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Can herbivore-induced volatiles protect plants by increasing the herbivores' susceptibility to natural pathogens?

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12 **Abstract**

13 In response to insect herbivory, plants mobilize various defences. Defence responses include the
14 release of herbivore-induced plant volatiles (HIPVs) that can serve as signals to alert undamaged
15 tissues and to attract natural enemies of the herbivores. Some HIPVs can have a direct negative
16 impact on herbivore survival, but it is not well understood by what mechanisms. Here we tested
17 the hypothesis that exposure to HIPVs renders insects more susceptible to natural pathogens.
18 Exposing caterpillars of the noctuid *Spodoptera exigua* to indole and linalool, but not exposure
19 to (*Z*)-3-hexenyl acetate increased the susceptibility to its nucleopolyhedrovirus (SeMNPV). We
20 also found that exposure to indole, but not exposure to linalool or (*Z*)-3-hexenyl acetate,
21 increased the pathogenicity of *Bacillus thuringiensis*. Additional experiments revealed
22 significant changes in microbiota composition after forty-eight hours of larval exposure to
23 indole. Overall, these results provide evidence that certain HIPVs can strongly enhance the
24 susceptibility of caterpillars to pathogens, possibly through effects on the insects' gut microbiota.
25 These findings suggest a novel mechanism by which HIPVs can protect plants from herbivorous
26 insects.

27

Importance

Multitrophic interactions involving insect pest, their natural enemies, microorganisms and plant hosts are increasingly being recognized as relevant factors in pest management. In response to herbivory attacks, plants activate a wide range of defences that aim to mitigate the damage. Attacked plants release herbivore-induced plant volatiles (HIPVs), which can act as priming signals for other plants, attract herbivore natural enemies and may have a direct negative impact on herbivore survival. In the present work, we show that exposure of the insects to the induced volatiles could increase the insects' susceptibility to the entomopathogens naturally occurring on the plant environment. These findings suggest a novel role for plant volatiles by influencing the insect interaction with natural pathogens, probably mediated by alterations in the insect microbiota composition. In addition, this work provides evidence for selectable plant traits (production of secondary metabolites) that can have an influence on the ecology of the pests and could be relevant in the improvement of pest management strategies using natural entomopathogens.

43 **Introduction**

44 Plants defend themselves against herbivores through the production of specific
45 metabolites and proteins with toxic, repellent, or antinutritive properties (1). These defence
46 compounds are either produced constitutively or induced in response to herbivore attack (2).
47 Induction is mainly mediated by the insect feeding and leads to the activation of multiple
48 signalling pathways that regulate the production of defensive proteins and metabolites (3-5).
49 Herbivores exhibit multiple feeding styles (e.g. chewing, sucking) and differ in the levels of
50 specialization to their host plants. Accordingly, the plant response can vary depending on the
51 type of herbivore and can involve a combination of responses in case of multiple attacks (6, 7).
52 Plant defence responses can also be elicited by other herbivore-related factors such as
53 oviposition by insects (8, 9) or even by the perception of volatiles emitted by neighbouring plants
54 in response to insect attack (10, 11).

55 Plant-emitted volatiles represent a group of specialized metabolites that play an important
56 role in the plant defence against herbivory. Attacked plants release herbivore-induced plant
57 volatiles (HIPVs) which can act as priming signals (10, 12, 13) or attract herbivore natural
58 enemies (14-16). HIPVs can also have direct benefits for the plant by repelling the herbivore or
59 reducing its growth and survival in the plant (17). For instance, the Green leaf volatile (Z)-3-
60 hexenol from infested neighbour plants was found to be converted to (Z)-3-hexenyl-vicianoside
61 in tomato (*Solanum lycopersicum*), reducing survival and growth of *Spodoptera litura*
62 caterpillars (18). More recently, it has also been shown that the HIPV indole increases weight
63 gain, but reduces food consumption and survival in *Spodoptera littoralis* (9).

64 In a multi-trophic context, the eventual outcome of the interaction between plant and
65 herbivore is also modulated by pathogenic microbes, which is assumed to be due to direct as well

66 as indirect effects of toxic phytochemicals on entomopathogen persistence and infectivity (19-
67 21). Although some authors have speculated about the possibility of plant promoting the action
68 or abundance of microbial entomopathogens (22) not much information is available about the
69 impact of HIPVs on the pathogenicity of entomopathogens. So far, only a few studies have
70 reported the influence of certain plant volatiles on the conidial germination rates of
71 entomopathogenic fungi (23, 24). To test this, we investigated the effect of specific HIPVs on the
72 pathogenicity of two types of entomopathogens that naturally infect the beet armyworm,
73 *Spodoptera exigua*. Larval mortality due to the *Spodoptera exigua nucleopolyhedrovirus*
74 (SeMNPV) and to *Bacillus thuringiensis* was measured during exposure of the insect to three of
75 the most common plant volatiles: indole, linalool, or hexenyl-acetate. Additionally, we evaluated
76 the effect of these volatiles on insect cellular immunity and gut microbiota composition. Indeed,
77 in addition to the well-studied direct interactions between pathogens and the insect immune
78 system, it is increasingly evident that the gut mutualistic and commensal communities can
79 enhance resistance or tolerance of insects to pathogens (25-27). The results reveal a novel
80 indirect defensive role for HIPVs by enhancing the pathogenicity of entomopathogens.
81

Results

HIPVs effects on the susceptibility to viral and bacterial pathogens

Compared with the control conditions in the absence of HIPVs, a significant increase in mortality due to baculovirus infection was observed when larvae were reared in the presence of indole (One-way ANOVA: F (2,6)=13.8, P=0.006; Newman-keuls post-test: q ($_{0\ 05,\ 2,\ 6}$) = 6.3) or linalool (One-way ANOVA: F (2,6)=12.5, P=0.007; Newman-keuls post-test: q ($_{0\ 05,\ 2,\ 6}$) = 5.8) (Fig. 1A and S1). No effect on SeMNPV pathogenicity was observed when the larvae were exposed to hexenyl-acetate (One-way ANOVA: F (2,6)= 2.71, P=0.40). Significant synergistic interaction was found between the SeMNPV virus and indole (χ^2 (1, *N*=82)=14.42, P=0.001) or linalool (χ^2 (1, *N*=82)=23.74, P<0.0001). At the SeMNPV dose that we used, no increase in virulence (measured as the mean of time to death by the viral infection) was observed for any of the HIPVs treatments (Fig. 1B and S1).

