Liquid Chromatography Mass Spectrometry-based High-Throughput, Unbiased Profiling of Upland and Lowland Rice Varieties Cultivated in Sabah

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ABSTRACT *Oryza sativa* L. commonly known as rice is one of the most cultivated cereal worldwide which sustained over 50% of the world's population. Malaysian rice cultivated in 2 systems namely lowland (irrigated rice) and upland (rain-fed rice). Rice varieties adapted different growth systems differ substantially from each other agronomic traits. It is challenging to distinguish from each other's using their morphological characteristics. Therefore, we aimed to propose a high-resolution mass spectrometry-based high-throughput, unbiased approach to distinguish rice species (upland or lowland cultivation) using the chemotaxonomy approach using whole rice (including barns). From our preliminary results, orthogonal partial least square discriminant analysis (OPLS-DA), a supervised pattern-recognition technique, successfully discriminates the differently cultivated rice species with R^2X, R^2Y, and Q^2 as 0.309, 0.914, and 0.871, respectively. Dendrogram demonstrates rice species were discerned from another. There are some plant-related metabolites and phospholipids species significantly differed between the cultivated rice species. Among the identified metabolites, the upland whole rice demonstrated a higher ratio of linoleic acid esters and glycerolipids including diacylglycerol lipids (DG), monoacylglycerol lipds (MG), and phosphocholine lipids (PC) compared to lowland whole rice. Interestingly, triacylglycerolipids were reduced in the upland as compared to lowland whole rice. It is suspected the rice expressed different levels of lipids contents play essential roles in rice germinations at adopted lands. Throughout such an approach, a systematic, scientific, evident-based approach could be established and proved an insight for the researcher to distinguish rice species and avoid nutrition facts exaggeration of specific rice species over the others.

KEYWORDS: Upland rice; Lowland rice; OPLS-DA; High-throughput; Unbiased; Profiling.
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INTRODUCTION

Rice is a staple food for nearly half of the world's population. However, the major challenges in rice identification are to prevent illegal rice trading by adulterating with low quality rice and mislabeling low quality rice with the premium one. By having the fingerprint of the origin, we can easily trace the product's genetic background.

Metabolomics is one of the "omics" studies used for acquiring biological information in rice. Metabolomics implies a qualitative and quantitative approach for all metabolites present in organisms. Mass spectrometer (MS) have become universal methods for proteomic studies. MS and nuclear magnetic resonance spectrometer (NMR) have been facilitating metabolomic studies (Oikawa *et al., 2008)*. Conventional genetic methods revealed biological functions of unknown genes such as rice grain production (Goff *et al.*, 2002). However, it is difficult to clarify the functions of all genes by only genetic technique because estimation of functions from genome sequences has limitation (Sasaki *et al.*, 2002). Metabolomics provides the possibility of clarifying gene functions directly connected to rice quality, since metabolome, implying the varieties and amounts of all metabolite is closely related to the important traits of rice.

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

Metabolomic analyses generally classified to two groups which is targeted and untargeted. The first generation of metabolomics research mainly focused on the development of methodology for untargeted metabolome analysis to analyse all detectable metabolites without the preselection of targets. To detect a wider range of metabolites, untargeted metabolome analysis has been performed while operating the mass spectrometer in full scan mode, time-of-flight (TOF) analyser are suitable for this because of the higher scan rate and sensitivity. The high resolution TOF analyser is preferred to characterise unknown metabolites by using accurate mass data. The difficulty in structural characterization of unknown metabolite signals based on high resolution data (Oikawa *et al*, 2008).

Targeted analyses focus on a specific group or defined groups of chemically characterized and biochemically annotated metabolites (Roberts, 2012). Targeted analyses are important for assessing the behaviour of a specific group of compounds though under determined conditions or the use of internal standards (Roberts, 2012). The targeted metabolomics require higher level of purification and selective extraction of metabolites. The targeted data analyses performed using the raw data produced by untargeted metabolome analysis. There was a study on targeted metabolites in rice metabolomics by targeted MS^2 to identify and annotate metabolites in rice. Metabolites identified including amino acids, flavonoids, Lysophosphatidylcholines, fatty acids, and some phytohormones (Chen *et al*., 2013).

