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Composition of Metabolites in Swamp Rice Varieties to Stress Tolerance Screening

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Abstract: Rice is an important food and produces metabolites which play several roles in the defense of the plant against abiotic and biotic stress. The development of stress tolerance variety is an importance for rice production. This study aims to analyze the metabolites of rice straw extract using Gas Chromatography-Mass Spectrometry (GC-MS). The analysis was carried out with 10 rice straw extracts at the flowering stage, while a total of 78 compounds were identified from the extract. The number of compounds in each variety ranged from 12-23. Fatty acid was the most dominant compound with 37%, followed by ester 26%, and steroid 14%. Palmitic, linoleic, and oleic acid were fatty acids found in many varieties. In addition, ethyl palmitate and 2-ethylhexyl hydrogen phthalate were also detected in all extracts. Based on the results, the highest content found in all varieties was 2-ethylhexyl hydrogen phthalate, except for Inpara 7 and 8 which predominantly contain linoleic acid. Inpara 4, 3, and 5 were included in one cluster that has a higher 2-ethylhexyl hydrogen phthalate, but a lower palmitic and linoleic acid content. The fatty acid can be used as one of the potential criteria in screening varieties for tolerance to stress, especially cold temperature, salinity, and blast disease.

Keywords: Swamp Rice, Metabolite, Tolerance, Abiotic and Biotic Stress

1. Introduction

Rice is an important food crop, consumed by the world's population, its production is influenced by genetic and environmental factors. Abiotic and biotic are environmental factors that affect rice production by reducing crop growth and productivity. Abiotic stress adversely affects crop growth via high and low temperature, drought, salinity, submergence, acid sulfate soils, greenhouse gases, and nutritional deficiencies [1]. Whereas, biotic include insect pest, fungi, bacteria, viruses, and herbicide toxicity. These factors have a huge impact and reduce yields by more than 50%. The paddy has specific mechanisms to respond to stress conditions. However, this varies depending on the severity of the stress, age of the plant, as well as inherent stress [2]. Moreover, plants produce secondary metabolites that play a role in response to changing environment [3]. These compounds induce disease resistance, anti-insect, and allelopathic activities against biotic and abiotic stress, or as plant growth regulators [4].

Secondary metabolites in rice have been identified with over 276 compounds which includes phenolic acids, flavonoids, terpenoids, steroids, alkaloids, and other derivates [5]. Phenolic acids constitute an important class in the phenolic fraction, and some of the derivatives detected in rice include salicylic, 4-hydroxybenzoic, gallic, vanillic, p-coumaric, caffeic, and ferulic acid, etc. Primary metabolites have a strong correlation with phenolic acids, and phenylalanine was found to be most significant in making phenolics prediction model for rice grain [6]. Furthermore, the major terpenoid compounds found in rice include limonene, trans-β-ocimene, β-cymene, and linalool [7]. The constitutive emissions of Eβf and limonene regulated by the constitutive expression of tps46 tends to play a crucial role in rice defense against *Rhopalosiphum padi* [8]. Meanwhile, accumulation of sterols, such as campesterol, β-sitosterol, and stigmasterol play a role in drought tolerance [9].

The identification of compound's content is done by various methods, including GC-MS (*Gas Chromatography-Mass Spectrometry*). GC-MS is used as a metabolomics analytical method due to its excellent chromatographic separation [10], high sensitivy and resolution [11]. This method is widely used as a separation technique for volatile organic molecules such as hydrocarbons, alcohols, steroids, fatty acids, etc. This study aims to identify secondary metabolites present in straw rice. The study outcomes provide an alternative in choosing varieties based on the content of for specific purposes, especially to abiotic and biotic stress tolerance.

2. Materials and Methods

2.1. Materials

A total of 10 swampy rice varieties were used including Inpara 1, 2, 3, 4, 5, 6, 7, Inpara 8 and 9 Agritan, as well as Inpara 10 BLB. All plants were cultivated under the same condition at Screen House, Indonesian Center for Rice Research, Indonesia. The stem and leaves were taken at the flowering stage and then oven-dried at 35°C and mashed while the sample was macerated using methanol. The injection of each sample was 5 µl.

