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ORIGINAL ARTICLE

Herbivore performance and plant defense after sequential attacks by inducing and suppressing herbivores

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Abstract It is well known that herbivore-induced plant defenses alter host plant quality and can affect the behavior and performance of later arriving herbivores. Effects of sequential attacks by herbivores that either suppress or induce plant defenses are less well studied. We sequentially infested leaves of tomato plants with a strain of the phytophagous spider mite *Tetranychus urticae* that induces plant defenses and the closely related *Tetranychus evansi*, which suppresses plant defenses. Plant quality was quantified through oviposition of both spider mite species and by measuring proteinase inhibitor activity using plant material that had been sequentially attacked by both herbivore species. Spider-mite oviposition data show that *T. evansi* could suppress an earlier induction of plant defenses by *T. urticae*, and *T. urticae* could induce defenses in plants previously attacked by *T. evansi* in 1 day. Longer attacks by the second species did not result in further changes in oviposition. Proteinase inhibitor activity levels showed that *T. evansi* suppressed the high activity levels induced by *T. urticae* to constitutive levels in 1 day, and further suppressed activity to levels similar to those in plants attacked by *T. evansi* alone. Attacks by *T. urticae* induced proteinase inhibitor activity in plants previously attacked by *T. evansi*, eventually to similar levels as induced by *T. urticae* alone. Hence, plant quality and plant defenses were significantly affected by sequential attacks and the order of attack does not affect subsequent performance, but does affect proteinase inhibitor activity levels. Based on our results, we discuss the evolution of suppression of plant defenses.

Key words plant defense; plant–herbivore interactions; plant quality; sequential attack; *Tetranychus evansi*; *Tetranychus urticae*

Introduction

Plants use several constitutive and induced strategies to defend themselves against pathogens and herbivorous arthropods (Karban & Baldwin, 1997; Walling, 2000; Dangl & Jones, 2001). Induced defenses result in changes of plant quality during and after a herbivore attack. They vary with the attacking herbivore species

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(Stout *et al.*, 1998; Voelckel & Baldwin, 2004; de Vos *et al.*, 2005; Rodriguez-Saona *et al.*, 2010; Soler *et al.*, 2013) and can even differ among herbivore strains (Kant *et al.*, 2008). Plants are commonly attacked by different species of herbivores (Futuyma & Gould, 1979; Strauss, 1991; Rodriguez-Saona *et al.*, 2010), and defenses induced by multiple species may differ from those induced by each species separately (Voelckel & Baldwin, 2004; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Rodriguez-Saona *et al.*, 2010). The defenses induced by an early-arriving herbivore affect the performance and colonization of other herbivores on the same plant (Karban & Carey, 1984; Karban & Baldwin, 1997; Stout *et al.*, 1998; Viswanathan *et al.*, 2005; Erb *et al.*, 2011;

Johnson *et al.*, 2012; Soler *et al.*, 2012; Erwin *et al.*, 2014; Glas *et al.*, 2014; Wang *et al.*, 2014; Godinho *et al.*, 2016). Hence, the order of arrival of different species on the same plant may determine the defense that is induced, and affect the outcome of interactions among herbivore species (Kessler & Baldwin, 2004; Voelckel & Baldwin, 2004; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Erb *et al.*, 2011).

Defenses induced by one herbivore species can result in increases or decreases of the performance of other herbivore species (Karban & Carey, 1984; Karban & Baldwin, 1997; Rodriguez-Saona & Thaler, 2005; Viswanathan *et al.*, 2005; Poelman *et al.*, 2008; Zhang *et al.*, 2009; Bruessow *et al.*, 2010; Soler *et al.*, 2013; Kant *et al.*, 2015). It has been suggested that species from the same feeding guild or sub-guild (Soler *et al.*, 2013) affect each other negatively through the elicitation of the same defensive pathways, whereas species from different feeding guilds may affect each other positively or not at all because of the negative cross-talk between the different defensive pathways that they induce (Rodriguez-Saona *et al.*, 2005; Howe & Jander, 2008; Soler *et al.*, 2013). Indeed, most of the studies on interactions of herbivores through the induction of plant defenses have concentrated on herbivores from different feeding guilds, and most studies dealt with herbivores that induce plant defenses.

Much less is known of the interaction between herbivores that induce plant defenses and herbivores that suppress them. Evidence is accumulating that herbivores may feed stealthily on plants, thus avoiding the induction of plant defenses (Walling, 2008), or may even suppress plant defenses (Kant *et al.*, 2008; Alba *et al.*, 2011; Sarmento *et al.*, 2011a; Consales *et al.*, 2012; Glas *et al.*, 2014; Alba *et al.*, 2015; see Kant *et al.*, 2015 for a review). Although this is to the benefit of the individuals that suppress plant defenses, it may also have positive effects on competitor species (Sarmento *et al.*, 2011b; Kant *et al.*, 2015; Godinho *et al.*, 2016).

