To be submitted to: Pest Management Science

# Zoophytophagous mirids provide pest control by inducing direct defences, antixenosis and attraction to parasitoids in sweet pepper plants

Running title: Induced defences by mirids in sweet peppers

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**Abstract**

BACKGROUND: In addition to their services as predators, mirid predators are able to induce plant defences by phytophagy. However, whether this induction occurs in sweet pepper and whether it could be an additional benefit to their role as biological control agent in this crop remains unknown. Here, these questions are investigated in two model insects, the mirids *Nesidiocoris tenuis* and *Macrolophus pygmaeus*.

RESULTS: Plant feeding behaviour was observed in both *N. tenuis* and *M. pygmaeus* on sweet pepper and occupied 33% and 14% of total time spent on the plant respectively. The punctures caused by mirid plant feeding induced the release of a blend of Volatile Organic Compounds (VOCs) which repelled the herbivore pests *Frankliniella occidentalis* and *Bemisia tabaci* and attracted the whitefly parasitoid *Encarsia formosa*. The repellent effect on *B. tabaci* was observed for at least 7 days after initial exposure of the plant to *N. tenuis,* and attraction of *E. formosa* remained functional for 14 days.

CONCLUSION: Feeding induced plant defences by mirid predators, their subsequent effects on both pests and natural enemy behaviour, and the persistence of these observed effects open the door to new control strategies in sweet pepper crop. Further application of this research is discussed, such as the vaccination of plants by zoophytophagous mirids in the nursery before transplantation.

**Key worlds:** *Nesidiocoris tenuis, Macrolophus pygmaeus,* phytophagy, HIPV's, plant response, vaccination

## 1 INTRODUCTION

Predatory mirid bugs (Hemiptera: Miridae) have been extensively studied in the last few decades for their ecological significance and role as predators of agricultural pests .[1,2] In recent studies, their importance as biocontrol agents in sweet pepper has been highlighted.[3-5] The use of generalist natural enemies in sweet pepper crops is widely common and has been proven successful.[6-8] If properly managed, the release and the conservation of the predatory mite, *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae) together with the anthocorid *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) can successfully manage the population of the key pepper pests; sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) and western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae).[9,10] Moreover, the polyphagous behaviour of *A. swirskii* and *O. laevigatus* contribute to the management of secondary pests, such as spider mites and Lepidoptera.[11,12] In this system aphids manage to evade the control of both predators[13,14] and so the multiple release of natural enemy species is usually practiced, which can have considerable implications in the final cost of the biocontrol programme in this crop.[3,15] Alternative biocontrol strategies in which mirid predators are included have hence been recently explored. *Nesidiocoris tenuis* (Reuter), *Macrolophus pygmaeus* (Rambur) and *Dicyphus maroccanus* (Wagner) (Hemiptera: Miridae) were shown to effectively control the aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) on sweet pepper.[5] Furthermore, *M. pygmaeus* was found to be the most effective agent for the control *M. persicae* in sweet pepper when compared with three other mirid species, *Dicyphus errans* (Wolff), *D. tamanii* (Wanger) and *Deraeocoris pallens* (Reuter).[4] Under combined release, intraguild interactions between *M. pygmaeus* and *O. laevigatus* did not result in population imbalances of either predatory species, but a better control strategy for both thrips and aphids on sweet pepper resulted.[16]

77 Within the mirids, omnivory is common (Wheeler, 2001) [2] and they are able to exploit
78 both plant and prey resources during the same developmental stage.[17] This flexibility in their
79 behaviour increases survival rates by taking advantage of plant resources when prey is either
80 less abundant or completely absent.[18-20] As in herbivores, the phytophagous behaviour of mirid
81 predators may also induce indirect plant defences.[21-25] It is well known that plants can respond
82 to the damage induced by phytophagous insects, involving several signal transduction
83 pathways that are mediated by phytohormones. Jasmonic acid (JA), salicylic acid (SA), abscisic
84 acid (ABA) and ethylene (ET) are the main targeted components and their accumulation in the
85 plant activates signalling cascades that regulate transcriptional response.[26-30] Indeed, plants
86 damaged by herbivores often produce a blend of volatiles, commonly referred to as herbivore-
87 induced plant volatiles (HIPV's).[31-34] These HIPV's consist of a mixture of the so-called green-
88 leaf volatiles ($C_6$ aldehydes, alcohols and esters), terpenes (monoterpenes, sesquiterpenes,
89 homoterpenes) and aromatic compounds among others.[35,36] Consequently, natural enemies
90 use the change in the composition and concentration of these released volatiles as a cue for
91 the presence of potential prey or hosts.[37-39]

92 In this work, the potential of *N. tenuis* and *M. pygmaeus* to induce plant defences in
93 sweet pepper is investigated and whether this could be an additional benefit to their role as a
94 biological control agent in this crop. The behaviour of *N. tenuis* and *M. pygmaeus* on sweet
95 pepper was first explored in order to quantify feeding activity on the crop. Secondly, the level
96 of the phytohormones involved in the plant defence and the expression of several marker
97 genes was evaluated, both in intact plants (without mirids punctures) and in mirid-punctured
98 plants. A non-targeted analysis of the volatile compounds differentially released by mirid-
99 punctured and intact plants was then performed by means of headspace solid phase
100 microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS).
101 Thirdly, the response of two key sweet pepper pests, *F. occidentalis* and *B. tabaci*, and the
102 whitefly parasitoid *E. formosa* to the odour emitted by mirid-punctured and intact plants was

tested under dual choice experiments. Finally, the persistence of the plant response induced by *N. tenuis* was observed.

## 2 EXPERIMENTAL METHODS

### 2.1 Plants and insects

Pesticide free *Capsicum annuum* (cv. Lipari) (Dulce italiano, Mascarell semillas S.L, Valencia, Spain) seedlings were used for the study. Two weeks after germination, seedlings were transplanted into a mixture of soil and local peat moss in plastic pots (8 x 8 x 8 cm), housed in climatic chambers at 25 ± 2 ºC, 60-80% RH and 16:8 h (L:D) photoperiod at Instituto Valenciano de Investigaciones Agrarias (IVIA). Plants with 6 fully-developed leaves (approximately 15 cm in height) were used for the study of *N. tenuis* and *M. pygmaeus* behaviour and for the rest of the experiments plants were used once 10 leaves had fully-developed (approximately 20 cm in height). Two sweet pepper plant treatments were required, mirid-punctured plants and intact plants (control plants free from any arthropod contact). Mirid-punctured plant were obtained by exposing sweet pepper plants to 25 adult *N. tenuis* or *M. pygmaeus* (sex ratio 1:1) for 24 hours in a 30 x 30 x 30-cm plastic cage (BugDorm-1 insect tents; MegaView Science Co., Ltd., Taichung, Taiwan).

*Nesidiocoris tenuis*, *M. pygmaeus* and *B. tabaci* adults, and *E. formosa* pupae, were provided by Koppert Biological Systems, S.L. (Águilas, Murcia, Spain). Cultures of *N. tenuis* and *M. pygmaeus* were maintained in climatic chamber at 25 ± 2ºC, 60-80% RH and 16:8 h (L:D) photoperiod 25 ± 2ºC, 60-80% RH and 16:8 h (L:D) photoperiod at IVIA. Both mirid cultures were separately caged on sweet pepper plants with access to *Ephestia kuehniella* Zeller eggs (Entofood®; Koppert B.S.) as supplemented food until their use in the bioassays. Five day old adult *N. tenuis* and *M. pygmaeus* were used in all the experiments. Newly emerged adult *B. tabaci* (less than 2 day old) were similarly reared on sweet pepper plants caged in 60 x 60 x 60-cm BugDorm-2 insect tents. In the case of *E. formosa*, pupae were enclosed in a Petri dish (9

129 cm diameter) and allowed to emerge under ambient laboratory conditions (25 ± 2ºC), with a
130 small drop of honey provided as food. Female *E. formosa* were used at less than two days old
131 all experiments.

