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Original Article

Volatile compounds from soybeans under multiple on herbivores infestations attract the predatory mite *Neoseiulus californicus* (Acari: Phytoseiidae)

Compostos voláteis da soja sob infestação múltipla de herbívoros atraem o ácaro predador Neoseiulus californicus (Acari: Phytoseiidae)

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Abstract

Plant-induced resistance can be an important component of soybean mites biological control programs. This work evaluates the preference of predatory mite *Neoseiulus californicus* (Acari: Phytoseiidae) to soybean plants under single and multiple herbivory conditions by two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae), and velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Using a Y olfactometer, the following scenarios were evaluated: soybean with no infestation and soybean infested with *A. gemmatalis*; soybean infested with *T. urticae* and *A. gemmatalis*, and soybean infested with *T. urticae* and with both *T. urticae* and *A. gemmatalis*. Volatile compounds released by plants were analyzed and identified by a Trace GC Ultra gas chromatograph coupled to a mass spectrometer with a solid phase micro-extraction ion-trap. The predatory mite *N. californicus* preferred soybean plants infested with *T. urticae* compared to those infested with *A. gemmatalis*. Multiple infestation did not interfere with its preference to *T. urticae*. Multiple herbivory of *T. urticae* and *A. gemmatalis* modified the chemical profile of volatile compounds emitted by soybean plants. However, it did not interfere with the search behavior of *N. californicus*. Out of the 29 identified compounds only five promoted predatory mite response. Thus, regardless of single or multiple herbivory by *T. urticae* with or without *A. gemmatalis*, the indirect induced resistance mechanisms operate similarly. As such, this mechanism contributes to an increase in the encounter rate between predator and prey for *N. Californicus* and *T. urticae*, and the efficacy of biological control of mites on soybean.

Keywords: indirect induced defense, chemical ecology, biological control, plant resistance, pest management.

Resumo

A resistência induzida por plantas pode ser um importante componente dos programas de controle biológico de ácaros da soja. Este trabalho avalia a preferência do ácaro predador Neoseiulus californicus (Acari: Phytoseiidae) às plantas de soja sob condições de herbivoria simples e múltipla pelo ácaro-rajado Tetranychus urticae (Acari: Tetranychidae) e pela lagarta-da-soja Anticarsia gemmatalis (Lepidoptera: Noctuidae). Utilizando o olfatômetro Y, foram avaliados os seguintes cenários: soja sem infestação e soja infestada com A. gemmatalis; soja infestada com T. urticae e A. gemmatalis, e soja infestada com T. urticae e com T. urticae e A. gemmatalis. Os compostos voláteis liberados pelas plantas foram analisados e identificados por um cromatógrafo gasoso Trace GC Ultra acoplado a um espectrômetro de massas com uma armadilha de íons de microextração em fase sólida. O ácaro predador N. californicus preferiu plantas de soja infestadas com T. urticae em relação àquelas infestadas com A. gemmatalis. A infestação múltipla não interferiu na preferência por T. urticae. A herbivoria múltipla de T. urticae e A. gemmatalis modificou o perfil químico de compostos voláteis emitidos por plantas de soja. No entanto, não interferiu no comportamento de busca de N. californicus. Dos 29 compostos identificados apenas cinco promoveram resposta de ácaros predadores. Assim, independentemente da herbivoria simples ou múltipla por T. urticae com ou sem A. gemmatalis, os mecanismos de resistência induzida indiretamente operam de forma semelhante. Assim, esse mecanismo biológico contribui para o aumento da taxa de encontro entre predador e presa para N. Californicus e T. urticae, e para a eficácia do controle de ácaros em soja.

Palavras-chave: defesa indireta induzida, ecologia química, controle biológico, resistência de plantas, manejo de pragas.