Several of the examples of suppression of plant defenses concern mites, which feed on host plants by piercing parenchyma cells and sucking out the contents. The feeding of some species or strains of species induces direct plant defenses of both the jasmonic acid and salicylic acid pathway, whereas other strains or species do not induce these defenses or even suppress them below constitutive levels (Kant *et al.*, 2004, 2008; Alba *et al.*, 2015; Godinho *et al.*, 2016; Schimmel *et al.*, 2017). Unlike many other cases, *T. evansi* suppresses plant defenses of both the jasmonic acid and salicylic acid pathway, thus this suppression is independent of the antagonism between these two pathways (Sarmento *et al.*, 2011a; Glas *et al.*, 2014; Alba *et al.*, 2015). We recently showed that effects of induction of plant defenses by the spider mite *Tetranychus urticae* and the suppression of defenses by *T. evansi* roughly cancel out in plants attacked by both species simultaneously (de Oliveira *et al.*, 2016), Likewise, Alba *et al.* (2015) showed that *T. urticae* that shared a leaflet with a suppressor had an higher oviposition rate than mites that shared this leaflet with mites that induced plant defenses. Hence, *T. urticae* would benefit from simultaneously attacking the same plant as *T. evansi*, but *T. evansi* would perform worse on plants with *T. urticae*. However, the probability that both passively dispersing species would arrive on a plant at the same time may be low, and we therefore studied the effect of sequential attacks.

Studies on plant defenses are characterized by two different experimental approaches (Underwood *et al.*, 2002): one approach focuses on consequences of plant defense on herbivore performance. Clearly, herbivore performance is not only affected by induced or suppressed defenses, but also by changes in plant quality due to loss of healthy plant tissue through herbivory. The other approach studies plant physiology and expression of genes thought to be involved in defenses, but the effects of these changes on herbivore performance are not always clear. These approaches also differ in the way sequential attacks of plants by herbivores are investigated. Physiological and genetic studies of sequential attacks specifically looked at changes in plant defenses after a first attack and a second attack, thus including the effects of the second attacker on defenses (Voelckel & Baldwin, 2004; Poelman *et al.*, 2008). In contrast, studies on herbivore performance have focused on the effects of a first attack on plant quality for a second attacker (Kessler & Baldwin, 2004; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Rodriguez-Saona *et al.*, 2010). Hence, the latter studies look at the consequences of a first attack for a subsequent attacker, and the former studies quantify the combined effects of a first and a second attack on plant defenses. Here, we were interested in the consequences of sequential attacks on herbivore performance, that is, how a first plus second attack changes plant quality and subsequent herbivore performance. The approach of the experiments described here is thus similar to experiments on the effects of a second attack on plant defense (Voelckel & Baldwin, 2004; Poelman *et al.*, 2008), but instead of evaluating gene expression after two sequential attacks, we evaluate herbivore performance after, not during, two such attacks. We therefore followed a somewhat different approach from earlier studies on herbivore performance as a measure of plant quality. We first infested a leaf of a tomato plant with a defense-inducing line of *T. urticae* or the suppressor *T. evansi*, and then infested it with the other species for different intervals. Subsequently, we independently measured plant

quality through an oviposition assay on these sequentially infested leaves, hence, after various periods of a second attack, as well as in controls. Specifically, we wanted to know whether (i) the defense-inducing and suppressing species are capable of inverting the effect of the other species on plant defenses and (ii) how long this would take. We furthermore measured levels of proteinase inhibitors (PI), which are commonly used as markers for plant defenses being induced. They hamper the digestion of proteins in the gut of herbivores, needed for acquiring amino acids (Ryan, 1990; Koiwa *et al.*, 1997). Earlier research found no clear correlation between PI activity and herbivore performance (Underwood *et al.*, 2002; da Silva *et al.*, 2015; de Oliveira *et al.*, 2016). The aim here was to further confirm this.

Materials and methods

Tomato plants (*Solanum lycopersicum* var. Santa Clara I-5300) were grown as described in de Oliveira *et al.* (2016). They were used for experiments and for spider mite cultures when 45 d old and having at least 4 completely developed leaves. Spider mites (*T. evansi* and *T. urticae*) were collected from naturally infested tomato plants of the same variety as above in a greenhouse at the Federal University of Viçosa, Brazil in 2002 (Sarmento *et al.*, 2011a,b; de Oliveira *et al.*, 2016). This strain of *T. urticae* induces defences in tomato plants, the strain of *T. evansi* suppresses defences (Sarmento *et al.*, 2011a). They were cultured on detached tomato leaves, of which the petiole was inserted in a PVC tube with water to maintain leaf turgor. The tubes 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 °C, 70%–90% relative humidity and 12 h light.

