



## Research article

## Uncovering metabolite changes in rice leaves (*Oryza sativa* L.) during vegetative stages using ethanolic extract analysis based on gas chromatography-mass spectrometry

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### Abstract

**Importance of the work:** This research contributes to understanding rice physiology and emphasizes the potential for utilizing metabolomic data in breeding programs aimed at enhancing crop resilience and yield.

**Objectives:** To investigate the metabolite profiles of rice leaves and to elucidate the catabolic pathway of chlorophyll during critical growth stages.

**Materials and Methods:** Two rice varieties, IR50404 (IR504) and Huyet Rong (HRO), were examined at 3 wk and 5 wk of growth to elucidate the changes in key metabolites using gas chromatography-mass spectrometry analysis. The total chlorophyll contents of rice leaves were measured to determine the shift in metabolite pathways.

**Results:** There were significant differences in the chlorophyll content, with both varieties having higher levels at 3 wk than at 5 wk. The profiling revealed that IR504 contained a greater number of metabolites than HRO at both time points. Notably, phytol and bis(2-ethylhexyl) phthalate (DEHP) were predominant compounds, with the phytol levels notably increasing in HRO by week 5. Additionally, there were metabolic shifts in the volatile organic compounds, with an increase in diterpenoids and a decrease in phthalate esters as the plants matured. Based on the results, the study proposed the metabolic pathway of chlorophyll degradation to produce important compounds (phytol, vitamin E and fatty acid derivatives) in rice leaves.

**Main finding:** Overall, the findings underscored the dynamic nature of rice leaf metabolism and provided insights into the biochemical adaptations that occur during critical growth stages.

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## Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops globally, providing sustenance for over one-half of the world's population (Muthayya et al., 2014). Understanding the physiological and biochemical changes in rice during its growth stages is crucial for optimizing cultivation practices and enhancing yield. For example, among the various developmental phases, the vegetative stage plays a vital role in determining the overall health and productivity of the plant as during this period, plants undergo major metabolic changes that are influenced by both genetic and environmental factors (Böttger et al., 2018; Zaynab et al., 2019). Other studies have highlighted the importance of specific metabolites in plants, linking them to various physiological traits such as drought resistance, nutrient uptake and overall plant vigor (Verma and Shukla, 2015; Ashraf et al., 2018).

Metabolomics, the comprehensive study of small molecules or metabolites within biological samples, has gained prominence as a means to elucidate the complex biochemical pathways that underpin plant growth and development (Patel et al., 2021). Metabolites are essential for numerous physiological functions, including energy production, stress response and the synthesis of secondary metabolites that provide protection against biotic and abiotic stressors (Wu et al., 2023). Profiling these metabolites allows researchers to gain insight into the metabolic shifts that occur as rice progresses through its vegetative stages. Gas chromatography-mass spectrometry (GC-MS) has emerged as a robust analytical technique for profiling and quantifying the complex mixtures of metabolites present in plant tissues that is particularly effective for analyzing volatile organic compounds (VOCs) and has been widely applied in metabolomics to understand plant biochemical profiles (Choudhury et al., 2022). While other studies have utilized GC-MS to identify key metabolites in rice, focusing primarily on seeds and grains (Llorente et al., 2019; Ashokkumar et al., 2020; Min et al., 2021), there remains a notable gap in research regarding the comprehensive profiling of rice leaves during the vegetative stages. This aspect is critical, as the vegetative phases are characterized by extensive metabolic activity that supports growth and prepares the plant for subsequent reproductive stages (Huang et al., 2024). Understanding the biochemical changes during these stages can provide valuable insights into enhancing crop yield and resilience against environmental stressors.

The Mekong Delta region is one of the three predominant rice growing areas, producing over one-half of the total rice production in Vietnam (Tho and Umetsu, 2022). IR50404 (IR504), non-pigmented (white) rice, was developed by the International Rice Research Institute and adopted in Vietnam, where it is characterized by short growth duration (85–90 d), high adaptability and stability to different cropping seasons, high tolerance to pathogens and high grain yield (6–8 t/ha; Phuoc et al., 2022). Huyet Rong (HRO), a native pigmented (red) rice variety of Vietnam, is characterized by long growth duration (175–180 d), high tolerance to pathogens and average yield (3.5–3.7 t/ha; Ta and Chu, 2022). HRO is known as an herbal rice because of its high nutritional value and pharmaceutical properties (Bhat and Riar, 2017).

Rice leaves are a rich source of various primary and secondary metabolites that play essential roles in physiological processes and defense mechanisms against pathogens and herbivores (Böttger et al., 2018; Zaynab et al., 2019). Recent findings have demonstrated that environmental factors, such as light intensity, significantly influence the metabolic pathways in rice leaves (Tho et al., 2024). However, the underlying mechanisms driving the synthesis and accumulation of phytochemicals in rice leaves, along with their contributions to plant health and development, remain inadequately explored. Therefore, the current study aimed to address these gaps by analyzing two rice varieties, IR504 and HRO, at 3 wk and 5 wk of growth. GC-MS was used to present an in-depth analysis of the metabolite profiles of rice leaves during the vegetative stages, uncovering the biochemical intricacies that support plant development. By elucidating the changes in key metabolites, this research should not only enhance fundamental understanding of rice biology but also highlight the roles of these metabolites in the plant's metabolic pathways. Ultimately, this work seeks to inform breeding and management strategies that could improve rice cultivation in the face of growing environmental challenges.

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## Materials and Methods

### *Plant conditions and extraction*

Two varieties of rice (*Oryza sativa* L.) commonly grown in Vietnam were utilized, specifically IR504 and HRO. Their seeds were sown in plastic trays measuring 65 cm in length, 42 cm in width, and 16 cm in depth, each containing 25 kg of alluvial soil. The seed density in each tray was 200 seeds

(about 500 seeds/m<sup>2</sup>, 1–2 cm distance between seeds). The trays were kept in a greenhouse with a temperature range of 24.5–32.6°C, a light intensity of 1125.76 μmol/m<sup>2</sup>s, and relative humidity levels in the range 70–80%. The rice plants were watered twice daily without any added fertilizers or pesticides. The physical and chemical properties of soil were: pH = 5.7; electrical conductivity (EC, σ) = 456 μS/cm; cation exchange capacity (CEC) = 24.7 cmol<sub>c</sub>/kg; oxidation-reduction potential = 107.5 mV; N = 0.41%; P = 0.1%; K = 1.2%; Ca = 1,089.02 mg/kg; and Mg = 297.75 mg/kg. Rice leaves were harvested at two developmental stages: 3 wk (21 d) and 5 wk (35 d) after planting.

