The tomato trichomes are deadly hurdles limiting the establishment of *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae)

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Abstract

Amblyseius swirskii is a predatory mite widely used in organic farming and in integrated management programs in conventional agriculture as well, for the control of very important pest species such as whiteflies and thrips. However, this species cannot become established on tomato crops, probably due to the negative effect of the plant trichomes and their exudates on its biological parameters. In this work, the effect of tomato plants on *A. swirskii* was evaluated at four different levels: a) the effect of volatile-mediated plant traits on mite preference, b) the effect of plant leaves on the development, predation and oviposition of predatory mites, c) the effect of stem trichomes on the dispersal and survival of mites, and d) the effect of secondary metabolites secreted by tomato trichomes on mite survival. The results showed that *A. swirskii* avoid tomato plants, even if they have been previously in contact with this plant. On the other hand, it was demostrated that survival of *A. swirskii* eggs and juveniles was not affected on tomato leaves but, adult survival was significantly reduced when tested on the whole plant. This is due to the impact of trichomes and their secondary metabolites, present in high concentration on the stems, which affected the mites attempting to disperse on the plant. Finally, it was demonstrated that among the secondary metabolites detected in tomato trichomes, the strongest negative effect was exerted by the acyl sugars. They were highly toxic against the mites and were also detected physically stuck to their bodies after walking on tomato plants. Altogether our results show evidence suggesting why *A. swirskii* is not an efficient biocontrol agent on tomato and set the basis to address new lines of research that would allow the use of this phytoseiid in tomato crops.

1. Introduction

Amblyseius swirskii Athias-Henriot (Acari: Phytoseiidae), previously known as *Typhlodromips swirskii*, is a generalist phytoseiid, which feeds and reproduces on various arthropods and it is currently the most widely used biological control agent in augmentative biological control (Knapp et al., 2018). This predatory mite was originally described in 1962 in Israel (Athias Henriot, 1962), where it has been found on various annual and perennial crops, such as citrus, grapes, vegetables and cotton, usually associated with whiteflies (Swirski & Amitai, 1997). Today, it is used to control economically important greenhouse pest species such as whiteflies, thrips and plant feeding mites in vegetables, fruits and ornamentals (Calvo et al., 2015). In addition to its high efficacy, controlling these groups of pests, *A. swirskii* can establish on crops in the absence of prey, using pollen or factitious prey as a food source (Nomikou et al., 2003). Moreover, it can develop under a wide range of temperatures, since it does not enter diapause (Lee & Gillespie, 2011), it can be combined with other biocontrol agents (eg. *Orius* sp and mirid predators) (Doğramaci et al., 2011; Bouagga et al., 2018) and it is easily mass-reared on factious prey (Calvo et al., 2015). All these biological attributes of *A. swirskii* have contributed to its success in augmentative biological control worldwide. The integration of this phytoseiid in Integrated Pest Management (IPM) strategies against whiteflies and thrips in protected sweet pepper crops, in South-eastern Spain, has contributed to the sharp decrease in the use of chemical pesticides and was the first successful showcase of its potential in augmentative biological control of pests in protected crops (Calvo et al., 2011; Calvo et al., 2015; Van Lenteren et al., 2018).

However, *A. swirskii* cannot establish on tomato (*Solanum lycopersicum*), the most important vegetable crop in Europe, with a production of 17,059,000 tons in 2018 (Eurostat, June 2019). On detached tomato leaflets, *A. swirskii* can attack and develop on common tomato pests, such as the tomato russet mite *Aculops lycopersici* Massee (Acari: Eriophyidae) (Momen and Abdel-Khalek, 2008; Park et al., 2010), the South America tomato pinworm *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) (Momen et al., 2013), the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) and the two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (personal observation). However, on tomato plants the survival and efficacy of phytoseiids are hindered, most likely due to the impact of tomato defenses mediated by the trichomes and their exudates (Kennedy 2003).

Tomato plants are covered by various types of trichomes which are not present on favorable host plants like the sweet pepper. Moreover, it has been shown that high trichomes density on host plants can be detrimental for insect predators (Riddick and Simmons, 2014). Tomato trichomes are diverse in terms of morphology and chemistry and are classified as glandular trichomes with types I, IV, VI and VII and as non-glandular trichomes with types II, III and V (Luckwill, 1934). Non-glandular trichomes are hair-like structures that cover the plant surface and function as physical barriers to arthropod dispersal and herbivory (Baur et al., 1991; Simmons and Gurr, 2005). Glandular trichomes have specialized cells on their tips that form the glandular heads and produce a variety of secondary metabolites with antibiotic and antixenotic effects against herbivores but also affecting natural enemies (Simmons and Gurr, 2005). Among glandular trichomes, the most abundant are type I and VI. They produce a wide array of compounds including high levels of the sticky acyl

sugars, but also terpenoids and methyl ketones (Schilmiller et al., 2010). Terpenoids are highly volatile and play an important role in indirect plant defense mostly by attracting predators and parasitoids and repelling herbivores (Dicke et al., 1998; Bleeker et al., 2009). Predatory mites can respond to volatiles and may associate them with positive or negative conditions such as the presence of prey or absence of prey that leads to starvation, respectively (Drukker et al., 2000). Methyl ketones can be toxic to phytophagous mites, such as the two spotted spider mite, but they are found only at trace levels on cultivated tomato (Chatzivasileiadis & Sabelis, 1997). Tomato glandular trichomes have been previously associated with the entrapment of small arthropods and have a negative impact on both, pests and natural enemies (Cédola and Sánchez, 2003). It has been shown that acyl sugars accumulate on the legs of aphids while walking on the plant, hampering their dispersal (Wagner et al., 2004), and that they can be toxic to mites at very low concentrations (Puterka et al., 2003). Also, these compounds reduce herbivore feeding, development and oviposition of various insect pests such as, the leafminer *Liriomyza trifollii* (Burgess) (Diptera, Agromyzidae), the moths *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), the thrips, *Frankliniella fusca* (Hinds) and *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Hawthorne et al., 1992; Juvik et al., 1994; Resende et al., 2006; Leckie et al., 2016).

Defensive plant traits against herbivory can be deleterious to natural enemies and subsequently, of great importance for designing effective integrated pest management strategies. In this work, the objective was to unravel the effect of the defensive traits of

tomato plants limiting the performance of the generalist predatory mite *A. swirskii* and to identify the secondary metabolites most likely responsible for these effects. To do this, first the behavioral response of *A. swirskii* to the volatiles emitted by tomato plants was evaluated, indicating how stressful the tomato plant is for the mite. Secondly, the mite performance on tomato leaves was measured as predation capacity and oviposition rate. These parameters were compared with those obtained on sweet pepper plants, a favorable host plant for this phytoseiid (Calvo et al., 2011). Thirdly, as the mites usually disperse through the stems to avoid kin competition and overexploitation, the effect of tomato stem trichomes on the dispersal and survival of *A. swirskii* was assessed. Also, the capacity of trichomes secretions to stick to the mites' body parts was assessed by microscopy. Finally, to move further on the identification of secondary metabolites influencing the deleterious effects of tomato, an extraction of such metabolites with several solvent fractions was performed and their toxicity on mites was characterized.