132 *Frankliniella occidentalis* adults were obtained from a culture established at IVIA in
133 2010, originally collected from Campo de Cartagena (Murcia, Spain). The thrips culture was
134 maintained on bean plants (*Phaseolus vulgaris* L.; Fabales: Fabaceae) and housed in a climatic
135 chamber at 25 ± 2ºC, 65 ± 10% RH and a 14:10 h (L:D) photoperiod at IVIA. All female *F.*
136 *occidentalis* used for experimentation were less than five days old.

137 **2.2 Mirid behaviour on sweet pepper**

138 Direct observations for both male and female *N. tenuis* and *M. pygmaeus* behaviour were
139 carried out on intact sweet pepper plant for 30 minutes under a hand magnifying glass (5cm of
140 diameter and a magnification of 2.5x-5x) (Entomopraxix, Barcelona, España). The experimental
141 arena consisted an intact sweet pepper plant inside a plastic cage 60 x 60 x 60-cm (BugDorm-2
142 insect tents). A single mirid predator (male or female) was then released onto the plant.
143 Recording began when the first behavioural activity was observed (typically: walking, though
144 any of the other recorded behaviours were also considered). Duration of each behaviour and
145 the corresponding location on the plant was noted. For each assay (species and sex), twenty
146 replications were carried out and the sweet pepper plant replaced by new intact plant for each
147 of the subsequent observations.

148 Observed behaviours were the following: Walking (W): Predator walking behaviour on the
149 different regions of the plant. Antennating (A): Stationary searching activity, characterised by
150 moving the antenna. Walking- Antennating (W-A): Non-stationary searching activity
151 characterised by moving the antennae and walking. Feeding (F): The predator uses labium to
152 probe the feeding sites and then inserts the stylet vertically into the plant. Oviposition (O): The
153 predator firstly probes the oviposition site with the labium, then the whole abdomen is
154 pressed onto the plant and the length of the ovipositor inserted into the plant, egg deposition

155 is visible. Grooming (G): Cleaning mouthparts with forelegs and/or cleaning another part of the
156 body. Flying (Fl): Flying movement typically from the plant to the cage walls or the opposite.
157 Resting (R): The predator is at rest, stationary and not carrying out any other described
158 behaviour

159 The plant locations visited by the predator during the observation were defined. One
160 location off-plant (plastic cage, plastic pot or soil) and two locations on-plant (apical region and
161 basal region) were defined. The apical region of sweet pepper plant was defined as the first 5
162 cm of the plant formed by apical stem, young developing leaves and 2 fully developed leaves.
163 The basal region was the rest of the plant, approximately 10-12 cm with 4 developed leaves,
164 basal stem and cotyledons.

165 **2.3 Phytohormone analysis and plant gene expression**

166 In order to identify the phytohormone profile of 1) *N. tenuis*-punctured plants, 2) *M.*
167 *pygmaeus*-punctured plants and 3) sweet pepper intact plants, the hormones, abscisic acid
168 (ABA), salicylic acid (SA), jasmonic acid (JA) and JA-isoleucine (JA-Ile) were analysed by ultra-
169 performance liquid chromatography coupled with mass spectrometry (UPLC-MS).[24,40,41]The
170 apical region of the pant, as defined previously, from each treatment was removed and stored
171 at -80°C until analysis. Five replicates were collected for each treatment. Analyses were carried
172 out using an Acquity ultra-performance liquid chromatography system (UPLC; Waters,
173 Mildford, MA, USA) and the chromatograph interfaced to a triple quadrupole mass
174 spectrometer (TQD, Waters, Manchester, UK). MassLynx NT software version 4.1 (Micromass)
175 was used to process the quantitative data from calibration standards and the plant samples.
176 The calibration curves were obtained by using solutions containing increasing amounts of ABA,
177 SA, JA and JA-Ile commercial standards (Sigma-Aldrich, http://www.sigma-aldrich. com/).

178 Expression of (i) *ASR1* (abscisic acid stress ripening protein 1) a marker gene for ABA, (ii)
179 *PIN2* (wound-induced proteinase inhibitor II precursor) a marker gene for JA, and (iii) *PR1*

(basic PR-1 protein precursor) a marker gene for the SA signalling pathway, were quantified for each of the three plant treatment samples taken from the apical region of the sweet pepper plants. Samples were cut and then ground in liquid nitrogen and a portion used for RNA extraction. Total RNA (1.5 µg) was extracted using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions.[21,24] Samples were homogenized with TRIzol™ Reagent and then chloroform was added to separate protein RNA and DNA. RNA was precipitated with the addition of isopropanol and 1.2 Mm NaCl. After quantification, the RNA was treated with the Turbo DNA-free DNase kit (Applied Biosystems) to eliminate any traces of genomic DNA, according to the manufacturer's protocol. cDNA was then synthesized using prime script™ RT reagent kit (perfect real time) (TAKARA Bio, CA, USA). The reaction mixture was then incubated in the thermo-cycler for 15 min at 37°C followed by 5 s at 85°C. Real-time PCR amplifications were performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA). *Capsicum annuum* specific forward and reverse primers (0.5 µl) were designed and added to 5 µl of Syber green/ROX qPCR MM and 1 µl of cDNA and then brought to 10 µl total volume with Milli-Q sterile water. PCR reactions were run in duplicate, in accordance with manufacturer recommendations. Quantitative PCR was carried out using the LightCycler® 480 System (Roche Molecular Systems, Inc., Switzerland), under the following amplification conditions, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. Melting curve analysis was performed at 95°C for 5 s, 60°C for 1 min and then a continuous increase of temperature up to 95°C to finalise the process. Data acquisition and calculation were performed with the thermal cycler's software and were then collected and analysed in Microsoft Excel. Each qPCR data point is the average of 8 independent experiments. EF1 (elongation factor-1) was used as a standard control gene for normalization. The nucleotide sequences of the gene specific primers are described in Table S1.

### 2.4 Determination of plant volatile compounds

206 Volatile compounds emitted from 1) *N. tenuis*-punctured sweet pepper plants, 2) *M.*
207 *pygmaeus*-punctured plants and 3) intact plants were collected using 5 L volume glass jars as
208 used in Y-tube olfactometery described below (Analytical Research Systems, Gainesville, FL).[1]
209 One sweet pepper plant was introduced (either intact or mirid-punctured) into each jar. After
210 closing the jar, an adsorbent-coated SPME fibre (PDMS/DVB (65 µm
211 polydimethylsiloxane/Divinylbenzene; Supelco, Bellefonte, PA, USA) was mounted on the fibre
212 holder, and injected through the first septum (top of the jar). Agitation of the atmosphere
213 inside the container was achieved by pumping at 5 ml/min using a syringe injected through the
214 second septum (bottom of the jar). The three jars were maintained in the Y-tube olfactometer
215 table at a light intensity of 2516 lx. The volatiles emitted were captured over a 3 hour period,
216 and 5 replicates per treatment were performed. After collection, the fibre was retracted into
217 the needle and the SPME device was removed from the container. The compounds absorbed
218 in the SPME fibre were separated and detected by means of GC-MS.

219 Desorption was performed by means of a CombiPAL autosampler (CTC Analytics) at 250°C
220 for a duration of 1 min in splitless mode in the injection port of a 6890N gas chromatograph
221 coupled to a 5975B mass spectrometer (Agilent Technologies). To prevent cross-
222 contamination, fibres were cleaned after desorption in an SPME fibre conditioning station (CTC
223 Analytics) at 250°C for 5 min under helium flow. Chromatography was performed on a DB-5ms
224 (60 m, 0.25 mm, 1.00 µm) column with helium as carrier gas at a constant flow of 1.2 ml/min.
225 GC interface and MS source temperatures were 260°C and 230°C respectively. Oven
226 programming conditions were 40°C for 2 min, 5°C/min ramp until 250°C, and a final hold at
227 250°C for 6 min. Data were recorded in the 35-300 m/z range at 5 scans/s, with electronic
228 impact ionization at 70 eV. Untargeted analysis of the chromatograms was performed by
229 means of the MetAlign software (WUR, http://www.metalign.nl).

Kovats retention indices (KI) were calculated for each of the compounds. Differentially emitted volatiles were first tentatively identified by comparing their mass spectra with those in the NIST 05 Mass Spectral Library. When available, identity was confirmed by coelution with the pure standards (Sigma-Aldrich). For quantitation of the selected compounds, one specific ion was selected for each compound, and the corresponding peak area from the extracted ion chromatogram was integrated by means of the ChemStation E.02.02 software (Agilent Technologies). The criteria for ion selection were the highest signal-to-noise ratio and being specific enough in that particular region of the chromatogram in order to provide good peak integration.