The extraction process used 80% ethanol as the solvent. Prior to extraction, the rice leaves were thoroughly washed and finely chopped. Then, these plant samples were subjected to sonication in an ethanol solution containing 1% hydrochloric acid, using a ratio of 10 g of plant material per 100 mL of solvent. The sonication process lasted for 30 min to ensure effective extraction of metabolites. Following sonication, the resulting samples were carefully passed through Whatman filter paper No. 1 to remove any remaining solid residues. Subsequently, the solvent was evaporated from the filtered samples using a rotary evaporator, set at a temperature below 45°C (Tamprasit et al., 2019). Then, the dried crude extract was stored at -20°C until further analysis.

### Total chlorophyll content

The method for evaluating the total chlorophyll content was performed according to Tamprasit et al. (2019), with some modifications. The dried extract of rice leaves was re-dissolved in 80% ethanol to achieve the original volume of 100 mL. Next, a 2 mL aliquot of the ethanol extract was scanned at wavelengths of 645 nm and 663 nm using a UV-Vis spectrophotometer (GENESYS 150; Thermo Scientific; Waltham, MA, USA). The total chlorophyll content (measured in micrograms per gram fresh weight of leaves) was calculated using the Equation 1:

$$\text{Total chlorophyll content} = [(20.2 \times A_{645}) + (8.02 \times A_{663})] \quad (1)$$

where  $A_{645}$  and  $A_{663}$  are the absorbance values at the specified wavelengths.

### Gas chromatography-mass spectrometry analysis

GC-MS analysis of the phytochemicals in the rice leaf extracts was performed following the methodology outlined by Tho et al. (2024), using an Agilent 7890B GC System (Agilent Technologies Inc.; USA) equipped with an Agilent 122-5532G column (40 m × 250 μm × 0.25 μm), with helium serving as the carrier gas. The split ratio was set to 25:1 and the split flow was maintained at 30 mL/min. The sample was injected into the GC inlet under pressure flow control mode, with the purge flow kept at 3 mL/min.

Initially, the column oven temperature was set at 70°C and then increased to 150°C at a rate of 25°C/min. Subsequently, the temperature was raised to 200°C at a rate of 10°C/min and finally increased to 280°C at a rate of 15°C/min, with a hold time of 5 min at the final temperature. A diluted sample (1 μL) was injected at 280°C and 10,546 kg/m<sup>2</sup> pressure, with a flow rate of 1.2 mL/min and an average velocity of 34.908 cm/sec. The total run time for the analysis was approximately 50 min. Detected compounds were identified by processing the raw GC-MS data against the NIST Mass Spectral Library, Version NIST05.L (<https://chemdata.nist.gov/>).

### Statistical analysis

All experimental results were expressed as mean ± SD values. Statistical analysis applied one-way analysis of variance and Duncan's test for statistical differences ( $p < 0.05$ ). Principal component analysis (PCA) was used to analyze the patterns and relationships from the GC-MS dataset in the Statgraphics Centurion XVIII (Version 18.1.12; Statgraphics Technologies, Inc., USA).

## Results

### Total chlorophyll content

Both the rice varieties had higher chlorophyll contents when harvested at 3 wk compared to 5 wk. Specifically, the chlorophyll content in the IR504 rice leaves was 1,319.59 ± 13.89 μg/g of fresh leaves at 3 wk, decreasing to 1,203.22 ± 10.26 μg/g of fresh leaves at 5 wk. Similarly, the HRO variety showed a slight decrease in the chlorophyll content from 1,279.36 ± 15.50 μg/g of fresh leaves at 3 wk to 1,232.90 ± 20.13 μg/g of fresh leaves at 5 wk. In addition, there were changes in the levels of chlorophyll a and chlorophyll b between 3 wk and 5 wk (Table 1).

**Table 1** Chlorophyll changes in rice (*Oryza sativa* L.) leaves during vegetative stages

Rice variety	Vegetative stage	Total chlorophyll	Chlorophyll a	Chlorophyll b	Chlorophyll <i>a/b</i> ratio
	(wk)	( $\mu\text{g/g}$ fresh leaf weight)	( $\mu\text{g/g}$ fresh leaf weight)	( $\mu\text{g/g}$ fresh leaf weight)	
IR504	3	1319.59 $\pm$ 13.89 <sup>a</sup>	532.78 $\pm$ 6.02 <sup>a</sup>	786.31 $\pm$ 13.98 <sup>b</sup>	1.476 $\pm$ 0.04 <sup>b</sup>
	5	1203.22 $\pm$ 10.26 <sup>b</sup>	390.31 $\pm$ 9.56 <sup>b</sup>	813.54 $\pm$ 6.99 <sup>a</sup>	2.086 $\pm$ 0.07 <sup>a</sup>
HRO	3	1279.36 $\pm$ 15.50 <sup>a</sup>	501.69 $\pm$ 10.48 <sup>a</sup>	777.93 $\pm$ 11.76 <sup>b</sup>	1.551 $\pm$ 0.02 <sup>b</sup>
	5	1232.90 $\pm$ 20.13 <sup>b</sup>	412.05 $\pm$ 7.23 <sup>b</sup>	820.69 $\pm$ 10.95 <sup>a</sup>	1.992 $\pm$ 0.05 <sup>a</sup>

Mean  $\pm$  SD within the same column with different lowercase superscripts indicate significant differences ( $p < 0.05$ ) between means in each rice variety, based on Duncan's multiple range test.

