Comparative Analysis of Bioactive Volatiles from Susceptible and Resistant Rice Varieties to the Major Rice Pest *Nilaparvata lugens* Stål

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Abstract

Infestation by herbivorous pests can change plant volatile profiles leading to increased foraging by natural enemies and reduced attack by the pests. Eight rice bioactive volatiles (semiochemicals) were identified by GC-EAG. The quantitative differences in the production of these volatiles between susceptible and resistant rice varieties to the rice pest Nilaparvata lugens (BPH) were then determined. There was no clear correlation in the production of these volatiles with the rice resistance levels against BPH. The total amount of these volatiles and the expression of genes associated with the biosynthesis of these volatiles were significantly higher in susceptible varieties than in resistant varieties, and as expected further upregulated upon BPH-infestation. In behavioural experiments, the un-infested rice volatiles (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 volatiles in the infested rice plants (IRVs). Furthermore, the 1:1 molar mixture of these volatiles identified from IRVs repelled BPHs. These results demonstrate as expected that UIRVs initially serve as attractive signals to rice insect pests. The pest-infestation changes the rice volatile profile to be less attractive, which pushes further colonization to un-infested plants nearby.

Summary Statement

In insect-plant systems, there is mounting evidence that plants change their volatile profiles during infestation and become more attractive to natural enemies of pests, yet the chemical identities of rice volatiles and roles of the volatiles from infested rice plants, particularly in the context of pest resistant rice varieties have been largely overlooked. Eight bioactive compounds were identified in this study. The differences in the production of these compounds were quantified among eight rive varieties. These compounds could serve as biomarkers of pest infestation or be used as semiochemicals to enhance the efficacy of chemical lures used to trap insect pests and in insect pest management. Our results suggest a mechanism for the spread of insect pest infestation to un-infested plants nearby in rice field, and that this indirect defence of the volatiles from infested rice plant can be used to manipulate the behaviour of insect pests and may also improve the performance of their natural enemies and predators for crop protection based on a push-pull strategy.

Keywords

Nilaparvata lugens , herbivores, behaviours, rice bioactive volatiles, semiochemical, pest colonization, GC-EAG, rice resistant variety.

Running title : Comparative analysis of rice bioactive volatiles.

Introduction

Despite the importance of volatile compounds emitted by un-infested plants for insect pests to initially locate their hosts, the research on plant volatiles in chemical ecology and plant defence has mainly focused on herbivore-induced plants volatiles (HIPV) either to repel insect pests or to attract their natural enemies as defence against the pests (1–4). HIPVs have also been implicated in signalling between plants and other organisms (5–10). HIPVs mainly can comprise terpenoids, fatty acid derivatives, phenylpropanoids and benzenoids (11, 12) and are emitted either at the site of damage or systemically from undamaged parts of affected plants (10). They are used as cues by parasitoids and predators of plant-feeding insects in locating prey (5, 13-16).

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 volatiles play an important role in host plant location for BPH (19) and in prey location by natural enemies of the rice insect pests (6, 20, 19–22). Lou and his co-workers examined the role of rice volatiles induced by the chewing lepidopterans herbivore *Chilo suppressalis* (Walker) (23), sucking feeders BHP*s* (19–20, 24) and the white-backed planthopper *Sogatella furcifera* (Horváth). Their studies focused on the attractiveness of HIPVs to the natural enemies of rice pests such as the egg parasitoid *Anagrus nilaparvatae* Pang et Wang (21, 25) and the light green mirid bug *Cyrtorhinus lividipennis* Reuter (26), and found that the attractiveness of HIPVs to these insects was significantly increased when rice stems were infested by BPH and other chewing insects (6).

Surprisingly, the chemical identities of rice volatiles induced by BPHs and the effects of BPH-induced volatiles on rice BPH behaviours have rarely been reported. The behavioural responses of BPH to rice volatiles induced by the caterpillars of the tobacco cutworm *Spodoptera litura* (Fabricius) were studied, which showed a clearly repellent effect of these volatiles on BPH female adults compared to the volatiles of un-infested plants (3, 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 on the adult BPHs.

