The changes of oil palm roots cell wall lipids during pathogenesis of *Ganoderma boninense*

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Abstract. One of the first physical defences of plants against fungal infection is their cell wall. Interaction between combinations of metabolism enzymes known as the "weapons" of pathogen and the host cell wall probably determines the fate of possible invasion of the pathogen in the host. The present work aims to study the biochemical changes of cell wall lipids of oil palm roots and to determine novel information on root cell wall composition during pathogenesis of *Ganoderma boninense* by using Gas Chromatography Mass Spectrometry. Based on Total Ion Chromatogram analysis, 67 compounds were found more abundant in the roots infected with *G. boninense* compared to the healthy roots (60 compounds). Interestingly, nine new compounds were identified from the cell wall lipids of roots infected with *G. boninense*. These includes Cyclohexane, 1,2-dimethyl-, Methyl 2-hydroxy 16-methyl-heptadecanoate, 2-Propenoic acid, methyl ester, Methyl 9-oxohexacosanoate, 5-[(3,7,11,15-Tetramethylhexadecyl)oxy]thiophene-2carboxylic acid, Ergosta-5,7,22,24(28)-tetraen-3beta-ol, 7-Hydroxy-3',4'-methylenedioxyflavan, Glycine and (S)-4'-Hydroxy-4-methoxydalbergione, this may involve as response to pathogen invasion. This paper provides an original comparative lipidomic analysis of oil palm roots cell wall lipids in plant defence during pathogenesis of *G. boninense*.

1. Introduction

Ganoderma boninense is a white rot fungus, which is responsible for destructive basal stem rot (BSR) disease in oil palm. This is a major problem in oil palm plantations in Southeast Asia, especially in Malaysia and Indonesia. The economic loss due to this disease is estimated up to 500 million USD per year in Malaysia [1, 2]. Effects of BSR disease either occur from death of palms or yield reduction from standing palms [3]. Various detection and control measures have been developed in managing this disease but none of them is satisfactory. Lack of understanding on the biology of pathogen and host-pathogen interaction during pathogenesis contributes to poor management of basal stem rot (BSR) disease.

Different approaches on metabolic and molecular processes in oil palm against *G. boninense* have been evaluated to further understand the pathogen-host relationship. However, no research reported

the changes in cell wall composition of oil palm during the interaction, despite, knowing its importance as the foremost interface during the interaction event.

The plant cell wall is the first lines of defensive barrier in plant immune response against infection, where initially all plant pathogens have to get-through in order to colonize the host. In order to penetrate this barrier, pathogenic fungi evolved arrays of wall degrading enzymes virulence factor for disease development. After the penetration, the cell wall becomes a battleground between the host and its pathogen. Cell wall is a dynamic reservoir of antimicrobial compounds and generates signaling molecules that trigger defense responses in the host [4, 5]. During plant-pathogen interaction, pathogens attempt to utilize the metabolism of host plants to suppress plant defense and to obtain nutrients for survival [6, 7]. At the same time, the plant synthesizes metabolites that serve as signals, sedatives or toxins to limit the proliferation of the pathogen [8]. The importance of plant cell wall integrity and cell wall-mediated resistance during plant-pathogen interaction has been well demonstrated, but the related components have not been fully elucidated [7].

Lately, lipids were reported to trigger multiple stages of plant-pathogen interaction which includes communication between the host pathogen, activation and implementation of plant defenses in response to pathogen attack [9, 10]. Previous study [11] suggested lipids metabolites involved in early defense mechanism of oil palm against *G. boninense*. Therefore, lipidomics has gained a lot of attention and became an emerging field of study. Thus, the aim of this study is to provide an insight of oil palm *Ganoderma*-BSR interaction at lipidomics level and to discover potential metabolites for early disease detection. Moreover, this information could also be harnessed in managing BSR disease in future.

2. Materials and methods

2.1. Plant material

Oil palm seedlings D x P (*Dura x pisifera*) of eight months old were purchased from Sawit Kinabalu Sdn. Bhd. and artificially inoculated with *G. boninense* [12]. The stock culture of *G. boninense* was obtained from Universiti Malaysia Sabah and identified prior before inoculation [13]. The seedlings were maintained in a nursery with 70% of shade protection. Control seedlings (healthy/uninoculated) and inoculated seedlings were watered and fertilized according to standard agronomic practices. The seedlings were uprooted after three months and washed under running tap water to remove all unwanted materials and air dried. The success of *Ganoderma* infection was re-confirmed by using *Ganoderma* Selective Media (GSM) [14] and through ergosterol analysis [15]. The dried roots were lyophilized for 24 hours and homogenized into fine powder by using a blender.