2.2. Analysis of GCMS

Gas chromatography analysis was performed using Agilent Technologies 7890 with autosampler and 5975 mass selective detector as well as chemstation data system. The column was a 30 m x 0.20 mm i.d x 0.11 µm film thickness HP Ultra with an initial temperature of 80°C for 0 minutes, increased at 3°C/min to 150°C for 1 minute, and then 20°C/min to 280°C for 26 minutes. Furthermore, the flow rate of helium (He) carrier gas was set at 1.2 ml/min while the temperature of the ion source was 230°C and the electron was set at 70 eV using electron impact ionization method. Compound identification was carried out using mass spectra library search and the results from this study were compared with the data from the literature.

3. Result and Discussion

Based on the GCMS analysis, the rice straw was identified to contain 78 compounds, however, the number of compounds for each variety was different. The highest was found in Inpara 2 (23 compounds), while the lowest was in Inpara 8 (12 compounds). Furthermore, the number of compounds found in Inpara 1, 3, 4, 5, 6, 7, 9, and 10 were 17, 17, 16, 22, 19, 21, 19, and 22 respectively (Table 1). Chemical compounds of a plant are affected by biotic and abiotic stress, however, the biosynthetic regulation of rice compounds is highly complex [4] and strictly regulated at the transcriptional, translation, and metabolomic levels [12]. Among distinct compounds, the peaks and metabolite were identified by comparing the mass spectra and retention times with the standard. Eight rice straw extracts (Inpara 1, 3, 4, 5, 6, 7, 9, and 10) were shown to have peaks at the same compounds, 2-ethylhexyl hydrogen phthalate (Phthalic acid, mono-(2-ethylhexyl) ester). Meanwhile, two extracts (Inpara 7 and 8) have peaks in linoleic acid. Phthalic acid is an aromatic dicarboxylic acid. 2-ethylhexyl hydrogen phthalate (MEHP) is an active metabolite of bis(2-ethylhexyl) phthalate (DEHP) and its hydrolyzes via the enzyme bis(2-ethylhexyl) phthalate acylhydrolase. Linoleic acid is an essential polyunsaturated fatty acid and precursor to oxidized product by auto oxidation or enzimatically via lipoxygenase (LOX), cyclooxygenase (COX), cytochrome P450 (cyp450), and soluble epoxide hydrolase (sEH) [13].

The compounds were classified into nine classes: alcohol, aldehyde, terpenoid, ester, fatty acid, hydrocarbon, ketone, and steroid and others (Figure 1). Rice straw extract was dominated by fatty acids 37%, followed by ester 26% and steroid 14%. Fatty acid dominated the components in 8 rice straw extracts i.e Inpara 1, 2, 4, 6, 7, 8, 9, and 10. However, Inpara 3 and 5 were dominated by esters. Along with other derivates it plays crucial roles in plant defense responses. In addition, fatty acid is a potential molecular marker useful for basic studies on cold tolerance [14]. Salinity stress triggers the biosynthesis of fatty acid, as certain content such as palmitic, stearic, oleic, and linoleic acid were higher under salinity stress compared to control at the vegetative stage, but lower at ripening stage [15]. Suppressing the gene for stearoyl acyl carrier protein fatty acid desaturase (SACPD) resulted in increased resistance to blast and leaf blight diseases in rice [16].

Tabel 2 shows the five most abundant compounds detected by GC-MS. All rice straw extracts contained 2-ethylhexyl hydrogen phthalate, while palmitic acid was also shown to be present in all extracts except Inpara 3. The phthalic acid esters are widely used in the medical, cosmetics, paint, and pesticide industries. Meanwhile, the accumulation of (2-ethylhexyl) phthalate (DEHP) in the soil induce adverse effects on plant and pose a risk to human health [17]. DEHP had toxic effects on physiological and biochemical parameters characterized by decreased chlorophyll and increased MDA content, altered levels of antioxidant system enzymes, and decreased soluble sugar and vitamin C content in *Brassica chinensis* [18]. Fatty acids were also found in rice bran, with the majority being palmitic, oleic, linoleic, and linolenic acid [19]. The unsaturated fatty acids act as ingredients and modulators of cellular membranes glycerolipids, a reserve of carbon and energy in triacylglycerol, precursors of bioactive molecules, regulators of stress signaling, and inducers of oxidative stress [20]. Also, oleic and linoleic acids were reported to be involved in regulated development, seed colonization, and mycotoxin production by *Aspergillus* spp [21].

