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Eliciting tomato plant defenses by exposure to herbivore induced plant volatiles

Short title: Inducing defenses by HIPVs

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28

29 **Abstract**

30 When zoophytophagous mirids (Hemiptera: Miridae) feed on tomato plants they activate both

31 direct and indirect defense mechanisms, which include the release of herbivore induced plant

32 volatiles (HIPVs). HIPVs are capable of activating defense mechanisms in healthy

33 neighboring plants. In this work, we investigated which of these mirid-induced HIPVs are

34 responsible for inducing plant defenses. Healthy tomato plants were individually exposed to

35 eight HIPVs [1-hexanol, (Z)-3-hexenol, (Z)-3-hexenyl acetate, (Z)-3-hexenyl propanoate, (Z)-

36 3-hexenyl butanoate, hexyl butanoate, methyl jasmonate and methyl salicylate] for 24 hours.

37 Then, the expression level of defensive genes was quantified. All HIPVs led to increased

38 expression of defensive genes by the plant when compared to unexposed tomato plants. In a

39 further step, (Z)-3-hexenyl propanoate and methyl salicylate were selected to study the

40 response of four tomato key pests and one natural enemy to tomato plants previously exposed

41 to both HIPVs relative to unexposed control plants. Plants previously exposed to both HIPVs

42 were repellent to *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), *Tuta absoluta*

43 (Meyrick) (Lepidoptera: Gelechiidae) and *Frankliniella occidentalis* Pergande (Thysanoptera:

44 Thripidae), attractive to the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae)

45 and indifferent to *Tetranychus urticae* Koch (Acari: Tetranychidae). The volatiles emitted by

46 plants previously exposed to both selected volatiles were also determined. Increased levels of

47 C5 and C6 fatty acid-derived volatile compounds and β-ionone were detected, confirming that

48 both HIPVs significantly activated the lipoxygenase pathway. These results are the starting

49 point to advance the use of volatile compounds as defense elicitors in tomato crops.

50 **Keywords:** (Z)-3-hexenyl propanoate; methyl salicylate; *Tuta absoluta*; *Bemisia tabaci;*

51 *Frankliniella occidentalis*; *Tetranychus urticae*; *Encarsia formosa*

Introduction

Over the last 20 years tomato pest management in Europe has experienced a radical change (Pérez-Hedo et al. 2017; Arnó et al. 2018; van Lenteren et al. 2020). Management has developed from pesticide dominant practices to integrated management based on the use of biological control agents. The use of predatory mirids, principally the two species *Nesidiocoris tenuis* Reuter and *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), as biological control agents has become common. Mirids are generalist predators which can feed on a wide range of prey; including the key tomato pests, the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and the South American pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Alomar et al. 2006; Urbaneja et al. 2009, 2012; Calvo et al. 2009, 2012; Mollá et al. 2014; Biondi et al. 2016; Sylla et al. 2016). The success has been such that other important tomato producing areas such as those in the Americas are trying to find native predatory mirids with which to establish biological control programs (Pérez- Hedo et al. 2020). Aside from their wide range of prey, their ability to obtain nutrition from the plant itself permits the mirids to inhabit crops with low levels of prey (Wheeler 2000; Arnó et al. 2010; Urbaneja-Bernat et al. 2019; Thomine et al. 2020). Furthermore, it has recently been confirmed that mirids can activate plant defenses in tomato (Pérez-Hedo et al. 2015b; Pappas et al. 2015, 2016), making mirid predators even more valuable in biological pest control programs (Pérez-Hedo and Urbaneja 2016; Pérez-Hedo et al. 2017; Pérez- Hedo et al. 2020). The phytophagous behavior of mirids is what activates various metabolic pathways related to plant defense responses, such as the salicylic and jasmonic acid pathways which trigger the release of Herbivore Induced Plant Volatiles (HIPVs) (Naselli et al. 2016; Zhang et al. 2018, 2019; Bouagga et al. 2018a, 2020; Pérez-Hedo et al. 2018a). Some of these volatiles are responsible for the repellence of herbivores and the attraction of natural enemies (Pérez-Hedo et al. 2018b).

77   Plants previously exposed to mirids may also induce defenses in intact plants without

78   previous exposure by communication through volatiles (Pérez-Hedo et al. 2015b). The

79   production of these volatiles is induced by herbivore injury and emitted by the plant thereafter

80   (Pare and Tumlinson 1997, 1999; Kessler 2001; Dicke and Baldwin 2010). Plants which

81   receive these volatile warning cues can set off a wide array of defensive responses, such as the

82   production of Proteinase inhibitors (PIs), the emission of volatile compounds, the production

83   of alkaloids, the formation of trichomes, and the secretion of extra floral nectar (Farag & Pare

84   2002; Choh & Takabayashi 2006; Frost et al. 2008; Heil & Ton 2008).

