Identification of some volatile compounds in rice infestation with brown planthopper (BPH)

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ABSTRACT

Rice volatile compounds induced by brown planthopper BPH were analysed using capillary gas chromatography-mass spectrometry (GC-MS).Fifty-three (53) different volatile compounds were found in the rice infested with BPH and the control.Twenty-nine (29) volatile compounds increased emission in the rice infested with BPH, including alkane (nonane, dodecane, cyclopentane, tetradecane, cyclohexane and nonacosane), ketone (ethanone), alkene (1-dodecene), alcohol (5-methyl-4,7,10,13-tetraoxatetradeca-2-ol, phytol), aldehyde (E-15-heptadecenal and pyrrole).Gamma and alpha tocopherol, neophytadiene, dibutyl phthalate and stigmasterol were found in the control than those the attacked rice, whereas naptho[2,3-b] furan-4,9-dione was found as a major constituent in resistant cultivar after the BPH infestation.The gene involved in volatile compound production,*OsHPL*, also increased significantly following BPH infestation. The volatile compounds incorporating the gene may be responsible for the characteristic of rice being resistant to BPH in the resistant cultivar.

Keywords: Naptho[2,3-b] furan-4,9-dione, brown planthopper, rice, volatile compounds, metabolites

INTRODUCTION

The emission of low molecular weight volatile organic compounds (VOCs) is one of the plants'defence responses to pathogens and insects (Zhuang, X. *et al*.,2012). The plant volatiles function as an indirect defence to pathogens by attracting the natural enemies of their herbivores (Turlings,T.C.J. and Wickens, F.L.,2004;Howe, G.A. and Jander, G., 2008).The parasitoid, *Anagrus nilaparvatae*, a major natural enemy of the rice BPH, was attracted to rice infested with BPH, whereas there was no attraction to volatiles which were released from undamaged plants (Lou,Y.G., *et al.*, 2005).Among the VOCs, green leaf volatiles (GLV) which are produced from green plants upon herbivory and pathogen infection play an important role in plant defence (Scala, A. *et al*., 2013).Previous studies revealed that herbivore attack induces a set of defensive responses in rice, activating the signalling pathways (jasmonate and ethylene signalling) by expressing defensive genes (Hao, Z., *et al*., 2011; Duan, C., *et al*., 2014;Zhang, F., *et al*., 2004). For example, rice plants

damaged by the feeding of the *Spodoptera frugiperda* (FAW) were shown to emit a blend of volatiles that are highly attractive to parasitic wasps (Yuan, J.S. *et al*., 2008).

Recently, Wang *et al*. (2015) reported that herbivore-induced plant VOCs play an important role in regulating interactions between plants, herbivores and their natural enemies. Moreover, the production of rice volatiles induced by BPH showed an equal attraction of the parasitoid (Yujie, L. *et al*., 2006).

The BPH, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), is a destructive insect pest of rice, causing reductions in rice yield and large economic losses (Jannoey, P. *et al*., 2015; Jannoey, P and Channei, D., 2015). BPH outbreaks occur in tropical regions, especially in the central and lower northern regions of Thailand. The outbreak areas were observed in seven provinces, Sukhothai, Phitsanulok, Chai Nat, Ang Thong, Suphan Buri, Nonthaburi and Nakhon Nayok, during the raining season. The BPH suck sap in the seedling and tender spike stage, resulting in yellowness, wilting and even death, these symptoms are called hopperburn.

Outbreaks of BPH in Thailand had a significant impact during the last four years (2012-2015), causing the loss of 2.68, 1.68 and 1.18 million hectares of the rice area, respectively. The economic loss in the last four years was more than 26,000 million baht.

VOCs have been reported to be a messenger protecting plants against herbivores and pathogens. A recent study showed that (E)-β-caryophyllene, one of the VOCs, has been reported to attract BPH and its egg parasitoid, *A. nilaparvatae* (Wang, Q. *et al*., 2015a).Cheng, A.X. *et al*. (2007) found that the transgenic plants of both rice and Arabidopsis with higher levels of rice (E)-β-caryophyllene synthase (OsTPS3) attracted more wasps of *A. nilaparvatae* Pang et Wang, a main egg parasitoid of rice BPH.