Table 1. *Metabolites of swamp rice straw extracts.*

No	Compound	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
1	Phytone	+	+	+	+	+	+	+	+	-	+
2	Ethyl palmitate	+	+	+	+	+	+	+	+	+	+
3	Palmitic acid	+	+	+	+	+	+	+	+	+	+
4	Cysteamine S-sulfate	+	-	-	-	-	-	-	-	-	-
5	Linoleic acid	+	+	-	-	-	+	+	+	+	+
6	2-Octylcyclopropaneoctanal	+	+	-	-	-	-	-	-	-	-
7	9,17-Octadecadienal	+	+	+	-	-	-	-	-	-	-
8	2-Octadecoxyethanol	+	+	-	-	-	-	-	-	-	-
9	2-ethylhexyl hydrogen phthalate	+	+	+	+	+	+	+	+	+	+
10	Heptadecyl bromide	+	-	-	-	-	-	-	-	-	-
11	Lignoceric acid methyl ester	+	+	-	-	-	-	-	-	-	-
12	Oleic acid	+	+	-	+	+	+	+	-	+	-
13	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	+	-	-	+	-	+	-	-	-	-
14	Z-14-nonacosane	+	-	-	-	-	-	-	-	-	-
15	5,6-Dihydroergosterol	+	-	-	-	-	-	-	+	-	-
16	Tremulone	+	+	-	-	+	+	+	+	-	+
17	Sitostenone	+		+	+	+	-	-	-	+	+
18	Glycerin	-	+	+	+	+	+	-	+	-	-
19	Iodooctadecane	-	+	-	-	-	-	-	-	-	-
20	9-Octadecenoic acid	-	+	-	-	-	-	-	-	-	-
21	(14β)-Pregnane	-	+	-	+	-	-	-	-	-	-
22	4,4-dimethylcholestan-3-one	-	+	-	-	-	-	-	-	-	-
23	Asclepic acid	-	+	+	-	-	+	-	-	-	-
24	Cholesta-6,22,24-triene,4,4-dimethyl-	-	+	-	-	-	-	-	-	-	-
25	Campesterol	-	+	-	-	+	+	-	-	-	-
26	Stigmasta-5,22-dien-ol	-	+	-	-	-		-	-	-	-
27	g-Sitosterol	-	+	-	-	-	+	+	-	-	+
28	Cholest-4-ene-3,24-dione	-	+	-	+	-	-	-	-	-	+
29	4,22-stigmastadiene-3-one	-	+	+	-	-	-	-	-	+	+
30	Neophytadiene	-	-	+	-	+	-	+	-	-	-
31	2-methyltetralin	-	-	+	-	-	-	-	-	-	-
32	2-methyl-Z-Z-,13-octadecadienol	-	-	+	-	-	-	-	-	-	-
33	Oleamid	-	-	+	-	+	-	-	-	-	-
34	Cholestan-3-one,4,4-dimethyl-,(5.alpha.)-	-	-	+	-	-	-	-	-	-	-
35	Stigmasterol	-	-	+	+	+	+	+	+	+	+
36	Ergost-4-en-3-one,(24R)-	-	-	+	-	+	-	-	-	+	-
37	13-octadecenal	-	-	+	-	-	-	-	-	-	-
38	Nopinone	-	-	-	+	-	-	-	-	-	-
39	(Z)-9,17-Octadecadienal	-	-	-	+	-	-	-	-	+	+
40	9-Octadecenoic acid	-	-	-	+	+	-	-	-	-	-
41	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	-	-	-	+	+	+	-	-	-	-
42	β-sitosterol	-	-	-	+	+	+	-	+	+	+
43	5,5-Dimethyl-3-heptyne	-	-	-	-	+	-	-	-	-	-
44	2(5H)-furanone, 5-ethyl-	-	-	-	-	+	-	-	-	-	-
45	4,6-cholestadienol	-	-	-	-	+	-	-	-	-	-
46	Propargyl alcohol, trifluoroacetate	-	-	-	-	+	-	-	-	-	-
47	Ethyl vallesiachotamate	-	-	-	-	+	-	-	-	-	-
48	Lauryl palmitate	-	-	-	-	+	-	-	-	-	-
49	Z,Z-10,12-Hexadecadien-1-ol acetate	-	-	-	-	-	+	-	-	-	-
50	Heptacosane,1-chloro	-	-	-	-	-	+	-	-	-	-
51	Nonacosane	-	-	-	-	-	+	-	-	-	-
52	Stigmastryl toslate	-	-	-	-	-	+	+	-	-	-
53	Pentadecanoic acid	-	-	-	-	-	-	+	-	-	-
54	10-methyl-9-nonadecene	-	-	-	-	-	-	+	-	-	-
55	1,2-15,16-diepoxyhexadecane	-	-	-	-	-	-	+	-	-	-
56	Ethyl oleate	-	-	-	-	-	-	+	-	-	-
57	trans squalene	-	-	-	-	-	-	+	-	-	-
58	Z-14-nonacosane	-	-	-	-	-	-	+	-	-	-
59	14-methylcholesta-2,8-dien-6-yl acetate	-	-	-	-	-	-	+	-	-	-

