METABOLITE PROFILING OF *Ganoderma*-INFECTED OIL PALM TREE GROWN ON ULTISOL AND OXISOL SOIL

MUHAMAD FADHIL BIN AHMAD

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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MUHAMAD FADHIL BIN AHMAD

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Field of Study: **SOIL SCIENCE AND METABOLOMICS**

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METABOLITE PROFILING OF *Ganoderma*-INFECTED OIL PALM TREE GROWN ON ULTISOL AND OXISOL SOIL

ABSTRACT

The oil palm industry is a pillar of the Malaysian economy and plays a pivotal role in feeding and fueling a growing global population. In 2018, oil palm contributed 37.9% or RM37.7 billion to the country's' Gross Domestic Products (GDP) of agriculture sector. Despite achieving significant achievements in the industry, *Ganoderma* sp. infection that cause basal stem rot (BSR) and upper stem rot (USR) disease are considered a threat. The palm oil industry is expected to experience significant losses, thereby affecting yield production and ultimately the dead of trees. Some factors have been reported to affect the occurrence of the disease such as the cultivation of the previous crop, replanting technique, age and number of individual palms per planted area, type of soil series, inoculum potential, shading and soil temperature, but research conducted on weathered tropical soils in Malaysia is still lacking. In Malaysia, Ultisol and Oxisol soil are very common which occupy about 72% of the country's land area. This research is therefore conducted with the objective to determine soil physicochemical properties of Ultisol and Oxisol soil and profile metabolites of healthy and *Ganoderma*-infected oil palm grown on both soils. Biological samples were extracted using methanol and analyzed by using liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS). Soil physicochemical properties analyzed using U-Mann Whitney revealed that Ultisol and Oxisol soil are different at pH and TC level but not for EC level. A chi-square test of independence classified Ultisol and Oxisol soil into sandy clay and clay respectively. For biological sample analysis, the initial Principal Component Analysis (PCA) model concluded that there was a difference between two main groups of metabolite profiles between healthy and *Ganoderma*-infected oil palm tree rachis samples. A strongly significant Partial Least Square-Discriminant Analysis (PLS-DA) model was acquired, indicating that the 12 biological variables indeed contained class separating information. The separation of the four classes was slightly superior compared with the previous PCA modelling attempts.

This model also fits the criteria for validity in permutation test. From the model, 17 potential biomarker panels with variable importance in project (VIP) more than 2.00 and p-value of analysis of variance (ANOVA) less than 0.05 were identified. Among metabolite of interest putatively identified are choline phosphate, p-fluorophenylalanine, 2-oxoglutaramate, 4-oxoglutaramate, 4-amino-4-cyanobutanoic acid, 2-amino-4-cyanobutanoic acid, 5-aminolevulinate, L-allohydroxyproline and N-acetyl-beta-alanine. These findings are crucial for further study on the pathogenicity characteristic of BSR and USR disease in penetrating oil palm tree in the future.

Keywords: Metabolomics, *Ganoderma*, Oil Palm, Ultisol, Oxisol

PEMPROFILAN METABOLIT KELAPA SAWIT TERJANGKIT *Ganoderma* YANG DI TANAM PADA TANAH ULTISOL DAN OXISOL

ABSTRAK

Industri kelapa sawit merupakan tonggak ekonomi Malaysia dan memainkan peranan penting dalam kelangsungan bekalan makan terhadap populasi global yang semakin meningkat. Pada tahun 2018, kelapa sawit menyumbang 37.9% atau RM37.7 bilion kepada keluaran dalam negara kasar (KDNK) kepada sektor pertanian. Walaupun memperoleh pencapaian yang tinggi dalam industri ini, jangkitan kulat *Ganoderma* yang menyebabkan penyakit reput pangkal batang (RPB) dan reput batang atas (RBA) dianggap sebagai satu ancaman. Industri minyak sawit dijangka mengalami kerugian yang ketara disebabkan pengeluaran hasil berkurang dan kematian pokok. Beberapa faktor telah dilaporkan menjadi penyebab kepada penyakit tersebut seperti penanaman tanaman sebelumnya, teknik penanaman semula, umur dan bilangan sawit dalam satu kawasan, jenis siri tanah, potensi inokulum, teduhan dan suhu tanah, tetapi penyelidikan yang dijalankan ke atas tanah tropika berluluhawa di Malaysia masih kurang. Di Malaysia, tanah Ultisol dan Oxisol mendominasi kira-kira 72% kawasan tanah negara. Oleh itu, kajian ini dijalankan dengan tujuan untuk menentukan sifat fizikokimia tanah Ultisol dan Oxisol dan memprofil metabolit kelapa sawit yang tumbuh pada kedua-dua tanah tersebut. Sampel biologi telah diekstrakkan dengan menggunakan metanol dan dianalisis dengan menggunakan Kromatografi Cecair-Spektroskopi Jisim Masa. Ciri-ciri fizikokimia tanah yang dianalisis menggunakan U-Mann Whitney mendedahkan bahawa tanah Ultisol dan Oxisol berbeza di tahap pH dan TC tetapi tidak untuk tahap EC. Ujian kebebasan chi-kuasa dua pula mengelaskan tanah Ultisol dan Oxisol masing-masing sebagai tanah liat berpasir dan tanah liat. Untuk analisis sampel biologi, model Analisis Komponen Utama (AKU) menyimpulkan bahawa terdapat perbezaan di antara dua kumpulan utama profil metabolit antara sampel pokok sawit yang sihat dan dijangkiti kulat *Ganoderma*. Model Kuasa Dua Terkecil Separa-Analisis Diskriminan (KDTS-AD) menunjukkan bahawa 12 pembolehubah biologi memang mengandungi kelas pemisahan yang tersendiri. Pemisahan

empat kelas direkodkan lebih tinggi berbanding dengan percubaan model AKU sebelumnya. Model ini juga melepasi kriteria kesahihan dalam ujian permutasi. Dari model ini juga, 17 panel penanda biologi yang berpotensi telah dikenalpasti berpandukan nilai VIP lebih daripada 2.00 dan p-nilai ANOVA kurang daripada 0.05. Antara metabolit yang dikenal pasti adalah kolin fosfat, p-fluorofenilalanina, 2-oxoglutaramat, 4-oxoglutaramat, asid 4-Amino-4-cyanobutanoik, asid 2-Amino-4-cyanobutanoik, 5-Aminolevulinat, L-Allohidroksiprolin dan N- Acetyl-beta-alanina. Penemuan ini adalah penting untuk kajian selanjutnya berkaitan ciri patogenik penyakit RPB dan RBA dalam menembusi pokok kelapa sawit pada masa akan datang.

Kata kunci: Metabolomik, *Ganoderma*, Kelapa Sawit, Ultisol, Oxisol

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
µL	:	Microlitre
µm	:	Micrometre
%	:	Percent
Af	:	Tropical Wet
ALA	:	5-Aminolevulinic Acid
ANOVA	:	Analysis of Variance
BBOT	:	2,5-(Bis(5-tert-butyl-2-benzo-oxazol-2-yl) Thiophene
CEC	:	Cation Exchange Capacity
cm	:	Centimetre
BSR	:	Basal Stem Rot
dH_2O	:	Distilled water
DoS	:	Department of Statistics
E	:	East
EC	:	Electrical Conductivity
ET	:	Ethylene
FDR	:	Bonferroni Corrected P-values
FFB	:	Fresh Fruit Bunches
FMF	:	Find Molecular Feature
g	:	Gram
GC-MS	:	Gas Chromatography-Mass Spectrometry
GDP	:	Gross Domestic Product
GM	:	Glutamate Metabolism
H_2O_2	:	Hydrogen Peroxide
HCl	:	Hydrochloric Acid
JA	:	Jasmonic Acid

KDNKK	:	Keluaran Dalam Negara Kasar
km	:	Kilometers
LC-MS	:	Liquid Chromatography-Mass Spectrometry
LC-TOF-MS	:	Liquid Chromatography-Time of Flight-Mass Spectrometry
mg	:	Milligram
ml	:	Millilitre
mm	:	Millimetre
ms/ms	:	Tandem Mass Spectrometry
m/z	:	Mass-to-charge Ratio
MPOB	:	Malaysian Palm Oil Board
MPOC	:	Malaysian Palm Oil Council
N	:	North
NMR	:	Nuclear Magnetic Resonance
PLS-DA	:	Partial Least Square Discriminate Analysis
RBA	:	Reput Batang Atas
RPB	:	Reput Pangkal Batang
rpm	:	Revolutions per Minute
RM	:	Malaysian Ringgit
RT	:	Retention Time
SA	:	Salicyclic Acid
SIMCA	:	Soft Independent Modelling of Class Analogy
SM	:	Secondary Metabolites
SOM	:	Soil Organic Matter
TC	:	Total Carbon
UHPLC	:	Ultra-High-Performance Liquid Chromatography
USDA	:	United State Department of Agriculture
USR	:	Upper Stem Rot

VIP : Variable Important in Project

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The oil palm industry in Malaysia started more than 100 years ago. It was first introduced to Malaysia as a commercial plant in 1917 at the Tennamaram Estate in Selangor, which effectively laid the foundation for the development of the oil palm industry in Malaysia (Nambiappan et al., 2018). The oil palm plantation had expanded phenomenally from a mere 55 000 ha in 1960, to 5.85 million hectares in 2018 (MPOB, 2013b).

In tandem with the area expansion, the production of crude palm oil also grew significantly from less than 100 000 tons in 1960 to about 19.52 million tons in 2018 (MPOB, 2013c). Now, the Malaysian oil palm industry has transformed to become one of the key contributors to Malaysia's GDP. Oil palm contributed 37.9% of the agriculture sector or RM37.7 billion to the country's GDP in 2018 (DoS, 2018).

Despite attaining significant achievements in both its palm oil production and exports, the oil palm industry is currently facing issues of the destructive diseases that can affect the continued growth of the oil palm industry. The oil palm ecosystems comprise a wide range of fungi, bacteria, insects, nematodes, and viruses that are significantly responsible for reducing crop productivity. One of the most destructive diseases of oil palm is BSR and USR diseases where the oil palm is infected by wood-decaying fungi known as *Ganoderma boninense* (Pornsuriya et al., 2013).

The infection caused a reduction in oil palm Fresh Fruit Bunches (FFB) yield and sometimes the death of the palms (Roslan & Idris, 2012). Some factors have been reported to affect the occurrence of BSR and USR disease such as the cultivation of the previous crop, replanting technique, age and number of individual palms per planted area, type of soil series, inoculum potential, shading and soil temperature (Azahar et al., 2014; Parthiban et al., 2016; Rees et al., 2007).