1. Introduction

Volatile organic compounds (VOCs) emitted by plants when damaged by herbivores can to attract natural enemies to their prey or hosts, resulting in plant indirect induced defense mechanisms (van Poecke and

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Dicke, 2004; Leitner et al., 2005; Aartsma et al., 2017). These VOCs vary according to presence of elicitor substances that depend on type of oral apparatus of herbivores (Heil, 2014). Multiple herbivores infestation can induce emission of different VOCs (Dicke et al., 2009). Chewing herbivores can commonly induce jasmonic acid (JA) – mediated signaling pathways, whereas sucking insects tend to trigger the route of salicylic acid (SA) (Leitner et al., 2005; Yoneya and Takabayashi, 2014; Aartsma et al., 2017). When a plant is damaged by multiple herbivores, the "crosstalk" phenomenon may be observed between chemical routes to induce defense, in order to adjust an appropriate mechanism against a specific herbivore, guiding foraging of predators or parasitoids (Pieterse et al., 2012; Heil, 2014).

Under multiple herbivory conditions, it is more complicated to predict the response of natural enemies regarding search behavior (Dicke et al., 2009), especially in situations where arthropods feed simultaneously on same plant (Zhang et al., 2009). It may modify the VOCs produced by this interaction (Dicke et al., 2009). Thus, interactions between herbivores associated with a crop are influenced by several factors and plants respond accordingly, to type and sequence of arthropods activity (de Rijk et al. 2013).

Research on arthropod-plant interactions, which aim to clarify how volatiles attract natural enemies to herbivore-infested plants, usually evaluate only one species of herbivore (Moraes et al., 2005). On the other hand, there are few studies on arthropods responses to plants after being submitted to multiple infestations, particularly, if they belong to different feeding guilds (Zhang et al., 2009). Moraes et al. (2005) evaluated the response of *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) to soybean plants under multiple infestations of *Euschistus heros* (F.) (Heteroptera: Pentatomidae) and *Anticarsia gemmatalis*. It was found that volatile compounds from the saliva of *E. heros* directed the preference of its parasitoid to infested plants. However, it did not respond to plants infested with *A. gemmatalis* only.

Understanding the mechanisms and the interactions between plants, herbivores and natural enemies under multiple infestation conditions is fundamental to selecting and managing crop varieties that are resilient to environmental adversities, characterized by the diversity of herbivore species (Giron et al., 2018). It is also important in order to clarify the effect of induced defense on the tritrophic interactions in the environment in which the agroecosystem is inserted (Dicke et al., 2009; Ponzio et al., 2016; Aartsma et al., 2017).

Soybean leaves are attacked by multiple herbivores such as several species of Lepidoptera (Moscardi et al., 2012), and by several species of mites such as green mite *Mononychellus planki*, two-spotted spider mite *Tetranychus urticae* among other species such as *Tetranychus desertorum*, *Tetranychus gigas* and *Tetranychus ludeni* (Guedes et al., 2007; Roggia et al., 2008). There are still few studies investigating the biological control of pest mites on soybean. The predatory mite *Neoseiulus californicus* (McGregor) (Acari: Phytoseiidae) may be a promising biological control agent.

Induced plant resistance, even if indirect, may be an important component of biological control success. In this context, the rate in which the predatory mite *Neoseiulus californicus* was attracted to soybean volatile compounds under single and multiple herbivory of *T. urticae* and *A. gemmatalis* was evaluated.

2. Material and Methods

Experiments were conducted at the Laboratory of Agricultural and Forestry Entomology of the Federal University of São João Del Rei (UFSJ) - *Campus* Sete Lagoas, Minas Gerais, Brazil. Chemical analyzes were carried out at the Chemistry Laboratory of the Federal University of Minas Gerais (UFMG), in Belo Horizonte, Minas Gerais, Brazil.

2.1. Arthropod material

In order to establish colonies of *T. urticae*, seeds of bean (*Canavalia ensiformis*) were germinated in plastic pots (6.3 L), filled with substrate (Terral Solo®), and kept in anti-aphids cages inside a greenhouse regulated to 25 ± 5 ºC. Mites were previously collected from sorghum leaves (*Sorghum bicolor*) at Embrapa Maize & Sorghum. Specimens of *T. urticae* were visualized under a stereoscopic microscope and transferred individually to the abaxial surface of *C. ensiformis* leaves without phytosanitary treatments, kept in an environment-controlled chamber (BOD) regulated to 25 ± 2ºC, 70 ± 10% of relative humidity and a 12 hour photoperiod. Plant leaves were left abaxial side facing up and coated with moistened cotton to prevent mites from escaping. Samples were maintained on foam embedded in non-distilled water inside of 20 x 30 x 5 cm plastic trays.