2. Materials and methods

2.1 Plants and mites

Tomato plants, *Solanum lycopersicum* cv. Raf Marmande and sweet pepper plants *Capsicum annuum* cv. Lipari were used in the olfactometer experiment and the estimation of life history parameters of *A. swirskii* was performed as described below. Seeds were sown in a mixture of soil and local peat moss. Two weeks after germination seedlings were individually transplanted into pots (8 × 8 × 8 cm). Plants were maintained undisturbed at 25 ± 2 ºC, 65 % ± 5% relative humidity (RH) and 14:10 h (Light: Dark) photoperiod. Pesticide-free plants with 6 fully developed leaves were used for the experiments.

Colonies of *A. swirskii* were initiated from specimens supplied by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). They were maintained in rearing units; a piece of hard black plastic on top of a water saturated sponge, which is placed in a plastic tray with water. The borders of the plastic were covered with water saturated tissue paper to ensure a constant water supply for the phytoseiids, to fix the plastic piece to the sponge, and to prevent phytoseiids from escaping (Abad-Moyano et al., 2009). Cotton threads 2 cm long were provided on the rearing units to serve as oviposition sites. Twice a week, mites were fed *ad libitum* with *Carpobrotus edulis* (L) (Caryophyllales: Aizoaceae) pollen (Ragusa & Swirski, 1975). The colonies were maintained at 25 ± 2 ºC in growth chambers at 14:10 h (Light: Dark) photoperiod and 80 ± 10 % RH.

2.2 Olfactory responses to tomato volatiles

The olfactory response to tomato of three different sources of experienced mites was investigated in a Y-tube olfactometer. Experienced mites were obtained by releasing adult female mites for 48 hours on either sweet pepper leaves, tomato leaves or plastic arenas, with *C. edulis* pollen as food source *ad libitum* and left undisturbed at 23 ± 2 °C, 60 ± 10 % RH. Before testing, the mites were collected from the three different arenas and kept for one hour on a clean Petri dish to remove traces of pollen from their bodies. Mites experienced on sweet pepper leaves were tested for their response towards the following experimental treatments: sweet pepper plants (known plant host) *vs* clean air (no plant host), and sweet pepper plants *vs* tomato plants (unknown plant host). Female mites experienced on tomato leaves were tested for their response towards the following experimental treatments: tomato plants (known plant host) *vs* clean air (no plant host), and tomato plants *vs* sweet pepper plant (unknown plant host). Finally, mites reared on plastic,

inexperienced to plants, were tested for their olfactory response towards the following experimental treatments: sweet pepper plant *vs* tomato plants, sweet pepper plant *vs* clean air and tomato plant *vs* clean air.

The Y-tube olfactometer consisted of two glass jars, 5 L volume, connected to a Y-shaped glass tube (2.4 cm diameter), containing a metal string of the same shape (Pérez-Hedo et al., 2015). Unidirectional humidified airflow was pumped at 150 ml/min in each glass jar. Each female mite was individually released on the metal string, at the entrance of the Y-tube and its choice was recorded once the female had walked up one of the Y-tube arms, within 10 minutes. After every 5 valid responses the metal string and the Y-tube was rinsed with water, soap and acetone and the left and right odor source tubes were interchanged in order to minimize any spatial effect on the olfactory response. All plants used as odor sources were replaced after recording the response of ten mites. A total of 30 valid responses were recorded for each type of mite experience (sweet pepper, tomato, plastic) and each pair of odor sources. The Y-tube experiment was conducted at the following environmental conditions, 23 ± 2 °C, 60 ± 10 % RH. Light was provided by four 60-cm-long fluorescent tubes (OSRAM, L18 W/765, OSRAM GmbH, Germany) positioned 40 cm above the Y-tube and its intensity was measured with a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc., Pullman, WA) at 2,516 lux (Pérez-Hedo et al., 2015).

2.3 Immature survival on tomato leaflets *vs* sweet pepper leaves

Approximately one hundred female *A. swirskii* mites from the colony were placed for 24 h on two clean plastic rearing units with cotton threads to allow oviposition (as described in 2.1). Then, with the help of a fine paint brush, the eggs were gently collected from both

arenas and transferred evenly to plants. Eggs were individually placed on either one tomato leaflet or on a pepper leaf, whose petioles were covered with tanglefoot odorless glue (The Tanglefoot Company, Grand Rapids, MI, USA) to prevent the escape of the young mites after hatching. In total, twenty eggs were individually placed on the tomato leaflets of two intact tomato plants and another 20 were placed individually on the leaves of four intact sweet pepper plants. Both pepper and tomato plants had 6 fully developed leaves and each fully developed tomato leaf had 5-7 leaflets. Egg hatching and mite survival were evaluated daily for one week. Pollen of *C. edulis* was added every two days on the leaflets and leaves as food source.

2.4 Predation and oviposition on tomato leaflets *vs* sweet pepper leaves

Presumably mated females from the colony were transferred to plastic rearing units with four cotton threads 2 cm long (as described in 2.1) and were allowed to oviposit for 24 hours. Later, the four cotton threads, with approximately 200 eggs in total (50 eggs on each approximately), were transferred to two rearing units with tomato leaflets and two rearing units with sweet pepper leaves (one cotton thread on each unit). The rearing units consisted of either detached tomato leaflets or sweet pepper leaves, placed on water saturated sponges that were covered with wet cotton, and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs added as food source *ad libitum*. After seven days, 50 females and males were collected from the latter rearing units. Couples of male and female were individually placed, isolated on either tomato or sweet pepper leaf discs with 4 cm diameter (25 pairs per host plant species), with *E. kuehniella* eggs as food source and observed every 24 hours until the first oviposition was detected. After the first oviposition, males were removed, and the number of *E. kuehniella* eggs preyed by *A. swirskii* females and the number of eggs

laid per female was counted every 24 hours, during six days. The first day of oviposition was excluded from the analysis because the mites were stressed from the change to a new environment (personal observation). A total of 25 females (replicates) per host plant (tomato or sweet pepper) were tested. The leaf discs were maintained fresh on 1 % agar (w/v) gel, inside plastic cups (5 cm on diameter) with 2 × 2 cm screens on the lid covered with a fine mesh for ventilation but preventing mite escaping. All rearing units and experimental set ups were maintained in growth chambers at 25 ± 2 ºC, 14:10 h (Light: Dark) photoperiod and 80 ± 10 % RH.

