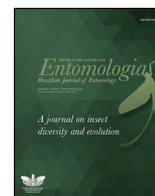




SOCIEDADE BRASILEIRA  
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Biological Control and Crop Protection

## Preference of *Neoseiulus californicus* (Acari: Phytoseiidae) for volatiles of Bt maize induced by multiple herbivory



Jéssica Mayara Coffler Botti , Mayara Loss Franzin ,  
Marcos Antonio Matiello Fadini \*, Júlio Onésio Ferreira Melo 

Universidade Federal de São João Del Rei, Campus Sete Lagoas, Sete Lagoas, MG, Brazil

### ARTICLE INFO

#### Article history:

Received 9 April 2019

Accepted 23 September 2019

Available online 17 October 2019

Associate Editor: Mariane A. Nickle

#### Keywords:

Indirect induced defense  
*Tetranychus urticae*  
Secondary plant compounds  
Plant resistance  
Fall armyworm

### ABSTRACT

Plant indirect induced defenses against herbivores are characterized by the production of plant volatiles that to attract natural enemies. The objective of this study was to evaluate whether the attack of the two-spotted spider mite *Tetranychus urticae* or the multiple herbivory of *T. urticae* together with the fall armyworm *Spodoptera frugiperda* are able to elicit indirect induced defense in conventional and Bt maize plants. The experiment was carried out in the laboratory using Y-tube olfactometer, evaluating the predatory mite *Neoseiulus californicus* olfactory preference for plant volatiles. The treatments involved: Clean Conventional Plant; Conventional Plant Infested with *T. urticae*; Clean Bt Plant; Bt Plant Infested with *T. urticae*; Conventional Plant Infested with *T. urticae* + fall armyworm; Bt Plant Infested with *T. urticae* + fall armyworm. For the chemical analyzes the Trace GC Ultra gas chromatograph was used, paired with the Polaris Q mass spectrometer, GC–MS system. *Neoseiulus californicus* was also unable to distinguish between volatiles from both conventional and Bt infested maize plants. Moreover, there was no discrimination of the predator mite between plants under single and multiple infestations, both in conventional and Bt maize. When comparing conventional and Bt plants, both with multiple infestation, the predator mite *N. californicus* had no preference among these sources of odors. However, there was observed chemical changes of the volatiles among the groups of plants studied. Thus, it is suggested that the groups of plants under study have chemical modifications, but they are not able to attract *N. californicus*. In addition, Bt plants response was similar to conventional plants on attracting *N. californicus*.

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### Introduction

The plants developed a diversity of defense mechanisms against different conditions, such as herbivorous attack (Bergelson et al., 2001). A mechanism against herbivores is the indirect induced defense, characterized by the production of plant volatiles after herbivory that are able to attract natural enemies of herbivores (Arimura et al., 2005; Schaller, 2008).

In addition to induced defenses, plants have constitutive defenses that are expressed continuously and independent of herbivorous attack (Price et al., 1980). One example is Bt plants, since they continuously express the defense protein. For example, 30F35 Hx maize expresses the Cry1F protein that confers lepidopteran resistance. However, not only the target herbivores are exposed to toxins, non-target herbivores and their natural enemies are

consequently exposed (Torres and Ruberson, 2008). Although the use of genetically modified plants has advantages (Kouser and Qaim, 2011), the impact of these plants on the foraging behavior of natural enemies has been questioned (Poppy and Sutherland, 2004). Hence, it is important to understand the influence of Bt plant proteins on non-target organisms.

Further, the cultivation of Bt plants promotes a decrease in the use of insecticides (Cattaneo et al., 2006). In this way, species of non-target pests of lesser economic importance, such as *Tetranychus urticae* (Koch) (Acari: Tetranychidae) in maize, may become more important, because the lower use of insecticides applied to target pests may increase their population.

Therefore, it is important to understand if Bt-based corn plants are capable able of releasing volatiles to attract the natural enemies of *T. urticae*. This questioning is performed because indirect

\* Corresponding author at: Universidade Federal de São João Del Rei. MG 424, km 47. Sete Lagoas, MG, 35701-970, Brazil.

E-mails: [jessicabotti@hotmail.com](mailto:jessicabotti@hotmail.com) (J.M. Botti), [mayarafranzin@gmail.com](mailto:mayarafranzin@gmail.com) (M.L. Franzin), [fadini@ufsj.edu.br](mailto:fadini@ufsj.edu.br) (M.A. Fadini), [onesiomelo@gmail.com](mailto:onesiomelo@gmail.com) (J.O. Melo).

induced defense has costs for the plants (Steppuhn and Baldwin, 2008). To trigger this defense, the plant needs to allocate physiological and ecological resources, which can affect relevant processes of the plant, such as growth and reproduction (Steppuhn and Baldwin, 2008).

