NMR Metabolite Profiling Analysis of Pigmented Rice Resistance to Rice Ear Bug

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Abstract

Rice ear bugs or Leptocorisa oratorius F. are major rice pests reported to reduce the rice yields severely. The present study aimed to screen local pigmented cultivars for resistance to rice ear bugs and perform metabolomics analysis to identify the metabolites responsible for the resistance character. For screening, eight cultivars including both black and red varieties were used. Rice ear bugs used for bioassay were collected from the field. The assay results showed that two cultivars of black rice and one cultivar of red rice exhibited high resistance characters as per the Standard Evaluation System guidelines for rice.

Metabolite profiling of rice seeds was performed at the milky stage using 500 Mhz NMR JEOL, followed by multivariate analysis with SIMCA ver 14. Metabolite profiling identified nine out of 15 metabolites, which were significantly different between the most resistant and susceptible cultivars. In the red rice, hydroxy-L-proline, threonine and formic acid and for black rice, valine, glutamate, α-glucose, β-glucose, galactinol and raffinose were identified as potential metabolites conferring the resistance character. This study identified the most resistant cultivars which can be used in the future to support the development of a novel line of cultivar resistant to rice ear bug.

Keywords: Biotic stress, metabolomics, multivariate analysis, *Leptocorisa oratorius*, black rice, red rice.

Introduction

Rice is among the three leading crops globally and is the staple food for more than half of its population. The fulfillment of rice increased global demands as staple food requires a fine balance between the final rice product's quantity and quality. In the past few years, pigmented rice has become quite popular among the Asian population including Indonesia's inhabitants. It has emerged as an alternative to white rice and this preference is majorly attributed to the health benefits conferred by pigmented rice. Pigmented rice is characterized by higher fiber levels, anthocyanin[34], micro-elements and macro-elements compared to white rice[19]. Pigmented rice was reported to have antidiabetic[5] and anticancer properties[17]. In addition to these, pigmented rice has been shown to exhibit a wide range of therapeutic activities including amelioration of iron deficiency anemia[7,56], antioxidant[18], anticarcinogenic, antiatherosclerosis and antiallergic activities[32,37]. All these properties of pigmented rice support its potency to be developed as a functional food.

Indonesia is home to a variety of local pigmented rice cultivars including both black and red rice[46]. In recent times, the pigmented rice agroindustry has emerged as an attractive alternative to increasing food demands. However, all the efforts aimed at increasing rice production suffer from issues of pests and diseases[6]. Chemical treatment using pesticides is not ideal as it does not fulfill the consumers' demand for healthy organic pigmented rice. Thus, there is an urgent requirement for developing a high-quality cultivar with high resistance to pests and diseases.

More than 70 species of insects including rice ear bugs have been identified to damage rice. Among these, only 20 species cause maximum damage and are considered as main pests[9]. The dominant rice ear bug species known to damage rice fields in Indonesia is *Leptocorisa oratorius* F.[40] The severity of this pest can be realized from previous reports, which stated that an outbreak of rice ear bug could result in complete devastation of rice crop, reducing rice yields up to/by 100 percent[21]. With current advances in agroindustry, an outbreak of rice ear bugs can still lose up to 50 percent. Thus, the rice ear bugs, *L. oratorius*, is one of the main pests demanding serious attention.

Since pigmented rice holds a side crop status, no official data is available for rice ear bug effects on pigmented rice yields. Rice ear bug was one of the most significant problems associated with pigmented rice farming based on observations made in the field and information provided by direct communication with local farmers. Thus, the present study aimed to screen pigmented rice cultivars for resistance to rice ear bugs and identify the metabolites involved in the resistance mechanism. For screening bioassay, eight pigmented rice cultivars were included in the study.

Metabolite profiling for the most resistant and susceptible cultivars was performed using NMR (Nuclear Magnetic Resonance)-based metabolomics approach. In recent years, the NMR metabolomics approach has been developed as a useful tool for screening stress-resistant traits among various plant populations[39]. The development of a premium variety of pigmented rice with resistance traits to various biotic stress is one of the strategies that will assist in establishing

pigmented rice as a primary crop for sustainable human consumption. Such measures will also support Integrated Pest Management (IPM) for green agriculture.