2.5 Dispersal on tomato stems

Adult female mites from the colony were used to investigate their dispersal on tomato plants with intact stem trichomes and tomato plants with removed stem trichomes. The trichomes of the stem were removed by mechanical pressure, after rubbing softly the stems with a paper tissue and verifying the removal of the trichomes under the stereoscope. The central part of the stem with three successive leaves was used for the observations and it was delimited by Tanglefoot ® (The Tanglefoot Company, Michigan, USA) glue barriers. On the part with the three successive leaves, the middle leaf was removed and pollen of *C. edulis* was added on the other two leaves. One day later, one female mite was released on the scar left at the stalk base and it was assessed whether the mite had reached the successive leaf or not after two hours (adapted from Van Haren et al., 1987). If the mite reached the successive leaf, the dispersal was recorded as successful. If the mite was stuck to the exudate of the trichomes, the survival was registered after 24 hours. Mites were considered alive if they moved after a gentle probe with a fine paint brush. Thirty-five

female mites were tested on each type of tomato plant, with and without trichomes, at 22 ± 3 °C and 50 ± 10 % relative humidity.

2.6 Isolation, purification and characterization of trichome secondary metabolites

Tomato plants cv Muchamiel were grown in a greenhouse at Instituto de Biología Molecular y Celular de Plantas at the Universitat Politécnica de Valencia under standard growing conditions. To isolate the tomato trichomes from tomato stems, leaves were removed, and the petioles and the stems were submerged in liquid nitrogen. Then, the frozen stems and petioles were softly rubbed with a fine brush inside a mortar with liquid nitrogen in order to remove and collect the frozen trichomes. Three grams of isolated trichomes were used to extract non-volatile compounds by adding 12 ml of isopropanol:acetonitrile:water (3:3:2 v/v/v). The extract was vortexed vigorously, sonicated for 10 min (Selecta 300683) and then centrifuged at 12,000 rpm for 15 min (Allegra 64R, Beckman Coulter, USA). The supernatant containing trichome secondary metabolites was concentrated by evaporation under vacuum. Then, the concentrated secondary metabolites were combined with Bondesil-C18 40 μM Silica gel (Varian), at a weight to weight ratio of 2:3, secondary metabolites to C18 Silica gel and were purified using a dry column vacuum chromatography protocol. Varian solid phase extraction columns were placed on a filter flask attached to a vacuum and the dried residue was then loaded on top. Columns were washed under vacuum pressure four times using 5 ml of water. Secondary metabolites were then eluted from the column with two 5 ml methanol washes of decreasing polarity (water containing 25, 50, 75, and 100 % methanol) and were named FII, FIII, FIV and FV, respectively (adapted by Leckie et al., 2016).

For chromatographic analyses, samples were diluted in 80 % methanol (LC/MS grade, supplemented with biochanin A as an internal standard for relative quantitation purposes) to reach a concentration of 1 mg/ml (respect of the dry residue amount).

UPLC/MS analyses were performed using an Acquity SDS LC system (Waters Corp., MA, USA) coupled to a Q-TOF Mass Spectrometer (Micromass Ltd., UK), similarly as described in Ghosh et al., (2014). Ten microliters of each trichome extract fraction (1 mg/ml) were injected. Separation was performed using a C18 Analytical HPLC column (Luna Omega 1.6 µm Polar C18, 2.1 × 100 mm,). The mobile phase consisted of aqueous 10 mM ammonium formate, pH 2.64 (Solvent A) and acetonitrile (Solvent B) using a linear gradient elution of 1 % B at 0–1 min, 1–80 % B at 1–100 min, 80–100 % B at 100–101 min, 100 % B at 101–105 min and 1 % B at 105–106 min. A 4 min re-equilibration time was used between analyses. During analyses, the solvent flow rate was 0.3 ml/min and the column temperature was 40 °C. Analyses were performed in positive and negative ion modes. Source parameters were as follows: capillary voltage 2500 V, sample cone voltage 30 V, desolvation temperature 350 °C, source temperature 120 °C, cone gas flow 40 l/h and desolvation gas flow 350 l/h for the negative ion mode. For positive ion mode, the capillary voltage was set to 3500 V and sample cone voltage was set at 30 V. Mass spectra acquisition was performed within the *m/z* 50 to 1,500 a.m.u. range in both positive and negative ion modes with a scan time of 0.2 s. The fragment ions were obtained by setting a second acquisition function within the same m/z range but including a collision-induced dissociation step (collision cell energy ramp was set between 5 and 60 eV). Accurate mass values were obtained by co-injecting Leu-enkephalin as a lockmass standard compound ($[M+H]^+$ 556.2771, $[M-H]^-$ 554.2615).

Tentative identification of acyl sugars was performed by comparison of both, mass spectra and retention time with those in the *Solanum* trichome metabolite database version 2015.MSU.004P (Jones, 2015). Identification of all other metabolites was performed by means of comparison of both mass spectra and retention time with those of authentic standards, when available, or by matching precursor and MS/MS mass spectrum with those available in literature or public databases (METLIN, HMDB or KNApSAcK).

2.7 Toxicity of trichome extracts

The fractions were concentrated by evaporation and their dry weight was estimated. Then they were diluted again in 75 % methanol to reach a concentration of 20 mg/ml. These solutions were then used to make working dilutions of 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml, for fractions FII – FV. Moreover, even aliquots of fractions FII and FIII were combined to form the more polar fraction (F-MP) while the same was done with fractions IV and V to form the less polar fraction (F-LP). A 1:1 mixture of two fractions was created by combining equal parts of both fraction solutions at 20 mg/ml, thereby creating a mixture with an overall concentration of 20 mg/ml, wherein each fraction was represented at 10 mg/ml upon application. F-MP and F-LP dilutions of 10 mg/ml and 5 mg/ml were tested for toxicity against *A. swirskii,* as well (adapted by Leckie et al., 2016).

To test the toxicity of dilutions of each fraction and the F-MP, F-LP mixtures, a 2 µl droplet was applied, with an automatic micropipette, on a single female mite for 1 minute inside a glass Petri dish. Afterwards, the droplet was dried with a paper tissue and the mortality was recorded after 30 minutes. For each fraction, 20 mites were tested at room temperature (23

± 2 ºC) and 50 ± 5 % RH. Also, a solution of 75 % methanol in water was tested on 30 mites as a blank control to test if the buffer solution caused mortality to the mites.

2.8 Detection of acyl sugars on mites

To stain the acyl sugars present in the trichomes, the middle part of tomato stems (10 cm long) was cut and the leaves removed. The stem sections were soaked in 0.2 % Rhodamine B (Sigma-Aldrich, St. Luis, Missouri, USA) for 60 minutes and then washed 4 times with distilled water to remove unbound stain (Lin and Wagner 1994). The stems were left to dry for 24 hours, and then adult female mites were released individually for approximately 12 hours on the stems. Then the mites were killed by freezing and they were subsequently observed under the fluorescence microscope (excitation 550 nm / emission 582 nm) (Wagner et al., 2004). All manipulations and solutions were performed at room temperature (22 ± 3 °C).