In Malaysia, Ultisol and Oxisol soil containing kaolinite, gibbsite, goethite and hematite in the clay fraction are very common especially in the upland areas, occupying about 72% of the country's land area. The soils are highly weathered as they exist under a tropical environment with high rainfall and temperature throughout the year, resulting in leaching of plant nutrients and accumulation of sesquioxides. They are by nature devoid of basic cations and available phosphorus and hence, their productivity is generally considered as low. The soils are mainly utilized for cultivation of oil palm and other economically important crops with great success due to excellent soil management practices (Shamshuddin & Daud, 2011).

Plants use numerous defense mechanisms against different types of environmental stresses and microbial pathogens (Mazid et al., 2011). In terms of anti-microbial secondary metabolites, some exist constitutively inside plants while some are induced by infection (Pusztahelyi et al., 2015). Metabolomics, the global analysis of a broad class of metabolites, is an important tool enabling investigations of the molecular basis of plant immunity in plant-pathogen interaction systems. The plant defense response is largely a product of the interaction of a diverse class of metabolites (Heuberger et al., 2014).

Metabolite profiling experiments should be designed to maximize the coverage of chemical classes to simultaneously assay for events related to both cells signaling and primary and secondary metabolism (Heuberger et al., 2014). The metabolite profiling of oil palm may provide an opportunity to understand the plant responses to biotic and abiotic stresses. This could serve as a platform for additional exploration as well as identification of metabolite markers for the early detection of BSR and USR diseases (Zain et al., 2013).

Metabolite profiling of oil palm has been carried out extensively to understand the BSR and USR diseases. Various palm tissues of both healthy and infected palms were used to analyze metabolites by using a metabolomics-based approach. Oil palm root, spear leaf and mesocarp tissues were used to compare metabolite profiles between partially tolerant and

susceptible palm extracts to uncover the biochemical pathway involved as well as in searching of metabolite biomarkers or phenotyping associated to BSR Disease in oil palm (Neoh et al., 2013; Rozali et al., 2017; Zain et al., 2013). Until today, no research on metabolomics has been conducted related to weathered tropical soils in Malaysia. Study on plant disease severity from different soil types has been conducted and it is suggested that the mechanism of disease suppression of soil-borne plant pathogens may vary strongly according to the soil types (Messiha et al., 2007), therefore, this research is conducted with the following objectives:

1. To determine soil physicochemical properties of Ultisol and Oxisol soil where healthy and *Ganoderma*-infected oil palm tree are grown.
2. To extract and profile metabolites of *Ganoderma*-infected oil palm grown on Ultisol and Oxisol soil.

CHAPTER 2: LITERATURE REVIEW

2.1 Malaysian Palm Oil

The commercial variety of oil palm planted in Malaysia is *Elaeis Guineensis* which originates from West Africa. The genus Elaeis belongs to the palm Family Arecaceae, an important member of the monocot group under the Order Arecales. Figure 2.1 shows the photo and taxonomic tree of *E. Guineensis* which was taken from the sampling site. The oil palm tree originates from West Africa where it grows in the wild and later was developed into an agricultural crop. It was introduced to Malaysia, then Malaya, by the British in the early 1870s as an ornamental plant.

In 1917, the first commercial planting took place in Tennamaran Estate in Selangor, laying the foundations for the vast oil palm plantations and the palm oil industry in Malaysia. The cultivation of oil palm increased at a fast pace in the early 1960s under the government's agricultural diversification programmed, which was introduced to reduce the country's economic dependence on rubber and tin. Later in the 1960s, the government introduced land settlement schemes for planting oil palm to eradicate poverty for the landless farmers and smallholders.

The oil palm plantations in Malaysia are largely based on the estate management system and the smallholder scheme (MPOC, 2019). Palm oil is now a major source of sustainable and renewable raw material for the world’s food, oleo chemical and biofuel industries. Involvement in cultivation or downstream activities has uplifted the quality of life of people and has helped alleviate poverty among landless farmers (Basiron, 2007).

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Arecales

Family: Arecaceae

Genus: Elaeis

Species: *Elaeis guineensis*

Figure 2.1: Photo and taxonomic tree of *Elaeis guineensis* (ISC, 2020).

2.1.1 Oil palm Properties

Oil palm has the highest productivity compared to other cultivated oleaginous crops as it gives the highest yield of oil per unit area. This crop can fulfil the growing global demand for vegetable oils which is estimated to reach 240 million tons by the year 2050. The oil palm produces two distinct oils called palm oil and palm kernel oil in which both are important in world trade (Barcelos et al., 2015). Palm oil and its products are very versatile edible oil which offers great potential both in food and non-food applications. Throughout the world, 90% of palm oil is used for edible purposes while the remaining 10% is used for soap and oleo-chemical manufacturing.

The main uses of palm oil and its products in food applications are for cooking or frying, shortening, margarines, cocoa butter substitutes, dairy fat replacers and animal fat replacers. Palm oil is also used to produce intermediate and final oleo chemicals products such as soap, surfactants and detergents, cosmetic and personal care, agriculture and industrial products. Another new growth area of great potential is oil palm biomass that can enhance the industry's growth, competitiveness and sustainability. Biomass from the oil palm can be used for manufacturing particle and medium density fiber board, plywood as well as fiber composites to make car body components (MPOB, 2013a). The saturated fatty acid to unsaturated fatty acid ratio of palm oil is close to unity with a high number of antioxidants, β-carotene, and vitamin E content. Palm oil also contains a high proportion of palmitic acid as well as considerable quantities of oleic and linoleic acids (Kouski et al., 2015).

2.1.2 Palm Oil Production in Malaysia

In Malaysia, oil palm planted area in 2018 reached 5.85 million hectares, an increase of 0.7% as against 5.81 million hectares the previous year. Sarawak overtook Sabah as the largest oil palm planted state, with 1.57 million hectares or 26.9% of the total Malaysian oil palm planted area, followed by Sabah with 1.55 million hectares or 26.5% and Peninsular Malaysia with 2.73 million hectares or 46.6% (Table 2.1).

The plantation area is dominated by private estates (61.0%), followed by independent smallholders (16.8%), Federal Land Development Authority or FELDA (12.3%), state schemes/government agencies (5.8%), Federal Land Consolidation and Rehabilitation Authority or FELCRA (3.1%) and Rubber Industry Smallholders Development Authority or RISDA (1.0%) ((MPOB), 2013b). The distribution of Malaysian oil palm planted area by category can be visualized as in Figure 2.2. Total Malaysian exports of oil palm products in 2018 amounted to 24.88 million tons, higher by 3.8% from 23.97 million tons exported in 2017 with total export revenue of RM67.49 billion. In 2018, palm oil export earnings alone are RM38.63 billion, with palm oil export volume of 16.49 million tons (MPOB, 2013a).

Table 2.1: Malaysian oil palm plantation area as at December 2018 (MPOB, 2013b).

STATE	MATURED	%	IMMATURED	%	TOTAL	%
Johor	680562	91	67000	9	747562	12.8
Kedah	82287	91.1	8007	8.9	90294	1.5
Kelantan	121085	77.9	34287	22.1	155372	2.7
Melaka	51237	90.2	5574	9.8	56811	1
Negeri Sembilan	167026	89.1	20425	10.9	187451	3.2
Pahang	653535	86.4	102614	13.6	756149	12.9
Perak	364090	88.1	49221	11.9	413311	7.1
Perlis	641	94.1	40	5.9	681	0
Pulau Pinang	14042	95.5	660	4.5	14702	0.3
Selangor	123139	90.3	13222	9.7	136361	2.3
Terengganu	149519	88.5	19395	11.5	168914	2.9
Peninsular Malaysia	**2403163**	**88.3**	**320445**	**11.7**	**2727608**	**46.6**
Sabah	1378655	89	170590	11	1549245	26.5
Sarawak	1403526	89.3	168951	10.7	1572477	26.9
Sabah & Sarawak	**2782181**	**89.1**	**339541**	**10.9**	**3121722**	**53.4**
Malaysia	**5189344**	**88.7**	**659986**	**11.3**	**5849330**	**100**

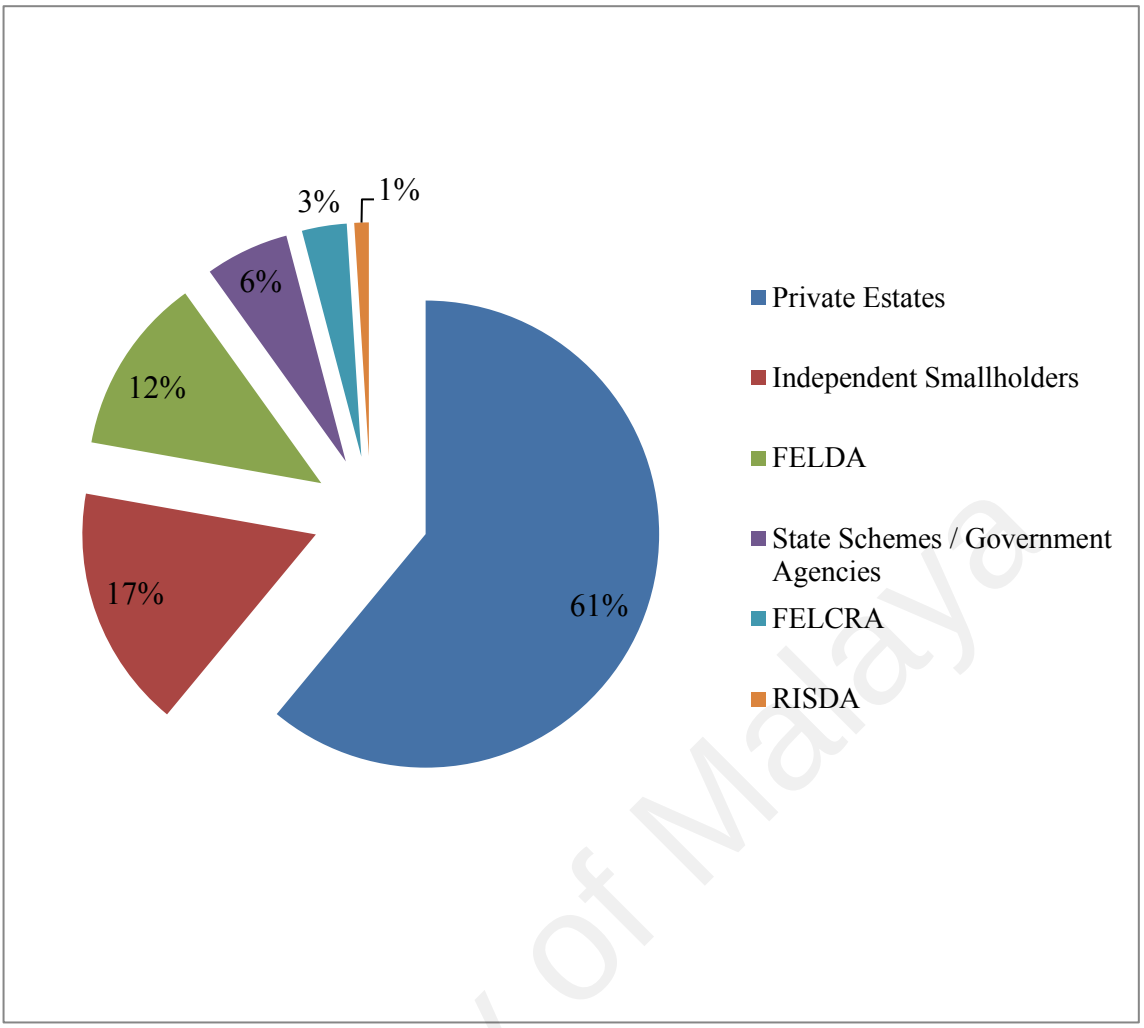


Figure 2.2: Malaysian oil palm plantation area by category 2018 (MPOB, 2013b)

2.1.3 Oil Palm Cultivation on Ultisol and Oxisol Soil

Soil scientists have developed a soil classification or taxonomy system in order to identify, understand, and manage soils. The most general level of classification in the United States system is the soil order. There are 12 soil orders categorized by the U.S. Department of Agriculture (USDA). The 12 soil orders are Gelisols, Histosols, Spodosols, Andisols, Oxisols, Vertisols, Aridisols, Ultisols, Mollisols, Alfisols, Inceptisols and Entisols. Each order is classified based on dominant physical, chemical, or biological properties that differentiate one to another (Ditzler & Hempel, 2016). Table 2.2 shows the lists of Malaysian soil series classified by USDA belong to Ultisol and Oxisol soil orders.