Material and Methods

Experimental design: Seeds of six pigmented rice cultivars were obtained from local breeders in the Yogyakarta region, Indonesia. For black rice, Melik, Pari Ireng, Cempo Ireng Gunung Kidul and Cempo Ireng Sleman cultivars were chosen as representatives, while for red rice included Inpari 24, Aek Sibundong, Segreng and RC 204 cultivars were used. All pigmented rice cultivars were grown in the screen house until the plants reached the milky stage period. For non-choice bioassay, cultivars were divided into three groups; one group was used for NMR-based metabolomics analysis without infestation, while the other two groups were infested with rice ear bug for NMR analysis.

Screening and determination of pigmented rice resistance status: The bioassay for screening resistance in local cultivars to rice ear bug infestation was performed in duplicate. For each bioassay, five replicates of each cultivar were arranged randomly. Rice ear bugs were collected from the paddy field in Yogyakarta and identification of bugs was performed by a trained entomologist from the Laboratory of Plant Pest, Faculty of Agriculture, Universitas Gadjah Mada. Rice ear bug infestation was performed at a ratio of 10:1, ten adult bugs for one plant. The bioassay commenced at the beginning of the milky rice stage and was continued for ten days. The severity of rice ear bug infestation was determined based on the formation of dark spots contributed by rice ear bugs activity. The scoring was performed according to SES (Standard Evaluation System for Rice, 2002) assessment developed by the International Rice Research Institute (IRRI), Los Banos, Philippines.

Extraction of plant material: The rice grains were ground into a fine powder using mortar and pestle under liquid nitrogen. The water was removed from the samples by freeze-drying. For NMR analysis, approximately 50 mg of powdered sample was used. The dried sample was further extracted using 1 ml of 50% $MeOD_4$ containing 0.01% w/w trimethylsilylpropanoic acid (TSP) [0.75 ml of $MeOD_4$ and KH_2PO_4 in D_2O phosphate buffer pH 6, 3-(trimethylsilyl) propionic-2,2,3,3-D_4 acid sodium salt, TSP]. The metabolites were extracted by vortexing the sample at room temperature for 2 min, followed by ultrasonication for 15 min. Further, the mixture was centrifuged at 13,000 rpm for 15 min to remove solid materials. For NMR, 800 µl of the resulting supernatant was transferred into a 5 mm NMR tube and analyzed in an NMR machine.

One- and two-dimensional NMR analysis: ^{1}H-NMR spectra were recorded at 500 MHz using JEOL NMR Spectrometer (JEOL, USA Inc.) according to the procedure reported by Nuringtyas et al.[39] Deuterated methanol was used as an internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans and 26 s acquisition time with a relaxation delay of 1.5 s. TSP was used as a reference at δ 0.00 ppm. Metabolite identification was conducted based on acquired ^{1}H-NMR spectra compared with the NMR spectra of the reference compounds and those available in published literature. Two-dimensional *J*-resolved NMR spectra were acquired using eight scans per 128 increments for the spin-spin coupling constant axis (f1) and 8 k for the chemical shift axis (f2) using spectral widths at 66 Hz and 5000 Hz respectively. A relaxation delay of 1.5 s was employed resulting in a total acquisition time of 56 min.

Processing NMR spectra and multivariate statistical analysis: Each of the acquired spectra was manually phased and baseline-corrected using MNOVA software version 11.02 (Mestrelab Research, Spain) and calibrated to the internal standard TSP. Mestrenova software was also used for bucketing analysis. All ^{1}H-NMR spectra were reduced and binned using a spectral width of δ 0.04 to form a region of δ -0.50–10.0. The regions corresponding to water (δ 4.70–4.90) and methanol (δ 3.23–3.36) were excluded. The clustering of the samples was performed using multivariate analysis. Principal Component Analysis (PCA) and Orthogonal Partial Least-Discriminant Analysis (OPLS-DA) were performed using SIMCA software version 14.0 (Umetrics, Sweden). OPLS-DA method was validated by permutation and CV-ANOVA. The scaling method used was the Pareto method.

Semi-quantitative data analysis: All metabolites were relatively quantified by plotting the mean area of metabolite peak relative to TSP using Mestrenova software (Escondido. CA, USA). The differences between resistant and susceptible cultivars of pigmented rice were evaluated by analysing variance (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Screening of pigmented rice resistance against rice ear bug: The bioassay was performed to screen pigmented rice cultivars for resistance against rice ear bug. Scoring data for all pigmented rice cultivars used in the study are summarized in table 1. Among eight rice cultivars screened in the study, two cultivars of black rice and one cultivar of red rice showed resistance to rice ear bug. Black rice cultivars showing the highest resistance character were Melik and Cempo Ireng Sleman. Among the red rice cultivars screened, Inpari 24 displayed the highest resistance character.