2.9 Statistical analysis

The olfactory responses to tomato and sweet pepper volatiles was analyzed using the Exact Binomial Test (R package), with Clopper-Pearson 95 % confidence interval, to compare the number of mites attracted to volatiles from sweet pepper, tomato or to clean air, against the null hypothesis that the probability of mites choosing any odour is equal. Immature survival probabilities on tomato and pepper plants were compared with a Fisher's Exact Test (R package), where p-values are obtained using the hypergeometric distribution, to test the null hypothesis that these probabilities are similar. The data on predation and oviposition on tomato leaflets *vs* sweet pepper leaves were fitted to a Generalized Linnear Model with quasipoisson distribution and the F-Test was applied to compare the variances.

A Fisher's Exact Test was used as well, to test the null hypothesis that the probabilities of dispersal and survival on tomato plants with trichomes were similar to the probabilities on the tomato plants without the trichomes. Toxicity data were fitted to a Generalized Linnear Model with binomial distribution and the χ^2 test was applied to compare the variances. Dose response curves were fitted to a model and the 50 % lethal concetration (LC_{50}) was estimated using the R package drc version 3.0. To test for effects of mixture of fractions and their interaction on the mite mortality, data were analyzed by a generalized linear model including the rate of each fraction in F-MP and F-LP respectively, and the interaction of these rates. Synergism or antagonism between the two components of each mixture would be indicated by a significant interaction term in the model fit. All the statistical analysis was performed on the software R version 3.5.1.

3. Results

3.1 Olfactory responses to tomato volatiles

Amblyseius swirskii mites, experienced on tomato leaves, preferred either the unknown host plant, sweet pepper ($P = 0.04$, $N = 30$, binom. test) or the absence of plant (clean air) ($P = 0.005$, $N = 30$, binom. test) showing a clear avoidance of the known environment, the tomato plant (Figure 1). On the contrary, mites experienced on sweet pepper plants preferred to move towards the known host plant, the sweet pepper, instead of the unknown host (tomato plant) ($P < 0.001$, $N = 30$, binom. test) or the absence of plant ($P = 0.001$, $N = 30$, binom. test) (Figure 1). Finally, when mites that did not have a previous experience on any host plant, were tested for their preference between two unknown host plants, the tomato and the sweet pepper, or between one host plant (either tomato or sweet pepper)

and the absence of plant, no statistically significant preference was identified, since the mites were choosing equally any of the two environments offered in each comparison ($P > 0.05$, $N = 30$, binom. test) (Figure 1).

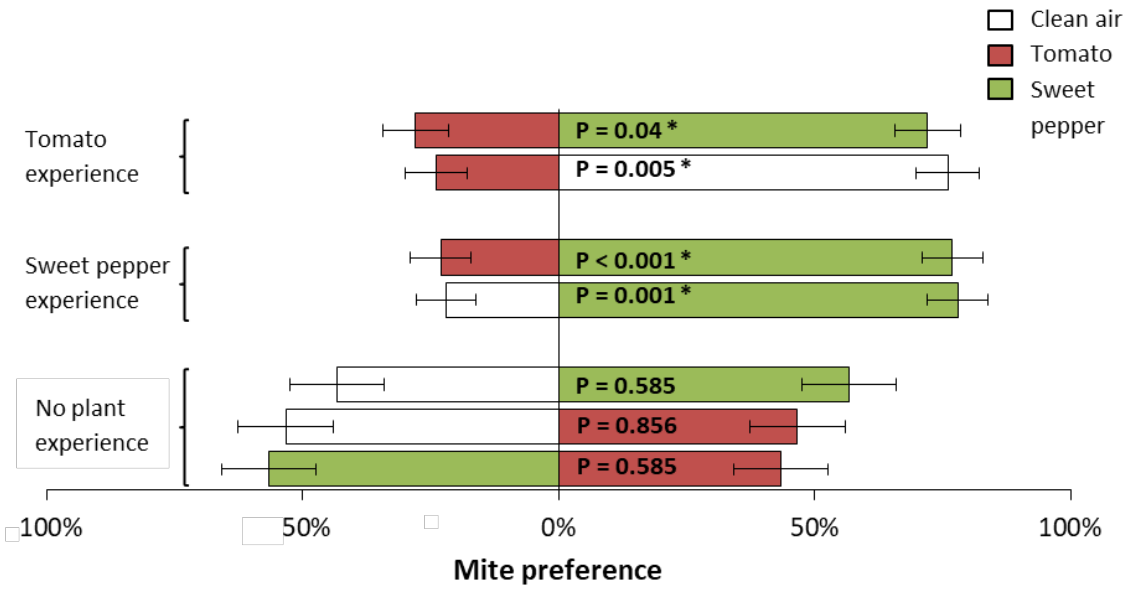


Figure 1 Olfactory response rate of *Amblyseius swirskii* females, inexperienced with plants, experienced on sweet pepper leaves and experienced on tomato leaves, towards the volatiles of sweet pepper, tomato or versus clean air. Red color indicates response to tomato volatiles, green color indicates the response to sweet pepper volatiles and white indicates the mite response to clean air. The true responses of 30 mites were collected from each two-side choice experiment. Significant differences, based on the Exact Binomial Test with Clopper-Pearson 95 % confidence interval, are marked with (*) (P<0.05).

3.2 Immature survival and adult performance on tomato leaflets

The egg hatching rate was 100 % on both, tomato and sweet pepper leaves and the pre-adult survival rate from egg to adult was 95 % and 100 % for mites reared on tomato and mites reared on sweet pepper leaves, respectively ($P = 1$, $N = 30$, Fisher's Exact Test). The oviposition rate of adult female mites on tomato and sweet pepper leaf discs was estimated

for the first five days after oviposition started and was found to be 2.05 ± 0.12 eggs/female/day and 1.8 ± 0.08 eggs/female/day, respectively, which were not significantly different between them (F = 2.80; df = 1, 156; P = 0.09) (Figure 2). The predation rate of *E. kuehniella* eggs by females of *A. swirskii* was similar in both cases, reaching 4.65 ± 0.22 eggs/female/day on sweet pepper and 4.22 ± 0.17 eggs/female/day on tomato leaf discs (F = 2.47; df = 1, 158; P = 0.11) (Figure 2).