### 2.5 Response to induced sweet pepper plants

The olfactory response of the sweet pepper pests *F. occidentalis and B. tabaci* and the whitefly parasitoid *E. formosa,* to both mirid punctured plants and intact plants was firstly investigated in the Y-tube olfactometer. The Y-tube olfactometer consisted of two 5 L volume jars connected with a 2.4 cm diameter Y-shaped glass tube.[5] with a 13.5-cm long base and two arms each 5.75 cm long. Both side arms were connected via high density polyethylene (HDPE) tubes to the two identical glass jars. Each glass jar was connected to an air pump that produced a unidirectional humidified airflow at 150 mL min-1.[5] Four 60-cm fluorescent tubes (OSRAM, L18W/765, OSRAM GmbH, Germany) were positioned 40 cm above the horizontal Y-shaped glass tube. The light intensity registered 2,516 lux over the Y-tube and was measured using a ceptometer (LP-80 AccuPAR, Decagon Devices, Inc. Pullman, WA, USA). All Y-tube experiments were conducted under the following environmental conditions, 23 ± 2°C, 60 ± 10% RH.

In the entrance of the Y-tube we individually introduced each of the tested females of either *B. tabaci*, *F. occidentalis* or *E. formosa.* The result of the choice test was only recorded

255 once the female had walked at least 3 cm up one of the arms or the assay was terminated
256 after 15 minutes had elapsed and excluded from the data analysis. A total of 30-40 valid
257 replicates from each species were recorded for each pair of odour sources. For each 5
258 collected responses the Y-tube was rinsed with soap, water and acetone and then left for 5
259 minutes to dry. Odour sources were switched between the left and right side arms to minimize
260 any spatial effect on choice. All test plants were replaced after recording 10 responses.
261 To confirm the Y-tube observation a second choice experiment was conducted using 16 plastic
262 cages (60 x 60 x 60 cm) (BugDorm-2 insect tents) maintained in a climatic chamber at 25 ± 2 ºC,
263 60-80% RH and 14:10 h (L:D) photoperiod. Inside each cage, three intact plants and three
264 mirid-punctured plants (either by *N. tenuis* or *M. pygmaeus*) were arranged alternately in a
265 circle. One hundred *F. occidentalis* or 100 *B. tabaci* adults were released separately in the
266 centre of the circle of plants. *Frankinella occidentalis* and *B. tabaci* were allowed to freely
267 forage within the cage for 24 hours, the number on each plant treatment group (intact or
268 mirid-punctured) were counted. The experiment was replicated four times for both *F.*
269 *occidentalis* and *B. tabaci* to test their response to *N. tenuis*-punctured plants and *M.*
270 *pygmaeus*-punctured plants.
271
272 **2.6 Persistence of plant induction**
273 In the Y-tube olfactometer we evaluated the persistence of the attraction or
274 antixenosis induced by *N. tenuis* in order to induce indirect defences. The response of *B. tabaci*
275 and *E. formosa* were tested at 4, 7 and 14 days after exposure to *N. tenuis*. Twenty-four hours
276 after activation, *N. tenuis* adults were removed and punctured plants were left in enclosed
277 plastic cages (30 x 30 x 30 cm) (BugDorm-1 insect tents) where the experiment was conducted.
278 A total of 30 responses were recorded.

279 RNA extraction and gene expression (*ASR1*, *PIN2* and *PR1*) (Table S1) was conducted to
280 confirm Y- tube results. Eight apical regions from intact plants and from *N. tenuis*-punctured
281 plants were collected. The same protocol as described above for quantitative PCR reaction was
282 followed. According to the olfactometer results, the relative expression of defensive genes was
283 performed 14 days after exposure to *N. tenuis* in comparison to intact plants.

284

285 **2.7 Statistical analysis**

286 Mirid behaviour on sweet pepper was analysed using two-way analysis of variance
287 (ANOVA) to differentiate between predator species and sex, followed by comparison of means
288 (Bonferroni post-tests) at $\alpha < 0.05$. One-tailed Student's *t*-test ($P<0.05$) was performed to
289 compare oviposition behaviour between the two mirid species. To compare between intact
290 plants, *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants, the volatile profile from
291 mirid-punctured plants and intact plants, phytohormone profile and defensive gene
292 expressions were normalized using a logarithmic transformation and then analysed using a one
293 way analysis of variance (ANOVA), followed by comparison of means (Tukey's test) at $\alpha < 0.05$.
294 In the no-choice experiment, the number of thrips and whiteflies was compared between
295 intact plant assays and mirid-punctured plant assays using a one-tailed Student's *t*-test
296 ($P<0.05$). To evaluate the persistence of plant defence induction, a one tailed t-test ($P<0.05$)
297 was performed to compare the quantified expression of defensive genes between intact plants
298 and *N. tenuis*-punctured-plants over the time increments. Chi-square ($\chi^2$) goodness of fit tests
299 based on a null model were used to analyse data collected from the olfactory responses where
300 the odour sources were selected with equal frequency. Individuals which did not make a
301 choice were excluded from the statistical analysis. Results were expressed as the mean ±
302 standard error.

303 **3 RESULTS**

### 3.1 Mirid behaviour on sweet pepper

Both mirid species were found to spend the most time feeding on the plant (Table 1), with *N. tenuis* spending significantly more time feeding than *M. pygmaeus* ($F_{1,76}$ = 22.37, $P$ < 0.0001). Feeding behaviour between the sexes was not significantly different ($F_{1,76}$ = 0.09, $P$ = 0.75). However, a significant interaction between sex and species was found ($F_{1,76}$ = 4.57, $P$ = 0.03) with *N. tenuis* males tending to feed on the plant for a longer duration than females, whereas the contrary was observed for *M. pygmaeus*. Time duration of walking activity was higher for *M. pygmaeus* than that of *N. tenuis* ($F_{1,76}$ = 8.46, $P$ = 0.0048) and males of both species spent significantly more time walking than females ($F_{1,76}$ = 9.137, $P$ = 0.0034). In contrast, females of both species spent significantly more time walking-antennating (walking accompanied by exploratory behaviour of the antenna) than males ($F_{1,76}$ = 4.034, $P$ = 0.0481), with no significant differences between species observed ($F_{1,76}$ = 1.55, $P$ = 0.22). For all other observed behaviours (antennating, grooming and flying), both mirid species and sexes behaved similarly, with no significant difference observed (Table 1 and Table S2).

Residency on different plant localities was found to vary between species. *Nesidiocoris tenuis* was found to spend a significantly longer duration of time in the apical region of the sweet pepper plant than *M. pygmaeus* ($F_{1,76}$ = 6.80, $P$ = 0.01), while the opposite occurred in the basal region ($F_{1,76}$ = 4.7, $P$ = 0.03). No differences were found in residency between the sexes either in the apical ($F_{1,76}$ = 3.739, $P$ = 0.05) or in the basal regions ($F_{1,76}$ = 0.80, $P$ = 0.37). Males of both species remained for significantly longer time periods off-plant than the females ($F_{1,76}$ = 4.75, $P$ = 0.03) with no significant difference observed in time spent off-plant between the two mirid species ($F_{1,76}$ = 6.21, $P$ = 0.01) (Table 1).

### 3.2 Mirids impact both ABA and JA signaling pathways

The feeding behaviour of both mirid predators significantly altered the hormonal profile of sweet pepper plants (Fig. 1). *Nesidiocoris tenuis* and *M. pygmaeus* feeding behaviour

significantly increased the levels of ABA, JA and JA-ILE when compared to intact plants ($F_{2,14}$= 20.27, $P$<0.0001 for ABA; $F_{2,14}$= 20.14; $P$<0.0001 for JA; $F_{2,14}$=9.36; $P$=0.004 for JA-ILE) (Figs. 1a, c, d). Furthermore, the level of ABA was significantly higher following inoculation with *N. tenuis* which may suggest a higher impact on plant's metabolism than *M. pygmaeus* feeding behaviour. In the case of SA, plants punctured by feeding behaviour of either mirid species displayed increased concentrations of this hormone, but these differences were not significantly different between species ($F_{2, 14}$= 3.26; $P$=0.074).