A second concern is the interaction among different types of plant defenses mechanisms (Kant et al., 2015; Price et al., 1980). Studies have demonstrated significant differences in the amount of volatiles emitted between Bt and non-Bt maize plants (Dean and De Moraes, 2006; Turlings et al., 2005). This shows that plants with constitutive defenses (e.g. Bt) can alter the production of volatiles and, consequently, the attraction of natural enemies. This suggests a negative interaction between these defenses (Shimoda, 2010).

Furthermore, another factor that can alter induced defense is the multiple attacks of herbivore species, affecting preference and performance of herbivores and of their natural enemies (Dicke et al., 2009). In nature, plants are not exposed to a single herbivore. The attack of multiple herbivores can alter the emission of compounds, both positively and negatively in the attractiveness of natural enemies (Kaplan et al., 2009; Rodriguez-Saona et al., 2005; Viswanathan et al., 2005). This work evaluated whether both conventional and Bt maize plants induce the production of volatile compounds attractive to *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) after herbivory of *T. urticae*. And if conventional and Bt maize plants induced by simple (*T. urticae*) and multiple herbivory (*T. urticae* + fall armyworm), produce volatile compounds attractive to the predatory mite *N. californicus*.

## Materials and methods

### Rearing of two-spotted spider mite, fall armyworm and predatory mite

Infested leaves of Sorghum plants with *T. urticae* were collected in the greenhouse at Embrapa Milho e Sorgo facilities, and taken to the Entomology Laboratories of the Universidade Federal de São João Del-Rei (UFSJ), in Sete Lagoas, Minas Gerais, Brazil. To establish the colony, adult mites were collected, individually using a stereomicroscope and a hairs brush, then transferred to jack bean plants (*Canavalia ensiformis*) with two completely expanded cotyledon leaves and without any phytosanitary treatment.

The bean plants used were planted in 1.0L plastic pots using Terral Solo® substrate. Infested plants were kept isolated in screened cages in order to prevent external infestation with other herbivores. Irrigation was done whenever necessary. Clean jack bean plants were added to the rearing colony when needed. The *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) was acquired from rearing colony at Embrapa Milho e Sorgo. The predatory mite, *N. californicus*, was acquired from the Koppert company.

### Planting of maize seeds

The maize seeds used for planting were the commercial hybrids Pioneer® 30F35 (conventional maize) and Pioneer® 30F35 Hx (Bt maize). Two seeds were sown in plastic pots with a volume of 1.0L, using Terral Solo® substrate and kept in a greenhouse. After 15 days of sowing, thinning and fertilization were provided with 0.2 g of ammonium sulphate per pot. The maize plants were split into 3 groups: clean plants, plants infested with *T. urticae* and plants infested simultaneously by *T. urticae* and fall armyworm. For *T. urticae* infestation, 20 days after sowing, 10 females were on one leaf, using entomological glue. The glue was applied at the leaf base, not allowing the females to move to the rest of the plant.

The group of plants infested with *T. urticae* + fall armyworm, besides the infestation of the mite that was identical to the previous group, a third instar larvae was isolated with a “clip cage”. This was carried out on a different leaf of *T. urticae* infestation, also 20 days after sowing.

### Olfactometer test

Plants after 48 h of infestation were used to carry out the tests in Y-tube olfactometer, with the following treatments: Clean Conventional Plant X Conventional Plant Infested with *T. urticae*; Clean Bt Plant X Bt Plant Infested with *T. urticae*; Conventional Plant Infested with *T. urticae* X Bt Plant Infested with *T. urticae*; Conventional Plant Infested with *T. urticae* X Conventional Plant Infested with *T. urticae* + fall armyworm; Plant Bt Infested with *T. urticae* X Bt Plant Infested with *T. urticae* + fall armyworm; Conventional Plant Infested with *T. urticae* + fall armyworm X Plant Bt Infested with *T. urticae* + fall armyworm. Each treatment consisted of three true replicates (i.e. odor sources) then 15 responses of predatory mites were obtained from each replicate, totaling 45 releases of predatory mites per treatment.