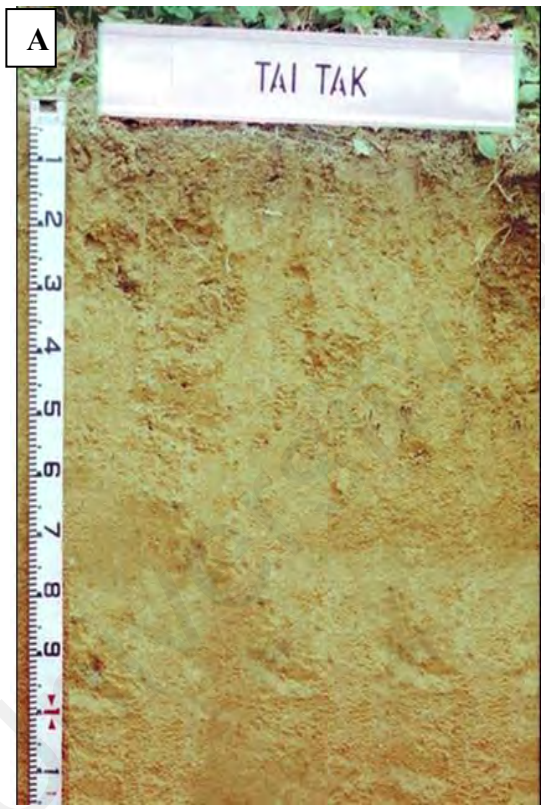
Ultisol soil formed in humid areas and intensely weathered. It contains a subsoil horizon that has an appreciable amount of translocated clay and are relatively acidic. Most nutrients are held in the upper centimeters of Ultisol soil, and this soil has low fertility but can become productive with the additions of fertilizer and lime. Ultisol soil makes up about 8% of the glacier-free land surface.

Oxisol soil originated from tropical and subtropical regions. It is dominated by iron oxides, quartz, and highly weathered clay minerals such as kaolinite. This soil can be found on gently sloping land surfaces of great age that have been stable for a long time. For the most part, it is nearly featureless soil without clearly marked layers, or horizons. Like Ultisol, Oxisol soil is highly weathered, has low natural fertility but can be made productive through wise use of fertilizer and lime, and can be found over about 8% of the glacier-free land surface too (Ditzler & Hempel, 2016).

Table 2.2: Malaysian Soil Series classified by USDA Soil Orders (Ultisol and Oxisol soil), Sub-orders and Great Groups (ARABIS, 2018).

Order	Sub-Order	Great Group	Soil Series
Ultisol	Aquults	Endoaquults	Inanam, Jabil
		Kandiaquults	Jabil, Lunas, Sogomana
		Kanhaplaquudults	Cherang Hangus
		Paleaquults	Inaanam/Poor, Jelutong
	Udults	Hapludults	Asahan, Batu Anam, Dagat, Durian, Kumansi, Pohoi
		Kandiudults	Batang, Bungor, Gajah Mati, Harimau, Holyrood, Kasau, Lambak, Lanchang, Langkawai, Lelau, Merbau, Rengam, Serdang, Sitiawan, Tai Tak, Tavy, Tebok, Tungau, Ulu Dong
		Kanhapludults	Apek, Gong Chenak, Kawang, Kening, Kuala Brang, Marang, Nami
		Paleudults	Abok, Bedup, Berkenu, Inanam/Imp, Kapilit, Kinabutan, Kulai, Lumisir, Merit, Musang, Nyalau, Stom Tanjong Lipat, Tok Yong, Yong Peng
		Plinthudults	Batang, Chuping, Sipit
		Rhodudults	Jakar, Sarekei
Oxisol	Udox	Acrudox	Jerangau, Kampong Kolam, Kuantan, Prang, Segamat, Senai, Sungei Mas, Table
		Eutrudox	Sagu, Sungei Mas
		Hapludox	Apas, Gading, Jarangan, Katong, Malacca, Munchong, Nobusu, Patang, Pinianakan, Tandak, Tarat
		Kandiudox	Batang Merbau, Bungor, Chat, Harimau, Lanchang, Rengam, Tai Tak, Ulu Dong

Malaysian soils dominantly fall into the Ultisol and Oxisol Orders in Soil Taxonomy. This is about 70% of the country's land area. These soils are acidic in nature, with pH values ranging from 4 to 5. These soils contain mainly sesquioxides and kaolinite both of which are essentially variable charge minerals. The phosphorus present in the soil system is highly fixed by the sesquioxides in the soil system. This means that these soils are lacking with phosphorus. In fact, phosphorus is considered as the most limiting nutrient for crop production in the tropics (Sung et al., 2017). Figure 2.3 shows the photos and classification of soils from Tai Tak and Segamat Series.



Soil Classification
FAO Unesco Legend: Typic Paleudult
USDA Soil Taxonomy: Clayey, kaolinitic, isohyperthermic, Dystric Nitosol

Soil Classification
FAO Unesco Legend: Haplic Acrisol
USDA Soil Taxonomy: Very Fine, Kaolinitic, isohyperthemic, Typic Hapludox

Figure 2.3: Physical characteristics and soil classification of Ultisol and Oxisol soil; (A) Ultisol soil and (B) Oxisol soil (JPM, 2008).

Ultisol and Oxisol soil also have very low basic cation status and effective cation exchange capacity (CEC). Accessions of acidity by these soils contribute to soil degradation as a result of reactions which liberate toxic levels of aluminum and manganese ions, reduce the CEC, increase the anion exchange capacity, and promote the loss of basic cations by leaching. The activities of soil organisms are generally reduced under such conditions. This can take a toll on crop yield and impair biological nitrogen fixation. Therefore, amelioration involves both the neutralization of exchangeable aluminum and manganese ions and restoration of higher levels of exchangeable basic cations such as calcium ion throughout the soil profile (Sung et al., 2017).

Figure 2.4 illustrates the industrial oil palm and other plantations in Peninsular Malaysia. The yield of oil palm grown on Ultisol and Oxisol soil ranges from 20 to 30 tons FFB /hectare/year with the rate of oil extracted is 20% of the FFB. The natural canopy of oil palm tree has maintained the soil moisture for optimum palm growth. Prior to cutting, the oil palm fronds are placed in between the planting rows so that the organic matter will decompose naturally. The increase in this soil nutrients composition could enhance susceptibility to other disorders and diseases from soil-borne plant pathogens (Shamshuddin & Daud, 2011).

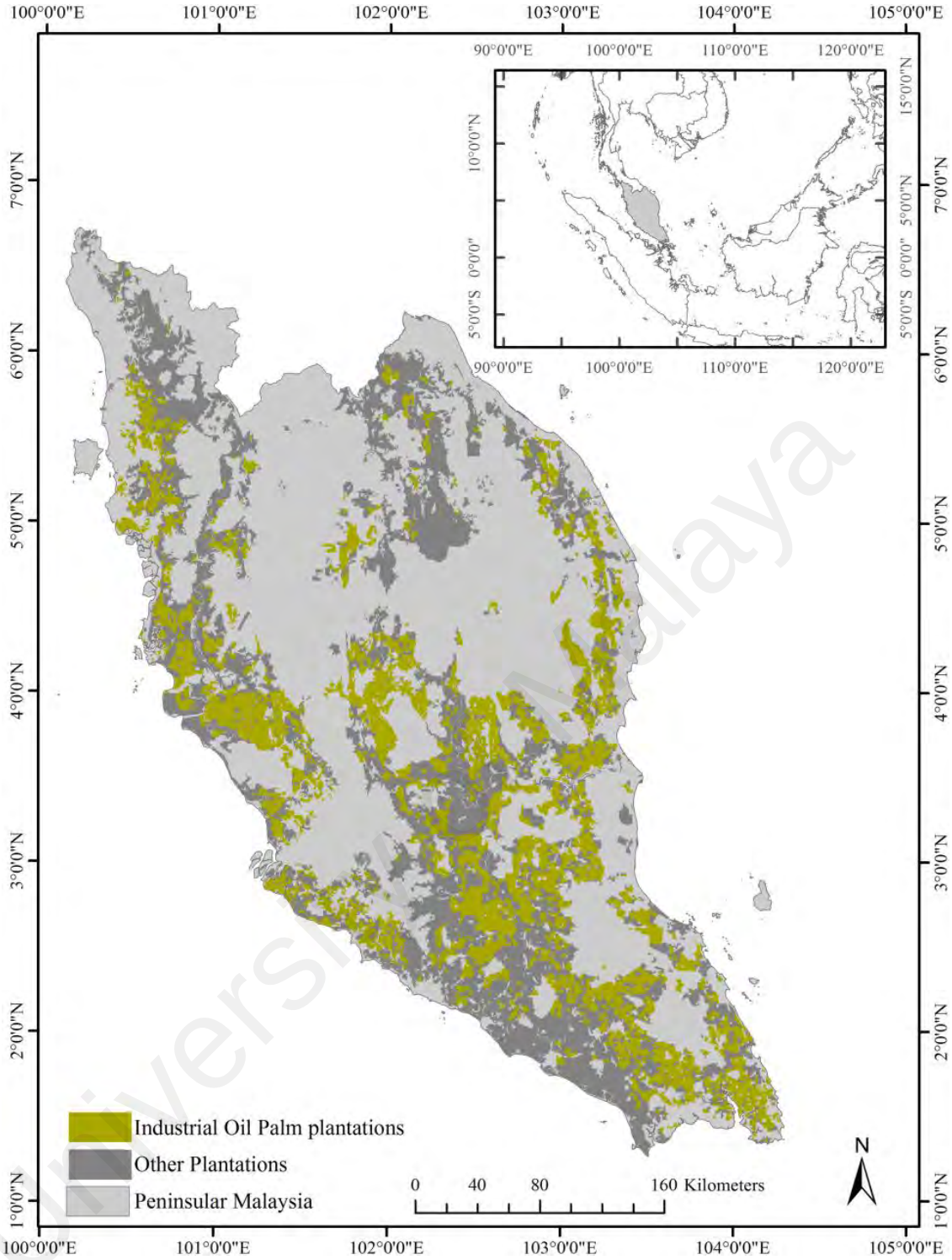


Figure 2.4: Industrial oil palm and other plantations in Peninsular Malaysia (Shevade & Loboda, 2019).