Untargeted analyses are the comprehensive analysis of all measurable analytes in a sample. This method has been used in the identification of possible fingerprints of biological phenomena such as plant by discriminative analyses. Discriminative analyses by creating statistical models or evaluating possible pathways that may elucidate differences (Roberts, 2012). Discrimination is usually achieved by use of multivariate data analysis (MVDA) techniques intended to maximize classification, orthogonal partial least square discriminant analysis (OPLS-DA) is using in this research with a supervised pattern-recognition technique, successfully discriminates the differently cultivated rice species.

A two-step LC/MS approach was employed to understand the mechanism of infection and immunity of rice to bacterial leaf blight, and to identify metabolites that are related to infection and resistance. Rapid differential expression analysis of samples using time-of-flight (TOF) mass spectrometry (MS) was followed by targeted identification of differentially expressed metabolites using quadrupole time-of-flight (Q-TOF) MS/MS. Based on the metabolites obtained, the rice and state of infection were clearly distinguishable (Fischer & Sana, 2007).

In this project, 3 types of lowland and upland rice each cultivated in Sabah were studied through metabolomic analysis. Throughout such an approach, we intended to apply high-throughput profiling on the rice extracts and chemotaxonomically classified each species based on profiled compounds. Throughout such study, researcher would be able to identify the different type of Sabah rice and establish chemical fingerprints which may assist the new researcher in identification of the new varieties.

METHODOLOGY

Sample collection and preparation

There were six rice samples used in this study, which was collected in Ranau, Sabah, Malaysia. Three upland rice samples collected were Sulug Tongod, and Bario Kg. Suminimpad and 3 lowland rice samples collected were TR8, TR9, and TN-1. All samples were ground together with the husk

and freeze-dried using freeze dryer (Labconco, Kansas City, MO, USA). Each sample was weighed ≈100 mg (±1%) and subsequently extracted using a 9 mL of methanol/acetonitrile/water (1:1:1, v/v/v) mixture. The mixtures were mixed using IKA Vortex Genius 3 and 1000 g centrifugation at 20˚C for 20 minutes. Then the entire lower layer was transferred into a falcon tube. Prior to the LC-MS/MS analysis, 2 mL of sample was transferred into a glass vial (Ling *et al*., 2014).

Ultra-high-performance Liquid Chromatography-Quadrupole time-of-flight (UHPLC-QTOF) mass spectrometry analysis of rice samples

In this study, the UHPLC-QTOF was used to analyze the rice samples. The mixture of 1% (v/v) of Solvent A organic mobile phase (methanol/acetonitrile (6:4, v/v)-0.01% (v/v) ammonium acetate-0.1% (v/v) formic acid) and 99% (v/v) of Solvent B aqueous mobile phase (H_2O-0.01% (v/v) ammonium acetate-0.1% (v/v) formic acid) served as loading and washing solutions and was delivered to 1.7 µl, 100 x 2.1 mm PFP Pentaflurophenyl column, by Thermo Scientific Vanquish Duo UHPLC Systems pump at flow rate of 600 µl/min, preheated at 40˚C column temperature, followed with gradient solvent A to 30% for the first 3 minutes, then increase gradient to 100% from 3 to 7 minutes and holding 100% solvent A with 1 minute. Finally, the system was flushed with washing solution for 2 minutes. During the profiling, quality control (QC) of the 10 µl sample was injected every 2 samples to assure the instrumental signal stability. The ultra-high resolution QTOF, Impact II (Bruker, Billerica, MA, USA) was used and mass spectrometer data acquisition was set at 50 to 1500 m/z. Voltage for positive electrospray ionization (EZI) was set as 3.5 kV, while the gas temperature of the ion source was set at 300˚C along with drying gas flow at 10 L/min, and nebulizer flow at 3.0 Bar (Ling *et al*., 2020).

Chemometric analysis

Acquired raw data were pre-processed using MZmine 2 software to compensate the variations in retention times and m/z value in each analysis (Pluskal *et al*., 2010). MZmine 2 was used to process both low-resolution (unit mass) and high-resolution (exact mass) data in mzXML file. Pre-processed data were exported as peak list table in CSV format, with rows representing the samples and columns representing the integrated and normalized peak areas. The pre-processed data processed the following information, raw data filtering, peak detection, removing of isotopes, normalization of the retention time, gap filling using the peak finder, normalization of peak height/area. The acquired data set were log-transformed and Pareto scaled prior to the multivariable analysis using open source software MetaboAnalyst 4.0 (Xia & Wishart, 2016) to determine the clustering of the rice samples. All data sets were subjected to principle components analysis (PCA), orthogonal partial least square discriminant analysis (OPLS-DA), a supervised pattern-recognition technique to discriminate the differently cultivated rice species (Puah *et al*., 2019).

