ELISA FARIA DE OLIVEIRA

INDUCTION AND REDUCTION OF TOMATO PLANT DEFENSES BY SPIDER MITES: EFFECTS OF SEQUENTIAL AND SIMULTANEOUS ATTACKS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2012

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

O48i
2012

Oliveira, Elisa Faria de, 1984-
Induction and reduction of tomato plant defenses by spider mites: effects of sequential and simultaneous attacks / Elisa Faria de Oliveira. – Viçosa, MG, 2012.
viii, 61f. : il. (algumas col.) ; 29cm.

Orientador: Angelo Pallini Filho.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Ácaro de plantas - Controle. 2. Tomate - Resistência a doenças e pragas. 3. Inibidores enzimáticos proteolíticos. 4. *Tetranychus evansi*. 5. *Tetranychus urticae*.
I. Universidade Federal de Viçosa. II. Título.

CDD 22. ed. 632.65423

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APROVADA: 17 de fevereiro de 2012.

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AGRADECIMENTOS

Agradeço especialmente aos meus pais Valdir e Odila e minhas irmãs Eliana e Larissa, pelo incentivo, amor e pela grande torcida.

Ao Daniel pelo carinho e dedicação durante todos esses anos.

Ao professor Angelo Pallini, pela orientação e ensinamentos e por toda a sua contribuição para a realização desse trabalho.

Ao professor Arne Janssen pela grande colaboração, disponibilidade e sugestões valiosas.

Aos coorientadores, Dra. Madelaine Venzon e Prof. Renato Sarmento pelas correções e sugestões.

Aos amigos do Laboratório de Acarologia, Juliana, Felipe, Aline, Lívia, João, Cleide, Aninha, Caelum e Cleber pelo convívio e companheirismo.

À Ludimila pelo compromisso e grande ajuda nos experimentos.

Aos amigos do Laboratório de Enzimologia, Bioquímica de Proteínas e Peptídeos, Fabrício, Camila, Eduardo, Rita, Francine e Anderson pela receptividade no laboratório e sugestões na realização dos experimentos.

À todos os amigos da Entomologia por todos os momentos compartilhados.

À Universidade Federal de Viçosa e ao Programa de Pós Graduação em Entomologia pela oportunidade de realização do curso.

À CAPES pela concessão da bolsa de estudo.

BIOGRAFIA

Elisa Faria de Oliveira, filha de Valdir de Oliveira e Odila de Fátima Faria de Moraes Oliveira, nasceu no dia 20 de dezembro de 1984 em Bauru, São Paulo.

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SUMÁRIO

RESUMO

OLIVEIRA, Elisa Faria de, M.Sc., Universidade Federal de Viçosa, fevereiro de 2012. **Indução e redução de defesas em plantas de tomate por ácaros fitófagos: efeitos de ataques sequenciais e simultâneos.** Orientador: Angelo Pallini Filho. Coorientadores: Arnoldus Rudolf Maria Janssen e Madelaine Venzon.

Neste estudo foi investigado o efeito das defesas induzidas diretas de plantas de tomate sobre parâmetros biológicos do ácaro vermelho *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) e do ácaro rajado *Tetranychus urticae* Koch (Acari: Tetranychidae), pelo fato desses ácaros serem pragas na cultura do tomateiro. No primeiro capítulo foram determinadas as mudanças temporais nas características químicas que são ativadas após a alimentação dos ácaros *T. evansi* e *T. urticae* em folhas de tomate e o efeito biológico sobre os fitófagos. Foi observado que a taxa de oviposição de fêmeas de *T. evansi* e *T. urticae* em folhas de tomate previamente danificadas por *T. evansi* foi significativamente maior que em folhas de plantas limpas após 1 e 2 dias de infestação. Também foi observado que a taxa de oviposição de fêmeas de *T. evansi* e *T. urticae* em folhas de tomate previamente danificadas por *T. urticae* foi significativamente menor que em folhas de plantas limpas nos 4 dias de infestação. Adicionalmente, observou-se um aumento nos níveis de inibidores de proteinases 24 horas após o dano de *T. urticae* nas folhas de tomate. Entretanto, observou-se que os níveis de inibidores de proteinase foram suprimidos 24 horas após o dano de *T. evansi*. Os resultados sugerem que ambas as espécies de ácaros fitófagos podem estar sendo afetadas pelas defesas induzidas por cada espécie em particular e consequentemente pela qualidade nutricional do alimento. No segundo capítulo, foi estudado como infestações simultâneas podem afetar as respostas induzidas de plantas de tomate e qual as consequências aos ácaros fitófagos. Observou-se que a fecundidade de *T. evansi e T. urticae* foi maior em plantas danificadas por *T. evansi*, intermediária em plantas danificadas por ambos os ácaros (*T. evansi* + *T. urticae*) e menor em plantas danificadas por *T. urticae*. Adicionalmente, observou-se que atividade de inibidores de proteinase nas plantas danificadas por ambos os ácaros não diferiu significativamente da atividade em plantas apenas danificadas por *T. urticae*, apresentando os maiores níveis de atividade. Porém, a atividade de inibidores foi significativamente menor em plantas

apenas danificadas por *T. evansi*. Estes resultados indicam que infestações simultâneas podem afetar a desempenho dos fitófagos devido aos efeitos antagônicos de cada espécie sobre o sistema de defesa da planta. No entanto, a infestação simultânea não mostrou efeitos aditivos sobre a atividade de inibidores de proteinase. No terceiro capítulo, observou-se o efeito de infestações sequenciais por *T. evansi* e *T. urticae* nas respostas induzidas por plantas de tomate e seus efeitos sobre os fitófagos. Os resultados mostraram que a fecundidade dos ácaros foi dependente das defesas induzidas por cada espécie e também foi fortemente influenciada pela segunda herbivoria. Os resultados da atividade de inibidores de proteinase mostraram que a atividade em plantas previamente danificadas por *T. urticae* e posteriormente danificadas por *T. evansi* não diferiu significativamente de plantas danificadas por *T. evansi* sem pré-infestação e mostraram os níveis mais baixos de atividade. As plantas danificadas por *T. urticae* sem pré-infestação mostraram os maiores níveis de atividade enzimática. Porém, plantas previamente danificadas por *T. evansi* e posteriormente danificadas por *T. urticae* mostraram um padrão intermediário na atividade de inibidores. Isto indica que pode acorrer o redirecionamento de defesas pelo segundo fitófago e que a última espécie de ácaro que se alimenta da planta pode ter um maior efeito na sua qualidade. Dessa forma, a sequência de chegada dos fitófagos na planta pode ter um importante efeito nas suas respostas defensivas e na sua qualidade nutricional.

ABSTRACT

OLIVEIRA, Elisa Faria de, M.Sc., Universidade Federal de Viçosa, February, 2012. **Induction and reduction of tomato plant defenses by spider mites: effects of sequential and simultaneous attacks.** Adviser: Angelo Pallini Fillho. Co-advisers: Arnoldus Rudolf Maria Janssen and Madelaine Venzon.

In this study, we investigated the effect of induced direct defenses of tomato plants on biological parameters of the red mite *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) and *Tetranychus urticae* Koch (Acari: Tetranychidae). These mites are important pests in tomato plants. In the first chapter, we determine the temporal changes in the chemical characteristics that are activated after the spider mites *T. evansi* and *T. urticae* feed on tomato leaves and the biological effect on these herbivores. It was observed that the oviposition rate of *T. evansi* and *T. urticae* was significantly higher on tomato leaves previously damaged by *T. evansi* than on leaves of clean plant after 1 and 2 days of infestation. It was also observed that the oviposition rate of *T. evansi* and *T. urticae* on tomato leaves previously damaged by *T. urticae* was significantly lower than on leaves of plants without infestation during the 4 days of infestation. Additionally, we showed an increase in the levels of proteinase inhibitors 24 hours after the damage of *T. urticae* on tomato leaves. However, we observed that the levels of proteinase inhibitors were suppressed 24 hours after the damage of *T. evansi*. The results suggest that both species of spider mites may be affected by induced defenses by each species and consequently by nutritional quality of food. In the second chapter, it was studied if simultaneous infestations can affect the induced response in tomato plants and what the consequences for spider mites are. It was observed that the fecundity of *T. evansi* and *T. urticae* was higher in plants damaged by *T. evansi*, intermediate in plants damaged by both mites (*T. evansi* + *T. urticae*) and lower in plants damaged by *T. urticae*. Additionally, it was observed that the activity of proteinase inhibitors in plants damaged by both mites did not significantly differ from the activity in plants damaged only by *T. urticae* and showed higher levels of activity. However, the inhibitor activity was significantly lower in plants damaged only by *T. evansi*. These results indicate that simultaneous infestations can affect the performance of herbivores, possibly caused by antagonistic effects of each species on the plant defense system. However, simultaneous infestation showed no additive effects on the activity of proteinase inhibitors. In the third chapter, we observed the effect of sequential infestations by *T. evansi* and *T. urticae* in tomato plants responses and their effects on herbivores. The results showed that the performance of spider mites was dependent of induced defenses by each species and it was also strongly influenced by second herbivory. The results of the activity of proteinase inhibitors showed that activity in plants previously damaged by *T. urticae* and subsequently damaged by *T. evansi* did not differ significantly from plants damaged by *T. evansi* without pre-infestation and showed lower levels of activity. Plants damaged by *T. urticae* without pre-infestation showed the highest levels of enzymatic activity. However, plants previously damaged by *T. evansi* and later damaged by *T. urticae* showed an intermediate pattern in inhibitor activity. This indicates a redirection of defenses by the second herbivore and that the last herbivore species that feeds on plant may have a greater effect on their quality. Thus, the sequence of herbivores arrival on tomato plant may have an important effect on plant defensive responses and their nutritional quality.

GENERAL INTRODUCTION

Many species of plants display a wide variety of constitutive and induced defenses (Karban & Baldwin, 1997). Such resistance mechanisms may negatively affect the growth and reproduction of the herbivore organisms (Walling, 2000; Wu & Baldwin, 2009).

The defense mechanisms used by plants may provide them with direct protection (Karban & Baldwin, 1997) for example, increasing levels or activities of allelochemicals, which are deleterious to arthropods (Stout et al., 1998a). However, plants also defend themselves indirectly by attracting natural enemies of herbivores. This type of defense is called indirect induced defense, where plants release attractive volatiles when attacked. These volatiles serve as a guide to natural enemies, signaling the presence of an attacking pest (Maffei et al., 2007).

Plants respond to herbivore attack according to their specificity of elicitation, i.e. the ability of the plant to produce distinct chemical responses to different herbivores (Stout et al., 1998b). The activation of biochemical pathways that trigger the production of defense compounds occurs by the recognition of signals through the specific binding of protein molecules (Mithöfer & Boland, 2008). Recognizing a substance that acts as a signaling molecule, the plant activates a biochemical pathway for the production of metabolic compounds involved in defense response in a specific manner (Walling, 2000; Mithöfer & Boland, 2008). Defenses (direct and indirect) are induced only when the constitutive defenses are not sufficient to prevent the damage caused by the herbivores (Kant et al., 2004; 2008).

Many plant responses may have a negative effect on inducers as well as on other herbivores (Inbar et al., 1999). In nature, most plants are likely to be attacked by several herbivore species at the same time (De Boer et al., 2008). Thus, it is necessary to consider the consequences of plant responses to multiple attackers on species interactions. The induced responses can negatively affect the inducing agent or other herbivores that feed simultaneously or sequentially (Inbar et al., 1999). Herbivores may affect interspecific competitors asymmetrically due to difference in susceptibility to induced plant responses or when herbivory alters the plant quality differentially for the type of herbivore (Poelman et al., 2008).

Therefore, the induced plant defenses may depend on the number and identity of herbivore species (Rodriguez-Saona et al., 2005) and even the order of arrival of each herbivore on the plant (Erb et al., 2011).

In tomato plants the induction of defensive responses is regulated by the expression of genes (Alborn et al., 1997) and the magnitude of induction depends on the damage caused (Pearce et al., 1993; Malone & Alarcon, 1995), the type of herbivore (Stout et al., 1998a,b) or the inducing agent (Botella et al., 1996).