Meanwhile, many 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 volatiles from these resistant rice varieties, nor the relationships between the known mechanisms of resistance (see Discussion) and the volatile production in these resistant rice plants. Furthermore, although the volatiles from un-infested rice plants act as the initial signals in attracting the rice pests, remarkably, very few studies have investigated the chemical identities of bioactive volatiles and the changes of their volatile profiles after BPH-infestation. No comparative study on the rice volatile production before and after BPH-infestations has been made between susceptible and resistant rice varieties.

Here, we hypothesize that infestation by BPH alters the volatile profiles and thereby negatively influences attractiveness of infested plants to BPH. Thus, we used analytical chemistry, antennal electrophysiology and behavioural assays to identify bioactive volatile components and to quantify the emission profile of the volatiles before and after BPH infestation in eight susceptible and resistant rice varieties. We report for the first time that the volatiles from un-infested rice plants become less attractive when they are mixed with the blend of electrophysiologically active volatiles 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: 8 h dark: night. 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

Eight rice varieties with different degrees of resistance against BPH, as described in Table 1, were chosen for BPH behaviour bioassays and headspace volatile collections. The rice seeds were supplied by Huazhong Agriculture University (HZAU), Wuhan, China; Guangdong Academy of Agricultural Sciences (GAAS), Guangzhou, China and Yunnan Academy of Agricultural Sciences (YAAS), Kunming, China, and sowed in small garden pots and were grown under greenhouse condition (temperature: 24 ± 2 °C; photoperiod 18 L: 6D). Three-week-old seedlings were used for the volatile collections by air entrainments.

Headspace collection of rice volatiles

The volatile compounds of eight rice varieties (TN1, Minghui63, PT1, IR64, ZH11, Hazu6, Rathu Heenati, Babawee) (Table 1) were collected from un-infested and infested rice plants with 200 BPHs by air entrainment (27, 28). A total of 48 rice volatile collections (2 treatments x 8 varieties x 3 duplicates) 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 volatiles in air entrainment experiments (15, 29) 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 to 10-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°C in a refrigerator for GC or GC-MS analysis.

Gas Chromatography-Coupled Electroantennographic Detection (GC-EAG)

The response of BPH antennae to rice volatiles of infested TN1 rice variety was studied by GC-EAG. The GC analyses were carried out using an Agilent Technologies gas chromatograph (GC), model 6890, 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 female BPHs with fine forceps and mounted in an antennal holder (Syntech Inn., German) 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. A total of ten antennae was tested with the volatile collection of infested rice variety TN1.

Y-tube Olfactometer Setup

The behavioural bioassays of BPH to the collected volatiles were conducted using a small glass Y-shape tube olfactometer (1cm in diameter, 7 cm length of the arms and 8 cm length of the stem) with a 50^o inside angle between two arms. Incoming air was filtered through activated charcoal and humidified with doubly distilled, deionized water, and split to the two arms of the olfactometer. The Y-tube setup was surrounded by a 50 × 70 × 60 cm black fabric enclosure, and the holding chambers containing the treatments were placed outside the enclosure to eliminate visual cues for insects. In the single-choice bioassays, one chamber served as a control (diethyl ether) and another chamber held the test materials (i.e. either one of the 16 volatile collections or a pure chemical or a mixture of rice headspace volatiles and pure chemicals). In the double-choice bioassays, two chambers held the different test materials and the behaviours of BPHs were measured against each other. The airflow through the system was maintained at 200 ml/min. A 60-cm long, wide-spectrum fluorescent lamp (flickering rate: 26000 Hz) was positioned 40 cm above the arms of the olfactometer. Before each trial, light intensity over each arm was measured with a light meter, and the tube was adjusted until the intensity was the same in both arms.