Pari Ireng, a black rice cultivar, was the most susceptible to cultivar among all the cultivars. Interestingly, none of the white rice cultivars showed resistance character against rice ear bug. This was following the reports of Maulana[33] and Suprihatno et al[50] which stated that there are no varieties of white rice having sufficient resistance to rice ear bug up till now. Thus, the resistant cultivar of pigmented rice obtained from this study can be used as a model strain to unravel the plant mechanism responsible for the observed resistance to rice ear bugs.

NMR metabolomics analysis of pigmented rice resistance against rice ear bug: The bioassay for the screening of resistant pigmented rice cultivars was followed by metabolomics analysis. Metabolomics analysis aimed to identify the potential metabolites involved in pigmented rice's resistance mechanism against rice ear bug. For metabolomics analysis, only the most resistant and susceptible cultivars were included in the study. For red rice, Inpari 24 and RC 204 were used while Melik, Cempo Ireng Sleman and Pari Ireng were included in the case of black rice. Previous studies from our lab reported that the type of metabolites present in rice and black rice differs. Thus, metabolomics analysis between red rice and black rice was performed separately[54].

Metabolomics analysis of red and black rice using ^{1}H-NMR identified 15 metabolites (Figure 1 and table 2). Two-dimensional analysis *J*-Resolved was also performed to resolve the overlapping signals. The identification of fifteen metabolites was performed by comparison of NMR signals with the previously published NMR studies.[8,10,20,24,23,31,38,39,41,52] Most of the identified metabolites were primary metabolites detected in the region δ 5.5–0.5. NMR analysis of pigmented rice samples was performed at the milky stage, an early stage in rice grain ripening. In this milky stage, the grains generally produce more primary metabolites required for proper growth and development[13].

Multivariate data analysis of ^{1}H NMR spectra and semi-quantitative analysis of metabolites present in red rice: The score plot analysis of the NMR spectra for red rice showed good separation between the resistant and susceptible cultivars with a variance value of PC1 57 % and PC2 22% (Figure 2A). The loading plot revealed the metabolites that contributed to the separation of red rice between susceptible and resistant cultivars. These included alanine, glutamate, valine, acetate, succinic acid, formic acid, fumaric acid, α-glucose, raffinose andadenosine (Figure 2B).

Table 1
The scores non-choice bioassay of pigmented rice resistance against *Leptocorisa oratorius* F.

S.N.	Cultivar	Colour	% Resistance	Status*
1	Pari Ireng	Black	26.77	Susceptible
2	IR 64	White	13.23	Susceptible
3	RC 204	Red	13.07	Susceptible
4	Ciherang	White	11.46	Moderate
5	Gunung Kidul	Black	10.21	Moderate
6	Aek Sibundong	Red	9.96	Moderate
7	Segreng	Red	9.89	Moderate
8	Inpari 24	Red	7.44	Resistant
9	Sleman	Black	6.63	Resistant
10	Melik	Black	5.22	Resistant

*the assessment was based on SES IRRI (2002)