The main volatiles in rice constituent are indole (Zhuang, X. *et al*., 2012), terpene including cinnamyl alcohol, myristicin, sesquiterpene alcohol (caryolan-1-ol), (E)-β-caryophyllene, sesquiterpenes (Forlania, G. *et al*., 2011; Wang,G. *et al*., 2015; Cheng, A.X. *et al*., 2007), alkanes, alkenes and long-chain alcohols (Forlania, G. *et al*., 2011) has been reported. Zhuang, X. *et al*. (2012) found that the indole synthesised by indole-3-glycerol phosphate lyase was emitted from rice-herbivory induction. Similarly, rice plants damaged by FAW (*Spodoptera frugiperda*) larvae emitted about 30 volatiles, including methyl salicylate and methyl benzoate. FAW-induced volatiles are highly attractive to female parasitic wasps (*Cotesia marginiventris*),carnivorous enemies of FAW (Zhao, N. *et al*., 2010).

Furthermore, the VOC included alkanes, alkenes and long-chain alcohols as well as cinnamyl alcohol, myristicin, sesquiterpene alcohol (caryolan-1-ol), 1-octanol and 1-decanol showed fungistatic activity against to *M. oryzae* (Forlania, G. *et al*., 2011).

Interestingly, the hydroperoxide lyase (*OsHPL*) gene is one of the genes encoded for hydroperoxide lyase (HPL) enzyme production.The HPL enzyme is the key enzyme for green volatiles biosynthesis, its activity represents a key function of volatile compounds in plants as an indirect defence.The expression of the *OsHPL* gene in Arabidopsis caused increased volatile compounds production, resulting in higher attraction of parasitic wraps to control their herbivores. The loss of mutant *Os-HPL3* function in rice showed lower VOCs emission (Scala, A. *et al*., 2013).

Although the release of herbivore-induced plant volatiles has been reported above, there is a little evidence that the volatile induced by BPH biotype in Thailand. Indeed, the volatile and BPH interaction are very few data in resistant cultivar.

The aim of this study was to analyse and identify volatile-induced BPH and *HPL* gene expression in resistant cultivar. Gas chromatography (GC) coupling with Mass spectrometry (MS) technique and Real-time PCR were employed to analyse volatile compounds and HPL gene expression, respectively. The volatile-induced BPH may function as an important signal for understanding rice and BPH interaction.

MATERIAL AND METHODS

Rice plant growth and insect treatments

Rice seeds (*O. sativa* sp. Indica) were germinated and grown in a greenhouse for two weeks. BPH were cultivated in a cage with rice seedlings as a food source until they hatched. The BPH nymphs were transferred to rice plants in the caged condition. Rice plants were infested with BPH in a ratio of 12:1 insects per rice. Rice phenotypes were monitored and collected for volatile compound analysis compared to the rice without BPH infestation (control).

Volatile compound analysis

GC conditions

Analysis of the volatiles of rice seedlings was performed by using 600 mg of tissues ground in liquid nitrogen. Rice samples were extracted with dichloromethane, centrifuge at 6,000 rpm for 30 minutes. The crude extract was collected in a 1.5 ml vial and sealed with a magnetic cap. The samples were placed in an autosampler tray and were maintained at room temperature until analysed. The initial temperature was held at 250^{o}C using HP-5MS capillary column (0.25mm×30m×0.25µm; Agilent 19091S-433).The GC oven temperature was held for 1 minute at 250^{o}C, then increased to 260^{o}C for 30 minutes at a rate of 5^{o}C/minute in splitless mode.

MS conditions

The interface temperature was 280°C, the ion source using the Electron Impact (EI) mode at 230°C, the electron energy was 70 eV and the mass scan range (M/Z) was 35-350 amu.

