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3

4 ***Orius laevigatus* strengthens its role as a biological control agent by inducing plant**
5 **defenses**

6

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35Abstract

36*Orius laevigatus* is a generalist predator that is widely used in augmentative strategies against
37the key pest of sweet pepper, *Frankliniella occidentalis*. Despite being a zoophytophagous
38predator, the phytophagous behavior of *O. laevigatus* has not been previously explored in
39depth, nor has the impact of phytophagy on plant physiology. Here, the hierarchical
40significance of *O. laevigatus* feeding on sweet pepper is compared with other behaviors. *Orius*
41*laevigatus* spends the majority of its time (38%) feeding on apical meristems and apical fresh
42leaves, which were also preferred residence locations. Here, the phytophagous feeding
43behavior of *O. laevigatus* on sweet pepper is shown to trigger defensive responses in the plant.
44These *O. laevigatus* plant induced defenses are then shown to contribute to the repellence or
45attraction of pests or natural enemies, respectively. Specifically, *O. laevigatus*-punctured sweet
46pepper plants induce repellency for the whitefly *Bemisia tabaci* and the thrips species *F.*
47*occidentalis*. In contrast, the whitefly parasitoid *Encarsia formosa* was significantly attracted to
48*O. laevigatus*-punctured plants. The plant responses to *O. laevigatus* punctures include the
49release of an altered blend of volatiles and activation of the jasmonate acid and salicylic acid
50signalling pathways. These results highlight an interesting facet to the biology of *O. laevigatus*,
51in which the ability of the predator to induce defensive responses in sweet pepper plants may
52serve to improve the biological control of both thrips and whiteflies.

53**Key words:** minute pirate bug, behavior, plant response, herbivore induced plant volatiles,
54sweet pepper

55

56Key message

57This is the first time a defensive response in sweet pepper plants induced by the phytophagy of
58the generalist predator, *Orius laevigatus*, has been described. The results further show how
59the plant's response to *O. laevigatus* increases emission of herbivore-induced plant volatiles
60(HIPVs) which can modulate the behavior of other arthropods (*Bemisia tabaci*, *Frankliniella*
61*occidentalis* and *Encarsia formosa*). The results could explain the great success achieved by
62IPM programs based on the release, establishment and conservation of *O. laevigatus* in sweet
63pepper crops.

64

65Introduction

66Many species of the genus *Orius* Wolff, 1811 are considered to be important beneficial insects
67for a variety of agroecosystems (Hernández and Stonedahl 1999). *Orius* species are highly
68polyphagous, yet show preferential tendencies for immature and adult thrips (Chambres et al.
691993; Ruidavet 1995; Frescata and Mexia 1996) and to a lesser extent eggs, nymphs and adult
70whiteflies (Gerling et al. 2001; Arnó et al. 2008). As a result of these prey preferences, some
71species of *Orius* are commercially produced for augmentative release as biological control
72agents in various crops worldwide (Chambers et al. 1993; Sanchez and Lacasa 2002; van
73Lenteren and Bueno 2003; van Lenteren et al. 2017). In Europe, the minute pirate bug *Orius*
74*laevigatus* (Fieber) (Hemiptera: Anthocoridae) has become the most successful *Orius* species
75for biological control, and it is hence widely used in augmentative release programs. The prey
76range of *O. laevigatus* is broad and includes agricultural pests such as aphids, whiteflies,
77lepidopteran eggs, mites, and thrips with a distinct preference for the latter group of pests
78(Venzon et al. 2002; van Lenteren and Bueno 2003). In south eastern Spain, the release of *O.*
79*laevigatus* together with the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari:
80Phytoseiidae) is key to successful pest management in more than 10,000 ha of protected

81sweet pepper crops (van der Blom et al. 1997; Sanchez et al. 2000; Calvo et al. 2009; van der
82Blom et al. 2009).