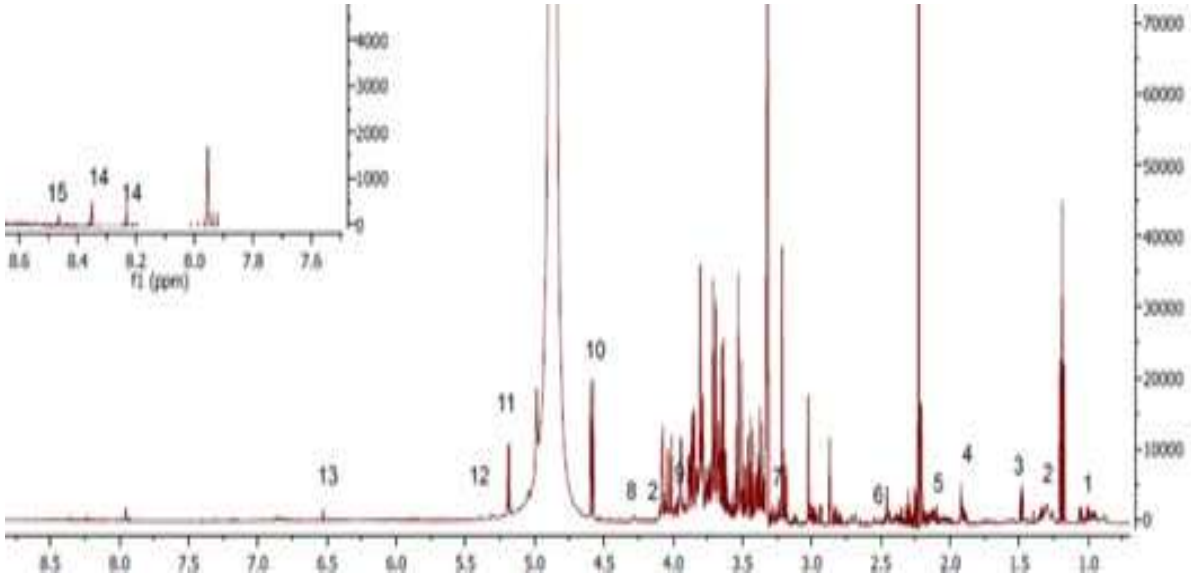
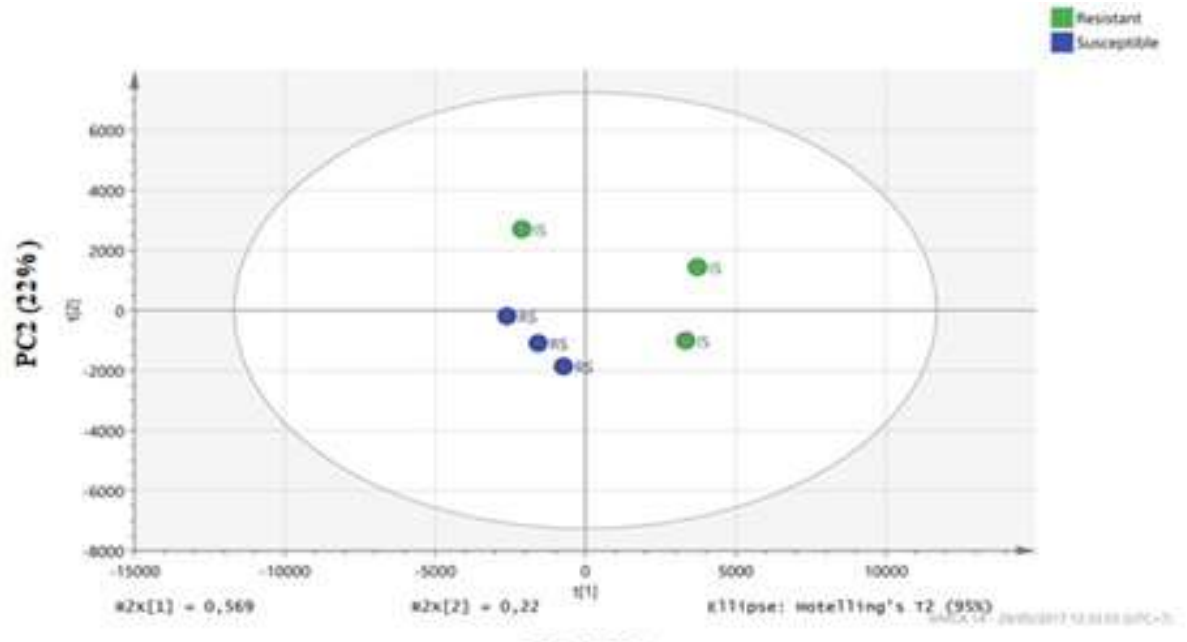
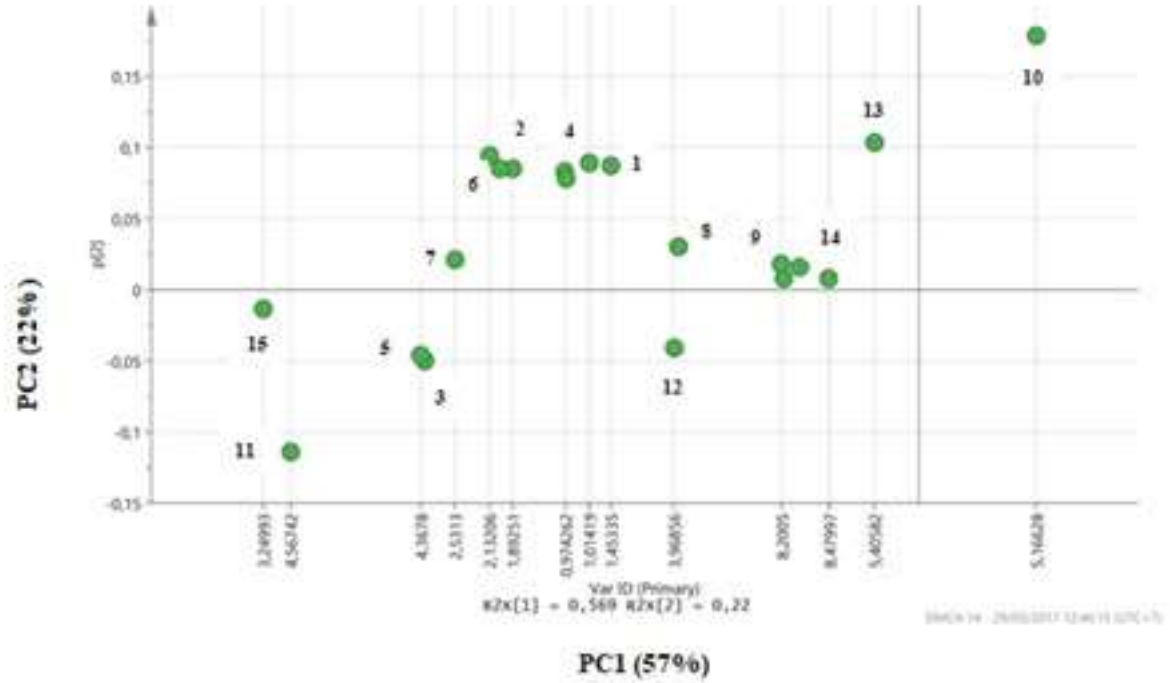