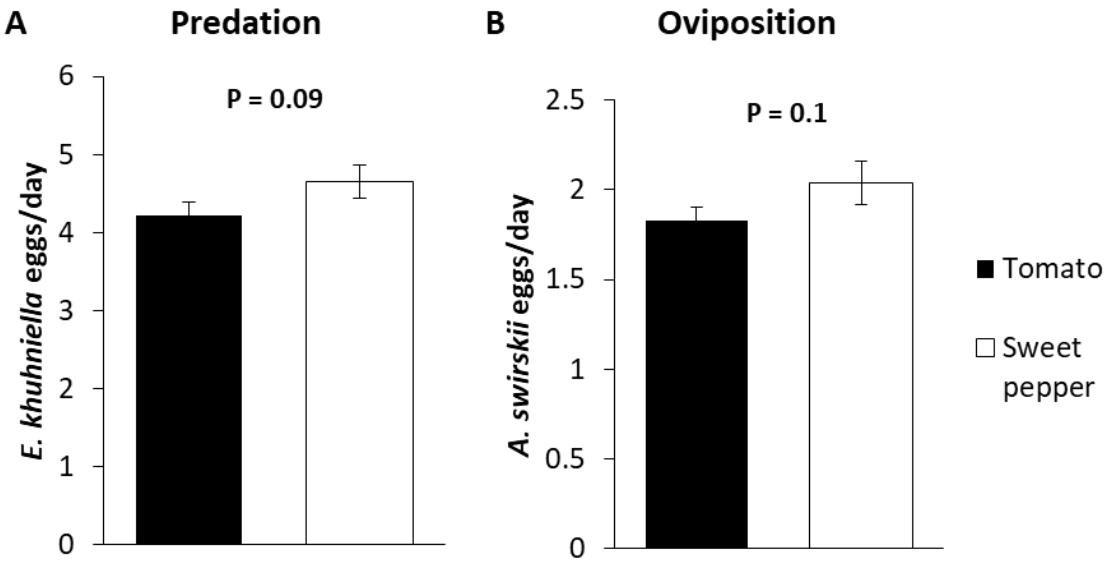


Figure 2. A: Mean predation of *A. swirskii* female mites and standard error (SE) on tomato and sweet pepper leaves for five days. B: Mean oviposition rate of *A. swirskii* female mites and SE on tomato and sweet pepper leaves for five days. The data for both parameters were collected from 25 mites for each plant, they were fitted to a generalized linear model and an F-test was applied to the model variances.

3.3 Dispersal on tomato stems

The dispersal rate of *A. swirski* adult female was tested on tomato stems with trichomes and it was found to be only 25 ± 9 %, in contrast to the 100 % dispersal rate recorded on

tomato plants with removed trichomes ($P < 0.001$, $N = 35$, Fisher's Exact Test) (Figure 3). On tomato plants without trichomes, all the mites survived for 24 hours, however only 38 ± 10 % of them survived on plants with trichomes (P <0.001 $N = 35$, Fisher's Exact Test) (Figure 3). In particular, on tomato plants with trichomes, the fraction of mites that survived for 24 hours includes the mites that successfully dispersed to an adjacent leaf (25 ± 9 %) and those that were entrapped on the trichomes but alive, being able to move at least one limb after 24 hours (13 %).

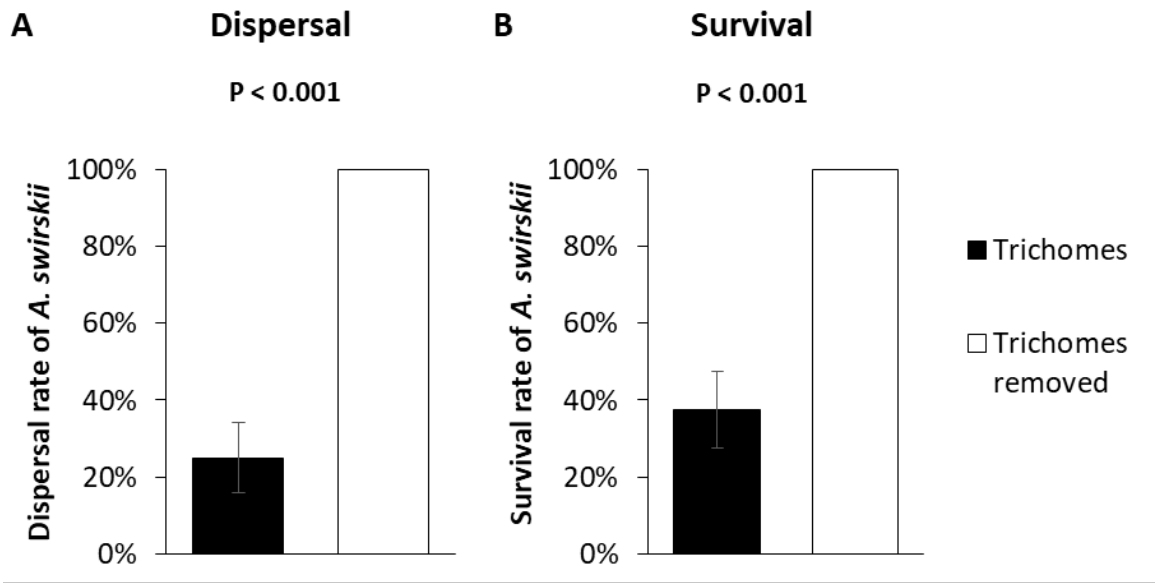


Figure 3. A: Mean dispersal and standard error (SE) on tomato stems with trichomes (black bars) and without trichomes (white bars) 2 hours after release. B: Mean survival rate of *A. swirskii* and SE on tomato stems with trichomes (black bars) and without trichomes (white bars) 24 hours after release. The data were analyzed with a Fisher´s Exact Test.

3.4 Toxicity of trichome extracts

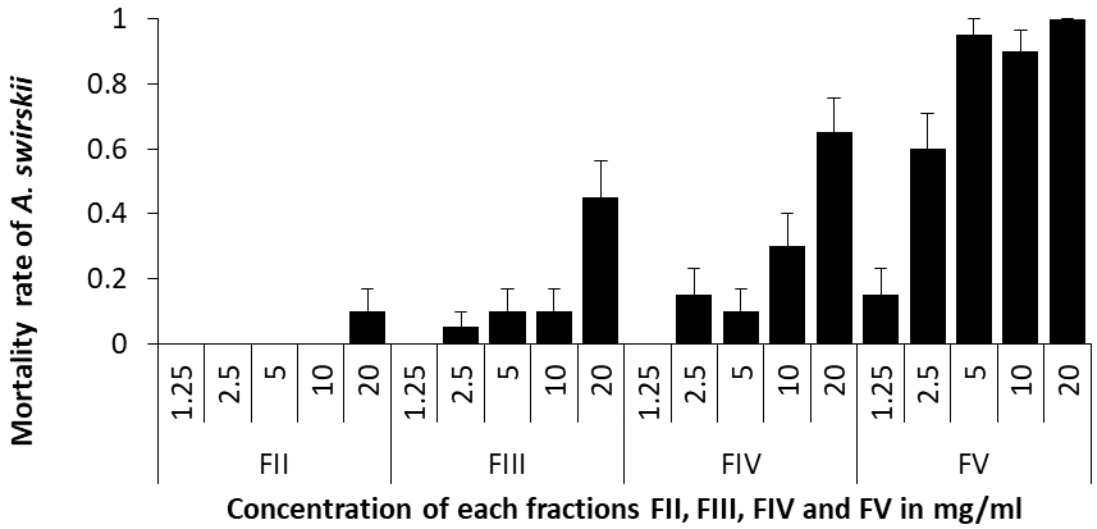
Trichome extracts of tomato plants were separated by dry column vacuum chromatography into four fractions, based on polarity, with the most polar fraction FII, followed by FIII,