83The genus *Orius* can also feed on plants, for example on the pollen and sap, which contributes
84to population survival in the absence of a primary source of protein-rich food in the crop
85(Cocuzza et al. 1997; Armer et al. 1998). Indeed, *O. laevigatus* can complete its development
86by feeding exclusively on fresh pollen from sweet pepper (Vacante et al. 1997). Armer et al.
87(1998) demonstrated that *Orius* species can also feed on xylem and mesophyll contents, which
88consists of mostly water with small amounts of sugars, starch and amino acids. The presence
89of amylase in *O. insidiosus* indicates that it can digest starch and therefore can take advantage
90of feeding directly on the plant (Zeng and Cohen 2000). Plants are not only a source of
91nutrients but also a substrate for oviposition. *Orius* females insert their eggs underneath the
92cuticle in the epidermal to subepidermal cell layers, which enhances offspring survival
93(Lundgren et al. 2008). During the early developmental stages, *O. laevigatus* can take nutrients
94from phloem tissue and survive solely from plant material for several days (Lundgren et al.
952008). All this supports that feeding on plant material is common and ecologically relevant for
96omnivorous *Orius*.

97Whereas plant-mediated interactions among herbivores have been relatively well-studied, the
98effect of phytophagy by omnivorous predators on plant defences and the subsequently
99induced insect behavior have not been investigated until recently. De Puyseleir et al. (2011)
100showed that increased tomato plant resistance to *F. occidentalis* feeding was produced via the
101jasmonic acid (JA)-mediated wound response during *O. laevigatus* ovipositing. More recently,
102several papers have demonstrated the capacity of different species of zoophytophagous
103species (plant-feeding carnivore), including mirid bugs (Hemiptera: Miridae), to induce plant
104responses in tomato plants (Pérez-Hedo et al. 2015a, b; Pappas et al. 2015, 2016).

105It is hence hypothesised that *O. laevigatus* would be able to induce plant responses in sweet
106pepper as has been demonstrated in other plant-zoophytophage systems. As a first step to

107better understand the interaction between *O. laevigatus* and sweet pepper, the behavior of *O.*
108*laevigatus* on the plants was studied and plant feeding behaviour quantified to compare
109general behaviors. A series of experiments were then conducted to determine whether *O.*
110*laevigatus* feeding punctures on sweet pepper induce plant defence responses and whether
111these in turn lead to behavioural responses in pest and natural enemy species. In parallel,
112targeted gene-expression analysis was used on plants previously exposed to *O. laevigatus* to
113ascertain which signaling pathways could be involved in plant defensive responses. Finally, the
114volatile compounds released as part of the plant response to *O. laevigatus* feeding punctures
115were characterized.

116

117Material and methods

118Insects and Plants

119Adult *O. laevigatus*, the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and the
120parasitoid *Encarsia formosa* (Gahan) (Hymenoptera: Aphelinidae) were supplied by Koppert
121Biological Systems, S.L. (Águilas, Murcia, Spain), all were less than 5 days old. *Frankliniella*
122*occidentalis* adults were obtained from a colony established at Instituto Valenciano de
123Investigaciones Agrarias (IVIA) in 2010 and originally collected from Campo de Cartagena
124(Murcia, Spain). Thrips colonies were maintained on the common bean (*Phaseolus vulgaris* L.
125Fabales: Fabaceae) and housed in a climatic chamber at 25 ± 2 °C, $65 \pm 10\%$ RH and a 14:10 h
126(L:D) photoperiod at IVIA.

127Pesticide-free sweet pepper seedlings cv ('Lipari') [*Capsicum annuum* (Solanaceae)] (Dulce
128italiano, Mascarell semillas S.L, Valencia, Spain) were individually transplanted to plastic pots
129(8 x 8 x 8 cm) containing a mixture of natural soil with local peat moss and housed undisturbed
130in climatic chamber at 25 ± 2 °C, 60-80% RH and 14:10 h (L:D) photoperiod at IVIA. Once the
131sweet pepper plants had approximately 6 fully developed leaves (approximately 15 cm in

132height) they were used for the study of *O. laevigatus* behavior, older plants of approximately
13320 cm in height were used for the rest of the experiments.