Larvae of velvetbean *A. gemmatali,* were obtained from a colony maintained in the laboratory. Velvetbean larvae and adults were fed an artificial diet of sugar solution (Vilela et al., 2014). Predatory mites *N. californicus* (SPICAL®) were purchased from Koppert.

2.2. Plant material

Plastic pots (1 L) containing Terral Solo® substrate were used to grow 100 soybean plants of the M6210IPRO variety, which express Bt protein Cry1Ac. Plants were protected in cages with anti-aphids screening inside a greenhouse and were used in the experiments at the V3 developmental stage.

2.3. Y-olfactometer analysis of predatory mite preference

The Y-shaped olfactometer (Sabelis and van de Baan, 1983) main and lateral tubes had 21 cm each in length and 3.5 cm of diameter. Before olfactometer experiments, soybean plants were individually infested with 100 adult females of *T. urticae* for 24 hours, and two larvae of *A. gemmatalis* at the fourth instar for 36 hours. Sample plants were taken into a small container along with a flow meter with velocity of 0.50 m/s. The flow meter was used to regulate the air flow from containers in the direction where predatory mites were released. The evaluated treatments were air x air; air x soybean without infestation; soybean without infestation x soybean infested with *T. urticae*; soybean without infestation x soybean infested with *A. gemmatalis*; soybean infested with *T. urticae* x soybean infested with *A. gemmatalis* and soybean infested with *T. urticae* x soybean infested with both, *T. urticae* and *A. gemmatalis*.

Tree replications were evaluated (i.e. each set of tested plants as a source of odors) with 20 readings of predatory mite responses for each replicate.

Predatory mites were individually released at end of tube (i.e. release area) on copper wire, to facilitate displacement of individuals. Predatory mite behavior was observed for 5 minutes, and response was considered positive after reaching 1/3 of one lateral tube extremity. Predatory mites that did not respond after 5 minutes were not computed in statistical analysis according to the methodology proposed by Janssen (1999). Individuals that left the copper wire or were lost of sight during test were removed and the test was ended. A new test started after washing the apparatus with tap water and drying. At every five responses, positions of the odor sources were inverted to avoid interference of olfactometer spatial positions on results.

2.4. Chemical analysis of volatiles

Chemical analysis was performed on Trace GC Ultra gas chromatograph coupled to a Polaris Q mass spectrometer (ThermoScientific, San Jose, CA), GC-MS system, with an ion-trap type analyzer in solid phase microextraction (SPME) in headspace mode (Merkle et al., 2015). Samples of volatiles, from same soybean plants used in the olfactometer, were captured in semi-polar polydimethylsiloxane / divinylbenzene (PDMS / DVB) by exposure in vials at 60 ºC for 20 minutes, which were sealed (25 mL headspace vial) and carefully identified. Subsequently, each sample was subjected to chromatographic analysis.

Chromatographic conditions for collection of soybean volatiles were as follows: temperature of injector, 200 ºC; injection in Splitless mode; "Splitless time", 5 minutes; temperature of ion source, 200 °C; interface temperature, 275 °C. Heating temperature of the equipment was 40 °C for 1 minute, gradient from 5 °C/min to 110 °C, maintenance of the isotherm for 3 minutes and then 7 °C/min to 220 °C, at which temperature the isotherm was maintained for 1 minute, and finally a gradient of 12 °C/min to 245 °C, at which temperature the isotherm was maintained for 1 minute. The detector was maintained in scan mode (fullscan, from 30 to 300), using the electron impact ionization (EI) technique, with energy of 70 eV. The chromatographic column used was the HP-5 MS capillary column (5% phenyl and 95% methylpolysiloxane), containing the following dimensions: 30 m long, 0.25 mm internal diameter and 0.25 µm film thickness (AgilentTechonolgies INC, Germany).