The quantification of *ASR1*, *PIN2* and *PR1* gene expression displayed upregulation of the *ASR1* and *PIN2* genes in plants punctured by either mirid species ($F_{2, 21=}$ 10.10, $P$= 0.001 for *ASR1* and $F_{2, 21=}$ 15.27, $P$= 0.0005 for *PIN2*), whereas only *N. tenuis* was able to upregulate the gene *PR1* ($F_{2, 21=}$ 10.29; $P$=0.0017) (Fig. 2) when compared with the intact sweet pepper plants.

### 3.3 Mirids significantly altered the volatile blend following inoculation

The untargeted analysis of the volatiles emitted by the tomato plants facilitated the identification of 14 compounds differentially emitted between mirid-punctured and intact plants (Table 2) based on their mass spectra and coelution with pure standards, when available. Compounds were identified as terpenoids (2 monoterpenoids, 3 sesquiterpenoids and 1 norisoprenoid), green leaf volatiles (5 esters ((Z)-3-hexenyl acetate, (Z)-3-hexenyl propanoate, (Z)-3-hexenyl butanoate, (Z)-3-hexenyl 3-methylbutanoate and (Z)-3-hexenyl benzoate) and their common precursor (Z)-3-hexenol), and two further compounds methyl salicylate and octyl acetate. Octyl acetate was only detected in *M. pygmaeus* punctured plants (Table 2). All of the identified compounds showed significantly increased emission in punctured plants when compared to intact, ranging from 9-fold to 130-fold.

### 3.4 Mirid infestation triggers parasitic wasp attraction and induces pest antixenosis

In the Y-tube olfactometer, the phytophagous species *F. occidentalis* and *B. tabaci* displayed a significant positive response towards the odour source emitted by intact sweet pepper plants when compared to either *N. tenuis*-punctured plants ($\chi^2$=10.90; *P*= 0.001 and $\chi^2$= 6.67; *P*= 0.0098, respectively) or *M. pygmaues* punctured plants ($\chi^2$= 10.45; *P*= 0.0012 and $\chi^2$= 10.45; *P*= 0.0012, respectively) (Fig. 3 a and b). In contrast, *E. formosa* displayed a significant attraction towards the sweet pepper plants punctured by *N. tenuis* ($\chi^2$= 6.48; *P*= 0.01) and *M. pygmaeus* ($\chi^2$= 11.08; *P*= 0.0009) relative to intact plants (Fig. 3).

In the cage experiments containing both *N. tenuis* punctured and intact plants a significantly lower number of *F. occidentalis* (*t*= 5.55; *P*= 0.0007) and *B. tabaci* (*t*= 3.60; *P*= 0.006) were found on *N. tenuis*-punctured plants than the control plants. In cage experiments containing *M. pygameus*-punctured plants and intact plants, again significantly lower numbers of *F. occidentalis* and *B. tabaci* individuals were found on *M. pygmaeus*-punctured plants (*t*= 5.07; *P*= 0.0011; *t*= 5.68; *P*= 0.0006, respectively) (Figs. 4a, b).

### 3.5 Indirect defences triggered by *N. tenuis* last for several weeks

The parasitoid, *E. formosa*, was significantly attracted to *N. tenuis*-punctured plants which were previously activated by the mirid, *N. tenuis,* 4, 7 and 14 days before ($\chi^2$= 9.60; *P*= 0.0019; $\chi^2$= 6.25; *P*= 0.0124; $\chi 2$= 4.27; *P*= 0.04 for 4, 7 and 14 days, respectively). In contrast, the phytophagous pest species *B. tabaci* was significantly repelled to *N. tenuis*-punctured plants, but only those plants activated 4 and 7 days before ($\chi^2$= 9.80; *P* = 0.0017; $\chi^2$= 4.27; *P*= 0.04 respectively). This repellent effect was not observed at day 14, where both plant treatments induced similar attraction response in *B. tabaci* ($\chi^2$ = 0.26; *P* =0.60) (Fig. 5a). The relative expression of the genes *ASR1*, *PIN2* and *PR1* quantified at day 14 after activation showed that the three genes were upregulated in *N. tenuis*-punctured plants when compared to intact plants (*t*= 4.51, *P*= 0.004; *t*= 4.101, *P*= 0.006 for *ASR1*, *PIN2* and *PR1*, respectively) (Fig. 5b).

381 **Discussion**

382 In this study, feeding activity by the zoophytophagous mirid predators, *N. tenuis* and
383 *M. pygmaeus* has been shown to induce defensive responses in sweet pepper plants for the
384 first time. Both predatory mirid species spent significantly more time feeding than any other
385 activity on sweet pepper plant, an important behaviour known to facilitate establishment of
386 the predator in the crop and maintain a population in periods of prey scarcity.[19,42] When
387 released after 24 hours of starvation,[43,44] _ENREF_51 *N. tenuis* feeding behaviour was observed
388 at 33% of total observed activity, more than double that of *M. pygmaeus* (14%). Both species
389 displayed a preference for feeding on the apical region of the sweet pepper plant, though the
390 strongest preference was observed in *N. tenuis,* with 93% of feeding activity occurring in this
391 region opposed to 64% in *M. pygmaeus.* These observations are in line with earlier studies
392 which showed that both predatory species occupy different strata of the tomato plant when
393 cohabitating the same plant, with *N. tenuis* spending significantly more time on the uppermost
394 region of the plant and *M. pygmaeus* on the lower leaves of apical region.[45]
395 Despite the large amount of time spent by both species of mirids feeding on the sweet pepper
396 plants, as of yet, neither of the two species have been described producing crop damage which
397 could affect yield.[19] Indeed, *M. pygmaeus* is considered a safe and efficient candidate to be
398 used for sweet pepper IPM strategies in Northern Europe to supplement aphid control.[4,5] The
399 use and conservation of *N. tenuis* as a biocontrol agent in sweet pepper is uncommon in
400 Europe, particularly in the Mediterranean basin where this mirid is naturally abundant.
401 However, in other pepper producing regions such as greenhouses in Kochi Prefecture, Japan,
402 *N. tenuis* is considered a key natural enemy against whiteflies, aphids and thrips, where
403 despite reaching high populations it has not been described to cause damage through plant
404 feeding. [46]

405 Despite significant differences in plant feeding behaviour between the two mirid
406 species, the level of cell wounding was sufficient in both species to activate the defence

407 mechanisms in sweet pepper, as has been described in tomato plants.[21-25] A significant
408 increase in ABA and JA signalling pathways was found in both *N. tenuis* and *M. pygmaeus*-
409 punctured plants which are co-regulated in response to wounding. This was in accordance with
410 the results of the relative expression of the target defensive genes, *ASR1* and *PIN2*,
411 respectively. Nevertheless, the levels of the phytohormone SA, which has been described as an
412 herbivore repellent in previous studies,[47-49] were not significantly different between mirid-
413 punctured plants and intact plants, although there was a tendency for it to be higher in
414 punctured plants. Furthermore, the related gene *PR-1* was upregulated for *N. tenuis*-punctured
415 plants but not for *M. pygmaeus*-punctured plants. *PR-1* has been recognised as a SA marker
416 gene, but it is also responsive to external stimuli and internal signals such as azelaic acid or
417 pipecolic acid which were not determined in the present study.[50,51] It is therefore likely that
418 mirid inoculation enhances the levels of other internal stimuli. In addition, MeSA, a compound
419 which plays an antagonistic role with free SA levels and a synergistic role with JA signaling,[52] is
420 significantly increased following inoculation by either mirid species.