2.1.4 Oil Palm Ecosystem

Oil palm ecosystems comprise a wide range of fungi, bacteria, insects, nematodes, and viruses that are significantly responsible for reducing crop productivity. One of the most destructive diseases of oil palm caused by *G. boninense*, a wood-decaying fungus, is BSR. The BSR causes the reduction of oil palm FFB yield and the collapse of the palms (Sahebi et al., 2017).

2.1.5 Economic Loss Due to BSR and USR Disease

The economic loss due to the infection is reported to be at 43.32% of the potential yields (Assis et al., 2016). The BSR Disease incidence rate in 1994 was estimated at 1.51% or 0.03 million hectares of affected areas from 2.14 million hectares of total matured plantation area as published in the Malaysian Palm Oil Board, MPOB Basal Stem Rot Census 1994-1995 Report. In 2009, the estimated BSR Disease incidence rate was estimated at 3.71% or 0.15 million hectares of affected areas from 4.70 million hectares of total matured plantation area. Therefore, the yearly growth rate of area affected over the 15-year period was 10.3% per year. If oil palm estates do not apply treatment and the disease infection follows the same growth pattern, it is estimated that the total oil palm plantation area affected by BSR Disease in 2020 would be around 0.44 million hectares involving about 65.6 million oil palm trees (Roslan & Idris, 2012). The never-ending problem of BSR and USR disease has affected the production of oil palm and burden planters especially smallholders and farmers (Chong et al., 2017).

2.1.6 BSR and USR Disease Caused by *Ganoderma* Sp. Infection

The genus *Ganoderma* belongs to the Family of Ganodermataceae, Order Polyporales and Class Agaricomycetes. The fungus is economically important and reported of being as helpful for medicinal purpose to as harmful as a pathogen of some precious crop plant including oil palm (Lee & Chang, 2016). In Malaysia, three common species of Ganoderma namely *Ganoderma boninense*, *Ganoderma zonatum* and *Ganoderma miniatocinctom* are the causal agents of BSR Disease in which the *G. boninense* has been reported to be the most harmful

that causes a great economical effect in oil palm industry (Wong, Bong, & Idris, 2012). Figure 2.5 shows the photo of *G. boninense* as captured at the sampling site as well as the taxonomic tree of the fungus.

Taxonomic Tree:

Domain: Eukaryota

Kingdom: Fungi

Phylum: Basidiomycota

Subphylum: Agaricomycotina

Class: Agaricomycetes

Subclass: Agaricomycetidae

Order: Polyporales

Family: Ganodermataceae

Genus: *Ganoderma*

Species: *Ganoderma boninense*

Figure 2.5: Photo and taxonomic tree of *G. boninense.* The arrows show the fruiting bodies of the *G. boninense* at the bottom of oil palm tree (ISC, 2020).

2.1.7 Mode of Infection and Symptoms of BSR and USR Disease

There are numerous modes of infection associated with the epidemiology of *G. boninense* in oil palm plantation, including in-contact roots with nearby infected palms and through airborne basidiospores. Deep insight on the route of infection and mycological pathogenicity behavior of the pathogen is the greatest priority in order to successfully develop effective management practices for disease control (Chong et al., 2017). The symptoms of BSR disease are decay of the bottom of the stem from where basidiocarps emerge and sometimes also decay of the roots. The rotting stem restricts the water and nutrients uptake from roots to the fronds and cause chlorosis. When the disease is more severe, the older fronds wilt and hang down to form a skirt around the trunk (Hushiarianet al., 2013). Other observable symptoms include flattening of the crown and spear leaves that have not opened. In some cases, the stem might even fracture (Rees et al., 2012). On the other hand, USR disease has symptoms like those of spear rot, bud rot and bunch rot in which lower leaves first become yellow and die from the tip to the base. This condition progresses to the middle of the crown, finally affecting the spear leaves. The stem tissues show a brown rot even when the roots of the palm are not affected (Hasan et al., 2005).

2.1.8 Control Measures for BSR and USR Disease

Some possible ways of controlling the disease have been implemented which includes soil mounding, surgery, sanitation or removal of diseased material, ploughing and harrowing, fallowing, planting legume cover crops, chemical treatments, application of fertilizer combination as well as biological control, but no single method has yet been able to halt the continuing spread of the disease (Hushiarian et al., 2013).

2.1.9 Factors affecting BSR and USR Disease

There are various factors were reported to influence the incidence of BSR and USR disease such as the cultivation of the previous crop, replanting technique, age and number of individual palms per planted area, inoculum potential, shading and soil temperature (Azahar

et al., 2014; Rees et al., 2007). Previous research visualized the geographical distribution of BSR disease incidence among oil palm smallholders in Selangor using Geographical Information System. Distribution pattern of the BSR disease revealed that most of the incidences were confined along the coastal area which might be due to the planting of oil palm in previous coconut stands. Meanwhile, the low pH of soil series showed a higher BSR incidence compared to the high pH of soil series that showed slightly lower BSR incidence. This research reported that the distribution of BSR was mostly confined to the coastal areas which might be due to the planting of oil palm in previous coconut stands rather than any relationship with the type of soil series (Parthiban et al., 2016). Other than that, research conducted on soil-borne plant host-pathogen interaction also suggested that the disease suppression could be affected by different type of soil due to the natural ecosystem in the soil and rhizosphere (Messiha et al., 2007).

Plants can respond to such soil condition due to the roots that are responsible for water and nutrients uptake. When there are difficult conditions of the restrictive environment in the soil, the roots will send inhibitory signals to the shoots. This behavior can be interpreted as feedforward responses to roots becoming infected with pathogens. The inhibitory signals may affect stomatal conductance, cell expansion, cell division and the rate of leaf appearance. A network of hormonal and other responses is involved in attuning growth and development of a plant to its environment (Passioura, 2002).

2.2 Metabolomics

The plant defense response is largely a product of the interaction of a diverse class of metabolites. Metabolite profiling experiments should be designed to maximize the coverage of chemical classes to simultaneously assay for events related to both cells signaling and primary and secondary metabolism. Metabolomics, the global analysis of a broad class of small molecule compounds, is, therefore, an important tool enabling investigations of the molecular basis of plant immunity in plant-pathogen interaction systems (Heuberger et al.,

2014). Metabolomics can be defined as a study that analyses endogenous and exogenous low molecular mass metabolites within a cell, tissue, or bio fluid of an organism in response to an external stressor such as disease, contaminant exposure, or nutritional imbalances (Saito & Matsuda, 2010). This field of science can generate comprehensive data sets of the sample being analyzed (Riekeberg & Powers, 2017).

Metabolomics strategies have been divided into untargeted and targeted metabolomics. Both approaches have their own inherent advantages and disadvantages. Untargeted metabolomics approach aims to gather information on as many metabolites as possible in each extract analyzed. In such untargeted approaches, all analytical information present in the profiles will be first transformed into coordinates based on mass, retention time and signal amplitude. These coordinates are then aligned across all samples. By applying appropriate statistical and multivariate analysis tools, differential mass peaks or mass peaks correlating with a specific trait can be filtered out and identified to some degree by using accurate mass, tandem mass spectrometry (MS/MS) fragmentation and then confirmed with standards when available (De Vos et al., 2007).

Targeted metabolomics is a measurement of defined groups of chemically characterized and biochemically annotated metabolites. The analysis can be carried out in a quantitative or semi-quantitative manner using internal standards. This approach takes advantage of the comprehensive understanding of a vast array of metabolic enzymes, their kinetics, end products, and the known biochemical pathways to which they contribute. When utilizing targeted metabolomics sample preparation can be optimized to reduce the dominance of high-abundance molecules in the analyses. In addition, since all analyzed species are clearly defined, analytical artefacts are not carried through to downstream analysis. When predefined lists of analytes are studied, novel associations between metabolites may be illuminated in the context of specific physiological states (Roberts et al., 2012).

2.2.1 NMR-based Metabolomics

Plants are rich with chemically diverse metabolites which are usually present in a large range of concentrations, and no single analytical technique is currently capable of extracting and detecting all the metabolites (Hall, 2006). Two main analytical techniques used in metabolomics study are nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Kopka et al., 2004). Several methodologies within these two analytical techniques are currently being developed specifically to deal with the types of complex samples analyzed in metabolomics studies. NMR spectroscopy is known as one of the premier methods for the analyses of multi-component mixtures as it requires little or no sample preparation. This technique provides a high reproducible result, is rapid, non-destructive and non-invasive. Peaks in the NMR spectra can be reliably assigned to specific metabolic species based on their chemical shifts and multiple patterns which directly provide a wealth of information on the identity and quantity of many metabolites in parallel from a single experiment (Gowda et al., 2008).

With advanced high-throughput NMR methodology, up to 200 samples can be measured within a day with the assistance of flow-injection probes and automated liquid handlers. The detection limit can also be decreased to 10's of nanogram using high field magnets, cryogenically cooled probes, micro coil probes equipped to handle very small samples, and methodologies that couple NMR to liquid chromatography and solid phase extraction (Gowda et al., 2008). Although NMR is in principle the most uniform detection technique and is essential for the unequivocal identification of unknown compounds, NMR-based metabolomics approaches still suffer from a relatively low sensitivity (Commisso et al., 2013).

2.2.2 MS-based Platforms

MS-based platforms have been the more popular choice in plant metabolomics (Jorge et al., 2016). The intrinsic high sensitivity of MS detection makes it an important method for

measuring metabolites in complex bio fluids. A variety of MS methods in combination with separation techniques such as gas and liquid chromatography or their variants have been used in numerous metabolomics investigations. This analytical technique covers a large variety of non-volatile metabolites, mainly those involved in primary metabolism, including organic and amino acids, sugars, sugar alcohols, phosphorylated intermediates in the polar fraction of extracts, lipophilic compounds such as fatty acids and sterols in the polar fraction, as well as plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivatives thereof (De Vos et al., 2007; Lisec et al., 2006).