85   In a previous work, the phytophagous behavior of *M. pygmaeus* and *N. tenuis*, triggered the

86   release of seven HIPVs in tomato plants: 1-hexanol, *(Z)*-3-hexenol, *(Z)*-3-hexenyl acetate, *(Z)*-

87   3-hexenyl propanoate, *(Z)*-3-hexenyl butanoate, hexyl butanoate and methyl salicylate (Pérez-

88   Hedo et al. 2018b). In this work, we investigated which of these volatiles, together with one

89   of the most studied plant defense activators methyl jasmonate, are responsible for inducing

90   defenses in adjacent intact plants with no previous exposure to mirids. For this, we exposed

91   individual tomato plants to each of these mirid-induced volatiles, for 24 hours, and studied the

92   effect of each HIPV on the expression of the basic pathogenesis-related protein precursor

93   (*PR1*), a marker gene for the SA signaling pathway, a marker for plant Proteinase Inhibitor I

94   (*Sl-PI-I*) and a marker gene for the JA signaling pathway (*PIN2*). Because the activation of

95   these plant defense genes on tomato plants triggers repellency or attraction to herbivores and

96   natural enemies (Pérez-Hedo et al. 2018b), we studied the response of four tomato key pests,

97   *T. absoluta*, *B. tabaci*, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) and

98   *Tetranychus urticae* Koch (Acari: Tetranychidae) and one parasitoid [*Encarsia*

99   *formosa* Gahan (Hymenoptera: Aphelinidae), in a Y-tube olfactometer to tomato plants

100   previously exposed to each of the eight HIPVs described above and to unexposed plants. For

101   the Y-tube study, two volatiles, (Z)-3-hexenyl propanoate and methyl salicylate, were selected

102   based on their ability to induce the PR1 and PIs molecular markers. Additionally, volatile

103   compounds from tomato plants primed with these two HIPVs were determined by headspace

104   solid-phase microextraction (HS-SPME) coupled to gas chromatography/mass spectrometry

105   (GC-MS).

106

Material and methods

108   **Plants and insects**

109   The tomato *Solanum lycopersicum* cv. Moneymaker was used in all experiments.

110   Moneymaker seeds were sown in soil in seedling trays and two weeks after germination,

111   seedlings were individually transplanted into pots ($8 \times 8 \times 8$ cm). Plants were maintained

112   undisturbed at 25 ± 2 ºC, with a constant relative humidity of $65\% \pm 5\%$ and a photoperiod of

113   14:10 h (light: dark). All tomato plants were pesticide-free. At four weeks of age

114   (approximately 20 cm high), plants were used for experimentation.

115   *Bemisia tabaci* adults and *E. formosa* pupae were provided by Koppert Biological Systems,

116   S.L. (Águilas, Murcia, Spain). Newly emerged adult *B. tabaci* (less than 2 day old) were

117   placed on tomato plants caged in 60 x 60 x 60 cm BugDorm-2 insect tents and housed in a

118   climate chamber at 25 ± 2ºC, $65 \pm 10\%$ RH and a 14:10 h (L:D) photoperiod at IVIA. Five

119   day old adult *B. tabaci* were used in all the experiments. In the case of *E. formosa*, pupae

120   were enclosed in a Petri dish (9 cm in diameter) and allowed to emerge under ambient

121   laboratory conditions (25 ± 2ºC), with a small drop of honey provided as food. Female *E.*

122   *formosa* were used at less than two days old in all experiments. *Frankliniella occidentalis*

123   adults were obtained from a culture established at IVIA in 2010, originally collected from

124   Campo de Cartagena (Murcia, Spain). The thrips culture was maintained in climatic chamber

125   on bean plants (*Phaseolus vulgaris* L.; Fabales: Fabaceae) under the same conditions

126   described above. All female *F. occidentalis* used for experimentation were less than five days

127   old. *Tetranychus urticae* adults were obtained from a culture established at IVIA in 2011

128   originally collected from the region of La Plana (Castelló, Spain). Mites were maintained on

129   tomato plants kept in a climatic chamber under the same climate and photoperiod described

130   above. *Tuta absoluta* females were obtained from tomato colonies maintained at IVIA in a

131   glasshouse located at IVIA at 25 ± 4ºC, 60 ± 15% RH and under natural photoperiod. Newly

132   emerged (less than 5 days old) adult females were used in all trials.