421 The results confirmed that the release of VOCs by punctured-sweet pepper induces the
422 observed repellency and attractiveness to the tested phytophage and natural enemy species.
423 Indeed, plants exposed to *N. tenuis* and *M. pygmaeus* feeding were associated with repellence
424 of both arthropod pests, *F. occidentalis* and *B. tabaci*, and attraction of the parasitoid *E.*
425 *formosa*. Similarly, the feeding activity of *N. tenuis* in tomato plants have been found to be
426 responsible for the repellence of *B. tabaci* and *Tuta absoluta* (Meyrick) (Lepidoptera:
427 Gelechiidae), and for the attraction of *E. formosa.*[24, 25] However, unlike the induced plant
428 response to *N. tenuis* feeding activities, those induced by *M. pygmaeus* and *Dicyphus*
429 *maroccanus* Wanger (Hemiptera: Miridae) were found not to repel *B. tabaci* and became
430 attractive to *T. absoluta*.[25] These results in tomato were found to be related to the
431 upregulation of ABA and JA signalling pathways, [24] and suggest that *M. pygmaeus* causes a

distinct response in tomato and pepper and are consequently capable of emitting different blends of volatiles.

The HIPVs identified in this work were classified in three important groups, green leaf volatiles (GLVs) involving the fatty acid/lipoxygenase biosynthesis pathway, terpenes (the isoprenoid pathway) and methyl salicylate, MeSA, (the shikimic acid pathway). A future step would be to identify the role of each HIPV within the blend and their capacity to repel and/or attract different sweet pepper pests. Of the identified volatile compounds, octyl acetate was only recorded in *M. pygmaeus*-punctured plants. Octyl acetate has been described as a specific compound acting as sexual pheromone emitted by females on some species of the Miridae family such as *Phytocoris* spp.[53-55] It could be that this compound was emitted by *M. pygmaeus* and traces were left on the plant. In any case, the role of this volatile on *M. pygmaeus* deserves further investigation.

Under cage conditions, choice experiments showed that *B. tabaci* and *F. occidentalis* were both less likely to reside on mirid-punctured plants than on intact plants. This lower preference might be a consequence of direct defence induction mediated by mirids. VOCs inside the box might be mixed-up, hence the consequence of unequal distribution of both pests may be attributed to the contact and feeding upon the plants with high content on JA, which can be a feeding deterrent for arthropod pests.[56,57] *Macrolophus pygmaeus*-punctured tomato plants were observed to increase locally and systematically the accumulation of transcripts and activity of protease inhibitors that are known to be involved in plant responses, resulting in the decreased life history traits of the two-spotted spider mite *Tetranychus urticae* (Koch) (Acari: Tetranychidae).[22] In the case of sweet pepper, further research should be done to elucidate these direct defence effects on subsequent herbivore development and reproduction.

Tomato plants exposed to *M. pygmaeus* with all individuals subsequently removed, as in this study, were previously described to remain vaccinated for up to two weeks.[22] The impact of *N. tenius* in sweet pepper is demonstrated to remain active for 7 to 14 days. The latter finding would be useful for growers applying a nursery release of *N. tenuis* as a vaccine, adopting such a practice on sweet pepper crops might increase resilience to pest attacks. This would be an added benefit of *N. tenuis* and *M. pygmaeus* in order to effectively manage the key sweet pepper pests, *B. tabaci* and *F. occidentalis*. After vaccination, mirids could become established in the crop so they could further contribute to the management of sweet pepper pests. However, the efficacy of *N. tenuis* and *M. pygmaeus* preying upon a mixed diet of sweet pepper pests and their compatibility with other natural enemies already adapted to sweet pepper, such as *A. swirskii* or *O. laevigatus* warrant further investigation. Another application derived from this study would be the ability to manipulate the attractant and repellent capacity of sweet pepper by exposure to HIPVs. As an example, the use of volatile dispensers to emit regular concentrations of one or a blend of these volatiles could result in saturated repellent and attractant environments for pests and natural enemies, respectively. Exploring the capacity to activate plant defences in intact sweet pepper by exposing the plants to these volatiles or volatile blends, would open the door to new ways of pest control in sweet pepper as successfully demonstrated in tomato plants [24].

**ACKNOWLEDGEMENTS**

The research leading to these results was partially funded by the Spanish Ministry of Economy and Competitiveness MINECO (AGL2014-55616-C3) and the Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana. The authors thank Miquel Alonso (IVIA) and Blas Agut and Jordi Gamir (UJI) for technical assistance, Dr. Javier Calvo and Dr. J.E. Belda (KOPPERT BS, Spain) for supplying the insects, Dr. Pablo Bielza (Universidad Politécnica de Cartagena, Spain) for thrips colonies, Dimitrios Afentoulis, Zoi Thanou and Konstantinos

Birmpilis (Erasmus + from Agricultural University of Athens) for providing support in the behaviour experiments and Alice Mockford (University of Worcester) for her constructive review of this manuscript. MP-H was the recipient of a research fellowship from the INIA Spain (Subprogram DOC-INIA-CCAA) and SB recipient a grant from Russell IPM Ltd. We thank the SCIC of the Universitat Jaume I for its technical support of the hormonal analysis.

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**Table 1.** Time in seconds (mean ± SE) spent by males and females of *N. tenuis* and *M. pygmaeus* in eight different behavioural states on sweet pepper plant, and the resident time (mean ± SE) of each predator on the three designed locations. Observation were performed during 30 minutes.

| | ***Nesidiocoris tenuis*** | | ***Macrolophus pygmaeus*** | |
|---|---|---|---|---|
| | **Female (n=20 )** | **Male (n=20 )** | **Female (n=20 )** | **Male (n= 20)** |
| **Behaviours** | | | | |
| Grooming | 105.1 ± 25.7 A | 72.5 ± 27.3 A | 72.7 ± 28.5 A | 31.8 ± 11.2 A |
| Feeding | 492.0 ± 74.1 A | 660.6 ± 86.9 A | 313.8 ± 74.1 B | 187.9 ± 46.7 B |
| Flying | 2.3 ± 1.0 A | 4.4 ± 2.0 A | 2.5 ± 1.5 A | 5.4 ± 1.8 A |
| Ovipositing | 28.4 ± 10.5 A | - | 78.6 ± 30.4 A | - |
| Resting | 504.9 ± 51.2 A | 555.6 ± 34.9 A | 605.4 ± 106.3 A | 728.3 ± 105.1 A |
| Antennating | 144.3 ± 41.5 A | 90.6± 24.0 A | 270.6 ± 76.0 A | 175.3 ± 59.8 A |
| Walking | 84.6 ± 20.7 B | 201.2 ± 49.8 B* | 193.6 ± 45.3 A | 483.8 ± 114.7 A* |
| Walk.- Antenn. | 338.0 ± 66.4 A | 186.5 ± 62.6 A* | 231.7 ± 63.6 A | 145.6 ± 40.3 A* |
| **Locations** | | | | |
| Apical region | 1407 ± 114.4 A | 1232 ± 159.7 A | 1134 ± 149.8 B | 749.7 ± 150.4 B |
| Basal region | 154.7 ± 83.4 B | 185.6 ± 89.9 B | 482.2 ± 131.7 A | 321.7 ± 113.5 A |
| Outside | 132.7 ± 85.2 A | 346.3 ± 117.5 A* | 136.6 ± 68.7 A | 677.3 ± 167.8 A* |

Values followed by different letters and * in each row were significantly different between predator species and sexes, respectively (ANOVA $P<0.05$)

**Table 2.** Significant relative levels (fold changes) of the volatiles emitted by *N. tenuis* and *M. pygmaeus* punctured sweet pepper plants relative to intact plants. One way ANOVA, Tukey's multiple comparison test $\alpha$<0.05.

| Type | Compound | Kovats RI | Fold change | | $F_{2,12}$ | P |
|---|---|---|---|---|---|---|
| | | | ***Nesidiocoris tenuis* vs control** | ***Macrolophus pygmaeus* vs control** | | |
| Terpenoids: monoterpenoids | Linalool* | 1103 | 9.10 | 22.90 | 33.3 | **0.0002** |
| | Monoterpene | 1050 | 15.12 | - | 5.5 | **0.0242** |
| Terpenoids: sesquiterpenoids | Sesquiterpene[1] | 1418 | 96.52 | 130.61 | 10.7 | **0.0033** |
| | (E)-nerolidol* | 1574 | 10.50 | 14.48 | 18.9 | **0.0004** |
| | Sesquiterpenoid | 1583 | 120.96 | 85.34 | 18.7 | **0.0004** |
| Terpenoids: norisoprenoids | Unknown [2] | 1098 | 46.51 | 54.40 | 40.6 | **P<0.0001** |
| Green leaf volatile esters | (Z)-3-hexenol* | 857 | - | 21.09 | 6.5 | **0.0156** |
| | (Z)-3-hexenyl acetate* | 1002 | 12.15 | 84.90 | 16.8 | **0.0006** |
| | (Z)-3-hexenyl propanoate* | 1096 | 17.07 | 12.54 | 5.5 | **0.0238** |
| | (Z)-3-hexenyl butanoate* | 1184 | 48.27 | 36.19 | 6 | **0.0195** |
| | (Z)-3-hexenyl 3-methylbutanoate* | 1236 | 54.81 | 35.75 | 12.7 | **0.0018** |
| | (Z)-3-hexenyl benzoate | 1596 | - | 24.43 | 5.1 | **0.0297** |
| Systemic acquired resistance (SAR) | Methyl salicylate* | 1215 | 28.89 | 34.50 | 34 | **P<0.0001** |
| Other | Octyl acetate | 1206 | - | ** | - | - |

Tentative identification based on mass spectra: [1] beta-elemene, [2]norisoprenoid C11H18.