Plants produce an extensive chemical diversity of metabolites, estimated to be between 100,000 and 200,000 compounds (Hill & Roessner, 2014). Gas chromatography-mass spectrometry (GC-MS) platform facilitates the identification and robust quantification of a few hundred metabolites in a single plant extract resulting in comprehensive coverage of the central pathways of primary metabolism. The main advantages of this technology are that it has long been used for metabolite profiling and thus there are therefore stable protocols for machine setup and maintenance, and chromatogram evaluation and interpretation (Lisec et al., 2006). Although no single analytical system can cover the whole metabolome, GC-MS has a relatively broad coverage of compound classes, including organic and amino acids, sugars, sugar alcohols, phosphorylated intermediates and lipophilic compounds. Recovery experiments of all measurable classes of compounds have been done during method validation. For unknown compounds, recovery rates can be determined by recombination experiments in which extracts of two plant species are evaluated both independently and after mixing (Lisec et al., 2006).

Liquid chromatography-mass spectrometry (LC–MS) is one of the major untargeted platforms to determine global metabolite profiles which aims at the identification and relative quantitation of all peaks from the ion chromatograms that are initially defined by retention

time and molecular mass (Hill & Roessner, 2014). The LCMS-based platform is expected to be of importance in plants research and is capable of analyzing a broad range of metabolites of plants which covers many semi-polar compounds not involved in primary metabolism in which several have been shown to have phenotypic and physiological importance (Goh et al., 2016; Zain et al., 2013). This includes the large and often economically important group of secondary metabolites such as alkaloids, benzoids, flavonoids, terpenes, isoprenes, glucosinolates and phenylpropanoids as well as highly polar and higher molecular weight molecules such as oligosaccharides and lipids (Hill & Roessner, 2014).

2.2.3 LC-TOF-MS

Beginning with the earliest concept of separating ionized molecules based on their flight times, time-of-flight mass spectrometry (TOF-MS) has shown valuable utility and uniqueness in its ability to perform extremely high-speed full-spectral measurements. Early work established the basic conditions to accomplish this intrinsic performance feature, spectral acquisition rate, with high resolving power. Over the past few years key developments in ionization, atmospheric sampling and ion optic and detection systems have led to the commercial success of analytical TOF systems capable of supporting the most demanding high-resolution chromatographic separations.

In addition to ongoing improvements in one-dimensional separations, multidimensional chromatography and ion mobility are adding additional analytical capabilities for which TOF is ideally suited. Concurrent to these instrumental developments researchers in many fields have made substantial progress in the development of non-targeted methodologies for the screening of low-level analytes in diverse matrices. Leveraging speed, dynamic range and resolution, TOF-MS has become well positioned to address a wide range of growing analytical needs (Fjeldsted, 2016).

2.2.4 Biotic Stress in Plants

Plants are faced with numerous biotic stresses and adverse environmental conditions. They respond to these stresses through several morphological, biochemical, and molecular mechanisms and evidence suggests that there are interactions among their respective signaling pathways (Nejat & Mantri, 2017). Among the downstream signaling pathways induced by effector-triggered and pathogen-associated molecular patterns-triggered immunity, three hormones stand out: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). While the SA pathway stimulates resistance responses to biotrophic and hemi-biotrophic pathogens, the JA and ethylene pathways are generally induced against necrotrophic pathogens and chewing insects (De Vleesschauwer et al., 2014).

SA, in turn, activates a systemic resistance response called Systemic Acquired Resistance that promotes the expression of pathogen related genes and provides long-term defense against a broad spectrum of pathogens (Grant & Lamb, 2006). Although SA, JA and ET defense signaling pathways have substantial differences in gene expression, they interact to help the plant choose the best defense strategy (Glazebrook, 2005). Other hormones such as abscisic acid, auxin, brassinosteriods, cytokinin, gibberellic acid and peptide hormones have also been reported as important regulators of immune responses (Bari & Jones, 2009).

Metabolite profiling of oil palm may provide an opportunity to understand the plant biosynthetic pathways in response to biotic and abiotic stresses and could serve as a platform for additional exploration as well as identification of metabolite markers for the early detection of BSR Disease (Sahebi et al., 2017).

2.3 Data Analysis

Metabolite profiling using LC-MS consists of a series of experiments which includes data pre-processing, statistical analysis, compound identification and data interpretation (Perez et al., 2017). Profile Analysis software provides an efficient pre-processing of complex LC-

TOF-MS data sets. The find molecular feature (FMF) makes use of a peak finding algorithm for quantitatively pinpointing relevant information as well as eliminates noise and outliers.

The system will later create bucket tables of data based on the extracted FMF compounds. The retention time alignment makes use of a shifting vector algorithm which will adjust all retention times in a representative run from the runs analyzed together. The software also provides different filtering, normalization and scaling options. All the data pre-processing steps will complete the basis for successful subsequent statistical analysis (Spicer et al., 2017).

2.3.1 Univariate Analysis: One-way ANOVA

The identification of significantly perturbed metabolites or metabolite peaks is probably the most basic task in metabolomic data analysis. A variety of strategies have been implemented in MetaboAnalyst 2.0 to help researchers select or identify compounds of interest and to facilitate different research objectives. Differential expression analysis has been extended to support multiple group analysis, can be used by users to identify compounds that are significantly different between two or more sets of experimental conditions or two or more populations under study.

MetaboAnalyst 2.0 supports both ordinary univariate methods and moderated t-statistic methods to compare means or medians of one variable across two or more groups. Because of the multiple-testing issue, Bonferroni corrected P-values (FDR) are also computed for these functions. Many standard statistical algorithms such as t-tests and ANOVA tests work under the assumption that the data being analyzed are normally distributed. If the data are not normally distributed or cannot be transformed into a normal distribution, then most standard statistical tests become unreliable. In addition to improving reliability and interpretability, data normalization can also help reduce any systematic bias in the data that may have arisen from instrumental or sampling problems (Xia et al., 2012).

2.3.2 Multivariate Analysis: PCA and PLSDA

Multivariate analysis such as PCA and PLS-DA will be implemented to study the multiple interactions between metabolites. The techniques provide an essential platform for rapid interpretation of information-rich spectral datasets for inferring biological conclusions (Worley & Powers, 2013). This research makes use of multivariate analysis software called SIMCA-P+ which uses soft independent modelling of class analogies (SIMCA). SIMCA is a classification method constructing separate PCA and PLS-DA models for each group enabling categorization of samples into groups.

CHAPTER 3: MATERIAL AND METHOD

3.1 Sampling Site

The soils used for this pot experiment were the Tai Tak and Segamat Series, which are classified as Ultisol and Oxisol soil respectively. Sample collections were carried out in June 2017, at Felda Ulu Belitong in Kluang (N1.91647° E103.48219°) and FGVPM Palong Timur 5 in Segamat (N2.79264° E102.70189°). The coordinate distance between study areas is 80.82 miles or 130.07 km. The study areas show very similar climatic conditions and the palms are grown under standard estate management practice. The climate in Kluang and Segamat are classified as tropical wet (Af) by the Kopper-Geiger system where precipitation occurs all year long. The monthly temperature variations in this climate are less than 3°C. Both study areas experienced the driest condition in July and wettest condition in December (CDO, 2019).

3.2 Soil Sample Analysis

Soil samples were collected from three different depths of 0-15 cm, 15-30 cm and 30-45 cm beneath the soil surface where the same biological samples of healthy and *Ganoderma*-infected palm trees were grown. Four technical replicates were chosen, and the soil samples were mixed, sealed and labelled accordingly. The soil samples were left to dry naturally for two months until analysis. The dried soil samples were ground using pestle and mortar, before sieved using 1.0 mm sieve as in Figure 3.1.

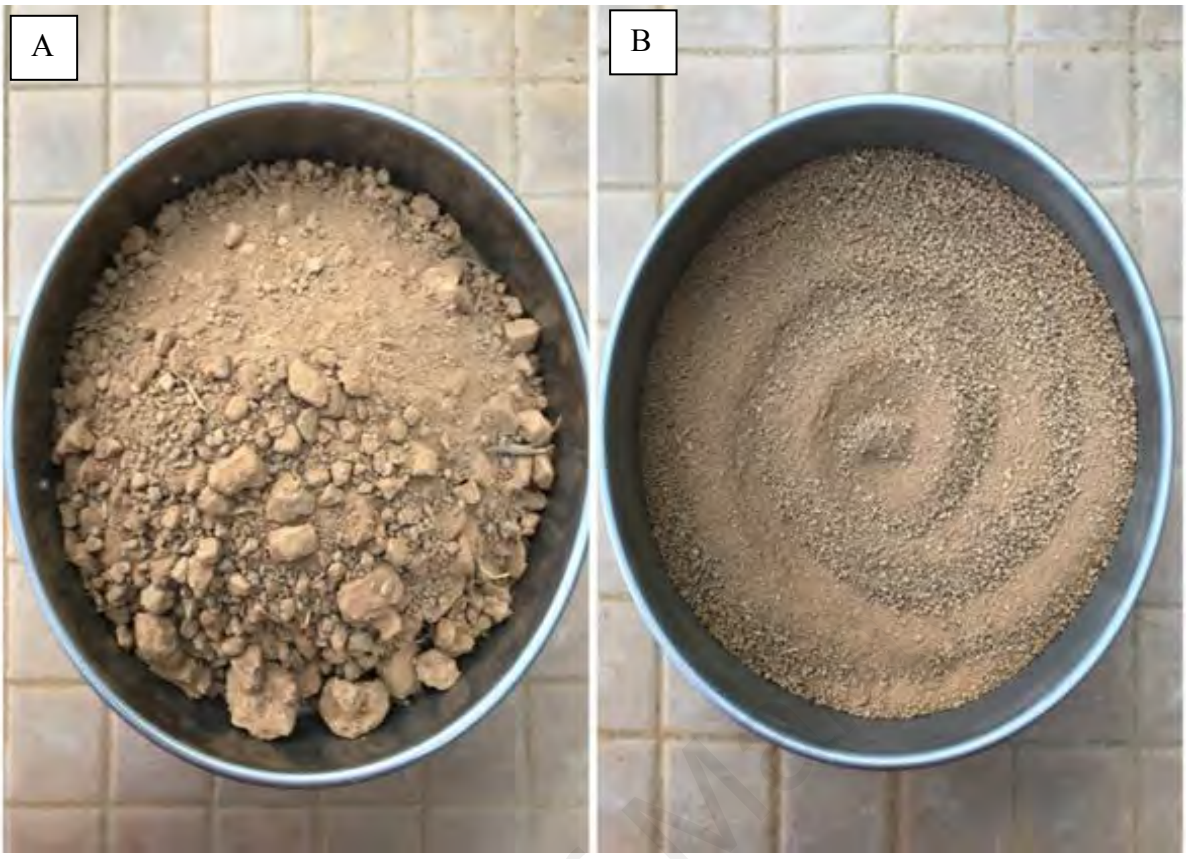


Figure 3.1: Soil sample preparation. A) The ground soil sample. B) The sieved soil samples.