134To obtain *O. laevigatus*-punctured plants, four intact sweet pepper plants were enclosed for
13524 hours in a 60 x 60 x 60-cm plastic cage (BugDorm-2 insect tents; MegaView Science Co.,
136Ltd., Taichung, Taiwan) and exposed to 100 *O. laevigatus* adults, all less than 4 days old (sex
137ratio 1:1). All individuals were removed from the plants before the experiment. Prior to their
138use, *O. laevigatus* were released into a plastic cage (30 x 30 x 30 cm) (BugDorm-1 insect tents;
139MegaView Science Co., Ltd., Taichung, Taiwan) with water supplied on soaked cotton plugs,
140and starved for twenty-four hours.

141

142Behavior of *O. laevigatus* on sweet pepper

143The behaviours of both male and female *O. laevigatus* on sweet pepper plants in the absence
144of prey items was observed during 30 minute assays. Less than 4-day-old males and
145presumably mated females of *O. laevigatus* were individually placed inside a plastic 5-ml vial
146and starved for 24 h before use. Water was supplied on soaked cotton plugs.

147A clean sweet pepper plant was placed inside a 60 x 60 x 60 cm plastic cage (BugDorm-2 insect
148tents). A single predatory *O. laevigatus* (male or female) was gently released and observations
149began once the individual walked freely onto the plant for the first time. The different
150observed behaviours were recorded continuously for 30 minutes. Behavioral observations
151were made by three researchers using a hand held magnifying glass after first quantifying their
152reliability. The sweet pepper plant was replaced before the start of each behavioural assay.
153Assays were repeated until 20 replicates for each sex had been observed.

154Locations were defined as off plant (plastic cage, plastic pot or soil) and on plant (distinguishing
155between apical and basal regions). The apical region was considered the first 5 cm of the plant
156formed by the apical stem, young leaves and 2 fully developed leaves. Conversely, the rest of

157the plant, approximately 10 cm with 4 fully developed leaves, basal stem and cotyledons,
158represented the basal region. Seven behavioral states were defined as follows:

159 Grooming (G): The predator's forelegs are used to clean mouthparts or another part of
160 the body.

161 Feeding (F): The predator inserts its stylet into the plant and stylet movements can be
162 observed.

163 Oviposition (O): The predator presses its whole abdomen onto plant and the ovipositor
164 is inserted into the plant.

165 Resting (R): The predator stands motionless.

166 Antennating (A): The predator is at rest but moves its antennae.

167 Walking (W): The predator walks on different regions of the plant without moving its
168 antennae.

169 Walking-Antennating (WA): The predator walks on different regions of the plant and
170 moves its antenna at the same time.

171Because the main interest was in quantifying the feeding behavior compared to other
172behaviors, only the analysis of the timed behaviorals grouped in the apical and basal regions of
173the plant are presented.

174

175*Frankiniella occidentalis* and *B. tabaci* plant selection mediated by *O. laevigatus*

176A Y-tube olfactometer experiment was conducted to test the olfactory responses of *B. tabaci*,
177*F. occidentalis* and *E. formosa* females to sweet pepper plants that were previously punctured
178by *O. laevigatus* relative to intact plants. The Y-tube olfactometer (Analytical Research

Systems, Gainesville, FL) consisted of a 2.4 cm diameter Y-shaped glass tube with a 13.5-cm long base and two arms each 5.75-cm long (Pérez-Hedo and Urbaneja 2015). Both side arms were connected via high density polyethylene (HDPE) tubes to two identical glass jars (5 l volume) each of which connected to an air pump that produced a unidirectional humidified airflow at 150 ml min⁻¹ (Pérez-Hedo and Urbaneja 2015). Four 60-cm fluorescent tubes (OSRAM, L18W/765, OSRAM GmbH, Germany) were positioned 40 cm above the horizontally disposed 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.

A single individual female of *B. tabaci*, *F. occidentalis* or *E. formosa* was introduced into the tube (entry array) and observed until she had walked at least 3 cm up one of the arms or until 15 min had elapsed. A minimum of 33 valid replicates from each species were recorded for each pair of odor sources. Females that did not choose a side arm within 15 min were recorded as 'no-choice' and were excluded from data analysis. After recording 5 responses, the Y-tube was rinsed with soap water and acetone and left to dry for 5 min. The odor sources were subsequently switched between the left and right side arms to minimize any spatial effect on choice. Both types of plants (intact and punctured) were used only once to test the response of 10 females and then were replaced with new plants.

