Chemical Identity and Functional Characterisation of Semiochemicals that Promote the Interactions Between Rice Plant and Rice Major Pest *Nilaparvata lugens*

Zhenfei Zhang[a,d,§], Yong Liu[b,d,§], Vincent Portaluri[c,d], Christine Woodcock[d], John A. Pickett[d,e], Senshan Wang[f], Jing-Jiang Zhou[d,f,g,*]

[a] Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, 510640, Guangzhou, China

[b] Plant Protection College, Shandong Agricultural University, 271018, Taian, China

[c] Eurofins Analytics France, Rue Pierre Adolphe Bobierre, 44323 Nantes, France

[d] Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, AL5 2JQ, UK

[e] School of Chemistry, Cardiff University, Cardiff, CF10 3AT, UK

[f] Biocontrol Engineering Laboratory of Crop Diseases and Pests of Gansu Province, College of Plant Protection, Gansu Agricultural University, Lanzhou, China

[g] State Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Huaxi District, Guiyang, 550025, China

[§] These authors contribute equally to the work.

*To whom correspondence may be addressed. E-mail: jjzhou@gsau.edu.cn

ABSTRACT

The interaction between food crops and insect pests is mediated by semiochemicals emitted from host plants. These semiochemicals are natural behavioural modifiers and act on insect olfactory system to locate hosts and preys. In this study, eight rice neuroactive semiochemicals were identified from rice varieties by GC-EAG and GC-MS. Their ability to modify rice pest behaviours was further studied as individual chemicals and physiologically relevant blend. The total amount of each semiochemical and the expression of their biosynthesis genes were significantly higher in pest susceptible variety than in pest resistant variety, and up-regulated by the infestation of the pest *Nilaparvata lugens* (BPH). The semiochemicals emitted by un-infested plants (UIRVs) were more attractive to BPHs. Interestingly, the attractiveness of UIRVs was significantly reduced by the addition of the blend that mimics the natural composition of these semiochemicals emitted by infested plants (IRVs). Our study suggests a mechanism for the spread of pest-infestation from infested plants to un-infested plants nearby. UIRVs initially serve as attractive signals to rice insect pests. The pest-infestation changes the rice semiochemical profile to be less attractive or even repellent, which pushes further colonization to un-infested plants nearby. The identified semiochemicals can be used for crop protection based on a push-pull strategy.

KEYWORDS: *semiochemical, terpenes, pest colonization, push-pull, Nilaparvata lugens, brown planthopper, rice, pest resistance.*

INTRODUCTION

Despite the importance of semiochemical compounds emitted by un-infested plants for insect pests in the initial host localisation, the research on plant semiochemicals in chemical

ecology and plant defence has mainly focused on semiochemicals from herbivore-induced plants either to repel insect pests or to attract their natural enemies as defence against the pests.[1-4] These semiochemicals have also been implicated in signalling between plants and other organisms. [5-10] They are also used as chemical cues by parasitoids and predators of plant-feeding insects in locating prey. [5, 11-14] They mainly comprise of terpenoids, fatty acid derivatives, phenylpropanoids and benzenoids [15,16] and are emitted after pest infestation either at the site of damage or systemically from undamaged parts of affected plants.[10]

The rice brown planthopper *Nilaparvata lugens* Stål (BPH) (Hemiptera: Delphacidae) is the most destructive pest of rice plants, resulting in a substantial loss in yield annually.[17] It also transmits both rice grassy stunt viruses (RGSV) and rice ragged stunt viruses (RRSV).[18] Previous studies have shown that rice semiochemicals play an important role in host plant location for BPH [6] and in prey location for the natural enemies of the rice insect pests.[19,20,21] The studies by Lou and his co-workers focused on the attractiveness of rice semiochemicals to the natural enemies of rice pests such as the egg parasitoid *Anagrus nilaparvatae* [22,24] and the light green mirid bug *Cyrtorhinus lividipennis* [25] and found that the attractiveness to these insects was significantly increased when rice stems were infested by herbivores. However, these studies did not further analysis of chemical identity of the bioactive components.

The behavioural response of BPH to rice semiochemicals induced by the caterpillars of the tobacco cutworm *Spodoptera litura* was studied.[6] In this study, sixteen components were reported in the headspace volatiles from rice seedlings and four of these compounds, methyl salicylate, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol and 2-heptanol had significantly repellent effects to adult BPHs. Surprisingly, the chemical identities of bioactive rice semiochemicals emitted from BPH-infested rice plants and the effects of BPH-induced semiochemicals on BPH behaviours have rarely been reported. Furthermore, although the semiochemicals from un-infested rice plants act as the initial signals in attracting the rice pests, remarkably, very few

studies have investigated their chemical identities and the emission profiles before and after BPH-infestation.

Meanwhile, many elite resistant rice varieties have been developed and their mechanisms of resistance against BPHs reported. However, little is known about the biologically active components and chemical identity of the semiochemicals from these resistant rice varieties, nor the relationships between the known mechanisms of resistance (see Discussion) and the semiochemical production in these resistant rice plants. No comparative study on the semiochemical production before and after BPH-infestations has been made between susceptible and resistant rice varieties.

Here, we hypothesize that there are differences in semiochemical profiles between un-infested and BPH-infested rice plants in both susceptible and resistant rice varieties. Thus, infestation by BPH alters the semiochemical profiles and thereby could influence attractiveness of rice plants to BPH. We use analytical chemistry, antennal electrophysiology and behavioural assays to identify semiochemical bioactive components and to quantify the emission profile of the semiochemicals before and after BPH infestation in the susceptible (TN1) and resistant rice (IR64) varieties. We report for the first time the chemical identities of bioactive components of the semiochemicals emitted from BPH-infested rice plants and demonstrate that the semiochemicals from un-infested rice plants become less attractive when they are mixed with the blend of electrophysiologically active semiochemical components from infested plants.

MATERIALS AND METHODS

Insect culture. The rice brown planthopper *Nilaparvata lugens* (BPH) was obtained from Bayer and maintained at the Insectary of Rothamsted Research, Harpenden, Hertfordshire, UK in a controlled environment room with a constant temperature of 27°C and relative

humidity of 65% under a photoperiod of 16 h light: 8 h dark. The insects were reared on the 40-day old susceptible rice variety Koshihikari in a netting container with 80 cm length × 60 cm width × 100 cm height. The rice plants were replaced every two weeks.

Plant materials. Two rice *Oryza sativa spp. Indica* varieties, the BPH susceptible variety Taichuang Native 1 (TN1) and the BPH resistant variety IR64, were used for BPH behaviour bioassays and headspace semiochemical analyses (Table 1). The rice seeds were supplied by Huazhong Agriculture University (HZAU), Wuhan, China and Guangdong Academy of Agricultural Sciences (GAAS), Guangzhou, China, respectively. They were sowed in small garden pots and grown under greenhouse condition (temperature: 24 ± 2 °C; photoperiod 18 L: 6D). Three-week old seedlings were used for the semiochemical collections by air entrainment.

Headspace collection of rice semiochemicals. The semiochemical compounds of two rice varieties (TN1 and IR64) were collected by air entrainment [26,27] from un-infested and infested rice plants with 200 BPHs. A total of 12 rice semiochemical collections (2 varieties × 2 treatments × 3 replicates) was obtained. All apparatus, including the air entrainment equipment, was scrupulously cleaned, and all glassware was heated at 200°C-230°C overnight before use. Solvents were rigorously purified by re-distillation. The Porapak Q (50-80 mesh) was used to trap semiochemicals in air entrainment experiments (Beale *et al*., 2006; Du *et al*., 1998) after it was washed with redistilled diethyl ether and conditioned by heating overnight in a stream of nitrogen at 180°C. The air entrainment experiments were accomplished in the Insectary because of the use of BPH. Plastic pots containing 3-week old rice plants, either un-infested or infested with 200 BPHs, were put separately into bell jars (20 litres) that were sealed with Teflon tape and connected to air flows. Air was purified by drawing through a molecular sieve (5 Å) and activated charcoal traps before entering the glass bell jars by the inlet pump. The inlet air flow was around 2 L/min and each outlet flow

was around 0.8 L/min. One air entrainment kit comprises one inlet pump and two outlet pumps. This allows to set up the air entrainment for both infested and un-infested plants side by side. Five rice seedlings per glass jar were used for each treatment and entrained for 120 hours. Then, the Porapak tubes were disconnected from the air entrainment kits. The samples were eluted from the Porapak into 2 mL glass vials with 750 µL (3 times of 250 µL) of redistilled diethyl ether, then concentrated with N_2 flux to about 100 µL of sample and stored at -20^{o}C in a refrigerator for GC or GC-MS analysis.