*Unequivocal identification (confirmed with a pure standard)

** only detected in *M. pygmaeus*-punctured plant

673 **Figure captions**

674 **Figure 1.**

675 Comparison between the phytohormone levels in the apical regions of intact sweet pepper
676 plants, *N. tenuis*-punctured plants and *M. pygmaeus*-punctured plants, (a) ABA, (b) SA, (c) JA
677 and (d) JA-Ile. The presented results are the mean hormone level of five independent analyses
678 ± *SE* (*n*=5). Bars with different letters are significantly different (ANOVA, Tukey's multiple
679 comparison test $\alpha$<0.05).

680 **Figure 2.**

681 Quantification of defensive genes in the apical regions of intact sweet pepper plants, *N. tenuis*-
682 punctured plants and *M. pygmaeus*-punctured plants, (a) *ASR1*, (b) *PIN2* and (c) *PR1.* Data are
683 presented as the mean of eight independent analyses of transcript expression relative to a
684 housekeeping gene ± SE (*n*=8). Bars with different letters are significantly different (ANOVA,
685 Tukey's multiple comparison test $\alpha$<0.05).

686 **Figure 3.**

687 Olfactory response of the selected insects to mirids-punctured plants in comparison to intact
688 plants. **(a)** Response of *F. occidentalis* (*n*=36)**,** *B. tabaci* (*n*=41) and *E. formosa* (*n*=30) in the Y-
689 tube olfactometer when exposed to intact sweet pepper plants and *N. tenuis*-punctured
690 plants. **(b)** Response of *F. occidentalis* (*n*=36)**,** *B. tabaci* (*n*=39) and *E. formosa* (*n*=30) in the Y-
691 tube olfactometer when exposed to intact sweet pepper plants and *M. pygmaeus*-punctured
692 plants. Significant differences based on a $\chi^2$-test are marked using * (*P* <0.05).

693 **Figure 4.**

694 Herbivores choice between mirid punctured plants and intact plants. **(a)** Number of *F.*
695 *occidentalis* adults per plant (X ± SE) captured 24 hours after releasing 100 *F. occidentalis* in the
696 centre of a cage containing 3 intact plants and 3 *N. tenuis* / *M. pygmaeus*-punctured plants. **(b)**
697 Number of *B. tabaci* adults per plant (X ± SE) captured 24 hours after releasing 100 *B. tabaci* in
698 the centre of a cage containing 3 intact plants and 3 *N. tenuis* / *M. pygmaeus*-punctured

699 plants. Both mirid species were in contact with the plants only 24 h and then removed.
700 Significant difference resulting from a one tailed *t*-test are marked with (*) ($P$<0.05).

701 **Figure 5.**

702 Persistence of sweet pepper induction following *N. tenuis* punctures. **(a)** Response of *E.*
703 *formosa* and *B. tabaci,* respectively to *N. tenuis*-punctured plants *vis* intact plants after 4 days,
704 7 days and 14 days exposure ended. Significant differences based on a $\chi^2$-test are marked using
705 * ($P$ <0.05). **(b)** Relative expression of defensives genes *ASR1*, *PIN2* and *PR1* in intact sweet
706 pepper plants with comparison to *N. tenuis*-punctures plants, 14 days after exposure ended.
707 Data are presented as the mean of eight independent analyses of transcript expression relative
708 to the constitutive *EF1* gene ± SE ($n$=8). Significant difference from a one tailed *t*-test are
709 marked with (*) ($P$<0.05).

710

**Figure 1.**

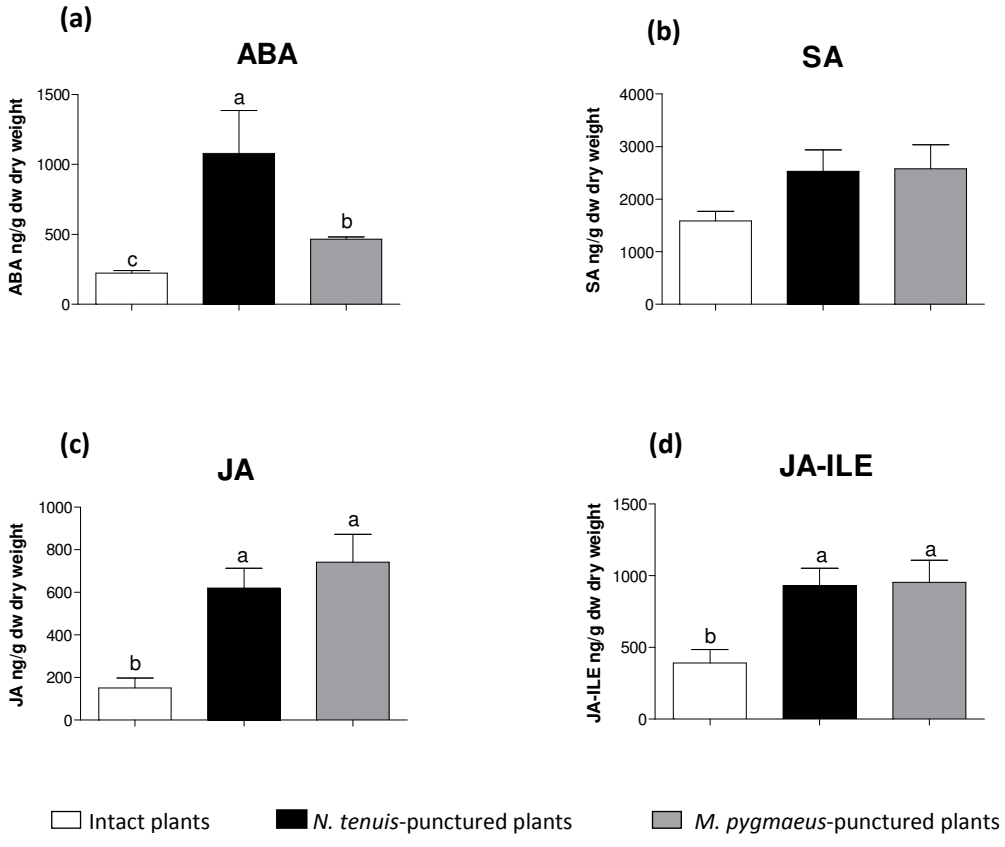

(a)
ABA
ABA ng/g dw dry weight
1500
1000
500
0
a
b
c
(b)
SA
SA ng/g dw dry weight
4000
3000
2000
1000
0
(c)
JA
JA ng/g dw dry weight
1000
800
600
400
200
0
a
a
b
(d)
JA-ILE
JA-ILE ng/g dw dry weight
1500
1000
500
0
a
a
b
Intact plants
N. tenuis-punctured plants
M. pygmaeus-punctured plants