### Gas chromatography-mass spectrometry profile

The rice leaves from the two varieties harvested at 3 wk and 5 wk were profiled using GC-MS. The mass spectra are provided in the supplementary material (ESM-1). The results

of the GC-MS analysis are presented in Tables 2 and 3. IR504 had a higher number of metabolites (22 identified at 3 wk and 28 at 5 wk) than HRO (16 and 11 metabolites at 3 wk and 5 wk respectively). Notably, there were 12 metabolites common to both rice varieties.

**Table 2** Volatile organic compounds in rice leaves of IR504 at 3 wk and 5 wk, based on gas chromatography-mass spectrometry

No.	Compound	Library number (s)*	Molecular formula (amu)	% Peak area		Chemical classification
				3 wk	5 wk	
1	Dodecanoic acid, methyl ester	67167	214.193	1.243	-	Fatty acid ester
2	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)-	16444	138.141	0.693	-	Monoterpene
3	Hexadecanoic acid, methyl ester	105644	270.256	5.462	-	Fatty acid ester
		105646	270.256	-	0.586	Fatty acid ester
4	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	119877	292.24	-	2.11	Fatty acid ester
5	Phytol	122405	296.308	-	24.925	Diterpenoid
		122407	296.308	6.629	-	Diterpenoid
6	9-Octadecenamide, (Z)-	112656	281.272	-	0.631	Fatty acid amide
7	1,2-Benzenedicarboxylic acid, diisooctyl ester	168519	390.277	51.507	-	Phthalate ester
		168521	390.277	-	0.472	Phthalate ester
8	Vitamin E	177303	430.381	-	2.489	Meroterpenoid
9	Methyl tetradecanoate	86753	242.225	0.737	-	Fatty acid ester
10	(-)-Spathulenol	71327	220.183	0.534	-	Sesquiterpenoid
11	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	121105	294.256	1.458	-	Fatty acid ester
		121106	294.256	-	1.778	Fatty acid ester
12	9-Octadecenoic acid, methyl ester	122299	296.272	5.074	-	Fatty acid ester
13	Octadecanoic acid, methyl ester	123708	298.287	1.595	-	Fatty acid ester
14	13-Docosenamide, (Z)-	146308	337.334	1.113	-	Fatty acid amide
15	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	60003	204.188	1.964	-	Sesquiterpenoid
16	Bicyclo[4.3.0]nonane, 7-methylene-2,4,4-trimethyl-2-vinyl-	59915	204.188	0.574	-	Sesquiterpenoid
17	Bicyclo[10.1.0]tridec-1-ene	41746	178.172	0.728	-	Cyclic hydrocarbon
18	Ergosta-5,8,22-trien-3-ol, (3.beta.,22E)-	170297	396.339	1.755	-	Steroid
19	Campesterol	171431	400.371	4.017	1.78	Steroid
20	Stigmasterol	173931	412.371	5.386	1.397	Steroid
21	Stigmasterol, 22,23-dihydro-	174408	414.386	5.201	1.675	Steroid
22	Cholest-5-en-3-ol, 24-propylidene-, (3.beta.)-	176594	426.386	0.692	-	Steroid
23	Pregn-4-ene-3,20-dione, (8.alpha.,10.alpha.)-	133591	314.225	1.086	-	Steroid
24	4,22-Stigmastadiene-3-one	173535	410.355	1.19	-	Steroid
25	Stigmast-4-en-3-one	173936	412.371	1.362	-	Steroid
26	Benzene, 1-methyl-3-[(2-methylpropyl)thio]-	43017	180.097	-	0.569	Phenol
27	1-Oxaspiro[4.5]decan-2-one, 6-isopropenyl-9-methyl-	62829	208.146	-	0.483	Heterocyclic compound

**Table 2** Continued

No.	Compound	Library number (s)*	Molecular formula (amu)	% Peak area		Chemical classification
				3 wk	5 wk	
27	Tetradecanenitrile	63714	209.214	-	1.143	Fatty nitrile
29	2-Chloroethyl linoleate	148820	342.233	-	0.452	Fatty acid
30	Bicyclo[5.1.0]oct-3-ene	5286	108.094	-	1.809	Cyclic hydrocarbon
31	9,12-Octadecadienoic acid (Z,Z)-	111993	280.24	-	0.816	Fatty acid
32	6-Octadecenoic acid, (Z)-	113359	282.256	-	1.107	Fatty acid
33	S-[2-[N,N-Dimethylamino]ethyl]morpholine-N-carbonylthiocarbohydroximate	99017	261.115	-	1.015	Heterocyclic compound
34	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	154542	354.277	-	2.118	Fatty acid ester
35	9-Octadecenal, (Z)-	102821	266.261	-	1.967	Fatty aldehyde
36	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	101506	264.245	-	0.922	Fatty alcohol
37	Bis(2-ethylhexyl) phthalate	168501	390.277	-	43.163	Phthalate ester
38	Squalene	173554	410.391	-	0.502	Triterpenoid
39	7,10-Hexadecadienoic acid, methyl ester	102730	266.225	-	0.642	Fatty acid ester
40	Cholest-5-en-24-one, 3-(acetyloxy)-, (3.β.)-	179014	442.345	-	0.639	Steroid
41	9,19-Cyclolanost-24-en-3-ol, acetate, (3.β.)-	181946	468.397	-	1.114	Triterpenoid
42	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.β.)-	178754	440.402	-	3.693	Triterpenoid

\* For some compounds, two library numbers are listed due to multiple matching spectra in the NIST library; - = not detected.

**Table 3** Volatile organic compounds in rice leaves of HRO at 3 wk and 5 wk, based on gas chromatography-mass spectrometry.