The effect of exposure to the indole on the SeMNPV infectivity was also tested at a higher viral dose ($5x10^4$ OBs/larvae, producing about 80-90% mortality). Under these conditions, no additional increase in mortality was observed in the presence of indole, however a significant increase in virulence of the virus was found, with mortality occurring 20% earlier in the indole-exposed insects (Fig. S2A and S2B). In a more controlled environment where indole was released at a similar rate as produced by maize plants (50 ng/h) (10), we also observed a significant increase in baculovirus virulence (Fig. S2C and S2D).

We also evaluated the effects of HIPVs on the insect's susceptibility to a bacterial pathogen (Fig. 2). Under our experimental conditions, mortality due to *B. thuringiensis* was affected by the exposure to indole (One-way ANOVA F (2,4)=9.34, P=0.03; Newman-keuls post-test: q ($_{0\ 05,\ 2,\ 4}$) = 5.2) and not affected by exposure to linalool or (*Z*)-3-hexenyl acetate. In this case, no

105 significant synergistic interaction was found between *B. thuringiensis* and indole (χ^2 (1, 106 N=74)=1.62, P=0.12) and the contribution of indole to the mortality by *B. thuringiensis* was only 107 additive.

108 *Immune status of insects exposed to volatiles*

109 To test if exposure to HIPVs affects the immunological status of *S. exigua*, we measured the 110 levels of two enzymatic key markers of the cellular immunity in insects, phenoloxidase (PO) and 111 phospholipase A2 (PLA2). PO is involved in the process of encapsulation and melanization (28), 112 whereas the enzyme PLA2 activates the eicosanoid pathway involved in the cellular immunity in 113 insects (29). Several studies have shown that the inhibition of eicosanoids increases insect 114 susceptibility to baculovirus (30, 31). PO activity was measured in the haemolymph of L3 larvae 115 exposed to the different HIPVs for 24 and 48 hours. Compared to controls, the exposure had no 116 effect on PO activity (Fig 3A). PLA2 activity was measured on the whole body extract of L3 117 larvae exposed to the three HIPVs for 24 and 48 hours (Fig 3B). Again, no effect on enzyme 118 activity was observed for any of the treatments.

119 *Changes in midgut microbiota after exposure to indole.*

120 Indole is known to be involved in bacterial processes, either by mediating bacterial 121 communication and quorum sensing (32), or through antimicrobial activity via RNA synthesis 122 inhibition (33). We therefore also evaluated, in side-by-side experiments, the effect of indole 123 exposure, as well as baculovirus infection on the larval gut microbiota load and composition. No 124 major effect of baculovirus infection on microbiota composition and diversity was observed 48 125 hours posts infection. However, exposure to indole had a significant effect on the microbiota 126 load, alpha diversity and composition (Fig 4). A multivariate canonical correspondence analysis 127 (CCA) showed a clearly different microbial profile (P=0.012) between the indole-exposed and

128 non-exposed group (Fig 4A). Forty-eight hours of exposure to indole, resulted in a significant
129 decrease in gut bacterial load ($P<0.019$; Fig 4B) and a significant increase in bacterial diversity
130 ($P=0.03$; based on the Shannon diversity index) (Fig 4C and D). The relative abundance in
131 percentage of the top genus in each sample as depicted in Fig 4C, suggests that changes in
132 diversity would be associated with the reduction in the relative abundance of bacteria of the
133 genus *Enterococcus* (Fig 4C). Linear discriminant analysis effect size (LEfSE) confirms this
134 differential abundance of the genus *Enterococcus* and revealed specific genera that were
135 differentially enriched in each group (Fig 4E). Among the most represented genera in the indole-
136 exposed group were *Faecalibacterium, Ruminococcus, Comanomonas*, *Chryseobacterium,*
137 *Providencia*, *Sphingobium* and unclassified *Oxalobacteriaceae,* while four different genera were
138 significantly overrepresented in the insects that were not exposed to indole.
139
140 **Discussion**
141 HIPVs play multiple roles in plant-herbivore interactions (17, 34, 35). Here we show that HIPVs,
142 in addition to their already known roles, can also have a role in affecting the susceptibility of
143 insect herbivores to viral and bacterial pathogens. These entomopathogens occur naturally in the
144 ecosystem (36-38), but are also used as active ingredients in biopesticides (39). Specifically, we
145 found that indole and linalool, two volatiles produced and released in response to herbivory by
146 various plant species such as maize, cotton, rice, tomato, tobacco etc (10, 35, 40-43), have a
147 synergistic effect on SeMNPV infectivity. To a lesser degree, the combination of indole with the
148 bacterium *B. thuringiensis* boosted mortality caused by the bacteria in an additive manner.
149 In the case of indole, its effect on the susceptibility of *S. exigua* to the baculovirus was observed
150 in different experimental settings and concentrations of the pathogen. Moreover, the synergistic

151 mortality effect was found at a viral dose that caused only sublethal infections in most of the
152 tested insects. At a higher viral dose, which caused mortality in most of the infected insects, the
153 effect of indole exposure was reflected in virus virulence. This increase in virulence in the
154 presence of indole was confirmed under more controlled conditions, where the insects were
155 exposed under a continuous airflow and at a realistic concentration of indole.