133   **Exposure of tomato plants to HIPVs in the laboratory and plant gene expression**

134   All synthetic standards of the tomato volatile compounds (1-hexanol, *(Z)*-3-hexenol, *(Z)*-3-

135   hexenyl acetate, *(Z)*-3-hexenyl propanoate, *(Z)*-3-hexenyl butanoate, hexyl butanoate, methyl

136   salicylate and methyl jasmonate) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

137   Volatile emitters were prepared from 2 x 2 cm filter paper impregnated each with 10 µl of the

138   corresponding volatile or the control (Pérez-Hedo et al. 2018b). The volatiles were firstly

139   diluted in methanol at 1:100 (v/v) and then further diluted in water at 1:100 (v:v; volatile

140   mix:water) so that the final test concentration was 1:10,000 (v/v). The control consisted of

141   1:100 methanol:water (v/v). Pérez-Hedo et al. (2018b) demonstrated that this volatile

142   concentration was very similar to those emitted by mirid-induced tomato plants, indeed, they

143   were of the same order of magnitude. Two impregnated volatile emitters were then placed in

144   the bottom part of a $30 \times 30 \times 30$ cm experimental cage (BugDorm-1 insect tents; MegaView

145   Science Co., Ltd, Taichung, Taiwan) together with an intact tomato plant. Plants and HIPV's

146   were kept undisturbed for 24 hours in isolated climatic chambers to avoid any volatile

147   interference and maintained at 25 ± 2ºC, 65 ± 10% RH and a 14:10 h (L:D) photoperiod. Each

148   plant, either exposed or intact, was used just once, either to quantify the plant gene expression

149   or in the Y-tube bioassays.

150   The transcriptional response of the *PR1*, *Sl-PI-I* and *PIN2* genes were studied 24 hours after

151   HIPVs exposition on six exposed tomato plants for each HIPV and on six intact tomato plants

152   (Lopez-Raez et al. 2010; Pappas et al. 2015; Pérez-Hedo et al. 2018a). Samples from the

153   apical part of the tomato plant were immediately ground in liquid nitrogen. Portions of the

154   ground samples were then used for RNA extraction. Total RNA (1.5 µg) was extracted using

155   a Plant RNA Kit (Omega Bio-Tek Inc., Doraville, GA, USA) and was treated with RNase-

156   free DNase (Promega Corporation, Madison, Wisconsin, USA) to eliminate genomic DNA

157   contamination. The RT reaction and the PCR SYBR reaction were performed as described by

158   Meritxell Pérez-Hedo et al. (2015b). Quantitative PCR was performed using the Smart Cycler

159   II (Cepheid, Sunnyvale, CA, USA) sequence detector with standard PCR conditions.

160   Expression of the gene *EF1* (Elongation factor-1) was used for normalization as housekeeping

161   gene. The nucleotide sequences of the gene specific primers are described in Table 1.

162   **Y-tube bioassays**

163   A Y-tube olfactometer experiment was conducted to test the olfactory responses of *F.*

164   *occidentalis, B. tabaci, T. urticae, T. absoluta* and *E. formosa* to tomato plants that were

165   previously exposed for 24 h to either *(Z)*-3-hexenyl propanoate or methyl salicylate relative to

166   intact unexposed plants. Plants were exposed to both volatiles as described above. The Y-tube

167   olfactometer (Analytical Research Systems, Gainesville, FL) consisted of a 2.4-cm-diameter

168   Y-shaped glass tube with a 13.5-cm long base and two arms each 5.75 cm long (Pérez-Hedo

169   and Urbaneja 2015). Both side arms were connected via high-density polyethylene (HDPE)

170   tubes to two identical glass jars (5-L volume), each of which contained a test odor source.

171   Each odor source was connected to an air pump that produced a unidirectional humidified

172   airflow at 150 ml/min. A single individual female was introduced into the tube (entry array)

173   and observed until she had walked at least 3 cm up one of the arms or until 15 min had

174   elapsed. Females that did not choose a side arm within 15 min were recorded as 'no-choice'

175   and were excluded from data analysis. A total of 40 valid replicates for each species were

176   recorded for each pair of odor sources. Each individual was tested only once. After recording

177   five responses, the Y-tube was rinsed with soapy water followed by acetone and left to dry for

178   5 min. The odor sources were subsequently switched between the left and right-side arms to

179   minimize any spatial effect on choice. The two types of plants (intact and exposed) were used

180   only once to test the response of 10 females and then were replaced with new plants. The Y-

181   tube experiment was conducted under the following environmental conditions: $23\pm2°C$ and

182    $60\pm10\%$ RH.

Analysis of volatile plant metabolites

184   Following the methodology described above, frozen plant material from apical part of 4

185   tomato plant exposed for 24 h to either *(Z)*-3-hexenyl propanoate or methyl salicylate and

186   intact plants was homogenized in liquid nitrogen with pestle and mortar, and the resulting

187   powder stored at -80ºC until analyzed. Volatile compounds were determined by means of

188   headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography/mass

189   spectrometry (GC-MS), as described in López-Gresa et al. (2017).