RESULT AND DISCUSSION

From our preliminary results, orthogonal partial least square discriminant analysis (OPLS-DA) successfully discriminates the differently cultivated rice samples with R^2X, R^2Y, and Q^2 as 0.309, 0.914, and 0.871, respectively as shown in Figure 1. With the predictive variation (p1) which correlated variation between X and Y, the separation between two group of upland and lowland rice was good with R^2Y=0.914. Dendrogram demonstrates rice samples were discerned from another as shown in Figure 2 with two groups of rice which is lowland rice (TN1, TR8, and TR9) and upland rice (Sulug Tongod, and Bario Kg. Suminimpad). In Figure 3, the PCA plot showed the summary of observations and variables for upland rice, lowland rice, and QC samples.

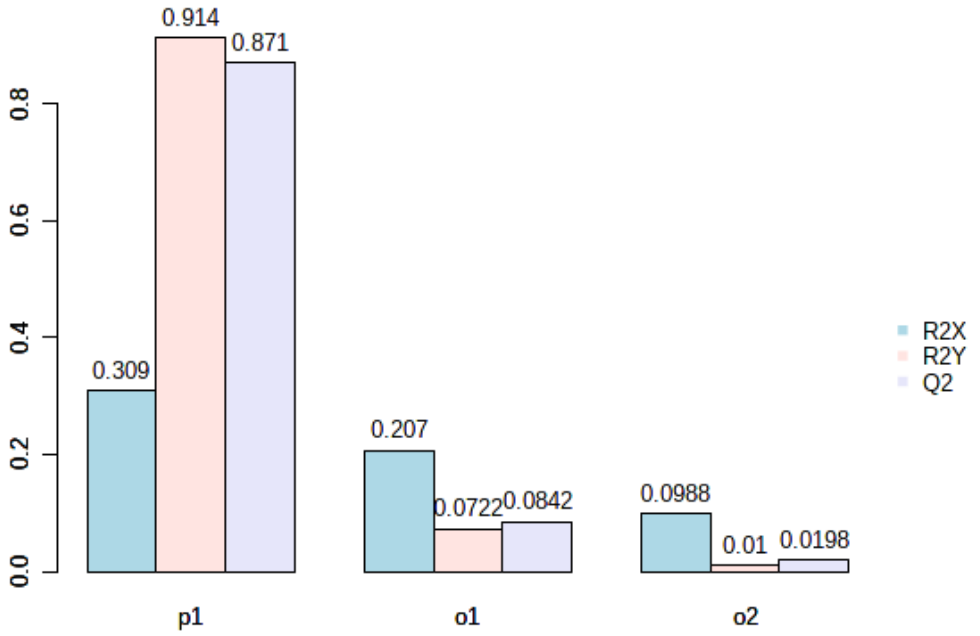

Figure 1. OPLS-DA models with corresponding values of R^2X, R^2Y, and Q^2.

