Monoterpenoid Allelochemicals in Resistance Rice Varieties against Brown Planthoppers, Nilaparvata Lugens (Stål)

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***Abstract*—Terpenoid compounds in resistant rice varieties that acted as allelochemicals affecting feeding behavior of the brown planthoppers (BPHs) were extracted by solid phase microextraction (SPME) and analyzed by using gas chromatography-mass spectrometry (GC-MS). Comparative study of terpenoid profiles of susceptible Khao Dawk Mali 105 (KDML105), resistant Rathu Heenati (RH), and their isogenic lines (IL) IL162, IL302, and IL283, was performed. Six monoterpenoids which were (E)-citral, citronellal, (E)-geraniol, β-citronellol, citronellyl acetate and geranelyl acetate, were detected only in leaves of the resistant and their 3 isogenic line rices. Antibiosis, antixenosis and tolerance experiments suggested that the RH and its 3 isogenic line rices had higher level of antibiosis, antixenosis and tolerance against BPHs than KDML105. Moreover, spraying 6 standard monoterpenoids on susceptible Taichung Native 1 (TN1) rice could decrease feeding activity of BPHs.**

***Index Terms*—brown planthopper, rice, GC-MS, monoterpenoid, antixenosis, antibiosis**

I. Introduction

Rice (genus *Oryza*) is one of the most important food for over half of the world population. In Thailand, most researches on rice are aimed to enhance production yield and to improve grain quality. In rice fields, insects and diseases cause problems and are the most constraints to rice production, brown planthoppers (*Nilaparvata lugens* (Stål), BPH) are a primary insect pest of cultivated rice (*Oryza sativar L.)* [1], [2]. The insects cause economic damage to the rice crop directly by feeding and also indirectly by transmitting grassy stunt and ragged stunt virus diseases [3]. Heavy BPHs infestations rapidly cause leaves to turn orange-yellow and then become brown and wither. Insecticides applied on seed beds to restrict plant hoppers poison the environment and ecosystem. Overdoes of insecticides and mono-culture of a single resistant rice are the main causes leading to numerous outbreaks of *N. lugens* [4]-[6]. Therefore, finding new resistant rice cultivars is possibly an alternative systematic approach to avoid or lessens the use of pesticides. Some researchers reported that expression of snowdrop lectin gene (Galanthus nivalis agglutinin; GNA) in transgenic rice plants may provide resistance to rice BPHs [7]. A more recent report suggested that multiple BPH resistant genes may be a further model for resistant rice breeding system [8].

In view of plant resistance based on chemicals defense, most previous studies have focused on the rice volatile synthesized by resistant cultivar [9]. Extensive studies of alleochemicals in rice plants from seeding [10], [11], root tissues [12]-[14], and hulls [15] have led to identification of a range of flavones, diterpenoids and other types of compounds. These findings suggest that rice may produce and release alleochemicals that aid in the defense system against weeds and pathogens. Rice allelopathy can potentially be used to improve weed and rice pathogen management in rice production. However, few studies on defensive phytochemicals have been conducted on the interaction between insects and rice crops.

Monoterpenes and sesquiterpenes are usually the majority of plant volatile compounds released after herbivore damage, and they may play a role in attracting arthropods that prey upon or parasitize herbivores, thus minimizing further damage to plant tissues and associated with the higher plant resistance to insects which have the effects of deterrence, antifeeding, and toxicities [16]. The identification and characterization of the volatile compounds usually involve with separation and subsequent analysis by gas chromatography-mass spectrometry (GC-MS).

This research was aimed to comparatively study the terpenoid profiles of susceptible, resistant and isogenic

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line rice varieties emphasizing on those defensive volatile compounds. Meanwhile, the behavioral responses of BPHs on each terpenoid compounds were subsequently investigated in terms of feeding activities. The chemical features of the resistant mechanism to BPH were discussed in detailed.

II. Materials and Methodology

A. Plant Materials and Insects

The rice samples used in this experiment consisted of susceptible varieties, Khao Dawk Mali 105 (KDML105) and Taichung Native 1 (TN1), and a resistant variety, Rathu Heenati (RH) rice. The IL162, IL283, and IL302 rices are isogenic lines derived from cross breeding between RH and KDML105. Rice plants at seedling and tillering stages were grown for 7 and 30 days, respectively in the experimental farm, Kampaengsang Campus, Kasatesart University, located in central Thailand. The temperature, photo-period and humidity was controlled inside the green house.