Figure 1: The NMR spectra of metabolites identified in pigmented rice. 1) Valin 2) Threonine 3) Alanine 4) Acetic acid 5) Glutamate 6) Succinic acid 7) Choline 8) Hydroxy-L-proline 9) Galactinol 10) β – Glucosa 11) α – Glucosa 12) Raffinose 13) Fumaric acid 14) Adenosine 15) Formic acid



(a)



(b)

Figure 2: A. The PCA Score Plot Analysis of Red Rice. IS = Inpari 24, RS = RC 204; B. PCA Loading Plot Analysis of Red Rice. 1) Alanine 2) Glutamate 3) Threonine 4) Valine 5) Hydroxy-L-proline 6) Acetate 7) Succinic acid 8) Formic acid 9) Fumaric acid 10) α-glucose 11) β-glucose 12) Galactinol 13) Raffinose 14) Adenosine 15) Choline

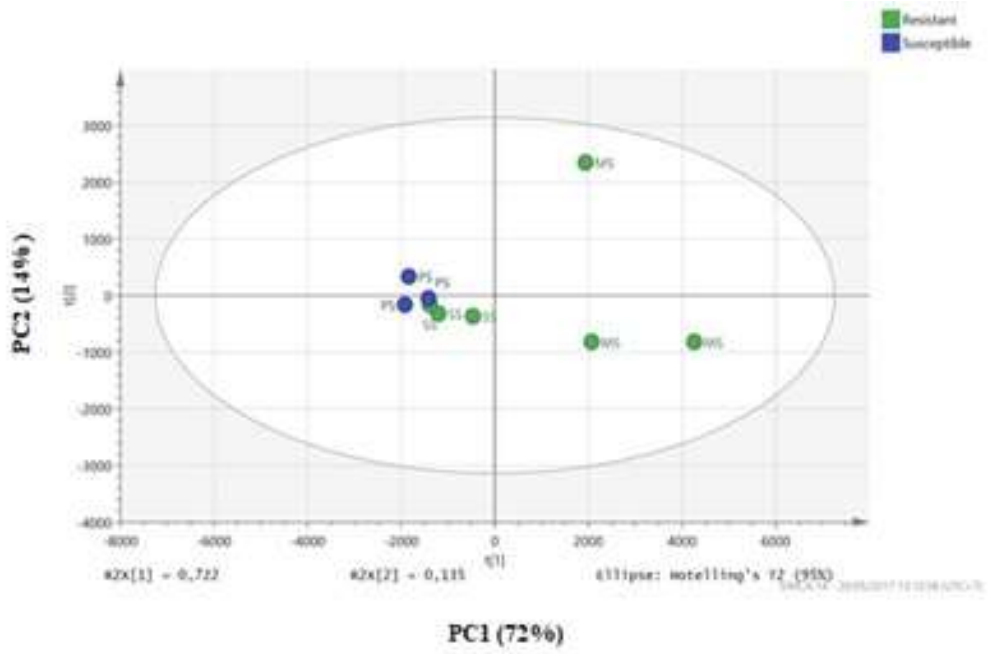
In NMR analysis, the concentration of a compound can be measured by calculating the corresponding compound's signal area compared to the area of an internal standard. Higher hydroxy-L-proline, threonine and formic acid levels were observed in Inpari 24 (a resistant cultivar) compared to the susceptible cultivar (Figure 4).