382 FIV and last the least polar fraction FV (Table 1). These fractions were tested for their
383 toxicity on adult mites in a range of concentrations from 1.25 to 20 mg/ml (Figure 4). The
384 mortality rates were fitted to a model with binomial distribution and it was found that some
385 fractions were significantly toxic to the mites (χ^2; df = 3, 16; $P < 0.001$). The highest
386 toxicity was recorded with fraction FV at 20 mg/ml where 100 % of the mites were killed.
387 In addition, this fraction caused 95 % mortality at a concentration as low as 5 mg/ml (Figure
388 4). Control testing on 30 mites, with blank 75 % methanol in water, did show any mortality.

389 **Table 1**. Estimated concentrations, in mg/ml, required to cause 50 % mortality (LC_{50}) and
390 their 95% delta confidence intervals (CI) for four fractions of trichome extracts. Trichome
391 secondary metabolites were extracted and fractioned according to decreasing polarity
392 (water containing 25, 50, 75, and 100 % methanol) and were named FII, FIII, FIV and FV,
393 respectively. Even aliquots of fractions FII and FIII were combined to form the more polar
394 fraction (F-MP) while the same was done with fractions IV and V to form the less polar
395 fraction (F-LP).

Fraction	LC_{50}	Std. Error	Lower CI	Upper CI
FII	26.72	47.74	0.00	120.28
FIII	28.52	11.07	6.83	50.21
FIV	15.23	3.62	8.13	22.34
FV	2.28	0.32	1.66	2.91
F-MP	29.37	134.47	0	292.91
F-LP	6.51	0.74	5.06	7.97

396

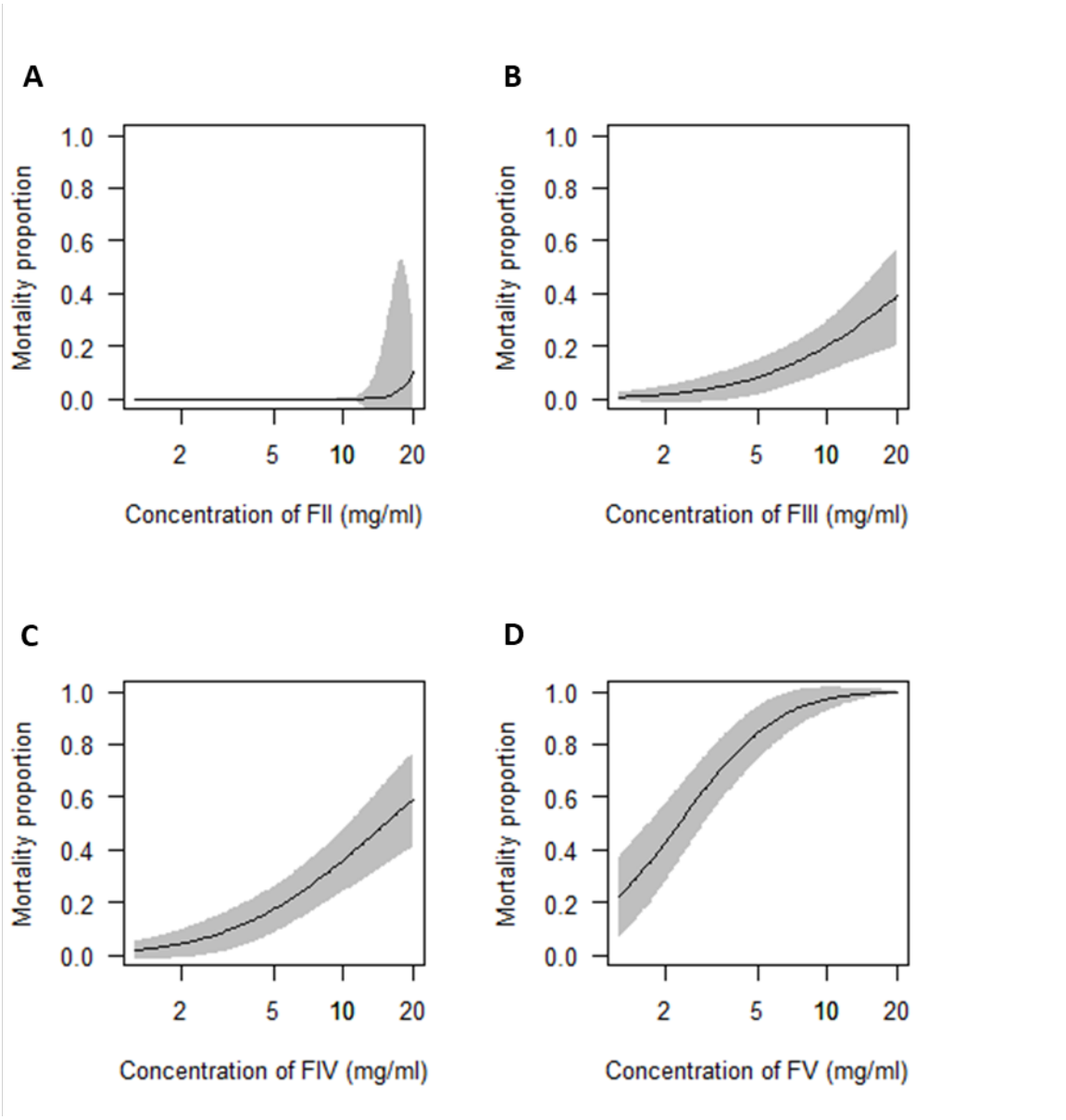


397

398 **Figure 4.** Mortality rate of *A. swirskii* mites exposed to four fractions of trichome extracts
399 with different polarity (FII, FIII, FIV, FV). Each fraction was tested at five concentrations
400 (1.25, 2.5, 5, 10, 20 mg/ml) on 20 mites.

401

402 Dose response curves were fit for each fraction, and the lethal concentration required to
403 kill 50 % of the mites (LC_{50}), with 95% delta confidence intervals, were estimated (Figure
404 5, Table 1). The lowest LC_{50} were observed with fraction FV at 2.28 ± 0.32 mg/ml,
405 followed by FIV at 15.23 ± 3.62 mg/ml, whereas the LC_{50} for the fractions FII and FIII
406 was not in the tested range of concentrations and was estimated by the model
407 approximately two times higher (Table 1).