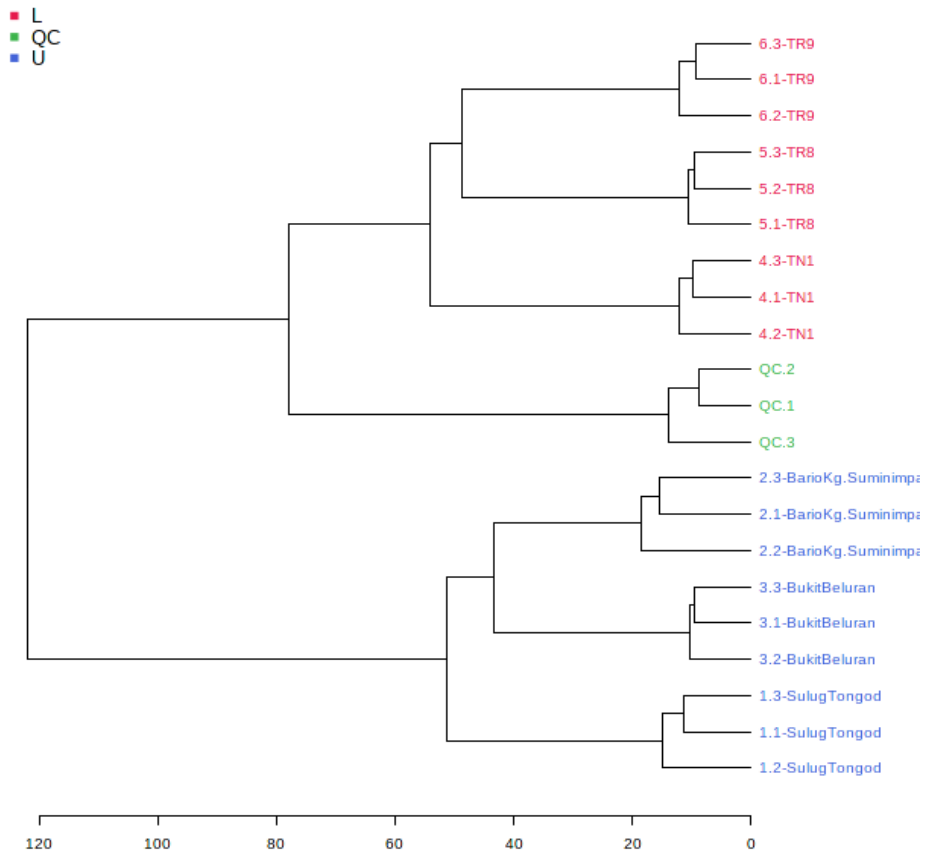

Figure 2. Dendrogram discriminate both upland and lowland rice with quality control (QC) samples.

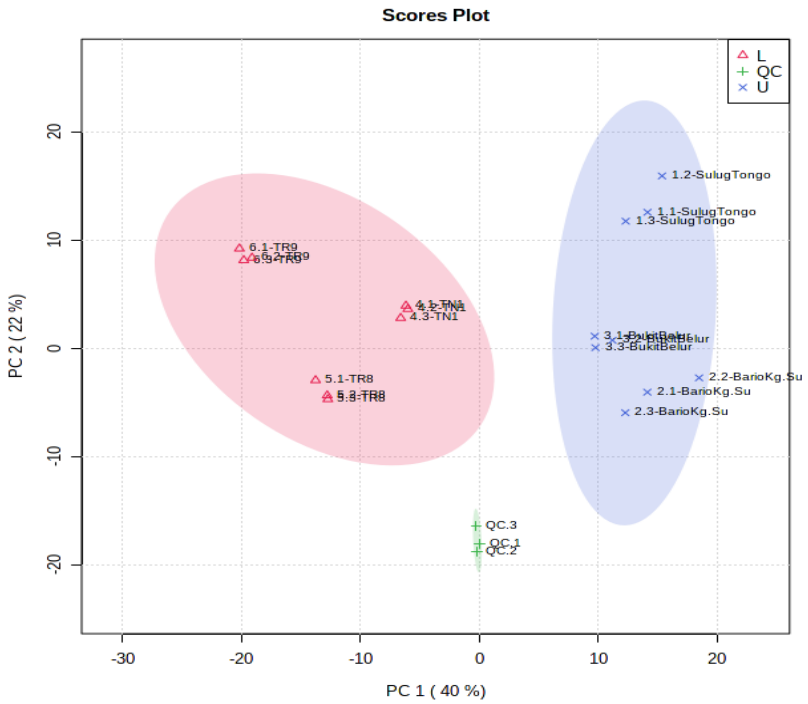

Figure 3. PCA plot showed the effectively differentiate of QC with lowland and upland rice.

There are some plant-related metabolites and phospholipids species significantly differed between the cultivated rice samples (Table 1). The upland whole rice demonstrated a higher ratio of linoleic acid esters and glycerolipids including diacylglycerol lipids (DG), monoacylglycerol lipids (MG), and phosphocholine lipids (PC) compared to lowland rice. DG is a prolific second messenger that activates proteins involved in a variety of signaling cascades. Because it can associate with a diverse set of proteins, DG potentially activates numerous signaling cascades. Thus, its accumulation needs to be strictly regulated. MG are esters of the trihydric alcohol glycerol in which only one of the hydroxyl groups is esterified with a long-chain fatty acid. They can exist in three stereochemical forms as illustrated. PC is a class of phospholipids that incorporate choline as a headgroup. They are a major component of biological membranes and can be easily obtained from a variety of readily available sources, such as egg yolk or soybeans, from which they are mechanically or chemically extracted using hexane.