Gas Chromatography-Coupled Electroantennographic Detection (GC-EAG). The response of BPH antennae to rice semiochemicals of infested TN1 plants was studied by GC-EAG. The GC analyses were carried out using the Hewlett-Packard 6890 of Agilent Technologies gas chromatograph (GC) equipped with a fused silica capillary column HP-1 (30 m × 0.2 mm) coated with Innowax (0.25 µm film thickness) (Agilent Technologies Inc., Santa Clara, CA, USA). For each run, a 2 µL sample was injected in splitless mode. Hydrogen was used as mobile phase at a linear velocity of 40 cm/sec. The oven temperature was programmed from 30°C (1 min hold), 5°C per min to 150°C (0.1 min hold), then 10°C per min to 230°C (22 min hold). Compounds eluting from the GC column were split into two at 1:1 ratio in a four-way splitter, with nitrogen as make-up gas (20 mL/min) and delivered spontaneously to the GC flame ionisation detector (FID) and the antenna respectively. The compounds were carried to the antenna through a glass tube by a charcoal-filtered and humidified air stream at 0.5 m/sec. Antenna was excised from a female BPH with fine forceps and mounted in an antenna holder (Syntech Inn., Germany) in a recording chamber. The signal was recorded with an electrode, amplified and analysed with GC-EAG software (UN-03b, Syntech, Hilversum, Netherlands). The EAG responses to the FID peaks were defined as repeatable deflections from the baseline of ten antennae.

149 **Y-tube Olfactometer Setup.** The behavioural bioassays of BPH to the collected
150 semiochemicals were conducted using a small glass Y-shape tube olfactometer (1 cm in
151 diameter, 7 cm length of the arms and 8 cm length of the stem) with a 50^{o} inside angle
152 between two arms. Incoming air was filtered through activated charcoal and humidified with
153 doubly distilled, deionized water, and split to the two arms of the olfactometer. The Y-tube
154 setup was surrounded by a 50 × 70 × 60 cm black fabric enclosure, and the holding chambers
155 containing the treatments were placed outside the enclosure to eliminate visual cues for
156 insects. In the single-choice bioassays, one chamber served as a control (diethyl ether) and
157 another chamber held the test materials (i.e., either one of the 12 semiochemical collections
158 without dilution or a pure chemical or a mixture of rice headspace semiochemicals and pure
159 chemicals). In the double-choice bioassays, two chambers held the different test materials
160 and the behaviours of BPHs were measured against each other. The airflow through the
161 system was maintained at 200 mL/min. A 60-cm long, wide-spectrum fluorescent lamp
162 (flickering rate: 26000 Hz) was positioned 40 cm above the arms of the olfactometer. Before
163 each trial, light intensity over each arm was measured with a light meter, and the tube was
164 adjusted until the intensity was the same in both arms.
165 **Behavioural Bioassays**. Approximately 1 h before behavioural trials, a one-day-old
166 female adult was placed inside a 2 mL plastic holding tube. The tubes containing insects were
167 then placed into a separate holding container, so the adults were not exposed to testing
168 semiochemicals and starved for 2 h before trials. For each trial, 1.25 µL of either one of the
169 12 semiochemical samples without dilution or a pure chemical or a mixture of EAG bioactive
170 semiochemicals was applied onto a small filter paper. They were then placed in the testing
171 chamber. At the beginning of each trial, the insect was released from the holding tube at the
172 downwind end of the Y-tube. Each insect was given 5 min to respond to the treatment, and
173 the first choice that the insect made for the left or right arm of the olfactometer was recorded.

The response was regarded as valid only if the insect went 1 cm into the arms across the Y junction. The following measurements were recorded for all individuals: the number of individuals which selected an arm of the Y-tube, the number of individuals that did not make any choice and the time stayed in an arm of the Y-tube. Temperature and relative humidity in the olfactometer were maintained at 27.0 ± 1 °C and 80 ± 3 %, respectively. Each individual insect was tested only once, and a clean Y-tube was used each time. Trials were replicated until a minimum of 20 individuals had responded for each treatment. The number of individuals selected an arm of the Y-tube between the different treatments were analysed with a Chi-square goodness of fit test. The time stayed in an arm of the Y-tube between treatments was compared by unpaired independent t-test.

Chemicals. Methyl benzoate, 2-nonanone, (*R,S*)-linalool, (*R*)-linalool, veratrole, methyl salicylate, β-ionone were purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO, USA). (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) were synthesized at Rothamsted Research.[28] All compounds were >=99% pure and dissolved with redistilled hexane for GC analysis.

Gas chromatography (GC) analysis. The rice semiochemicals were separated by a Hewlett-Packard 6890 gas chromatograph with hydrogen as the carrier gas through a cool-on-column injector of a 50 m × 0.32 mm ID methyl silicone-boned phase fused silica capillary column (HP-1) and detected with a flame ionisation detector (FID). The oven temperature for the HP-1 column was maintained at 40°C for 5 min and then programmed to increase at 5°C/min to 150°C, then at 10°C /min to 250°C. A total of 4 µL of each semiochemical sample was injected and analysed. The co-injection technique with authentic standards was used for quantitative characterization of bioactive compounds in the semiochemical collections.

198 **Gas Chromatography-Mass Spectrometry (GC-MS)**. A capillary column (50 m x 0.32
199 mm ID HP-1) fitted in the Hewlett Packard 6890 gas chromatograph was directly coupled to
200 the mass spectrometer and integrated data system (70- 250 VG Analytical and VG Autospec,
201 Fisons Instruments). Ionization was by electron impact at 70 eV and 230°C. The gas
202 chromatograph was maintained at 30°C for 5 min and then programmed to increase at
203 5°C/min to 180°C. Tentative identifications of each EAG-active chemicals by GC-MS were
204 confirmed by using Kovats Indices (KI) coupled with co-injection and peak enhancement
205 with authentic standards on two GC columns of different polarity.[29]
206 Enantiomeric determination of linalool in the rice headspace semiochemicals from
207 infested rice variety TN1 plants was achieved by GC using a chiral column. Briefly, chiral
208 separations were performed on the Hewlett Packard 6890 gas chromatograph equipped with
209 an alkylated ß-cyclodextrin (Restek, Bellefonte, PA; Rt-ßDEXsm) fused silica capillary
210 column (30 m × 0.25 mm i. d.; 0.25 µm film thickness). Injector and detector temperatures
211 were 230°C. Initial temperature was kept at 40°C for 5 min, and then programmed to increase
212 at 5°C/min to 150°C, then at 10°C/min to 250°C. Helium was used as the carrier gas at a
213 flow rate of 1 mL/min. A split ratio of 1:50 was used. Masses between 45 and 450 *m/z* were
214 recorded. The separated peaks were compared with those of enantiomerically authentic
215 standards and identified by using KIs and peak enhancement with authentic standards.
216 The quantification of the eight EAG active compounds before and after BPH infestation
217 allowed the analysis of the difference in their natural composition in the semiochemicals
218 from infested TN1 plants (IRV). Based on GC-MS analysis with authentic standard of each
219 compound (Figure S2 and S5), the difference in the natural composition of each compound
220 in the concentrated samples before and after BPH infestation was determined as 1.8 ng/µL
221 (0.9%) for methyl benzoate, 19.4 ng/µL (9.5%) for 2-nonanone, 161.2 ng/µL (78.8%) for
222 linalool, 2.3 ng/µL (1.1%) for DMNT, 0.2 ng/µL (0.1%) for veratrole, 17.2 ng/µL (8.4%) for

methyl salicylate, 2.1 ng/µL (1.0%) for β-ionone, 0.5 ng/µL (0.2%) for TMTT. These were added to UIRV to make UIRV+blend that mimics the natural composition of the eight EAG active compounds after BPH infestation and used in the behavioural experiments.

Total RNA isolation and cDNA synthesis. Frozen samples of the rice plants were ground to fine powder in liquid nitrogen with a pestle and mortar. The total RNA was extracted from 100 mg of each macerate plant tissue using the RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop^{TM} Spectrophotometer ND-1000 (Thermo Scientific), and the integrity of RNA was assessed by 1% agarose gel electrophoresis and ethidium bromide staining. The absence of contaminant DNA in the RNA samples was verified by PCR using primers spanning two exons and gel electrophoresis analysis. The absence of spurious product of amplification caused by genomic DNA was also continuously checked by the verification of RT-qPCR dissociation profile. Both tests showed that the RNeasy kit efficiently removed contaminant DNA from the RNA samples. cDNAs were synthesized by adding 50 µM of Oligo (dT 18) primer and 10 mM of each deoxyribonucleoside 5'-triphosphate (dNTPs) to 1 µg of total RNA. The mixture was incubated at 65°C for five minutes, and briefly chilled on ice more than 1 minute. First Strand Buffer, 20 mM of dithiothreitol (DTT) and 200 units of Superscript Ⅲ (Invitrogen) were then added to the prior mixture to a total reaction volume of 20 µL and incubated at 50°C for 50 min following manufacturer's instructions. Inactivation of the reverse transcriptase was done by incubating the mixture at 85°C for 5 min and the cDNA solution was stored at -20°C.