OsHPL gene expression analysis

RNA extraction and cDNA synthesis

The total RNA was extracted with E.Z.N.A.® Total RNA Kit (Omega) according to the manufacturer's instructions and then quantified using a NaNoDrop ND-100 spectrophotometer.The total RNA was converted to cDNA using Tetro cDNA Synthesis Kit (Bioline).The cDNA synthesis reactions were incubated at 37^{o}C for 15 minutes and 85^{o}C for 5 seconds. The cDNA was obtained and then amplified in a further step.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SensiFAST™ SYBR® Kits (Bioline). The PCR reaction was carried out in triplicate in 48-well plates. The thermal profile of the real-time system was set as one step at 95^{o}C for 30 seconds, followed by 40 cycles at 95^{o}C for five seconds (denaturation) and 60^{o}C for 30 seconds (annealing

and extension), followed by added dissociation pattern. The actin gene was used as the internal standard.

Data analysis

The quantification of the relative expressions level was determined from the average threshold cycles (CT).The ΔCT value was calculated by subtracting the average CT value of the interested gene from the CT value of the actin gene. $2^{-\Delta\Delta CT}$ was calculated to estimate the relative expression level.

RESULTS AND DISCUSSION

Rice phenotype during BPH infestation

Rice seedlings were infested with BPH nymphs, and hopperburn symptoms were observed on different infestation days (Table1).Following infestation, continuous feeding by BPH nymphs caused wilting of the seedlings, leading to hopperburn (browning of stem and leaves) (Figure 1).During early infestation (0-7 days after infestation, DAI), damage symptoms were not detected on infested plants except the TN1 cultivar. This is probably due to the fact that the BPH did not cause enough damage and plants were able to overcome the low level of insect stress (Sangh, J.S. *et al*., 2013) and some cultivars contained BPH-resistant genes.The difference in the phenotype among the cultivars was more obvious at 8-15 DAI, respectively. However, the results in Table 1 categorised the rice's resistance to BPH ability into three groups.The highest resistance to the BPH cultivar was the 67-111, PTB33, Rathu Heenati, PSL2 and RD49 cultivars, whereas RD41 and RD47 were categorised into moderately BPH-resistance ability. Finally, the susceptible cultivar was found in TN1, SP90 and PT1. Therefore, PTB33 was chosen to extract to identify *OsHPL* gene and the total volatile compounds in this study. The identified metabolites may be useful for understanding the mechanism involved in the rice's resistance to BPH in the resistant cultivar.

Comparison of volatile compounds emitted from rice-treated BPH

The volatile compounds analysis was collected from dichloromethane fraction. Fifty-three different components were found and classified on the basis of their structure (Table 2). Alkane compounds (nonane, dodecane, cyclopentane, tetradecane, cyclohexane, undecane, hexadecane, 2,5,8,11,14,17-hexaoxaoctadecane and nonacosane) were found to be the most abundant components, followed by alkene (1-dodecene), ketone (ethanone), aldehyde (E-15-Heptadecenal) and ester (1-methylethyl tetradecanoate). Moreover, neophytadiene, dibutyl phthalate, 2H-cyclopenta[b]furan-2-one, 7,9-di-tert-butyl-1-oxaspiro[4,5]-deca-6,9-diene-2,8-dione, gamma-tocopherol, alpha-tocopherol, stigmasta-5,23-dien-3-beta-ol and stigmasterol were found in the control than those in rice treated with BPH (Figure 2). Venkata *et al*. (2012) reported that phytol, в-tocopherol and stigmasterol were also found in aqueous extracts of *E.odoratum* plants. Those compounds were considered as antimicrobial, anti-cancer, anti-inflammatory, antidiuretic, immunostimulatory, anti-diabetic, antioxidant and radical scavenging activity (Venkata, R.B. *et al*., 2012). However, these compounds reduced emission in rice after BPH infestation, Similarly, Lou *et al*. (2005) found that the BPH infestation induced increasing of the tetradecane, pentadecane and linalool, subsequently attractive to the BPH's natural enemy. Moreover, previous reports found that most compounds of heptanone, 2-heptanol, limonene, linalool and (E)-4,8-dimethyl-1,3,7-nonatriene were released in