Behavioural Bioassays

Approximately 1 h before behavioural trials, a nymph or fifth-instar female adult was placed inside a 2 ml plastic holding tube. The tubes containing insects were then placed into a separate holding container, so nymphs and adults would not be exposed to testing volatiles and starved for 2 h before trials. For each trial, 1.25 µl of either one of the volatile collections or a pure chemical or a mixture of EAG bioactive volatiles was applied onto a small filter paper. Then they were placed in the chamber. At the beginning of each trial, the insect was released from the holding tube at the downwind end of the Y-tube. Each insect was given 5 min to respond to the treatment, and 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 selected an arm of the Y-tube, the number of individuals 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. A fifth-instar female 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

Most chemicals were obtained commercially. 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. All compounds were >=99% pure and dissolved with redistilled hexane for GC analysis.

Gas chromatography (GC) analysis

The rice volatiles were separated by the 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 volatile sample was injected and analysed. The co-injection technique with authentic standards was used for quantitative characterization of bioactive compounds of volatile collections.

Gas Chromatography-Mass Spectrometry (GC-MS)

A capillary column (50 m x 0.32 mm ID HP-1) fitted in a Hewlett Packard 6890 gas chromatograph was directly coupled to the mass spectrometer and integrated data system (70- 250 VG Analytical and VG Au-

tospec, Fisons Instruments). Ionization was by electron impact at 70 eV and 230°C. The gas chromatograph was maintained at 30°C for 5 min and then programmed to increase at 5°C/min to 180°C. Tentative identifications of each EAG-active chemicals by GC-MS were confirmed by using Kovats Indices (KI) coupled with co-injection and peak enhancement with authentic standards on two GC columns of different polarity (29).

Enantiomeric determination of linalool in the rice headspace volatile collection from infested rice variety TN1 plants was achieved by GC using a chiral column. Chiral separations were performed on the Hewlett Packard 6890 gas chromatograph equipped with an alkylated ß-cyclodextrin (Restek, Bellefonte, PA; Rt-ßDEXsm) fused silica capillary column (30 m × 0.25 mm i. d.; 0.25 μm film thickness). Injector and detector temperatures were 230°C. Initial temperature was kept 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. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. A split ratio of 1:50 was used. Masses between 45 and 450 *m/z* were recorded. The separated peaks were compared with those of enantiomerically authentic standards and identified by using KIs and peak enhancement with authentic standards.

Total RNA isolation and cDNA synthesis

Frozen samples were ground to fine powder in liquid nitrogen with a pestle and mortar. The total RNA extractions were performed from 100 mg of each macerate plant tissue in liquid nitrogen, using Plant RNA easy Mini Kit (QIAGEN, German) 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 also assessed by 1% agarose gel electrophoresis and ethidium bromide staining. The presence of contaminant DNA in the RNA samples was verified by PCR using primers spanning two exon and gel electrophoresis analysis. The presence 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 Rnasy DNA efficiently removed contaminant DNA from 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 unites of Superscript III (Invitrogen) were added to the prior mixture and the total volume (20 μL) was 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 for RT-qPCR using a SYBR Green JumpStart Taq ReadMix (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 genes. Primers were 5'-GTTTGATGAGCCTGCGTAGTATT-3' (Forward) and 5'-GCTGCTGGCACGGAGTTAG-3' (Reverse) for *Os18s* used in this study. For checking the expressions of biosynthesis genes of volatiles compounds*S* -linalool and methyl salicylate in different rice varieties before and after BPH infestation, the expression of synthase genes of*S* -linalool (*LIS*), the salicylic acid carboxyl methyltransferase gene (*SAMT*), and a methyl salicylate esterase gene (*SABP2*) in rice plant was determined using RT-qPCR. The primers of the biosynthesis genes are listed in Supplementary Table 1.