408

409 **Figure 5.** The dose response curve and the 95 % delta confidence interval of *A. swirskii*
410 mortality to A) fraction FII, B) fraction FIII, C) fraction FIV and D) fraction FV of
411 trichomes extract.

412

413 To investigate possible synergistic or antagonistic effects between fractions, the mixtures
414 F-MP and F-LP were derived from the combination of FII - FIII and FIV-FV, respectively.
415 In the presence of either synergy or antagonism, the mixture should cause a different

response than that expected in case of no interactions between the components of the mixture. At a concentration of 20 mg/ml, fraction F-MP, the 1:1 mixture of fractions FII and FIII, caused 5 % mite mortality, while FII and FIII caused 0 % and 10 %, respectively. Hence, the effect of the mixture F-MP was not stronger than that for the sum of the mixture's components in the toxicity test, neither the interaction effect of FII × FIII rates ($P = 0.19$, χ^2 test). Fraction F-LP, the 1:1 mixture of the fractions FIV and FV, caused 95 % mortality at a concentration of 20 mg/ml, consistent with the combination of the effects of FIV and FV (65% and 100%, respectively). The interaction effect of FIV × FV rates in Fr-LP was not significant ($P = 0.08$, χ^2 test) as well, and only the rate of FV in the mixture had a significant effect on mite mortality ($P < 0.001$, χ^2 test). Hence, there was no synergism or antagonism detected with fraction combinations. This finding is also depicted on the LC_{50} values for F-MP and F-LP, which are not very different from the values found for their individual components (Table 1).

3.5 Isolation, purification and characterization of trichome secondary metabolites

LC-MS characterization of each fraction revealed that fractions FIV and FV contained several acyl sugars and that the concentration of these compounds was higher in FV (most toxic) by one order of magnitude (Table 2). In total, 16 acyl sugars of sucrose were identified in the trichome extract fractions as described on Table 2. On the other hand, acyl sugars were not detected in the least toxic fractions, FII and FIII. Some other compounds, such as rutin, kaempferol, tomatine, were detected at similar levels in all fractions.

Table 2. The list of acyl sugars identified and characterized by mass spectroscopy with negative electrospray ionization. For each identified acyl sugar, tentative identity,

according to the Michigan State University database and its nomenclature, empiric formula, molecular mass, retention time (RT) and its abundance in each of the four fractions (FII, FIII, FIV and FV), measured as peak height of the chromatogram.

Identity	Empiric Formula	Molecular Mass	RT (min)	Abundance (peak height)			
				F V	F IV	F III	F II
S4:16	$C_{28}H_{46}O_{15}$	622.2837	50.75	39	nd	nd	nd
S4:17	$C_{29}H_{48}O_{15}$	636.2993	54.27	750	29	nd	nd
S4:17	$C_{29}H_{48}O_{15}$	636.2993	53.95	104	nd	nd	nd
S3:20	$C_{32}H_{56}O_{14}$	664.3306	68.57	149	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	73.35	92	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	71.66	32	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	58.08	57	nd	nd	nd
S3:21	$C_{33}H_{58}O_{14}$	678.3463	59.42	45	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	74.58	65	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	76.26	1990	nd	nd	nd
S3:22	$C_{34}H_{60}O_{14}$	692.3619	76.48	1420	nd	nd	nd
S4:22	$C_{34}H_{58}O_{15}$	706.3776	73.88	59	nd	nd	nd
S4:22	$C_{34}H_{58}O_{15}$	706.3776	79.22	43	nd	nd	nd
S4:23	$C_{35}H_{60}O_{15}$	720.3932	78.68	tr	nd	nd	nd
S4:24	$C_{36}H_{62}O_{15}$	734.4088	75.68	66	nd	nd	nd
S4:24	$C_{36}H_{62}O_{15}$	734.4088	81.75	337	nd	nd	nd

* In Identity: letter = sugar type (S=sucrose), number = number of esters with acyl groups : number = total number of carbons. In Abundance: nd= not detected, tr= found at trace levels.

3.6 Detection of acyl sugars on mites

The fluorescent microscopy of the type VI trichomes indicated the presence of sugar esters in the glandular heads, most likely the acyl sugars produced and stored by the glandular cells (Figure 6). Moreover, the images of mites that walked on the stems with the stained trichomes revealed that the acyl sugars are released from the trichomes and accumulate on the mite cuticle and in the limb joints and mouth parts from where it is likely to penetrate under the cuticle (Figure 6).

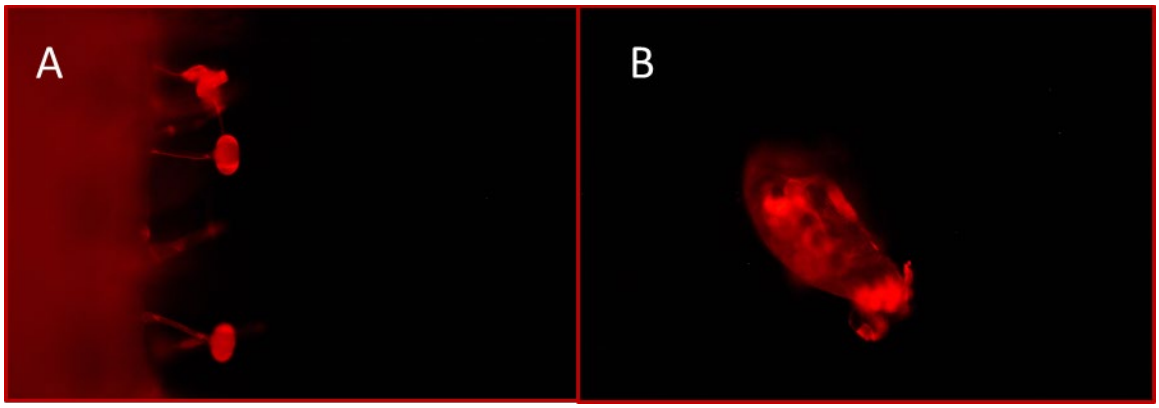


Figure 6. Fluorescent microscopy of (A) type VI tomato trichomes and (B) *A. swirskii* mites which were exposed to stained tomato stems for 12 hours.

4 Discussion and conclusions

Predatory mites can associate volatile plant cues with positive or negative conditions on the host plant, a process called associative learning (Drukker et al., 2000). In predatory mites, negative associative learning using volatile plant cues has been previously associated with stressful conditions such as starvation, but never with the plant trait itself (Drukker et al., 2000). Here, it was demonstrated that predatory mites experienced with tomato leaves, preferred a new, unknown environment with or without plant, instead of the tomato plant. Therefore, the avoidance of this host indicates a negative, stressful experience of the mites, associated with tomato plants regardless the presence of prey.