significantly higher amounts from BPH-JA-treated rice plants compared to the control. The volatiles emitted from JA-treated rice plants attracted the BPH to the parasitoid and enhanced the parasitism of *N. lugens* eggs in the greenhouse and the field (Thaler, J.S. 1999; Kessler and Baldwin, 2001). Furthermore, it was previously shown that linalool is attractive to *A. Nilaparvatae*. A difference in the attractiveness of linalool to BPH was found between JA-induced plants compared to the buffer solution. However, Wang, Q. *et al*. (2015) suggested that the rice white-backed planthopper (*Sogatella furcifera*) preferred feeding and ovipositing on wild-type plants to feeding on mutants with low levels of (E)-β-caryophyllene, whereas the other studies indicate that (E)-β-caryophyllene may function as an important signal by which herbivores on rice locate their host.

In contrast with the previous reports, GLV have not been found in this study. GLV constituents are composed of E-2-hexenal, E-2-hexenol, E-2-hexenal-acetate, Z-3-hexenal, Z-3-hexenol, Z-3-hexyl acetate, n-hexanal, n-hexanol and n-hexenyl acetate. These compounds are semi-chemicals used by insects to find their food source, while plants use GLV as an indirect defence and a direct effect on pests (Scala, A. *et al*., 2013). Many studies have shown that the predator and parasitoids are indeed attracted to GLV. For example, the aliphatic aldehyde (hexanal and E-2-hexenal) attract female BPH more than the other volatile compounds including leaf alcohol (Youan, N.Y. 2002). The previous report by Qiang et al. (2003) showed that the GLV compounds (E-2-hexenal, E-2-hexen-1-ol) had a significantly repellent effect on the adult BPH at higher concentration, resulting in increased susceptibility of rice to white-black BPH. Other reports found, however, that the E-2-hexen-1-ol, 2-heptanone, limonene, ocimene, linalool, β-caryophyllene and (E)-nerolidol had no significant repellent effect on BPH (Qiang et al., 2003).

Naphtho[2,3-b]furan-4,9-dione was only found in resistant cultivar due to BPH attack in this study (Figure 2). It is an important bioactive compound extracted from the trunk and inner bark of *Tabebuia billbergii* (Gómez-Estrada, H. *et al*., 2012; Ogawa,M. *et al*., 2009). Ogawa, M. *et al*. (2009) reported that several naphtho[2,3-b]furan-4,9-diones isolated from plants and synthesized having biological activities. For example, 2-acetylnaphtho[2,3-b]furan-4,9-dione isolated from *Tabebuia cassinoides* (Lam.) DC (Bignoniaceae) exhibited cytotoxic activity of tumour-specific cytotoxicity. The authors suggested that the hydroxyl radical was the active intermediate of these agents involved in the cytotoxic mechanism of naphtho[2,3-b]furan-4,9-diones. Moreover, the naphtho-furan-diones showed important inhibitory activity when assayed *in vitro* against *Plasmodium berghei*, especially in comparison to the activity exhibited by chloroquine, which was used as the control substance (Gómez-Estrada, H. *et al*., 2012). These compounds has been reported as a fungicides (botanical fungicides), "santonin" is approved as a common name in China.

Naphtho[2,3-b]furan-4,9-diones is also related to a mixture of novel pyrazole compounds formula for combatting invertebrate pests such as insects, arachnids or nematodes on plants, preventing such plants being infested with pests (WO 2013189801 A1). It is possible that naphtho[2,3-b]furan-4,9-dione may play a role in protecting rice plants from BPH attacks. Therefore, this finding indicates that the volatile compounds emitted from rice treated BPH may be responsible for BPH defence mechanism in resistant cultivar. However, the volatile compounds against BPH mechanism (indirect or direct) had not been proved in this study.