Volatile compounds were identified by comparisons between mass spectra present in the libraries based on values of retention time obtained from the Xcalibur 1.4 software of ThermoElectron Corporation.

2.5. Statistical analysis

Using Y olfactometer test, three experiments were carried out with a completely randomized design and three replicates. In each replicate the response of 20 predatory mites was evaluated in relation to sources of odors analyzed. These results were submitted to chi-square tests (α = 5%) for categorical data (Crawley, 2013). Percentages of relative areas, which represented amount of each identified compound, from each sample, were submitted to principal component analysis (PCA). Graphs and analyzes were carried out in R (R Development Core Team, 2014).

3. Results

3.1. Olfactometer test

No significant difference was observed in number of predatory mite *N. californicus* in olfactometer test when comparing air x air (Figure 1A), and clean plants x air (Figure 1B), indicating that there was no preference in the olfactometer, and that it was well calibrated and satisfactory for conducting the evaluations. However, a significant difference was noted in the number of *N. californicus* observed between plants infested by *T. urticae* and clean plants (Figure 1C).

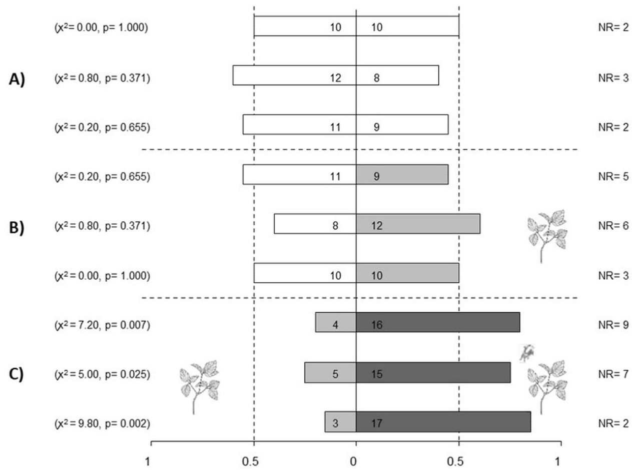

Figure 1. Proportion of the predator mite number, *N. Californicus*, responding to the following treatments evaluated in Y olfactometer: (A) air x air; (B) clean air x soybean plants without any infestation; (C) soybean plants without infestation x soybean plants infested by *T. urticae*. The number of mites without response to the treatments (NR), after 5 minutes, was eliminated from the statistical analysis.

No significant difference was observed in number of predatory mite *N. californicus* in olfactometer test when comparing soybean plant x soybean infested with *A. gemmatalis* only (Figure 2A). The number of predatory mites *N. californicus* was higher in *T. urticae*-infested soybean plants compared with plants infested with *A. gemmatalis* alone (Figure 2B).

No significant difference was observed in the number of *N. californicus* among *T. urticae*-infested soybean plants compared with plants under multiple infestations of both *T. urticae* and *A. gemmatalis* (Figure 2C).

The preference of predatory mite *N. californicus* for soybean infested by *T. urticae* did not change when *A. gemmatalis* was also present on same plant.

3.2. Chromatographic analysis

Twenty-nine compounds were identified of which 14 were found in clean plants, 13 in *T. urticae*-infested plants, 16 in plants infested with *A. gemmatalis* and 9 in plants with multiple infestations of both *T. urticae* and *A. gemmatalis* (Table 1).

Chemical parameters related to composition of volatiles compound from clean soybean and with the respective infestations explained 43.5% of the data variation (Figure 3).

Alcohols (4-pentenoic acid, 2,4-dimethyl methyl ester, $C_8H_{14}O_2$), an aldehyde (Benzene Acetaldehyde; C_8H_8O) and a monoterpene (Nerol; $C_{10}H_{16}O$) were more present in plants infested only with *T. urticae* and in the multiple infestations with both *T. urticae* and *A. gemmatalis*. The compounds vector representation is shown in Figure 4.