2.2. Isolation of cell wall

The cell wall composition of control and infected roots were extracted according to procedures described by [16] with slight modification. All procedures were conducted at 4°C unless mentioned otherwise. Three gram of powdered oil palm root tissues were agitated overnight in 50 mL of pre-cooled extraction buffer (5 mM acetate, 0.4 M sucrose, pH 4.6) added with 1% of Polyvinylpolypyrrolidone using an orbital shaker at 180rpm. Cell walls were separated from soluble cytoplasmic fluid by centrifuging at 1,000 xg for 15 min. The pellet was resuspended in 50 mL of 5 mM acetate buffer at pH 4.6 with 0.6 M sucrose. The slurry was centrifuged at 1,000 xg for 15 min. The pellet was further washed in 50 mL of 5 mM acetate buffer at pH 4.6 with 1 M sucrose and centrifuged at 1,000 xg for 15 min. The residue was washed extensively with 50 mL of 5 mM acetate buffer at pH 4.6 and centrifuged at 1,000 xg for 15 min, thrice. The supernatant was discarded and final pellet was lyophilized overnight. Most intercellular components were removed from the cell wall given the advantage of sucrose gradients and extensive washing with low ionic strength of acidic buffer.

2.3. Cell wall lipids extraction

A small-scale modified protocol was adopted from [17] using 50 mL reaction tubes with screw-cap lids for extraction of cell wall lipids. Three replicates of one gram of each control and infected samples (six replicates) were added to the reaction tubes along with 20 mL of solvent mixture (Chloroform: Methanol; 2:1; v/v). The tubes were vortexed and agitated on an orbital shaker for one hour at room temperature. The tubes were then centrifuged at 2000 rpm for five min to recover the liquid phases. The solvent mixture were washed with 0.2 volume of miliQ® water and vortexed for few seconds. The tubes were centrifuged again at 2000 rpm to separate the mixture into two phases. The aqueous upper phases were removed and the lower chloroform phases containing lipids were kept for further use. The extracts were later dried under a stream of nitrogen gaseous at room temperature.

2.4. Mild methylation of fatty acid methyl esters (FAMEs)

Prior to Gas Chromatography (GC) analysis, extracted cell wall lipids samples were subjected to metholysis [18] and analysed for FAMEs. Lipids samples were dissolved in 0.20 mL of Toulene and vortexed for few seconds. Methanol (1.5 mL) and 8% HCL solutions (0.3 mL) were added to the lipids solution. The mixtures were vortexed and incubated at 45˚C for 18 hours for mild methanolysis. To extract FAMES, the samples were cooled to room temperature and one mL of each hexane and miliQ water was added respectively to the samples. The mixtures were vortexed and hexane layers were directly analyzed by using GC after filtered through 0.22 µm PTFE filters.

2.5. Gas chromatography-mass spectrometry (GC-MS) analysis of FAMEs

FAMEs were analyzed by using GC-MS (Agilent, 5975C) with J & W GC column (30 m x 250 µm×0.25 µm; Agilent). The injector temperature maintained at 250°C. The column temperature was initially kept at 150°C for two mins and then increased at a rate of 12°C/min up to 200°C and held at this temperature for 15 mins. The temperature was increased to final temperature of 252°C and held for 10 mins at a rate of 3°C/min. Helium was used as the carrier gas at a flow rate of one mL/min and one µL of sample was injected using the splitless mode. The spectra were collected at 50-650 *m/z* range and were analyzed by using MZmine 2.21 software for mass detection, chromatogram builder and deconvolution. The detected peaks were subjected to compound identification using multiple online lipid and metabolomics database (NIST MS Search 2.0, METLIN, LipidMaps, KEGG and ChemSpider Databases).