Real-time quantitative polymerase chain reaction (RT-qPCR). Above cDNA samples were used in RT-qPCR using a SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, MO, USA) on an Applied Biosystems QuantStudio 3&5 Real-Time PCR System (Thermo Fisher Scientific, MA, USA). Samples were run in the technical triplicates on the qPCR system with

following protocol: 1 activation cycle of 5 min at 95°C; 40 amplification cycles of 30 s at 95°C, 30 s at 62°C and 30 s at 72°C; 1 melting curve cycle measuring from 65°C to 95°C. Fluorescence values were exported from the QuantStudio 3&5 Real-Time PCR program whereupon Ct values, normalization factors and primer efficiencies were calculated using *Oryza sativa* Japonica Group 18S ribosomal RNA (*Os18S*) gene as reference gene. The *Os18S* primers used in this study were 5'-GTTTGATGAGCCTGCGTAGTATT-3' (Forward) and 5'-GCTGCTGGCACGGAGTTAG-3' (Reverse). For the expression profiling of the biosynthesis genes for *S*-linalool and methyl salicylate in TN1 and IR64 rice plants, the expression of the *S*-linalool synthase gene (*LIS*), the salicylic acid carboxyl methyltransferase gene (*SAMT*), and the methyl salicylate esterase gene (*SABP2*) was determined using RT-qPCR before and after BPH infestation. The primers of the biosynthesis genes are listed in Table S1.

RESULTS

Rice semiochemicals from TN1 rice plants are attractive to BPHs. The headspace semiochemicals emitted by the susceptible variety TN1 before and after 5-day BPH-infestation were collected, named as UIRV for un-infested rice semiochemicals and IRV for infested rice semiochemicals. BPHs were significantly attracted to the UIRVs (χ^2 = 4.263, *P* = 0.039) and rested more time in the arms treated with the UIRVs (top bars in Figure 1A and 1B) in the single choice bioassays. This attraction was not significant when BPHs were tested behaviourally with the IRVs (middle bars in Figure 1A and 1B). In the double choice bioassays, BPHs were attracted significantly to the UIRV-treated arm over the IRV-treated arm (χ^2=3.857, *P*=0.050) (bottom bars in Figure 1A and 1B).

Eight rice semiochemicals are neuroactive to BPH female antennae. The headspace volatiles of BPH-infested TN1 plants (IRVs) consistently elicited GC-EAG responses on the

female antennae of BPHs (Figure 2). Eight compounds were identified by GC-MS and peak enhancement (Figure S1). These bioactive semiochemicals include methyl benzoate, 2-nonanone, linalool, DMNT, veratrole, methyl salicylate, β-ionone, and TMTT (Figure S2). There were two enantiomers *R*-linalool and *S*-linalool presented in the rice headspace semiochemicals and emitted at the ratio of 2.3:1 in IRVs after BPH infestation (Figure S3).

Eight EAG active semiochemicals contribute differently to the behaviour of BPH. The behavioural responses of BPHs to individual EAG active compounds as well as the 1:1 molar ratio mixture were then tested for their contribution to the BPH behaviours in single choice bioassays using a Y-tube olfactometer against the solvent diethyl ether (DE). The compounds methyl salicylate, β-ionone, TMTT and the 1:1 molar mixture repelled significantly BPHs (Figure 3). In contrast, 2-nonanone and veratrole attracted only slightly more BPHs than DE control but not statistically significantly. Methyl benzoate, linalool and DMNT had no effect on the behaviour of BPHs, i.e., similar number of BPHs was found responding to the semiochemicals and the control (Figure 3).

Interestingly, the addition of the blend into UIRV to mimic the natural composition of the eight EAG active compounds from IRV reduced significantly the attractiveness of the UIRV ($\chi^2 = 4.167$, $P = 0.041$) (Figure 4A) and BPHs spent much less time in the arms treated with UIRV plus the natural blend compared with the arms treated with the UIRV alone (Figure 4B).

The response of BPH is different to the semiochemicals from susceptible and resistant rice varieties. The behavioural study was further repeated with the semiochemicals from the BPH resistant rice variety IR64 to evaluate the ecological potentials of the EAG active semiochemicals. Unlike TN1 plant where the UIRVs attracted more BPHs than the solvent control diethyl ether (DE) (Figure 1A) and BPHs spent significantly more time in UIRV-treated arm (Figure S4A), the IRVs from the resistant variety IR64 attracted more BPHs than

298 the controls (Figure 5). However, there was no difference in the time that BPHs spent in the
299 IRV-treated arm and in the DE-treated arm (Figure S4A). For both varieties, there was no
300 difference in the time that BPHs spent between both control and IRV-treated arms (Figure
301 S4B). Furthermore, in double choice bioassays, there was no significant effect between
302 UIRV and IRV of IR64 plants on the BPH behaviours (Figure S4C). However, the UIRV
303 from TN1 plants was more attractive than IRV (Figure 1 and Figure 5C) and BPHs spent
304 significantly more time in the UIRV-treated arm (Figure S4C).

305 **Quantification and relative amount of the EAG active compounds in rice varieties**.
306 The emissions of the EAG active compounds in the susceptible variety TN1 and in the BPH
307 resistant rice variety IR64 were then qualified using Kovats Indices (KI) coupled with co-
308 injection technique. For both varieties, three compounds (2-nonanone, linalool and methyl
309 salicylate) were the main semiochemical compounds (collectively 80~95% of total EAG
310 active compounds) before and after the 5-day BPH-infestation (Figure 6).

311 The TN1 plants emitted significantly higher amount of the EAG-active compounds than
312 the IR64 plants (Figure 6 and Figure S5). The semiochemical emissions were strongly
313 induced by the 5-day BPH-infestation (Figure 6). The emissions from same weight of the
314 susceptible rice variety TN1 plant material were induced from 4.5 ng/mL to 24.0 ng/mL for
315 2-nonanone, from 24.5 ng/mL to 185.7 ng/mL for linalool, from 0.6 ng/mL to 2.9 ng/mL for
316 DMNT and from 2.4 ng/mL to 19.6 ng/mL for methyl salicylate. The emission of linalool
317 was increased by 7.6 folds in the susceptible variety TN1 and 2.5 folds in the resistant variety
318 IR64 after BPH-infestation (Figure 6 and Figure S5). Although the susceptible variety TN1
319 emitted much higher amount of the EAG active compounds than the resistant variety IR64
320 (Figure 6), the relative percentage change of each active compounds in total headspace
321 semiochemical collection before and after BPH-infestation was smaller than those of the
322 resistant variety IR64 (Figure S6).

Two semiochemicals, linalool and methyl salicylate, were detected as the main semiochemical compounds and emitted differentially between two varieties. The expression of these biosynthesis genes was highly upregulated by BPH-infestation, particularly in the susceptible variety TN1, of which the *OsLIS* expression was increased by 266.1 folds, while 15-fold upregulation by the infestation was obtained in the resistant variety IR64. The *OsSAMT* and *OsSABP2* expressions were increased by 8.4 and 6.0 folds, respectively in TN1 variety and <2 folds in IR64 variety (Figure 7A).

The expressions of *S*-linalool synthase (*OsLIS*) (OS02g02930.1), *OsSAMT* (Os11g15040.1) and *OsSABP2* (Os01g37650.1) were significantly lower in the resistant variety IR64 than their expressions in the susceptible variety TN1 by the fold changes of 5.3, 11.3 and 1.5 before the infestation and 94.1, 95.6 and 8.6 after the infestation (Figure 7B).

In summary, in order to identify bioactive semiochemical components associated with damage by BPH, the electrophysiological responses of BPH female antennae to the headspace semiochemicals of the BPH-infested susceptible rice variety TN1 were measured by GC-EAG. The EAG active compounds were identified and quantified for the first time as methyl benzoate, 2-nonanone, linalool, DMNT, veratrole, methyl salicylate, β-ionone, and TMTT (Figure 2). These compounds were also present in the resistant variety IR64 (Figure 6) and other resistant varieties (data not shown). Three compounds (2-nonanone, linalool and methyl salicylate) were the main components with the highest emission among eight EAG active compounds, and their emissions were increased significantly after BPH-infestation in both TN1 and IR64 varieties (Figure 6).