190   Then, 1 mL of a 5M $CaCl_2$ solution and 150 μL of a 500 mM EDTA solution (adjusted to pH

191   7.5 with NaOH) were added and mixed gently to inhibit endogenous enzyme activity and

192   drive the volatiles into the headspace by increasing polarity in the liquid phase. The vial was

193   then closed and sonicated for 5 min. Extraction of volatile compounds was performed from

194   the vial headspace by means of a 65 μm PDMS/DVB solid phase microextraction fiber

195   (SUPELCO). Vials were incubated at 50ºC for 10 min, under continuous 500 rpm agitation.

196   The SPME fiber was then introduced in the vial and exposed to the headspace for 20 min,

197   with identical conditions of agitation and temperature. The volatile compounds adsorbed in

198   the fiber were desorbed in the injection port of the gas chromatograph at 250ºC for 1 min in

199   splitless mode. Incubation, extraction and injection were performed by means of a CombiPAL

200   autosampler (CTC Analytics). Chromatography was performed on a 6890N gas

201   chromatograph (Agilent) with a DB-5ms (60 m, 0.25 mm, 1.00 µm) capillary column (J&W),

202   with helium as carrier gas at a constant flow of 1.2 mL/min. Oven ramp conditions were:

203   40ºC for 2 min, 5ºC/min ramp until 250ºC and a final hold at 250ºC for 5 min. GC interface

204   and MS source temperatures were 260ºC and 230ºC respectively. Data was recorded in a

205   5975B mass spectrometer (Agilent) in the 35-300 m/z range at 6.2 scans/s, with electronic 70

206   eV impact ionization. Data were recorded by the Enhanced ChemStation E.02.02 software.

207   Untargeted analysis of the chromatograms was performed by means of the MetAlign software

208   (WUR-PRI). When standards were available, unequivocal identification of compounds was

209   performed by the comparison of both retention time and mass spectrum with those of pure

210   standards. All the standards were provided by Sigma-Aldrich. In the case of the remaining

211   compounds, tentative identification was performed by their comparison of their mass spectra

212   and Kovats retention index with those in the NIST 05 mass spectral library.

213   **Data analysis**

214   The results of the transcriptional responses with markers were normalized using a logarithmic

215   transformation and then then analyzed using a one-way ANOVA, followed by comparison of

216   means (Tukey's test) at $P < 0.05$. Chi-square (χ^2) goodness of fit tests based on a null model

217   were used to analyze data collected from the olfactory responses (number of individuals)

218   where the odor sources were selected with equal frequency. χ^2-tests were conducted with the

219   responses from a sample size of 40 individuals. Previous to χ^2-test, a logistic regression

220   showed the factor "Plant" as non-significant in any of the Y-tube experiments. Individuals

221   that did not make a choice were excluded from the statistical analysis. Statistically significant

222   differences in volatiles between treatments and the control were achieved by means of the

223   Student's t test.

224

Results

An over expression of the three markers studied in the plants exposed to the volatiles was observed (Fig. 1 **A**, **B**, **C**). The expression of *PR1* was significantly greater after the exposure to all the volatiles with the exception of methyl salicylate, *(Z)*-3-hexenol, and 1-hexanol. Even though methyl salicylate, *(Z)*-3-hexenol, and 1-hexanol provided expressions at slightly higher levels than the control, the differences were not significant ($F_{8,14} = 3.370$; $p = 0.0055$). However, the expression of *Sl-PI-I* and *PIN2* increased significantly in plants after their exposure to all of the volatiles tested in comparison to the control plant expression. In the case of *Sl-PI-I*, the volatile *(Z)*-3-hexenyl propanoate elicited the greatest expression ($F_{8,14} = 11.57$; $p < 0.0001$). In the case of *PIN2*, the volatile methyl jasmonate induced the greatest expression ($F_{8,14} = 9.771$; $p < 0.0001$).

Based on its ability to induce the *PR1, Sl-PI-I* and *PIN2* molecular markers the (Z)-3-hexenyl propanoate was selected together with methyl salicylate to be used in the olfactometer. Plants exposed for 24 h to each of the two volatiles became repellent to *T. absoluta* ($\chi^2 = 9.80$; $P = 0.0017$ and $\chi^2 = 7.200$; $P = 0.0073$, respectively), *B. tabaci* ($\chi^2 = 12.80$; $P = 0.0003$ and $\chi^2 = 16.20$; $P < 0.0001$, respectively) and *F. occidentalis* ($\chi^2 = 5.00$; $P = 0.0253$ and $\chi^2 = 12.80$; $P = 0.0003$, respectively) (Fig. 2 **A**, **B**). Furthermore, both treatments made the plants more attractive to the parasitoid *E. formosa* ($\chi^2 = 5.00$; $P = 0.0253$ and $\chi^2 = 5.00$; $P = 0.0253$, respectively). On the other hand, *T. urticae* (Acari: Tetranychidae) showed no preference to the treatments ($\chi^2 = 1.80$; $P = 0.1797$ and $\chi^2 = 0.80$; $P = 0.3711$, respectively).