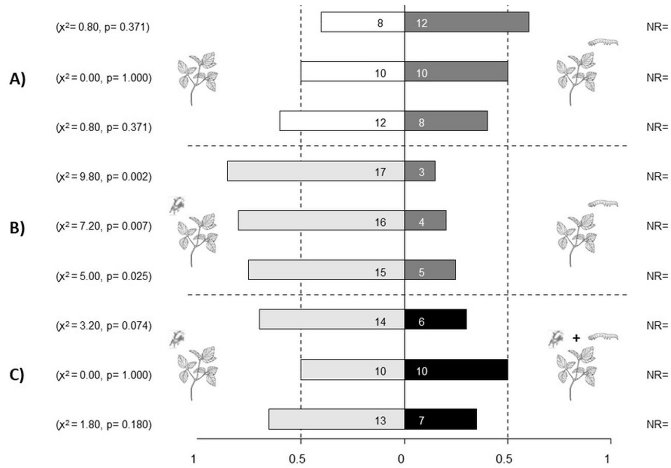

Figure 2. Proportion of predator mite number, *N. Californicus*, responding to the following treatments evaluated in Y-olfactometer: (A) clean soybean plants x plants with *A. gemmatalis* infestation; (B) soybean plants infested with *T. urticae* x plants infested with *A. gemmatalis*; (C) soybean plants infested with *T. urticae* x plants infested with *T. urticae* and *A. gemmatalis* on the same plant. The number of mites without response to the treatments (NR), after 5 minutes, was eliminated from the statistical analysis.

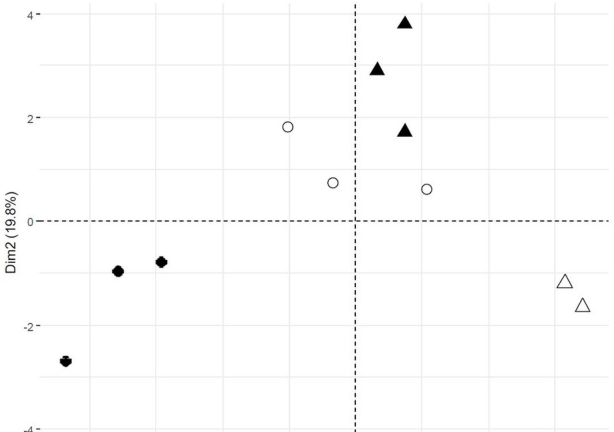

Figure 3. Principal Component Analysis (PCA) of the volatile compounds, detected by GC/MS, induced in soybean plants under the following treatments: plants without infestation (○); after infestation by *T. urticae* (△), after infestation by *A. gemmatalis* (●) and plants after multiple infestation with *T. urticae* and *A. gemmatalis* (▲).

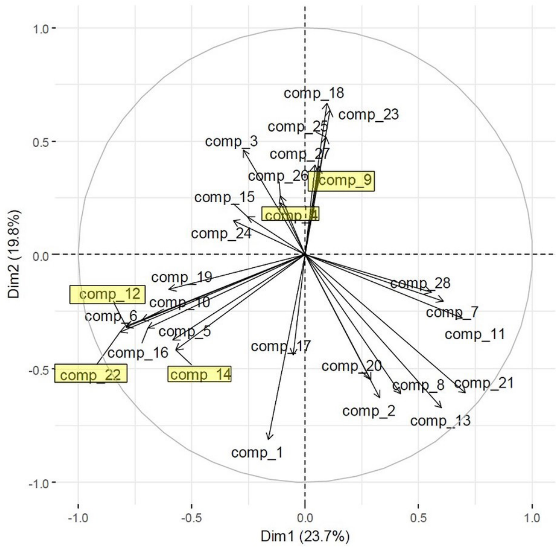

Figure 4. Principal component analysis (PCA) of the volatile compounds (identified in Table 1), detected by GC/MS, induced in soybean plants after simple infestation *T. urticae* and by *A. gemmatalis* and under multiple infestation with *T. urticae* and *A gemmatalis*.