In plants, hydroxy-L-proline is known to be one of the crucial metabolites playing a role in the cell wall structure[2]. Previous studies have suggested that the high hydroxy-L-proline levels might support the formation of a stronger cell wall, thus making plants more resistant to pests and pathogens.

In addition to hydroxy-L-proline, Inpari 24 also showed high levels of threonine and formic acid. Threonine has been previously reported to improve plant defense against pathogens[48]. In comparison to this, formic acid was reported

to exhibit antibacterial activity[12,44]. These results suggested these metabolites' role in plant defense mechanisms against biotic stress, especially rice ear bugs infestation.

Multivariate data analysis of ^{1}H NMR spectra and semi-quantitative analysis of metabolites present in black rice: Score plot of the NMR spectra for black rice showed good separation between the resistant and susceptible cultivars with a variance value of PC1 72 % and PC2 14 % resulting in a total variance of 86 % (Figure 3A). Melik (resistant cultivar) was clustered away from Pari Ireng (susceptible cultivar). However, a clear separation was not achieved in the case of Cempo Ireng Sleman (the resistant cultivar) and the susceptible cultivar. This might be contributed by the use of different defense strategies by these cultivars. Besides chemical defense, the plant also utilizes other defense strategies against biotic stress including genetic and structural defense systems[26].



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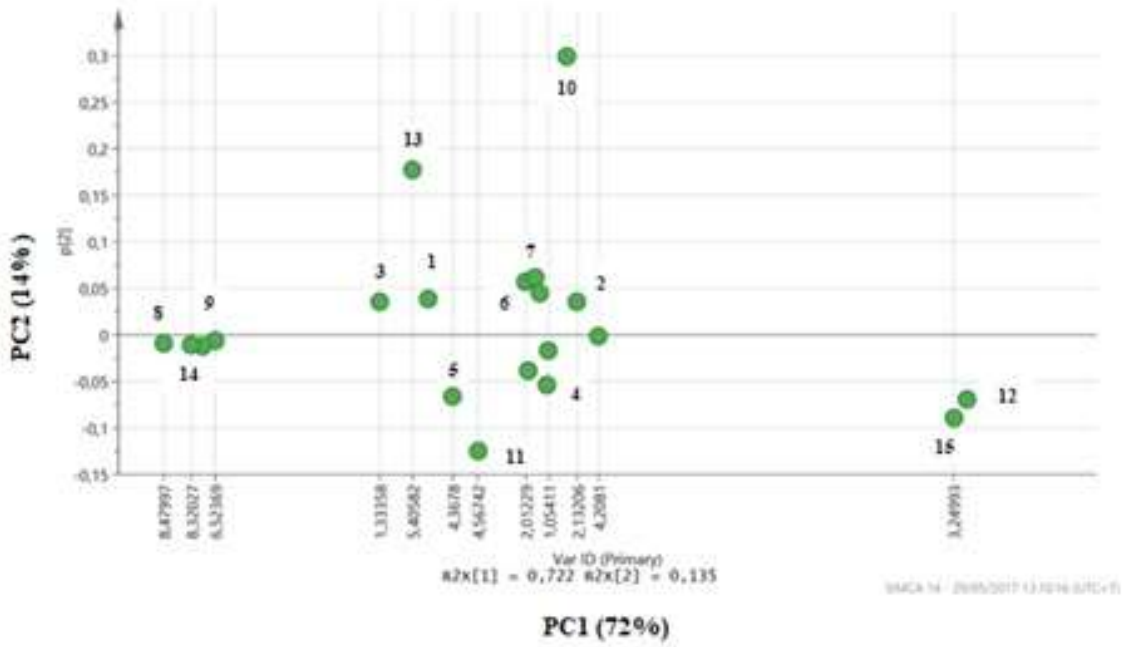

(b)