A Y-tube olfactometer was used to study the response of *N. californicus*. The glass Y-Tube (with dimensions of 21.0 cm of length of each arm and 3.5 cm of diameter) had a Y-shaped metal wire inside to facilitate the movement of mites. Containers consisting of a plastic box were attached to the Y-tube both arms. The Y-tube base was connected to a vacuum pump that directed the flow from the containers to the Y-tube arms. A flowmeter was connected to the containers air outlet and was used to calibrate the air inlet speed in each olfactometer arm. The wind speed was 0.50 m/s on each Y-tube arm. The predators were individually introduced at the starting point on the steel wire and then observed until a predator reached 1/3 of an arm, where the mite response was considered. Predatory mites who did not pass 1/3 of either arm within 5 min were accounted unresponsive. After every five responses, the odor sources positions were reversed.

### Chemical analysis of plant volatiles

For chemical analysis, a leaf sample of 0.250 g of each plant was taken. The chemical analysis was performed using solid phase microextraction (SPME) in headspace mode and Trace GC Ultra gas chromatograph (ThermoScientific, San Jose, CA) paired with the Polaris Q mass spectrometer (ThermoScientific, San Jose, CA), GC-MS system, with an ion-trap type analyzer (Garcia et al., 2016). For this analysis a true replicate was drawn on which a leaf sample (0.250 g) was withdrawn from each plant, which were transferred into a headspace flask (25 mL) and macerated with a glass stick. The flasks were sealed with aluminum seal and rubber septum and left in the freezer at  $-18^{\circ}\text{C}$  until analysis. Each sample was heated for 5 min at  $65^{\circ}\text{C}$  before exposure of the solid phase microextraction fiber (SPME). Then, above the macerated maize leaf, the semi-polar polydimethylsiloxane / divinylbenzene (PDMS / DVB) 65  $\mu\text{m}$  fiber was exposed to the gas phase in the headspace flasks at  $65^{\circ}\text{C}$  for 20 min. After exposure of the PDMS / DVB fiber with the sample, the same was left inserted in the injector of the chromatograph at  $200^{\circ}\text{C}$ , for 5 min, for desorption of the volatiles collected.

The conditions for the gas chromatograph are injector temperature,  $200^{\circ}\text{C}$ ; injection in splitless mode; splitless time, 5 min; ion source temperature,  $200^{\circ}\text{C}$ ; interface temperature,  $275^{\circ}\text{C}$ ; programmed temperature heating ( $40^{\circ}\text{C}$  for 2 min, then a gradient of  $8^{\circ}\text{C} / \text{min}$  to  $100^{\circ}\text{C}$ , at which temperature the isotherm was maintained for 2 min and then  $15^{\circ}\text{C} / \text{min}$  to  $180^{\circ}\text{C}$ , at which temperature it maintained the isotherm for 2 min, and finally a gradient of  $15^{\circ}\text{C} / \text{min}$  to  $245^{\circ}\text{C}$ , at which temperature the isotherm was maintained for 3 min). The detector was kept in

scan mode (full scan, from 30 to 300), using the electron-impact ionization (EI) technique, with energy of 70 eV. The chromatographic column used was HP-5 MS capillary column (5% phenyl and 95% methylpolysiloxane), with the following dimensions: length (30 m), internal diameter (0.25 mm) and film thickness (0.25  $\mu\text{m}$ ) (Agilent Technologies INC, Germany), specific for mass spectrometry analysis. The components identification was performed by comparing the mass spectra present in the NIST / EPA / NIH (2005) libraries.

### Statistical analyzes

For the olfactometer tests, the statistical design was completely randomized, with 6 treatments and 3 replicates per treatment.

For the chemical data, the retention time and peak intensity values were taken from the ThermoElectron Corporation Xcalibur 1.4 software. The peaks were then selected by cutting nine compounds of larger area in each chromatogram. A compound was related to each retention time based on mass spectra and retention indexes. A table was created with samples in the rows and compounds in the columns and inside the matrix were placed the relative intensities of the peaks, obtained in the chromatographic analysis. The data were assessed by analysis of the main PCA components (Crawley, 2013).

Predatory mite behavior data on olfactometer were submitted to chi-square tests for categorical data (Crawley, 2013). Predatory mites that did not choose one of the olfactometer arms after five minutes were not considered for analysis. The R (R Development Core Team 2014) software was used to perform the statistical analyzes and plot the graphs.

## Results

### Olfactory preference of *N. Californicus*

Predatory mites did not discriminate between odors from non-infested and infested maize plants, both in conventional and Bt

plants. The *N. californicus* was also unable to distinguish between the odors from infested conventional and Bt maize plants (Fig. 1).