For example, the damage of *Tetranychus urticae* Koch (Acari: Tetranychidae) on tomato plants induces the expression of genes responsible for the activation of biochemical pathways in secondary plant metabolism, leading to accumulation of proteinase inhibitors (Kant et al., 2004). Li et al. (2002) observed that the mite *T. urticae* induces the expression of genes regulated by jasmonic acid, responsible for a rapid defensive response in tomato plants. Recently, Sarmento et al. (2011) observed that the mite *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) is able to interfere in the plant defense system: the performance of *T. evansi* increased significantly on tomato plants previously attacked by conspecifics mites than un-damaged plants. In addition, it was observed that the levels of proteinase inhibitors in the leaves of tomato plants were reduced. Proteinase inhibitors are chemical compounds, which occur in a wide variety of plants. Interactions of the inhibitors with digestive proteases interfere with the normal degradation of the amino acids component from the proteins consumed (Ryan, 1990). Increased levels of these compounds have been directly related to a reduction in development and reproduction of many herbivorous arthropods (Green & Ryan, 1972; Koiwa et al., 1997; Lawrence & Koundal, 2002; Kant et al., 2004).

Hence, there are two closely related mite species, of which one (*T. urticae*) induces plant defenses and the other (*T. evansi*) reduces plant defenses. This offers ideal opportunities to study the response of plants to multiple herbivory, which is the topic of this thesis.

Spider mites are important herbivores on tomato plants and often are pests in commercial tomato (De Moraes & Flechtmann, 2008). The red spider mite *T. evansi* occurs in plants of the Solanaceae family (Moraes & McMurtry, 1985), especially in tomato crops, and it has been recorded in South and North America, Africa, Europe and Asia (Ramalho & Flechtmann, 1979; Blair, 1989; Ferragut & Escudero, 1999; Ho et al., 2004; Tsagkarakou et al., 2007; Gotoh et al., 2010). A striking characteristic of *T. evansi* is its high production of web, covering its host plants, and its rapid population growth (Ferragut & Escudero, 1999).

The two-spotted spider mite *T. urticae* is a generalist herbivore; it has been recorded from over 900 plant species comprising 124 different plant families (Bolland et al., 1998; Egas et al., 2003) and is an economically important pest of a wide range of plants (Helle & Sabelis 1985), and perhaps the most serious pest in greenhouses around the world (Lange & Bronson, 1981).

For feeding, *Tetranychus* mites use specialized stylets to puncture epidermal cells subsequently removing cellular contents of the host tissue (Li et al., 2002). As a result, spider mite feeding leads to a collapse of the underlying mesophyll tissue and formation of a chlorotic lesion in the area restricted to where feeding took place (De Moraes & Flechtmann, 2008). The destruction of plant tissues promotes a decrease of plant photosynthesis activity and consequently a reduction in crop productivity.

The two spider mite species studied here are distributed over large agricultural areas where they may inhabit the same crops or even the same plants (Escudero & Ferragut, 2005).

In this dissertation, we investigate how the spider mites *T. evansi* and *T. urticae* affect induced direct defenses of tomato plants. In chapter 1, we determine the temporal changes in the chemical characteristics that occur after these herbivores feed on tomato plants and the biological effects of the induced changes on the performance of these herbivores. In chapter 2, we investigate the simultaneous impacts of suppression (*T. evansi*) and induction (*T. urticae*) of defenses on host plant quality and how they affect the biology of these two herbivores. In chapter 3, we observe response of a plant upon sequential attack and whether its defensive products may be enhanced or redirected towards defense against the new attacker.

Thus, this dissertation proposes to study the effects of infestation by *T. evansi* and *T. urticae* in tomato plants, elucidating how the mechanisms of plant defenses affect development and behavior of these phytophagous mites.

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CHAPTER 1

Temporal dynamics of herbivore-induced responses in tomato

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ABSTRACT

The attack of plants by herbivorous arthropods can result in considerable changes in the plant responses. The timing of these responses is important to have optimal effect on the attackers and may influence the impact of induced resistance on herbivore populations. Here we measured the temporal dynamics of induced responses to *Tetranychus urticae* and *Tetranychus evansi* herbivory in tomato plants. We assessed the levels of proteinase inhibitors in damaged and clean tomato leaves. Additionally, we evaluated biological effects of the induced changes on the performance of these spider mites. Proteinase inhibitors were found to increase 24h after damaged by *T. urticae*. However, the levels of these chemical compounds decreased 24h upon damaged by *T. evansi*, showing that the induction by these mites is rapid. Herbivores performance bioassays coincided with the chemical quality of the plants. The oviposition rate of both species (*T. evansi* and *T. urticae*) was higher on tomato leaf discs that had received damaged by *T. evansi* and lower on leaf discs damaged by *T. urticae* as compared to leaf discs from undamaged plants. These results show that these spider mites differently induce tomato plants and can affect the herbivore species according to the specificity of induction and time of infestation.

Key words: Timing of responses, induced plant defense, specificity, proteinase inhibitors induction, spider mites, *Tetranychus evansi*, *Tetranychus urticae*.

INTRODUCTION

Plants can employ diverse strategies to reduce arthropod herbivory, some of which can be expressed constitutively. Such strategies are defined as constitutive defenses. However, once under attack by an herbivore, plants may increase the concentrations of existing chemicals compounds and this mechanism is defined as induced defense (Karban & Baldwin, 1997). The expression of induced responses depends on the capacity of the plant to detect and recognize its attackers (Wu & Baldwin, 2009). After feeding on a plant, an herbivore causes mechanical damage, leading to a wound response (Schaller & Frasson, 2001). This response also can be activated by products in the herbivore saliva that are delivered from the feeding organism into the wounded tissue (Mithöfer & Boland, 2008).

In tomato, the herbivore-induced defense response is characterized by an increase in signaling compounds (Walling, 2000) controlled by expression of genes (Alborn et al., 1997) and the magnitude of induction is dependent on damage caused (Pearce et al., 1993; Alarcon & Malone, 1995) and on inductive species (Stout et al., 1998).

These induced responses may provide an immediate plant resistance, but also may provide a late plant resistance, arising from the delay in the induction of biochemical pathways (Mathur et al., 2011). However, this time delay between the initiation of herbivore damage and the mounting of the complete defensive condition is normally a critical disadvantage of induced defensive strategies (DeWitt et al., 1998; Underwood, 1999; van Dam et al., 2001). Examples of inducible chemical compounds that play an important role in plant protection are proteinase inhibitors (Lawrence & Koundal, 2002). Interactions of these inhibitors with proteolytic enzymes in the gut of herbivores interfere with the normal digestive physiology and consequently the assimilation of nutrients (Ryan, 1990) and can affect the feeding, growth and reproduction of the herbivore (Broadway & Duffey, 1986).

Although there are many reports of pathogens and herbivores that can induce responses in plants (Ryan, 1990; Lawrence & Koundal, 2002; Amirhusin et al., 2007) recently some studies showed examples of arthropod herbivores that can attenuate the direct defensive compounds (Musser et al., 2002; Bede et al., 2006; Lawrence et al., 2008). For example, Kant et al. (2008) showed that one population of spider mite *Tetranychus urticae*, harbours distinct intraspecific variation in plant-defense suppression. Another herbivorous spider mite, *Tetranychus evansi* was found to down-regulate tomato defenses and in a much stronger and possibly different fashion than observed for other herbivores (Sarmento et al., 2011a). As a result of this manipulation, attacked plants may become even better resources for the herbivores than non-damaged plants resulting in an increased performance of the mites on damaged plants (Sarmento et al., 2011a).

Inducible defenses have their own dynamics and ecological consequences (Mathur et al., 2011) but little is known about the timing of expression or suppression of tomato defenses over a time period of several days after attack (but see Kant et al., 2004). Knowledge of the temporal dynamics of chemical responses, together with changes in performance of herbivores, is therefore important for understanding the eventual effects on pest spider mites threatening tomato cultures.

In the present study, we combined all these aspects and determined the temporal changes in the chemical characteristics that occur after spider mite feeding in tomato leaves and examined their effect on herbivores. The chemical responses that were measured consisted of proteinase inhibitors. We used the spider mite species *T. evansi* and *T. urticae* to induce the tomato plants. Furthermore, the biological effects of the induced changes on the performance of these spider mites were determined to verify whether these induced changes may result in resistance to different herbivore species.

MATERIALS AND METHODS

Plant material

Tomato seeds (*Solanum lycopersicum* var Santa Clara I-5300) were weekly sown in a commercial plant substrate (Bioplant®, Bioplant Misturadora Agrícola LTDA) in a polystyrene tray (8 x 16 cells), and kept inside a cage with a fine mesh in a greenhouse to avoid infestation with herbivores. After 21 days, plants were transferred to plastic pots (2 L) that contained a mixture of soil plus fertilizer (4-14-8 N-P-K). Tomato plants were further grown in mite-proof screen cages in a greenhouse until they were 45 days old and had at least four completely developed leaves. Subsequently, plants were used either for the experiments or for spider mite rearing.

Mite rearing

Spider mites (*T. evansi* and *T. urticae*) were obtained in 2002 from naturally infested tomato plants of the same variety mentioned above in a greenhouse at the Federal University of Viçosa, Brazil. Both species were cultured on detached tomato leaves, of which the petiole was inserted in a PVC tube with water to maintain leaf turgor. Tubes with infested leaves were kept in PVC trays filled with detergent and water (1:25, v / v), which served to prevent mite escapes and invasion of mites and other non-flying arthropods. The mass culture was maintained in a room at 25 ± 3 ºC, 70–90% relative humidity and 12 h light.

Spider mite fecundity

The oviposition rate of *T. evansi* and *T. urticae* was measured on damaged leaves of plants infested by *T. evansi* and *T. urticae* and clean leaves of un-infested plants (controls). One leaf of four randomly selected tomato plants of 45 days old was infested for 1, 2, 3 or 4 days with 100 adult females of *T. urticae* or *T. evansi*, while the other leaves were kept clean. Insect glue (Cola Entomológica; Bio-Controle, São Paulo, Brazil) was applied to the petioles of the leaf on which mites were released to prevent them from moving to another leaf. Control plants from the same batch and the same age were also treated with glue. Plants were kept inside mite-proof screen cages in a greenhouse during the experiment. After infestation for 1 – 4 days, the mites, their web and eggs were removed, and 10 leaf discs (12 mm Ø) were made, for each species of spider mite, from leaves damaged by *T. evansi* or *T. urticae* and from corresponding leaves of non-infested control plants. All leaf discs were inspected with a stereoscopic microscope to ensure that no spider mite eggs were present. Discs were subsequently kept in Petri dishes (8 cm Ø) containing wet cotton wool.

Because the oviposition rate of spider mites varies with age (Sabelis, 1991), female mites of similar age were used to measured oviposition. To obtain such cohorts, several adult females were allowed to lay eggs on detached tomato leaves on wet cotton wool to produce an egg-wave. The adults were removed after 24 h and the eggs were reared to adulthood. One randomly selected adult female of *T. evansi* or *T. urticae*, 2 days old since turning adult, was placed individually on each disc. After 4 days (28 ± 2 ºC; 70 ± 10% RH; 12 h light), the oviposition rate was measured. Each replicate consisted of a group of 10 leaf discs and each treatment was replicated 4 times.

Differences in mean oviposition rates per plant among treatments were tested with a Generalized Linear Model with a normal error distribution. The experiments with damaged plants infested by *T. evansi* and *T. urticae* could not be carried out at the same time for logistical reasons. Treatments could therefore only be compared to controls within the same experiment.

Proteinase inhibitor assays

The proteinase inhibitor activity was measured in the same leaves used for the oviposition experiments. For this, 2 leaflets were separated per leaf. Crude protein was extracted from these leaflets of tomato plants. The leaflets were frozen in liquid nitrogen and stored at -80 ºC. Subsequently, each sample was ground with mortar and pestle and a crude extract was obtained as described by Otha et al. (1986). Essentially, the leaves were homogenized in extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$; 1:3 w/v) and the homogenate centrifuged at 17200 g for 30 min at 4 ºC and the supernatant was collected. The resulting supernatant was used for determining the protein content and all other assays. Protein concentration was determined by the method described by Bradford (1976), using a solution of 0.2 mg/ml bovine serum albumin (BSA) as standard.

A standard spectrophotometric assay was used to measure trypsin inhibitory activity in the supernatant. A 100 μL aliquot of trypsin (4.7×10^{-5} M) was mixed with 100 μL of the supernatant and 500 μL extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$). The mixture was incubated at room temperature for 5 min. Controls consisted of 600 μL extraction buffer and 100 μL of trypsin (4.7×10^{5} M). A 700 μL aliquot of the mixture (tests and controls) was added to 500 μL extraction buffer and 500 μL D,L-BApNA (1.2 mM). Trypsin activity was monitored for 150 seconds at intervals of 30 seconds at 410 nm absorbance on a spectrophotometer. The difference between the absorbance measured at 150 and 60 seconds was used to determine the trypsin activity. Measurements were performed in triplicate per sample. The results obtained were converted to milligrams trypsin inhibited per gram of protein according to the following equation: mg trypsin inhibited per gram of protein = AB/1000PC with A = enzyme control – absorbance at 410 nm of the extract; B = sample dilution; P = protein concentration of the extracts, in g/mL; and C = trypsin factor, the result from the activity of 1 µg of trypsin on the substrate D,L-BApNA measured at 410 nm, for the combination of trypsin and D,L-BApNA = 0,019 (Kakade et al., 1974). Differences among treatments were analyzed with a Generalized Linear Model with a normal error distribution.