Figure 3: A. The PCA Score Plot Analysis of Black Rice. MS = Melik, SS = Sleman, PS = Pari Ireng and B. The PCA Loading Plot Analysis of Black Rice. 1) Alanine 2) Glutamate 3) Threonine 4) Valine 5) Hydroxy-L-proline 6) Acetate 7) Succinic acid 8) Formic acid 9) Fumaric acid 10) α-glucose 11) β-glucose 12) Galactinol 13) Raffinose 14) Adenosine 15) Choline

Table 2
The chemical shift of metabolites identified from H NMR spectra of Pigmented rice leaves extracted using $MeOD_4$

S.N.	Metabolite	Chemical shift
1	Valine	δ 1.02 (d, *J*=7.0 Hz), δ 1.06 (d, *J*=7.0 Hz), δ 1.00 (d, *J*=6.8 Hz)
2	Threonine	δ 1.34 (d, *J*=7.0 Hz), δ 4.22 (m)
3	Alanine	δ 1.46 (d, *J*=7.0 Hz)
4	Acetate	δ 1.90 (s)
5	Glutamate	δ 2.10 – 2.16 (m), δ 1.98 – 2.06 (m)
6	Succinic acid	δ 2.54 (s)
7	Choline	δ 3.24 (s)
8	Hydroxy-$_L$-proline	δ 4.33 – 4.38 (m)
9	Galactinol	δ 3.97 (m)
10	β – Glucose	δ 4.58 (d, *J*=7.8 Hz)
11	α – Glucose	δ 5.18 (d, *J*=3.8 Hz)
12	Raffinose	δ 5.42 (d, *J*=3.93 Hz)
13	Fumaric acid	δ 6.53 (s)
14	Adenosine	δ 8.33 (s), δ 8.20 (s)
15	Formic acid	δ 8.46 (s)
d = doublet; m = multiplet; s = singlet		