DISCUSSION

In insect-plant systems, there is mounting evidence that plants change their semiochemical profiles during infestation and become more attractive to natural enemies of

pests, yet the chemical identities and the roles of semiochemical bioactive components from infested rice plants to the behaviours of the major insect pest (BPH) of the stable food crop rice, particularly in the context of pest resistant rice varieties, have been largely overlooked. This study provides evidence to support previous suggestions [19,20,22,30-32] that the semiochemicals from rice plants could serve as chemical fingerprint for BPH-infestation and play an important biological function in mediating the interaction between insect pest BPH.[33,34] These compounds could serve as biomarkers of pest infestation or be used as behavioural modifiers to enhance the efficacy of chemical lures or repellents to trap or repel insect pests and in insect pest management.

Linalool was emitted in the largest amount among the eight EAG active compounds and elicited a strong EAG response (Figure 1) but had no effect on the behaviour of BPH in the Y-tube bioassay (Figure 3). It was reported that *S*-linalool from a different rice variety had a repellent effect on BPH behaviours.[20,30,32] It was also found that *R*-linalool had a repellent effect to aphids at un-naturally high concentrations.[35,36] A further analysis confirmed that the emitted linalool from TN1 variety was a mixture of *R*-linalool and *S*-linalool at a 2.3:1 ratio (Figure S3), this may explain the none-responsiveness of BPHs to linalool in this study.

Furthermore, the BPH behavioural responses to these semiochemicals were determined as individual chemicals and an equal molar mixture (Figure 3) as well as the blend (Figure 4). The results are in agreement with previous studies where the rice semiochemicals induced by BPH [3] and the tobacco cutworm *Spodoptera litura* [6] had a stronger repellent effect on BPH female adults compared to the semiochemicals of un-infested plants. This finding is consistent with a role of rice semiochemicals in mediating pest behaviour as an important signal in plant indirect defence against insect pests [3,19,37-39].

Although it has not been exhaustively studied, the behavioural results demonstrated a strong attraction of BPHs to the headspace semiochemicals of the un-infested susceptible

373 rice variety TN1 plants (UIRV) (Figure 1). The attractiveness of UIRV was dramatically
374 reduced by the addition of the blend of infested plants (Figure 4). Thus, the UIRV of the
375 susceptible rice variety TN1 which initially was highly attractive to BPH become repellent
376 when the blend of the eight EAG active compounds from the infested rice plants was added.
377 This could be one of factors in rice field to naturally push BPHs away to further colonise
378 nearby un-infested rice plants. Previous study suggested that the changes of the proportions
379 among the compounds in the blend after BPH infestation provide specific information on
380 host habitat quality for parasitoid wasps.[19]
381 There seems a clear relationship between the pest tolerance ability of the rice varieties
382 and the emission of the EAG active compounds, i.e., the stronger pest tolerance ability the
383 rice varieties have (IR64 > TN1), the less semiochemicals they emit (Figure 6). There is a
384 considerable variation in semiochemical profiles between TN1 and IR64 rice varieties in
385 terms of BPH behaviours (Figure 5), semiochemical emission ability (Figure 6) and
386 expression levels of biosynthesis genes for these semiochemicals (Figure 7). Interestedly, the
387 IRVs from susceptible rice variety TN1 (repellent) and resistant rice variety IR64 (attractive)
388 even triggered opposite behavioural responses. The expressions of *LIS*, *SAMT* and *SABP2*
389 were lower by fold changes of -5.3±0.5, -11.3±2.4 and -1.5±0.2 in IR64 variety relative to
390 those of the susceptible variety TN1. The resistance mechanism of IR64 resistant rice variety
391 through the resistant gene *bph3* seems unrelated to the semiochemical productions and the
392 expression of biosynthesis genes, and also does not correlate to the behavioural response of
393 BPHs. Thus, the emission rates of semiochemicals from resistant rice varieties appear not to
394 provide the resistant mechanisms against the rice pest BPH. Plant tolerance to insects by
395 breeding is so far a major target for breeding resistant varieties against herbivore infestation.
396 However, the ability of the resistant rice varieties to regulate semiochemical emission against
397 BPH (indirect defence) might have been reduced by the breeding programs for their direct

defence mechanisms. This study opens up an opportunity to further improve the defence of elite resistant rice varieties by enhancing the emission of biologically active semiochemicals.

In summary, our study reports for the first time that electrophysiologically active rice semiochemicals from the infested susceptible variety TN1 plants could reduce the attractiveness of un-infested rice plants. The stronger upregulation of the biosynthesis genes for methyl salicylate and *S*-linalool in the susceptible variety TN1 compared to the resistant varieties (Figure 7) [40], and the reduced attractive effect of UIRVs by the semiochemical blend of pest infested plants (Figure 4), support the view that semiochemicals of un-infested rice plants may only serve as initial attractive signals for rice pests. These same semiochemicals caused repellence to BPHs when the rice plants are infested by BPH, which may lead to the spread of the insects to un-infested plants nearby. The higher semiochemical emission and the stronger upregulation of semiochemical biosynthesis genes in the susceptible variety TN1 may serve to offset its susceptibility to rice pests. Such indirect defence by plant semiochemicals against insect pests cannot serve as a direct defence mechanism against insect pests but could be explored further to manipulate insect pest behaviour [6,41-43] and to attract natural enemies [5,11,12,20,44] and predators [25] in integrated pest managements.

ASSOCIATED CONTENT

Supporting Information Available

Table S1. Primers of RT-qPCR used in this study.

Figure S1. Confirmation of tentative identification of eight EAG-active rice volatiles.

Figure S2. GC- FID trace of the headspace volatiles of infested rice variety TN1.

Figure S3. GC analysis of authentic linalool.

Figure S4. Time spent by BPH females in the Y-tube arms.

Figure S5. Chemical fingerprint of EAG-active compounds in different rice varieties.

Figure S6. Relative amount as percentage of the EAG active compounds in rice plants.

AUTHOR INFORMATION

Corresponding Autor

Jing-Jiang Zhou - State Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Huaxi District, Guiyang, 550025, China;

orcid.org/0000-0002-8203-601X;

Email: jjzhou@gsau.edu.cn

Authors

Zhenfei Zhang - *Plant Protection Research Institute, Guangdong Academy of Agricultural Sciences, 510640, Guangzhou, China*

Yong Liu - *Plant Protection College, Shandong Agricultural University, 271018, Taian, China*

Vincent Portaluri - *Eurofins Analytics France, Rue Pierre Adolphe Bobierre, 44323 Nantes, France*

Christine Woodcock - *Department of Biointeractions and Crop Protection, Rothamsted Research, Harpenden, AL5 2JQ, UK*

John A. Pickett - *School of Chemistry, Cardiff University, Cardiff, CF10 3AT, UK*

Senshan Wang - *Biocontrol Engineering Laboratory of Crop Diseases and Pests of Gansu Province, College of Plant Protection, Gansu Agricultural University, Lanzhou, China*

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Notes

The authors declare no competing financial interest.

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594

595 **Figure legends**

596 **Figure 1.** Choice responses of at least 19 individual one-day-old BPH females in a Y-tube
597 olfactometer between the arm treated with the control solvent diethyl ether (DE) and the arm