The egg hatching, juvenile survival, oviposition and predation rates of predatory mites were not significantly different on tomato leaves compared to sweet pepper leaves under the given experimental conditions. The oviposition rate of *A. swirskii* fed on *E. kuehniella* eggs estimated in this study was higher than the lifelong oviposition rate with the same

food source (1.48 eggs/female/day at 23 ºC) (Nguye et al., 2013). This is because oviposition was recorded during the first five days where the peak rate occurs, a methodology widely used to evaluate this parameter in predatory mites, since these estimates are closed to those obtained from full lifetable analysis (Abad-Moyano, 2009, Argolo et al. 2013, Sabelis and Janssen 1992). A negative effect of the tomato host plant on various biological parameters of other predators, including phytoseiids, has been previously found. *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae) survival, adult longevity and predation rate on *T. absoluta* were negatively impacted on tomato plants with high densities of glandular trichomes (Benites Bottega et al., 2017). High density of tomato trichomes was also correlated to lower walking speed and fecundity rate of the predator *Delphastus (Pusillus) catalinae* Horn (Coleoptera: Coccinellidae) (Heinz and Zalom 1996). The movement and predation rate of the predatory larvae *Episyrphus balteatus* De Geer (Diptera: Syrphidae) and *Adalia bipuncata* Linnaeus (Coleoptera: Coccinellidae) was drastically reduced by the tomato trichomes (Shah et al., 1982; Verheggen et al., 2009) and *Podisus maculiventris* Say (Hemiptera: Pentatomidae) experienced high nymphal mortality on tomato plants (Lambert 2007). *Neoseiulus californicus* developmental time and sex ratio were similar on tomato and strawberry, but juvenile survival and oviposition were lower on tomato (Castagnoli et al., 1999). Oviposition rate of *N. californicus* on tomato leaves was negatively affected on tomato leaves, both directly and indirectly through the prey, when compared to bean leaves (Koller et al., 2007). *Phytoseiulus macropilis* and *P. longipes* walking, predation and oviposition rates were reduced on tomato leaves, when compared to strawberry (Sato et al., 2011). *Amblydromalus limonicus* Garman & McGregor (Acari: Phytoseiidae) mites preyed fewer

493 *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) psyllid nymphs per day on tomato
494 than on sweet pepper, but the mite survival was similar on the leaves of both plants
495 (Davidson et al., 2016). *Amblyseius swirskii* walked slower on plant species with increasing
496 trichome density and on tomato leaves their walking speed was lower when compared to
497 rose plants (Buitenhous et al., 2014). On tomato leaves, the trichomes and their exudates
498 seem to affect the phytoseiid predation rate and their oviposition rate. In this study, an
499 effect of leaf pubescence of different host plants, on predation and oviposition rates of *A.*
500 *swirskii* was not found. It is possible that offering food *add libitum* and the limited leaf
501 surface used in the experiment could have masked any small differences on predation rate
502 between different host plants.

503 The detrimental effect of tomato plants on *A. swirskii* dispersal and survival was observed
504 on the stems. This effect was in direct correlation with the presence of trichomes. The
505 profound impact of the glandular trichome density on mite dispersal and survival on tomato
506 stems has been previously shown for *Phytoseiulus persimilis* Athias-Henriot (Acari:
507 Phytoseiidae) as well, with entrapment and mortality rates estimated at 61 % and 73 %,
508 respectively (Van Haren et al., 1987). Also, mite entrapment rate has been positively
509 correlated to the size of glandular of trichome heads, which is influenced by light intensity
510 (Nihoul, 1993). The rates of entrapment and mortality of *A. swirskii* and *P. persimilis* are
511 rather similar and the small differences observed might be explained either by differences
512 in the trichome densities of different tomato varieties used in the experiments or by
513 differences in the mite morphologies, such as the length of legs. Adults are more affected
514 by the glandular trichomes present on the stems than juvenile developmental stages,
515 because young adults disperse in order to mate and avoid prey overexploitation, whereas

516 the juveniles usually stay at the natal patch until molting is completed (Pels and Sabelis,
517 1999).

518 Staining of the epicuticular sugar esters of the tomato stems revealed a high concentration
519 of sugar esters in the glandular trichomes type VI. The most abundant secondary metabolite
520 produced by those trichomes, the acyl sugars, are polyesters of glucose or sucrose
521 (Schilmiller et al., 2010). After walking on the stained tomato trichomes the predators, the
522 acyl sugars were released, and accumulated on their cuticle and mostly on their mouth parts
523 and limb joints. Similarly, the staining of the tobacco acyl sugars has shown their
524 accumulation on the body of aphids after walking on the plant surface (Wagner et al.,
525 2004). This study is the first to demonstrate the attachment and accumulation of acyl sugars
526 on the body of phytoseiids and pinpoints which secondary metabolites are most likely
527 hindering the establishment of predatory mites on tomato plants. Moreover, two main
528 mechanisms of insecticidal action for acyl sugars have been proposed; first, insects
529 suffocate when acyl sugars cover the openings on their cuticle; second, the insects become
530 desiccated when their cellular membranes under their cuticle are disrupted by the fatty acid
531 moiety of acyl sugars (Puterka et al., 2003.).

532 An additional fact supporting a role of acyl sugars in mite mortality is that acyl sugars were
533 identified in the trichome extract fraction FV that was the most toxic for the predatory
534 mites, and less in the FIV, that was accordingly less toxic for the mites. Other trichome
535 secondary metabolites were identified in similar levels in several of the fractions, hence
536 they were not considered responsible for the observed toxicity. High toxicity of acyl sugars

of *Nicotiana gossei* to pear psylla, *Cacopsylla pyricola* (Foerster) (Homoptera: Psyllidae), adults and nymphs has been observed as well (Puterka & Severson, 1995).

High densities of glandular trichomes on the host plant stem are clearly detrimental to the life history of the predatory mites, interfering with the biological control of the pests (Castagnoli et al., 1999; Cédola et al., 2001). Tomato pests on the other hand, have the morphological and behavioral adaptations to avoid the trichomes or the glandular exudates and so, can reproduce on a predator free environment. It is necessary to investigate how plant characters that render resistance against arthropods have fitness tradeoffs, because they provide enemy-free space to herbivores that are adapted to these defenses. Hence, more research is required to understand the influence of the tomato acyl sugars on herbivores and their key predators. Tomato plants with minimal levels of acyl sugars could be used in future studies to understand the effect of those secondary metabolites on the plant fitness in the presence of biological control. Last, the effect of the plant physiology on natural enemies is important for the application of biocontrol programs on the crops and it should be taken into account by plant-breeding programs.

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Author Contribution Statement

AP, JLR, MPLG, JGC and AU conceived the work and interpreted the data; AP collected the data and wrote the article; AP, JLRN, MPLG ~~VA~~ and AGC performed both SPE and LC-MS chromatographic analysis; JLR, MPLG, JGC and AU critically revised the article. All authors read and approved the manuscript.

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