RESULTS

Spider mite fecundity

The oviposition rate of *T. evansi* on leaves damaged by conspecifics varied with the time of infestation (**Figure 1**, $F_{4,15} = 95.5$, $P < 0.0001$).

The oviposition rate of *T. evansi* was significantly higher on tomato leaves that had received damaged by conspecifics for 1 or 2 days than on leaves that had been damaged for 3 or 4 days. Oviposition on the undamaged leaves was the lowest (**Figure 1**). The oviposition of *T. urticae* on leaves damaged by *T. evansi* also varied with the time of infestation (**Figure 2**, $F_{4,15} = 9.5$, $P < 0.001$), and showed a similar patter through time as the oviposition of *T. evansi* (cf. **Figure 1** with **Figure 2**): oviposition rate of *T. urticae* was significantly higher on tomato leaves that had received damaged by *T. evansi* for 1 or 2 days than on leaves with 3 or 4 days of damage, whereas the latter two treatments did not differ from the control (**Figure 2**).

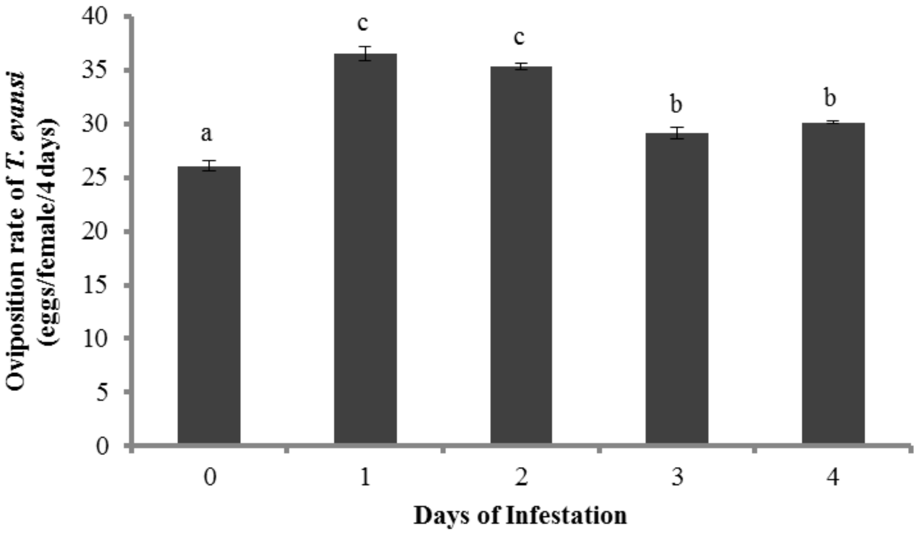

Figure 1 Effect of induction by *T. evansi* through the time. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* on tomato leaves that were previously damaged for 0, 1, 2, 3 or 4 days by conspecific mites. Different letters denote significant differences among treatments.

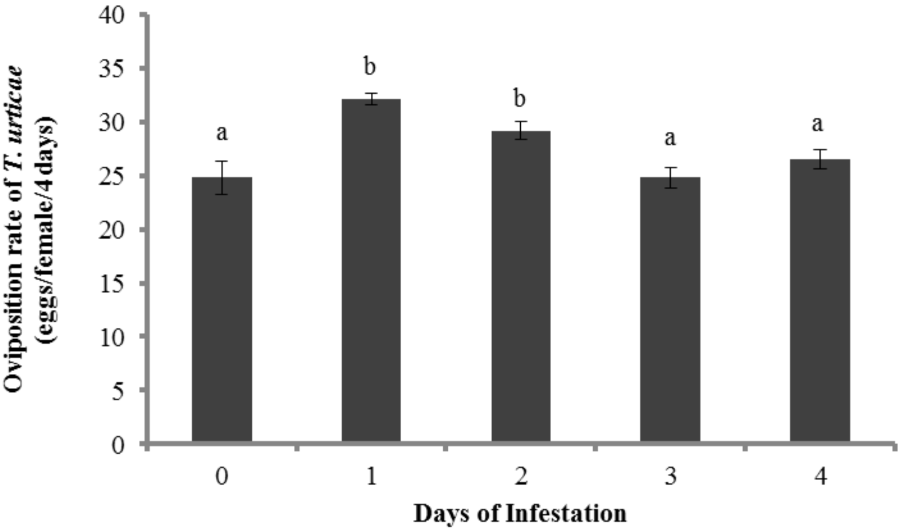

Figure 2 Effect of induction by *T. evansi* through the time. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. urticae* on tomato leaves that were previously damaged for 0, 1, 2, 3 or 4 days by *T. evansi*. Different letters denote significant differences among treatments.

There was a significant effect of time of infestation by *T. urticae* on the oviposition rate of *T. evansi* (**Figure 3**, $F_{4,15} = 25.7$, $P < 0.0001$) and *T. urticae* (**Figure 4**, $F_{4,15} = 57.5$, $P < 0.0001$). The oviposition of *T. evansi* was lower on leaf discs damaged for 1 or 4 days, intermediate on leaf discs damaged for 2 or 3 days, and highest on clean leaf discs (**Figure 3**). The rate of oviposition of *T. urticae* on leaves damaged by conspecifics was significantly lower on tomato leaves damaged by conspecifics for 1 day, followed by leaves damaged for 2, 3 and 4 days (**Figure 4**). The highest rate of oviposition was again found on leaf discs from clean plants.

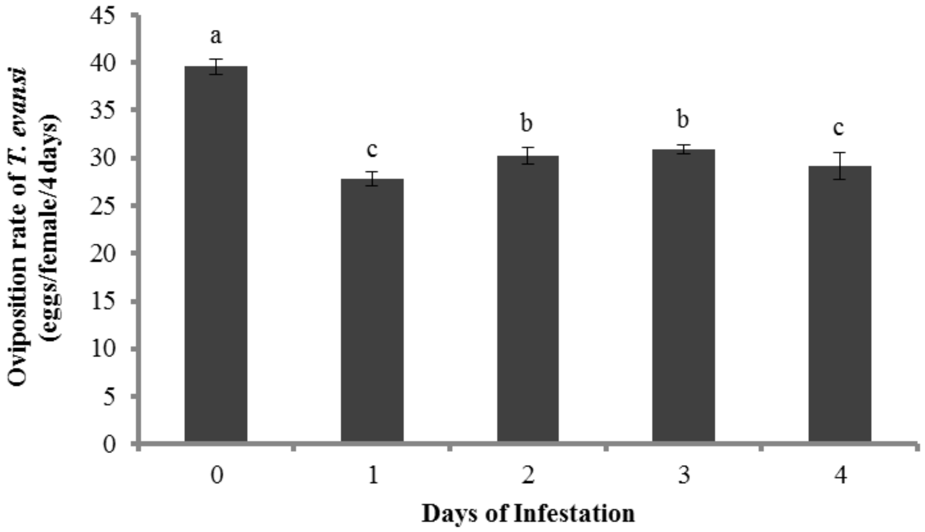

Figure 3 Effect of induction by *T. urticae* through the time. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* on tomato leaves that were previously damaged for 0, 1, 2, 3 or 4 days by *T. urticae*. Different letters denote significant differences among treatments.

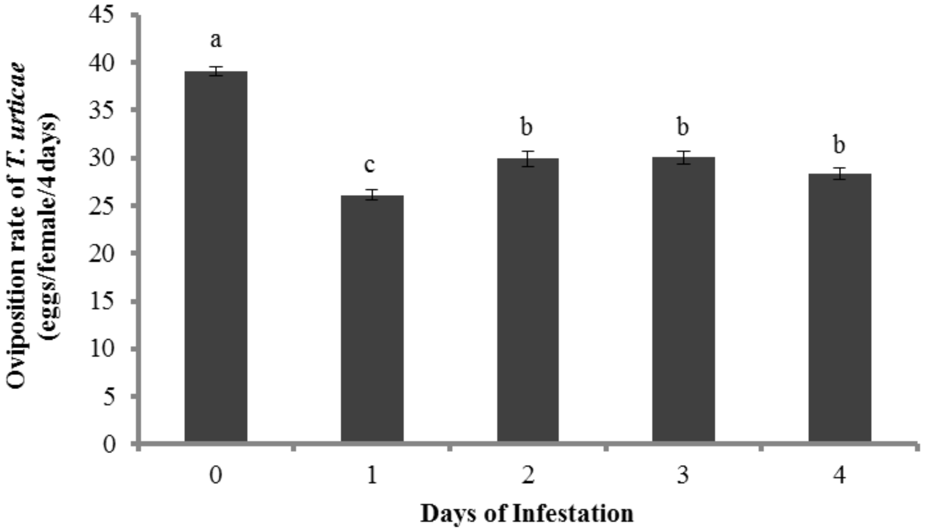

Figure 4 Effect of induction by *T. urticae* through the time. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. urticae* on tomato leaves that were previously damaged for 0, 1, 2, 3 or 4 days by conspecific mites. Different letters denote significant differences among treatments.

Trypsin inhibitor activity

The trypsin activity was determined in leaves from tomato plants that were damaged by *T. evansi* or *T. urticae* for 0, 1, 2, 3 or 4 days, obtained as in the experiment above. Proteinase inhibitors levels were significantly affected by herbivory and the numbers of days that the plant was damaged (**Figure 5**, $F_{4,15} = 3.2$, $P < 0.05$; **Figure 6**, $F_{4,15} = 8.6$, $P < 0.0001$). Levels of PI activity in leaves attacked by *T. evansi* were lower than activity levels in leaves of non-damaged plants after 1 day of induction onwards. The maximum decrease in activity was seen on the 4th day after damage **(Figure 5)**. PI activity in leaves attacked by *T. urticae* was higher than in non-damaged leaves **(Figure 6)**, suggesting that the proteinase inhibitor activity levels began to increase 1 day after infestation.

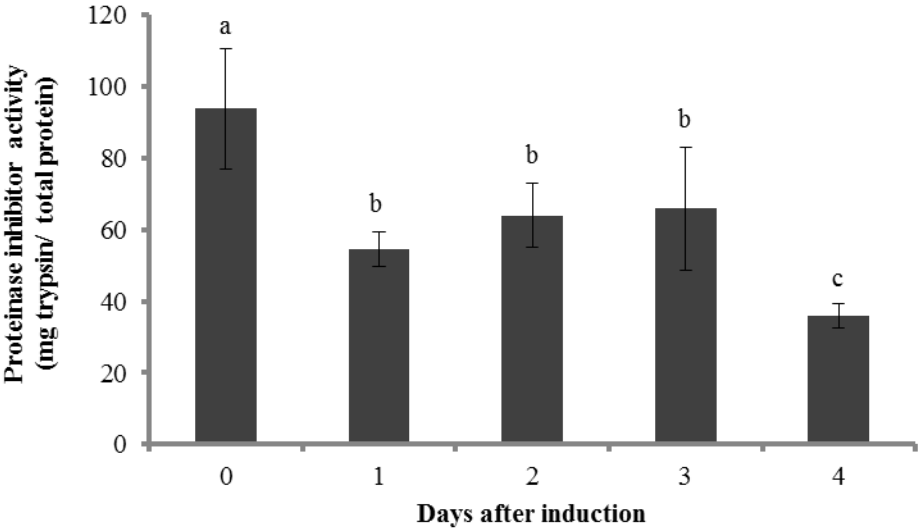

Figure 5 Proteinase inhibitor activity. Mean (+ SEM, n = 4 plants) proteinase inhibitor (PI) activity in leaves damaged for 0, 1, 2, 3 or 4 days by *T. evansi*. Bars labeled with different letters are significantly different.