There was no preference of *N. californicus* predatory mites among the volatiles of *T. urticae* infested maize plants versus *T. urticae* + *S. frugiperda* multiple infestation, both in conventional maize plants and in Bt plants. The same occurred in the preference of volatiles of plants of conventional and Bt maize on multiple infestation (mite + fall armyworm) (Fig. 2).

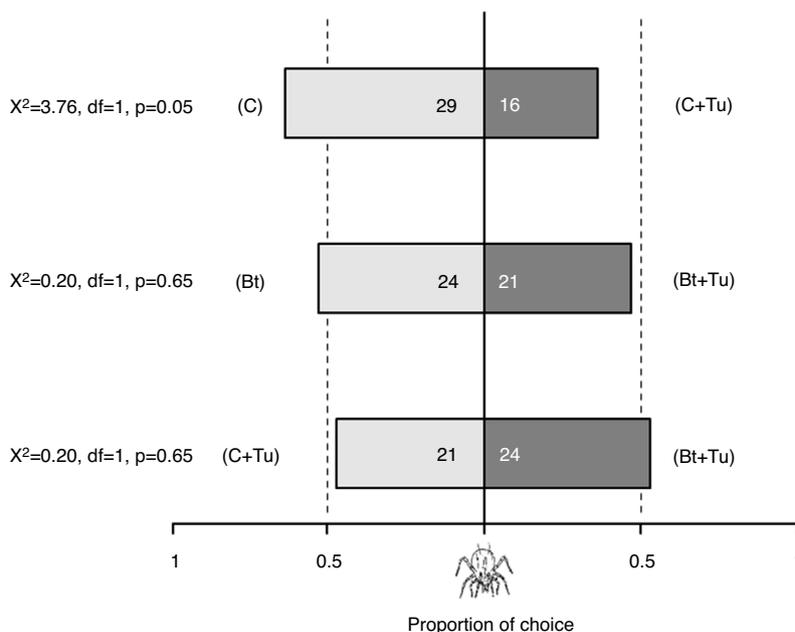
### Chemical parameters

For the analysis of maize leaves volatile chemical profile, nine compounds with the highest amount in each sample were selected. In clean plants versus infestation with only *T. urticae* 15 volatile compounds were found, 11 of which were pre-identified belonging to the *terpenoid* group (3 monoterpenes and 1 sesquiterpene), 3 aldehydes, 1 ether, 1 ketone, 1 alcohol and 1 ester (Fig. 3). In the simple versus multiple infestations, 13 compounds were found in the four groups of plants, of which 10 were pre-identified as 3 monoterpenes, 3 aldehydes, 1 ether, 1 ketone, 1 alcohol and 1 ester (Fig. 4).

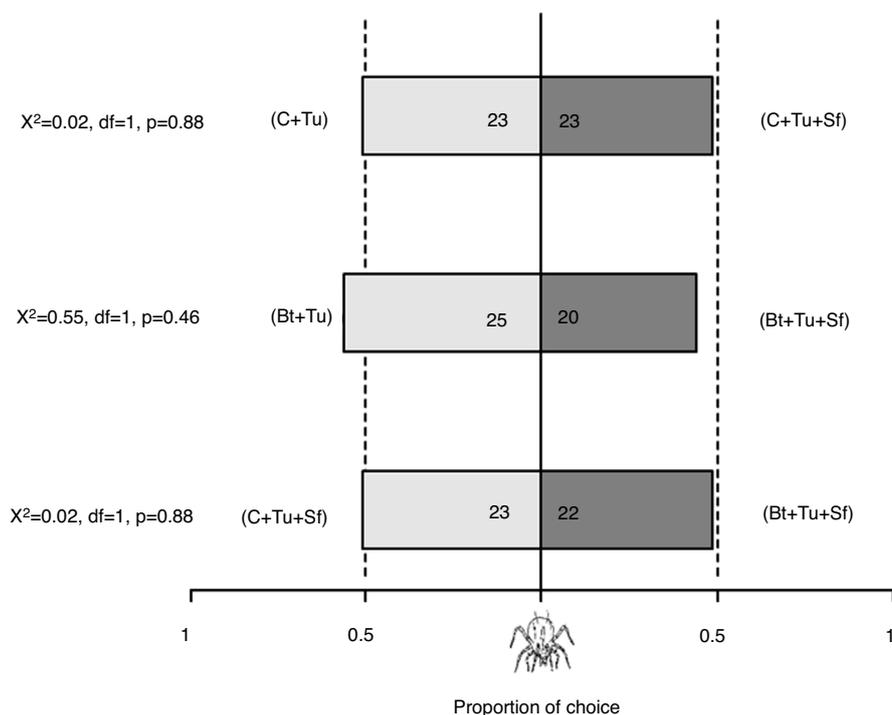
The principal component analysis of volatile composition in conventional and Bt leaves, cleaned and infested with *T. urticae* explained 65.0% of the variation (Fig. 5). Principal component 1 separated the clean Bt plants from the others. The principal component 2 separated the infested Bt plants from the rest. The principal component analysis (PCA) of volatile composition of conventional and Bt maize leaves, with single and multiple infestations, explained 49.8% of the variation (Fig. 6). Principal component 1 separated conventional plants with single infestation from Bt with single infestation. However, the main component 2 did not separate the groups of plants.