3.2.1 Soil pH and EC

About 10 g of the soil sample was mixed with 25 ml of distilled water (dH_2O) following the ratio of soil:dH_2O of 1:2.5. The mixture was then stirred for 30 minutes and left to rest for another 30 minutes. Prior to the calibration process, the pH value was measured using pH meter (Eutech pH 2700 Meter). After that, 25 ml of dH_2O was added to the mixture to measure its EC value. The reading was measured using an EC meter (HI-2211 Bench Top pH & mV Meter). The same steps were repeated for all soil samples and all data collected were tabulated.

3.2.2 Soil TC

The CNS LECO TruMac (Figure 3.2) Method system utilized a combustion technique that provided a result for carbon element within five minutes for each sample. Prior to soil sample analysis, 2,5-(Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT) was used as the

certified reference material where its carbon% was 72.48 ± 0.25. The dried soil samples were sieved again using 60 μm sieve. Each soil sample weighed 0.2 mg was placed into a large ceramic boat and loaded into the purge chamber located in the front of the horizontal ceramic high temperature furnace. The step was repeated until all soil samples were loaded accordingly.

After the entrained atmospheric gas was purge from the sample, the ceramic boat was introduced into the furnace regulated at a temperature of 1350℃. Complete oxidation of the soil sample was ensured by a pure oxygen environment within the furnace, with additional oxygen being directed onto the sample via a ceramic lance. The ceramic boat and all ash from the sample were removed from the furnace at the end of combustion, leaving the furnace free of ash build-up. The moisture was removed from the furnace by Anhydrone. The remaining combustion gases were collected and equilibrated in ballast where aliquot gases were swept through an infrared detector for carbon determination. External PC Windows-based operating software managed all the quantitative calculations and all data were displayed as a weight percentage and tabulated.

Figure 3.2: The CNS LECO TruMac analyzer used for soil TC analysis

3.2.3 Soil Particle Size

The method used for particle-size analysis is the pipet method. The same dried and sieved soil samples were used where 20 g of the soil sample was put into a tall beaker of 1000 ml. About 100 ml of hydrogen peroxide, H_2O_2, was added and leaved overnight. After that, the beaker was heated on a hot plate and further quantities of H_2O_2 were added until no more frothing occurs. A total of 100 ml 0.2 N HCl was added, followed by distilled water until the volume reached 400 ml. The mixture was heated again on the hot plate for 15 minutes and allowed to cool. The supernatant liquid was siphoned off and washed three times with 400 ml of dH_2O. About 50 ml Calgon Solution was added into the contents of the beaker and stirred for 15 minutes using a mechanical stirrer.

After that, the contents were sieved through a 50 μm sieve and transferred into 1000 ml cylinder. The contents in the cylinder were the clay and silt particles while the particles left on the 50 μm sieve were the sand fraction. The mechanical stirrer blades were washed with distilled water until no traces of the soil were left and the washed water was introduced in the cylinder through the sieve. The content of the cylinder was made up to 1000 ml. The cylinder was introduced in the water bath at a constant 23℃ temperature. A plunger was used to mix the contents thoroughly for one minute. The suspension was left to settle for six hours and 39 minutes. A pipette was introduced at a depth of 10 cm to pipet an aliquot. The contents of the pipette were transferred into an aluminum dish and oven-dried at 105℃ for 24 hours. The weight recorded as A.

After oven-dried, the aluminum dish could cool in a desiccator before weighing. Meanwhile, the sand fraction on the 50 μm sieve was oven-dried at 105℃ and were then transferred onto a nest of sieves arranged from top to bottom with decreasing sizes of 1000-, 500-, 250-, 100-, and 50 μm. All sieves were shaken for five minutes using a mechanical stirrer. Each sand fraction in the nest of sieves was weighed and the total weight of all sand fractions was recorded as B. As Calgon Solution was added before, the weight of clay particles was corrected by pipetting 50 ml of Calgon Solution into three separate dishes. They were placed in oven to be dried and cooled. The mean weight of the replicates was recorded as C. The calculations to determine % of clay, sand and silt are as follow:

% clay = ((A ×1000)/ (volume of pipette) -C) ×100/ (weight of soil)

% sand =B×100/ (weight of soil)

% silt =100-% sand -% clay

3.2.4 Data Analysis for Soil Physico-chemical Properties

Mann-Whitney U test is a non-parametric statistical technique. It is used to analyses differences between the medians of two data sets. It can be used in place of a t-test for independent samples in cases where the values within the sample do not follow the normal or t-distribution but also when the distribution of values is unknown. For the Mann-Whitney U test to be applied, values need to be measurable on an ordinary scale and comparable in size. The fact that all values are compared makes it distinct from the t-test, which compares the sample means.

The Mann-Whitney U is also used to test the null hypothesis, subject to both samples coming from the same basic set or having the same median value. The Mann-Whitney U test in the SPSS statistical program is performed in two parts. The first part represents the main part of the Mann-Whitney U test, and the second part the calculation of the median of each group (Milenovic, 2011). In this study, the Mann-Whitney U test will be carried out to see whether there are significant differences between Ultisol and Oxisol soil based on selected physico-chemical properties such as soil pH, EC, TC. The statistical data analyses will be performed using SPSS version 21.0 (SPSS, 2012).

3.3 Biological Sample Preparation

The study was carried out by using three biological replicates and three technical replicates (Figure 3.3). Biological replicates were harvested from oil palm of healthy and *Ganoderma*-infected 12-years old palms. The biological replicate means the sample was harvested from different oil palm tree while the technical replicate means three samples were collected from the same biological sample. For field experiment, the oil palm frond number 17 basal fronds or commonly known as rachis has been selected as biological sample. This is because it absorbed the most nutrients from the roots where the infection started (Jayaselan et al., 2018).

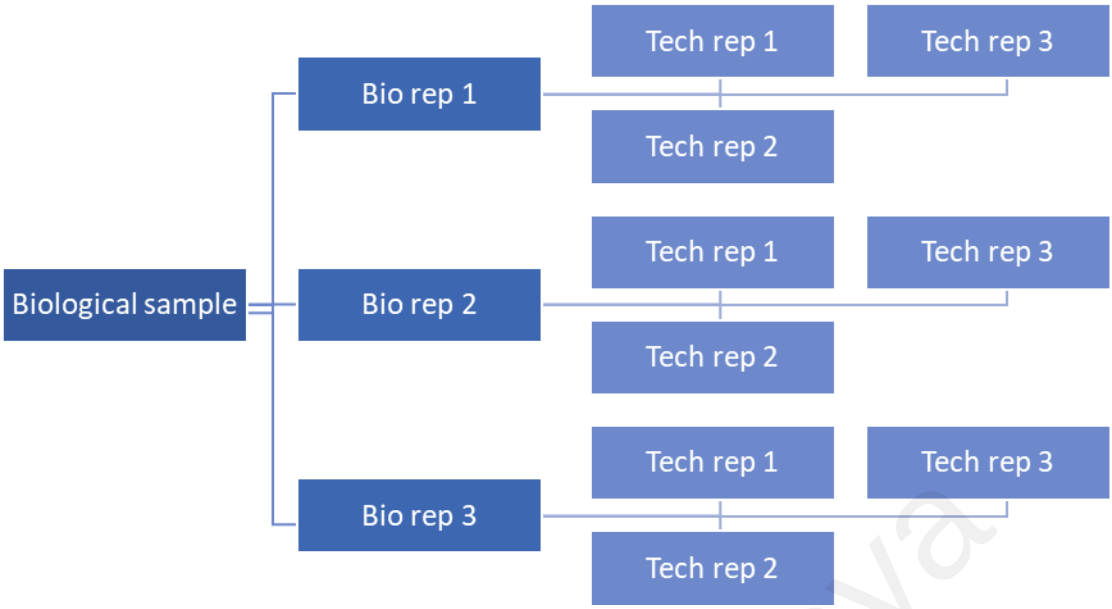


Figure 3.3: The number of biological and technical replicates used in this research.

Prior to harvesting, the excised rachises were wrapped using aluminum foil before storing at -80°C freezer in the laboratory on the same day of harvesting. For oil palm rachis sample preparation (Figure 3.4), the oil palm rachises were cut into smaller pieces and ground into powdered form using pestle and mortar. Liquid nitrogen was poured in between grinding process to prevent samples from thawing. The samples were left to dry via freeze-dry method to remove the remaining water. The samples were then stored in -80°C until extraction.

Figure 3.4: Oil palm rachis sample preparation. A) Sample was cut into smaller pieces. B) Sample was ground into powdered form.

3.4 Extraction Method

Three experimental designs with slight modifications were selected based on the established literature review on plant metabolomics protocols to choose the best extraction method for oil palm rachis samples. The similarities and differences between extraction methods are summarized as in Table 3.1. Extraction efficiencies of these three methods were evaluated.

For first method, metabolite extraction from oil palm rachis sample was performed according to Tatli (2015). Pre-cooled 4.25 ml extraction medium of chloroform:methanol:water (1:2.5:1 v/v/v) was added to 500 mg samples. All mixtures were vortexed and left on ice for 30 minutes. Approximately 2 ml of pre-cooled dH_2O was added and vortexed again. The mixtures were then centrifuged at a setting of 4000 rpm for 30 minutes. The supernatant was collected, and the pellet was re-extracted again using the same amount of extraction medium. The resulting supernatant was collected and combined with the first one collected into vials.

For second method, metabolites were extracted from samples according to the protocol of Cadahía (2015). About 500 mg samples were extracted using 2 ml pre-cooled chloroform following ultrasonication bathing for 15 minutes at 30 °C. A total of 8 mL of pre-cooled 75% aqueous methanol was added, and the mixtures were put into ultrasonication bathing again for 20 minutes at 30 °C. The mixtures were then centrifuged at a setting of 4000 rpm for 15 minutes at 25 °C. A total of 2 ml of supernatant (clear solution) from each sample was collected and evaporated using nitrogen stream. The resulting dried supernatants were mixed with 4 ml 50% aqueous methanol and centrifuged again. Every 1 ml of the supernatant was collected and put into vials.

For third method, metabolites extraction from samples followed from Zain (2013). About 500 mg samples were extracted using 5 ml pre-cooled 80% aqueous methanol following ultrasonication bathing for 30 minutes at 30 °C. The mixtures were then centrifuged at a setting of 4000 rpm for 15 minutes at 25 °C. 2 ml of supernatant from each sample was collected and evaporated using nitrogen stream. The resulting dried supernatants were mixed with 3 ml dH_2O and centrifuged again. Every 1 ml of the supernatant was collected and put into vials.

Table 3.1: The similarities and differences between extraction methods.