No.	Compound	Library number (s)*	Molecular formula (amu)	% Peak area		Chemical classification
				3 wk	5 wk	
1	Phenol, 2,4-bis(1,1-dimethylethyl)-	61449	206.167	0.205	-	Phenol
2	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	16398	138.141	0.481	-	Monoterpenoid
3	1,4-Eicosadiene	110849	278.297	0.167	-	Alkene
4	Sulfurous acid, 2-ethylhexyl tridecyl ester	163815	376.301	0.181	-	Organosulfate
5	Phytol	122405	296.308	2.246	38.495	Diterpenoid
		122407	296.308	-	6.022	Diterpenoid
6	Bis(2-ethylhexyl) phthalate	168501	390.277	-	39.397	Phthalate ester
7	Stigmasterol, 22,23-dihydro-	174408	414.386	0.568	1.29	Steroid
8	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	119876	292.24	0.162	-	Fatty acid ester
		119875	292.24	-	2.194	Fatty acid ester
9	1,2-Benzenedicarboxylic acid, diisooctyl ester	168521	390.277	0.329	-	Phthalate ester
		168519	390.277	91.751	-	Phthalate ester
10	13-Docosenamide, (Z)-	146308	337.334	0.394	-	Fatty acid amide
11	Ergosterol	170280	396.339	0.274	-	Steroid
12	Campesterol	171432	400.371	0.592	1.263	Steroid
13	Stigmasterol	173931	412.371	1.286	1.547	Steroid
14	Eicosane	113492	282.329	0.181	-	Alkane
15	3-Cyclohexene-1-carboxaldehyde, 4-methyl-	10249	124.089	0.331	-	Monoterpenoid
16	Spinasterone	173532	410.355	0.564	-	Steroid
17	Stigmast-4-en-3-one	173936	412.371	0.288	-	Steroid
18	1,3-Benzenediol, 5-pentyl-	42884	180.115	-	0.854	Phenol
19	Tetradecanenitrile	63712	209.214	-	2.164	Fatty nitrile
20	Bicyclo[5.1.0]oct-3-ene	5286	108.094	-	3.4	Cyclic hydrocarbon
21	2,4-Dinitrophenyl hydrazone of 4-trichloromethylbenzaldehyde	171683	401.969	-	2.495	Benzaldehyde
22	Ethanamine, 2,2'-oxybis[N,N-dimethyl]-	29957	160.158	-	0.878	Aliphatic Amine

\* For some compounds, two library numbers are listed due to multiple matching spectra in the NIST library; - = not detected.

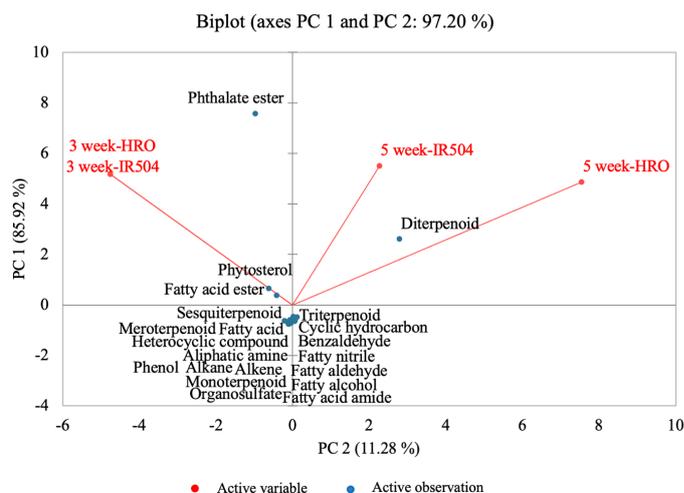
In addition, the rice leaves of IR504 had a higher abundance of compounds classified as fatty acid esters, steroids (or sterols) and terpenoids (or terpenes). In contrast, HRO predominantly contained steroid-derived compounds. Over two-thirds of the total metabolites in the rice leaves of IR504 were different between the two harvest times of 3 wk and 5 wk, whereas more than one-half of the total metabolites in the rice leaves of HRO were present in only one of the same time points.

Both rice varieties contained similar major compounds with the highest percentages of peak areas. At week 3, phytol and 1,2-benzenedicarboxylic acid, diisooctyl ester (DIOP) predominated, while at week 5, phytol and bis(2-ethylhexyl) phthalate (DEHP) were more abundant. In the HRO variety, the peak area of phytol significantly increased from 2.246% at week 3 to 44.517% at week 5. DIOP accounted for the highest peak at week 3, reaching 92.080%; however, unexpectedly, it was absent at week 5. Conversely, DEHP was not detected at week 3 but appeared as the highest peak at week 5, with a peak area of 39.397% in HRO.

Similarly, in the IR504 variety, phytol's peak area was four times higher at week 5 than in week 3. In addition, DIOP had the highest peak at week 3, with a peak area of 51.507%; however, it was present only in trace amounts at week 5 (0.472%). In contrast, in IR504, DEHP peaked at week 5, with a peak area of 43.163%. Additionally, other major compounds with high peak areas in IR504 were hexadecanoic acid methyl ester, campesterol, stigmasterol, 22,23-dihydro-stigmasterol, 9-octadecenoic acid methyl ester, 9,12,15-octadecatrienoic acid methyl ester, vitamin E and 9,19-cyclolanostan-3-ol, 24-methylene-(3.β.). In HRO, tetradecanenitrile and stigmasterol had high peak areas. Overall, based on the GC-MS results, there were notable differences and diversification in metabolite composition, influenced by both rice variety and harvest time.

### Principal component analysis

As illustrated in Fig. 1, the biplot revealed both differences and similarities for both rice varieties in the synthesis and accumulation of VOCs in the rice leaves between weeks 3 and 5. Principal components (PCs) 1 and 2 accounted for 97.20% of the variance observed in the GC-MS data. The loading scores, categorized by week and the nature of the VOCs, are presented in Table 4.



**Fig. 1** 2-Dimensional biplot of principal component analysis (PCA) using total proportion of chemical groups from gas chromatography-mass spectrometry dataset in rice leaves of IR504 and HRO at 3 wk and 5 wk.