## Discussion

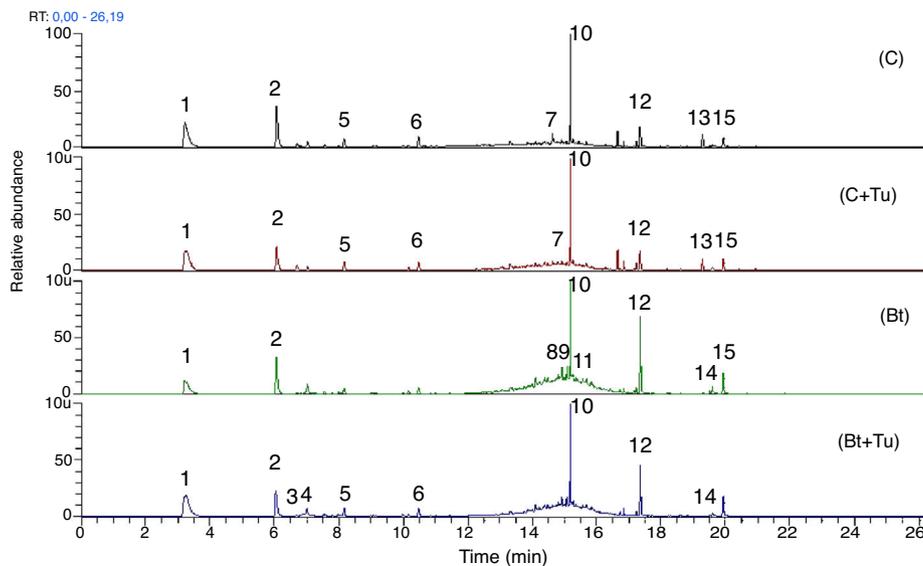
The mite did not distinguish among the odors from infested and uninfested plants by *T. urticae*, both conventional and Bt. It



**Fig. 1.** Predatory mite *Neoseiulus californicus* preference for volatile compounds in two-choice olfactometer tests, comparing: conventional maize plants uninfested (C); conventional maize plants infested by *Tetranychus urticae* (C+Tu); Bt maize plants uninfested (Bt); and Bt maize plants infested by *T. urticae* (Bt+Tu); Numbers in bars represent individual predatory mites that choose the indicated odor. No significant  $p \geq 0.05$ .



**Fig. 2.** Predatory mite *Neoseiulus californicus* preference for volatile compounds in two-choice olfactometer tests, comparing: conventional maize plants infested by *Tetranychus urticae* (C + Tu); conventional maize plants infested by *Tetranychus urticae* and *Spodoptera frugiperda* (C + Tu + Sf); Bt maize plants infested by *T. urticae* (Bt + Tu); Bt maize plants infested by *T. urticae* and *S. frugiperda* (Bt + Tu + Sf). Numbers in bars represent individual predatory mites that choose the indicated odor. No significant  $p \geq 0.05$ .