Table 1. Volatile organic compounds (VOCs) captured and identified in soybean plants (M6210IPRO) submitted to the following treatments: *Tetranychus urticae* (SA) infested with two larva of *A. gemmatalis* (SL) and plants with multiple infestation by *T. urticae* + *A. gemmatalis* (SAL).

N°	VOCs	Class	Formula	[1]S	[2]SA	[3]SL	[4]SAL
1	2-Hepten-1-ol	Alcohol	$C_7H_{14}O$		X	X	
2	2-Hexen-1-ol	Alcohol	$C_6H_{12}O$	X	X	X	
3	2-Hexenal	Aldehyde	$C_6H_{10}O$	X		X	
4	2-hexyn-1-ol	Alcohol	$C_6H_{10}O$		X		X
5	2,4-Hexadien-1-ol	Alcohol	$C_6H_{10}O$	X			
6	2,4-Decadien-1-ol	Alcohol	$C_{10}H_{18}O$			X	
7	2-Octenal	Aldehyde	$C_8H_{14}O$		X	X	X
8	4-hexen-1-ol	Alcohol	$C_{10}H_{18}O$	X	X		
9	3- Octanone	ketone	$C_8H_{16}O$		X		
10	Octan-3-ol	Alcohol	$C_8H_{18}O$	X		X	X
11	1,3,7-Octatriene,2,7- dimetil	Monoterpene	$C_{10}H_{16}$			X	
12	4-pentenoic acid, 2,4-dimethyl metil ester	Ester	$C_8H_{14}O_2$		X		X
13	Felandral	Monoterpene	$C_{10}H_{16}O$	X		X	
14	Benzeno Acetaldeido	Aldehyde	C_8H_8O		X		
15	5-Octen-1-ol	Alcohol	$C_8H_{16}O$	X	X	X	
16	3-Hexen-1-ol	Alcohol	$C_6H_{12}O$	X		X	
17	3,5-Octadieno	Monoterpene	$C_{10}H_{18}O$			X	
18	Linalol	Monoterpene	$C_{10}H_{18}O$	X	X	X	
19	α- Carofileno	Sesquiterpene	$C_{15}H_{24}$	X			X
20	Citronelol	Monoterpene	$C10H_{20}$			X	X
21	Isobormeol	Monoterpene	$C_{10}H_{18}O$		X	X	
22	Nerol	Monoterpene	$C_{10}H_{18}O$		X		
23	Pulegona	Monoterpene	$C_{10}H_{16}O$			X	
24	Cis-o-mentha-2,8-dien-1-ol	Monoterpeno	$C_{10}H_{16}O$				X
25	B-Ocimeno	Monoterpene	$C_{10}H_{16}$	X		X	
26	Etanona	Monoterpene	$C_{10}H_{16}O$	X			
27	Beta Ionona	Ketone	$C_{13}H_{20}O$				X
28	Ionona	Ketone	$C_{13}H_{20}O$	X			
29	Fenol2,4,6-tris(1-metiletil)	Sesquiterpene	$C_{15}H_{24}O$	X	X		
	Total of compounds			14	13	16	9

[1]Soybean clean (S) and [2]with two spotted spider mite infestation (SA), by[3]velvetbean larva (SL) and [4]both two spotted spider mite and velvetbean larva (SAL) in the same plant.

Compounds 12, 14 and 22, which promoted positive responses of predatory mites *N. californicus* were grouped in the same quadrant. Compounds four and nine are dispersed in two other quadrants.