156 Baculovirus infections are very common in natural populations of Lepidoptera (44). In the case
157 of *S. exigua*, 54% of larvae in the field have a non-lethal infection of their baculovirus, SeMNPV
158 (37). The dynamics of pathogen–host interactions in insects are determined primarily by host and
159 pathogen density, but also by the virulence of the pathogen (impact on infected individuals
160 ranging from slightly debilitating to lethal) (45). Our results suggest that exposure to indole and
161 linalool can increase pathogen virulence to SeMNPV to a degree that normally sublethal doses of
162 the virus become lethal. This may have an important impact in the context of crop protection and
163 could help to significantly decrease pest densities in the field and consequently reduce crop
164 damage. Recent studies have started to provide evidence for selectable plant traits that enhance
165 the ability of pathogens to control insect pests (46). Our data further confirm the potential of
166 plant traits to enhance the efficacy of entomopathogens as biocontrol agents. It is likely that from
167 the extensive arsenal of metabolites produced by plants (47), many others could also synergize
168 the pest management potential of entomopathogens that are naturally found in the ecosystem or
169 artificially released as pest control agents.

170 We also explored the molecular basis that underlies the effect of HIPVs on the susceptibility to
171 entomopathogens. Indole and oxindole have previously been found to be produced by
172 entomopathogenic bacteria and to inhibit the *in vitro* activity of PLA2, one of the key enzymes
173 from the eicosanoids pathway that is involved in the cellular immunity (48, 49). It has been

174 shown that certain inhibitors of the eicosanoids pathway (including a PLA2 inhibitor), when
175 added to the rearing diet of caterpillars (at concentrations of about 30-50 mM), can increase their
176 susceptibility to nucleopolyhedroviruses (30). In our study, however, when we analysed the
177 effect of HIPV exposure on PLA2 activity of the exposed larvae, we did not detect any reduction
178 in the enzymatic activity for any of the three volatiles. Similarly, no effect on the PO activity, an
179 enzyme involved in cellular and humoral defence, was observed in the insects exposed to the
180 three HIPVs. Even though we cannot fully exclude that the volatiles have a direct negative effect
181 on other aspects of the insects' immune system, these results strongly suggest that another
182 mechanism, different from the direct interference with the insect's cellular immunity, mediates
183 the enhanced susceptibility after exposure to indole and linalool. One such mechanism could
184 involve changes in the gut microbiota caused by the HIPVs. We and others have previously
185 shown that changes in the gut microbiota composition can affect an insect's susceptibility to
186 bacterial (26, 50) and viral (51) pathogens. Insect's gut microbiota composition and homeostasis
187 depend on the diet (52) and its immune system (53), but also relies on the microbial synthesis
188 and secretion of metabolites and enzymes that contribute to the establishment of the interactions
189 with the host and other microbes (54). Gut microbiota influences in insect development and
190 physiology (55), and consequently, dysbiosis in microbiota composition may have important
191 effects on gut physiology and homeostasis leading to enhanced success of viral infections.
192 The changes that we observed in the gut microbial composition after indole exposure may be
193 caused by direct effects of the indole on the microbiota, or by changes in physiological
194 parameters of the larvae that might indirectly affect an insect's microbiota. Given the known role
195 of indole in microbial processes (56), it is possible that the observed changes are the result of
196 direct exposure of the gut microbes to indole. More than 85 bacterial species (Gram-negative as

197 well as gram-positive) can synthesize indole (57), and as an intercellular signal molecule, indole
198 controls diverse aspects of bacterial physiology, such as spore formation, plasmid stability, drug
199 resistance, biofilm formation, and virulence in indole-producing bacteria (56, 57). In our
200 measurements species from the genus *Enterococcus* were the most dominant in the microbiota
201 community of the *S. exigua* larvae. It is likely that indole exposure interfered with normal growth
202 of *Enterococcus spp*, thereby possibly promoting the growth of other bacterial species that could
203 affect the insects' physiology in a way that it lowers their resistance to entomopathogens. Vega
204 *et al.,* (58) have shown that bacterial communication through indole signalling induces
205 persistence, a phenomenon that allows a subset of an isogenic bacterial population to tolerate
206 antibiotic treatment. It is possible that the observed indole-induced increase in microbial
207 diversity involves a similar mechanism, in this case leading to enhanced susceptibility to the
208 pathogen. Indeed, it has been shown that indole has a minor beneficial effect on *E. coli* when it is
209 cultured with *Enterococcccus faecalis* (59). It would be interesting to test the effect of indole on
210 the growth of specific *Enterococcus spp* isolates from the larval gut.

211 In summary, our results support a novel role for the HIPVs in the plant-insect-microbe
212 interaction. In addition to their function in direct defence, signalling between plant tissues, and
213 multitrophic interactions (60), HIPVs may mediate interactions between insects and their
214 pathogens. These interactions are likely affected by altered gut microbiota composition as a
215 result of indole exposure. The observed increase in susceptibility to viral and bacterial pathogens
216 provides an additional element to the possible application of HIPVs to regulate the abundance
217 and dynamics of insect pests. Further experiments using other insect-pathogens combinations
218 and other HIPVs are needed to determine the prevalence of the phenomenon and to further
219 resolve the underlying genetic and physiological mechanisms.