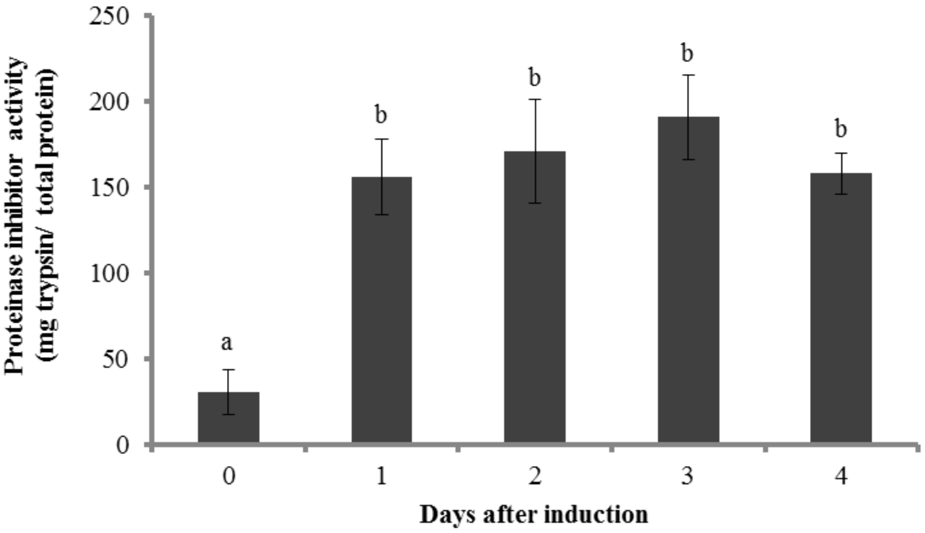

Figure 6 Proteinase inhibitor activity. Mean (+ SEM, n = 4 plants) proteinase inhibitor (PI) activity in leaves damaged for 0, 1, 2, 3 or 4 days by *T. urticae*. Bars labeled with different letters are significantly different.

DISCUSSION

Tomato plants have been intensively studied regarding both direct and indirect defenses (Karban & Baldwin, 1997). Studies have shown that herbivory induces responses, which can reduce their suitability to herbivores. In tomato plants, both herbivore feeding and mechanical damage induce systemic responses (Kessler & Baldwin, 2002, Rodriguez-Saona et al., 2005). Several studies have provided evidence for the adverse effect of induced plant defenses on arthropod feeding, growth and development (Karban & Baldwin, 1997; Mattiacci et al., 2001; Agrell et al., 2003; Soler et al., 2007).

Proteinase inhibitors are believed to serve as direct defenses against herbivore attack, primarily by interfering with absorption of dietary nutrients in the gut (Koiwa et al. 1997). In this study, we showed a rapid and stable increase in levels of these inhibitors after damage by *T. urticae*, with higher protein activities evident in damaged leaves after 1 to 4 days of infestation, suggesting that the proteinase inhibitor activity levels began to increase 1 day after damage. For phytophagous performance bioassays, we observed a lower oviposition rate for both spider mites species tested here on plants that were previously attacked than on clean plants, showing that they were negatively affected by the defenses induced by *T. urticae* over 4 days. These results are in agreement with the induction of plant defenses as reported for many herbivores and plants, where herbivore performance is lower on previously attacked plants than on non-damaged plants (Walling, 2000).

However, only recently it has been shown that herbivores can suppress wound-induced anti-herbivore defenses of their host plants (Musser et al., 2005; Bede et al., 2006; Lawrence et al., 2008; Kant et al., 2008) and exhibit higher rates of growth and survival (Sarmento et al., 2011a,b). We found that chemical responses of tomato were suppressed 1 to 4 days after the damage by *T. evansi*; the strongest decrease was seen on the 4th day after induction. Additionally, the oviposition rate of *T. evansi* was higher in plants damaged by *T. evansi* for 1 to 4 days than on clean plants. However, the oviposition rate of *T. urticae* on these plants was significantly higher only in the 2 first days of damaged by *T. evansi* when compared to clean plants. This suggests that both mite species can benefit from the down-regulated defense by *T. evansi*, which results in an improved food quality of the plant.

Our results are in accordance with Sarmento et al. (2011a), who showed that the spider mite *T. evansi* had a higher oviposition rate and a higher adult survival on plants that were previously attacked by conspecifics than on non-damaged plants. This manipulation may entail physiological costs, but ecological costs may also be involved. For example, the down-regulation of plant defenses may also benefit other herbivores (such as *T. urticae*) that compete with *T. evansi*. Therefore, the success of *T. evansi* in reducing plant defense compounds increases its own fitness but also the fitness of the other herbivore and it may also affect the community structure of herbivores on plants attacked by *T. evansi.*

In this experiment, the plant resistance observed is likely due to induction or reduction of chemical defenses, so changes in plant chemistry may have negative or positive effects on herbivore performance. In conclusion, the phytophagous mites *T. evansi* and *T. urticae* seem to affect plant defenses in a different manner, where such responses are induced by both species with only 1 day of infestation, directly influencing the plant resistance. The spider mite *T. urticae* increases the level of plant defense and one chemical compound that is thought to be associated with this defense, whereas *T. evansi* decreases both defenses and proteinase inhibitor activity. But it is not clear what might cause these responses in tomato plants.

These different effects of both closely related herbivores on plant defense may in theory be caused by the type of feeding damage (Felton et al., 1994; Stout et al. 1994), salivary constituents (Turlings et al., 1990; Mattiacci et al., 1995; Alborn et al., 1997), and the extent of damage imposed by the herbivore (Karban 1987, Agrawall 2000). However, differences in induction between herbivore species are normally related between herbivores in different feeding guilds (Walling 2000). Yet the two spider mites in this study are cell-content feeders and members of the family Tetranychidae.

Some studies have demonstrated that herbivore oral secretions elicit distinct inducible defenses of plants (Mattiacci et al., 1995; Alborn et al., 1997; McCloud & Baldwin, 1997; Musser et al., 2005). These salivary secretions may play a physiological role by manipulating host defensive responses and may to stimulate or suppress plant defenses. Here we hypothesize that salivary compounds released into the host cells through the stylets could be inducing different plant responses. However, there are few examples of collection and analysis of mite salivary secretions (but see Lillo & Monfreda, 2004).

A strategy that also may be being used by *T. evansi* to overcome plant defenses is the association with micro-organisms. The digestive system of herbivores holds a wide diversity of micro-organisms due to their continuous exposure to the external environment. Once in the herbivore gut, these micro-organisms can grow or can be excreted (Walker et al., 1999). It is known that the nutritional contribution of the intestinal microbiotic can occur improving the efficiency of digestion, acquisition of digestive enzymes and increasing supply of vitamins (Dillon & Dillon, 2004). Furthermore, there is evidence that metabolic products of some herbivore gut micro-organism are involved in the synthesis of defensive compounds in plants (Spiteller et al., 2000) that suggest that micro-organisms should be included as another trophic level in studies of induce plant defense by herbivores.

This study thus highlights the complexity of plant-herbivore interactions showing that induced responses depend on the species or strain of herbivores (Sarmento et al., 2011a; Kant et al., 2008), whereas different herbivores can affect each other's performance through induction or reduction of plant defenses. Our results also showed that the induction by mites *T. evansi* and *T. urticae* in tomato plants is rapid; occurring with only 1 day of infestation. Additionally, the plant resistance may be associated with chemical characteristics induced by each species of phytophagous mites during periods of herbivory.

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CHAPTER 2

Induced responses of tomato plants to multiple herbivores

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ABSTRACT

Induced plant responses to attacks by a single herbivore have been intensively studied. Much less is known about responses when plants are simultaneously attacked by more than one species. Here we determined the effect of simultaneous infestation on tomato plants by two species of spider (*T. evansi* and *T. urticae*) and its consequences for these herbivores. We assessed the levels of proteinase inhibitors in tomato leaves upon multi-species herbivory in comparison to single-species herbivory. It was also evaluated the effect of defense response on the fecundity of these spider mites. The results showed that the performance (oviposition) of both herbivores was higher on plants damaged only by *T. evansi*, lower in plants damaged only by *T. urticae* and intermediate on dual-damaged plants. Furthermore, upon herbivory by multiple-species, tomato plants induced higher levels of proteinase inhibitors compared with plants infested by *T. evansi* alone and did not differ significantly in the levels of proteinase inhibitors on plants infested only by *T. urticae*. Our results provide evidence that simultaneous herbivory by these two species of spider mites can affect their performance on tomato plants.

Key words: Simultaneous herbivory, specificity of plant responses, proteinase inhibitors, spider mites, *Tetranychus evansi*, *Tetranychus urticae*.

INTRODUCTION

Many species of plants employ a wide range of induced defense mechanisms in response to herbivore attack. These mechanisms are characterized by morphological changes and synthesis of secondary metabolites (Ament et al., 2004) that consequently can decrease herbivore performance.

The induction of plant defenses depends on the ability of the plant to identify and recognize its attackers (Wu & Baldwin, 2009). Thus, herbivore damage can cause chemical and morphological modifications in the plant physiology, which are specific to the type of arthropod species (Rodriguez-Saona et al., 2005). For example, caterpillars and aphids induce different responses in tomato plants; *Spodoptera exigua* induces the production of a variety of plant defenses including proteinase inhibitors (Broadway et al., 1986; Stout et al., 1998a) while the aphid *Macrosiphum euphorbiae* induces peroxidase and lipoxygenase activities but not proteinase inhibitor activities (Stout et al., 1998a; Fidantsef et al., 1999). Furthermore, leaves previously damaged by aphids were better sources of food for *S. exigua* than leaves which had not been fed upon by aphids (Stout et al., 1998a).

In tomato, it was also demonstrated that the spider mite *Tetranychus urticae* elicit different plant responses than the red spider mite *Tetranychus evansi*. For *T. urticae*, it was demonstrated that the mite induces defense responses, resulting in a decrease of plant quality (Kant et al., 2004), whereas *T. evansi* represses this plant defenses, resulting in an increase of plant quality (Sarmento et al., 2011).

Ecologically, the ability of the plant to generate distinct chemical responses to different damage types is important because damage by a single herbivore is rare; more commonly, plants are attacked by different species of herbivores at the same time (Rodriguez-Saona et al., 2005).

However, there are few studies that show what happens when plants are simultaneously attacked by more than one species and its consequences for herbivores (Rodriguez-Saona et al., 2005). To date, studies on the production of herbivore-induced plant volatiles have attempted analyzing the chemical composition of volatile blends upon multi-species herbivory (Shiojiri et al., 2001; Rodriguez-Saona et al., 2003; Zhang et al., 2009). These volatiles play an important role in the interactions between plants, herbivores, and natural enemies in food webs (De Boer et al., 2008) and can be affected by simultaneous herbivory (Shiojiri et al., 2001; Dicke et al., 2009, Rodriguez-Saona et al., 2010).

In addition, plant responses to one species of herbivore can be affected by the plant's response to other species of herbivores on the plant. Consequently, the effects of the feeding of multiple herbivores can differentially affect components of the plant defense system (Rodriguez-Saona et al., 2005).

The purpose of this chapter was to determine whether simultaneous infestation of tomato plants by two species of spider mites (*T. urticae* and *T. evansi*) had additive, synergistic or antagonistic effects in plant responses. Given the specificity of tomato plant responses to spider mites and the potential of interactions between these responses, in this chapter, we investigated the effects of simultaneous herbivory by *T. urticae* and *T. evansi* on host plant quality. In particular, we evaluated the performance of these two herbivores in these plants. Subsequently, we evaluated the activity of proteinase inhibitors in plants attacked by either of the two or both species. These proteins have the capacity to inhibit proteolytic enzymes of arthropod herbivores (Green & Ryan, 1972) and can significantly reduce their growth and development (Broadway & Duffey, 1986). Therefore, this chemical compound may be involved and play an important role in tomato plants defense against spider mites.

MATERIALS AND METHODS

Plant material

Tomato seeds (*Solanum lycopersicum* var Santa Clara I-5300) were weekly sown in a commercial plant substrate (Bioplant®, Bioplant Misturadora Agrícola LTDA) in a polystyrene tray (8 x 16 cells), and kept inside a cage with a fine mesh in a greenhouse to avoid infestation with herbivores. After 21 days, plants were transferred to plastic pots (2 L) that contained a mixture of soil plus fertilizer (4-14-8 N-P-K). Tomato plants were further grown in mite-proof screen cages in a greenhouse until they were 45 days old and had at least four completely developed leaves. Subsequently, plants were used either for the experiments or for spider mite rearing.

Mite rearing

Spider mites (*T. evansi* and *T. urticae*) were obtained in 2002 from naturally infested tomato plants of the same variety mentioned above in a greenhouse at the Federal University of Viçosa, Brazil. Both species were cultured on detached tomato leaves, of which the petiole was inserted in a PVC tube with water to maintain leaf turgor. Tubes with infested leaves were kept in PVC trays filled with detergent and water (1:25, v ⁄ v), which served to prevent mite escapes and invasion of mites and other nonflying arthropods. The mass culture was maintained in a room at 25 ± 3 ºC, 70–90% relative humidity and 12 h light.