Triacylglycerolipids and lyso (lysoPC) were reduced in the upland as compared to lowland rice. It is suspected the rice expressed different levels of lipid contents play essential roles in rice germination at adopted lands (Liu *et al.*, 2013). TG(18:1(9Z)/18:1(9Z)/18:1(9Z)) also named as triolein is a triglyceride formed by esterification of the three hydroxy groups of glycerol with oleic acid. Triolein is one of the two components of Lorenzo's oil. It has a role as a plant metabolite and a Caenorhabditis elegans metabolite. It derives from an oleic acid. The lyso (lysoPC) forms in rice endosperm represent the major starch lipid, and may form inclusion complexes with amylose, affecting the physicochemical properties and digestibility of starch, and hence it's cooking and eating quality (Lin *et al.*, 2017). LysoPC(18:1(9Z)), LysoPC(18:2(9Z,12Z)), and LysoPC(16:0) are a lysophospholipid (LyP). It is a monoglycerophospholipid in which a phosphorylcholine moiety occupies a glycerol substitution site. Lysophosphatidylcholines can have different combinations of fatty acids of varying lengths and saturation attached at the C-1 (sn-1) position. Fatty acids containing 16, 18 and 20 carbons are the most common. LysoPC(18:19Z)), in particular, consists of one chain of oleic acid at the C-1 position. The oleic acid moiety, an omega-9 fatty acid, is derived from various animal and vegetable sources such as olive oil, acai and grapeseed oil. LysoPC(18:2(9Z, 12Z)), in particular, consists of one chain of linoleic acid at the C-1 position. The linoleic acid moiety

is derived from seed oils. LysoPC(16:0), in particular, consists of one chain of palmitic acid at the C-1 position. The palmitic acid moiety is derived from fish oils, milk fats, vegetable oils and animal fats.

Table 1. Metabolites identified from the rice.

M/Z	Molecular Formulae	Molecular Structure	Mass Error	p value	log2(FC)
319.2253	C18H32O3	12,13-EpOME	-3.3905	***	-2.5638
295.2275	C18H28O3	12-oxo-PDA	4.9664	***	-3.6418
317.2095	C18H30O3	3-Oxo-2-(2-entenyl)cyclopentaneoctanoic acid;OPC-8:0	-4.5017	***	-3.5311
393.262	C23H36O5	6alpha-Malonyloxymanoyl oxide	3.061	***	-2.4552
339.2904	C22H43NO	6-cis-Docosenamide	-2.8417	*	-1.5331
295.2276	C18H30O3	9(10)-EpODE	-3.2245	***	-3.6763
279.2327	C18H30O2	Alpha-Linolenic acid	-3.2467	***	-2.5672
327.2282	C20H32O2	Arachidonic acid	4.4734	**	-1.3857
377.3232	C23H36O4	Ardisiphenol A	3.1258	***	7.2039
409.2564	C23H36O6	Australifungin	2.3645	***	-3.7282
599.4297	C35H60O6	beta-Sitosterol 3-O-beta-D-galactopyranoside	-2.3935	***	-1.0971
599.5022	C37H68O4	Cohibin C	-2.4607	***	-4.7982
351.2152	C18H32O5	Corchorifatty acid F	-1.4173	**	-1.546
277.217	C18H28O2	decylplastoquinone	-4.3931	***	-5.703
615.4976	C39H66O5	DG(18:4(6Z,9Z,12Z,15Z)/18:1(11Z)/0:0)	1.4186	***	-6.2328
633.5577	C44H72O2	ergosteryl palmitoleate	2.0836	***	-5.5946
587.5027	C36H68O4	FAHFA(18:1(9Z)/5-O-18:0)	-0.2278	**	13.3
317.2095	C20H28O3	Gibberellin A12 7-aldehyde	3.4193	***	-3.0452
939.8147	C49H88O15	Glycerol 2-(9Z,12Z-octadecadienoate) 1-hexadecanoate 3-O-[alpha-D-galactopyranosyl-(1->6)-beta-D-galactopyranoside]	-0.1824	***	4.3757
599.4297	C37H58O6	Hericenone G	1.7145	***	-1.0978
522.3563	C26H52NO7P	LysoPC(18:1(9Z))	-3.2683	***	-1.5422
520.3404	C26H50NO7P	LysoPC(18:2(9Z,12Z))	-2.3954	**	-1.8813
496.3406	C24H50NO7P	LysoPC(16:0)	-2.9152	*	-1.8854
379.2828	C21H40O4	MG(0:0/18:1(9Z)/0:0)	-2.693	**	-1.2331
784.5869	C44H82NO8P	PC(18:2(9Z,12Z)/18:1(9Z))	-3.8902	**	-1.1979
782.5711	C44H80NO8P	PC(18:2(9Z,12Z)/18:2(9Z,12Z))	-3.3118	**	-1.1439
335.2200	C20H30O4	Phytocassane B	2.8601	***	-4.4407
321.2408	C18H39NO3	Phytosphingosine	-3.4334	***	-2.3557
449.1084	C21H20O11	Quercitrin	-2.1694	**	-11.039
265.1550	C14H20N2O3	Subaphylline	-1.8967	*	-1.8284
902.8195	C57H104O6	TG(18:1(9Z)/18:1(9Z)/18:1(9Z))	-2.7164	***	4.4657