Because the oviposition rate of spider mites varies with age (Sabelis, 1991) female mites of similar age were used to measure oviposition. To obtain such cohorts, several adult females were allowed to lay eggs on detached tomato leaves on wet cotton wool. The adults were removed after 24 h and the eggs were reared to adulthood. Females used for the experiments were adults for 2 d.

Infestation of plants

We were interested in the effects of sequential attacks by spider mites that either induced or suppressed plant defenses on subsequent herbivore performance and PI activity. Plants therefore received two sequential treatments. The first treatment consisted of an infestation with

either *T. evansi* or *T. urticae*. The third leaf, counted from below, of 1 group of 5 plants was infested with *T. evansi* (100 adult mites), and the third leaf of another group of 5 plants was infested with *T. urticae* (100 adult mites). Insect glue (Cola Entomológica; Bio-Controle, São Paulo, Brazil) was applied to the petiole of the leaf with mites to prevent them from moving to other leaves, which were kept clean. Plants were kept inside mite-proof screen cages in a greenhouse. After 1 d, the mites, their web and eggs were removed from the infested plants under a stereomicroscope with a fine brush. This time of infestation is sufficient for the spider mites to induce (*T. urticae*) or suppress (*T. evansi*) plant defenses (de Oliveira *et al.*, 2016). Two other groups of 5 plants served as control and were not infested with spider mites, but did receive a glue barrier and were also kept in cages.

The second treatment was applied after 1 d, immediately after removing the mites and their web and eggs. The previously infested leaf of 4 plants of the 2 groups of infested plants was reinfested with 100 adult female mites of the other species (Table 1). The fifth plant of these groups was not infested, and was used to confirm the effect of the first treatment. Four plants of the 2 groups that were not infested during the first treatment also received 100 adult female mites, either *T. urticae* or *T. evansi* on the third leaf. They served as controls for the second infestation (Table 1). The remaining plant of these 2 groups were not infested (i.e., they had never received spider mites) and served as a control for the first infestation (Table 1). The experiments were done in 4 blocks through time, each block with 1 plant of each combination of first and second treatment (20 plants per block).

Of each group of plants, 1 plant was harvested every day, the infested leaf was cleaned and was used for the oviposition experiment and measurements of PI activity outlined below. Hence, this yielded a series of leaves of plants that had had a second attack for 1–4 d (Table 1).

Ideally, the assessment of oviposition rates and PI activity of all plants of all treatments should be carried out simultaneously. However, this would require that plants with different duration of the second treatment be treated on different days, thus they would be of different age or be planted on different days. Because plants were grown in a greenhouse where conditions were not completely constant, this could affect the plant response to herbivory. The oviposition rates were measured under more controlled laboratory conditions with less interference of external conditions. We therefore chose to use plants of the same age and of the same batches, and evaluating oviposition and PI activity on different days. Despite these slight

Table 1 Schematic set-up of the plant treatments before the oviposition experiment and PI activity assessment.

First treatment	Second treatment	Duration second treatment (d)	Number of plants
T. urticae	*T. evansi*	1–4	4 per Duration (16 total)
T. evansi	*T. urticae*	1–4	4 per Duration (16 total)
T. urticae	None	0	4
T. evansi	None	0	4
None	*T. urticae*	1–4	4 per Duration (16 total)
None	*T. evansi*	1–4	4 per Duration (16 total)
None	None	0	4

The third leaf of tomato plants was infested with spider mites (*T. urticae* or *T. evansi*) during 1 d (first treatment), or were not infested during this day (None). Subsequently, spider mites, their eggs and web were removed from the infested leaves and the same leaves were infested with the other species (*T. evansi* or *T. urticae*) for 1–4 d (Duration second treatment). Control groups did not receive a second infestation (None). Subsequently, leaf discs were made from the third leaves, which were used for an oviposition test or for assessment of proteinase inhibitor activity. The third column gives the number of days from the first treatment until leaf discs were made for further experimentation; this corresponds to the horizontal axis labels in the figures. See text for further explanation and justification.

differences in the time of assessment of oviposition rates and PI activity levels, this experimental set up allows for assessment of the effect of the second attack through time.

Oviposition

After the second treatment (1–4 d), the mites, web and eggs were removed from the infested leaves and 20 leaf discs (12 mm Ø) were made from each infested leaf. From the control plants that did not receive a second treatment, leaf discs were made from the corresponding leaves immediately after the first treatment. Other leaflets of the same leaf were collected for measurements of PI activity (see next section for details). Hence, this resulted in leaf discs that had either received a first treatment with mites or not, and subsequently received a second treatment with mites for 1–4 d, or no mites (None, Table 1). This allowed us to study the effect of sequential

attacks, as well as of the duration of the second attack, on plant quality. All leaf discs were inspected with a stereo-scopic microscope to ensure that no spider mite eggs had remained behind. Discs were subsequently placed on wet cotton wool in Petri dishes (8 cm Ø) and were used to measure the performance of spider mites. We used the oviposition rate of spider mites as a measure of performance, which is strongly related to the fitness of spider mites (Sabelis, 1991), and is affected by induction and suppression of plant defenses (Sarmento *et al.*, 2011a).