**Figure 2.**

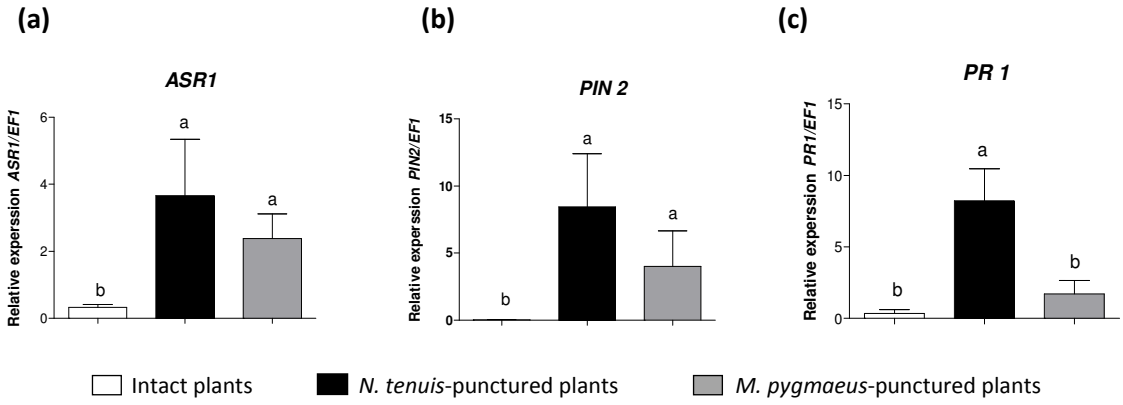
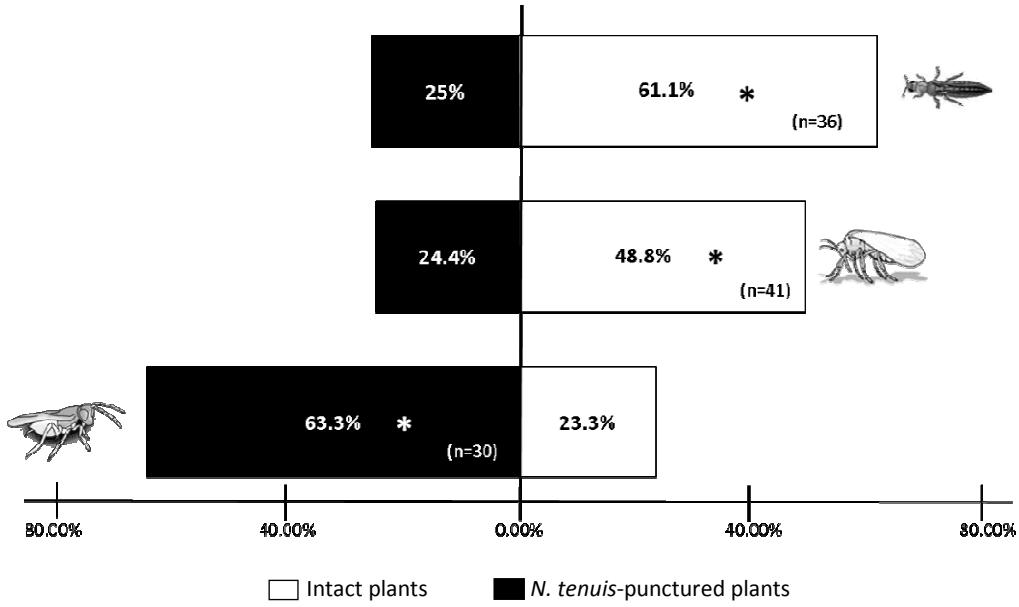

(a)
ASR1
Relative expression ASR1/EF1
6
4
2
0
a
a
b
(b)
PIN 2
Relative expression PIN2/EF1
15
10
5
0
a
a
b
(c)
PR 1
Relative expression PR1/EF1
15
10
5
0
a
b
b
Intact plants
N. tenuis-punctured plants
M. pygmaeus-punctured plants

**Figure 3.**

**(a)**


25%
61.1%
*
(n=36)
24.4%
48.8%
*
(n=41)
63.3%
*
(n=30)
23.3%
80.00%
40.00%
0.00%
40.00%
80.00%
Intact plants
N. tenuis-punctured plants

**(b)**

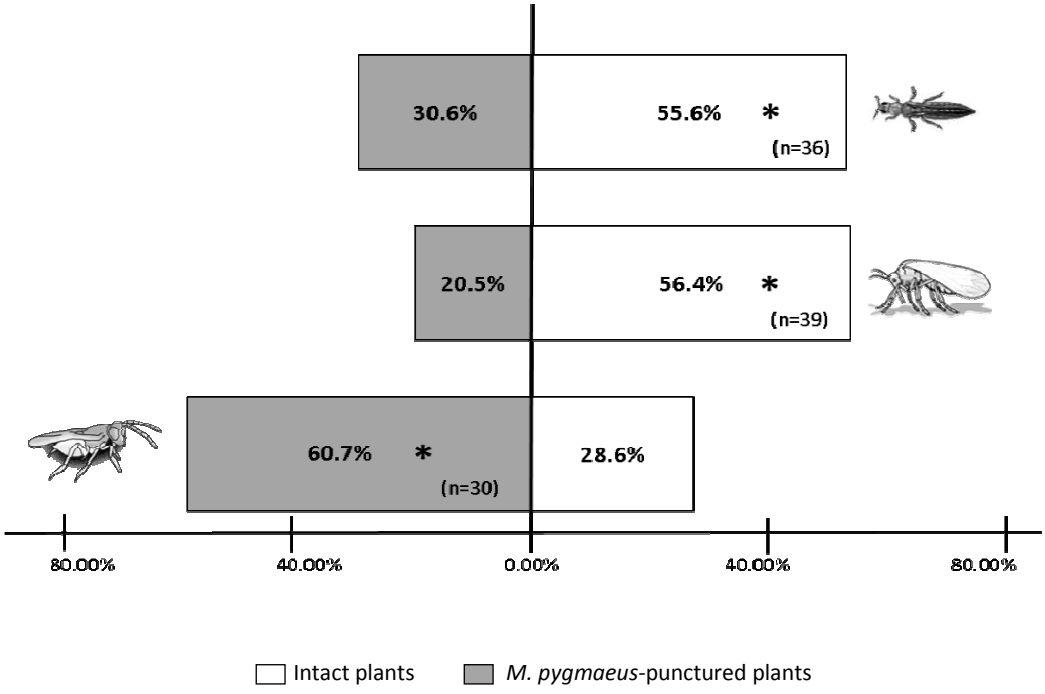

30.6%
55.6%
*
(n=36)
20.5%
56.4%
*
(n=39)
60.7%
*
(n=30)
28.6%
80.00%
40.00%
0.00%
40.00%
80.00%
Intact plants
M. pygmaeus-punctured plants