220

221 **Experimental procedures**

222 ***Insects and chemicals***

223 The *Spodoptera exigua* colony was established with eggs that were provided by Andermatt
224 Biocontrol AG (Grossdietwil, Switzerland) and was continuously reared on artificial diet (61) at
225 25 ± 3°C with 70 ± 5% relative humidity and a photoperiod of LD 16:8 h.
226 The synthetic volatiles used in the bioassays (indole, linalool and (Z)-3-hexenyl acetate) were
227 purchased from Sigma-Aldrich.

228 ***Effect of the HIPVs on SeMNPV infectivity***

229 For the exposure to selected HIPVs we prepared 0.2 ml micro-centrifuge tube to which we added
230 4 mg of indole powder or 10 µl of 10% of linalool or 10% (Z)-3-hexenyl acetate (in distilled
231 water). After perforating the lid of a tube with a G25 needle it was placed in a rearing well (a. 2
232 cm X 2 cm X 2 cm) that contained an individual larva and a piece of artificial diet. The well was
233 then sealed with micro perforated adhesive tape (Frontier Agricultural Sciences, Product# 9074-
234 L).
235 Aiming to assess the effect of the selected HIPVs on the SeMNPV, third instar (first day) *S.*
236 *exigua* larvae were orally infected and reared in presence or absence of one of the volatiles. For
237 this, larvae were fed individually with diet plugs (about 0.4 mm^3) containing different amounts
238 (10^2 or $5x10^4$) of occlusion bodies (OB) from the SeMNPV. Larvae were kept for 24 hours with
239 the virus-contaminated food. After that, larvae that completely consumed the food were selected
240 for the bioassay and fed with virus-free artificial diet. Larval mortality was then recorded every
241 12 hours until the death or pupation of all the larvae. Then mortality curves were assessed using
242 the Kaplan-Meier method and compared using the log-rank analysis (Mentel-cox test) and the

243 GraphPad Prism program (GraphPad software Inc., San Diego, CA, USA). In addition, and due
244 to the different levels of mortality for each treatment, changes in virulence were estimated by
245 comparison of the mean time to death. The statistical differences were assessed using either the
246 student's t-test or One-way ANOVA with the Newman-keuls post-test (GraphPad Prism). Three
247 independent replicates were performed using 16 larvae per treatment and replicate.
248 In a second experiment, newly molted third instar larvae were exposed to the volatile indole at
249 approximately 50 ng /h, similar to what is released by caterpillar-infested maize plants (9, 10).
250 For this purpose, volatile dispensers that consisted of 2 ml amber glass vials (Supelco, Sigma-
251 Aldrich) supplied with 20 mg of synthetic indole were used. The vials were closed with an open
252 screw cap with rubber septum. The septum was pierced with 2 µl microcaps® (Drummond
253 Scientific, Broomall, PA, USA) through which indole diffused at a constant rate. Groups of
254 caterpillars (5 to 6) were placed in individual plastic cages (5 cm diameter, 2 cm height) covered
255 with a nylon mesh and fed with a cube of artificial diet contaminated with 50 µl of 10^4 OBs / ml,
256 then kept into glass vessels which contained control or indole-releasing dispenser. Purified air
257 entered these vessels via Teflon tubing at a rate of 0.3 l min^{-1} to avoid indole over-accumulation.
258 The larvae were reared at 22 ± 2°C and supplied with fresh diet every 48 hours. Mortality curves
259 and mean time to death were assessed as described above. Three independent replicates were
260 performed using 16 larvae per treatment and replicate.
261 ***Effect of the different HIPVs on susceptibility to B. thuringiensis infection***
262 Effect of the selected HIPVs on the entomopathogenic bacterium *Bacillus thuringiensis* was
263 tested using the surface contamination bioassay method (62). In these experiments, a formulation
264 of wettable granules containing *B. thuringiensis* subsp. *aizawai* (Xentari ®, Kenogard S.A,
265 Spain) was tested. Surface contamination assays were employed with first instar *S. exigua* larvae,

266 and the larvae were exposed to the different HIPVs as described in the first experiments. Briefly,
267 a volume of 50 µL of the bacterial suspension was applied on the surface of the diet in individual
268 wells (0.5 ng/cm^2) and left to dry for 30-60 min in a flow hood. Then, first instar larvae were
269 placed individually in each well together with the tube containing the respective volatile and
270 mortality was recorded after five days. Statistical analysis was performed using One-way
271 ANOVA with Newman-keuls post-test (GraphPad Prism). Three independent replicates were
272 performed using 16 larvae per treatment and replicate.

273 ***Analyses of the interaction of entomopathogens with the different HIPVs***

274 Possible antagonistic/synergistic interactions between entomopathogens and each of the selected
275 HIPVs were determined using the mortality values at seven and five days post infection for the
276 SeMNPV and *B. thuringiensis* treatment, respectively. Mortality percentages were corrected
277 using the Abbott correction (63). Then the expected mortality was calculated with the response
278 addition model (64), which is used to evaluate mixtures of substances that have different modes
279 of action employing the following equation:

280 $E(c_{MIX}) = E(c_A) + E(c_B) - [E(c_A) * E(c_B)]$, where $E(c_{MIX})$ is the prediction of a total effect of
281 the mixture (mortality in our case) and $E(c_A)$ and $E(c_B)$ are the observed effect caused by
282 individual SeMNPV or *B. thuringiensis* and the volatile, respectively (64). Significance of the
283 deviations between the observed and expected mortality values was assessed using Chi-square
284 test (GraphPad Prism).