Induction of plants

The experiment consisted of the following treatments: i) plants simultaneously damaged by *T. evansi* e *T. urticae*; ii) plants damaged by *T. evansi*; iii) plants damaged by *T. urticae*; and iv) non-damaged plants. To obtain damaged plants, 100 adult mites were put on each plant. In the first treatment, 100 adult mites of each species were put on each plant, hence 200 adult mites per plant. Previous experiments showed that the difference in the number of individuals did not affect the performance of spider mites (**Figure 1**, $F_{1,14} = 0.06$, $P = 0.80$ and **Figure 2,** $F_{1,14} = 1.14$, $P = 0.30$). One leaf of four randomly selected 45 days old tomato plants was infested for 1 day as described above, while the other leaves were kept clean. This time was sufficient for these spider mites to induce biochemical responses in the plants (Chapter 1). Insect glue (Cola Entomológica; Bio-Controle, São Paulo, Brazil) was applied to the petioles of the leaf on which the mites were released to prevent them from moving to another leaf. Control plants from the same batch and the same age were also treated with glue. Plants were kept inside mite-proof screen cages in a greenhouse during the experiment.

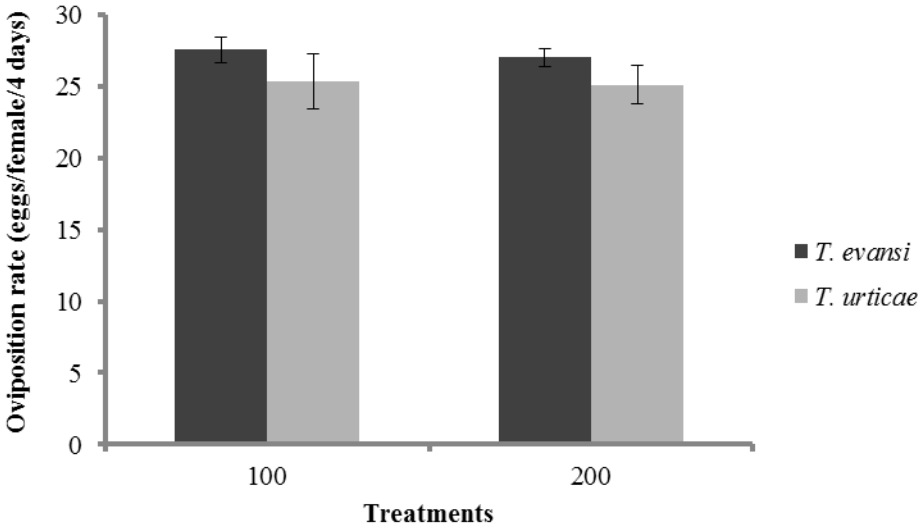

Figure 1 Performance of *Tetranychus evansi* and *Tetranychus urticae*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* and *T. urticae* on leaves that were damaged by 100 and 200 females of *T. evansi* (GLM, P > 0.05).

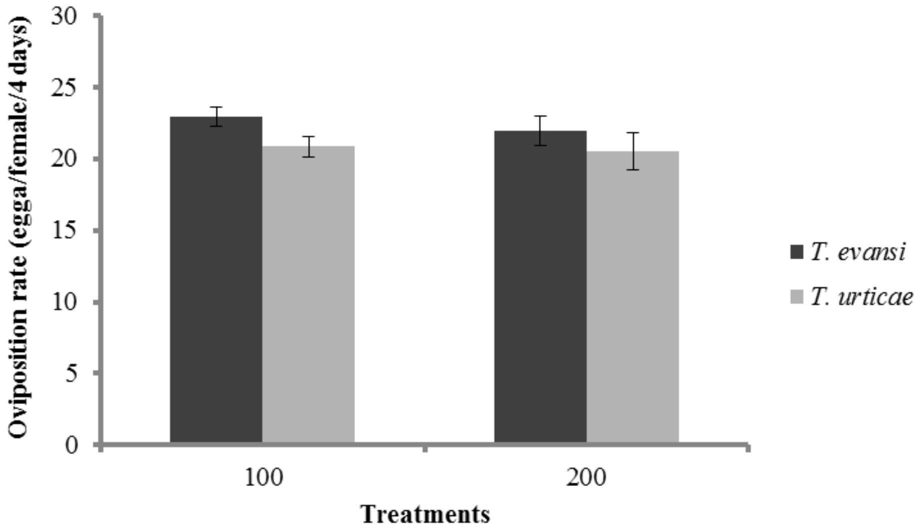

Figure 2 Performance of *Tetranychus evansi* and *Tetranychus urticae*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* and *T. urticae* on leaves that were damaged by 100 and 200 females of *T. urticae* (GLM, P > 0.05).

Spider mite fecundity

The oviposition rate of *T. evansi* and *T. urticae* was measured on damaged leaves of plants infested by *T. evansi* and *T. urticae* and on leaves of undamaged plants (controls). After infestation for 1 day, the mites, their web and eggs were removed, and 10 leaf discs (12 mm Ø) were made from the damaged leaves and from corresponding leaves of non-infested control plants. All leaf discs were inspected with a stereoscopic microscope to ensure that no spider mite eggs were present. Discs were subsequently kept in Petri dishes (8 cm Ø) containing wet cotton wool.

One randomly selected adult female of *T. evansi* or *T. urticae*, 2 days old since turning adult, was individually placed on each disc, at 28 ± 2 ºC; 70 ± 10% RH and 12 h light. The oviposition rate was evaluated every 24 h for a period of 4 days. Each replicate consisted of a group of 10 leaf discs and each treatment was replicated 4 times.

Differences in mean oviposition rates per plant among treatments were analyzed using a linear mixed-effects model (lme) with treatment as fixed effect and replicate as a random factor.

Proteinase inhibitor assays

The proteinase inhibitor activity was measured in the same leaves used for the oviposition experiments. For this, it was separated 2 leaflets per leaf. Crude protein was extracted from these leaflets of tomato plants. The leaflets were frozen in liquid nitrogen and stored at -80 ºC. Subsequently, each sample was ground with in a mortar and pestle and a crude extract was obtained as described by Otha et al. (1986). Essentially, the leaves were homogenized in extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$; 1:3 w⁄v) and the homogenate centrifuged at 17200 g for 30 min at 4 ºC and the supernatant was collected. The resulting supernatant was used to determine the protein content and all other assays. Protein concentration was determined by the method described by Bradford (1976), using a solution of 0.2 mg/ml bovine serum albumin (BSA) as standard.

A standard spectrophotometric assay was used to measure trypsin inhibitory activity in the supernatant. A 100 µL aliquot of trypsin (4.7×10^{-5} M) was mixed with 100 µL of the supernatant and 500 µL extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$). The mixture was incubated at room temperature for 5 min. Controls consisted of 600 µL extraction buffer and 100 µL of trypsin (4.7×10^{5} M). A 700 µL aliquot of the mixture (tests and controls) was added to 500 µL extraction buffer and 500 µL D,L-BApNA (1.2 mM). Trypsin activity was monitored for 150 seconds at intervals of 30 seconds at 410 nm absorbance on a spectrophotometer. The difference between the absorbance measured at 150 and 60 seconds was used to determine the trypsin activity. Measurements were performed in triplicate per sample.

The results obtained were converted to milligrams trypsin inhibited per gram of protein according to the following equation: mg trypsin inhibited per gram of protein = AB/1000PC with A = enzyme control – absorbance at 410 nm of the extract; B = sample dilution; P = protein concentration of the extracts, in g/mL; and C = trypsin factor, the result from the activity of 1 µg of trypsin on the substrate D,L-BApNA measured at 410 nm, for the combination of trypsin and D,L-BApNA = 0,019 (Kakade et al., 1974). Differences among treatments were analyzed with a Generalized Linear Model with a normal error distribution.

RESULTS

Spider mite fecundity

The rate of oviposition of *T. evansi* varied with time (**Figure 3**, $F_{3,45} = 66.2$, $P < 0.0001$) and there was a significant effect of the treatments (**Figure 3**, $F_{3,45} = 87.6$, $P < 0.0001$). Furthermore, a significant interaction ($F_{9,45} = 9.6$, $P < 0.0001$) was observed between treatment and time.

The oviposition rate of *T. evansi* was significantly higher on tomato leaves that had received damaged by conspecifics than on leaves of plants infested by *T. urticae*, *T. evansi* + *T. urticae* and clean plants (**Figure 3,** Log-likelihood-ratio-test, $P < 0.0001$). Additionally, the oviposition rate was significantly higher on leaves from clean plants than on leaves damaged by *T. evansi* + *T. urticae* and leaves damaged by *T. urticae* (**Figure 3**, Log-likelihood-ratio-test, $P < 0.005$ and $P < 0.0001$, respectively). It also was significantly higher on tomato leaves damaged by *T. evansi* + *T. urticae* than on leaves damaged by *T. urticae* (**Figure 3**, Log-likelihood-ratio-test, $P < 0.0001$).

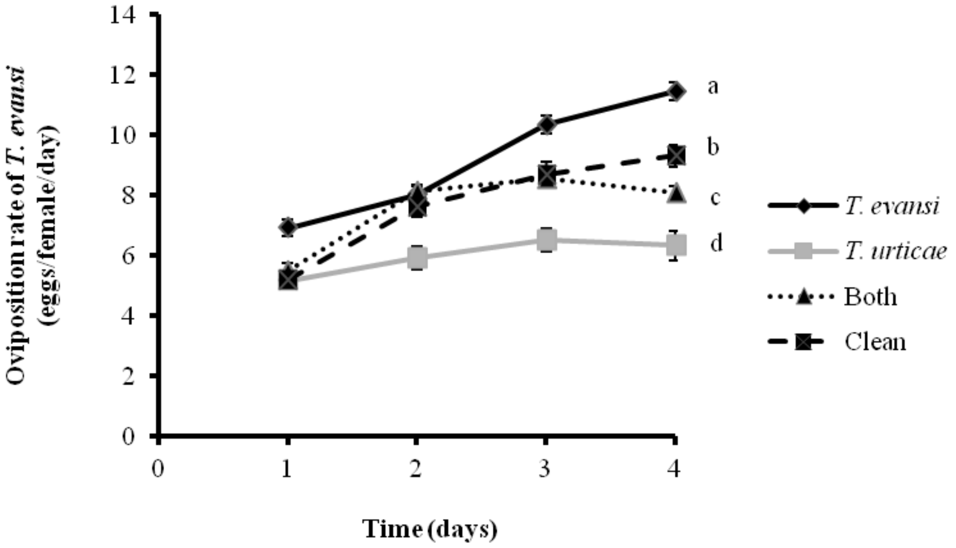

Figure 3 Performance of *Tetranychus evansi*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* on tomato leaves that were damaged by *T. evansi*; *T. urticae*; both (*T. evansi* + *T. urticae*) and leaves from clean plants. Different letters denote significant differences among treatments through time.

The oviposition of *T. urticae* also varied with time (**Figure 4**, $F_{3,45} = 41.9$, $P < 0.0001$) and there was a significant effect of the treatments (**Figure 4**, $F_{3,45} = 120.4$, $P < 0.0001$). There was a significant interaction ($F_{9,45} = 6.9$, $P < 0.0001$) between treatment and sampling time. The oviposition rate of *T. urticae* was significantly higher on tomato leaves that were damaged by *T. evansi* than on leaves infested by *T. urticae*, by both mites and clean plants (**Figure 4**, Log-likelihood-ratio-test, $P < 0.0001$).

The oviposition of *T. urticae* was significantly higher on leaves from clean plants than on leaves damaged by *T. evansi* + *T. urticae* and leaves damaged by *T. urticae* (**Figure 4**, Log-likelihood-ratio-test, $P < 0.001$ and $P < 0.0001$, respectively). It was also significantly higher on tomato leaves damaged by *T. evansi* + *T. urticae* than on leaves damaged by conspecifics (**Figure 4**, Log-likelihood-ratio-test, P < 0.0001).