*, p value < 0.05; **, p value < 0.01 and ***, p value < 0.001 using OPLS-DA

Phytosphingosine is a phospholipid which a major component of all biological membranes such as sphingosine and ceramide. The phytosphingosine content was higher in upland rice as compared to lowland rice. Phytosphingosine is bioactive molecule, when in over-expression and stress responses will caused increase in sphingosine and ceramide (Begum *et al*., 2016). Refer to table 1, upland rice showed higher ratio of alpha-linolenic acid compare to lowland rice. This is a type of fatty acid found in plants (rice bran oil). The rice bran oil was extracted from the hard-outer brown layer of rice called chaff (rice husk).

There are another 3 metabolites, Ardisiphenol A, FAHFA(18:1(9Z)/5-O-18:0) and Glycerol 2-(9Z,12Z-octadecadienoate) 1-hexadecanoate 3-O-[alpha-D-galactopyranosyl-(1->6)-beta-D-galactopyranoside] showed higher ratio in lowland rice. Ardisiphenol A is an acetate ester obtained from the formal condensation of acetic acid with the hydroxyl group at position 1 of 6-pentadecylbenzene-1, 2, 4-triol with molecular formula $C_{23}H_{36}O_4$. Branched fatty acid esters of hydroxy fatty acids (FAHFAs) are endogenous lipids found in adipose tissue and serum that correlate with insulin sensitivity and are reduced in insulin-resistant humans. Structurally, they are characterized by a branched ester linkage between a fatty acid and a hydroxy-fatty acid (Ma *et al.*, 2015). Glycerol 2-(9Z,12Z-octadecadienoate) 1-hexadecanoate 3-O-[alpha-D-galactopyranosyl-(1->6)-beta-D-galactopyranoside] is found in cereals and cereal products and constituent of wheat flour.

There are significant metabolites discriminated the upland rice from lowland rice (Table 1 with fold change of down regulated values). The fatty acid, 6-cis-Docosenamide, 12,13-EpOME and 9(10)-EpODE are significant higher in upland rice. 6-cis-Docosenamide is a primary fatty amide. 9(10)-EpODE is an epoxy fatty acid are termed 'leukotoxins' (the term includes a range of diverse compounds), because they produce their primary toxic effects against leukocytes. At high dosages, they have toxic cardiovascular effects, which can even result in death (Sharmila *et al.*, 2017). 12,13-EpOME is a very hydrophobic molecule, practically insoluble (in water), and relatively neutral. 12,13-EpOME have been identified in seed oil as well as the rice plant, it characterized as self-defense substances (Hildreth *et al.*, 2020). Besides that, unsaturated fatty acid also showed higher ratio in upland rice, 12-oxo-PDA, 3-Oxo-2-(2-entenyl)cyclopentaneoctanoic acid;OPC-8:0 and arachidonic acid. 12-oxo-PDA and 3-Oxo-2-(2-entenyl)cyclopentaneoctanoic acid;OPC-8:0 are unsaturated carboxylic acids and based upon the fatty acid arachidonic acid, it can be found in corn which makes this a potential biomarker for the consumption of this food product. 6alpha-Malonyloxymanoyl oxide; (+)-6alpha-Malonyloxymanoyl oxide is type of anti-bacterial agent and malonate ester of a labdane diterpenoid also found higher ratio in upland rice together with Australifungin which is an antifungal agent and carbobicyclic compound or a HIV-1 integrase inhibitor.