285 ***Effect of the HIPVs on the insect immunity***

286 In order to study the effect of the HIPVs on the immune system of *S. exigua*, the enzymatic
287 activities of the phenoloxidase (PO) and phospholipase A2 (PLA2), two markers of the cellular
288 immunity, were measured. For the PO assay, haemolymph of L3 larvae exposed to a volatile or

289 not (same conditions as above) was extracted 24 and 48 h after exposure and centrifuged at 500 g
290 for 2 min at 4ºC to remove the haemocytes. Four microliters of cell-free haemolymph, 46 µl of
291 PBS 1X and 50 µl of the substrate L-dopamine (100 µg/ml in PBS 1X) were added to each wells
292 in a 96-well microtiter plate. PO activity was determined by monitoring the increase of
293 absorbance at 492 nm for 30 min using the Infinite 200 PRO multimode plate reader (TECAN
294 Group Ltd., Switzerland). The activity of the enzyme was represented as the initial velocity (Vo)
295 of the reaction, measuring the change in absorbance per second. To perform the assay of PLA2
296 activity, bodies of the L3 larvae mentioned above were homogenized in Tris-HCl 50 mM (pH
297 7.0) and centrifuged at maximum speed for 5 min at 4ºC. The protein concentration was
298 determined using the Bradford (1972) assay, with bovine serum albumin (BSA) as a standard.
299 The enzymatic reaction was done with 136 µl of Tris-HCl 50 mM (pH 7.0), 1 µl of $CaCl_2$, 150
300 mM, 1.5 µl of BSA 10%, 10 µl of larval extract and 1.5 µl of pyrene-labeled substrate (1-
301 Hexadecanoyl-2-(1-Pyrenedecanoyl)-sn-Glycero-3-Phosphocholine; ThermoFisher) (10 mM in
302 ethanol). A multimode plate reader (TECAN) was used to measure fluorescence intensity by
303 excitation at 345 nm and emission at 398 nm. The activity of PLA2 was then calculated as the
304 changes in fluorescence per second. Due to the intrinsic variability between biological replicates,
305 values for each enzyme and treatment were calculated as the difference in percentage of activity
306 with unexposed insects within each replicate.

307 ***Microbiota composition and diversity***

308 To determine if exposure to indole and/or infection with the baculovirus influence the gut
309 microbiota of *S. exigua*, third instar (first day) larvae were exposed to indole and infected with
310 SeMNPV as described above. After 48 hours, larval midguts from each treatment were dissected,
311 pooled by treatment and homogenized in Luria-Bertani (LB) medium supplemented with 10% of

312 glycerol. A fraction of the homogenized guts was used for total DNA extraction using the
313 MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA). Three replicates were
314 performed using 5 larvae per treatment and replicate and for each replicate the different
315 treatments were applied simultaneously in a side-by-side manner. PCR amplification of the 16S
316 rRNA (V3-V4 region) and sequencing were carried out using 2 x 300 pb paired-end run (MiSeq
317 Reagent kit v3) on a Illumina MiSeq sequencing platform at the Foundation for the Promotion of
318 Health and Biomedical Research (FISABIO, Valencia). Quality assessment of obtained reads
319 was done with the prinseq-lite program (65) with defined parameters (i.e., min_length: 50,
320 trim_qual_right: 20, trim_qual_type: mean, trim_qual_window: 20). Paired reads from Illumina
321 sequencing where joined using *fastq-join* from ea-tools suite (66). Filtered and demultiplexed
322 sequences were then processed with the open-source software QIIME v.1.9. (67) using default
323 parameters. A total of 12 samples were sequenced. One sequence showed fewer than 1000 reads
324 and was removed for further analysis. The sequences were then binned into Operational
325 Taxonomic Units (OTUs) using de novo OTU picking based on 97% identity and filtering the
326 Unassigned taxa. Bacterial composition was also determined filtering the Unassigned,
327 Chloroflexi and Cyanobacteria taxa, and the 20 most abundant genera were represented in a bar
328 graphic using Excel software. Calypso version 8.2 (68) was used with the OTU table data
329 transformed by CSS + log with total sum normalization, to generate Canonical Correspondence
330 Analysis (CCA) plot for multivariate analysis at genus level, and indole exposure as factor.
331 Alpha diversity using Shannon index and linear discriminant analysis effect size (LEfSE) (69)
332 were determined at genus level, and again indole exposure as factor.

333 Total DNA was also used to determine the bacterial load by specific qPCR using universal
334 primers for the 16S rDNA gene (70). The qPCRs were carried out in StepOnePlus Real-Time

335 PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed using 5x
336 HOT FIREPOL EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) in a total
337 volume of 20 µl. The bacterial concentration in each sample was calculated by comparison with
338 the Ct values obtained from a standard curve of known bacterial DNA concentration. These were
339 generated using serial 10-fold dilutions of DNA extracted from *E. coli* bacteria. Bacterial loads
340 were statistically compared with the student's *t*-test (GraphPad Prism).

341

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349

350 **Competing interests**

351 The authors declare that they have no competing interests

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354

355

356 **Figures legends**

357 **Fig. 1.** Effect of the tested HIPVs on the *S. exigua* susceptibility to SeMNPV infection (10^2
358 OBs/larvae). Ind (indole 4 mg), Lin (linalool 10%), and Hac (hexenyl acetate 10%.) A)
359 Percentage of larval mortality for the different combinations. Observed mortality (O) and
360 Expected mortality (E) assuming the additive model. Statistical analyses were performed using
361 One-way ANOVA with Newman-keuls post-test to compare the mortalities and the Chi-square
362 test was used to check whether there is synergism or additive effect between the different
363 treatments. B) Mean time to death produced by baculovirus in the presence/absence of the
364 corresponding HIPV. Values were statistical compared using student's t-test.