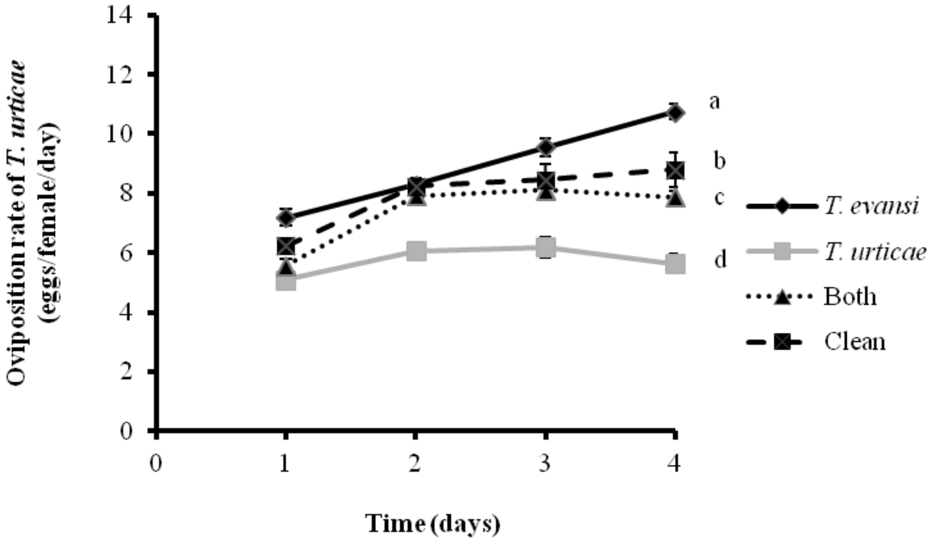

Figure 4 Performance of *Tetranychus urticae*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. urticae* on tomato leaves that were damaged by *T. evansi*; *T. urticae*; both (*T. evansi* + *T. urticae*) and leaves from clean plants. Different letters denote significant differences among treatments through time.

Trypsin inhibitor activity

The trypsin activity was determined in leaves from tomato plants that were damaged by *T. evansi*, *T. urticae*, *T. evansi* + *T. urticae* and leaves from clean plants. There was a significant effect of the treatments (**Figure 5**, Chi^2 = 17071, d.f. = 3, P < 0.005). Post-hoc comparisons showed that leaves damaged by *T. evansi* and leaves from clean plants had the lowest inhibitor activity, whereas leaves damaged by *T. evansi* + *T. urticae* and leaves damaged by *T. urticae* showed the highest inhibitor activity against trypsin **(Figure 5)**. There was no significant difference in proteinase inhibitor activity between leaves that were damaged by *T. evansi* and clean leaves (Chi-Test, P = 0.5264), and between leaves that were damaged by *T. evansi* + *T. urticae* and leaves damaged by *T. urticae* (Chi-Test, P = 0.5098).

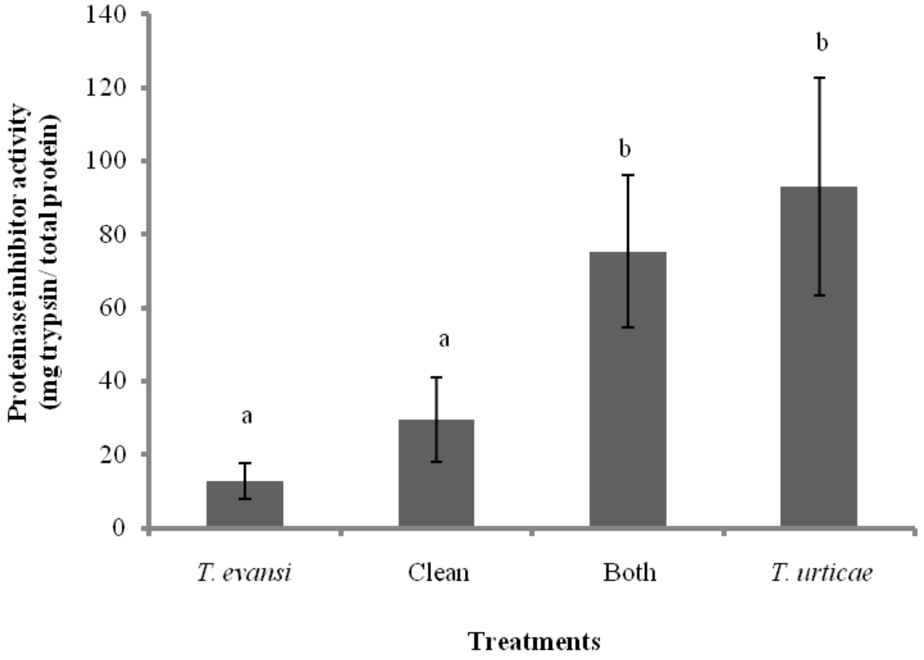

Figure 5 Proteinase inhibitor activity. Mean (+ SEM, n = 4 plants) proteinase inhibitor (PI) activity in leaves damaged by *T. evansi*; *T. urticae*; both (*T. evansi* + *T. urticae*) and leaves from clean plants. Within each panel, bars labeled with different letters are significantly different.

DISCUSSION

In natural ecosystems, plants are commonly attacked by more than one species of herbivore (Strauss et al., 1991; Delphia et al., 2007; De Boer et al., 2008). Interactions between plants and multiple species of herbivores may affect the preference and performance of herbivores and their natural enemies (Shiojiri et al., 2001; Vos et al., 2001). These responses depend on the identity and quantity of arthropod species feeding on the plant (Rodriguez-Saona et al., 2005).

In the phytophagous performance bioassays, it was observed that *T. urticae* and *T. evansi* induce different levels of plant resistance, but that dual-damaged plants were phenotypically distinct from plants damaged by either herbivore alone. For example, the rate of oviposition of *T. evansi* and *T. urticae* was higher on plants damaged only by *T. evansi*, and it was lower on plants damaged only by *T. urticae*. We observed an intermediate oviposition rate for both species (*T. evansi* and *T. urticae*) on dual-damaged plants compared with plants that were induced by each of the herbivores separately (**Figure 3** and **4**). Thus, we hypothesized that the plant defensive response to *T. urticae* was weakened when *T. evansi* also feed on the plant, possibly caused by antagonistic effects of each species of spider mite.

Recent studies demonstrated that most volatile compounds induced by multi-species herbivory quantitatively differ from that of the sum of each of the herbivores feeding separately (De Boer et al., 2008; Dicke et al., 2009), indicating that biosynthetic pathways may act synergistically or antagonistically. Thus, plant responses to feeding by a single herbivore species can differ from response to attack by multiple herbivores (Shiojiri et al., 2001; Rodriguez-Saona et al., 2003) and can have both positive and negative effects on preference and performance of subsequent herbivores, as well as natural enemies of such herbivores (Cardoza et al., 2002; Shiojiri et al., 2002; Rodriguez-Saona et al., 2005).

In this study, we also showed that plants induced by multiple-species herbivory (*T. evansi* and *T. urticae*) significantly differed in the levels of proteinase inhibitors compared to plants infested with a single herbivore species (*T. evansi*). Furthermore, plants induced by multiple-species herbivory did not significantly differ in the levels of proteinase inhibitors to the plants infested with a single herbivore by *T. urticae*. Our data show that *T. urticae* induced high PI enzymatic activity; plants damaged by *T. urticae* had three times higher PI enzyme activity compared to control plants (**Figure 5**), while *T. evansi* had no effect on PI activity. However, the presence of *T. evansi* did not significantly affect the PI activity induced in response to *T. urticae* damage. This suggests that the two herbivores feeding simultaneously on the same plant do not have an additive effect on proteinase inhibitor activity, potentially indicating that the spider mite *T. urticae* can exert a greater effect on its host plant.

In summary, plant responses to dual-damage may outcome in additive responses due to lack of response specificity to different herbivores or may also result in specificity in the elicitation of response. Thus, the plant may respond to each herbivore differently but the induced responses to each herbivore species when the plant is damaged by both herbivores can be entire or attenuated (Rodriguez-Saona et al., 2010). Here, we show an example of specificity in the plant response; there was an attenuation of the response in the levels of proteinase inhibitors when the plant is dual-damaged. However, the performance of these two herbivores showed that there was an antagonistic effect of the specific response of each species of spider mite.

Thus, we suspect that other compounds in the plant defense system may have influenced resistance to herbivores.

Responses to herbivore feeding in tomato plants are numerous and include increase in peroxidase, polyphenol oxidase, proteinase inhibitors and lipoxygenase activities (Schaller & Ryan, 1995; Stout et al., 1996; Stout et al., 1998a,b). All induced chemical compounds comprise the overall defensive plant response and this is the reason the difficulty in assigning specific roles in induced resistance to the individual compounds (Stout et al., 1998b). In this study, other defensive chemicals may have been involved in the defense of tomato plants, but only the levels of proteinase inhibitors were evaluated.

Our results suggest that exposure of tomato plants to more than one herbivore induces more complex phenotypic changes in a plant than attacks by single species of herbivores, which affect the performance of these herbivores (van Zandt & Agrawal, 2004; Dicke et al., 2009).

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CHAPTER 3

Sequential feeding by spider mites affects plant defense responses

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ABSTRACT

Induced responses by one herbivore can alter host plant quality and affect the behavior and performance of subsequent herbivores. In this chapter, we investigated how sequential attacks by the spider mites *T. evansi* and *T. urticae* can interfere in chemical responses of tomato plants and also affect the performance of both species. We assessed the levels of proteinase inhibitors activity in response to early herbivory and the interaction of these chemical responses with responses to secondary herbivory. It was also evaluated the fecundity of both spider mites on these plants. We observed that spider mites induced different plant resistance after sequential attacks. The results showed that the last herbivore that fed on a plant has a greater effect on its quality. We showed that the rate of oviposition of both spider mites was dependent on the defense response elicited by each species before and after sequential attack. The results also indicated that the sequence of herbivores arrival is an important determinant of PI activity. We showed that leaves damaged by *T. urticae* had the highest inhibitor activity but subsequent herbivory on these leaves by *T. evansi* significantly decreased the levels of PI. Furthermore, leaves damaged by *T. evansi* had the lowest inhibitor activity but subsequent herbivory by *T. urticae* on leaves firstly damaged by *T. evansi* showed intermediate levels of PI. Remarkably, the second spider mite to feed on tomato plants may modify induced resistance of the first attacker.

Key words: Plant-herbivore interactions, sequential attack, plant quality, plant defense.

INTRODUCTION

Plants use several constitutive and induced strategies to defend against damage caused by pathogens and herbivorous arthropods (Thaler et al., 1999). These plant responses have emerged as a determinant factor of plant quality (Denno et al., 1995; Viswanathan et al., 2007). Changes in plant metabolites induced by arthropod feeding may modify the nutritional value and alter the physiology of the host plant and these changes may affect other herbivores (Inbar et al., 1999). Thus, the plant defenses induced by a first herbivore can affect interspecific competition with other herbivores (Moayeri et al., 2007; Kaplan & Denno, 2007).

Furthermore, plants are commonly attacked by different species of herbivores (Voelckel & Baldwin, 2004; Rodriguez-Saona et al., 2010), and the order of arrival of different species on the same plant may determine the defense that is induced and therefore, the outcome of interactions among herbivore species. For example, plant defense may be influenced more by the first herbivore to feed on a plant and this may limit the plant response to subsequent attackers (Viswanathan et al., 2007). In contrast, the plant response may be more affected by the last herbivore to fed on a plant. In this situation, a plant may change the induction generated by the first herbivore and redirect the responses to subsequent herbivores (Viswanathan et al., 2007). And finally, the plant response may be influenced by all herbivores that fed on plant, thus the induced defenses may differ from those expected for each herbivore separately due to an interaction between the responses induced sequentially (Poelman et al., 2008; Voelckel & Baldwin, 2004; Viswanathan et al., 2007).

Recent studies showed the effect of feeding by multiple species on induction of plant defenses (Inbar & Gerling, 2008, Poelman et al., 2008; Rodriguez-Saona et al., 2010). For example, Voelckel & Baldwin (2004) found a different transcriptional expression of defensive responses in tobacco plants after different forms and orders of attacks. These studies show that plant responses are specific to species of attackers and that the defenses induced by multiple and sequential herbivory are different from the defenses induced by each herbivore individually (Shioriji et al., 2001; Rodriguez-Saona et al., 2003; Viswanathan et al., 2007).

We demonstrated in a previous chapter that the temporal dynamics of induced responses in tomato plants depend on the inducing species. For example, the mite *Tetranychus urticae* induced direct defenses in tomato plants resulting in lower performance of both *T. urticae* and the closely related *T. evansi*. In contrast, the performance of the herbivores was higher on plants previously damaged by *T. evansi* (Chapter 1). Parallel to this, the attacks by *T. urticae* resulted in increased levels of proteinase inhibitors whereas *T. evansi* suppressed proteinase inhibitors levels. The performance of the herbivores was also influenced on dual-damaged plants compared with plants that were induced by each of the spider mites separately (Chapter 2).