In addition, beta-Sitosterol 3-O-beta-D-galactopyranoside, Cohibin C, Subaphylline, Corchorifatty acid F, decylplastoquinone, ergosteryl palmitoleate, Gibberellin A12 7-aldehyde, Hericenone G, Phytocassane B, and Quercitrin also shown higher ratio in upland rice. Beta-Sitosterol 3-O-beta-D-galactopyranoside, Cohibin C, and Subaphylline were belongs to the class of organic compound. The beta-Sitosterol 3-O-beta-D-galactopyranoside belongs to the class of organic compounds known as stigmastanes and derivatives. These are sterol lipids with a structure based on the stigmastane skeleton, which consists of a cholestane moiety bearing an ethyl group at the carbon atom C24 (Mizushina *et al.*, 2006). Cohibin C belongs to the class of organic compounds known as annonaceous acetogenins. These are waxy derivatives of fatty acids (usually C32 or C34), containing a terminal carboxylic acid combined with a 2-propanol unit at the C-2 position to form a methyl-substituted alpha, beta-unsaturated-gamma-lactone. One of their interesting structural features is a single, adjacent, or nonadjacent tetrahydrofuran (THF) or tetrahydropyran (THP) system with one or two flanking hydroxyl group(s) at the center of a long hydrocarbon chain (Gleye *et al.*, 2000). Cohibin C is an extremely weak basic (essentially neutral) compound (based on its pKa). Corchorifatty acid F is found in cereals and cereal products. Subaphylline belongs to the class of organic compounds known as hydroxycinnamic acids and derivatives. Hydroxycinnamic acids and derivatives are compounds containing a cinnamic acid (or a derivative thereof) where the benzene ring is hydroxylated. Subaphylline is a very strong basic compound (based on its pKa). Subaphylline is found, on average, in the highest concentration within sweet oranges. Subaphylline has also been detected, but not quantified in, several different foods, such as corns, avocado, fruits, mandarin

orange (clementine, tangerine), grapefruit leaves, and juice (Killiny & Nehela, 2020). This could make subaphylline a potential biomarker for the consumption of these foods. Corchorifatty acid F is isolated from rice infected with blast disease and confers activity against rice blast disease. Decylplastoquinone is a member of the class of 1,4-benzoquinones in which the quinone ring is substituted at positions 2 and 3 by methyl groups and at position 5 by a decyl group. It has a role as a cofactor. Ergosteryl palmitoleate is an ergosteryl ester. It has a role as a *Saccharomyces cerevisiae* metabolite. Gibberellin A12 7-aldehyde is found in pulses. Gibberellin A12 7-aldehyde is a are a large family of tetracyclic diterpenoid plant hormones that induce a wide range of plant growth responses, including seed germination, stem elongation, leaf expansion, induction of flowering, and pollen maturation. Hericenone G is found in mushrooms. Hericenone G is a constituent of the edible lion's mane mushroom (*Hericium erinaceum*). Quercitrin is a quercetin O-glycoside that is quercetin substituted by alpha-L-rhamnosyl moiety at position 3 via a glycosidic linkage. It has a role as an antioxidant, an antileishmanial agent, an EC 1.1.1.184 [carbonyl reductase (NADPH)] inhibitor, an EC 1.1.1.21 (aldehyde reductase) inhibitor, an EC 1.14.18.1 (tyrosinase) inhibitor and a plant metabolite. It is a monosaccharide derivative, a tetrahydroxyflavone, an alpha-L-rhamnoside and a quercetin O-glycoside. It is a conjugate acid of a quercitrin-7-olate.

CONCLUSION

We used UHPLC-TOF to comparatively analyze metabolites in upland and lowland rice. As results, we can cluster two different types of rice by using the metabolites identified from the rice. There are 4 major compounds shown upregulated higher ratio in lowland rice compare to upland rice. These compounds are the acetate ester of Ardisiphenol A, fatty acid esters of FAHFA(18:1(9Z)/5-O-18:0), Glycerol 2-(9Z,12Z-octadecadienoate) 1-hexadecanoate 3-O-[alpha-D-galactopyranosyl-(1->6)-beta-D-galactopyranoside], and Triacylglycerolipids of TG(18:1(9Z)/18:1(9Z)/18:1(9Z)). Throughout such an approach, a systematic, scientific, evident-based approach could be established to identify different types of Sabah rice varieties and distinguish the rice varieties based on profiled metabolite compounds.

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