365

366 **Fig. 2.** Effect of the tested HIPVs on the *S. exigua* susceptibility to *B. thuringiensis* (Xentari ®).
367 Ind (indole 4 mg), Lin (linalool 0.1%) and Hac (hexenyl acetate 0.1%.). Percentage of larval
368 mortality for the different combinations. Observed mortality (O) and Expected mortality (E)
369 assuming the additive model. Statistical analyses were performed using One-way ANOVA with
370 Newman-keuls post-test to compare the mortalities and the Chi-square test was used to check
371 whether there is synergism or additive effect between the different treatments.

372

373 **Fig. 3.** Effect of the tested HIPVs on two enzymatic markers of the cellular immunity of *S.*
374 *exigua.* A) Relative Phenoloxidase activity in the haemolymph of insects exposed to selected
375 volatiles at 24 and 48 hours after exposure. B) Relative PLA2 activity in the fat body of insects
376 exposed to selected volatiles at 24 and 48 hours after exposure. For both markers, the activity is
377 normalized according to the activity obtained for the non-exposed insects.

378

379 **Fig. 4.** Effect of the exposure to indole on the gut microbiota composition of the *S. exigua* larvae.
380 A) Canonical correspondence analysis (CCA) showing the relationship between gut microbiome
381 composition (genus level) in the indole-exposed and non-exposed insects. B) Bacterial load
382 calculated for the samples from the indole-exposed and non-exposed insects. C) Relative