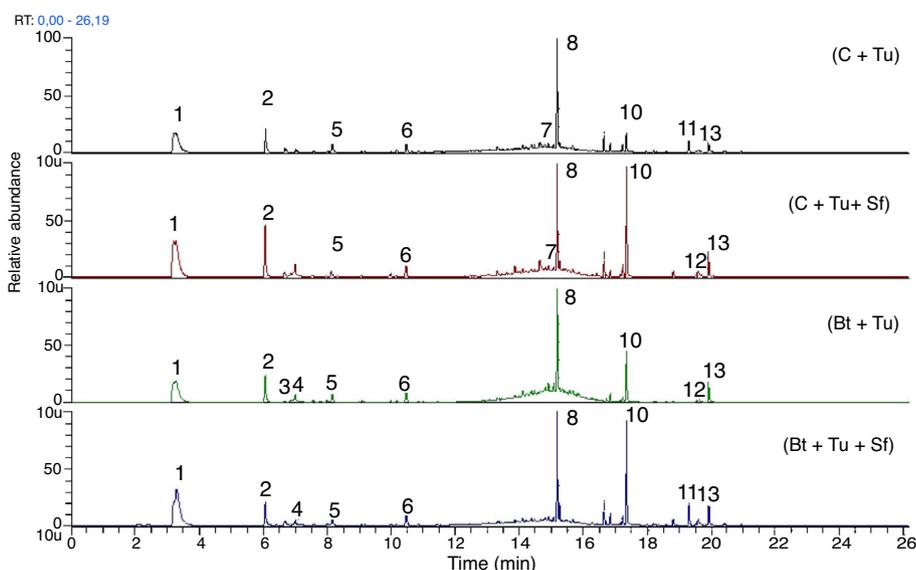
**Fig. 3.** Chromatograms of volatile compounds extracted from leaves of conventional maize plants unfested (C); conventional maize plants infested by *Tetranychus urticae* (C + Tu); Bt maize plants unfested (Bt); and Bt maize plants infested by *T. urticae* (Bt + Tu). The compounds observed in analysis were 1 = (E)-Hex-2-enal; 2 = heptanal; 3 = (E)-Oct-2-enal; 4 =  $\alpha$ -pineno; 5 =  $\beta$ -cisocimeno; 6 =  $\beta$ -Ciclocitral; 7 = 1-metil-6-(3-metilbuta-1,3-dienil)-7-oxabicyclo[4,1,0]heptano; 8 = no identified = C8; 9 = no identified = C9; 10 =  $\beta$ -ionona; 11 = Ciclosativena; 12 = (E)-7-tetradecen-1-ol; 13 = no identified = C13; 14 = Linolenic acid ethyl ester; 15 = no identified = C15.

did not distinguish between odors from conventional infested and unfested plants with *T. urticae*.

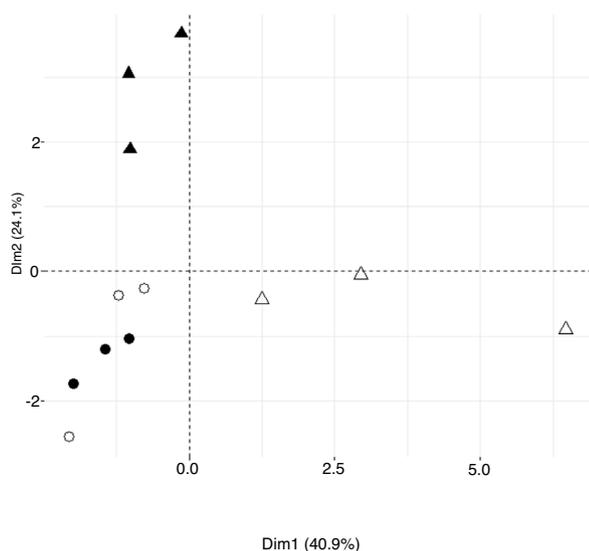
Studies have shown that plants infested with *T. urticae* produce volatiles capable of attracting natural enemies. One example was the attraction of the predatory mite *N. californicus* to bean plants infested with *T. urticae* (Shimoda et al., 2005). This shows that the same herbivore may or may not trigger defenses on different plants. In addition, Gardiner et al. (2005) studied *Humulus lupulus* (L.) (Rosales: Cannabaceae) hops plants infested by *T. urticae* versus clean plants, verified that in the same plant differentiation in the pref-

erence of distinct predators can occur. Where the predatory mite *Neoseiulus fallacis* (Garman) (Acari: Phytoseiidae) was attracted by infested plants, but the predatory mite *Galendromus occidentalis* (Nesbitt) (Acari: Phytoseiidae) was not. This allows further research on the foraging of other natural enemies by *T. urticae* infested maize plants.