Table 1 Comparative resistance of rice cultivar to BPH at different times for 19 days.

Rice cultivar	BPH damage scale (1–9 score)																		
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Highly Resistant																			
67-111	0	0	0	0	0	0	0	1	1	2	3	3	3	3	4	4	4	4	4
PTB33	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2
Rathu	0	0	0	0	0	0	1	1	1	1	2	2	2	2	2	2	3	3	3
PSL2	0	0	0	0	0	0	1	2	2	3	4	4	4	4	4	4	4	4	4
RD 49	0	0	0	0	0	1	1	2	2	3	4	4	4	4	4	4	4	4	4
Moderately Resistant																			
RD 41	0	0	0	0	1	1	1	2	2	2	3	3	3	3	4	4	4	4	4
RD 47	0	0	0	0	0	0	1	2	2	2	3	4	4	4	4	5	5	5	5
Susceptible																			
TN1	0	0	0	1	2	3	5	8	9	9	9	9	9	9	9	9	9	9	9
PT 1	0	0	0	0	0	0	0	1	1	2	3	4	5	5	5	6	6	7	7
SPL 90	0	0	0	0	0	0	1	3	3	4	5	5	6	6	6	7	7	7	7

**The infested plants were observed for BPH feeding damage rated using a 1-9 scale (1=no damage symptoms; 3=slight damage, pale outer leaves; 4-5=wilting on 50% leaves, slight stunting; recovery possible if insects removed; 7=Severe hopperburn, only one or two leaves green, no recovery possible; 9=Highly susceptible, complete wilting)

(a) (b) (c)

Figure 1 Rice cultivars infested with BPH in cages conditions for 19 days (a) Resistant cultivars (b) Moderately resistant cultivars (c) susceptible cultivars.