598 treated with either the headspace semiochemicals from the un-infested (UIRV) or the
599 headspace semiochemicals from infested (IRV) TN1 plants. (A) shows the number of BPH
600 in the arms of Y-tube and (B) shows the time that BPH spent in the arms of Y-tube. The
601 negative numbers indicate the number of BPHs responded to the arm treated with UIRV,
602 IRV and IRV. The positive numbers indicate the number of BPHs responded to the arm
603 treated with DE, DE and UIRV. The numbers in the white bars indicate the number of insects
604 that did not make any choice between the two arms of the Y-tube olfactometer. The
605 significant difference ($P \leq 0.05$) between two arms was indicated by * and analysed with a
606 Chi-square goodness of fit test in (A) for the numbers of responded insects between two
607 arms and unpaired independent t-test in (B) for the spent time between two arms.
608 **Figure 2**. Simultaneous recording of flame ionization detector (FID) (upper trace) and
609 responses of the female antennae (EAG) of the rice brown planthopper *Nilaparvata lugens*
610 (BPH) (lower trace) to the headspace semiochemicals of 3-week-old susceptible rice variety
611 TN1 infested by BPHs. The compounds were identified as methyl benzoate (1), 2-nonanone
612 (2), (*R*/*S*)-linalool (3:1) (3), (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) (4), veratrole (5),
613 methyl salicylate (6), β-ionone (7), and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
614 (TMTT) (8) by GC-MS and confirmed by using Kovats Indices (KI) coupled with co-
615 injection and peak enhancement with authentic standards (Figure S1 and S2).
616 **Figure 3**. Responses of one-day-old BPH females in a Y-tube olfactometer to the EAG active
617 semiochemical compounds and their 1:1 molar mixture (left dark bars) against the control
618 solvent (diethyl ether) (right light dark bars) over at least 20 individual insects per treatment.
619 Linalool(x) indicates that it is a mixture of *R*-linalool enantiomer and *S*-linalool enantiomer
620 at 2.3:1 ratio (Figure S3). The 1:1 molar mixture is made of eight EAG active semiochemical
621 compounds at equal molar ratio. The numbers in the dark grey bars (negative x-axis) show
622 the number of BPHs responded to individual semiochemical and the 1:1 molar mixture. The

623 numbers in the light grey bars (positive x-axis) show the number of BPHs responded to
624 solvent control (diethyl ether). The numbers in the white bar indicate the number of insects
625 that did not make any choice (no choice) between the semiochemical-treated and solvent-
626 treated arms. The significant difference in the numbers between semiochemical-treated and
627 solvent-treated arms was analysed with a Chi-square goodness of fit test and * indicates $P \leq$
628 0.05 significance.
629 **Figure 4.** Dual choice responses of at least 19 individual one-day-old BPH females in a Y-
630 tube olfactometer between the arm treated with the headspace semiochemicals from un-
631 infested TN1 plants (UIRV) and the arm treated with UIRV plus the semiochemical blend
632 (UIRV+blend). The blend was made of the eight EAG active compounds at the ratio
633 determined as described in Figure 6 and Figure S2 so that the (UIRV+blend) mixture mimics
634 the natural composition of these compounds in the IRV (see detail in the MM). The numbers
635 in the white bars indicate the number of insects that did not make any choice between the
636 two arms of the Y-tube olfactometer. The significant difference ($P \leq 0.05$) between two arms
637 was indicated by * and analysed with a Chi-square goodness of fit test in (A) for the numbers
638 of responded insects between two arms and unpaired independent t-test in (B) for the spent
639 time between two arms of Y-tube.
640 **Figure 5.** The responses of one-day-old female BPHs in a Y-tube olfactometer to the
641 headspace volatiles of the resistant rice variety IR64 in single choice bioassays (**A** and **B**)
642 and dual choice bioassays (**C**) in comparison with those of the susceptible variety TN1 (data
643 from Figure 1). The comparison was made (**A**) between the number of BPHs responded to
644 the control (diethyl ether, DE) (negative x-axis) and the number of BPHs responded to the
645 semiochemicals from un-infested rice plants (UIRV) (positive x-axis), (**B**) between the
646 number of BPHs responded to the control (DE) (negative x-axis) and the number of BPHs
647 responded to the IRV from infested rice plants (negative x-axis), and (**C**) between the number

of BPHs responded to the IRV from infested rice plants (negative x-axis) and the number of BPHs responded to the UIRV from un-infested rice plants (positive x-axis). The numbers in the white bars indicate the numbers of insects that did not make choice between treatments and control (no choice). The significant difference was analysed with a Chi-square goodness of fit test. * indicates a significance at $P < 0.05$ and ** indicates a significance at $P < 0.01$.

Figure 6. Quantification of eight EAG active semiochemicals from un-infested and infested rice varieties TN1 (left panel) and IR64 (right panel). A total of 4 µL of each semiochemical sample was injected and analyzed with a 50 m × 0.32 mm ID methyl silicone-boned phase fused silica capillary column (HP-1). The co-injection technique with authentic standards was used for the quantification of bioactive compounds in each semiochemical collection (as in Figure S1). The concentrations of the semiochemicals between un-infested and infested rice samples were compared with paired t-test and * indicate significant difference between un-infested and infested rice plants at $P < 0.05$.

Figure 7. Relative expression of genes *S*-linalool synthase (*OsLIS*), salicylic acid carboxyl methyltransferase (*OsSAMT*) and methyl salicylate esterase gene (*OsSABP2*) associated with the biosynthesis of *S*-linalool, methyl salicylic acid, and salicylic acid in susceptible rice variety TN1 and resistant rice varieties IR64. The expression levels of the biosynthesis genes in infested and un-infested plants were determined by RT-qPCR and normalised to the expression of the endogenous gene *Os18S*. The expression levels were presented (**A**) as fold changes by BPH infestation and (**B**) as fold changes relative to their expressions in the susceptible rice variety TN1. The negative numbers indicate lower expression in IR64 than in TN1. The gene expressions between un-infested and infested rice samples were compared with paired t-test and ** indicate significant difference between un-infested and infested rice plants at $P < 0.01$.

Table 1. Species, Phenotype and seed source of the rice varieties in this study

Rice varieties	Species	Resistant Phenotype [a]	Name in Text	Source [b]
Koshihikari [c]	*Oryza sativa spp. Japonica*	Susceptible	n.a	*RRes*
Taichuang Native 1	*Oryza sativa ssp. Indica*	Susceptible	TN1	*HZAU*
IR64	*Oryza sativa spp. Indica*	Resistant with *bph3*	IR64	*GAAS*

[a]The resistance level of rice varieties to BPH had been checking by the seeds providers.

[b] HZAU, Huazhong Agricultural University; GAAS, Guangdong Academy of Agricultural Sciences.

[c] This susceptible rice variety was used for rearing the stock culture *N. lugens*.

Figure 1

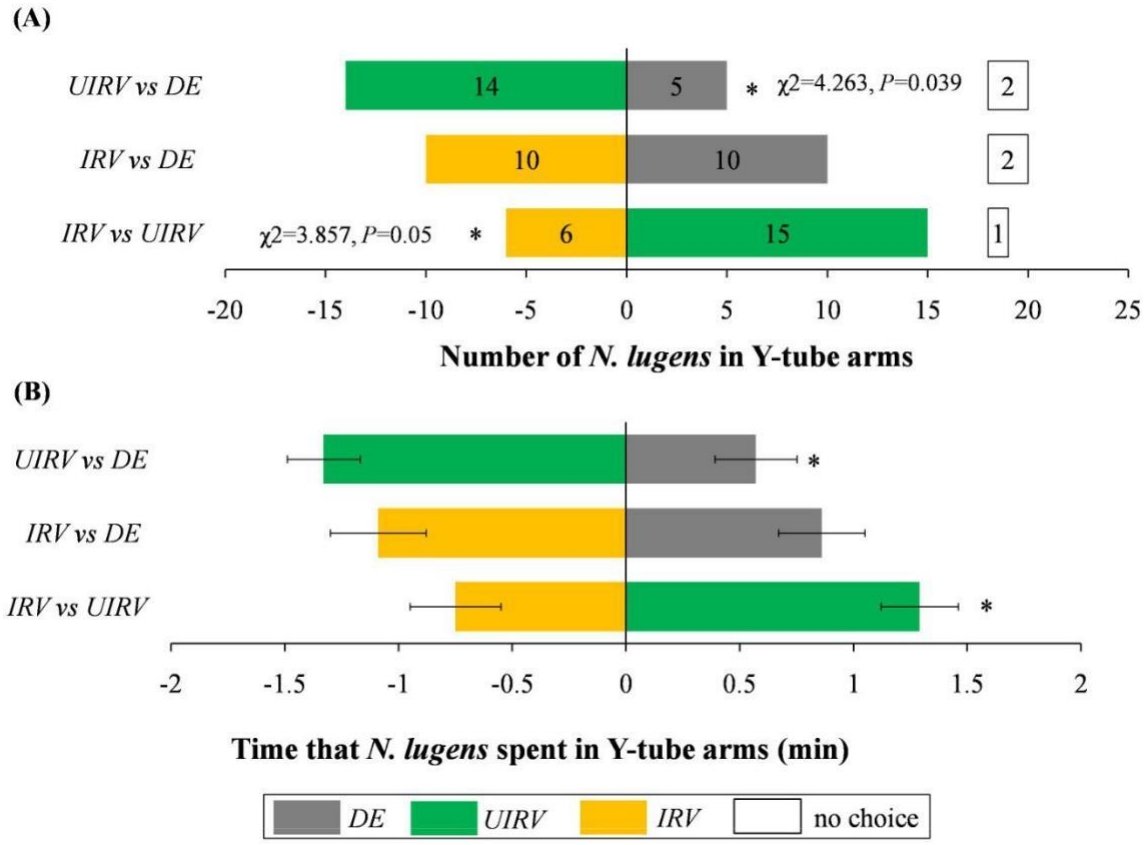


Figure 1. Choice responses of at least 19 individual one-day-old BPH females in a Y-tube olfactometer between the arm treated with the control solvent diethyl ether (DE) and the arm treated with either the headspace volatiles from the un-infested (UIRV) or the headspace volatiles from infested (IRV) TN1 plants. (A) shows the number of BPH in the arms of Y-tube and (B) shows the time that BPH spent in the arms of Y-tube. The negative numbers indicate the number of BPHs responded to the arm treated with UIRV, IRV and IRV. the positive numbers indicate the number of BPHs responded to the arm treated with DE, DE and UIRV. The numbers in the white bars indicate the number of insects that did not make any choice between the two arms of the Y-tube olfactometer. The significant difference ($P <= 0.05$) between two arms was indicated by * and analysed with a Chi-square goodness of fit test in (A) for the numbers of responded insects between two arms and unpaired independent t-test in (B) for the spent time between two arms.