Reference	Method 1	Method 2	Method 3
Sample mass (mg)	500	500	500
Solvent ratio (v/v/v)	chloroform:methanol:dH_2O 1:2.5:1	chloroform:methanol:dH_2O 1:1:3	methanol:dH_2O 1:4
Ultrasonification bath	Absent	Present	Present
Centrifugation	Present	Present	Present

3.5 Metabolomics Data Analysis

Metabolite profiling using LC-MS consists of a series of experiments which includes data pre-processing, statistical analysis, compound identification and data interpretation (Perez et al., 2017). Profile Analysis software provides an efficient pre-processing of complex LC-TOF-MS data sets. The FMF makes use of a peak finding algorithm for quantitatively pinpointing relevant information as well as eliminates noise and outliers.

The system will later create bucket tables of data based on the extracted FMF compounds. The retention time alignment makes use of a shifting vector algorithm which will adjust all retention times in a representative run from the runs analyzed together. The software also provides different filtering, normalization and scaling options. All the data pre-processing steps will complete the basis for successful subsequent statistical analysis (Spicer et al., 2017).

For LC-TOF-MS Analysis, the separation was performed on Thermo Scientific C18 column (AcclaimTM Polar Advantage II, 3 x 150mm, 3um particle size) on an UltiMate 3000 UHPLC system (Dionex). Gradient elution was performed at 0.4ml/minute and 40°C using H_2O + 0.1% Formic Acid (A) and 100% Acetonitrile (B) with 22 minutes total run time. The injection volume of sample was 1µL. The gradient started at 5% v/v B (0-3minutes); 80% v/v B (3-10minutes); 80% B (10-15minutes) and 5% B (15-22minutes). High-resolution MS was carried out using a MicroTOF QIII Bruker Daltonic (Bremen, Germany) using an electrospray positive ionization with the settings of 4500 V capillary voltage, 1.2 bar nebulizer pressure, and 8 L/min drying gas at 200 °C. The mass range was at 50-1000 m/z. For data processing, the accurate mass data of the molecular ions, provided by the TOF analyzer, were processed by Compass Data Analysis software (Bruker Daltonik GmbH) set at signal to noise ratio of 5 and smoothing width of 1.

3.5.1 Univariate Analysis: One-way ANOVA

Univariate analysis methods are the most common methods used for exploratory data analysis. For multigroup analysis, MetaboAnalyst provides one-way ANOVA. As ANOVA only tells whether the overall comparison is significant or not, it is usually followed by post-hoc analyses to identify which two levels are different. MetaboAnalyst provides two most commonly used methods for this purpose - Fisher's least significant difference method (Fisher's LSD) and Tukey's Honestly Significant Difference (Tukey's HSD). The univariate analyses provide a preliminary overview about features that are potentially significant in discriminating the conditions under study. Figure 2 shows the important features identified by ANOVA analysis. Table 2 shows the details of these features. The post-hoc Sig. Comparison column shows the comparisons between different levels that are significant given the p value threshold.

Univariate One-way ANOVA is performed using Metaboanalyst Software where significant m/z features were found based on the selected p-value cut off 0.05. Metabolite putative identification of the oil palm rachis is determined by using METLIN database search functions. In addition to more than 1 million metabolites and other small molecules in the database, METLIN has incorporated tools to automate the identification process of known and unknown molecules by use of experimental MS/MS data. For example, once the m/z of a feature of interest is defined, the Simple Search menu allows users to perform an exact mass search and thus obtain putative molecules within a user-defined mass tolerance window. This search menu also offers the possibility to take into consideration different adducts of the molecule that could match the selected m/z.

Normalized m/z features from previous analysis are used for multivariate data analysis. The PCA and PLS-DA was performed with the SIMCA-P+ Software (v. 12.0, Umetrics, Umea, Sweden) using scaling based on Pareto to enhance slow abundant peaks without significant amplification of noise. The analysis conducted is based on the first two

components, will reveal the cluster distribution of metabolites detected to present in between groups.

3.5.2 Multivariate Analysis: PCA and PLSDA

In SIMCA-P+ m/z and retention time are selected as primary and secondary variable IDs respectively in order to carry out multivariate analysis. PCA is unsupervised multivariate analysis where the modelling does not involve any user intervention and is based only on the explanatory variables leaving any responses optional for later stages of data analysis. PLS-DA on the other hand is a supervised multivariate analysis where the user can predefine the groups within the samples in order to see how the underlying variables affect the separation between two groups (Trivedi & Iles, 2012).

3.5.3 Permutations Plot for PLS-DA Model

The Permutations Plot helps to assess the risk that the current PLS or PLS-DA model is spurious. The idea of this validation is to compare the goodness of fit (R2 and Q2) of the original model with the goodness of fit of several models based on data where the order of the Y-observations has been randomly permuted, while the X-matrix has been kept intact. R2 is the percent of variation of the training set-X with PCA - explained by the model. It is a measure of fit, i.e. how well the model fits the data. Q2 is the percent of variation of the training set - X with PCA - predicted by the model according to cross validation. Q2 indicates how well the model predicts new data. A large Q2 ($Q2 > 0.5$) indicates good predictivity.

The plot shows, for a selected Y-variable, on the vertical axis the values of R2 and Q2 for the original model (far to the right) and of the Y-permuted models further to the left. The horizontal axis shows the correlation between the permuted Y-vectors and the original Y-vector for the selected Y. The original Y has the correlation 1.0 with itself, defining the high point on the horizontal axis.

The criteria for validity are:

i) All blue Q2-values to the left are lower than the original points to the right.

or

ii) The blue regression line of the Q2-points intersects the vertical axis (on the left) at, or below zero.

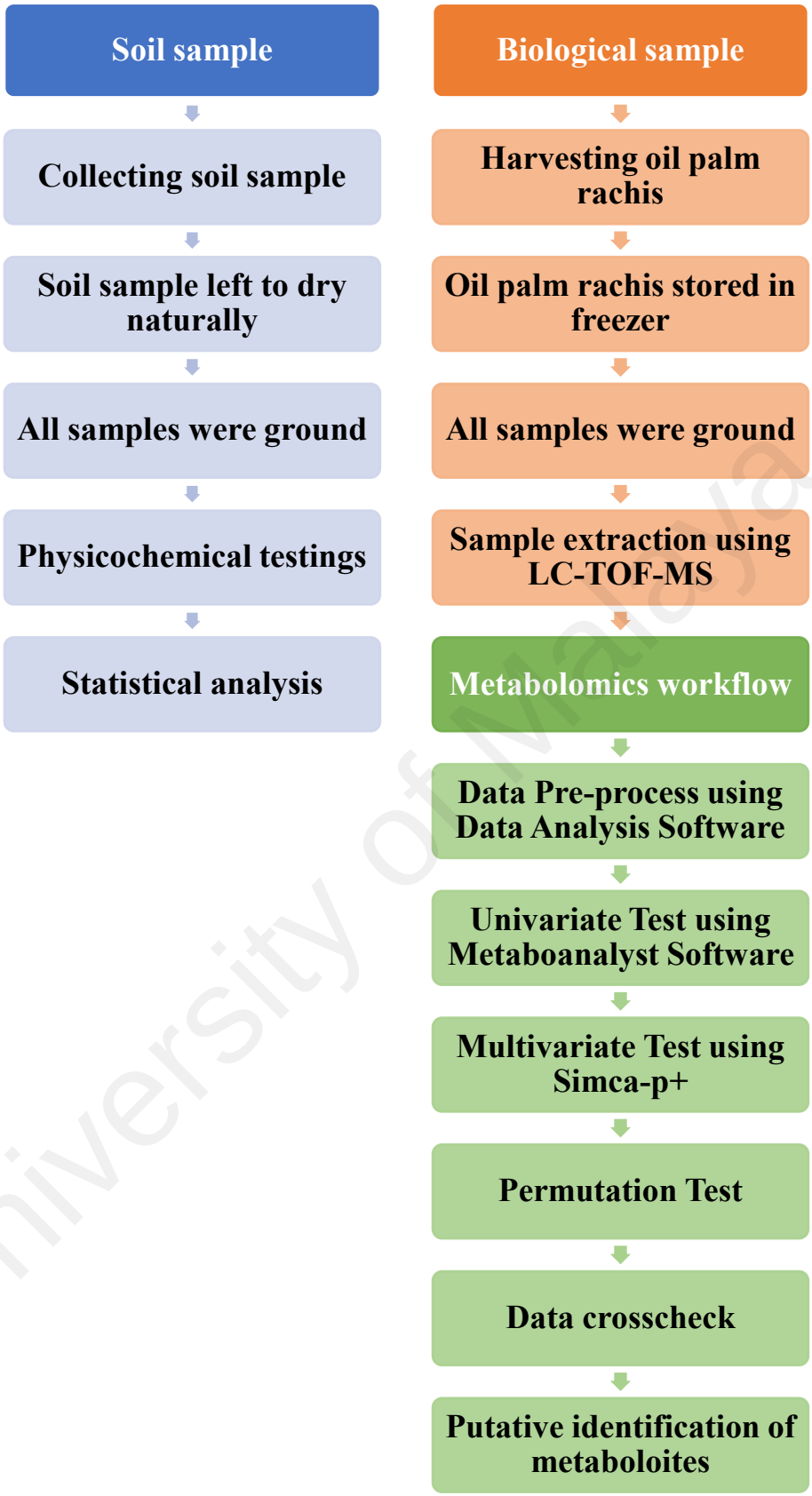


Figure 3.5: General method workflow of this research. This figure summarizes the steps used in this research.

CHAPTER 4: RESULTS

4.1 Soil Physico-chemical Properties

For analyzing the physico-chemical properties of Ultisol and Oxisol soil, four parameters were tested which are soil pH, TC, EC and texture. Soil pH, EC and TC data sets were analyzed using U-Mann Whitney since the sample size was small, n=9, and not normally distributed. The statistical data analyses were performed using SPSS version 21.0 (Spss, 2012) in between three parameters of: 1) Oxisol soil where control and infected oil palm were grown, 2) Ultisol soil where control and infected oil palm were grown and 3) Ultisol and Oxisol soil where infected oil palm were grown. The third parameter was used in order to standardize the physico-chemical properties of soil as both soil orders were collected from *Ganoderma*-infected oil palm. A significant value was set at α=0.05 and based on the findings, there is a significant difference in soil pH (p=0.017) and TC (0.001) between Ultisol and Oxisol soil while there is no significant difference in soil EC (p=0.691) between the soil orders. The results were tabulated in Table 4.1. For soil texture analysis, a chi-square test of independence revealed a significant association (p=0.000) between soil order and texture. Ultisol soil samples were classified as sandy clay while Oxisol soil samples were classified as clay. The soil particle size analysis between the groups was tabulated as in Table 4.2.

Table 4.1: U-Mann Whitney test on soil pH, EC and TC.