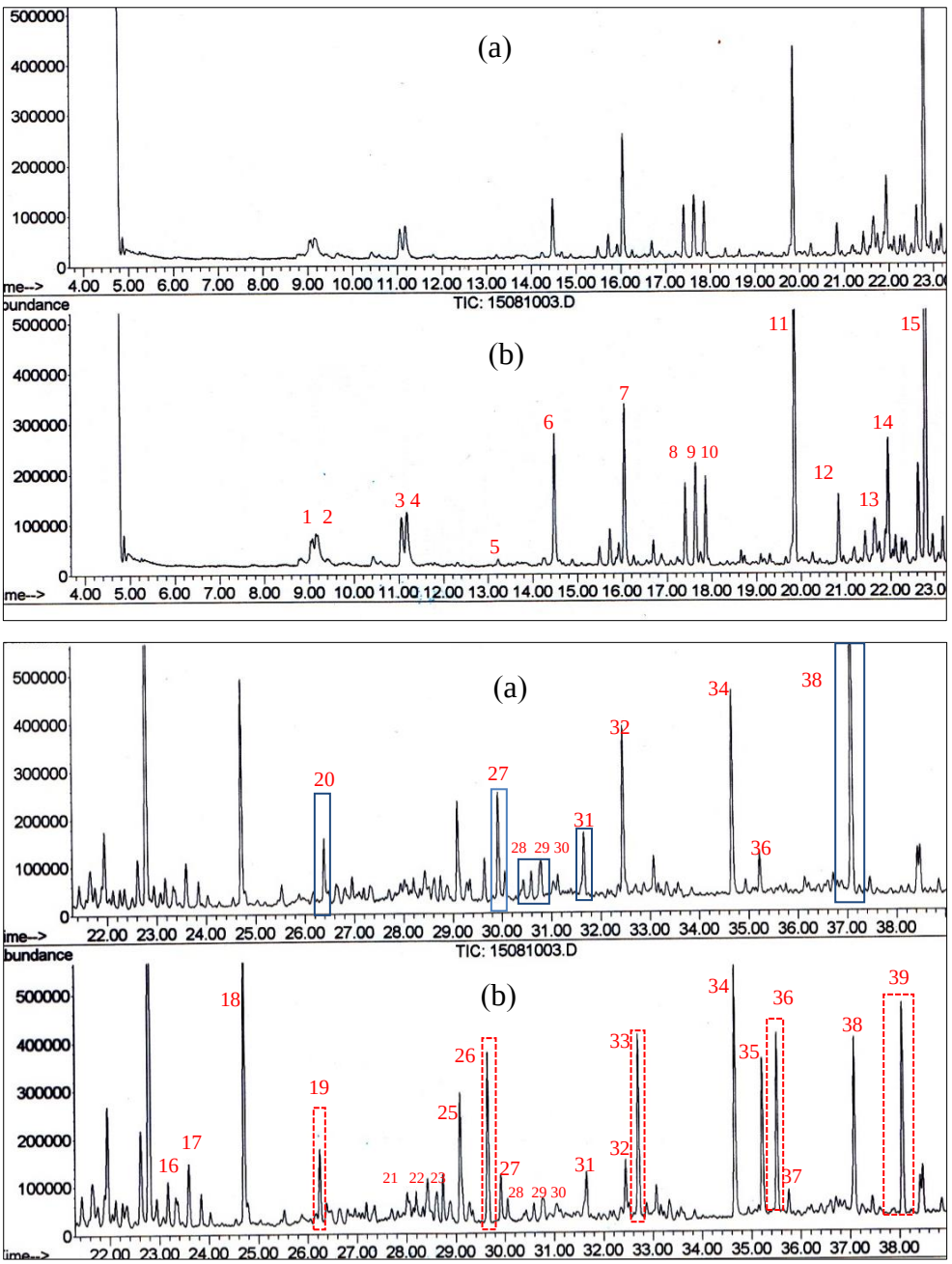

Figure 2 Chromatogram of differential emission of volatile compounds of resistant cultivar (PTB 33) (a) without BPH infestation (b) BPH infestation for 19 days at tiller stage. Solid line (____) represent the increase of volatile emission in rice infestation with BPH; Dot line (.....) represent the increase volatile emission in rice without BPH infestation (control).