Results

Behavioural response of BPH to the headspace volatiles of the TN1 variety

The headspace volatiles emitted by the susceptible variety TN1 before and after 5-day BPH-infestation were collected, named as UIRV for un-infested rice volatiles and IRV for infested rice volatiles, respectively, for

further behavioural and chemical studies. BPHs were significantly attracted to the UIRVs (Figure 1A) and rested more time in the arms treated with the UIRVs (Figure 1B). This attraction was not significant when BPHs were tested behaviourally with the IRVs in the single choice bio-assays. As expected, BPHs were attracted significantly to the UIRV-treated arm when BPHs were given a choice between UIRV and IRV in double choice bioassays.

Identification of electrophysiologically active volatiles by GC-EAG

The volatiles from the headspace collection of BPH-infested rice variety TN1 consistently elicited GC-EAG responses on the female antennae of BPHs (Figure 2). Eight compounds were identified as bioactive volatiles by GC-MS and peak enhancement (Supplementary Figure S2). These include methyl benzoate, 2-nonanone, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), veratrole, methyl salicylate, β-ionone, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). There were two enantiomers *R*- linalool and *S*- linalool presented in the headspace volatiles used in this study and emitted at the ratio of 3:1 after BPH infestation (Supplementary Figure S1).

Behavioural response of BPH with the blend of EAG compounds

The headspace volatiles emitted by the susceptible variety TN1 after BPH-infestation were then analysed for the natural composition of the eight EAG active compounds in the volatiles from infested TN1 plants (IRV) (Figure 3 and Supplementary Figure S2). In the behavioural bioassays using a Y-tube olfactometer (Figure 4), the addition of the blend that mimics the natural composition of the eight EAG active compounds from IRV into UIRV reduced the attractiveness of the UIRV (Figure 4A) and BPHs spent much more time in the arms treated the UIRV alone compared with the arms treated with UIRV plus the natural blend from the IRVs (Figure 4B).

Behavioural response of BPH to the individual and 1:1 molar ratio of the EAG active compounds

The behavioural responses of BPHs to individual EAG active compounds and a mixture at a 1:1 molar ratio of these eight compounds 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) (Figure 5). The compounds methyl salicylate, β-ionone, TMTT repelled significantly BPHs. 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 volatiles and the control (Figure 5). The 1:1 molar mixture also repelled BHPs.

Comparison of BPH responses to the headspace volatiles of rice varieties

The behavioural study was extended using other seven rice varieties (Hzau6, ZH11, Minghui63, PT1, Babawee, IR64 and Rathu Heenati) which have different degrees of resistance and mechanisms against BPHs (Table 1) together with the variety TN1 to evaluate the ecological potentials of the EAG active compounds. The results indicated significant variations among the rice varieties. The single choice bioassays showed that the UIRVs of un-infested susceptible variety TN1 as well as resistant varieties ZH11, Hzau6, Babawee, PT1 and RH attracted more BPHs than the solvent control diethyl ether (DE) (Figure 6A; Supplementary Figure S3A). By contrast, the UIRVs of un-infested resistant variety IR64 attracted less BPHs than the controls (Figures 6B) and the BPHs spent longer time in the UIRV-treated arm than in the DE-treated arm (Supplementary Figure S3B). The IRVs of all varieties expect TN1 attracted more BPHs than DE (Figure 6B). Furthermore, in double choice bioassays, the responses of BPHs were measured with UIRV against IRV (Figure 6C). In this case, the UIRVs from the susceptible variety Hzau6 and the resistant variety IR64 had similarly attractive effects on the BPH behaviours as the UIRV of the susceptible variety TN1. For the resistant variety Babawee, the URIVs and IRVs had similar effects on the BPH behaviours. Interestedly, the IRVs from susceptible rice variety TN1 (repellent) and resistant rice variety IR64 (attractive) even triggered opposite behavioural responses (Figure 6B and Supplementary Figure S3B). Similarly, the IRVs from the rice varieties ZH11 and MH63 attracted more BPHs, and those from the rice varieties PT1 and RH attracted

significantly more BPHs (Figures 6C and Supplementary Figure S3C) than UIRVs, an opposite effect as that of UIRVs from the susceptible rice variety TN1, i.e. the UIRV-treated arm attracted significantly more BPHs and BPHs spent longer times in the UIRV-treated arm (Figure 1).