Considering the fact that these spider mites can differently affect defense responses in tomato plants, here we investigate how sequential attacks by these spider mites can affect the performance of both species and interfere in the chemical responses of tomato plants.

MATERIALS AND METHODS

Plant material

Tomato seeds (*Solanum lycopersicum* var Santa Clara I-5300) were weekly sown in a commercial plant substrate (Bioplant®, Bioplant Misturadora Agrícola LTDA) in a polystyrene tray (8 x 16 cells), and kept inside a cage with a fine mesh in a greenhouse to avoid infestation with herbivores. After 21 days, plants were transferred to plastic pots (2 L) that contained a mixture of soil plus fertilizer (4-14-8 N-P-K). Tomato plants were further grown in mite-proof screen cages in a greenhouse until they were 45 days old and had at least four completely developed leaves. Subsequently, plants were used either for the experiments or for spider mite rearing.

Mite rearing

Spider mites (*T. evansi* and *T. urticae*) were obtained in 2002 from naturally infested tomato plants of the same variety mentioned above in a greenhouse at the Federal University of Viçosa, Brazil. Both species were cultured on detached tomato leaves, of which the petiole was inserted in a PVC tube with water to maintain leaf turgor. Tubes with infested leaves were kept in PVC trays filled with detergent and water (1:25, v ⁄ v), which served to prevent mite escapes and invasion of mites and other non-flying arthropods. The mass culture was maintained in a room at 25 ± 3 ºC, 70–90% relative humidity and 12 h light.

Induction of plants

In this experiment, firstly a group of plants was infested with *T. evansi* (100 adult mites/plant) and another group of plants was infested with *T. urticae* (100 adult mites/plant). One leaf of four randomly selected tomato plants was infested for 1 day, while the other leaves were kept clean. This time was sufficient for these spider mites to induce host plant biochemical responses (Chapter 1). Insect glue (Cola Entomológica; Bio-Controle, São Paulo, Brazil) was applied to the petioles of the leaf on which mites were released to prevent them from moving to another leaf.

After 1 day of induction, the mites, their web and eggs were removed. The same leaf was submitted to re-infestation by hetero-specific mites (100 adult mites/plant). For example, the group of plants were firstly infested by *T. evansi* were re-infested by *T. urticae* and vice versa. Controls consisted of a group of plants that were first left clean and were then infested by *T. evansi* or *T. urticae*. The re-infestation occurred during 1, 2, 3 or 4 days, hence, leaves were collected on the day of re-infestation (0), 1 d (+1), 2 d (+2), 3 d (+3) or 4 d (+4) after re-infestation (**Figure 1**). Plants were kept inside mite-proof screen cages in a greenhouse during the experiment.

Spider mite fecundity

The oviposition rate of *T. evansi* and *T. urticae* was measured on leaves of plants as described above. After removal of mites, web and eggs, 10 leaf discs (12 mm Ø) were made from damaged leaves and from corresponding leaves of control plants. All leaf discs were inspected with a stereoscopic microscope to ensure that no spider mite eggs were present. Discs were subsequently kept in Petri dishes (8 cm Ø) containing wet cotton wool.

One randomly selected adult female of *T. evansi* or *T. urticae*, 2 days old since turning adult, was placed individually on each disc. After 4 days (28 ± 2 ºC; 70 ± 10% RH; 12 h light) the oviposition rate was measured. Each replicate consisted of a group of 10 leaf discs and each treatment was replicated 4 times.

Differences in mean oviposition rates per plant among treatments were analyzed using Generalized Linear Model with a gamma error distribution.

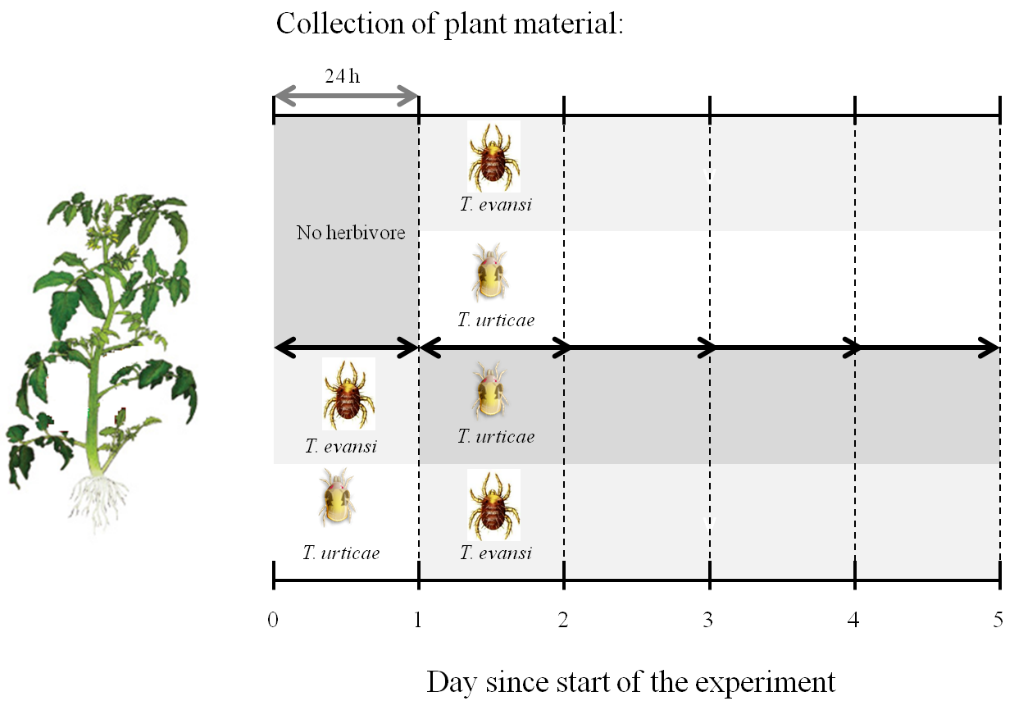

Figure 1 Experimental setup of plant induction. Plants were exposed to *T. evansi* or *T. urticae* herbivory for 1 day; control plants were kept clean. Mites, web and eggs were removed after 1 day. Afterwards, induced and control plants were infested with spiders mites of one of the two subsequent herbivores; the time of infestation lasted 1 to 4 days. Plant material was collected at times indicated by dotted lines.

Proteinase inhibitor assays

The proteinase inhibitor activity was measured in the same leaves used for the oviposition experiments. For this, it was separated 2 leaflets per leaf. Crude protein was extracted from these leaflets of tomato plants. The leaflets were frozen in liquid nitrogen and stored at -80 ºC. Subsequently, each sample was ground with mortar and pestle and a crude extract was obtained as described by Otha et al. (1986). Essentially, the leaves were homogenized in extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$; 1:3 w/v) and the homogenate centrifuged at 17200 g for 30 min at 4 ºC and the supernatant was collected. The resulting supernatant was used for determining the protein content and all other assays. Protein concentration was determined by the method described by Bradford (1976), using a solution of 0.2 mg/ml bovine serum albumin (BSA) as standard.

A standard spectrophotometric assay was used to measure trypsin inhibitory activity in the supernatant. A 100 µL aliquot of trypsin (4.7 $\times 10^{-5}$ M) was mixed with 100 µL of the supernatant and 500 µL extraction buffer (0.1 M Tris-HCl buffer, pH 8.2 and 20 mM $CaCl_2$). The mixture was incubated at room temperature for 5 min. Controls consisted of 600 µL extraction buffer and 100 µL of trypsin (4.7 $\times 10^{5}$ M). A 700 µL aliquot of the mixture (tests and controls) was added to 500 µL extraction buffer and 500 µL D,L-BApNA (1.2 mM). Trypsin activity was monitored for 150 seconds at intervals of 30 seconds at 410 nm absorbance on a spectrophotometer. The difference between the absorbance measured at 150 and 60 seconds was used to determine the trypsin activity. Measurements were performed in triplicate per sample.

The results obtained were converted to milligrams trypsin inhibited per gram of protein according to the following equation: mg trypsin inhibited per gram of protein = AB/1000PC with A = enzyme control – absorbance at 410 nm of the extract; B = sample dilution; P = protein concentration of the extracts, in g/mL; and C = trypsin factor, the result from the activity of 1 µg of trypsin on the substrate D,L-BApNA measured at 410 nm, for the combination of trypsin and D,L-BApNA = 0,019 (Kakade et al., 1974). Results were analyzed with a Generalized Linear Model with a normal error distribution.

RESULTS

Spider mite fecundity

The rate of oviposition of *T. evansi* varied with time of infestation (**Figure 2,** Deviance = 0.14, d.f. = 4, P < 0.05) and there was a significant effect of the treatments (**Figure 2,** Deviance = 1.06, d.f. = 3, P < 0.0001). Furthermore, a significant interaction (Deviance = 0.84, d.f. = 12, P < 0.0001) was observed between treatment and time of infestation.

The results show that prior to the second infestation, the oviposition rate of *T. evansi* was higher on plants attacked by *T. evansi*, intermediate on clean plants and lower on plants damaged by *T. urticae* (cf. bars of 0, **Figure 2**). After the second infestation, the oviposition rate on plants that were first clean and later damaged by *T. evansi* was significantly increased. In contrast, the rate of oviposition on plants that were first clean and later damaged by *T. urticae* showed a decreased in oviposition, but this effect was not significant. However, the oviposition of *T. evansi* on plants that were first attacked by *T. evansi* and subsequently damaged by *T. urticae* showed a significant decrease from day 0 to day 5, whereas the oviposition in plants first attacked by *T. urticae* and subsequently damaged by *T. evansi* showed a significant increase with time.

The oviposition rate of *T. evansi* was also affected by the treatments according to the time of re-infestation (**Figure 2**).

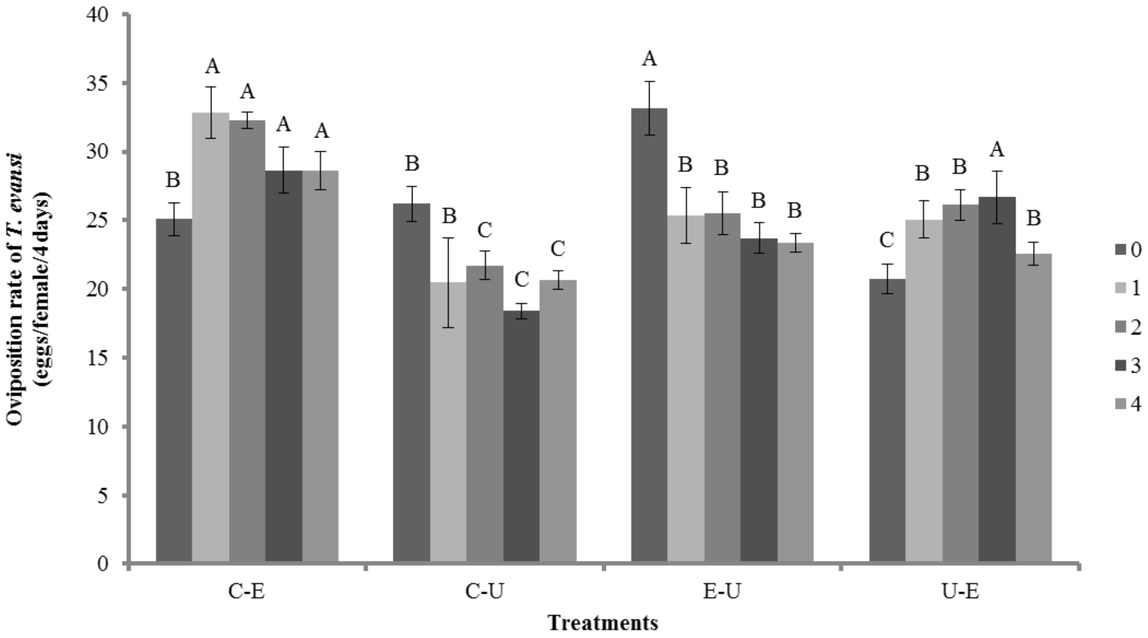

Figure 2 Performance of *Tetranychus evansi*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. evansi* on tomato leaves that were firstly damaged by *T. evansi* or *T. urticae* and afterwards re-infested by mites of the other species (E-U and U-E) and tomato leaves that were left clean at first and were infested by *T. evansi* or *T. urticae* later (C-E and C-U). Lowercase letters compare the time of re-infestation (0, 1, 2, 3 and 4 days) of each treatment and uppercase letters compare treatments in each time.