Plant gene expression analysis

The apical region of the sweet pepper plants, as defined above, were subjected to targeted gene expression analysis to detect: (i) *ASR* (abscisic acid stress ripening protein 1) a marker gene for ABA, (ii) *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. Samples of the sweet pepper apical region were collected from both intact and *O. laevigatus*-

205punctured plants. After homogenization in liquid nitrogen, total RNA was extracted using Trizol
206(Invitrogen, CA, USA) according to the manufacturer's instructions (Pérez-Hedo et al. 2015a;
207Naselli et al. 2016). After homogenizing the sample with TRIzol™ Reagent, chloroform was
208added to separate RNA of protein and DNA, and subsequent isopropanol and 1.2 Mm NaCl was
209added to precipitate the RNA. The RNA pellets resulting from the precipitation were washed
210twice with 70% ethanol, dried at room temperature and eluted in water. The RNA was
211quantified and then treated with the Turbo DNA-free DNase kit (Applied Biosystems) to
212eliminate any traces of genomic DNA, according to the manufacturer's protocol. cDNA was
213synthesized by adding to the samples (1 µg/µl), RT buffer, 10 µM Oligo dT and prime script™ RT
214reagent kit (perfect real time) (TAKARA Bio, CA, USA). The reaction mixture was then incubated
215in the thermo-cycler for 15 min at 37°C and for 5 s at 85°C. Real-time PCR amplifications were
216performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, MA, USA).
217*Capsicum annuum* forward and reverse specific primers (0.5 µl) were designed and added to 5
218µl of Syber green/ROX qPCR MM and 1 µl of cDNA and then brought to 10 µl total volume with
219Milli-Q sterile water. PCR reactions were run in duplicate according to manufacturer
220recommendations. Quantitative PCR was carried out using the LightCycler® 480 System (Roche
221Molecular Systems, Inc., Switzerland) and the protocol consisted in 95°C for 10 min followed
222by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s. Melting curve analysis was
223performed at 95°C for 5 s, 60°C for 1 min and then a continuous increase of temperature until
22495°C finalizing the process. Data acquisition and calculation were performed with the thermal
225cycler's software and then were collected and analyzed in Microsoft Excel. Each qPCR data
226point is the average of 8 independent experiments. EF1 (elongation factor-1) was used as a
227standard control gene for normalization. The nucleotide sequences of the gene-specific
228primers are described in Table 1.

229

230Composition of volatile blends

231 Volatile compounds were collected by means of solid phase microextraction (SPME) and
232 separated and detected by means of gas chromatography coupled to mass spectrometry (GC/
233 MS). Volatiles were adsorbed in a 65- μ m PDMS/DVB SPME fiber
234 (polydimethylsiloxane/divinylbenzene; Supelco, Bellefonte, PA, USA). The adsorbent-coated
235 fiber was mounted on an SPME fiber holder, and injected through the first septum of the
236 sample container. Agitation of the atmosphere inside the container was achieved by pumping
237 at 5 ml/min using an injected syringe through the second septum of the sample container.
238 Each sample was performed for 3 hours. Four replicates for the intact plant and three
239 replicates for *O. laevigatus*-punctured plant treatments were conducted. After collection, the
240 fiber was retracted into the needle and the SPME device was removed from the container for
241 GC-MS analysis.

242 Desorption was performed by means of a CombiPAL autosampler (CTC Analytics) at 250°C for 1
243 min in splitless mode in the injection port of a 6890N gas chromatograph coupled to a 5975B
244 mass spectrometer (Agilent Technologies). To prevent cross-contamination, fibers were
245 cleaned after desorption in an SPME fiber conditioning station (CTC Analytics) at 250°C for 5
246 min under helium flow. Chromatography was performed on a DB-5ms (60 m, 0.25 mm, 1.00
247 μ m) column with helium as carrier gas, at a constant flow of 1.2 ml/min. The GC interface and
248 MS source temperatures were 260°C and 230°C, respectively. Oven programming conditions
249 were 40°C for 2 min, 5°C/min ramp until 250°C, and a final hold at 250°C for 6 min. Data were
250 recorded in the 35-300 m/z range at 5 scans/s, with electronic impact ionization at 70 eV.
251 Untargeted analysis of the chromatograms was performed with MetAlign software (WUR,
252 <http://www.metalign.nl>).