The GC-MS analysis identified increased levels in C_5 and C_6 fatty acid-derived volatile compounds and β-ionone (Table 2). In addition, increased levels in the phenylpropanoid Eugenol and a yet unidentified norisoprenoid were also detected but with greater abundance in MeSA elicited plants.

Discussion

In this work, methyl jasmonate, methyl salicylate and the six green leaf volatiles (GLVs) induced defenses when exposed individually to healthy tomato plants. All the tested volatiles elicited a response in the HIPVs-exposed plants. These HIPVs are released when damage occurs on the leaves (Dicke and Baldwin 2010) and in our study were selected since they are emitted by plants after the plant feeding activity of zoophytophogous predators (Bouagga et al. 2018a,b; Pérez-Hedo et al. 2018b). Previous work has demonstrated that the phytophagy of some mirid species, such as *N. tenuis*, *M. pygmaeus* and *Dicyphus bolivari* (Lindberg), induces direct and indirect defenses in sweet pepper and tomato plants that trigger the release of volatile compounds that result in the repellency of herbivore pests and the attraction of natural enemies (Pappas et al. 2015; Pérez-Hedo et al. 2015a; Zhang et al. 2019; Silva et al. 2021). Pérez-Hedo et al. (2018) showed that all the differentially expressed volatiles from *N. tenuis* or *M. pygmaeus*-induced tomato plants were capable of repelling *B. tabaci* and *T. absoluta* and attracting *E. formosa* when those HIPVs were tested individually in a Y-tube experiment. In an additional step, this work demonstrates that exposure of tomato plants to each of these volatiles induces defenses on healthy tomato plants and makes them repellent and attractive to these organisms. Interestingly, after the HIPV exposure, the number of volatiles that are triggered in those exposed plants is significantly higher than those detected on mirid-punctured tomato plants which release 7 HIPVs (Pérez-Hedo et al. 2018b). In our case of (Z)-3-hexenyl propanoate-exposed plants, twenty-two volatiles were significantly more abundant when compared to unexposed plants and nineteen volatiles in the case of methyl salicylate-exposed plants. This difference could be due to the different volatile collection method used in both studies. However, the volatiles detected in both studies belong to the same groups. Most of the volatile compounds detected were C5 and C6 fatty acid-derived volatile compounds and β-ionone, which demonstrates that both HIPVs activated the

274   lipoxygenase pathway in a similar manner (Feussner & Wasternack 2002). These groups of

275   compounds were previously described as causing these repellency / attraction effects

276   (Turlings & Erb 2018). Future works should address whether some of the volatiles found in

277   this work which are different from those identified by Pérez-Hedo et al. (2018) could also be

278   responsible for the induction of defenses.

279   HIPVs can induce defenses in non-attacked parts of the same plant and even, as we have been

280   demonstrated in this work, nearby healthy plants (Heil & Silva Bueno 2007; Heil 2008; Heil

281   & Ton 2008). Here, exposure to (Z)-3-hexenyl propanoate upregulated *PR1* and *PIN2* gene

282   markers, indicating that both the salicylic acid and jasmonic acid metabolic pathways were

283   activated. Both routes are closely related to the ability of the plant to resist attacks from pests

284   and diseases (Kessler & Baldwin 2002). Many previous studies have demonstrated that

285   HIPVs can elicit defences just by exposure (Turlings & Erb 2018). For example, in corn the

286   potential of green leaf volatiles (GLV) for inducing defenses in undamaged plants was

287   demonstrated. The exposure of corn plants to a pure synthetic GLV chemical such as (Z)-3-

288   hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate was reported to induce defenses against

289   two generalist caterpillars (Lepidoptera: Noctuidae), *Spodoptera exigua* (Hübner) (Engelberth

290   et al. 2004) and *Spodoptera littoralis* Boisduval (Ton et al. 2006) upon activating the JA

291   pathway. In tomato plants, exposure to the GLV (Z)-3-hexanol induced jasmonic acid- and

292   salicylic acid-mediated defence responses. These defence responses decreased the oviposition

293   and negatively influenced the feeding behaviour of *B. tabaci* and increased the attraction of

294   the parasitoid *E. formosa* which improved its parasitism on *B. tabaci* (Yang et al. 2020). A

295   further step in our line of research will be to study whether plants exposed to any of these

296   volatiles are capable of not only reducing pest infestation, but also the multiplication of plant

297   diseases. In sweet pepper plants the upregulation of the jasmonate acid pathway triggered by

298   mirid phytophagy reduced tomato spotted wilt virus (TSWV) accumulation in mirid-

299 punctured plants (Bouagga et al. 2020). Additionally, tomato plants with high expression of

300 methyl jasmonate are less likely to be infected with the Tomato yellow leaf curl virus

301 (TYLCV) (Escobar-Bravo et al. 2016).