**Table 4** Results of principle component (PC) analysis based on gas chromatography-mass spectrometry dataset for analysis of metabolite changes in rice leaves at 3 wk and 5 wk

	PC 1	PC2
Eigenvalue	3.437	0.451
Variance (%)	85.925	11.280
Vegetative stage	Factor loading	
3 week-IR504	0.925	-0.308
5 week-IR504	0.984	0.147
3 week-HRO	0.926	-0.308
5 week-HRO	0.870	0.489
VOC nature	Factor score	
Cyclic hydrocarbon	-0.499	0.124
Diterpenoid	2.599	2.802
Fatty acid	-0.632	-0.039
Fatty acid amide	-0.665	-0.129
Fatty acid ester	0.371	-0.414
Fatty alcohol	-0.707	-0.070
Fatty aldehyde	-0.653	-0.048
Fatty nitrile	-0.612	0.064
Heterocyclic compound	-0.677	-0.058
Meroterpenoid	-0.596	-0.064
Phenol	-0.688	-0.031
Phthalate ester	7.574	-0.965
Sesquiterpenoid	-0.625	-0.210
Phytosterol	0.658	-0.617
Triterpenoid	-0.479	0.024
Aliphatic amine	-0.721	-0.037
Alkane	-0.751	-0.094
Alkene	-0.751	-0.094
Benzaldehyde	-0.659	0.060
Monoterpenoid	-0.735	-0.109
Organosulfate	-0.751	-0.094

VOC = volatile organic compound.

Notable disparities in VOC accumulation were observed between weeks 3 and 5 in the rice leaves of both IR504 and HRO. At week 3, the rice leaves of HRO and IR504 aligned closely in the biplot, indicating similar accumulation patterns. Specifically, the factor loadings for both IR504 and HRO at week 3 were nearly identical, with the same score for PC 2 (-0.308) and very similar scores for PC 1 (0.925 and 0.926, respectively). In contrast, the loading scores for week 5 were different between the two varieties, with IR504 having PC1 and PC2 scores of 0.984 and 0.147, respectively, while HRO had PC1 and PC2 scores of 0.870 and 0.489, respectively. However, despite these differences, both rice varieties had similar trends on the right side of the vertical axis.

There were abundant level of key metabolites (identified as phthalate esters, diterpenoids, phytosterols, and fatty acid esters) synthesized during both weeks 3 and 5. The VOCs in the diterpenoid group accumulated at higher concentrations in week 5 than in week 3, whereas the VOCs classified as phthalate esters were more abundant in week 3 than in week 5. Additionally, for both rice varieties, leaves at week 3 produced higher levels of phytosterol and fatty acid ester compounds than those at week 5. These findings illustrated the metabolic distinctions between weeks 3 and 5 in the rice leaves during the vegetative stage.

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## Discussion

The results of this study highlight that at different growth stages, there were major differences in the metabolic profiles of the rice leaves of the IR504 and HRO varieties. The biplot analysis (Fig. 1) effectively demonstrates how the synthesis and accumulation of VOCs varied between weeks 3 and 5, with PC 1 and PC 2 accounting for a notable 97.20% of the variance in the GC-MS data, indicating that these two components had captured almost all of the metabolic variation between the samples.

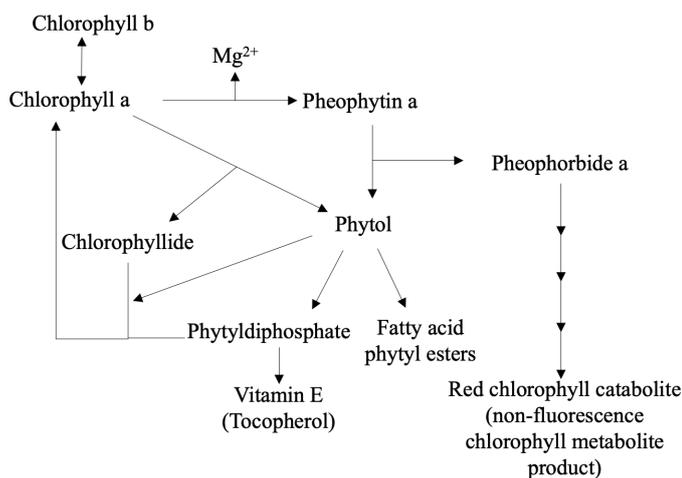
The observed accumulation patterns of the VOCs revealed distinct metabolic shifts as the plants matured. At week 3, both rice varieties had similar profiles, suggesting that their metabolic pathways were closely aligned during the early vegetative stage. The nearly identical loading scores for PC 1 and PC 2 in both varieties at this time point indicated a shared response to environmental factors or developmental cues. However, by week 5, the metabolic profiles diverged considerably. The different loading scores for IR504 and HRO suggested that each variety may have responded uniquely to the demands of later growth stages, possibly due to genetic

differences or varying environmental adaptations. Similarly, differences in the expression of phytochemical composition between the two rice cultivars under abiotic stresses have been reported due to genetic background (Du et al., 2021). This divergence underscored the complexity of plant metabolic processes and the influence of developmental timing on metabolite accumulation.

The identification of key metabolites (phthalate esters, diterpenoids, phytosterols and fatty acid esters) provides insights into the functional roles these compounds may play in plant health and development. For example, the higher accumulation of diterpenoids in week 5 suggested an enhanced role of these compounds in stress responses or developmental signaling as the plant prepared for reproductive phases. Diterpenoids are known for their roles in plant defence and may enhance resilience against biotic and abiotic stressors (Chen et al., 2021; Jan et al., 2021). Conversely, while it remains unclear whether rice plants synthesize phthalate esters or acquire them from external environmental sources, the elevated levels of these compounds observed at week 3 suggested their potential importance during early developmental stages. This may have been related to growth regulation or defensive responses against pests. Babu and Wu (2010) reported that the biosynthesis of phthalate esters in algae and cyanobacteria may act as a pheromone to defend against aquatic organisms and as a cell membrane regulator to maintain cell flexibilities. Comprehensive literature reviews have indicated that natural phthalate esters have allelopathic, antimicrobial and insecticidal activities essential for plant growth and development (Roy, 2020; Huang et al., 2021). Therefore, the decline in these compounds by week 5 in the current study suggested a shift in metabolic priorities as the plant matured.