Brown planthoppers (BPHs) were originated from the insect population collected from Ubon Ratchathani province, the north-east Thailand. The insects were reared on young seedlings of a susceptible cultivar, TN1 following the method of Yushima *et al.* [17]. All insect cultures were maintained under controlled conditions (approx. 25 ℃ and 70% relative humidity) inside a nylon mesh cage. For mass rearing of BPH populations, adults BPH were reared on seedling of TN 1 inside a wire screen cage measuring 1 ×1 ×1 m in the greenhouse.

B. Extraction, Separation and Identification of Rice Terpenoids

1) Solid phase microextraction (SPME)

A 50/30 μm divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/PDMS) fiber was used to extract the terpenoids of rice samples. This fiber was thermally conditioned prior to desorption at 230 ℃ in an injection port of GC for 30 min to reduce bleeding and to prevent the sample contamination. Five grams of rice leaves was placed in a 250 ml Duran bottle.The sample was extracted at 75 ℃ for 20 min. Then, the fiber was inserted to absorb the volatile compounds for 30 min and was subjected to GC-MS for further component analysis.

2) GC-MS analysis

The capillary column used was AT-5MS (%5 phenylmethylpolysiloxane) with dimension of 30 m × 0.25mm i.d. and 0.25 μm film thickness. The oven temperature was initially held at 45 ℃, then increased at a rate of 2 ℃/min to a final temperature of 230 ℃ which was maintained for 13 min. The injector temperature was 230 ℃. Purified helium was used as the carrier gas at a flow rate of 1 ml/min. Electron impact (EI) mass spectra were collected at 70 eV ionization voltages over the range of m/z 29-550. The ion source and quadrupole temperatures were set at 230 and 150 ℃, respectively.

C. Antixenosis, Antibiosis and Tolerance Activities

1) Antixenosis of seedling

Antixenosis experiment was modified from the previous method by Khan and Saxena [18]. An experiment of orientation response of BPHs to the rice verities was performed by comparing four rice varieties at seedling stage with KDML105. The three seedlings of each set of two different rice cultivars were oppositely planted in the chamber. At 10 days, 10 second instar nymphs were released on the seedlings. The number of nymphs settling on each seedling was counted at 0, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, and 96 hrs.

2) Antibiosis of tillering rices

Antibiosis was determined by collecting their honeydew droplets on the filter papers disk [19]. The five BPH adults were allowed to feed on each rice plant for 24 hrs. At the end of experiment, the filter papers were collected and total areas of blue-green spots resulted from honeydew deposition were measured using Motic picture advance program for windows.

3) Assessment of plant tolerance

Standard Evaluation System for Rice (SES) was applied to estimate the initial damage of the BPHs. The SES is the BPH damage score to rice plant that ranged from 0 to 9 and can be considered by the investigator using the following scale: 0 = no damage, 1 = very slight damage, 3 = first and second leaves of most plants partially yellowing, 5 = pronounced yellowing and stunting or about 10 to 25% of the plants wilting, 7 = more than half of the plants wilting or dead and remaining plants severely stunted or dying, 9 = all plants dead. Ten insects at the same age were collected from the cage using aspirator. The insects were oven dried at 70 ℃ for 48 hrs and weighed. The infested and un-infested plants were removed from all cage within cubed sponge and their roots. The plant height was also observed as the co-factor of specifically different rice plant characteristics. The plant samples were air dried for 3 hrs, and oven dried at 70 ℃ for 48 hrs and weighed. Evaluation of tolerance level was calculated using functional plant loss index (FPLI), tolerance index (TI) as described by Panda and Heinrichs [20]. The FPLI and TI were calculated as followed:

FPLI = [1-(Dry weight of infested plant/Dry weight of un-infested plant)] × 100

TI = BPH dry weight on test line/BPH dry weight on susceptible check, KDML105

D. Effect of Defence Volatile Compounds on Feeding Rate of BPHs

Some selected terpenoid compounds identified in leaves of the resistant rice that have potential of being defensive chemicals were subjected to further investigation based on the above method for antibiosis of tillering. Honeydew collections were performed for these standard compounds by varying amount at 0.1-5.0 mg/mL spread on tillering of TN 1 rice. IC_{50} of terpenoid compounds against feeding of BPHs were calculated.