No	Compound	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
60	3-Eicosene,(E)-	-	-	-	-	-	-	+	-	-	-
61	11,13-Dimethyl-12-tetradecen-1-ol acetate	-	-	-	-	-	-	+	-	-	-
62	Cyclopropaneoctanal acid	-	-	-	-	-	-	+	+	-	-
63	Heptadecanoic acid	-	-	-	-	-	-	-	+	+	-
64	3-ethoxy-1,2-propanediol	-	-	-	-	-	-	-	-	+	-
65	(2S,5R)-2-isopropyl-5 methylhept-6-en-1-ol	-	-	-	-	-	-	-	-	+	-
66	Elaidic acid ethyl ester	-	-	-	-	-	-	-	-	+	-
67	Ethyl margarate	-	-	-	-	-	-	-	-	+	+
68	Ethyl tetradecanoate	-	-	-	-	-	-	-	-	+	-
69	1,3-dioxane,2,2,4,5-tetramethyl-6-(1-methyloctadecyl)-	-	-	-	-	-	-	-	-	+	-
70	4-vinylcholesta-3-ol	-	-	-	-	-	-	-	-	+	-
71	Erythritol	-	-	-	-	-	-	-	-	-	+
72	Monoelaidin	-	-	-	-	-	-	-	-	-	+
73	Eicosane	-	-	-	-	-	-	-	-	-	+
74	Nonadecane	-	-	-	-	-	-	-	-	-	+
75	Octacosyl acetate	-	-	-	-	-	-	-	-	-	+
76	1,3-cyclohexadecanedione	-	-	-	-	-	-	-	-	-	+
77	13-Methyl-Z-14-nonacosane	-	-	-	-	-	-	-	-	-	+
78	Cyclo laudenol	-	-	-	-	-	-	-	-	-	+

Note: V1= Inpara 1, V2=Inpara 2, V3=Inpara 3, V4= Inpara 4, V5= Inpara 5, V6= Inpara 6, V7= Inpara 7, V8= Inpara 8, V9= Inpara 9, V10= Inpara 10
(+) indicated the compound was detected, (-) indicated the compound wasn't detected