Quantification and relative amount of the EAG active compounds in rice varieties

The emission of the EAG active compounds (methyl benzoate, 2-nonanone, linalool, DMNT, veratrole, methyl salicylate, β-ionone, and TMTT) in all eight rice varieties before and after BPH-infestation was determined by using Kovats Indices (KI) coupled with co-injection technique. The susceptible variety TN1 emitted the largest amount of the EAG-active compounds (Figure 7A and Supplementary Figure S4), and the resistant varieties IR64 and RH emitted the least amount of these volatiles (Figures 7G and 7H). Three compounds (2-nonanone, linalool and methyl salicylate) were the main volatile compounds (collectively 80˜95% of total EAG active compounds) among the eight EAG active compounds in all varieties.

The volatile emissions were strongly induced by the 5-day BPH-infestation (Figure 7). The compounds 2-nonanone, linalool and methyl salicylate were still dominant in both susceptible and resistant varieties after the infestation. The emissions from same weight of the susceptible rice variety TN1 plant material were induced from 4.5 ng/ml to 24.0 ng/ml for 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 DMNT and from 2.4 ng/ml to 19.6 ng/ml for methyl salicylate (Figure 7A). The emissions of linalool and methyl salicylate in all varieties except the variety RH (Figure 7H) were significantly induced by the infestation (Figure 7). The emission of linalool was 8-fold and 2-fold higher in the susceptible variety TN1 and in the variety PT1, respectively, after BPH-infestation (Figure 7 and Supplementary Figure S4). The emission of 2-nonanone was also significantly induced in all varieties except MH63 and Babawee varieties (Figure 7D and 7F).

Apart from the differences in the quantity of the volatiles emitted, the relative profiles of the EAG active volatiles were also very different between the susceptible and resistant varieties (Supplementary Figure S5). The relative percentage of each EAG active compound in total volatile emission was changed dramatically and differentially by BPH-infestation among rice varieties. Although the susceptible variety TN1 emitted the largest amount of the EAG active compounds (Figure 7), the relative percentage change of each active compounds in total headspace volatile collection was minimum before and after BPH-infestation (Supplementary Figure S5).

Expression of the biosynthesis genes of methyl salicylate and linalool

Two volatiles, linalool and methyl salicylate, were detected as the main compounds from UIRVs and IRVs of rice varieties. Further to confirm their possible involvement in the behaviours of the BPHs toward rice plants, we compared the expression levels of their biosynthesis genes between the susceptible rice variety TN1 and the resistant varieties Babawee, IR64 and RH before and after BPH-infestation. These include genes that encode for *S* -linalool synthase (*LIS*), salicylic acid methyl transferase (*SAMT*) and salicylic acid-binding protein 2 (*SABP2*).

The expressions of these biosynthesis genes is as expected highly upregulated by BPH-infestation, particularly in the susceptible variety TN1, of which the *LIS* expression was increased by 266.1 folds, the *SAMT* and *SABP2* expressions were increased by 8.4 and 6.0 folds, respectively, after BPH-infestation (Figure 8). However, for the highly resistant variety RH, the expressions of *LIS* ,*SAMT* and *SABP2* were less variable before and after BPH-infestation with fold changes less than 2 (Figure 8).

On the other hand, without BPH-infestation, the resistant variety Babawee expressed significantly higher level of *LIS* and*SABP2* with a fold change of 9.6±0.5 and 4.5± 0.7, respectively, relative to their expressions in un-infested susceptible variety TN1 (Figure 9). However, its *SAMT* expression was significantly lower than that of the susceptible variety TN1. For the resistant varieties IR64 and RH, the expressions of *LIS* , *SAMT* and *SABP2*were lower relative to those of the susceptible variety TN1 with the fold changes of -5.3±0.5, -11.3±2.4 and -1.5±0.2 in IR64 variety and -13.2±1.8, -1299.9±277.8 and -28.8±20.3 in RH variety, respectively (Figure 9A). After BPH-infestation, all resistant varieties expressed these genes much lower than

the susceptible variety TN1 (Figure 9B).