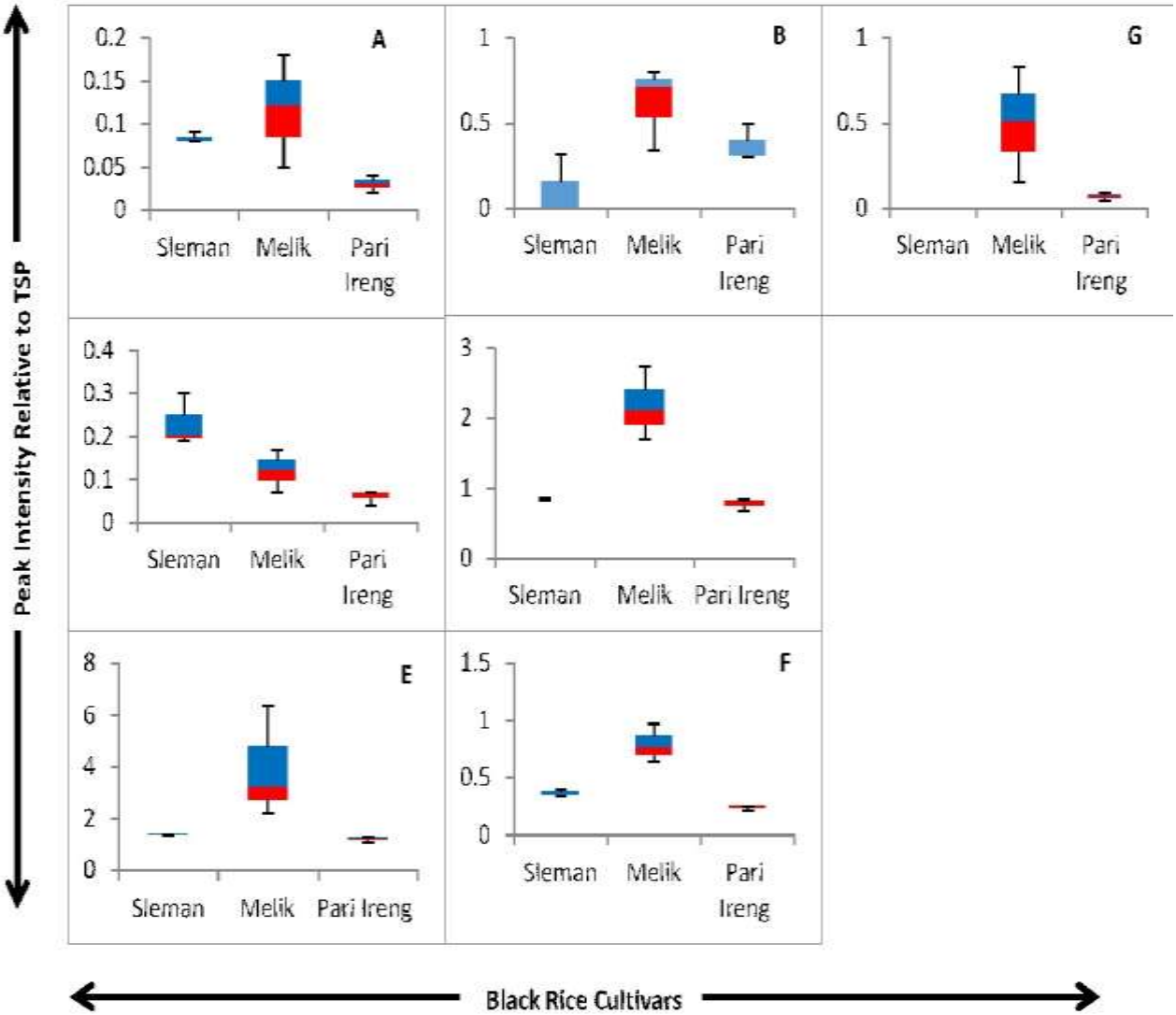

Figure 4: Box plot of black rice metabolite. A) Valine B) Glutamate C) Acetate D) α-Glucose E) β-Glucose F) Galactinol G) Raffinose

The loading plot revealed the influence of alanine, glutamate, threonine, valine, hydroxy-L-proline, acetate, succinic acid, formic acid, fumaric acid, α-glucose, β-glucose, galactinol, raffinose, adenosine and choline in the separation of black-pigmented resistant and susceptible cultivars (Figure 3B). The semi-quantitative analysis showed that only valine, glutamate, α-glucose, β-glucose, galactinol and raffinose were significantly different between the resistant and susceptible cultivars (Figure 4).

Previous studies reported the role of valine and glutamate in plant resistance mechanisms against biotic stress[47]. Valine is also crucial for the maintenance of nitrogen balance in plants[3,27]. Glutamate acts as a precursor for arginine, spermine and α-aminobutyric acid which highly correlates to its involvement in plant defense[11,15,30]. Glutamate was previously shown to contribute to the resistance mechanism of rice against sheath blight disease[49].

Galactinol, α-glucose, β-glucose and raffinose are part of the sugar group. Sugar is a significant energy source in plants and it also acts as a signaling molecule in various metabolic processes including defense mechanisms against biotic stress and abiotic stress[4,14,35,45].

A positive correlation was observed between glucose levels in plant tissue and higher resistance character against the pathogen[35]. The contribution of sugar can be observed in defense against pathogens, especially during the early stages of the attack. They are involved in the increased oxidative burst, increased lignification of the cell wall and stimulation of flavonoid synthesis and induce specific PR proteins for inducing defense against pathogens. In addition to this, glucose (α-glucose and β-glucose) has been shown to activate plant defense mechanism via SA-dependant pathway[43,51].

Another member of the sugar group, which was successfully identified in NMR studies was galactinol. Galactinol is a precursor for raffinose synthesis which is catalysed by raffinose synthase. Previous studies have shown that galactinol and raffinose act as signals to stimulate the plant's immune system upon attack by pathogens and insects.

In tobacco, galactinol was reported to activate genes PR1a, PR1b and NtACS1, associated with the plant defense mechanisms[22]. Raffinose is also known to contribute to plant resistance to nematode infections[16].

In the present study, nine metabolites were identified to have different concentrations in the resistant pigmented rice than the susceptible ones. Further investigations are required to confirm the findings in this study. The exogenous treatment with some of these compounds, especially the ones that are commercially available and inexpensive, is strongly recommended. A study on the anatomical structure of leaves and grains might help better understand the pigmented rice's resistance mechanism against rice ear bugs.

Conclusion

The present study screened eight pigmented rice cultivars for resistance to rice ear bug. Inpari 24 (red rice), Melik (black rice) and Cempo Ireng Sleman (black rice) were found to be the most resistant cultivars. NMR-based metabolomics analysis identified nine metabolites contributing to the defense mechanism of pigment rice cultivars. Metabolites that played a role in red rice resistance were hydroxy-L-proline, threonine and formic acid.

In black rice, valine, glutamate, α-glucose, β-glucose, galactinol and raffinose were significant contributors. These results suggested the suitability of these resistant cultivars to be used as parental lines to develop premium rice cultivars to support the concept of green agriculture based on IPM.

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