253 Kovats retention indexes (KIs) were calculated for the compounds. Differentially emitted
254 compounds were first tentatively identified by the comparison of their mass spectra with those
255 in the NIST 05 Mass Spectral Library. When available, identity was confirmed by coelution with

256pure standards (Sigma-Aldrich). To quantify the selected compounds, one specific ion was
257selected for each compound, and the corresponding peak area from the extracted ion
258chromatogram was integrated by means of the ChemStation E.02.02 software (Agilent
259Technologies). The criteria for ion selection were the highest signal-to-noise ratio and
260specificity to that particular region of the chromatogram to provide good peak intergration.

261

262Data analysis

263The different behaviors of *O. laevigatus* on sweet pepper plants and the predator location
264were analyzed using three-way analysis of variance (ANOVA), taking as factors, sex, observer
265researcher and either behavior or location. Tukey's test was used for mean separation at $\alpha <$
2660.05. Since only three of the 20 tested female *O. laevigatus* oviposited on plants, this behavior
267was excluded from the analysis. The data from the olfactory responses were analyzed using a
268 χ^2 goodness of fit test based on a null model where the odor sources were selected with equal
269frequency. Individuals who did not make a choice were excluded from the statistical analysis.
270The data from gene expression analyses and volatile profiling were analyzed using one-tailed
271Student's *t*-test ($P < 0.05$). For volatile profiling, discriminant compounds were determined by
272the analysis of the data obtained from the untargeted chromatogram and the results
273expressed as the means $\pm$ standard error.

274Results

275Behaviors and locations of *O. laevigatus* on sweet pepper

276*Orius laevigatus* showed preference to the apical region of the sweet pepper plants where the
277majority of the observed behaviours were performed ($F_{2, 110} = 108.33$; $P < 0.0001$) (Fig. 1).
278Neither sex nor the observer researcher was found to be significant ($F_{1, 110} = 0.11$; $P = 0.7415$
279and $F_{2, 110} = 0.06$; $P = 0.9374$, respectively).

O. laevigatus spent 38% of their time feeding on plant tissues, which was significantly higher than the time spent on all other activities ($F_{5, 221} = 21.01$; $P < 0.0001$) (Fig. 2). Neither the sex nor the observer were significant in the statistical analysis of the behaviors, ($F_{1, 221} = 0.12$; $P = 0.724$ and $F_{2, 221} = 0.48$; $P = 0.6211$, respectively).

Frankliniella occidentalis and *B. tabaci* plant selection mediated by *O. laevigatus*

In the Y-tube experiment, females of *F. occidentalis* showed preference for the odor emitted from intact plants over that from *O. laevigatus*-punctured plants ($\chi^2 = 19.931$; $P < 0.0001$) (Fig. 2883). In the case of *B. tabaci*, tested females were also attracted to the odor emitted by intact sweet pepper plants in comparison to *O. laevigatus*-punctured plants ($\chi^2 = 17.071$; $P < 0.0001$) (Fig. 3). In contrast to both phytophagous insects, the parasitoid *E. formosa* significantly chose *O. laevigatus*-punctured plants over intact plants in the olfactometer assay ($\chi^2 = 6.250$; $P = 0.0124$) (Fig. 3).

Plant gene expression analysis

Transcriptional analysis showed that plant feeding by *O. laevigatus* on the apical portion of sweet pepper plants increased the expression of the *PIN2* gene (JA pathway) ($t = 2.161$, $P = 0.019$) and the *PR1* gene (SA pathway) ($t = 1.835$, $P = 0.039$) (Fig. 4a, b). In contrast, the *ASR1* gene (ABA pathway) was not affected in *O. laevigatus*-punctured plants ($t = 0.113$, $P = 0.455$) (Fig. 4c).