Discussion

In order to identify volatile rice components associated with damage by BPH in susceptible and resistant rice varieties, the BPH behaviour to the volatiles of un-infested susceptible rice variety TN1 was determined, and the electrophysiological responses of BPH female antennae to the headspace volatiles of the BPH-infested susceptible rice variety TN1 were measured by GC-EAG. The EAG active compounds were identified as methyl benzoate, 2-nonanone, linalool, DMNT, veratrole, methyl salicylate, β-ionone, and TMTT (Figure 2). These compounds were present in all of eight tested rice varieties and induced by the BPH infestation (Figure 7). 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 most rice varieties (Figure 7). As previously suggested in the studies on the interactions between parasitoid wasps and a rice variety, the volatiles 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 and rice varieties. Further behavioural studies demonstrated that the compounds methyl salicylate, ß-ionone, TMTT and, more interestingly, the 1:1 molar mixture of eight EAG-active compounds triggered a significant repellent behaviour to BPHs (Figure 5).

Linalool was emitted in the largest amount among the eight EAG active compounds and elicited a strong EAG response but had no effect on the behaviour of BPH in the Y-tube bioassay (Figure 5). It was reported that*S*- linalool from a different rice variety had a repellent effect on BPH behaviours (20, 33, 34). 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 3:1 ratio (Supplementary Figure S1). Further studies should determine the relationship of two linalool enantiomers individually in BPH behaviours, particularly to R- linalool.

The behavioural results of the current study demonstrated a strong attraction of BPHs to the headspace volatiles of the un-infested susceptible rice variety TN1 plants (UIRV) (Figure 1). Although it has not been exhaustively studied, this finding is consistent with a role of rice volatiles in mediating pest behaviour as an important signal in plant indirect defence against insect pests (3, 19, 30–32). The attractiveness of UIRV was dramatically reduced by the addition of the blend of eight EAG active compounds at the ratio that mimics the natural compositions in the IRVs from the BPH-infested TN1 plants (Figure 4). Thus, the UIRV of the susceptible rice variety TN1 which initially was highly attractant to BPH become repellent when the blend of the eight EAG active compounds from the infested rice plants. This could be one of factors to naturally push BPHs further colonise nearby un-infested rice plants.

There is a considerable variation in volatile profiles and BPH behaviours between rice varieties (Figure 7 and Supplementary Figure S3), which is consistent with the suggestion of previous studies that the changes in volatile profiles serve as specific information on host habitat quality for wasp attractive behaviours (20, 37, 38). Our results provide such evidence that this could also be applied to the rice pests and the variations of key EAG active compounds in the headspace volatiles among rice varieties could be a determining factor in mediating BPH behaviours. It is likely that, although individual volatiles contribute differently (Figure 5), the differences in pest behaviours to rice varieties are dependent on the volatile emission profiles of rice plants before and after pest infestation rather than individual volatile compounds. This may explain the different behaviours of BPH to the headspace volatiles of different rice varieties observed in the current study (Figure 6). Furthermore, as expected, we found no correlation between the mechanisms of resistance in the rice varieties so far developed and the BPHs responses to their headspace volatiles (Figure 6 and Supplementary Figure S3). The resistance mechanisms of these resistant rice varieties are not relevant to the behavioural response of BPHs to these plants and unrelated to the volatile productions. Thus, the emission rates of volatiles from resistant rice varieties appear not to provide the resistant mechanisms against the rice pest BPH.