4. Discussion

There was no significant difference in number of predatory mites *N. californicus* in soybean plants without infestation and infested only by *A. gemmatalis*, indicating no preference by the predator mite between these two treatments. On the other hand, there was significant difference when comparing number of predatory mite choice to infested plants with *T. urticae* and any other treatment. Plants infested with *T. urticae* attracted the predatory mite *N. californicus* indicating that volatile compounds released by the plants directed the predatory mite to move towards to its prey. The attraction of the predatory mite *Phytoseiulus persimilis* to volatile compound of bean plants with herbivory of *T. urticae* or by the larva of *Spodoptera exigua*, and they reported the attraction by plants infested with *T. urticae*. *N. californicus* was able to locate plants infested by *T. urticae*. Oliveira et al. (2009) found similar results evaluating the behavior of *Phytoseiulus macropilis* in clean and *T. urticae*-infested strawberry plants. They observed a significant stronger attraction by the predatory mite to the infested plants either with the olfactometer or in arena test. In greenhouses, Janssen (1999) also observed the preference of *P. persimilis* to volatile compounds from cucumber infested by *T. urticae*. These results suggest that three different species of predatory mites can locate infested plants with prey by its odors in different environments.

The larval injury of *A. gemmatalis* on soybean leaves did not interfere in the choice for the predatory mite *N. californicus* on plants under single or multiple infestations. It did not repel or attract the *N. californicus*, so equivalent results were observed from plants with single infestation and multiple infestations with both *T. urticae* and *A. gemmatalis.* Thus, the damage caused by *A. gemmatalis* on soybean plants, despite promoting alteration of the chemical profile of the volatile compounds, did not affect the response of the predatory mite *N. californicus*. Therefore, the mechanism of indirect induced resistance in plants, releasing volatile compounds to attract the natural enemies toward the infested plants with its prey, continues to operate despite the multiple infestations by herbivores.

As such, it suggests that mechanism of indirect induced resistance would increases the efficacy of *T. urticae* biological control by the predatory mite *N. californicus*. Results from olfactometer confronted with volatiles compound released by plants with multiple infestations, suggests that *N. californicus* can be used as a biological control agent in both situations, since its preference is not compromised by the presence of the *A. gemmatalis*. Consequently, a multiple-herbivore infestation would not reduce the encounter rate of the predatory mite *N. californicus* with its prey *T. urticae*.

Compounds 2-hexene-1-ol (2), 2-hexenal (3), 3-Octanone (9), 3-Octanol (10), 3-Hexen-1-ol (16), Linalol Benzene Acetaldehyde (14), Beta-Oximene (25) and α-Carophilene (19) were also identified from soybean plants in other publications evaluating the emission of volatile compounds (del Rosario et al., 1984; Liu et al., 1989; Damiani et al., 2000; Boue et al., 2003; De Boer et al., 2004; Moraes et al., 2005; Zhu and Park 2005; Rostás and Eggert 2008; Michereff et al., 2011; Cai et al., 2015). The activity of these compounds on the predatory mite *N. californicus* should be evaluated in future, in pure or in mixture to explain compound combinations and dose response. There is a potential for use of these compounds as adjuvants in a field biological control program of *T. urticae*.

T. urticae feeding promotes a response of soybean plants to produce volatiles that attract the predatory mite *N. californicus*. Multiple herbivory of *T. urticae* and *A. gemmatalis* modifies the chemical profile of the volatile compounds emitted by soybean plants. However, it does not interfere in the search behavior of the predatory mite *N. californicus*. Volatile compounds responsible for the attraction of the predator mite *N. californicus* from soybean plants submitted to *T. urticae* herbivory are: 2-hexyn-1-ol ($C_6H_{10}O$); 3-Octanone ($C_8H_{16}O$); 4-pentenoic acid, 2,4-dimethyl methyl ester ($C_8H_{14}O_2$); Benzene Acetaldehyde (C_8H_8O) and Nerol ($C_{10}H_{16}O$).

Independently of simple or multiple herbivory conditions by *T. urticae* and *A. gemmatalis*, indirect induced resistance mechanisms are operating and contributing to increase the efficacy of the predatory mite *N. californicus* as a biological control agent of *T. urticae* on soybean.

5. Conclusion

Simple or multiple herbivory by *T. urticae* and *A. gemmatalis* induces indirect resistance mechanism, increasing preference of predatory mite *N. Californicus* on biological control of phytophagous mites on soybean.

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