One randomly selected adult female of *T. evansi* or *T. urticae* from a cohort of mites that were 2 d old since turning adult, was placed on a leaf disc (10 females per species per plant). After 4 d (28 ± 2 °C; 70% ± 10% RH; 12 h light), eggs were counted and the survival of the females was assessed. To avoid pseudoreplication, we subsequently averaged the oviposition rate per spider mite species over all leaf discs that originated from the same plant, excluding oviposition of females that had not survived the 4 d of the oviposition test. Hence, this yielded 1 average measure of oviposition for *T. evansi* and 1 for *T. urticae* per plant. This still included some pseudoreplication because the oviposition rates of the 2 species were measured on leaf discs of the same plants. We therefore first tested whether the 2 species were differentially affected by the treatments by assessing the significance of the interaction between species, treatment and duration of the second treatment with a generalized linear model (GLM) with a Gaussian error distribution. Upon not detecting such a significant interaction, we averaged the oviposition rates of the 2 species, yielding 1 oviposition rate per plant. These oviposition rates were then further analyzed as follows. We first tested whether the first treatments had resulted in the expected differences in oviposition rates, using the data of plants that did not receive a second treatment (Table 1, second treatment: None) with a GLM as above. Subsequently, we assessed the immediate effect of the second treatment by specifically comparing the average oviposition rates on plants that received no second treatment (Table 1, second treatment: None) with those on plants that received the second treatment for 1 d (Table 1, Duration second treatment 1), using a GLM as above. Lastly, we assessed how oviposition rates were affected by the period of the second treatment, so excluding the plants that did not receive a second treatment (i.e., excluding second treatment None) and including all plants that received a second treatment, also when they did not receive a first treatment. Contrasts were assessed with the glht function with *P* values adjusted with the Tukey method (package lsmeans of R, Lenth, 2016). Differences in survival of the adult females during the oviposition

experiment were tested with a GLM with a quasi-binomial error distribution. All models were checked by plotting residuals against fitted values and checking normality of the error distributions.

Proteinase inhibitor activity

The proteinase inhibitor (PI) activity was measured in leaflets of the same leaves and the same plants as used for oviposition, collected at the same time that leaf discs were made for oviposition experiments. They were frozen in liquid nitrogen and stored at –80 °C. Subsequently, each sample was ground with mortar and pestle and a crude protein extract was obtained as described by Ohta *et al.* (1986). Essentially, the leaves were homogenized in extraction buffer (0.1 mol/L Tris-HCl buffer, pH 8.2 and 20 mmol/L $CaCl_2$; 1 : 3 w : v) and the homogenate centrifuged at $17\,200 \times g$ for 30 min at 4 °C 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 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} mol/L) was mixed with 100 μL of the supernatant and 500 μL extraction buffer (0.1 mol/L Tris-HCl buffer, pH 8.2 and 20 mmol/L $CaCl_2$). 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} mol/L). 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 mmol/L). Trypsin activity was monitored for 150 s at intervals of 30 s at 410 nm absorbance on a spectrophotometer. The difference between the absorbance measured at 150 and 60 s was used to determine the trypsin activity. Measurements were performed in triplicate per sample.

Measurements were converted to milligrams trypsin inhibited per gram of protein according to the following equation: mg trypsin inhibited per gram of protein $= AB/(1000PC)$, where A is the enzyme control— absorbance at 410 nm of the extract; B is the sample dilution; P is the protein concentration of the extracts in g/mL; and C is the trypsin factor, the result from the activity of 1 μg of trypsin on the substrate $_{D,L}$-BApNA measured at 410 nm, and is equal to 0.019 (Kakade *et al.*, 1974). Results were analyzed with a GLM with log-transformed PI activity as above.

Results

We first compared the effects of the plant treatments on the 2 species. Overall, the oviposition rates of both species were similarly affected by the first and second treatments (Fig. 1, interaction among ovipositing species, treatment and duration of the second treatment: $F_{3,144} = 0.87$, $P = 0.459$). Because the oviposition of 2 spider mite species was tested on leaf discs of the same plant, we subsequently averaged the oviposition rates over the 2 species, thus avoiding pseudo-replication. For completeness, we show the oviposition rates of the 2 species separately (Fig. 1).