3. Results and discussion

The findings from this study showed lipids composition of oil palm roots consists of FAMEs, sterols, neutral glycolipids and phospholipids. Full-scan spectra showed peak profiles were almost similar between control and *Ganoderma*-Infected roots. Approximately 56 compounds were detected in both control and *Ganoderma*-Infected roots as shown in table 1. These compounds are associated with normal metabolic activity of oil palm. Pentadecanoic acid, 14-methyl-, methyl ester (*m/z* 270.400; $C_{17}H_{34}O_2$) was detected at RT 16.66, which was the most abundant FAME in both control and *Ganoderma*-Infected cell wall roots. On the other hand, several compounds were detected at high abundance in *Ganoderma*-infected roots according to their base peak intensity data in Total Ion Chromatogram (TIC). Two fatty acid compounds; 3-methyl-6-(1-methyl-ethyl)-3, 9-decadien-1-ol (*m/z* 211.2; $C_{14}H_{26}O$) and 10, 13-Octadecadienoic acid, methyl ester (*m/z* 294.4; $C_{19}H_{34}O_2$) were more abundant in *Ganoderma*-Infected roots compared to controls. These compounds play significant roles in pathogen defence in response to various stresses [19]. A lipid phosphate derivatives bind with cyclic Guanosine Monophosphate (GMP), N'-Phosphoguanidinoethyl methyl phosphate (*m/z* 277; $C_4H_{13}N_3O_7P$) increased after the infection of *Ganoderma*. This compound is critically implicated in responses to both abiotic and biotic stress [20] such as gating of channels [21, 22], plant hormone signal transduction [23, 24], nitric oxide (NO)-dependent signalling [25] and regulation of transcription. Hence, the increase of this compound is proportional to the stress level in oil palm root tissue infected with *G. boninense*. Increment of phenolic compound, trans-trismethoxy Resveratrol

(*m/z* 270; $C_{17}H_{18}O_3$) in *Ganoderma*-infected roots also supports the indication of these compounds involvement in oil palm resistance against *Ganoderma* [11, 26]. Although the increment of hexadehydrovitamin D3/25-hydroxy-16,17,23,23,24,24-hexadehydrocholecalciferol (*m/z* 395.3; $C_{27}H_{38}O_2$) is not fully understood, this vitamin precursor might involve in cell-cell signalling to initiate defense mechanism in plant against stress and acts as an antioxidant compound to protect the plant from reactive oxygen species (ROS). Several lipids compounds were also detected in low amounts in roots infected with *G. boninense* (table 1).

Table 1. List of compounds that present in both control (healthy) and *Ganoderma*-Infected cell wall lipids of oil palm roots.

No	Compounds	RT (Min)	M/Z (30ppm tolerant)	Molecular Formula
1	2-methoxy-Ethanamine	6.87	74.900	C_3H_9NO
2	2-methyl-1-Pentene	8.01	85.000	C_6H_{12}
3	1,3,5-Trioxane	8.25	90.900	$C_3H_6O_3$
4	2-Propenal	8.29	56.900	C_3H_4O
5	1,2-Butadiene	8.46	54.900	C_4H_6
6	2-Propenamide	9.32	70.900	C_3H_5NO
7	* 3-methyl-6-(1-methyl-ethyl)-3,9-decadien-1-ol	11.38	211.200	$C_{14}H_{26}O$
8	p-methoxystilbene	11.46	211.100	$C_{15}H_{14}O$
9	2-Hexene	12.07	85.000	C_6H_{12}
10	* Propanenitrile	12.74	55.900	C_3H_5N
11	2-methyl-1,3-Butadiene	13.01	68.900	C_5H_8
12	* * Pentadecanoic acid, methyl ester	13.62	256.200	$C_{16}H_{32}O_2$
13	* N'-Phosphoguanidinoethyl methyl phosphate	14.63	277.000	$C_4H_{13}N_3O_7P_2$
14	* trans-trismethoxy Resveratrol	15.40	270.100	$C_{17}H_{18}O_3$
15	9-Hexadecenoic acid, methyl ester, (Z)-	16.29	268.100	$C_{17}H_{32}O_2$
16	* * Pentadecanoic acid, 14-methyl-, methyl ester	16.66	270.400	$C_{17}H_{34}O_2$
17	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	17.56	292.400	$C_{18}H_{28}O_3$
18	1-Butanol, 3-methyl-, acetate	18.06	114.900	$C_7H_{14}O_2$
19	1,2-Benzenedicarboxylic acid, diheptyl ester	18.30	148.800	$C_{22}H_{34}O_4$
20	5-Hydroxy-7-methoxy-8-C-methylflavanone	19.05	284.100	$C_{17}H1_6O_4$
21	* * Heptadecanoic acid, methyl ester	20.75	284.200	$C_{18}H_{36}O_2$
22	4-Methoxy potassium salicylate	23.69	207.000	$C_8H_8KO_4$
23	* 10,13-Octadecadienoic acid, methyl ester	24.38	294.400	$C_{19}H_{34}O_2$
24	* * 9-Octadecenoic acid, methyl ester, (E)-	24.66	296.400	$C_{19}H_{36}O_2$
25	* * 1-Dodecene	24.89	168.300	$C_{12}H_{24}$
26	* * Methyl stearate	25.80	298.500	$C_{19}H_{38}O_2$
27	3-Methoxymandelic acid-4-O-sulfate	26.67	278.000	$C_9H_{10}O_8S$
28	Benzenamine, N-phenyl-4-(phenylazo)-	27.75	273.300	$C_{18}H_{15}N3$
29	2(3H)-Furanone, dihydro-5-methyl-	28.20	100.100	$C_5H_8O_2$
30	(6Z,9Z,11E,13S)-13-Hydroperoxy-6,9,11-octadecatrienoic acid	28.99	310.200	$C_{18}H_{30}O_4$
31	* 2-Acetylthiazole	30.05	127.000	C_5H_5NOS
32	* *Octadecanoic acid, 2-hydroxy-, methyl ester	30.14	359.600	$C_{22}H_{47}O_3$