The oviposition rate of *T. urticae* did not vary with time of infestation, but there was a significant effect of the treatments (**Figure 3**, Deviance = 0.57, d.f. = 3, $P < 0.0001$). Also, a significant interaction (Deviance = 0.30, d.f. = 12, $P < 0.001$) was observed between treatment and time of infestation. The results showed that prior to the second infestation, the oviposition rate of *T. urticae* was higher on plants pre-infested by *T. evansi* and clean plants and lower on plants pre-infested by *T. urticae* (cf. bars of 0, **Figure 3**). After the second infestation, the oviposition rate of *T. urticae* on clean plants (without pre-infestation) that were subsequently damaged by

T. urticae showed a significant decrease from day 0 to day 5, whereas the oviposition in plants firstly attacked by *T. urticae* and subsequently damaged by *T. evansi* showed a significant increase with time. This effect was not observed in other treatments. However, the oviposition rate of *T. urticae* was affected by the treatments according to the time of re-infestation (**Figure 3**)

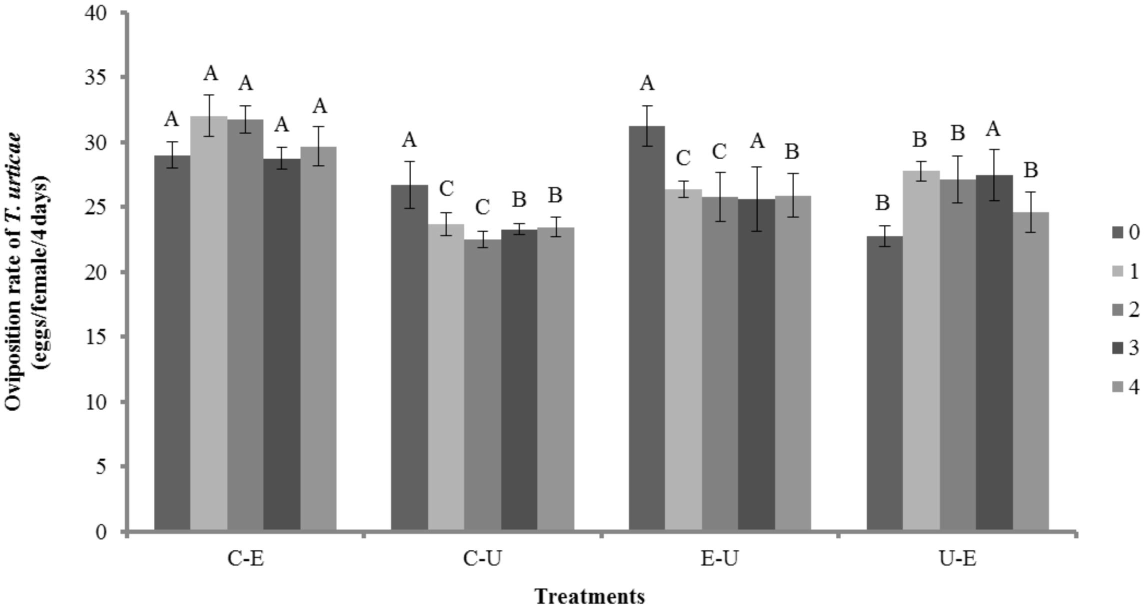

Figure 3 Performance of *Tetranychus urticae*. Mean (± SEM, four plants, 10 mites per plant) oviposition of *T. urticae* on tomato leaves that were firstly damaged by *T. evansi* or *T. urticae* and afterwards re-infested by mites of the other species (E-U and U-E) and tomato leaves that were left clean at first and were infested by *T. evansi* or *T. urticae* later (C-E and C-U). Lowercase letters compare the time of re-infestation (0, 1, 2, 3 and 4 days) of each treatment and uppercase letters compare treatments in each time.

Trypsin inhibitor activity

The trypsin activity was determined in leaves from tomato plants that received the same treatments as above. Proteinase inhibitor levels were significantly affected by treatments and the numbers of days elapsed after re-infestation (**Figure 4**, Deviance = 167169, d.f. = 3, P < 0.001; Deviance = 70408, d.f. = 4, P < 0.01). Post-hoc comparisons showed that after introducing *T. urticae* as a second herbivore, pre-infested plants produced lower levels of proteinase inhibitors compared to plants without pre-infestation. However, post-hoc comparisons showed that after introducing *T. evansi* as a second herbivore, pre-infested plants did not produce significant difference in levels of proteinase inhibitors compared to plants without pre-infestation (Chi-Test, P = 0.0947).

Therefore, the highest levels of proteinase inhibitors occurred on leaves of plants only damaged by *T. urticae*, and the lowest levels were on plants that were posteriorly damaged by *T. evansi* (**Figure 4)**.

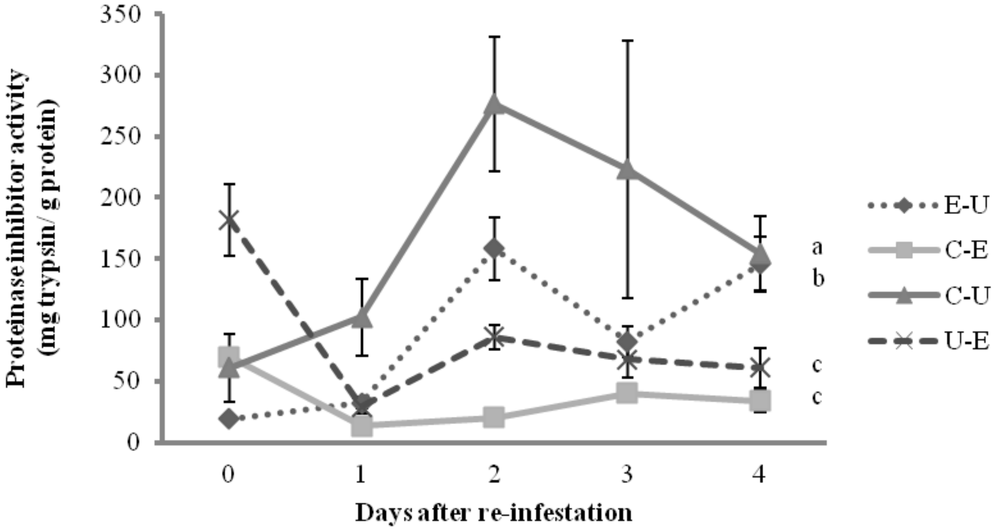

Figure 4 Proteinase inhibitor activity. Mean (+ SEM, n = 4 plants) proteinase inhibitor (PI) activity in tomato leaves that were first damaged by *T. evansi* or *T. urticae* and later re-infested by hetero-specific mites (E-U and U-E) and tomato leaves that were first clean and later were infested by *T. evansi* or *T. urticae* (C-E and C-U).

DISCUSSION

Induced plant defenses by first herbivory are known to differentially affect secondary herbivores (Kaplan & Denno, 2007; Poelman et al., 2008). Although many herbivores avoid plants that are damaged by other herbivore species (Sato et al., 1999; De Moraes et al., 2001; Pallini et al., 1997) they can learn to distinguish differences in food quality (Egas et al., 2003) and are therefore perhaps capable of locating plants with defenses weakened by earlier attacks.

Recent studies demonstrated that *T. evansi* down-regulates tomato plant defenses whereas *T. urticae* up-regulates it (Kant et al., 2004; Sarmento et al., 2011). Here we demonstrate that these spider mites that elicit specific plant responses can induce a similar pattern of responses after sequential attacks. The results show that the effects on plant quality and defense of the last herbivore to attack the plant eventually overruled effects of the first herbivore. For example, *T. urticae* induced defenses in the plant which decreased the performance of later-arriving herbivores. But even with the up-regulated of defenses, *T. evansi* can manipulate and suppress these responses resulting in increased of the herbivore performance. In contrast, the suppression of defenses caused by *T. evansi* improved the plant quality for mite species, but *T. urticae* was able to reverse this manipulation and increase the plant resistance and consequently negatively affect the performance of herbivores (**Figure 2** and **3**).

Therefore the herbivore performance was determined by the identity of the attackers and herbivory by subsequent attackers altered the quality of initial plant responses; this may be the result of redirection of defenses by the second herbivore.

Conversely, Viswanathan et al. (2007) showed that the second herbivore to feed on plants did not modify induced resistance of the first attacker; they suggested that the first herbivory may canalize the plant responses, in this way suppressing the response to secondary herbivory. The canalization or redirection of defenses by a plant may depend on their specificity induced responses to different attackers; resulting in an important mechanism in expression of plant responses under multiple attacks (Poelman et al., 2008).

Interactions between herbivores that are mediated by plant quality and the induction of plant defenses may not only depend on the identity of herbivores, but also on their sequence of arrival (Erb et al., 2011). For example, pre-infestation of maize plants by *Spodoptera frugiperda* had a significant negative effect on the development and colonization by *Diabrotica virgifera* larvae, but only when *S.*

frugiperda arrived on the plant before the root herbivore. When *S. frugiperda* arrived after the root herbivore had established, no negative effects on larval performance of *D. virgifera* were detected (Erb et al., 2011).

Here, we show that the activity of proteinase inhibitors in plants that were previously damaged by *T. urticae* and subsequently damaged by *T. evansi* did not differ significantly from plants damaged by *T. evansi* without pre-infestation and showed lower PI enzymatic activity (**Figure 4**). This indicates that responses induced by the initial herbivore did not make plants less responsive to subsequent attack. It also shows that *T. evansi* is capable of down-regulating plant defenses, even when they were first induced. Additionally, the plants damaged by *T. urticae* without pre-infestation showed higher PI enzymatic activity. However, the responses to secondary herbivory by *T. urticae* interacted with the response to the initial herbivory by *T. evansi* and consequently the levels of proteinase inhibitors were intermediate between the responses induced by each herbivore individually (**Figure 4**). This result indicates that *T. urticae* may be able to induce PI enzymatic activity, but this induction was decreased when plants had already responded to the previous damage by *T. evansi*.

Previous studies also showed that the combination of herbivores that attack the plant may affect their transcriptional response (Voelckel & Baldwin, 2004) and when plants exhibit specificity in the elicitation of induced responses such effects of initial or final herbivory may depend on the order of herbivore arrival (Erb et al., 2011). However, none of these studies utilize herbivores with traits that enable them to exploit their host plant reducing the detrimental effects of plant resistance.

In conclusion, we showed that plants induce different responses after sequential attack and these responses elicited by each species of spider mite influence the herbivore performance and the dynamics of proteinase inhibitor activity. Our results also demonstrate that the mechanisms of suppression and induction of plant defense seen operate independently and these induced changes in the host plant can be an important determinant in interactions between spider mites and tomato plants.

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GENERAL CONCLUSIONS

The induction by spider mites *T. evansi* and *T. urticae* in tomato plants is rapid; occurring with only 1 day of infestation. Proteinase inhibitor activities and the performances of both mites were significantly higher in plants damaged by *T. evansi* and significantly lower in plants damaged by *T. urticae* compared to clean plants, during 4 days of infestation.

Plant resistance may be associated with chemical characteristics induced by each species of phytophagous mites during periods of herbivory.

Dual-damaged plants by *T. evansi* and *T. urticae* were phenotypically distinct from plants damaged by either herbivore alone. The performance of *T. evansi* and *T. urticae* was higher in plants damaged by *T. evansi*, intermediate in plants damaged by both mites (*T. evansi* + *T. urticae*) and lower in plants damaged by *T. urticae*, possibly caused by antagonistic effects of each species of spider mite.

The activity of proteinase inhibitors in plants damaged by both mites did not significantly differ from the activity in plants damaged only by *T. urticae* and showed higher levels of activity. However, the inhibitor activity was significantly lower in plants damaged only by *T. evansi*.

These two herbivores feeding simultaneously on the same plant do not have an additive effect on proteinase inhibitor activity, potentially indicating that the spider mite *T. urticae* can exert a greater effect on its host plant.

These spider mites that elicit specific plant responses can induce a similar pattern of responses after sequential attacks, showing that the effects on plant quality and defense of the last herbivore to attack the plant eventually overruled effects of the first herbivore.

The mite *T. urticae* induced defenses in the plant which decreased the performance of later-arriving herbivores. But even with the up-regulated of defenses, *T. evansi* can manipulate and suppress these responses resulting in increased of the herbivore performance. In contrast, the suppression of defenses caused by *T. evansi* improved the plant quality for mite species, but *T. urticae* was able to reverse this manipulation and increase the plant resistance and consequently negatively affect the performance of herbivores.

The activity of proteinase inhibitors indicates that responses induced by the initial herbivore did not make plants less responsive to subsequent attack. The herbivory by subsequent attackers altered the quality of initial plant responses; this may be the result of redirection of defenses by the second herbivore.