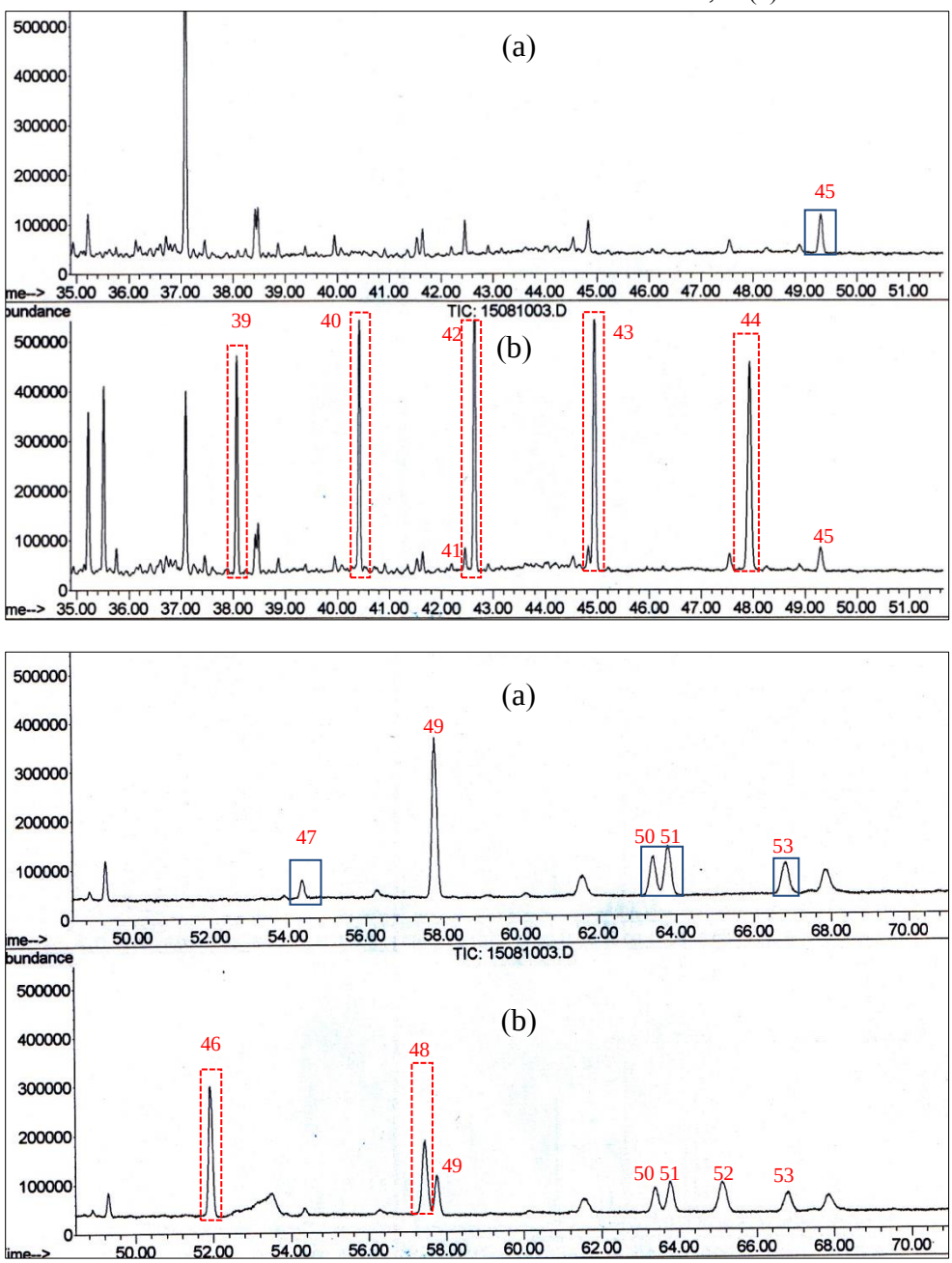

Figure 2 (continued) Chromatogram of differential emission of volatile compounds of resistant cultivar (PTB 33) (a) without BPH infestation (b) BPH infestation for 19 days at tiller stage. Solid line (____) represent the increase of volatile emission in rice infestation with BPH; Dot line (.....) represent the increase volatile emission in rice without BPH infestation (control).

Table 2 Identification of volatile compound in resistant cultivars (PTB 33) after BPH infestation compared to the control

Peak number	RT	Chemical name	Emission level	
			Control	infestation
1	9.064	Nonane	✓	✓I*
2,6	9.128,14.479	Dodecane	✓	✓I
3	11.055	1-Dodecene	✓	✓I
4	11.177	Ethanone	✓	✓I
5	13.221	Naphtho[2,3-b]furan-4,9-dione	✓	✓I
7	16.045	Benzene or/(1,3-bis(1,1-dimethylethyl)-	✓	✓I
8	17.401	1-Undecene	✓	✓I
9-10	17.628,17.85	Cyclopentane	✓	✓I
11	19.847	Tetradecane	✓	✓I
12	20.836	Ethanone	✓	✓I
13	21.948	N-METHYL-2-THIENYL-PYRROLE	✓	✓I
14	22.618	5-Methyl-4,7,10,13-tetraoxatetradecan-2-ol	✓	✓I
15	22.764	Phenol or / 2,4-bis(1,1-dimethylethyl)	✓	✓I
16,21	23.171 28.009	Cyclohexane	✓	✓I
17	23.596	Undecane	✓	✓I
18	24.720	Hexadecane	✓	✓I
19	26.245	naphtho[2,3-b]furan-4,9-dione	ND.	✓
20	26.379	1,6,6-Trimethyl-8-oxabicyclo[3.2.1]octan-2-one	✓	ND
22	28.429	Cyclohexane	✓	✓I
23	28.621	Cyclopentane	✓	✓I
24	28.743	2,5,8,11,14,17-Hexaoxaoctadecane	✓	✓I
25	29.092	Octadecane	✓	✓I
26	29.645	1-methylethyl tetradecanoate	✓	✓I
27,28, 38	29.919,30.42, 37.069	NEOPHYTADIENE	✓I	✓