383 abundance in percentage of the top genus in samples from the indole-exposed and non-exposed
384 insects. The exposition to the viral infection is indicated as + in the top of the panel. D)
385 Microbial diversity calculated as the Shannon index in the samples from the indole-exposed and
386 non-exposed insects. E) LefSe (Linear discriminant analysis effect size) results, reporting the
387 more significantly overrepresented taxa for the indole and no indole group.

388

389 **Fig. S1.** Time course mortality of L3 larvae exposed to Indole (A), Linalool (B), and Hexenyl
390 acetate (C) and the SeMNPV. Final mortality values are reported in Fig. 1.

391

392 **Fig. S2.** Time course mortality and calculated mean time to death of L3 larvae exposed to indole
393 at a concentration of SeMNPV producing about 80% mortality. In A) and B), insects were
394 exposed to the indole by placing 4 mg of indole in 0.2 ml tube punched with a G25 needle in
395 their lid into the rearing well. In C) and D), insects were exposed to a continuous rate of indole
396 (a.50 ng /h) using a volatile dispenser. * refers to P-value<0.01

397

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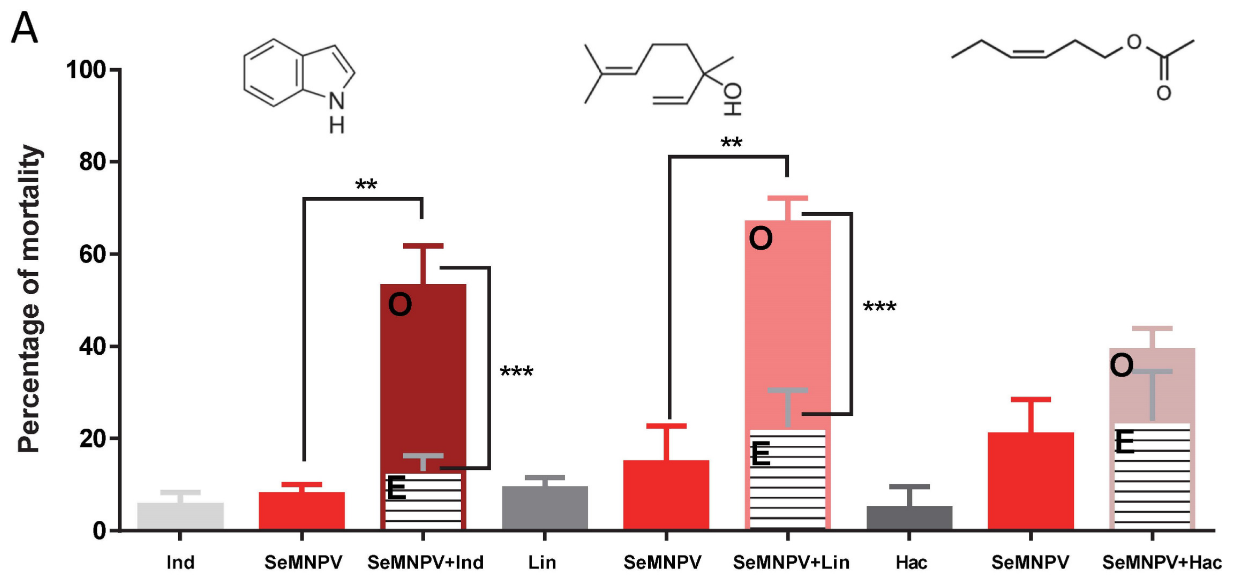
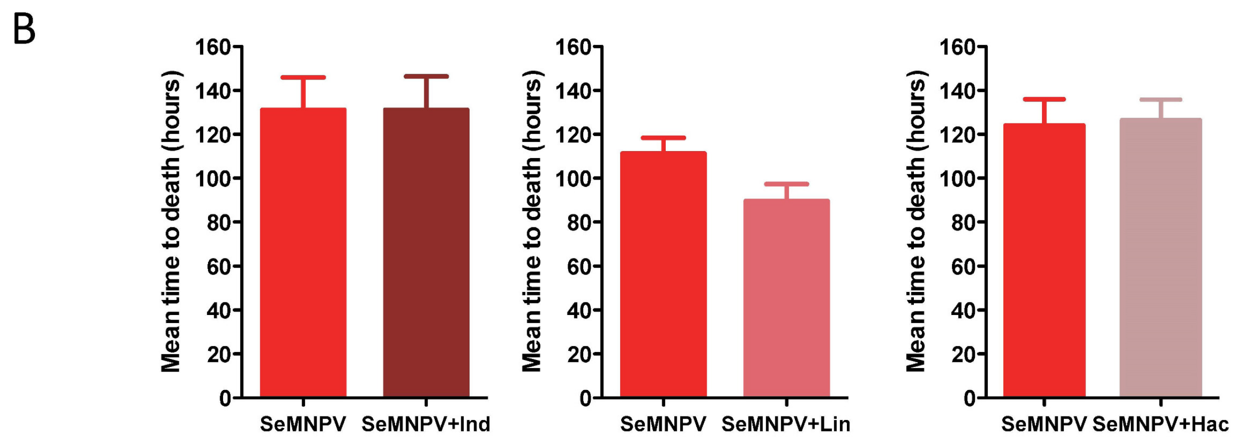
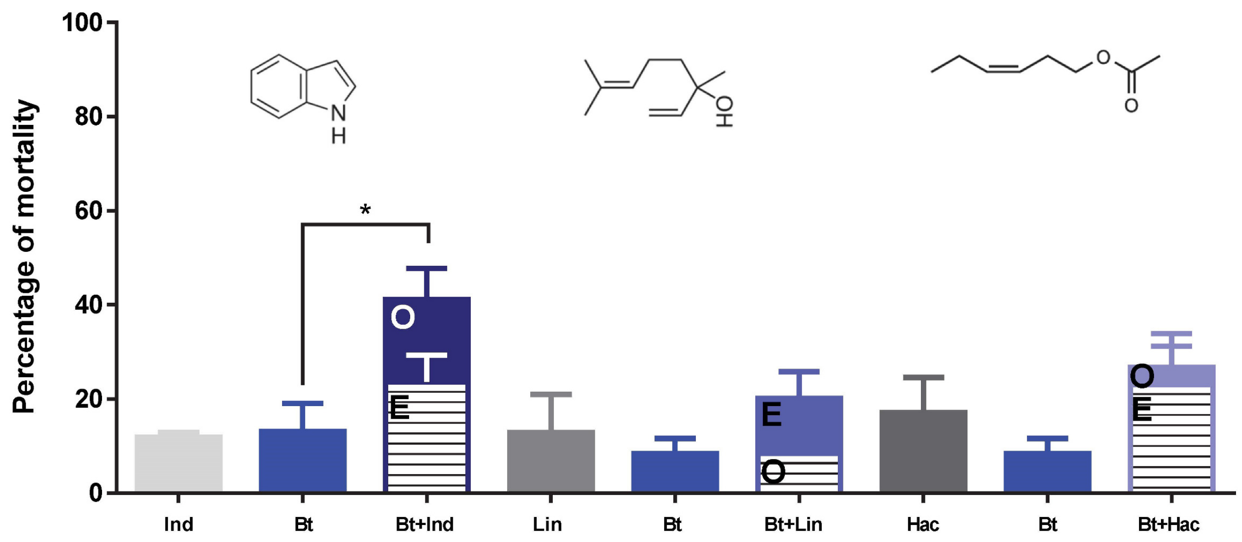
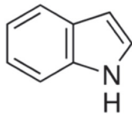
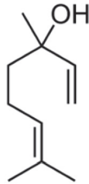