Figure 2

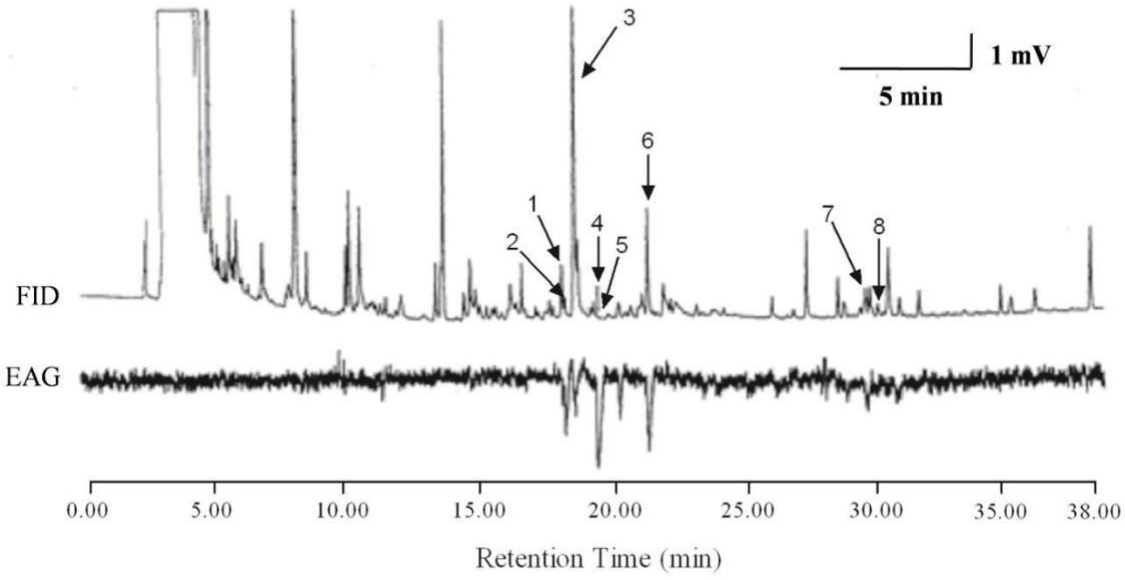


Figure 2. Simultaneous recording of flame ionization detector (FID) (upper trace) and responses of the female antennae (EAG) of the rice brown planthopper *Nilaparvata lugens* (BPH) (lower trace) to the headspace volatiles of 3-week-old susceptible rice variety TN1 infested by BPHs. The compounds were identified as methyl benzoate (1), 2-nonanone (2), (*R*/*S*)-linalool (3:1) (3), (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT) (4), veratrole (5), methyl salicylate (6), β-ionone (7), and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (8) by GC-MS and confirmed by using Kovats Indices (KI) coupled with co-injection and peak enhancement with authentic standards (Figure S1 and S2).

Figure 3

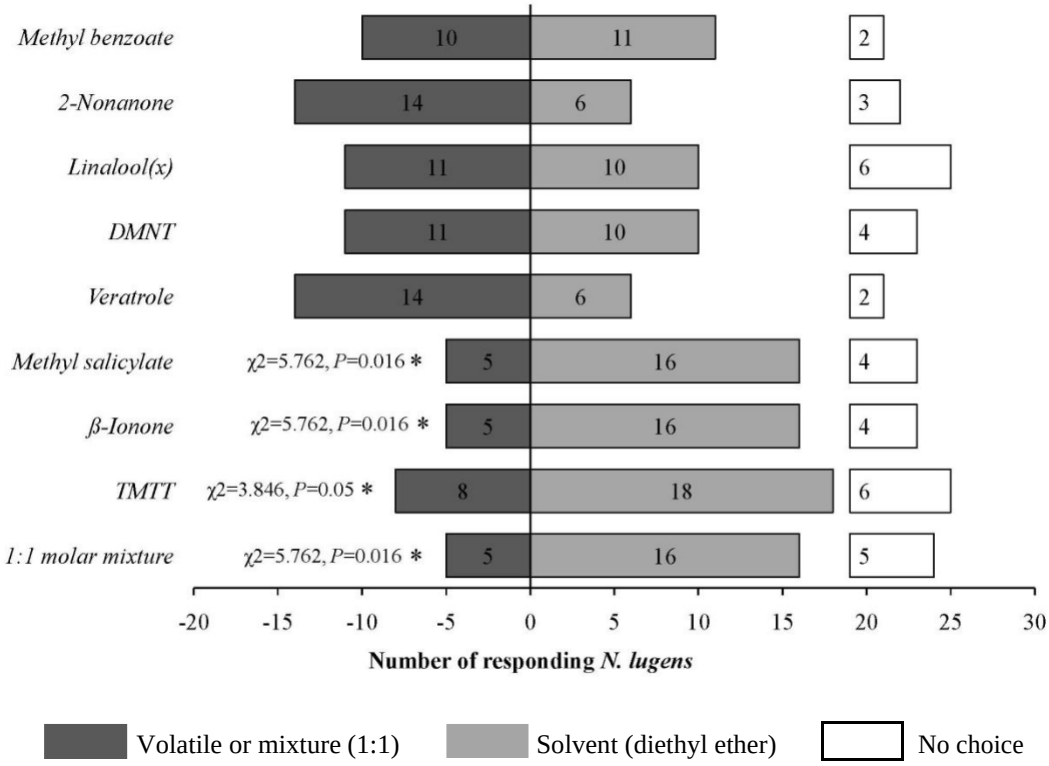


Figure 3. Responses of one-day-old BPH females in a Y-tube olfactometer to the EAG active volatile compounds and their 1:1 molar mixture (left dark bars) against the control solvent (diethyl ether) (right light dark bars) over at least 20 individual insects per treatment. Linalool(x) indicates that it is a mixture of *R*-linalool enantiomer and *S*-linalool enantiomer at 2.3:1 ratio (Figure S3). The 1:1 molar mixture is made of eight EAG active volatile compounds at equal molar ratio. The numbers in the dark grey bars (negative x-axis) show the number of BPHs responded to individual volatile and the 1:1 molar mixture. The numbers in the light grey bars (positive x-axis) show the number of BPHs responded to solvent control (diethyl ether). The numbers in the white bar indicate the number of insects that did not make any choice (no choice) between the volatile-treated and solvent-treated arms. The significant difference in the numbers between volatile-treated and solvent-treated arms was analysed with a Chi-square goodness of fit test and * indicates $P <= 0.05$ significance.