E. Statistical Analysis

Analysis of variance was used to find the level of significant differences due to orientational responses and feeding activity of BPH on resistant and susceptible rice cultivars were employed with the level of significance set for $P < 0.05$. All statistical analysis were conducted using variance (ANOVA) test of CropStat version 7.2.3 (CropStat, IRRI).

TABLE I. PERCENTAGE OF RELATIVE ABUNDANCE OF THE IDENTIFIED TERPENOID COMPOUNDS OF LEAVES OF SUSCEPTIBLE RICE CULTIVARS, KDML105, RESISTANCE RICE CULTIVAR, RATHU HEENATI, AND THE 3 ISOGENIC LINE RICE CULTIVARS, IL162, IL283, IL302.

Assignment compounds	Tr	I[a]	I[b]	% Relative abundance				
				Rathu Heenati	IL162	IL283	IL302	KDML105
Linalool	19.600	1101	1097	0.0104	0.0004	0.0235	0.0412	0.0003
Isopulegol	22.690	1146	1150	0.0021	0.0004	0.0002	0.0077	-
Citronellal	23.140	1153	1153	0.1884	0.0022	0.0003	0.1190	-
β-cyclocitral	27.280	1215	-	0.0033	0.0087	0.0082	0.5550	0.0001
β-citronellol	28.270	1229	1226	0.0015	0.0003	0.0002	0.0455	-
(E)-citral	28.740	1236	1238	0.0004	0.0030	0.0014	0.0453	-
(E)-Geraniol	29.790	1252	1253	0.0051	0.0007	0.0008	0.0478	-
Geranial	30.765	1266	1267	0.0011	0.0021	0.0027	0.0033	-
α-Cubebene	35.628	1340	1351	0.0042	0.0011	0.0010	0.0175	0.0002
Citronellyl acetate	36.086	1347	1353	0.0052	0.0028	0.0033	0.0077	-
α-Copaene	37.416	1367	1377	0.0140	0.0003	0.0022	0.0217	0.0001
Geranyl acetate	37.921	1375	1381	0.0075	0.0002	0.0019	0.0029	-
β-Cubebene	38.218	1380	1388	0.0135	0.0022	-	-	-
β-Elemene	38.390	1382	1391	0.0002	-	-	0.0231	0.0027
(E)-Caryophyllene	40.106	1409	1409	0.0326	0.0036	0.0025	0.0865	0.0029
Epi-bicyclosesquiphellene	40.759	1420	1435	0.0009	0.0002	0.0001	0.0517	0.0002
α-Bergamotene	41.092	1425	1435	0.0002	0.0004	0.0004	0.0040	0.0004
(E)-Geraylactone	42.214	1443	1455	0.0007	-	-	0.0667	-
α-Humulene	42.303	1445	1445	0.0022	0.0034	0.0033	-	-
(E)-β-farnesene	42.529	1448	1457	0.0007	0.0008	0.0006	0.0305	0.0060
γ-Muurlene	43.633	1466	1480	0.0012	0.0005	0.0003	0.0285	0.0011
β-Ionone	43.946	1471	1489	0.7	0.0103	0.0172	1.5612	0.0230
β-Ionone epoxide	44.099	1474	1485	0.0021	-	-	0.2451	0.0073
Aromadendreane	44.524	1481	1493	0.0002	0.0002	0.0022	0.0409	0.0073
α-Zingiberence	44.999	1487	1495	0.0009	0.0013	0.0011	0.0191	0.0003
α-Muurolene	45.391	1494	1500	0.0002	0.0004	0.0003	0.2261	0.0002
β-Bisabolene	45.914	1503	1506	0.0021	0.0010	0.0008	0.0087	0.0015
δ-Cadinene	46.359	1511	1514	0.0058	0.0818	0.1072	-	0.0355
1s-Calamene	46.531	1514	1514	0.0016	-	-	0.0296	-
β-Sesquiphellendreane	46.721	1517	1523	0.0008	0.0021	0.002	0.0061	0.0022
(E)-γ-Bisabolene	46.846	1519	1531	0.0005	0.0003	0.0002	0.0660	0.0003
Calacorene	47.582	1532	1546	0.0003	0.003	0.0024	0.0315	0.0001
Nerolidol	49.037	1557	1563	0.0009	0.0005	0.0005	0.0168	0.0022