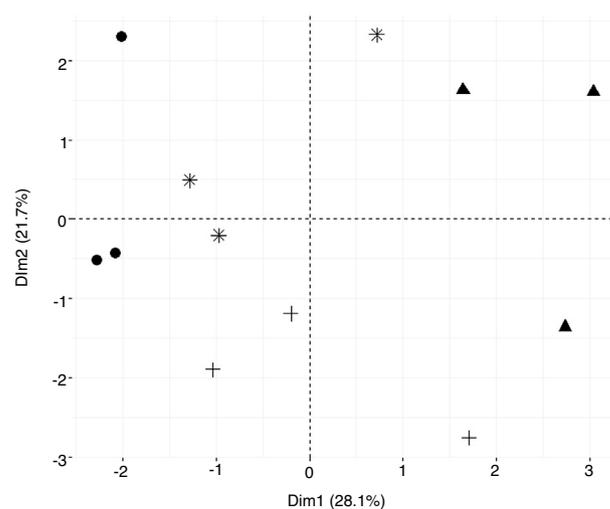
The cultivar is another factor that is correlated with indirect defense induction (Dicke et al., 1990). Tahmasebi et al. (2014) studied four bean cultivars infested with *T. urticae* and observed that only the cultivars Naz and G11867 were able to attract the



**Fig. 4.** Chromatograms of volatile compounds extracted from leaves of conventional maize plants infested by *Tetranychus urticae* (C + Tu); conventional maize plants infested by *Tetranychus urticae* and *Spodoptera frugiperda* (C + Tu + Sf = +); Bt maize plants infested by *T. urticae* (Bt + Tu); Bt maize plants infested by *T. urticae* and *S. frugiperda* (Bt + Tu + Sf). The compounds observed in analysis were 1 = (*E*)-Hex-2-enal; 2 = heptanal; 3 = (*E*)-Oct-2-enal; 4 =  $\alpha$ -pineno; 5 =  $\beta$ -cisocimeno; 6 =  $\beta$ -Ciclocitral; 7 = 1-metil-6-(3-metilbuta-1,3-dienil)-7-oxabiciclo[4,1,0]heptano; 8 =  $\beta$ -ionona; 9 = no identificated = C9; 10 = (*E*)-7-tetradecen-1-ol; 11 = no identificated = C11; 12 = Linolenic acid ethyl ester; 13 = no identificated = C13.



**Fig. 5.** Projection to principal component analysis (PCA) based on the headspace composition of volatiles of conventional maize plants uninfested (C = o); conventional maize plants infested by *Tetranychus urticae* (C + Tu = ●); Bt maize plants uninfested (Bt =  $\Delta$ ); and Bt maize plants infested by *T. urticae* (Bt + Tu =  $\blacktriangle$ ), using the first two principal components (Dim) with explained variance in brackets.



**Fig. 6.** Projection to principal component analysis (PCA) based on the headspace composition of volatiles of conventional maize plants infested by *Tetranychus urticae* (C + Tu = ●); conventional maize plants infested by *Tetranychus urticae* and *Spodoptera frugiperda* (C + Tu + Sf = +); Bt maize plants infested by *T. urticae* (Bt + Tu =  $\blacktriangle$ ); Bt maize plants infested by *T. urticae* and *S. frugiperda* (Bt + Tu + Sf = \*), using the first two principal components (Dim) with explained variance in brackets.

predatory mite *Phytoseiulus persimilis* (Athias-Henriot) (Acari: Phytoseiidae). This demonstrates a variation in the production of volatiles within the same plant species. So, this results indicate the need for further studies with various others cultivars.

For the analysis of conventional and Bt plants with single and multiple infestation, the predator mite *N. californicus* did not present a preference between volatiles. This suggests that multiple herbivory does not alter the predator response, or the predatory mite did not distinguish between volatiles from conventional and Bt plants with multiple infestation. Studies have shown that plants may respond differently when attacked by one or more herbivores (Dicke et al., 2009). The attraction of the natural enemy can increase when the plant is under multiple herbivory. For example, pepper

plants infested with only one herbivore, *T. urticae* or green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), were less attractive to the predator *Macrolophus caliginosus* (Wagner) (Heteroptera: Miridae) than plants infested with the two herbivores simultaneously. This shows that the volatiles produced by multiple herbivory are more attractive to the predator than the volatiles induced by only one herbivore (Moayeri et al., 2007).

Besides that, multiple infestations may not alter the attractiveness of natural enemies, as found in the present study. A study comparing neutrality in the attraction of natural enemies shows that the predatory mite *Neoseiulus baraki* (Athias-Henriot) (Acari: Phytoseiidae) does not distinguish fruit odors from coconut trees infested by *Aceria guerreronis* (Keifer) (Acari: Eriophyidae) or *Steneotarsonemus concavuscutum* (Lofego and Gondim Jr.) (Acari:

Tarsonemidae) from odors of the fruit infested simultaneously by these herbivores (Lima et al., 2016).