Verification of induction and suppression

To verify that the first treatment by either of the 2 species resulted in the expected changes in plant quality (i.e., higher oviposition and lower PI activity levels on plants previously attacked by *T. evansi* and the opposite for plants previously attacked by *T. urticae*), we first compared plants that did not receive a second treatment but only a first treatment (0 d of second treatment, Fig. 1, Table 1). Oviposition varied significantly with the first treatment (GLM, $F_{3,12} = 10.92$ $P < 0.001$). It was significantly higher on plants that were previously infested with *T. evansi* than on clean plants and significantly lower on plants previously infested with *T. urticae* (0 d of second treatment, Fig. 1). Likewise, the PI activity differed significantly among plants that received only a first treatment (Table 1, Fig. 2, GLM: $F_{3,12} = 16.2$, $P < 0.001$). Together, these results confirm that *T. evansi* suppressed plant defenses relative to clean plants and that *T. urticae* induced plant defenses.

Effect of second infestation

To see the immediate effect of the second treatment we compared plants that received no second treatment versus plants that received 1 d of the second treatment (Table 1, Fig. 1, 0 d vs. 1 d of second treatment). There was a significant interaction between the first treatment and the second treatment (0 d vs. 1 d) ($F_{3,24} = 10.07$, $P = 0.00018$). The high oviposition on plants with a first treatment with *T. evansi* was no longer observed after a second treatment of 1 d with *T. urticae* (Fig. 1, closed circles of day 0 and 1). In contrast, the low oviposition rate on plants with a first treatment with *T. urticae* was no longer observed after a second treatment of 1 d with *T. evansi* (Fig. 1, closed triangles of day 0 and 1). This shows that the effects of the induction of plant defenses by *T. urticae*
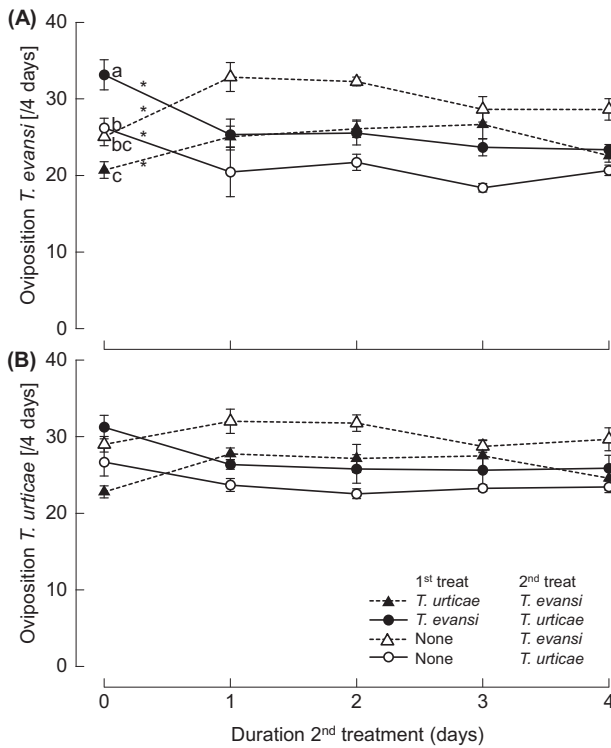
Fig. 1 Oviposition by *Tetranychus evansi* (A) and *T. urticae* (B) on leaf discs from tomato plants that received 2 treatments. The first treatment (1st treat) consisted of *T. evansi* (closed circles) or *T. urticae* (closed triangles) damaging 1 leaf of these plants during 1 d. Controls (open symbols) did not receive damage. The second treatment (2nd treat) consisted of allowing the other species (*T. evansi*: triangles; *T. urticae*: circles) to damage the same leaf. This second treatment lasted from 1 to 4 d (Duration second treatment). A group of control plants did not receive a second attack (0 d of second treatment), resulting in plants that received either the first or the second treatment, plants that received both treatments and plants that received no treatment. See Table 1 for treatments. Shown are average oviposition (± s.e.) of *T. evansi* (A) and *T. urticae* (B) per plant (4 plants per data point) during 4 d. Because oviposition of *T. evansi* and *T. urticae* was assessed on leaf discs coming from the same plants, the statistics shown in (A) were done with the average oviposition of the 2 species combined to avoid pseudo-replication. Different lowercase letters show significant differences among treatments per duration of second treatment. Asterisks near the lines connecting points of 0 and 1 d of second attack indicate significant differences between the 2 connected data points.