Tr: Retention time using AT-5MS column

I[a]: Retention indiece using AT-5MS column

I[b]: Retention indiece using KI and Literature

III. Results and Discussions

A. Identified Terpenoids Involving in Rice Resistance

The identification of terpenoid compounds was mainly performed by mass spectral data comparison with database libraries, viz. Adams 2001, NIST 98 and Wiley 275, supported by the linear temperature program retention indices data (LTPRI), which were calculated from retention times on the GC-MS. In addition, retention indices (RI) calculated by applying the C_8 to C_{22} n-paraffim hydrocarbon mixtures diluted in dichloromethane were compared with the published Kovates indices [21], to aid structural confirmation. The results showed separation of at least 33 terpenoids in all rice samples (Table I). These identified terpenoids were classified in groups of monoterpenoids and susquiterpenoids including linalool, isopulegol, citronellal, β-cyclocitral, β-citronellol, (E)-citral, (E)-geraniol, geranial, α-cucebene, citronellyl acetate, α-copaene, geranyl acetate, β-cubebene, β-elemen, (E)-caryophyllene, epi bicyclosesquiphellene, α-bergamotene, (E)-geraylactone, α-humulene, (E)-β-farnsene, γ-muurlene, β-ionone, β-ionone epoxide, aromadendreane, α-zigiberene, α-muurolene, β-bisabolene, δ-cadinene, 1s-calamine, β-sesquiphellendreane, (E)-γ-bisabolene, calacorene and nerolidol. Interestingly, some potential repellants, such as (E)-citral, (E)-geraniol, β-citronellol, β-citronellal, citronellyl acetate and geranelyl acetate were consistently detected in leaves of the resistant rice, RH and the 3 isogenic line rices. Previous studies suggested that these 6 terpenoids have been considered as attractive chemicals communicating to other insects. It is commercially well known that these terpenoids, possess activity against mosquitoes, lice, and some other household insects [22]-[24]. The prevalence of such chemicals in the resistant rice, RH and the 3 isogenic line, clearly support their roles in the defense mechanism of rice against insect pests.

B. Antixenosis, Antibiosis and Tolerance Activities

1) Antixenosis of seedling

The antixenosis activity of the seedlings was evaluated in terms of orientation response of BPHs. The proportions of insects in a chamber within 2 choices of rice plants should be 1:1 and no surrounding factors affected the insect's movement ability except the host plant factor itself. The percentage of BPHs was calculated from the ratio of numbers of settled BPHs on each rice variety. Orientation responses of BPH to KDML105, IL162, IL283, IL302, and RH rice seedling are presented in Fig. 1. As for the results, at the initial time, there was no significant difference in clustering of the insects on each rice cultivar. For RH and KDML105, BPHs started to move to KDML105 rice seedling after 4 hrs. But for isogenic rice varieties, BPHs tended to relocate from isogenic rice to KDML105 after 36 hours of the experiment. The different numbers of BPHs between the two varieties were increased until 96 hrs, which no BPH was observed on the resistant varieties.