Composition of volatile blends

Quantitative differences were recorded in the herbivore-induced plant volatiles (HIPVs) profile of *O. laevigatus*-punctured and intact sweet pepper plants. Untargeted analysis of the volatiles emitted allowed the identification of ten compounds with significantly increased levels in

punctured plants, while no compounds with decreased levels were identified. The emission of discriminant compounds increased in the range of 2 to 100-fold and corresponded to terpenoids (1 monoterpenoid, 4 sesquiterpenoids and 1 norisoprenoid), a set of two (Z)-3-hexenyl esters, methyl salicylate and another unknown compound (Table 2).

Discussion

Here, for the first time *O. laevigatus* induced defensive response in sweet pepper plants due to phytophagous behavior is described. This is of particular interest as the predator *O. laevigatus* has been one of the most studied and successfully used augmentative biological control agents in sweet pepper (van Lenteren et al. 2017). Furthermore, the modulated behavior of both pest species (*B. tabaci* and *F. occidentalis*) and a natural enemy (*E. formosa*) associated with sweet pepper, in response to an induced plant response by the zoophytophage *O. laevigatus*, is documented for the first time.

Previous studies on the behavior of several species of *Orius* under different conditions (availability of prey and/or pollen, quality of the plant as a feeding and/or oviposition substrate, architecture of the plant) have been reported (Fritsche and Tamó 2000; Yano et al. 2005; Jonathan and Fergen 2006; Lundgren et al. 2008). However, to our knowledge, no other study has quantified the time that a specific *Orius* species spends feeding on sweet pepper. Here, *O. laevigatus* feeding behaviors are described and quantified, and reveal both females and males feed on plant tissue by inserting their stylets, a behavior which represents 38% of time spent on the plant. When comparing this plant feeding behavior with the other observed behaviors under experimental conditions, plant feeding was the preferred activity by *O. laevigatus*, occupying the greatest proportion of time. Individuals tested in the behavioral experiment were starved 24 hours, with access to just water, before their release into the experimental arenas. The reason for both female and male *O. laevigatus* plant feeding behavior upon their release could well be due to the need to obtain nutrients from the plant

and not so much because of the need for hydration. Indeed, the sequential behavior of importance was searching for prey (the sum of the Antennating-A and “Walking-Antennating-WA behaviors). The experimental conditions (starved *O. laevigatus*, the size of sweet pepper plants and the absence of prey after release) were standardised according to conditions *O. laevigatus* would encounter in the field when released in augmentative biological control programs. The next step would be to quantify the plant feeding behavior in the presence of prey in sweet pepper plants, and whether under this situation, defensive plant responses are also activated.

It is known that predators avoid laying eggs where prey are scarce or absent as in our study (Evans and Dixon 1986; Hemptinne et al. 1992). Nakashima and Hirose (2002) observed that females of *O. sauteri* (Poppius) oviposited at higher rates in prey-rich patches than in prey-deficient patches. *O. laevigatus* individuals used in this study were subjected to a very similar procedure before their use (starvation with access to water for 24 h) as in Nakashima and Hirose (2002), and only three of the 20 tested females of *O. laevigatus* oviposited on plants. The time represented by oviposition occupied just 0.2% of total observation time. These results suggest that plant defence response can be induced by plant feeding behavior in addition to the oviposition behavior described by De Puyseleyer et al (2011) in tomatoes. Recently, Naselli et al. (2016) demonstrated that all motile stages of the mirid *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) can induce plant responses in tomato plants, although such responses may differ slightly depending on the stage considered. Similarly, knowing if immature *O. laevigatus* individuals can trigger defensive responses in sweet peppers would also be of interest.