The volatiles released in response to herbivory are generally detectable and indicators of the presence of herbivores which may potentially attract natural enemies near their prey (De Moraes et al., 2000). However, as *T. urticae* is not a pest that causes severe damage to the maize crop, these plants may not coevolve to produce efficient compounds to attract predatory mites. Moreover, when the plants were under multiple herbivory, a change occurred in the production of compounds, which were not attractive to the predator mite *N. californicus*, but this change may be specific to some natural enemy of the fall armyworm, which the maize plant has a greater interaction and consequently the coevolution process is more likely.

In the present study, the Bt technology did not alter the foraging of the predatory mite *N. californicus*, but studies show that Bt toxins may have indirect effects on natural enemies, an example is having the nutritionally inadequate being prey (Dively et al., 2004).

Conventional and Bt maize, cleaned and infested by *T. urticae*, showed a chemical differentiation of the groups of plant under study. However, this difference was not able to attract the predatory mite. Compounds detected in conventional infested plants were: (E)-Hex-2-enal; heptanal;  $\beta$ -cisocymene;  $\beta$ -Cyclocitral; 1-methyl-6-(3-methylbut-1,3-dienyl)-7-oxabicyclo [4.1.0] heptane;  $\beta$ -ionone; (E)-7-tetradecen-1-ol. While, in Bt were: (E)-Hex-2-enal; heptanal; (E)-Oct-2-enal;  $\alpha$ -pinene;  $\beta$ -cisocymene;  $\beta$ -Cyclocitral;  $\beta$ -ionone; (E)-7-tetradecen-1-ol; Linolenic acid ethyl ester. These chemical results are not compatible with *T. urticae* induced volatiles in bean plants where it was detected the following compounds (E)-Hex-2-enal, (Z)-3-hexen-1-ol, (Z)-3-hexenyl acetate, methylene salicylate, linalool, (Z)- $\beta$ -ocimene, (Z)-4,8-dimethyl-1,3,7-nonatriene, (E)-4,8-dimethyl-1,3,7-nonatriene,  $\beta$ -caryophyllene, (E, E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. This set of compounds was able to attract the predatory mite *N. californicus* (Shimoda et al., 2005). This indicates that, even though maize plants have changed chemically after *T. urticae* infestation, the volatiles produced are unable to attract *N. californicus*. This chemical change allows new studies with different predators, since the volatiles produced can be attractive to other natural enemies.

Conventional and Bt maize leaves with single and multiple infestations showed a chemical differentiation of the plant groups with single infestation. This indicates that Bt plants differ chemically when they are on *T. urticae* herbivory. However, the predator mite *N. californicus* had no preference among these groups of plants. The Bt plants require energy resources to produce the Cry1F toxin that may alter the investment of secondary defense compounds such as volatiles, possibly the chemical profile difference of these plants were due to the interaction of constitutive and induced defenses (Kant et al., 2015; Price et al., 1980). However, the predator does not distinguish between the odors released by plants in single and multiple infestations, which indicates that the constitutive defense (Bt Maize) did not alter the foraging of the predator under study. Nonetheless, this chemical change found allows further studies with other natural enemies.

Besides the maize volatiles already mentioned in the literature, in the present study 1-methyl-6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo [4.1.0] heptane, (E)-7-tetradecen-1-ol and linolenic acid ethyl ester were first identified in maize. This was possibly due to the fact that there were no reports in the volatile literature of Pioneer 30F35 and 30F35 Hx corn. Degen et al. (2004) showed that among 31 varieties of maize there is a differentiation in total amount of volatiles and volatile profiles are highly variable in each genotype.

The evidence from this study indicates a chemical differentiation in the groups of plants studied. However, the *N. californicus* are not able to distinguish them. This fact potentiates the chance

of *T. urticae* to reach pest status in maize by reducing the chance of encounter between predator and prey. *N. californicus* does not differ between single and multiple infestations in maize plants. This fact indicates that multiple infestations does not alter foraging of the predator mite.

## Acknowledgments

The authors thank the Graduate Program in Agricultural Sciences, of Federal University of São João del-Rei (UFSJ / PPGCA), for the scientific support. We thank Embrapa Milho e Sorgo for the structure provided for the experiment, and Fapemig (CAG-APQ-00556-16) and Capes for the financial support. Also, the authors thank Dr. J.M. Waquil, for the English review and suggestions in original manuscript.

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