Table 2 (continued) Identification of volatile compound in resistant cultivars (PTB 33) after BPH infestation compared to the control

Peak number	RT	Chemical name	Emission level	
			Control	infestation
29	30.583	Dibutyl phthalate	✓I	✓
30	30.781	2H-Cyclopenta[b]furan-2-one	✓I	✓
31	31.654	7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione	✓I	✓
32-33,37	32.714 32.735 35.753	Naphtho[2,3-b]furan-4,9-dione	ND	✓
34	34.647	E-15-Heptadecenal	✓	✓I
35	35.211	Phytol	ND	✓I
36	35.508	1,13-dibrom-7-thiatrideca-3,10-diyne	ND	✓
39-40 43-44	38.059 40.393 44.911 47.886	Naphtho[2,3-b]furan-4,9-dione	ND	✓
41	42.454	1,2-Benzenedicarboxylic acid	ND	✓
42	42.606	1,13-dibrom-7-thiatrideca-3,10-diyne	ND	✓
45	49.301	Nonacosane	✓	✓I
46,48,52	51.915 57.441 65.091	Naphtho[2,3-b]furan-4,9-dione	ND	✓
47	54.349	gamma.-Tocopherol	✓I	✓
49	57.784	alpha.-Tocopherol	✓I	✓
50	63.391	Stigmasta-5,23-dien-3.beta.-ol	✓I	✓
51	63.781	1,30-Triacontanediol	✓	✓I
53	66.809	Stigmasterol	✓I	✓

I = BPH-induced volatile increased emission; ND = not detectable;
✓ = volatile emissions were found in both cultivars.

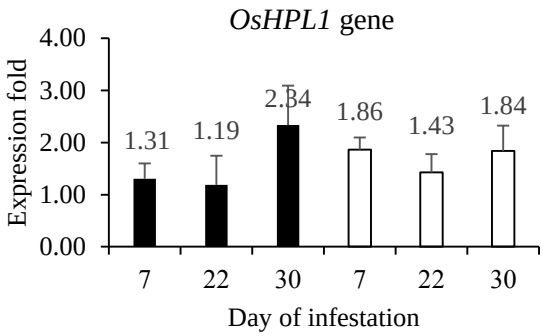

Figure 3 *OsHPL* gene expression ratio compare to the control during rice infested by BPH ■ = PTB33 cultivar (resistant cultivar), □= TN1 cultivar (susceptible cultivar)

We also found that the rice *HPL* gene up-regulated in response to BPH infestation (**Figure 3**). The HPL gene expression ratio of rice infested with BPH increased 1.31,1.19 and 2.34-fold at 7, 22 and 30 days of infestation, respectively. Similarly, Wang, B. et al. (2015b) reported the up-regulated *HPL* gene in rice response to BPH. The antisense expression vector of OsHPL-3 (*as*-HPL reduce the gene expression by 48.31-52.56% (Wang, B. et al., 2015b).

CONCLUSIONS

This research successfully identified induced volatile compounds emitted from rice-treated BPH. Alkane (nonane, dodecane, cyclopentane, tetradecane, cyclohexane and nonacosane) is the main component of volatile compounds, while naphtho[2,3-b]furan-4,9-dione was significantly only found in rice-treated BPH. Those functions may induce the volatiles to attract the egg parasitoids or they directly attack BPH as a pesticide. The HPL gene showed increased expression, but GLV was not found in this study, however.

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