Particularly noteworthy are the findings regarding phytosterols and fatty acid esters. The higher levels of these compounds in the leaves aged 3 wk compared to those aged 5 wk suggested that these metabolites were crucial during the early stages of growth. Phytosterols are vital for maintaining cell membrane integrity and fluidity, which is essential for the growth and development of young tissues (Rogowska and Szakiel, 2020; Zhang et al., 2020). Similarly, fatty acid esters play important roles in energy storage and membrane structure (Dyall et al., 2022; Ali and Szabó, 2023). The decline in these metabolites at week 5 in the current study may have indicated a metabolic transition as the plants reallocated resources to support other developmental needs, emphasizing the dynamic nature of plant metabolic processes and the need for further research to understand the regulatory mechanisms underlying these changes.

Chlorophyll is essential for photosynthesis, the process through which plants convert light energy into chemical energy (Liu et al., 2024). The observed decline in chlorophyll content from week 3 to week 5 in both rice varieties suggested a possible transition in the plant from primary growth to reproductive development. This shift may involve reallocating resources away from chlorophyll synthesis as the plant prepares for flowering and seed production (Ohmiya et al., 2014; Kuai et al., 2018). Phytol, a long-chain alcohol derived from the degradation of chlorophyll, plays a crucial role in the integrity of chlorophyll molecules (Gutbrod et al., 2019). As the chlorophyll content decreases, it is expected that phytol levels may also fluctuate (Durrett and Welti, 2021). The significant increase in phytol observed in week 5 (Fig. 2), particularly in HRO, may indicate its dual role: not only as a breakdown product of chlorophyll but also as a precursor for other vital metabolites, including tocopherols such as vitamin E (Rise et al., 1989; Dorp et al., 2015). This transformation can be seen as a metabolic adaptation, where the plant repurposes phytol for protective functions as chlorophyll diminishes. Furthermore another reason for the reduction of chlorophyll content could be the lack of nitrogen supply, which resulted in chlorophyll degradation during leaf chlorosis (Chen et al., 2024).



**Fig. 2** Chlorophyll degradation and turnover in rice leaves

The interrelationships among chlorophyll, phytol, vitamin E and fatty acids highlight a complex network of metabolic pathways. The decline in chlorophyll may trigger a compensatory increase in phytol and vitamin E, which work together to mitigate oxidative stress and maintain membrane integrity (Cahoon et al., 2003; Chaudhary and Khurana, 2009). Furthermore, the dynamics of fatty acid metabolism suggest

that energy storage and structural needs are carefully balanced with the plant's developmental stage (He et al., 2020). These findings have major implications for understanding plant responses to environmental stressors. For example, enhanced levels of vitamin E and phytol in the later stages may indicate a protective mechanism against oxidative stress, thereby improving the plant's resilience. Additionally, the metabolic shifts observed could inform breeding strategies aimed at developing rice varieties with improved stress tolerance and yield potential.

Regarding the biological activities of the main phytochemical groups, high numbers of phytosterols and fatty acid esters are presented in both rice varieties. Phytosterols belong to the class of triterpenoids and are predominantly accumulated in nuts, legumes, cereals, plant oils and fruits (Veza et al., 2020). Several reports have mentioned that similar phytosterols are present in other species, having antifungal, antioxidant, anti-inflammatory and anti-carcinogenic activities (Khan and Javaid, 2020; Khor et al., 2021; Guo et al., 2022). Furthermore, abundant amounts of fatty acid esters and phytosterols present in the extract of *Pleurotus ostreatus* were reported to have good antioxidant, antimicrobial and antitumor activities (Mishra et al., 2022). More recently, Nguyen et al. (2024) reported on potential uses of rice leaves or rice grass at the vegetative stage in the food and pharmaceutical industries. Similarly, the current study demonstrated that rice leaves, particularly at age 5 wk, synthesized diverse phytochemical compounds with potential for these applications.

## Conclusion

A comprehensive analysis was undertaken of the metabolite profiles of rice leaves (*Oryza sativa* L.) during the vegetative stages, utilizing GC-MS to elucidate the dynamic changes in key metabolites. There were significant differences in chlorophyll content, VOCs and essential metabolites such as phytol, vitamin E and fatty acids between the IR504 and HRO rice varieties, at 3 wk and 5 wk of growth. Based on the results, as the rice plants transitioned from early to later vegetative stages, there was a notable shift in metabolic priorities. The decrease in chlorophyll content correlated with increased levels of phytol and vitamin E, suggesting adaptive mechanisms to mitigate oxidative stress and maintain cellular integrity during critical growth transitions. Furthermore, the variation in the fatty acid profiles highlighted the plant's changing energy and structural requirements. Overall, this research should

not only enhance understanding of the biochemical pathways involved in rice leaf development but also underscore the importance of metabolomic profiling in agricultural science. The insights gained from this study should inform breeding programs aimed at developing resilient rice varieties capable of thriving under varying environmental conditions. Future investigations should focus on the functional roles of the identified metabolites and their interactions, contributing to the broader knowledge of plant physiology and sustainable agricultural practices.

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### Conflict of interest

The authors declare no conflicts of interest.