Most of the plant defense mechanisms against insects are activated by signal transduction pathways mediated by JA, SA and ethylene (Shivaji et al. 2010; Gill et al. 2010). Little had previously been described on the subject of plant responses to the feeding of *O. laevigatus* before this study. It is shown here that activation of the JA and SA pathways in *O. laevigatus*-

356punctured plants is associated with aversion of both arthropod pests, *B. tabaci* and *F.*
 357*occidentalis* and by contrast, the whitefly parasitoid *E. formosa* was significantly attracted to *O.*
 358*laevigatus*-punctured plants. Pérez-Hedo et al. (2015a) observed that the feeding activity of
 359the predatory mirid *N. tenuis* on tomato plants activated the abscisic acid (ABA) and jasmonic
 360acid (JA) signaling pathways, which made plants exposed to mirids less attractive to *B. tabaci*
 361and more attractive to *E. formosa*. Additionally, Pérez-Hedo et al. (2015b) showed that three
 362different zoophytophagous predators [*N. tenuis*, *Macrolophus pygmaeus* (Rambur) and
 363*Dicyphus maroccanus* (Wagner) (Hemiptera: Miridae)] had different capacities to induce
 364specific responses in tomato plants. Tomato plants exposed to *N. tenuis* were less attractive to
 365*B. tabaci* as mentioned above but also to the lepidopteran *Tuta absoluta* (Meyrick)
 366(Lepidoptera: Gelechiidae). By contrast, tomato plants exposed to *M. pygmaeus* and *D.*
 367*maroccanus* were not able to repel *B. tabaci* and, more interestingly, became more attractive
 368to *T. absoluta*. All three zoophytophagous mirid predators activated the JA signaling pathways,
 369which resulted in the attraction of the parasitoid *E. formosa* to punctured tomato plants.
 370Pappas et al. (2015, 2016) observed that the performance of *Tetranychus urticae* (Koch) (Acari:
 371Tetranychidae) decreased as a result of the prior exposure of tomato plants to *M. pygmaeus*.
 372This was accompanied by a locally and systematically increased accumulation of transcripts
 373and activity of proteinase inhibitors that are known to be involved in plant responses. Similarly,
 374a further step in the research with *O. laevigatus* will be to investigate the performance, by
 375evaluating different life history traits, of *B. tabaci* and *F. occidentalis* on *O. laevigatus*-
 376punctured plants.

377It is well documented that non-consumptive effects triggered by arthropod predators can
 378modulate behavior, physiology, development and morphological traits of a subsequently
 379infesting herbivore (Werner and Peacor 2003). This non-consumptive effect relies on the ability
 380of prey to perceive chemicals and visual cues directly emitted by predators, before being
 381preyed. Escape, avoidance, reduced oviposition, reproduction and reduced feeding are some

382of the solutions prey employ to avoid predation risk (Nomikou et al. 2003; Sendoya et al. 2009;
383Ninkovic et al. 2013; Wasserberg et al. 2013; Lee et al. 2014). No chemical compound has yet
384been isolated which could be related to an *O. laevigatus* chemical cue, since all differentially
385identified chemical compounds were emitted from the plant. Therefore, the responses of *B.*
386*tabaci*, *F. occidentalis* and *E. formosa* were a direct consequence to the odor emitted from the
387*O. laevigatus*-punctured sweet pepper plants.

388The results presented here showed increased emission of HIPVs in sweet pepper punctured by
389*O. laevigatus* compared to intact plants. These volatiles belong to already identified volatile
390groups activated in plants being injured by true phytophagy, the green leaf volatiles group
391(GLVs), terpenoids and methyl salicylate (Kessler and Baldwin 2001; Kessler and Baldwin 2002;
392War et al. 2011). It is known that HIPVs are released by most plant species (McCormick et al.
3932014). However, each species of plant could emit a specific blend of volatiles and that the
394relative amounts of HIPVs could vary widely between species and with the type of damage
395(Kigathi et al. 2009; Ponzio et al. 2014; Ardanuy et al. 2016). In this paper, it has been shown
396that the particular volatile profile emitted by the *O. laevigatus*-punctured plant repelled *B.*
397*tabaci* and *F. occidentalis* and attracted *E. formosa*.

398In summary, these results show that the effectiveness of *O. laevigatus* as a biological control
399agent in sweet pepper is due to not just its predatory role but also to its ability to induce plant
400defensive responses. These results open the doors to new management methods for *B. tabaci*
401and *F. occidentalis*. Once the volatile(s) responsible for the repellency of both pests have been
402identified, new repellent products can be developed. In parallel, plant breeding programs
403aimed at obtaining plants with an ability for greater emission of these volatiles could be
404implemented.