**Figure 4.**

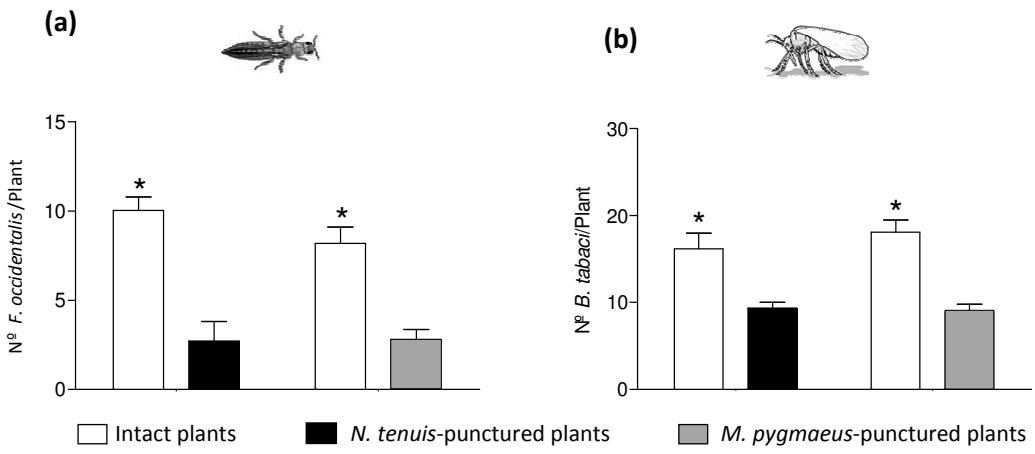
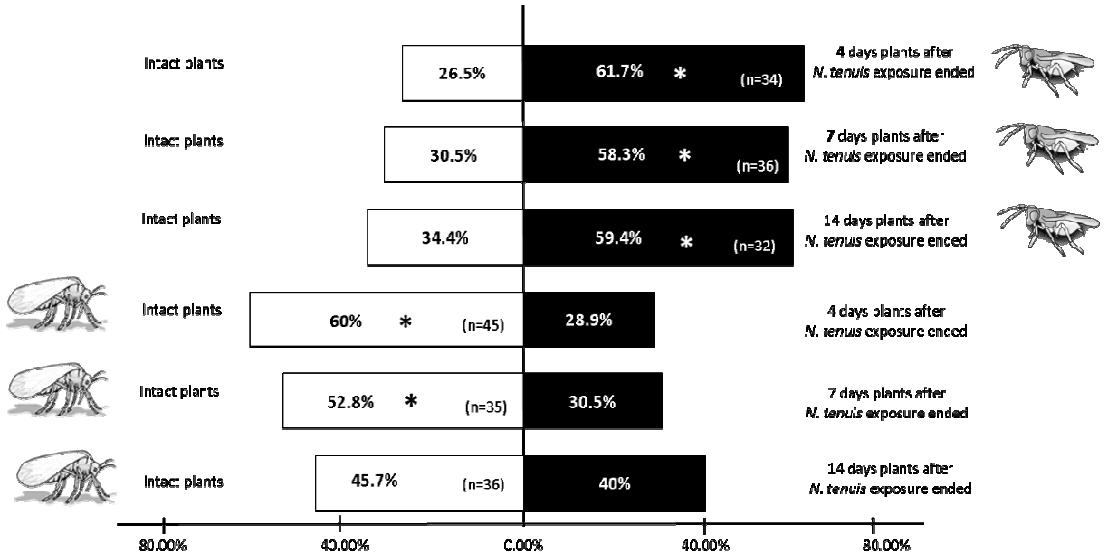
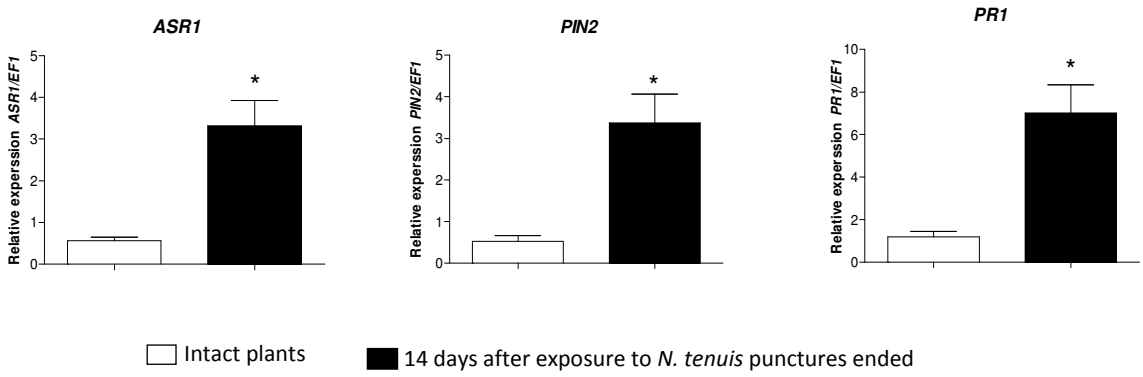

(a)
(b)
Nº F. occidentalis/Plant
Nº B. tabaci/Plant
15
10
5
0
30
20
10
0
*
Intact plants
N. tenuis-punctured plants
M. pygmaeus-punctured plants

**Figure 5.**

**(a)**


Intact plants
26.5%
61.7%
*
(n=34)
4 days plants after
N. tenuis exposure ended
Intact plants
30.5%
58.3%
*
(n=36)
7 days plants after
N. tenuis exposure ended
Intact plants
34.4%
59.4%
*
(n=32)
14 days plants after
N. tenuis exposure enced
Intact plants
60%
*
(n=45)
28.9%
4 days plants after
N. tenuis exposure enced
Intact plants
52.8%
*
(n=35)
30.5%
7 days plants after
N. tenuis exposure ended
Intact plants
45.7%
(n=36)
40%
14 days plants after
N. tenuis exposure ended
80.00%
40.00%
0.00%
40.00%
80.00%

**(b)**


ASR1
Relative expression ASR1/EF1
PIN2
Relative expression PIN2/EF1
PR1
Relative expression PR1/EF1
*
Intact plants
14 days after exposure to N. tenuis punctures ended

800

801 **Supporting information**

802 **Table S1**. Forward and reverse sequences of *ASR1* (abscisic acid stress ripening protein 1),
803 marker gene for ABA*, PIN2* (Wound-induced proteinase inhibitor II precursor), marker gene for
804 JA, *PR1* (basic PR-1 protein precursor)*,* and the constitutive gene *EF1.*

| Primers | Forward | Reverse |
|---|---|---|
| ***ASR1*** | 5'-TGTGCAATTTGTCTTGTGGAA-3' | 5'-CGGACATGACGAGTTCGATA-3' |
| ***PIN2*** | 5'-CTTGCCCCAAGAATTGTGAT-3' | 5'-GCCCTAGCGTATTACGGAGA-3' |
| ***PR1*** | 5'-ACGTCTTGGTTGTGCTAGGG-3' | 5'-CCATACGGACGTTGTCCTCT-3' |
| ***EF1*** | 5'-CCTGGACAGATTGGAAATGG-3' | 5'-GACCACCTGTCGATCTTGGT-3' |

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808 **Table S2.** Statistics (P, F and degree freedom values) for the two-way ANOVA comparison of
809 time spent by males and females of *N. tenuis* and *M. pygmaeus* in eight different behavioural
810 states on sweet pepper plant, and the resident time of each predator and sex on the three
811 designed locations.

| | Species | Sex | Species * Sex |
|---|---|---|---|
| **Behaviours** | | | |
| **Grooming** | $F_{1,76}$ = 2.28, $P$= 0.13 | $F_{1,76}$ = 2.30, $P$ = 0.13 | $F_{1,76}$ = 0.03, $P$ = 0.86 |
| **Feeding** | $F_{1,76}$ = 22.37, $P$ < 0.0001 | $F_{1,76}$ = 0.10, $P$ = 0.76 | $F_{1,76}$ = 4.58, $P$ = 0.03 |
| **Flying** | $F_{1,76}$ = 0.11, $P$= 0.74 | $F_{1,76}$ = 2.30, $P$ = 0.13 | $F_{1,76}$ = 0.07, $P$ = 0.78 |
| **Ovipositing** | $t_{1,38}$ = 1.56, $P$= 0.13 | / | / |
| **Resting** | $F_{1,76}$ = 2.85, $P$= 0.09 | $F_{1,76}$ = 1.15, $P$ = 0.29 | $F_{1,76}$ = 0.20, $P$ = 0.66 |
| **Antennating** | $F_{1,76}$ = 3.82, $P$= 0.05 | $F_{1,76}$ = 1.90, $P$ = 0.17 | $F_{1,76}$ = 0.15, $P$ = 0.70 |
| **Walking** | $F_{1,76}$ = 8.46, $P$= 0.0048 | $F_{1,76}$ = 9.14, $P$ = 0.003 | $F_{1,76}$ = 1.67, $P$ = 0.20 |
| **Walk.- Anten.** | $F_{1,76}$ = 1.55, $P$= 0.22 | $F_{1,76}$ = 4.03, $P$ = 0.05 | $F_{1,76}$ = 0.31, $P$ = 0.58 |
| **Locations** | | | |
| **Apical region** | $F_{1,76}$ = 6.81, $P$= 0.01 | $F_{1,76}$ = 3.47, $P$ = 0.06 | $F_{1,76}$ = 0.52, $P$ = 0.47 |
| **Basal region** | $F_{1,76}$ = 4.75, $P$= 0.03 | $F_{1,76}$ = 0.81, $P$ = 0.37 | $F_{1,76}$ = 0.37, $P$ = 0.54 |
| **Outside** | $F_{1,76}$ = 2.61, $P$= 0.11 | $F_{1,76}$ = 4.75, $P$ = 0.03 | $F_{1,76}$ = 6.28, $P$ = 0.014 |

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