302 The defense activation in the plant by exposure to volatiles could imply a metabolic cost

303 (Agrawal et al. 2002). In tomato plants a negative correlation between the induction of

304 defenses and constitutive levels of *PI-II* protein was observed in primed plants with the

305 volatiles triggered by plants attacked by *Spodoptera exigua* (Hübner) (Lepidoptera:

306 Noctuidae) (Zhang et al. 2020). Therefore, it is critical to know if the defense activation of

307 tomato plants through exposure to some of these volatiles can cause any negative

308 physiological effect under controlled conditions. In particular, it would be interesting to carry

309 out trade-off studies focused on plant fitness.

310 In summary, our study suggests that tomato plants undergo strong defense activation when

311 detecting the presence of both HIPVs. Interest in the potential of HIPVs to induce plant

312 defenses and their application in the field has increased greatly in recent years (Turlings &

313 Erb 2018). However, to date and to the best of our knowledge, there is no control method

314 applied to the management of pests and diseases based on communication between plants.

315 This work opens the doors to the commercial application of HIPVs as defense elicitors in

316 tomato crops. For example, the application of dispenser in field conditions loaded with one of

317 some of these HIPVs could result in a new biorational pest management method to be

318 carefully considered. Preliminary work in our research group points in this direction.

319

326 **Competing interests:** M.P.-H., A.U., M.A.-V., V.N.-LL., S.V., J.R. and A.G. are inventors

327 on the requested Spanish Patent No. P202030330 entitled "Uso de propanoato de (Z)-3-

328 hexenilo y método para proteger plantas frente a plagas" E.K.-H.C. The other authors declare

329 no conflict of interest.

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510

Figure legends

Figure 1. Transcriptional response of the defensive genes *PR1* (a marker gene for the SA signaling pathway) (**A**), *Sl-PI-I* (a marker for plant Proteinase Inhibitor I) (**B**), and *PIN2* (a marker gene for the JA signaling pathway) (**C**) in tomato plants exposed to methyl salicylate [MeSA], methyl jasmonate [MeJA], (Z)-3-hexenyl propanoate [(Z)-3-HP], (Z)-3-hexenyl butanoate [(Z)-3-HB], (Z)-3-hexenol [(Z)-3-H], (Z)-3-hexenyl acetate [(Z)-3-HA], 1-hexanol [1-H] and hexyl butanoate [HB]. Data are presented as the mean of eight independent analyses of transcript expressions relative to a housekeeping gene ± SE (n = 6). Bars with different letters are significantly different (ANOVA with Tukey's multiple comparison test (P <0.05).

Figure 2. Response (% ± SE) of *Encarsia formosa*, *Tuta absoluta*, *Tetranychus urticae*, *Bemisia tabaci* and *Frankliniella occidentalis* females in a Y-tube olfactometer when exposed to control (1:10,000 methanol:water, v/v) and the two synthetic HIPVs *(Z)*-3-hexenyl propanoate [(Z)-3-HP] (**A**) and methyl salicylate [MeSA] (**B**) (1:10,000 volatile:water, v/v). "nc" indicates the number of tested females that did not make a choice. Asterisks indicate significant differences in the distribution of side-arm choices (χ^2 tests; $P < 0.05$).

529   **Table 1.**

530   **Table 1**. Primers used for quantification of *EF1* (elongation factor-1), *PR1* (pathogenesis-

531   related protein precursor), *SI-PI-I* (Proteinase Inhibitor I) and *PIN2* (JA-regulated defense

532   protein) genes.

Gene	Primer forward (5' → 3')	Primer reverse (5' → 3')
EF1	5-GATTGGTGGTATTGGAACTGTC-3	5-AGCTTCGTGGTGCATCTC-3
PR1	5-CTCATATGAGACGTCGAGAAG-3	5-GGAAACAAGAAGATGCAGTACTTAA-3
Sl-PI-I	5-TGAAACTCTCATGGCACGAA-3	5-TTTTGACATATTGTGGCTGCTT-3
PIN2	5-GAAAATCGTTAATTTATCCCAC-3	5-ACATACAAACTTTCCATCTTTA-3

533

534

535 **Table 2.** Volatile compounds significantly altered by *(Z)*-3-hexenyl propanoate [(Z)-3-HP] or methyl salicylate [MeSA] exposure in tomato