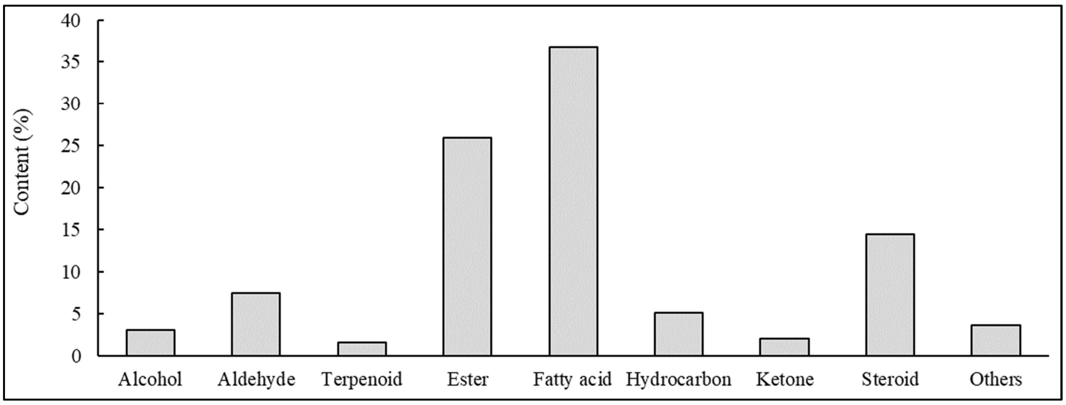

Figure 1. *The compound content of swamp rice straw extract.*

Table 2. *Top five compounds in swamp rice straw extract.*

No	Inpara 1	Inpara 2	Inpara 3	Inpara 4	Inpara 5
1	2-ethylhexyl hydrogen phthalate	2-ethylhexyl hydrogen phthalate	2-ethylhexyl hydrogen phthalate	2-ethylhexyl hydrogen phthalate	2-ethylhexyl hydrogen phthalate
2	Linoleic acid	Palmitic acid	Stigmast-4-en-3-one	Oleic acid	Glycerin
3	Palmitic acid	Linoleic acid	4,22-stigmastadiene-3-one	Palmitic acid	Palmitic acid
4	Z-14-Nonacosane	(14β)-Pregnane	Glycerin	9-Octadecenoic acid	Ergost-4-en-3-one,(24R)-
5	9,17-Octadecadienal	9,17-Octadecadienal	Ergost-4-en-3-one,(24R)-	(14β)-Pregnane	Stigmast-4-en-3-one

Table 2. *Continued.*

No	Inpara 6	Inpara 7	Inpara 8	Inpara 9	Inpara 10
1	2-ethylhexyl hydrogen phthalate	Linoleic acid	Linoleic acid	2-ethylhexyl hydrogen phthalate	2-ethylhexyl hydrogen phthalate
2	Palmitic acid	Palmitic acid	2-ethylhexyl hydrogen phthalate	Linoleic acid	Linoleic acid
3	Oleic acid	2-ethylhexyl hydrogen phthalate	Palmitic acid	Palmitic acid	Palmitic acid
4	Linoleic acid	Z-14-nonacosane	Cyclopropaneoctanal acid	3-ethoxy-1,2-propanediol	Octacosyl acetate
5	Nonacosane	Oleic acid	Glycerin	(Z)-9,17-Octadecadienal	Heptadecanoic acid, ethyl ester

Furthermore, the compound contained in each extracts variety varied. There were 10 dominant compounds in 10 rice straw extracts (Tabel 3). The compounds found in all extracts include ethyl palmitate, 2-ethylhexyl hydrogen phthalate, and palmitic acid. Meanwhile, the highest average concentration was found in 2-ethylhexyl hydrogen phthalate (20.35), followed by linoleic (15.04) and palmitic acid (12.41). Compounds with less than 5% prevalence include glycerin (3.74), ethyl palmitate (2.59), phytone (1.94), tremulone (1.42), stigmasterol (2.84), and β sitosterol (2.60). Secondary metabolites are widely used as chemicals for drugs, flavors, fragrances, insecticides, dyes, and have a great economic value [22]. For example, glycerin is used in the medical, pharmaceutical, cosmetic, and food industry. In addition, rice bran glycerin extract is potentially useful as natural antidiabetic and antiglycation [23]. The appressoria of rice blast fungus (*Magnaporthe grisea*) reportedly used glycerin to generate pressure which ruptures plant cuticles [24] and collectively drive turgor penetration of the rice leaf [25].

Heatmap and cluster analysis was used to classify varieties and compounds according to content. Cluster analysis was carried out using average linkage with Euclidean distance. Figure 2 showed two clusters varieties, the first cluster has three varieties, i.e Inpara 4, 3, and 5 whereas the second cluster consist of seven varieties, i.e Inpara 8, 7, 6, 1, 2, 9, and 10. Based on compound content, 2-ethylhexyl hydrogen phthalate was the highest and was included in one cluster while palmitic and linoleic acid belong to the same cluster. Both compounds were included in fatty acids group with C16 and C18 respectively.