405

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416

417**Author contribution statement**

418SB, AU and MP-H conceived and designed the research. All authors performed the research,
419and SB, AU and MP-H wrote the paper. SB, AU, JLR and MP-H analyzed the data. All authors
420wrote the manuscript.

421

422**Compliance with Ethical Standards**

423**Conflict of interest** The authors declare that they have no conflict of interest.

424**Informed consent** Informed consent was obtained from all individual participants included in
425the study.

426**Research involving human participants and/or animals** This article does not contain any
427studies with human participants or animals performed by any of the authors.

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Figure Legends

Fig. 1. Residence time (mean $\pm$ SE) of *O. laevigatus* adults on sweet pepper plants. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test $\alpha < 0.05$) (n=40).

Fig. 2 Time (mean $\pm$ SE) spent exhibiting different behaviors by *O. laevigatus* adults on sweet pepper plants during a period of 30 min. Bars with different letters are significantly different (ANOVA, Tukey's multiple comparison test $\alpha < 0.05$) (n=40).

Fig. 3 Response in Y-tube olfactometer of females of *F. occidentalis* (n=33), *B. tabaci* (n=33) and *E. formosa* (n=34) to the odor emitted by intact sweet pepper and by sweet pepper plants previously exposed to *O. laevigatus*. Significant differences based on a χ^2 -test are marked using * ($P < 0.05$).

Fig. 4 Transcriptional response of the defensive genes *ASR1* (a), *PIN2* (b) and *PR1* (c) responsible for the change in level of the phytohormones ABA, JA, and SA, respectively, in *O. laevigatus*- punctured plants. Data are presented as the mean of eight independent analyses of transcript expression relative to a housekeeping gene $\pm$ SE (n = 8). Significant differences based on t- test are marked with (*) ($P < 0.05$).

571**Table 1** Forward and reverse sequences of *ASR*, *PIN2*, *PR1*, and the constitutive gene *EF1*

Primers	Forward	Reverse
<i>ASR</i>	5'-TGTGCAATTTGTCTTGTGGAA -3'	5'-CGGACATGACGAGTTCGATA-3'
<i>PIN2</i>	5'-CTTGCCCCAAGAATTGTGAT-3'	5'-GCCCTAGCGTATTACGGAGA-3'
<i>PR1</i>	5'-ACGTCTTGGTTGTGCTAGGG-3'	5'-CCATACGGACGTTGTCCTCT-3'
<i>EF1</i>	5'-CCTGGACAGATTGGAAATGG-3'	5'-GACCACCTGTCGATCTTGGT-3'

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574 **Table 2.** Significant relative levels (fold changes) of the volatiles emitted by *O. laevigatus* punctured plants relative to intact plants. Student's t-test (P<0.05).

Type	Compound	Kovats RI	Fold change	t	P
Terpenoids: monoterpenoids	Linalool*	1103	6.42	2.5353	0.0327
Terpenoids: sesquiterpenoids	Sesquiterpene ¹	1418	33.80	4.593	0.0029
	Sesquiterpene ²	1459	4.30	6.739	0.0005
	(E)-nerolidol*	1574	7.55	7.036	0.0004
	Sesquiterpenoid	1583	97.95	2.557	0.0254
Terpenoids: norisoprenoids	Unknown ³	1117	4.30	2.71	0.0211
Green leaf volatile esters	(Z)-3-hexenyl propanoate*	1096	3.04	2.353	0.0327
	(Z)-3-hexenyl benzoate*	1596	3.10	5.722	0.0011
Systemic acquired resistance (SAR)	Methyl salicylate*	1215	17.01	3.47	0.0089
Other	Unknown ⁴	1664	2.52	2.151	0.0421

575*Unequivocal identification (confirmed with a pure standard); Tentative identification based on mass spectra: ¹ beta-elemene, ² alpha-bergamotene, ³ 576norisoprenoid (C11H18) and ⁴ methyl dihydrojasmonate.

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578**Fig. 1**

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586Fig. 2

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Fig. 3

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