Nevertheless, there seems a clear relationship between the pest tolerance ability of the rice varieties and

the emission of the EAG active compounds, i.e. the stronger pest tolerance ability the rice varieties have, the less volatiles they emit (Figure 7 and Table 1). Plant tolerance to insects by breeding is proposed as a major target for resistant varieties against herbivore infestation. The rice variety Rathu Heenati (RH) is highly tolerant to BPH with its *bph3*resistant gene, functioning as lectin receptor kinases (39) and*bph6* resistant gene, functioning as an exocyst-localized protein (40), but it has the lowest total volatile emission among eight tested rice varieties, which is less than 1/30 of the volatiles emitted by the susceptible variety TN1 (Figure 7 and Supplementary Figure S4-S5). Babawee, another highly tolerant rice variety with the *bph4*resistant gene whose molecular functions have not been reported, also emitted much less bioactive volatiles than the susceptible variety TN1 (Figure 7). Furthermore, the expression of biosynthesis genes for the key EAG active compounds methyl salicylate and S -linalool in resistant varieties was much lower than those of the susceptible variety TN1 (Figure 8), and the fold changes of these genes after BPH-infestation in the resistant varieties were less than 2 folds relative to those before pest infestation (Figure 9). Unlike all varieties, the emission of linalool and methyl salicylate in the resistant variety RH did not change by the BPH-infestation. Similarly, the emission of 2-nonanone of the resistant variety Babawee was not induced. These results add a further confirmation that the volatile productions of resistant rice plants are not relevant to the resistance mechanisms of the resistant rice varieties against BPH and the BPH behaviours to rice plants. The ability of the resistant rice varieties to regulate indirect defence mechanisms against BPH might have been reduced by the breeding programs for their direct defence mechanisms. This selection process for insect pest resistance might have even enhanced the attractiveness of BPHs in some resistant varieties.

In summary, our results demonstrate that volatile emission rates are different between rice varieties and upregulated by BPH-infestation. However, such variations are not correlated to known BPH resistance mechanisms in rice varieties. Our study reports for the first time that electrophysiologically active rice volatiles 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 8 and 9, also see 41), and the reduced attractive effect of UIRVs by the volatile blend of IRV (Figure 4), support the view that volatiles of un-infested rice plants may only serve as initial attractive signals for rice pests. However, these same volatiles caused repellency when the rice plants are infested by BPH, which may lead to the spread of the insects to un-infested plants nearby. The higher volatile emission and the stronger upregulation of volatile biosynthesis genes in the susceptible variety TN1 may serve to offset its susceptibility to rice pests. Such indirect defence by plant volatiles against insect pests may not serve as a direct defence mechanisms against insect pests but could be explored further to manipulate insect pest behaviour (6, 42–44) and to attract natural enemies (5, 13, 14, 20) and predators (26) in integrated pest managements.

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Figure legends

Figure 1. Dual choice responses of at least 19 individual fifth-instar BPH females in a Y-tube olfactometer to the arms treated with the headspace volatiles UIRV from the un-infested susceptible TN1 plants and IRV from infested TN1 plants. DE (diethyl ether) is the control solvent. (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 BPHs responded to the arm treated with UIRV, IRV and IRV against the arm treated with DE, DE and IRV, respectively, indicated by the positive numbers. 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 . 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 (Supplementary Figure S2).

Figure 3 . Partial trace (10˜35 min) of gas chromatography FID response of the headspace volatile compounds of the rice variety TN1 infested by BPH*s* . This is used to quantify the eight EAG active compounds and the identity of each indicated compound confirmed as in Supplementary Figure S2.

