.93 ± 18.88	86.03 ± 18.74

Table 2: Total quantity of volatiles (ng g^{-1} dry plant tissue) released by undamaged plants, plants damaged by *Bt*-resistant larvae of *Spodoptera frugiperda* and regurgitant-treated plants of transgenic and isogenic maize.

Treatment	Genotype	Total volatiles	*P* value
Undamaged	Isogenic	1545.79 ± 382.74	0.935
	Transgenic	1585.82 ± 282.15	
Herbivore-damaged	Isogenic	10595.37 ± 4102.37	0.939
	Transgenic	10981.71 ± 2733.80	
Regurgitant-treated	Isogenic	4108.76 ± 945.2285	0.472
	Transgenic	3129.13 ± 908.4743	

P values (GLM, test *F*) compared plant genotypes in each treatment.