33	5-Hydroxy-7-methoxyisoflavone	30.17	269.100	$C_{16}H_{12}O_4$
34	Cyclopentanol	30.51	86.100	$C_5H_{10}O$
35	Heptane, 2,2,4,6,6-pentamethyl-	30.51	170.300	$C_{12}H_{26}$
36	Benzene, (1-methoxyethyl)-	31.25	136.000	$C_9H_{12}O$
37	2-chloromuconic acid	31.45	177.000	$C_6H_5O_4Cl$
38	Methyl 9.cis.,11.trans.t,13.trans.-octadecatrienoate	32.34	292.400	$C_{19}H_{32}O_2$
39	* * 2-oxo-4-methylthio-butanoic acid	32.47	149.000	$C_5H_8O_3S$
40	* * 2H-Pyran, 2,5-diethenyltetrahydro-	33.08	138.000	$C_9H_{14}O$
41	Eicosanoic acid, methyl ester	34.02	326.500	$C_{21}H_{42}O_2$
42	* * 2-Butenoic acid, methyl ester, (E)-	36.45	100.000	$C_5H_8O_2$
43	3-bromo-2Z-heptenoic acid	37.42	207.000	$C_7H_{11}BrO_2$
44	4-Acetoxy-3-methoxyacetophenone	37.90	160.000	$C_{11}H_{12}O_4$
45	* * 3,4-Hexanediol, 3,4-bis(4-hydroxyphenyl)-	37.97	302.000	$C_{18}H_{22}O_4$
46	gamma.-Sitosterol	39.52	414.700	$C_{29}H_{50}O$
47	* * 24-keto-25dehydrocholesterol	39.77	399.300	$C_{27}H_{42}O_2$
48	* * Docosanoic acid, methyl ester	40.56	354.600	$C_{23}H_{46}O_2$
49	stigmast-22E-en-3beta-o	41.22	414.400	$C_{29}H_{50}O$
50	* * 4-Hydroxy-2,3,3',4',5-pentachlorobiphenyl	42.39	339.900	$C_{12}H_5C_{l5}O$
51	Tricosanoic acid, methyl ester	44.07	368.600	$C_{24}H_{48}O_2$
52	* 25-hydroxy-16,17,23,23,24,24-hexadehydrovitamin D3 / 25-hydroxy-16,17,23,23,24,24-hexadehydrocholecalciferol	49.48	395.300	$C_{27}H_{38}O_2$
53	2-Propenoic acid, ethenyl ester	50.70	100.100	$C_5H_8O_2$
54	Pentacosanoic acid, methyl ester	53.62	396.700	$C_{26}H_{52}O_2$
55	2-methylene-4-oxo-pentanedioic acid	54.04	159.000	$C_6H_6O_5$
56	Methyl 2-hydroxy-tetracosanoate	55.59	398.700	$C_{25}H_{50}O_3$

Note: * More abundant in infected tissues ** Less abundant in infected tissues based on base peak intensity in Total Ion Chromatogram (TIC).

In the present work, TIC chromatogram of control and *Ganoderma*-infected cell wall lipids revealed different peaks profiles as shown in figure 1.

Based on the GC-MS analysis, several peaks were not detected in *Ganoderma*-Infected roots and were only present in control oil palm cell wall lipids. The identified peaks are shown in table 2. After the roots were infected with *G. boninense,* these compounds may possible being suppressed or degraded root cells may unable to metabolize the respective compounds. Several factors might contribute to the suppression of metabolic activity in plant cell, which include stress factors and infection.