536 leaves. Data are expressed as a ratio to the control (untreated) plants. Statistically significant values (t test; $P < 0.05$) are highlighted.
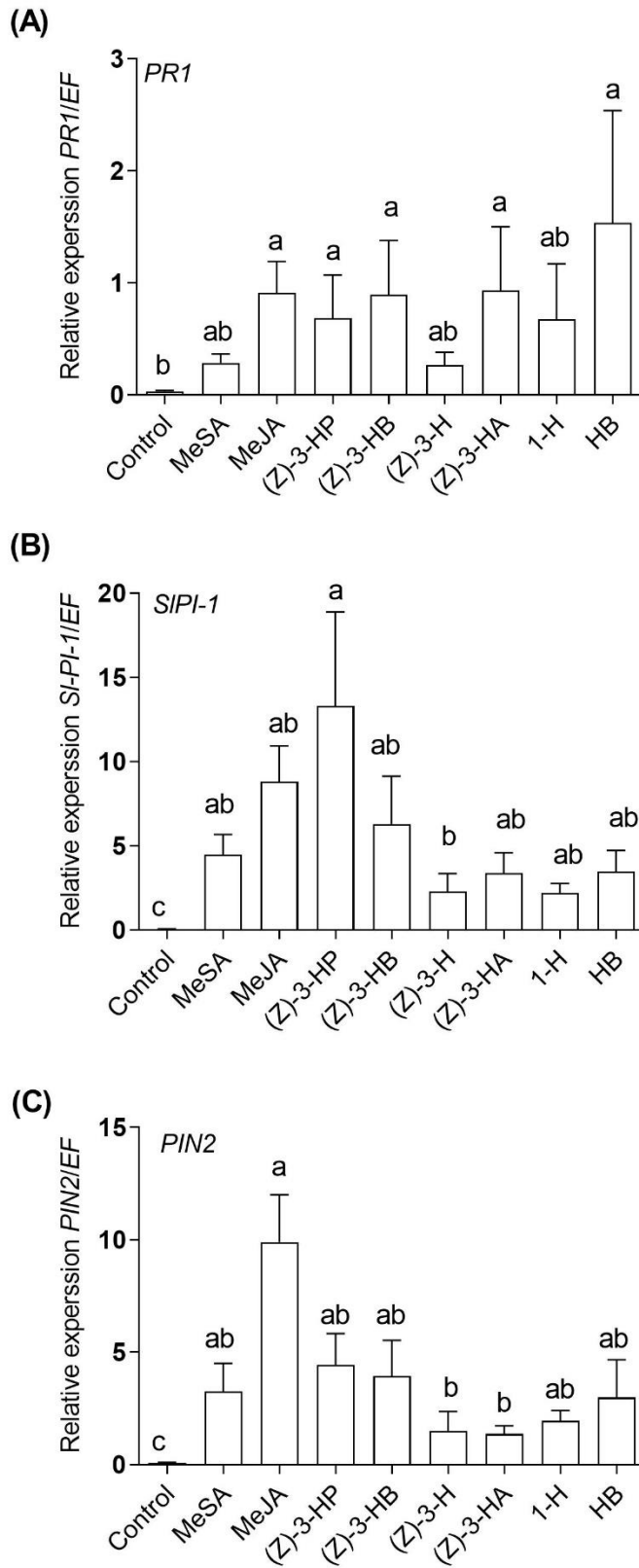
Metabolic pathway	Compound	Retention time (min)	Mass	Match	Empiric formula	Fold change		t test (*p-value*)	
						(Z)-3-HP vs control	MeSa vs control	(Z)-3-HP vs control	MeSa vs control
Apocarotenoids	β-ionone[a]	38.75	177	STD		1.60	1.79	0.00483	0.00247
Fatty acid derivatives	Hexanal[a]	15.85	72	STD		2.72	2.96	0.00005	0.00001
	2-methyl-4-pentenal[b]	16.00	56	771, 827	C6H10O	2.33	2.53	0.00012	0.00001
	(E)-2-pentenal[a]	14.10	55	STD		1.88	2.24	0.02464	0.00721
	(E)-3-hexenoic acid[b]	22.43	114	895, 922	C6H10O2	-	2.22	0.08337	0.01217
	1-penten-3-one[a]	11.35	55	STD		-	1.95	0.08417	0.04667
	(E)-2-hexenal[a]	17.99	83	STD		1.66	1.91	0.00062	0.00018
	(Z)-2-hexenal[b]	17.65	83	912, 916	C6H10O	1.51	1.54	0.00112	0.00195
	(E-E)-2,4-hexadienal[a]	20.33	81	STD		1.52	1.44	0.00015	0.00303
	2-hexen-4-olide[b]	21.88	112	823, 827	C6H8O2	1.52	1.42	0.02254	0.04021
	2-ethylfuran[a]	11.93	81	STD		1.40	1.35	0.01631	0.03437
	Unknown[b]	25.98	95	757, 789	C8H12O	1.38	1.33	0.03562	0.02399
	(E)-4-hexenoic acid[b]	22.45	112	895, 922	C6H10O2	1.40	-	0.02193	0.05485
	(Z)-3-hexenal[a]	15.77	69	STD		-	1.27	0.05505	0.04630
Monoterpenes	Hydrocarbon monoterpene[b]	27.85	115	864, 878	C10H14	0.74	-	0.03598	0.17791
	Hydrocarbon monoterpene[b]	28.76	134	854, 867	C10H14	0.75	-	0.03830	0.19592
Monoterpenoids	2,2,6-trimethyl-6-vinyltetrahydropyran[b]	22.75	139	789, 842	C10H16O	-	1.47	0.06314	0.00905
	2-isopropyl-5-methyl-3-Cyclohexen-1-one[b]	32.54	82	837, 849	C10H16O	0.76	-	0.03435	0.58528