Figure 4

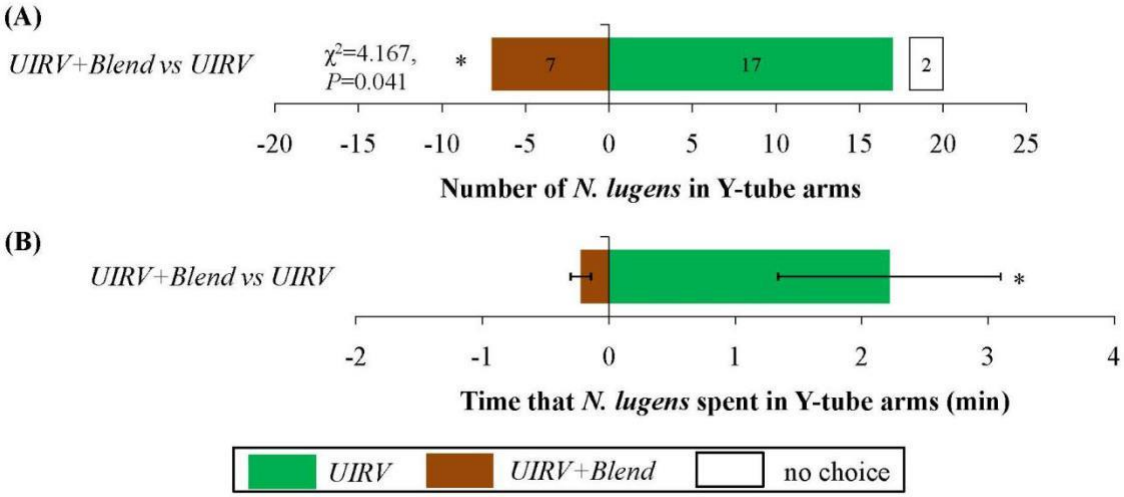


Figure 4. Dual choice responses of at least 19 individual one-day-old BPH females in a Y-tube olfactometer between the arm treated with the headspace volatiles from un-infested TN1 plants (UIRV) and the arm treated with UIRV plus the volatile blend (UIRV+blend). The blend was made of the eight EAG active compounds at the ratio determined as described in Figure 6 and Figure S2 so that the (UIRV+blend) mixture mimics the natural composition of these compounds in the IRV (see detail in the MM). The numbers in the white bars indicate the number of insects that did not make any choice between the two arms of the Y-tube olfactometer. The significant difference ($P <= 0.05$) between two arms was indicated by * and analysed with a Chi-square goodness of fit test in (A) for the numbers of responded insects between two arms and unpaired independent t-test in (B) for the spent time between two arms of Y-tube.

Figure 5

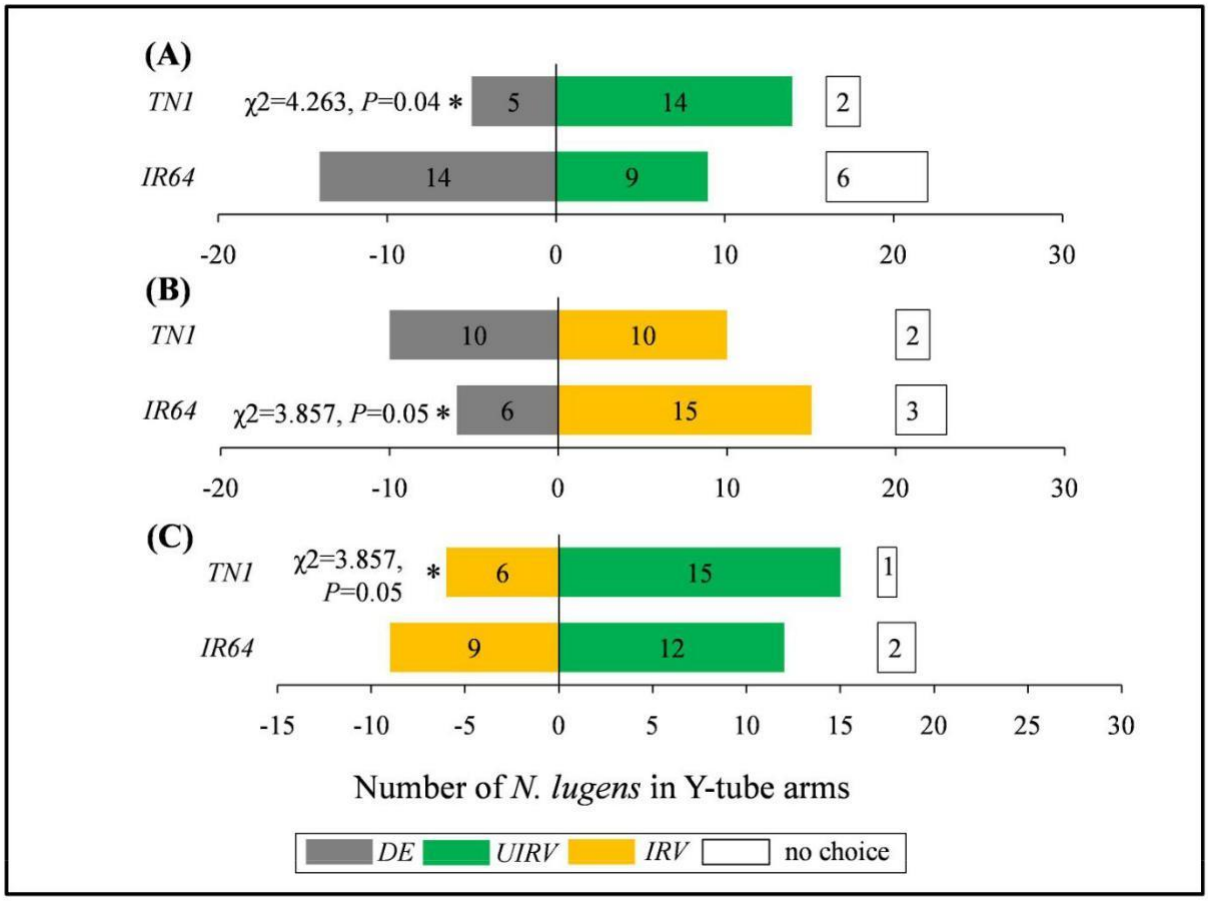


Figure 5. The responses of one-day-old female BPHs in a Y-tube olfactometer to the headspace volatiles of the resistant rice variety IR64 in single choice bioassays (**A** and **B**) and dual choice bioassays (**C**) in comparison with those of the susceptible variety TN1 (data from Figure 1). The comparison was made (**A**) between the number of BPHs responded to the control (diethyl ether, DE) (negative x-axis) and the number of BPHs responded to the volatiles from un-infested rice plants (UIRV) (positive x-axis), (**B**) between the number of BPHs responded to the control (DE) (negative x-axis) and the number of BPHs responded to the IRV from infested rice plants (negative x-axis), and (**C**) between the number of BPHs responded to the IRV from infested rice plants (negative x-axis) and the number of BPHs responded to the UIRV from un-infested rice plants (positive x-axis). The numbers in the white bars indicate the numbers of insects that did not make choice between treatments and control (no choice). The significant difference was analysed with a Chi-square goodness of fit test. * indicates a significance at $P < 0.05$ and ** indicates a significance at $P < 0.01$.