Table 2. List of compounds that were detected only in control (healthy) oil palm roots.

No	**Compounds**	**RT (Min)**	**M/Z (30ppm tolerant)**	**Molecular Formula**
1	Benzoic acid, 3-methyl-, methyl ester	15.96	152.000	$C_9H_{10}O_2$
2	Malonic acid	30.74	105.000	$C_3H_4O_4$
3	Methyl 2-hydroxy-eicosanoate	38.28	342.500	$C_{21}H_{42}O_3$
4	Tetracosanoic acid, methyl ester	48.34	382.700	$C_{25}H_{50}O_2$

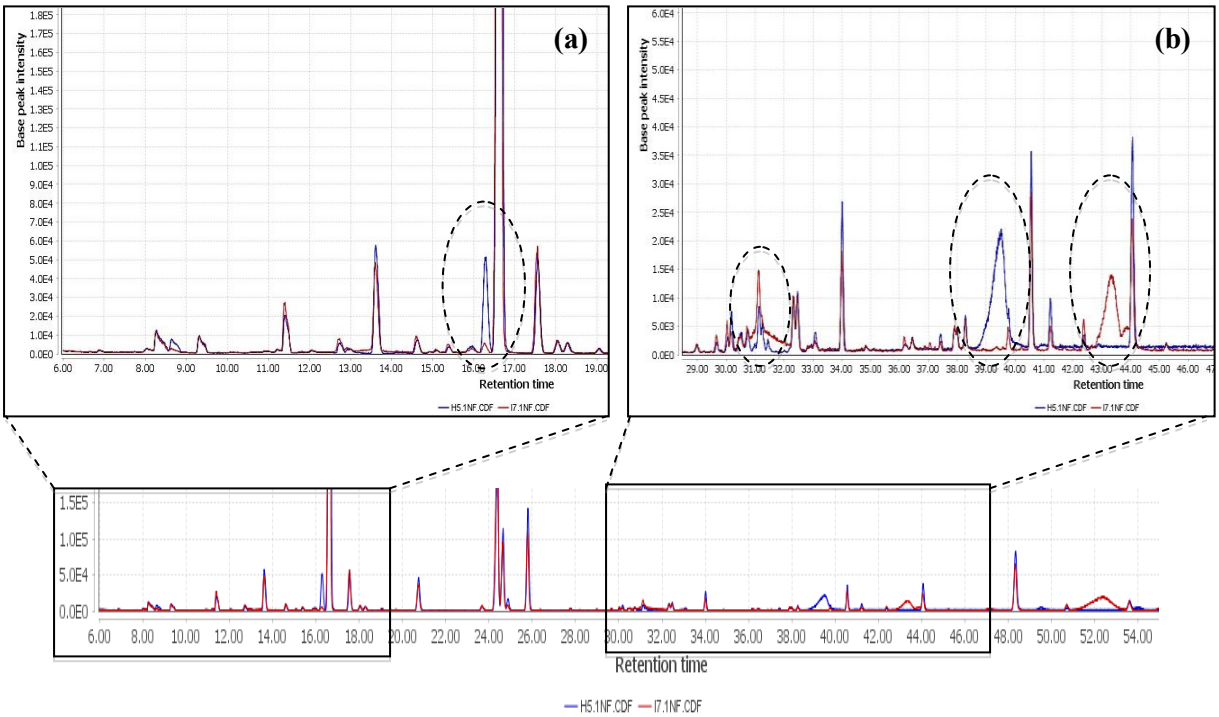

Figure 1. Overlayed GC-MS chromatogram of control (healthy) (blue line) and *Ganoderma*-infected (red line) oil palm roots cell wall lipids. (A) Enlarged area from RT 0-19 min. (B) Enlarged area from RT 29-47 min. Major different peaks are circled.

Based on the GC-MS analysis, several new peaks were detected and identified in the cell walls of *Ganoderma*-infected roots as summarized in table 3. However, these peaks were absent in healthy roots. These lipids may act as a key for plant defense response via signaling pathways of root tissues or play a key role in physiological changes and/or metabolic switch. Cyclohexane, 1, 2-dimethyl- is an aliphatic lipid component and by-product of lipid metabolism in plant. Increase of aliphatic compounds was reported previously in potato tuber infected by *Erwinia carotivora* [27].