and their suppression by *T. evansi* roughly cancelled out when plants were sequentially attacked by the 2 species. A second treatment of plants that did not receive a first treatment with mites (Fig. 1, open symbols) resulted in the expected effects on oviposition rates: a treatment of plants
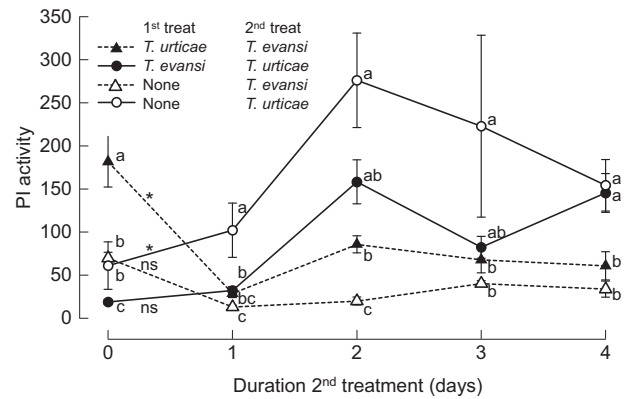
Fig. 2 Proteinase inhibitor activity in leaves used for the oviposition experiment (Fig. 1). See legend to Fig. 1 and Table 1 for explanation of the treatments and of significance. Shown is mean (+ SEM, $n = 4$ plants) proteinase inhibitor (PI) activity.

with *T. evansi* during 1 d resulted in a significantly higher oviposition rate and a treatment with *T. urticae* resulted in a significantly lower oviposition rate (Fig. 1, cf. open symbols on day 0 and 1). Analysis of the effects of the duration of the second treatment on oviposition rates (i.e., excluding plants with second treatment "None," Table 1) showed that giving the plants a second treatment for more than 1 d did not result in any further significant changes in oviposition rates (cf. days 1–4 in Fig. 1: GLM, $F_{3,57} = 2.35$, $P = 0.082$).

Changes in PI activity from day 0 to day 1 showed a somewhat different pattern from the changes in oviposition rate (Fig. 2). There was a significant interaction between the duration of the second treatment (0 d vs. 1 d) and the first treatment ($F_{3,24} = 18.6$, $P < 0.0001$). The high PI activity in plants with a first treatment with *T. urticae* was no longer observed after a second treatment of 1 d with *T. evansi* (Fig. 2, cf. closed triangles of day 0 and 1). The low PI activity in plants with a first treatment with *T. evansi* was no longer observed after a second treatment of 1 d with *T. urticae* (Fig. 2, cf. closed circles of day 0 and 1). Plants that had not received mite damage during the first treatment responded as expected after the second treatment: a second treatment with *T. urticae* resulted in a significant increase of PI activity within 1 d, and a second treatment with *T. evansi* resulted in a decrease of PI activity, but this was not significant after 1 d of the second treatment. Proteinase inhibitor activity levels varied considerably with duration of the second treatment (from day 1–4: GLM: $F_{3,57} = 16.2$, $P < 0.0001$), and there was a marginally significant interaction of the duration of the second treatment with the first treatment ($F_{9,48} = 2.06$, $P = 0.052$), especially for those with a second treatment

with *T. urticae*. After 4 d of the second treatment, activity levels of plants with a second treatment with *T. evansi* (triangles) were low and activity levels of plants that received a second treatment with *T. urticae* (circles) were high (Fig. 2).

There was no significant effect of treatment or time of second attack on survival of the ovipositing females (GLM, *T. evansi*: treatment: $F_{3,76} = 0.72$, $P = 0.54$, time: $F_{4,72} = 0.69$, $P = 0.60$; *T. urticae*: treatment: $F_{3,76} = 0.59$, $P = 0.62$, time $F_{4,72} = 2.05$, $P = 0.10$).

Discussion

We investigated how sequential induction of plant defenses by one herbivore species and suppression of defenses by a closely related species interfere. Our results show that induction of defenses by *T. urticae* and prior or subsequent suppression of defenses by *T. evansi* roughly cancel out. Earlier, we showed that simultaneous attacks by these two spider mites also resulted in intermediate defense levels (de Oliveira *et al.*, 2016). This suggests that the induction and reduction of plant defenses by these two spider mites are, at least partially, revertible and similar biochemical pathways may be involved in the induction and reduction. Alternatively, it is possible that feeding by the 2 herbivores causes effects on plant defenses only at the feeding site, resulting in a patchwork of leaf tissue with induced, suppressed or unchanged defense levels (Schimmel *et al.*, 2017). Spider mites feed by piercing leaf cells and sucking out their contents, and this indeed results in localized damage (Kant *et al.*, 2004). However, defenses induced by *T. urticae* are known to be systemic (Karban & Carey, 1984; Kant *et al.*, 2008), and it was recently shown that the suppression of defenses by *T. evansi* in one half of a tomato leaflet increased the performance of mites isolated from them on the other leaflet half (Alba *et al.*, 2015), suggesting that the suppression is not restricted to the feeding site. Recently, some candidate effectors associated with the salivary glands of a suppressor strain of *T. urticae* and of *T. evansi* were isolated, which are possibly involved in suppressing plant defenses (Villarroel *et al.*, 2016). It would be interesting to study whether these effectors affect plant defenses beyond the feeding site.