A
N
H
OH
O
O
**
**
***
***
O
E
O
E
O
E
Percentage of mortality
100
80
60
40
20
0
Ind
SeMNPV
SeMNPV+Ind
Lin
SeMNPV
SeMNPV+Lin
Hac
SeMNPV
SeMNPV+Hac

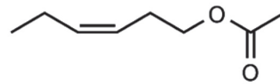

B
Mean time to death (hours)
160
140
120
100
80
60
40
20
0
SeMNPV
SeMNPV+Ind
SeMNPV
SeMNPV+Lin
SeMNPV
SeMNPV+Hac



Percentage of mortality
100
80
60
40
20
0
*
O
T
E
E
O
O
E
N
H
OH
O
O
Ind
Bt
Bt+Ind
Lin
Bt
Bt+Lin
Hac
Bt
Bt+Hac


N
H


OH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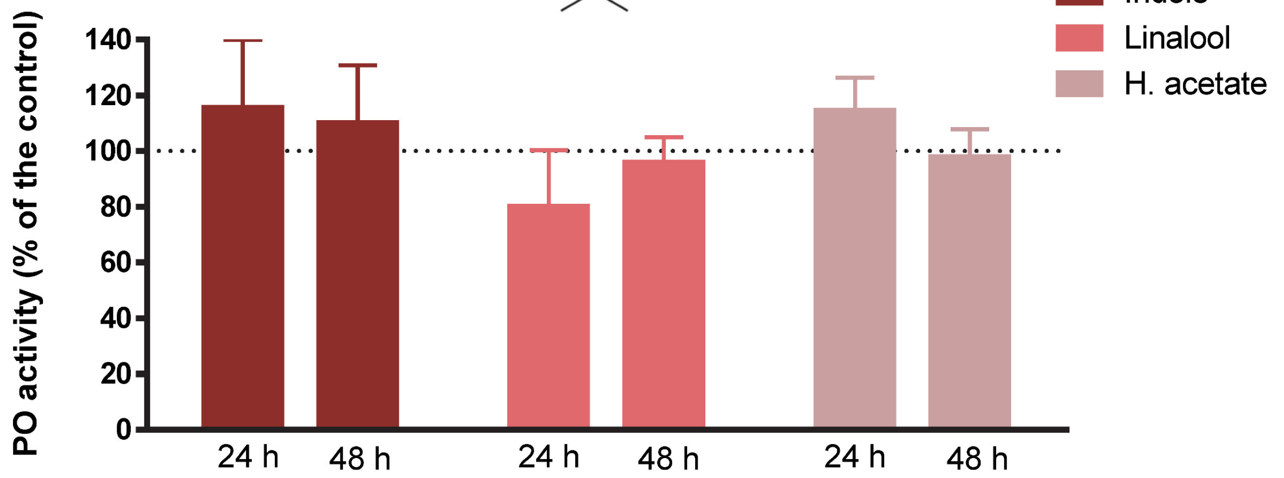
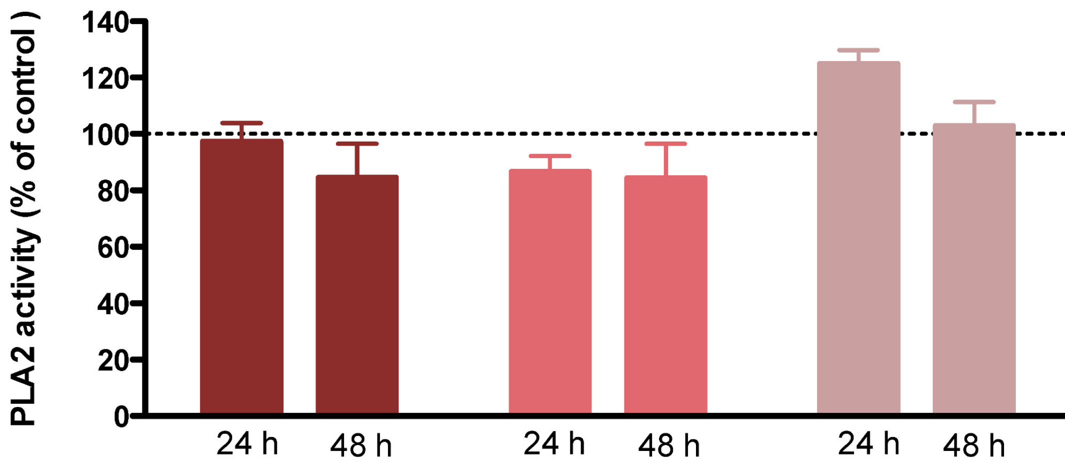
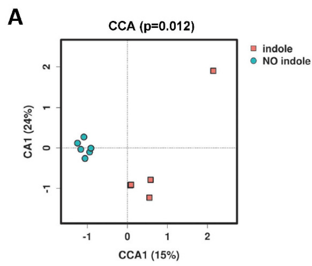
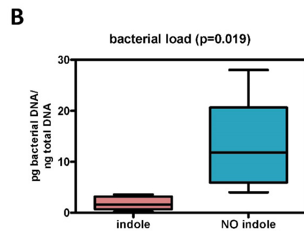
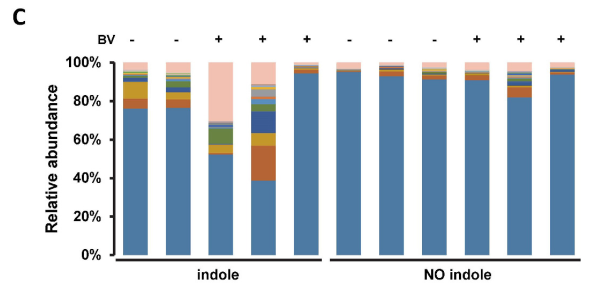
O

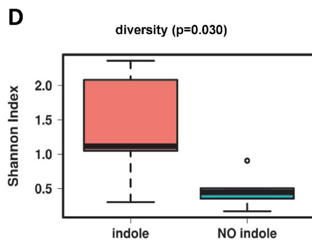

Indole
Linalool
H. acetate
PO activity (% of the control)
140
120
100
80
60
40
20
0
24 h
48 h
24 h
48 h
24 h
48 h

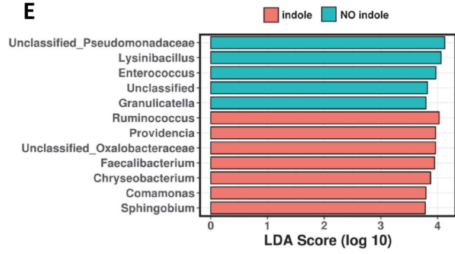

PLA2 activity (% of control)
140
120
100
80
60
40
20
0
24 h
48 h
24 h
48 h
24 h
48 h

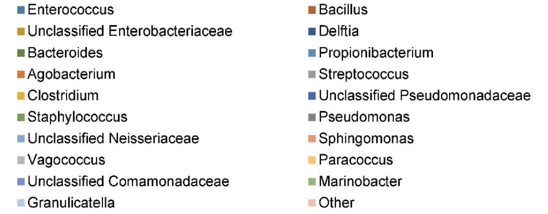

A
CCA (p=0.012)
indole
NO indole
CA1 (24%)
CCA1 (15%)


B
bacterial load (p=0.019)
pg bacterial DNA/
ng total DNA
indole
NO indole


C
BV
Relative abundance
indole
NO indole


D
diversity (p=0.030)
Shannon Index
indole
NO indole


E
indole
NO indole
Unclassified_Pseudomonadaceae
Lysinibacillus
Enterococcus
Unclassified
Granulicatella
Ruminococcus
Providencia
Unclassified_Oxalobacteraceae
Faecalibacterium
Chryseobacterium
Comamonas
Sphingobium
LDA Score (log 10)


Enterococcus
Bacillus
Unclassified Enterobacteriaceae
Delftia
Bacteroides
Propionibacterium
Agobacterium
Streptococcus
Clostridium
Unclassified Pseudomonadaceae
Staphylococcus
Pseudomonas
Unclassified Neisseriaceae
Sphingomonas
Vagococcus
Paracoccus
Unclassified Comamonadaceae
Marinobacter
Granulicatella
Other