Figure 4. Dual choice responses of at least 19 individual fifth-instar BPH females in a Y-tube olfactometer to the arms treated with the headspace volatiles from un-infested TN1 plants (UIRV) and 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 3 and Supplementary Figure S2 so that it mimics the natural composition of these compounds in the IRV. 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. Responses of fifth-instar BPH females in a Y-tube olfactometer to the EAG active volatile compounds and their 1:1 molar mixture (left dark bars) against the 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 3:1 ratio (Supplementary Figure S1). The 1:1 molar mixture is made of eight EAG active volatile compounds at equal molar ratio. The numbers in the dark bars (negative x-axis) show the number of BPHs responded to individual volatile and the 1:1 molar mixture. The numbers in the 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 6 . The responses of fifth-instar female BPHs in a Y-tube olfactometer to the headspace volatiles of rice varieties TN1, Hzau6, ZH11, MH63, PT1, Babawee, IR64 and RH (see Table 1) in single choice bioassays (**A** and **B**) and dual choice bioassays (**C**). 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 7 . Quantification of eight EAG active volatiles from un-infested and infested rice varieties TN1 (A), Hzau6 (B), ZH11 (C), MH63 (D), PT1 (E), Babawee (F), IR64 (G) and RH (H). 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 Supplementary Figure S2). The concentrations of HIPVs 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 8. Relative expression of genes *LIS* , *SAMT*and *SABP2* associated with the biosynthesis of *S* -linalool (*LIS*), methyl salicylic acid (*SAMT*), and salicylic acid-binding protein 2 (*SABP2*) in infested susceptible rice variety TN1 and resistant rice varieties Babawee, IR64 and RH after BPH-infestation. The expression levels were determined by RT-qPCR, normalised to those of the endogenous gene *Os18s* and presented as expression fold changes relative to their expression in un-infested plants. A positive value indicates expression upregulation, and a negative value indicates expression downregulation by BPH-infestation.

Figure 9. Effect of BPH-infestation on the expression of biosynthesis genes of *S* -linalool synthase (*LIS*), the salicylic acid carboxyl methyltransferase (*SAMT*), and a methyl salicylate esterase (*SABP2*). The expressions were determined by RT-qPCR with three biological replicates per sample of resistant rice varieties (Babawee, IR64, RH) before (A) and after (B) BPH-infestation. The expression levels were normalised to the expression of the endogenous gene *Os18s* and presented as expression fold changes relative to their expressions in the susceptible rice variety TN1. The positive numbers indicate the upregulation (higher expression than in TN1) and the negative numbers indicate the downregulation (lower expression than in TN1).

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*
Hzau6	*Oryza sativa spp. Indica*	Susceptible	Hzau6	*HZAU*
ZH11	*Oryza sativa spp. Japonica*	weakly resistant	ZH11	*HZAU*
Minghui63	*Oryza sativa spp. Indica*	weakly resistant	MH63	*HZAU*
Perennial 1	*Oryza sativa spp. Indica* (*O. sativa RD23 /O.longistaminata RILs*)	resistant	PT1	*YAAS*
Babawee	*Oryza sativa spp. Indica*	resistant with *bph4*	Babawee	*GAAS*
Rathu Heenati	*Oryza sativa spp. Indica*	high resistant with *bph3*	RH	*GAAS*
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; YNAAS, YunNan Academy of Agricultural Sciences.

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

Supplementary Table 1. Primers of RT-qPCR used in this study

Genebank Accession	Gene name	Primer sequeces	Gene description
OS02g02930.1	*OsLIS*	Forward primer (5'-3') GC-CCAGCAAAAATCTTGA-GACT Reverse primer (5'-3') AATCCTTCTTGTGCCTCATCCTT	S-linalool synthase gene
Os03g42530.1	*Os18S*	Forward primer (5'-3') GTTTGATGAGCCT-GCGTAGTATT Reverse primer (5'-3') GCTGCTGGCACGGAGTTAG	Oryza sativa Japonica Group 18S ribosomal RNA
Os11g15040.1	*OsSAMT*	Forward primer (5'-3')TTCCAGTGTACTGCCGTTGA Reverse primer (5'-3') GGACTCTGTCGCCACCCTTA	salicylic acid methyl transferase
Os01g37650.1	*OsSABP2*	Forward primer (5'-3')CGCTTTCTTTTCCTCTTATTTCG Reverse primer (5'-3') CCACCACATACACCTTTCTCACG	salicylic acid-binding protein 2

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