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### References

- Ali, O., Szabó, A. 2023. Review of eukaryote cellular membrane lipid composition, with special attention to the fatty acids. *Int. J. Mol. Sci.* 24: 15693. doi.org/10.3390/ijms242115693
- Ashokkumar, K., Govindaraj, M., Vellaikumar, S., Shobhana, V.G., Karthikeyan, A., Akilan, M., Sathishkumar, J. 2020. Comparative profiling of volatile compounds in popular South Indian traditional and modern rice varieties by gas chromatography–mass spectrometry analysis. *Front. Nutr.* 7: 599119. doi.org/10.3389/fnut.2020.599119
- Ashraf, M.A., Iqbal, M., Rasheed, R., Hussain, I., Riaz, M., Arif, M.S. 2018. Chapter 8 - Environmental stress and secondary metabolites in plants: an overview, in: Ahmad, P., Ahanger, M.A., Singh, V.P., Tripathi, D.K., Alam, P., Alyemeni, M.N. (Eds.), *Plant Metabolites and Regulation Under Environmental Stress*. Academic Press. Cambridge MA, USA. pp.153–167. doi.org/10.1016/B978-0-12-812689-9.00008-X
- Babu, B., Wu, J.T. 2010. Production of phthalate esters by nuisance freshwater algae and cyanobacteria. *Sci. Total Environ.* 408: 4969–4975. doi.org/10.1016/j.scitotenv.2010.07.032
- Bhat, F.M., Riar, C.S. 2017. Characterizing the pigmented traditional rice cultivars grown in temperate regions of Kashmir (India) for free and bound phenolics compounds and *in vitro* antioxidant properties. *J. Cereal Sci.* 76: 253–262. doi.org/10.1016/j.jcs.2017.06.018
- Böttger, A., Vothknecht, U., Bolle, C., Wolf, A. 2018. Plant secondary metabolites and their general function in plants, in: Böttger, A., Vothknecht, U., Bolle, C., Wolf, A. (Eds.), *Lessons on Caffeine, Cannabis and Co: Plant-Derived Drugs and Their Interaction with Human Receptors*. Springer International Publishing. Cham, Switzerland. pp. 3–17. doi.org/10.1007/978-3-319-99546-5\_1
- Cahoon, E.B., Hall, S.E., Ripp, K.G., Ganzke, T.S., Hitz, W.D., Coughlan, S.J. 2003. Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. *Nat. Biotechnol.* 21: 1082–1087. doi.org/10.1038/nbt853
- Chaudhary, N., Khurana, P. 2009. Vitamin E biosynthesis genes in rice: Molecular characterization, expression profiling and comparative phylogenetic analysis. *Plant Sci.* 177: 479–491. doi.org/10.1016/j.plantsci.2009.07.014
- Chen, L.H., Xu, M., Cheng, Z., Yang, L.T., 2024. Effects of nitrogen deficiency on the photosynthesis, chlorophyll a fluorescence, antioxidant system, and sulfur compounds in *Oryza sativa*. *Int. J. Mol. Sci.* 25: 10409. doi.org/10.3390/ijms251910409.
- Chen, R., Bu, Y., Ren, J., Pelot, K.A., Hu, X., Diao, Y., Chen, W., Zerbe, P., Zhang, L. 2021. Discovery and modulation of diterpenoid metabolism improves glandular trichome formation, artemisinin production and stress resilience in *Artemisia annua*. *New Phytol.* 230: 2387–2403. doi.org/10.1111/nph.17351
- Choudhury, F.K., Pandey, P., Meitei, R., Cardona, D., Gujar, A.C., Shulaev, V. 2022. GC-MS/MS profiling of plant metabolites. *Methods Mol. Biol. Clifton NJ.* 2396: 101–115. doi.org/10.1007/978-1-0716-1822-6\_9
- Dorp, K., Hölzl, G., Plohmman, C., Eisenhut, M., Abraham, M., Weber, A.P.M., Hanson, A.D., Dörmann, P. 2015. Remobilization of phytol from chlorophyll degradation is essential for tocopherol synthesis and growth of *Arabidopsis*. *Plant Cell.* 27: 2846–2859. doi.org/10.1105/tpc.15.00395
- Du, S., Huang, X., Cai, Y., Hao, Y., Qiu, S., Liu, L., Cui, M., Luo, L. 2021. Differential antioxidant compounds and activities in seedlings of two rice cultivars under chilling treatment. *Front. Plant Sci.* 12: 631421. doi.org/10.3389/fpls.2021.631421
- Durrett, T.P., Welti, R. 2021. The tail of chlorophyll: Fates for phytol. *J. Biol. Chem.* 296: 100802. doi.org/10.1016/j.jbc.2021.100802
- Dyall, S.C., Balas, L., Bazan, N.G., et al. 2022. Polyunsaturated fatty acids and fatty acid-derived lipid mediators: Recent advances in the understanding of their biosynthesis, structures, and functions. *Prog. Lipid Res.* 86: 101165. doi.org/10.1016/j.plipres.2022.101165
- Guo, H., Chariyakornkul, A., Phannasorn, W., Mahatheeranont, S., Wongpoomchai, R. 2022. Phytochemical profile and chemopreventive properties of cooked glutinous purple rice extracts using cell-based assays and rat model. *Foods.* 11: 23–33. doi.org/10.3390/foods11152333
- Gutbrod, K., Romer, J., Dörmann, P. 2019. Phytol metabolism in plants. *Prog. Lipid Res.* 74: 1–17. doi.org/10.1016/j.plipres.2019.01.002
- He, M., Qin, C.X., Wang, X., Ding, N.Z. 2020. Plant unsaturated fatty acids: Biosynthesis and regulation. *Front. Plant Sci.* 11: 390. doi.org/10.3389/fpls.2020.00390
- Huang, L., Zhu, X., Zhou, S., Cheng, Z., Shi, K., Zhang, C., Shao, H. 2021. Phthalic acid esters: natural sources and biological activities. *Toxins.* 13: 495. doi.org/10.3390/toxins13070495
- Huang, X., Zhu, Y., Su, W., Song, S. et al. 2024. Widely-targeted metabolomics and transcriptomics identify metabolites associated with flowering regulation of Choy Sum. *Sci. Rep.* 14: 10682
- Jan, R., Asaf, S., Numan, M., Lubna, Kim, K.M. 2021. Plant secondary metabolite biosynthesis and transcriptional regulation in response to biotic and abiotic stress conditions. *Agronomy.* 11: 968. doi.org/10.3390/agronomy11050968