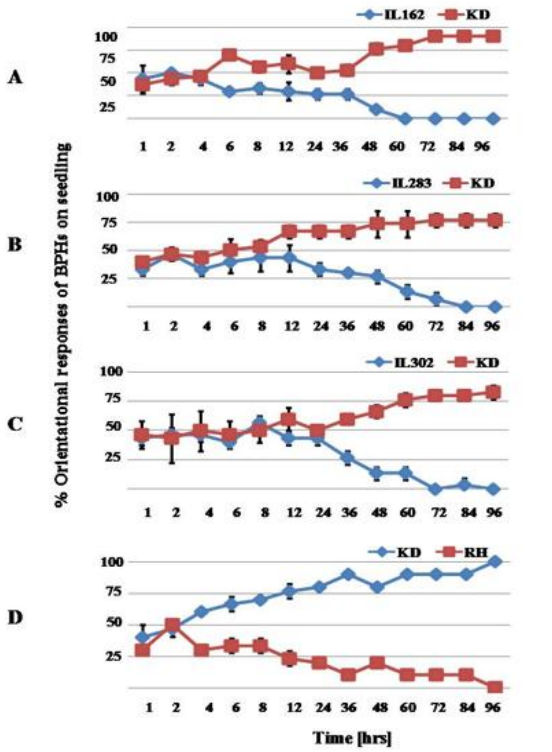

Figure 1. Orientational responses of BPHs of rice. A: IL162: KD (KDML105), B: IL283: KD (KDML105), C: IL302:KD (KDML105), D: RH:KD(KDML105).

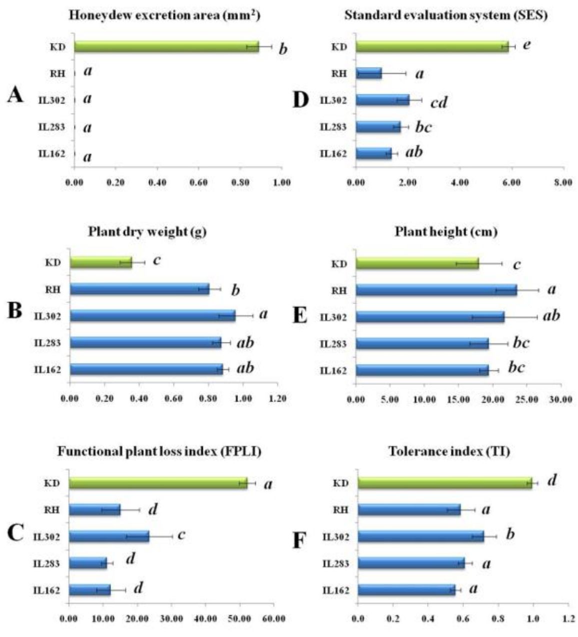

Figure 2. Comparison of antibiosis of seedling, standard evaluation system (SES), plant dry weight, plant height, functional plant loss index (FPLI) and tolerance index (TI) on difference varieties.

2) Antibiosis of tillering rices

The BPH feeding rate was determined by the amounts of the excreted honeydew. The excreted honeydew is directly proportional to the feeding rate. Comparison of blue-green spot area on filter paper disks showed that female adult BPHs fed on control susceptible TN1 rice (1.62±0.03) and KDML105 rice (1.27±0.08). Whereas the feeding activity of female adults BPHs significantly decreased on resistant RH and the 3 isogenic line. The results suggested that the rate of honeydew excretion of KDML105 was higher than the resistance rice and its isogenic lines.

3) Assessment of plant tolerance

FPLI and TI were applied in this study to investigate the BPH damages (Fig. 2 and Table II). FPLI revealed the loss of rice plant biomass due to the BPH attack. TI also gave the conviction of the FPLI by pointing to the facilitation of plant to insect growth. The SES of damaged rice by BPH was applied as one of co-factors to the FPLI. After 7 days of infestation, the experiment was stopped since all susceptible TN1 died. The KDML105 revealed the highest amount of damage by BPH with a SES value at 5.58±0.25 followed by IL302 at 2.05±0.48, IL283 at 1.72±0.28, IL162 at 1.37±0.22, and RH rice at 1.00±0.92. The lowest FPLI was noticed in IL283 at 11.12±1.71%. KDML105 showed the highest percentage of FPLI at 52.20±2.40%. The TI was calculated from the BPH dry weight on test line divided by the BPH dry weight on susceptible TN1. The results revealed that RH (0.58±0.07), IL162 (0.55±0.02), IL283 (0.6±0.04), and IL302 (0.71±0.06) could defy the BPH infestation. Nevertheless, TN1 and KDML105 showed the lowest tolerance at the same value as 0.99±0.02. The highest FPLI and TI values not only were observed in TN1 but also in KDML105. From this experiment, the results proposed that IL162, IL283, and RH rice could resist BPH attack at the same level ($p<0.05$), whereas, IL302 also withstand the infestation at the lower level than those 3 rice cultivars.