Table 3. *The dominant compounds of the swamp rice straw extract.*

Compounds	Formula	Peak area (%)									
		V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Glycerin	$C_3H_8O_3$	-	2.09	6.56	1.04	8.38	1.72	-	2.64	-	-
Ethyl palmitate	$C_{18}H_{36}O_2$	3.42	3.12	1.49	3.73	1.63	3.32	2.30	2.39	2.37	2.14
2-ethylhexyl hydrogen phthalate	$C_{16}H_{22}O_4$	20.19	16.03	34.76	17.74	31.36	15.40	12.23	22.67	18.55	14.61
Palmitic acid	$C_{16}H_{32}O_2$	16.12	14.67	3.60	14.18	5.42	14.77	15.14	20.57	7.97	11.67
Linoleic acid	$C_{18}H_{32}O_2$	17.52	8.41	-	-	-	10.34	17.23	26.43	11.83	13.52
Oleic acid	$C_{18}H_{34}O_2$	1.74	2.76	-	16.6	3.31	10.66	5.87	-	2.82	-
Phytone	$C_{18}H_{36}O$	2.18	1.68	1.43	2.02	1.54	1.91	2.19	2.63	-	1.88
Tremulone	$C_{29}H_{46}O$	1.55	1.15	-	-	1.95	1.16	1.25	1.06	-	1.84
Stigmasterol	$C_{29}H_{48}O$	-	-	2.69	1.19	3.05	3.05	4.62	2.03	2.33	3.75
β sitosterol	$C_{16}H_{22}O_4$	-	-	-	1.48	3.31	2.50	-	2.40	2.35	3.56

Note: V1= Inpara 1, V2=Inpara 2, V3=Inpara 3, V4= Inpara 4, V5= Inpara 5, V6= Inpara 6, V7= Inpara 7, V8= Inpara 8, V9= Inpara 9, V10= Inpara 10

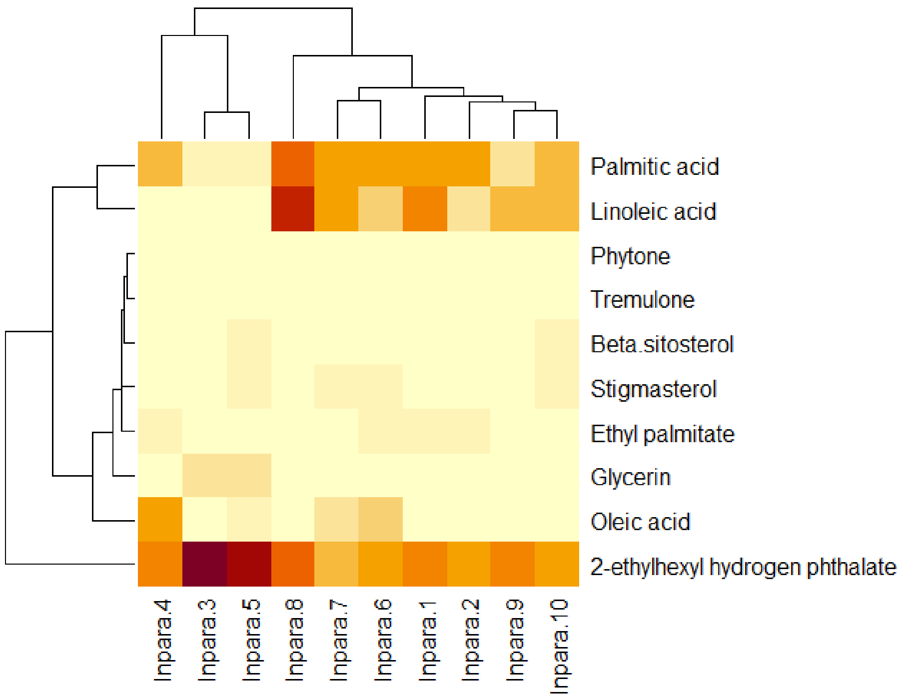

Figure 2. *Heatmap and cluster analysis of the compound in swamp rice straw extract.*

4. Conclusion

Based on the result, rice straw extract contains different amounts of metabolites, ranging between 12 to 23 compounds. The identified compounds were categorized into several groups which include alcohol, aldehyde, terpenoid, ester, fatty acid, hydrocarbon, ketone, steroid and others. 2-ethylhexyl hydrogen phthalate was the most dominant compound in all varieties, except Inpara 7 and 8 which predominantly contain linoleic acid. The fatty acids are potential metabolites useful for screening of rice tolerance to cold temperature, salinity, and blast disease.

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