Several studies evaluated the effects of a first and second attack on activity of defensive compounds and gene expression (e.g., Voelckel & Baldwin, 2004; Poelman *et al.*, 2008). Many studies (see citations in Introduction) evaluated how first attacks affected the performance of herbivores attacking the plant subsequently. It is unknown, however, how these subsequent attacks further

affect plant quality and herbivore performance, because the performance of the second attacker was measured after a first attack in these experiments, but the effects of the second attack on plant quality was not measured independently. Here, we show that the second attacker can further affect plant quality and plant defenses, even within 1 d.

It is known that the order of arrival of different herbivore species determines the defense that is induced (Voelckel & Baldwin, 2004; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Erb *et al.*, 2011). Here, we did not find such an effect on the performance of the herbivores (Fig. 1). Defenses of plants that were sequentially attacked were intermediate between those of plants attacked by either of the 2 species separately. A first attack by *T. evansi* suppressed defenses to levels below that in unattacked plants (Fig. 1), but a second attack by this species suppressed defense levels of plants previously induced by an attack of *T. urticae* only to levels roughly equal to that in unattacked plants (Figs. 1 and 2). Likewise, a secondary attack by *T. urticae* reduced oviposition rates on plants previously attacked by *T. evansi* to levels comparable to those on clean plants, but not lower (Fig. 1). This was true even when the second attack lasted 4 times longer than the first attack, showing that there is a lasting effect of a relatively short previous attack on plant defense levels after a second attack. It thus seems that neither first nor second attacks exclusively determine the final plant quality.

Proteinase inhibitor activity showed a slightly different picture: leaves attacked first by *T. evansi* and then by *T. urticae* showed a trend towards higher PI activity than leaves attacked first by *T. urticae* and then by *T. evansi*, although this difference was not always significant (Fig. 2). Proteinase inhibitor activity levels in plants after 4 d were mainly determined by the last attack, independent of the first attacker (Fig. 2). The levels of PI activity did not completely correlate with the oviposition rates found on leaf discs of the same leaves (cf. Figs. 1 and 2), and PI activity showed much more variation with the time of second attack than did oviposition. We have shown elsewhere that oviposition rates of *T. urticae* and *T. evansi* and PI activity levels indeed do not correlate well (de Oliveira *et al.*, 2016), suggesting that PI is not a good stand-in measure for defenses experienced by herbivores (see also Underwood *et al.*, 2002; da Silva *et al.*, 2015).

Alba *et al.* (2015) studied the time course of defense induction and suppression by several strains of *T. urticae* and the strain of *T. evansi* used here. They found accumulation of phytohormones and increased expression of genes known to be involved in plant defenses in tomato plants from 1 to 7 d after the onset of the attack by

an inducing strain of *T. urticae*. In contrast, *T. evansi* did not significantly induce phytohormones, even after 7 d, but did induce changes in expression of some plant defensive genes, especially shortly after initiation of the attack (Alba *et al.*, 2015). We show here and elsewhere (de Oliveira *et al.*, 2016) that feeding by *T. evansi* does not result in decreased herbivore performance, so the question is whether the changes in gene expression are important for performance of the two mites studied here.

We show here that 1 d of feeding by *T. evansi* reduced defense levels previously induced by *T. urticae* to roughly constitutive levels. Alba *et al.* (2015) showed that *T. evansi* did not suppress the accumulation of the phytohormones JA-isoleucine and SA induced by *T. urticae* on the same leaf, but that the expression of downstream marker genes involved in PI activity and the synthesis of a pathogenesis-related protein were suppressed, suggesting that *T. evansi* can suppress defense downstream from phytohormone accumulation. This would explain its capacity to suppress defenses induced by *T. urticae* (Fig. 1). However, this suppression is only partial, never reaching the low levels of suppression achieved by *T. evansi* alone. We therefore suggest that the suppression of plant defenses by *T. evansi* is partly due to the suppression of accumulation of defensive plant hormones, and partly due to downstream suppression.

Schimmel *et al.* (2017) showed that *T. evansi* suppressed tomato plant defenses more strongly when a leaflet was coinfested with *T. urticae* than when the leaflet was coinfested with *T. evansi* or when it was clean. This was correlated with higher expression levels of effector-coding genes in *T. evansi*, and with higher oviposition rates of *T. evansi* in the presence of its competitor on the same leaflet. Here, we did not find such higher oviposition by *T. evansi* after sequential infestations by both species, and elsewhere we report on the lack of such higher oviposition after simultaneous infestations (de Oliveira *et al.*, 2016). The reasons for these seemingly contradictory results may be that the spider mites in the experiments reported here and elsewhere (de Oliveira *et al.*, 2016) occupied the same leaf area, whereas they were spatially separated on leaflets in the experiments by Schimmel *et al.* (2017).