Fatty acid methyl ester (FAME) compounds exhibited antimicrobial activity against pathogens. In this study, three FAMEs were identified as Methyl 2-hydroxy-16-methyl-heptadecanoate, 2-Propenoic acid, methyl ester and Methyl 9-oxohexacosanoate which possibly produced by oil palm roots to suppress *G. boninense* colonization. Similarly, previous study [28] found other fatty acids in oil palm tissue infected by *Ganoderma*. A heterocyclic aromatic compound, 5-[(3,7,11,15 Tetramethylhexadecyl)oxy] thiophene-2-carboxylic detected in oil palm tissues are usually produced by anaerobic microorganisms which exists endophytically in the plant tissues [29]. The presence of this compound in *Ganoderma*-infected tissue might indicate *G. boninense* as a facultative anaerobic fungi and lives endophytically in oil palm. Ergosta-5,7,22,24(28)-tetraen-3beta-ol is a derivative of fungal sterol found in the cell membrane. Thus, the presence of this compound confirmed *G. boninense* infection in root tissues. Previous studies suggested the importance of ergosterol in oil palm tissue for quantification of *Ganoderma* colonization [15, 30]. Another new compound detected after the infection of *Ganoderma*, is 7-Hydroxy-3',4'-methylenedioxyflavan, a benzopyran derivative and previously had been isolated from *Zephyranthes candida* [31]. It had been demonstrated that this compound in *Z. candida* was merely for defence and tolerant mechanism against biotic and abiotic stress. Therefore, this compound might play the similar role in *Ganoderma*-Infected root tissues. The presence of glycine in *Ganoderma*-Infected roots sample is quite fascinating. Previously, glycine-containing lipoamine acid was reported in *Cytophaga johnsonae* and its compound a combination of iso-3-hydroxy heptadecanoic acid, amide linked to glycine [32]. However, in this work glycine might be dissociated from its original gylcine-containing lipoamine acid structure due to the degradation by

high temperature during GC analysis. This study is the first to report the presence of glycine in cell wall extract of oil palm roots infected with *G. boninense*. Glycine-containing lipoamine acid could also be developed as biomarker for BSR early detection. A flavonoid compound, (S)-4'-Hydroxy-4-methoxydalbergione, which is lipid-soluble, was detected in cell wall lipids extract of *Ganoderma*-Infected roots. This compound plays important role in plant resistance against pathogens. Flavonoid compounds are transported to the site of infection and induce the hypersensitivity reaction, which is the earliest defense mechanism employed by the infected plants and programmed cell death [33]. This compound might accumulate in cell walls during interaction between oil palm and *G. boninense*. Previous study also found the accumulation of flavonoid compounds in cotton cell wall after inoculated with *Xanthomonas campestris* pv. *malvacearum* [34].

Table 3. List of compounds which were detected only in *Ganoderma*-Infected oil palm roots samples.

No	Compounds	RT (Min)	M/Z (30ppm tolerant)	MF
1	Cyclohexane, 1,2-dimethyl-	29.66	112.000	C_8H_{16}
2	Methyl 2-hydroxy-16-methyl-heptadecanoate	31.11	299.500	$C_{18}H_{35}O_3^-$
3	2-Propenoic acid, methyl ester	36.18	86.000	$C_4H_6O_2$
4	Methyl 9-oxohexacosanoate	37.05	424.400	$C_{27}H_{52}O_3$
5	5-[(3,7,11,15-Tetramethylhexadecyl)oxy]thiophene-2-carboxylic acid	37.23	424.300	$C_{25}H_{44}O_3S$
6	Ergosta-5,7,22,24(28)-tetraen-3beta-ol	43.34	394.200	$C_{21}H_{28}O_3$
7	7-Hydroxy-3',4'-methylenedioxy flavan	47.11	271.100	$C_{16}H_{14}O_4$
8	Glycine	52.39	75.000	$C_2H_5NO_2$
9	(S)-4'-Hydroxy-4-methoxydalbergione	55.1	271.100	$C_{16}H_{14}O_4$

4. Conclusion

In brief, this work presented a comparison of cell wall lipids composition between the healthy and *Ganoderma*-infected oil palm root tissue extracts. Through GC-MS analysis, some lipids compounds were only detected in *G.boninense* infected oil palm roots. New lipids and some lipid-linked compounds associated with plant defense response were discovered in this study. The fluctuation of several compounds between healthy and *Ganoderma*-Infected root tissue were also revealed. Further investigation is needed to explore the roles or mechanism of action of newly found compounds during pathogenesis of *Ganoderma* in oil palm. These compounds has valuable potential for development of biomarker and that might lead to better control strategies for combating *Ganoderma* infection in oil palm.

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