- Khan, I.H., Javaid, A. 2020. Antifungal activity and GC-MS analysis of n-butanol extract of quinoa (*Chenopodium quinoa* Willd.) leaves. *Bangladesh J. Bot.* 49: 1045–1051. doi.org/10.3329/bjb.v49i4.52537
- Khor, B.K., Chear, N.J.Y., Azizi, J., Khaw, K.Y. 2021. Chemical composition, antioxidant and cytoprotective potentials of *Carica papaya* leaf extracts: a comparison of supercritical fluid and conventional extraction methods. *Molecules.* 26: 1489. doi.org/10.3390/molecules26051489
- Kuai, B., Chen, J., Hörtensteiner, S. 2018. The biochemistry and molecular biology of chlorophyll breakdown. *J. Exp. Bot.* 69: 751–767. doi.org/10.1093/jxb/erx322
- Liu, J., Liu, Y., Li, Q., Wang, P., Li, Y., Liu, Q., Li, Z. 2024. Perspectives on improving photosynthesis to increase crop yield. *Plant Cell* 36: 3944–3956. doi.org/10.1093/plcell/koad281
- Llorente, C., Jimenez, R., Jackie, Brotman, Y., Fernie, A.R., Sreenivasulu, N. (2019). Rice Grain Quality Benchmarking Through Profiling of Volatiles and Metabolites in Grains Using Gas Chromatography Mass Spectrometry. In: Sreenivasulu, N. (eds) *Rice Grain Quality. Methods in Molecular Biology*, vol 1892. Humana Press, New York, NY, USA. https://doi.org/10.1007/978-1-4939-8914-0\_11
- Min, X., Xu, H., Huang, F., Wei, Y., Lin, W., Zhang, Z. 2021. GC-MS-based metabolite profiling of key differential metabolites between superior and inferior spikelets of rice during the grain filling stage. *BMC Plant Biol.* 21: 439. doi.org/10.1186/s12870-021-03219-8
- Mishra, V., Tomar, S., Yadav, P., Vishwakarma, S., Singh, M.P. 2022. Elemental analysis, phytochemical screening and evaluation of antioxidant, antibacterial and anticancer activity of *Pleurotus ostreatus* through *in-vitro* and *in-silico* approaches. *Metabolites.* 12: 821. doi.org/10.3390/metabo12090821
- Muthayya, S., Sugimoto, J.D., Montgomery, S., Maberly, G.F. 2014. An overview of global rice production, supply, trade, and consumption. *Ann. N. Y. Acad. Sci.* 1324: 7–14. doi.org/10.1111/nyas.12540
- Nguyen, T.T.U., Nguyen, P.T., Nguyen, T.T., Nguyen, T.P.T., Nguyen, H.T. 2024. Rice leaves or ricegrass—available biomaterial with potential biological activities for different industrial applications: a review. *Discov. Food.* 4: 106. doi.org/10.1007/s44187-024-00187-4
- Ohmiya, A., Hirashima, M., Yagi, M., Tanase, K., Yamamizo, C. 2014. Identification of genes associated with chlorophyll accumulation in flower petals. *PLOS ONE.* 9: e113738. doi.org/10.1371/journal.pone.0113738
- Patel, M.K., Pandey, S., Kumar, M., Haque, M.I., Pal, S., Yadav, N.S. 2021. *Plants Metabolome Study: Emerging Tools and Techniques.* *Plants* 10: 2409. doi.org/10.3390/plants10112409
- Phuoc, L.H., Suliansyah, I., Arlius, F., Chaniago, I., Xuan, N.T.T., Quan, P.V. 2022. Responses of growth and grain yield of IR50404 rice to temperature stress. *Int. J. Agric. Sci.* 6: 9–18. doi.org/10.25077/ijasc.6.1.9-18.2022
- Rise, M., Cojocar, M., Gottlieb, H.E., Goldschmidt, E.E. 1989. Accumulation of  $\alpha$ -tocopherol in senescing organs as related to chlorophyll degradation. *Plant Physiol.* 89: 1028–1030. doi.org/10.1104/pp.89.4.1028
- Rogowska, A., Szakiel, A. 2020. The role of sterols in plant response to abiotic stress. *Phytochem. Rev.* 19: 1525–1538. doi.org/10.1007/s11101-020-09708-2
- Roy, R.N. 2020. Bioactive natural derivatives of phthalate ester. *Crit. Rev. Biotechnol.* 40: 913–929. doi.org/10.1080/07388551.2020.1789838
- Ta, H.L., Chu, D.H. 2022. Study on the cultivation practices for “Huyet Rong” herbal rice variety in Quang Tri province. *TNU J. Sci. Technol.* 227: 3–9. doi.org/10.34238/tnu-jst.4989
- Tamprasit, K., Weerapreeyakul, N., Sutthanut, K., Thukhammee, W., Wattanathorn, J. 2019. Harvest age effect on phytochemical content of white and black glutinous rice cultivars. *Molecules.* 24: 4432. doi.org/10.3390/molecules24244432
- Tho, L.C.B., Umetsu, C. 2022. Rice variety and sustainable farming: A case study in the Mekong Delta, Vietnam. *Environ. Chall.* 8: 100532. doi.org/10.1016/j.envc.2022.100532
- Tho, N.P., Uyen, N.T.T., Thao, N.T.P., Thanh, N.H., Thien, D.C., Nhut, P.M., Tho, N.T. 2024. Insights into the metabolism of rice leaves (*Oryza sativa* L.) under shade stress by investigating the metabolite profile using gas chromatography-mass spectrometry (GC-MS) analysis. *Russ. J. Plant Physiol.* 71: 126. doi.org/10.1134/S102144372460630X
- Verma, N., Shukla, S. 2015. Impact of various factors responsible for fluctuation in plant secondary metabolites. *J. Appl. Res. Med. Aromat. Plants.* 2: 105–113. doi.org/10.1016/j.jarmap.2015.09.002
- Vezza, T., Canet, F., de Marañón, A.M., Bañuls, C., Rocha, M., Victor, V.M. 2020. Phytosterols: nutritional health players in the management of obesity and its related disorders. *Antioxid. Basel Switz.* 9: 1266. doi.org/10.3390/antiox9121266
- Wu, M., Northen, T.R., Ding, Y. 2023. Stressing the importance of plant specialized metabolites: omics-based approaches for discovering specialized metabolism in plant stress responses. *Front. Plant Sci.* 14: 1272363. doi.org/10.3389/fpls.2023.1272363
- Zaynab, M., Fatima, M., Sharif, Y., Zafar, M.H., Ali, H., Khan, K.A. 2019. Role of primary metabolites in plant defense against pathogens. *Microb. Pathog.* 137: 103728. doi.org/10.1016/j.micpath.2019.103728
- Zhang, X., Lin, K., Li, Y. 2020. Highlights to phytosterols accumulation and equilibrium in plants: Biosynthetic pathway and feedback regulation. *Plant Physiol. Biochem.* 155: 637–649. doi.org/10.1016/j.plaphy.2020.08.021