Defense suppression does occur in various herbivore species, but it is unclear why it does not occur more often, especially because it results in better performance of the suppressing herbivores. Variation in the capacity to suppress or induce plant defenses exists within herbivore populations (Kant *et al.*, 2008), and it is possible to select for herbivore lines that suppress plant defenses (Alba *et al.*, 2015). If herbivores can be selected to suppress plant defenses, then why does the majority of herbivores

studied so far induce defenses instead of suppressing them? Possibly, this is because other herbivores can profit from the suppression of defenses, whereas suppressing herbivores may suffer from the induction of defenses by these other herbivores, as was shown here and elsewhere (Kant *et al.*, 2008; Sarmento *et al.*, 2011b; Glas *et al.*, 2014; Alba *et al.*, 2015). Our results show that it is in principle advantageous for the inducing strain of *T. urticae* to coinfest plants in which *T. evansi* downregulates plant defenses, whereas it is detrimental for *T. evansi* to coinfest plants in which *T. urticae* induces plant defenses (de Oliveira *et al.*, 2016). Nevertheless, *T. evansi* has invaded Africa and Europe, where *T. urticae* was already abundantly present (Boubou *et al.*, 2011; Navajas *et al.*, 2013), and the one study on competition between these two species showed that *T. evansi* outcompeted *T. urticae* by far (Sarmento *et al.*, 2011b). The competitive superiority of *T. evansi* is at least partly caused by the dense web that it produces, which impedes feeding by *T. urticae* (Sarmento *et al.*, 2011b), allowing *T. evansi* to monopolize its feeding site. Furthermore, reproductive interference of *T. evansi* with *T. urticae* may also reduce the population growth rate of the latter species (Sato *et al.*, 2014). Such monopolization only works when defense suppression is a local phenomenon, for which there are indeed some indications for *T. evansi* (Sarmento *et al.*, 2011a), but, as argued above, this suppression is not entirely local (Alba *et al.*, 2015).

The monopolization of leaf areas by producing dense web may keep heterospecific competitors away from areas with suppressed defenses, but does not protect against conspecific free riders. It has been suggested that strategies that increase the quantity or quality of local resource levels can only be evolutionary stable if the probability that the same resource is attacked by organisms with different strategies is low, that is, when populations are not well mixed but consist of local populations of related organisms with low dispersal among populations (van Baalen & Sabelis, 1995; Pels *et al.*, 2002). Indeed, many spider mite species have these characteristics: they disperse passively and often overexploit their host plant (Nachman, 1991; Sabelis *et al.*, 1991; Janssen *et al.*, 1997; Ellner *et al.*, 2001). The question is then why spider mites of the species *T. urticae* mostly induce plant defenses while there is genetic variation in the capacity to suppress (Kant *et al.*, 2008; Alba *et al.*, 2015), whereas the two populations of *T. evansi* that have been studied so far suppress plant defenses (Sarmento *et al.*, 2011a; Alba *et al.*, 2015). Perhaps the probability of secondary attacks of plants by unrelated conspecifics differs significantly between these two species. Whereas *T. urticae* attacks over 1100 plant species, *T. evansi* attacks primarily solanaceous plants

(Bolland *et al.*, 1998; Migeon & Dorkeld, 2015). It seems reasonable to assume that the total density of host plants covaries with the number of host plant species, implying that the density of host plants of *T. evansi* is lower than that of *T. urticae*. Because the mites disperse passively on air currents, this would result in a lower probability of secondary attacks for *T. evansi*. Remarkably, *Tetranychus ludeni* and the eriophyid mite *Aculops lycopersici* also suppress plant defenses in tomato (Glas *et al.*, 2014; Godinho *et al.*, 2016), and both also attack primarily solanaceous plants (Lindquist *et al.*, 1996; Migeon & Dorkeld, 2015), indicating that their host plant densities are also lower than that of *T. urticae*. We suggest that this may be a general pattern: suppression of plant defenses may occur more frequently in species where the probability of a secondary infestation with conspecifics is low. This could coincide with species with relatively low densities of host plants (i.e., species that have restricted host plant ranges), and with a metapopulation structure (i.e., where local populations do not persist for long periods). This would offer an alternative explanation for the idea that generalist and specialist herbivores may be differently affected and may differently induce host plant defenses (Ali & Agrawal, 2012). From the point of view of the plant, direct defenses against rare herbivores may not be subject to strong selection, in contrast with direct defenses against common herbivores.

Acknowledgments

Author contributions

EFdO and AJ conceived and designed the experiments. EFdO performed the experiments. AJ and EFdO analysed the data. AJ, EFdO, and AP wrote the manuscript.

Disclosure

The authors declare no conflicts of interest.

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