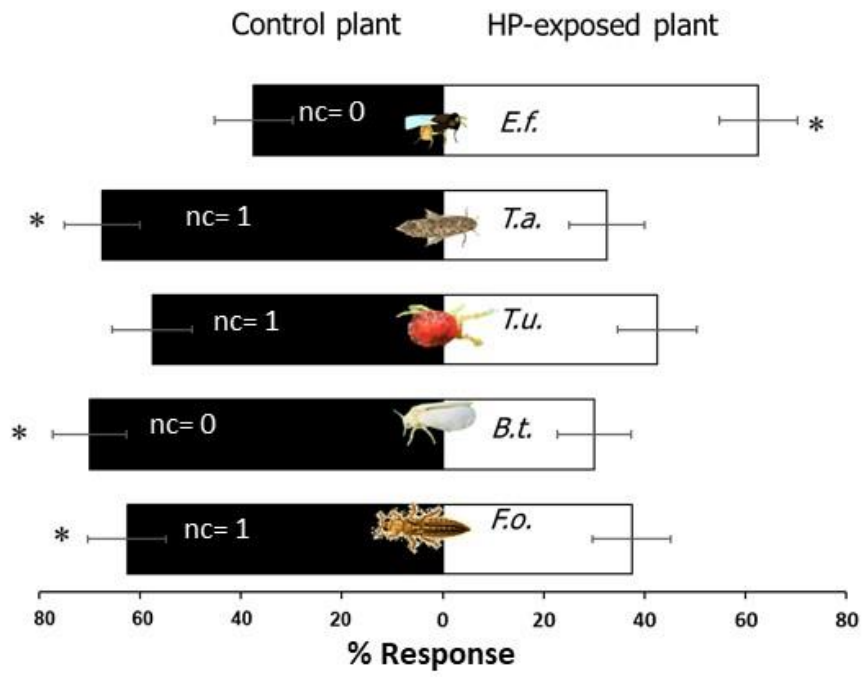|								
	3,6-Dimethyl-2,3,3a,4,5,7a-hexahydro-1-benzofuran[b]	30.44	137	854, 878	C10H16O	0.72	-	0.04640	0.23610
	Monoterpenoid[b]	29.83	79	777, 816	C10H16O	0.64	-	0.02199	0.15009
Norisoprenoid	Unknown[b]	35.47	107		C12H18O	3.71	5.16	0.00057	0.00116
Phenylpropanoids	Eugenol[a]	35.22	164	STD		-	7.65	0.20163	0.00029
	Methyl salicylate[a]	30.66	120	STD		1.18	1.18	0.03516	0.03210
Sulfur compounds	3-ethyl-thiophene[b]	18.77	97	912, 913	C6H8S	1.34	1.53	0.01877	0.02163
	2-(methylthio)-thiophene[b]	19.05	115	654, 679	C5H6S2	1.59	1.45	0.03050	0.03834

537   [a] Unequivocal identification (confirmed with a pure standard).
538   [b] Tentative identification based on mass spectra

539   **Figure 1**

(A)

(B)

(C)

540

541   **Figure 2**

(A)

Control plant   HP-exposed plant

nc= 0   *E.f.*   *

*   nc= 1   *T.a.*

nc= 1   *T.u.*

*   nc= 0   *B.t.*

*   nc= 1   *F.o.*

80 60 40 20 0 20 40 60 80

% Response

(B)

Control plant   MeSA-exposed plant

nc= 4   *E.f.*   *

*   nc= 1   *T.a.*

nc= 2   *T.u.*

*   nc= 4   *B.t.*

*   nc= 0   *F.o.*

80 60 40 20 0 20 40 60 80

% Response

542