4) Effect of some volatile compounds on feeding rate of BPHs

The predominant 6 volatile terpenoids identified in the leaves of rice resistant and their isogenic lines were individually subjected to further investigate of antibiosis. These were (E)-citral, (E)-geraniol, β-citronellol, β-citronellal, citronellyl acetate and geranelyl acetate. Each was sprayed separately on susceptible TN1 with concentration range of 0.1-5.0 mg/mL and analyzed for their IC_{50} from the excreted honeydew from female adult BPHs (Fig. 3). IC_{50} values of the 6 terpenoid compounds are presented in Table III. It could be concluded that, in terms of biological defense mechanism, the 6 terpenoids have their ability to reduce rate of BPHs feeding.

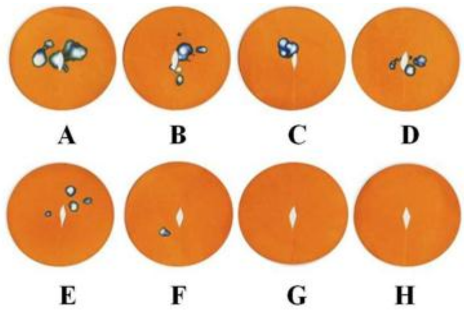

Figure 3. Visual demonstration of the honeydew spots on filter papers after female BPH adults fed on susceptible TN1 rice overspread (E)-citral at concentration A: TN1 (control), B: 0.1, C: 0.5, D: 1.0, E: 2.0, F: 3.0, G: 4.0and H: 5.0 mg/mL.

TABLE II. ANTIBIOSIS, FUNCTIONAL PLANT LOSS INDEX, TOLERANCE INDEX, PLANT HEIGHT, AND PLANT DRY WEIGHT OF RICE VARIETIES.

Rice varieties	Antibiosis of rices	Plant Tolerance				
	Area of HD (mm^2)	SES	Plant height	Plant dry weight	FPLI	TI
IL162	0.00±0.00^{a}	1.37±0.22^{ab}	19.42±1.35^{bc}	0.88±0.03^{ab}	12.26±4.16^{d}	0.55±0.02^{a}
IL283	0.00±0.00^{a}	1.72±0.28^{bc}	19.40±2.70^{bc}	0.87±0.05^{ab}	11.12±1.71^{d}	0.60±0.04^{a}
IL302	0.00±0.00^{a}	2.05±4.73^{ab}	21.70±4.73^{ab}	0.95±0.09^{a}	23.53±6.67^{c}	0.71±0.06^{b}
Rathu Heenati	0.00±0.00^{a}	1.00±0.92^{a}	23.55±3.13^{a}	0.80±0.06^{b}	15.11±5.51^{d}	0.58±0.07^{a}
KDML105	0.89±0.06^{b}	5.87±0.25^{e}	17.95±3.36^{c}	0.36±0.07^{c}	52.20±2.40^{a}	0.99±0.02^{d}

TABLE III. IC50 OF TERPENOIDS COMPOUNDS AGAINTS THE BPH.

Terpenoids (Defense volatile compounds)	IC_{50} (mg/mL)
Geranul acetate	0.6393
β-Citronellal	0.5382
(E)-Geraniol	0.5109
(E)-Citral	0.3593
Citronellyl acetate	0.1763
β-Citronellol	0.1099

IV. CONCLUSION

Volatile terpenoids in plants crucially take part in rice-BPH interaction. The results of chemical interactions between some rice varieties and the insect pest BPHs were evidenced in this study. It is revealed that the released volatiles in group of terpenoids from resistant rice possibly acted as alleochemicals and might be involved in the defense mechanism against BPHs. The GC-MS analysis of chemical components from leaves of RH and their isogenic cultivars showed the dominance of β-citronellal, follow by geranelyl acetate, citronellyl acetate, and (E)-geraniol, β-citronellol, and (E) citral. The existence of these terpenoids in RH and its 3 isogenic lines indicated their important role in the resistant mechanism of rice to BPHs. This may offer an alternative way to improve the resistance of rice to BPHs through the rice breeding program.

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