Figure 6

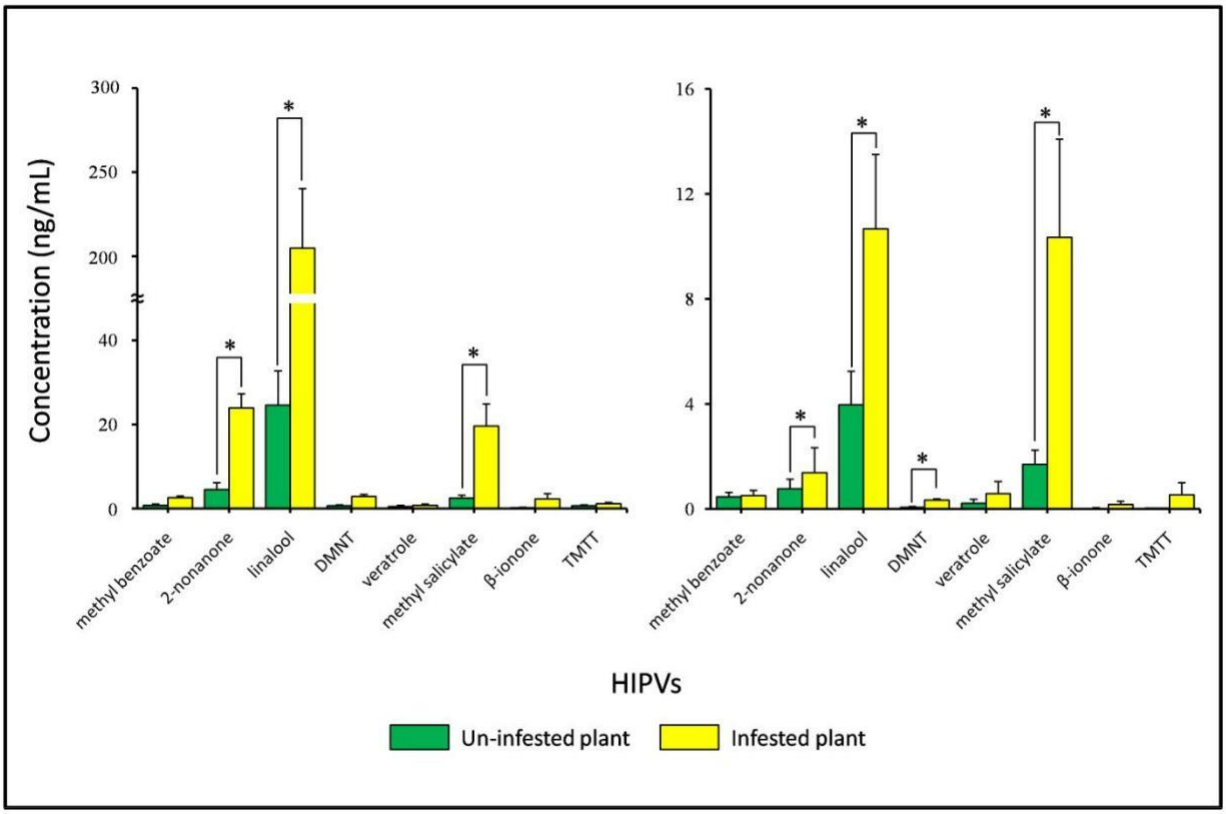


Figure 6. Quantification of eight EAG active volatiles from un-infested and infested rice varieties TN1 (left panel) and IR64 (right panel). A total of 4 µL of each volatile sample was injected and analyzed with a 50 m × 0.32 mm ID methyl silicone-boned phase fused silica capillary column (HP-1). The co-injection technique with authentic standards was used for the quantification of bioactive compounds in each volatile collection (as in Figure S1). The concentrations of the volatiles between un-infested and infested rice samples were compared with paired t-test and * indicate significant difference between un-infested and infested rice plants at $P < 0.05$.

Figure 7

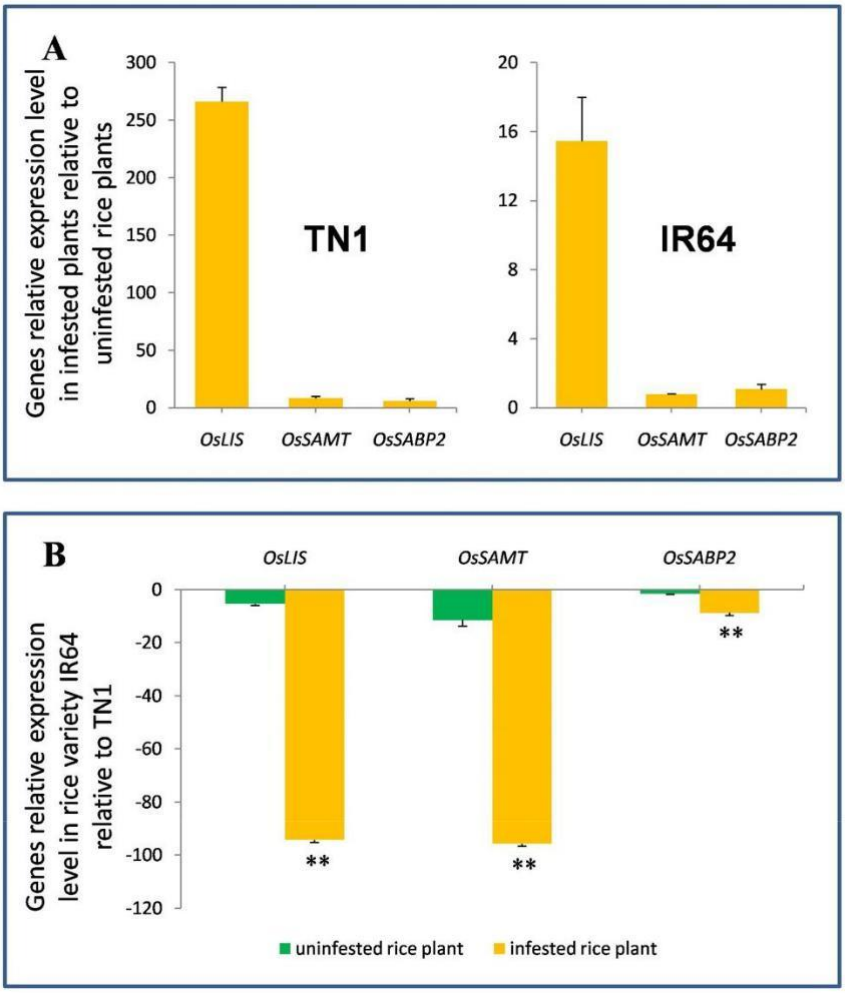
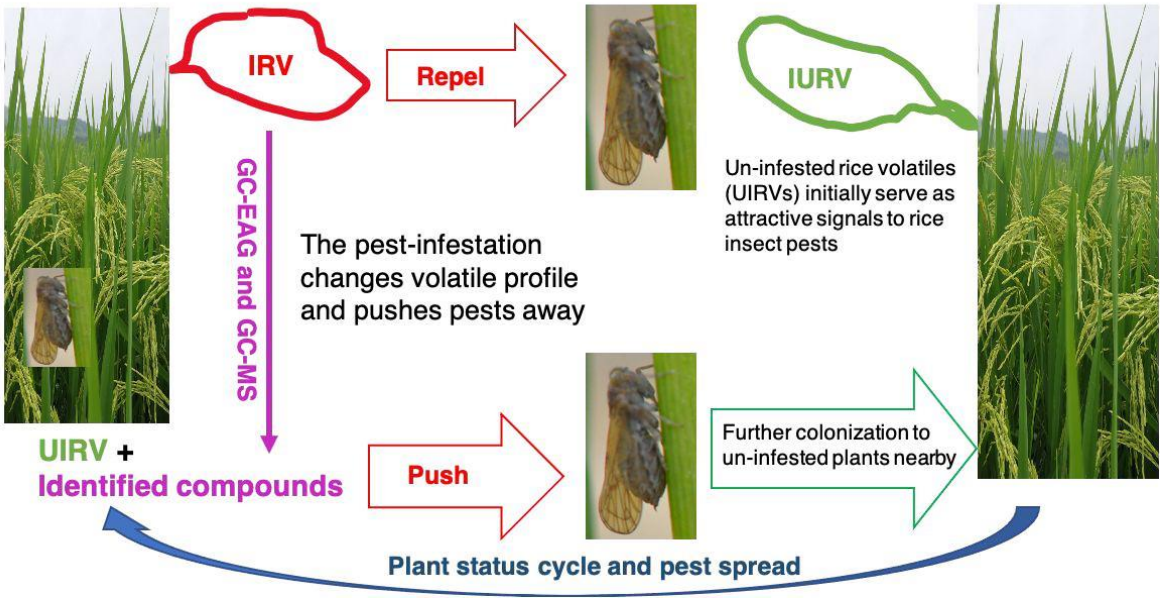


Figure 7. Relative expression of genes *S*-linalool synthase (*OsLIS*), salicylic acid carboxyl methyltransferase (*OsSAMT*) and methyl salicylate esterase gene (*OsSABP2*) associated with the biosynthesis of *S*-linalool, methyl salicylic acid, and salicylic acid in susceptible rice variety TN1 and resistant rice varieties IR64. The expression levels of the biosynthesis genes in infested and uninfested plants were determined by RT-qPCR and normalised to the expression of the endogenous gene *Os18S*. The expression levels were presented (**A**) as fold changes by BPH infestation and (**B**) as fold changes relative to their expressions in the susceptible rice variety TN1. The negative numbers indicate lower expression in IR64 than in TN1. The gene expressions between un-infested and infested rice samples were compared with paired t-test and ** indicate significant difference between un-infested and infested rice plants at $P < 0.01$.

Table of Contents Graphic

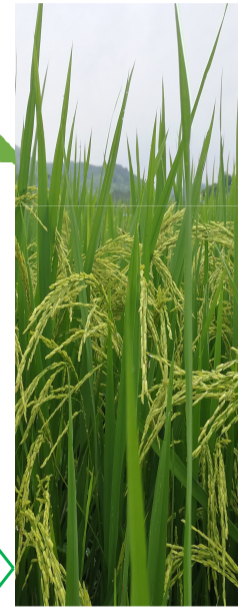


IRV

Repel

IURV

Un-infested rice volatiles (UIRVs) initially serve as attractive signals to rice insect pests



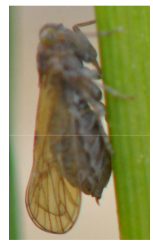
EAG-GC and MS-GC

The pest-infestation changes volatile profile and pushes pests away

UIRV +

Identified compounds

Push



Further colonization to un-infested plants nearby

Plant status cycle and pest spread