Parameter	Soil Order/Group		Soil Properties	Mean	Median	p-value
1	Oxisol soil	Control	pH	4.28	4.27	0.965
		Infected		4.26	4.23	
		Control	EC	130.67	123.00	0.171
		Infected		152.00	135.00	
		Control	TC	1.19	1.18	0.965
		Infected		1.23	1.11	
2	Ultisol soil	Control	pH	4.41	4.37	0.401
		Infected		4.45	4.48	
		Control	EC	167.89	168.00	0.145
		Infected		146.67	143.00	
		Control	TC	0.66	0.54	0.895
		Infected		0.58	0.55	
3	Infected	Oxisol soil	pH	4.26	4.23	0.017
		Ultisol soil		4.45	4.48	
		Oxisol soil	EC	152.00	135.00	0.691
		Ultisol soil		146.67	143.00	
		Oxisol soil	TC	1.23	1.11	0.001
		Ultisol soil		0.58	0.55	

Table 4.2: Chi-square Test of Independence on soil texture.

Texture analysis		Control						Infected					
		Oxisol soil			Ultisol soil			Oxisol soil			Ultisol soil		
		Soil depth (cm)			Soil depth (cm)			Soil depth (cm)			Soil depth (cm)		
		0-15	15-30	30-45	0-15	15-30	30-45	0-15	15-30	30-45	0-15	15-30	30-45
Rep 1	%Sand	7.15	5.60	4.89	38.81	36.74	39.40	24.48	21.87	16.49	48.87	48.30	47.99
	%Clay	75.15	85.00	86.70	47.95	51.15	48.48	54.53	57.93	65.80	36.90	43.40	40.92
	%Silt	17.70	9.40	8.41	13.24	12.11	12.13	21.00	20.21	17.71	14.23	8.30	11.08
	Total	100.00	100.00	100.00	100.00	100.00	100.01	100.01	100.01	100.00	100.00	100.00	99.99
	Texture	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Sandy Clay**	**Sandy Clay**	**Sandy Clay**
Rep 2	%Sand	7.52	6.68	6.15	42.48	33.23	35.45	5.51	3.74	3.71	48.08	46.78	47.81
	%Clay	79.02	81.88	83.07	43.30	54.40	52.23	77.35	81.90	81.60	40.80	41.28	41.25
	%Silt	13.45	11.45	10.78	14.22	12.37	12.33	17.14	14.36	14.69	11.12	11.94	10.94
	Total	99.99	100.01	100.00	100.00	100.00	100.01	100.00	100.00	100.00	100.00	100.00	100.00
	Texture	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Sandy Clay**	**Sandy Clay**	**Sandy Clay**
Rep 3	%Sand	5.56	4.83	4.76	38.10	37.11	37.44	4.18	3.94	3.79	50.21	51.36	47.51
	%Clay	81.77	83.70	83.90	48.75	51.30	50.37	76.68	78.65	74.92	37.25	37.03	39.68
	%Silt	12.67	11.47	11.34	13.15	11.59	12.19	19.15	17.41	21.28	12.54	11.62	12.81
	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.01	100.00	99.99	100.00	100.01	100.00
	Texture	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Clay**	**Sandy Clay**	**Sandy Clay**	**Sandy Clay**

4.2 Extraction Method

The three established literature reviews describing plant metabolomics protocols were chosen because the metabolite profiling of plant samples from all protocols revealed a wide range of plants sugars and phenolics such as hydrocinnamic acid derivatives, flavon-3-ols, proanthocyanidins, flavonols, sedoheptulose, procyanidin B1, pinocembrin malonyl hexoside and many other identified and unidentified secondary metabolites. From all protocols, different solvents combination and technical steps have resulted in a different number of metabolites detected by LC-TOF-MS. Thus, it is important to choose the right solvents combination when targeting certain groups of plant secondary metabolites. Table 4.3 summarized the number of base peaks of biological samples runs in LC-TOF-MS. The base peak chromatogram can be found from Appendix A.

Table 4.3: Number of base peaks from three extraction methods.

Extraction method	1		2		3	
Number of base peaks	Control	Infected	Control	Infected	Control	Infected
	138	144	416	458	541	572

4.3 Overview on PCA and PLS-DA Models

The PCA score plot of the two first PCs of the metabolites datasets provides a map of how the samples relate to each other. In the t/t, score plot (Figure 4.1) the four categories of biological samples are not well separated. The model shows that the samples of healthy oil palm rachis from both Ultisol and Oxisol soil cluster in groups near the origin. Group contains samples of *Ganoderma*-infected oil palm rachis from Ultisol soil cluster in the upper right-hand quadrant while group contains samples from Oxisol soil cluster in the lower left-hand quadrant (except for IU3).

Figure 4.2 and 4.3 show the t/t, score plot and loading plots of the PLS-DA model. The score plot is a summary of the relationships among the observations (biological samples) and the loading plot a similar summary of the variables (plant metabolites). The loading plot is a means to interpret the patterns seen in the score plot. The two plots are complementary and superimposable, and a direction in one plot corresponds to the same direction in the other plot. On the other hand, to validate the PLS-DA model, the results of the permutation tests (after 200 permutations) of the two-component model are plotted in Figure 4.4.

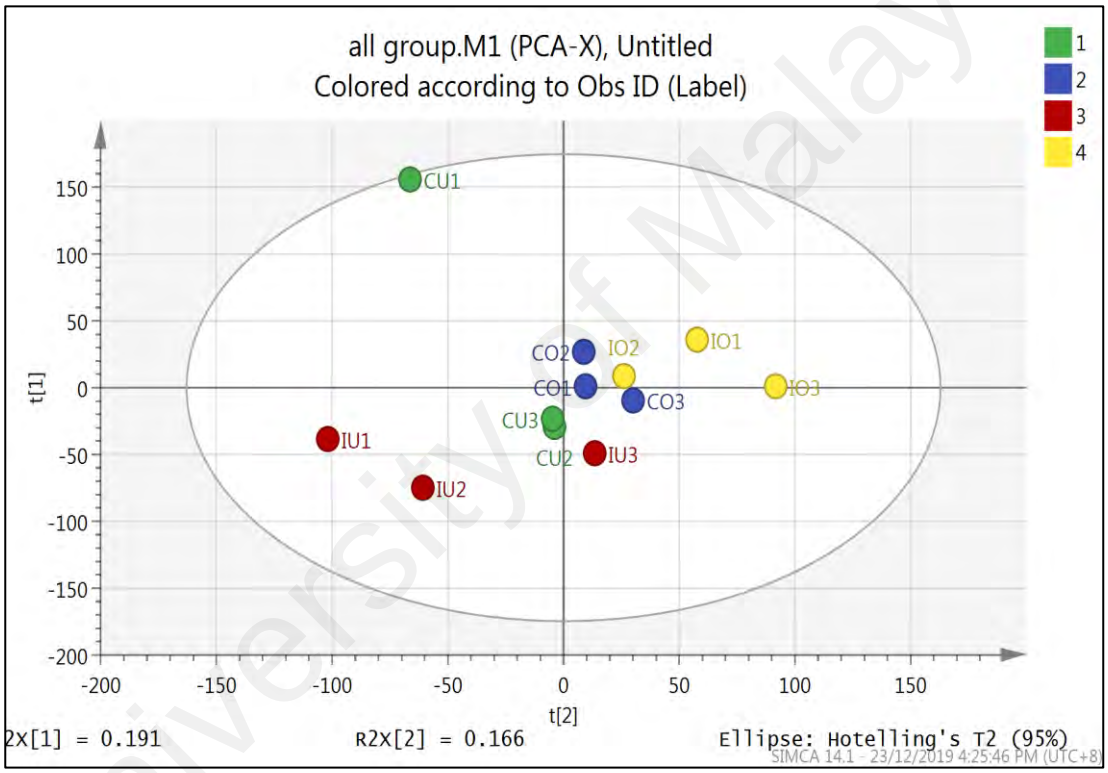


Figure 4.1: PCA score plot of the two first PCs of the biological sample datasets of control Ultisol (CU), infected Ultisol (IU), control Oxisol (CO) and infected Oxisol (IO).

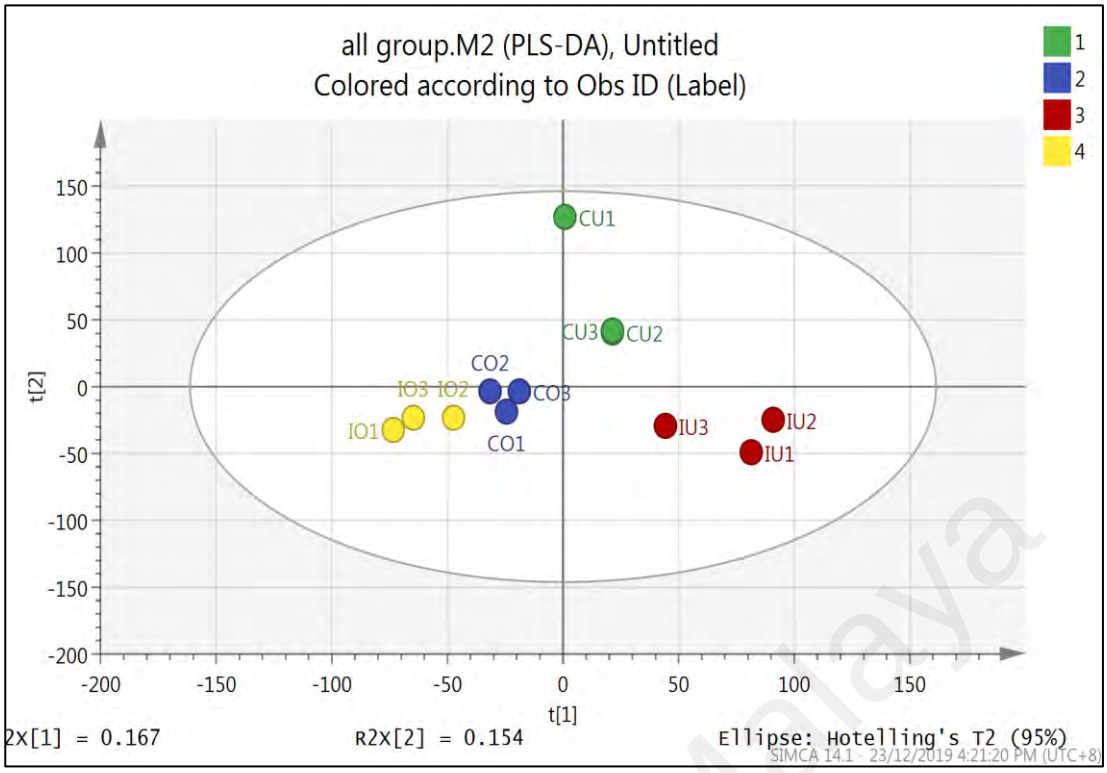


Figure 4.2: PLS-DA score plot containing biological replicates of control Ultisol (CU), infected Ultisol (IU), control Oxisol (CO) and infected Oxisol (IO). The four classes of biological samples are clearly discriminated.

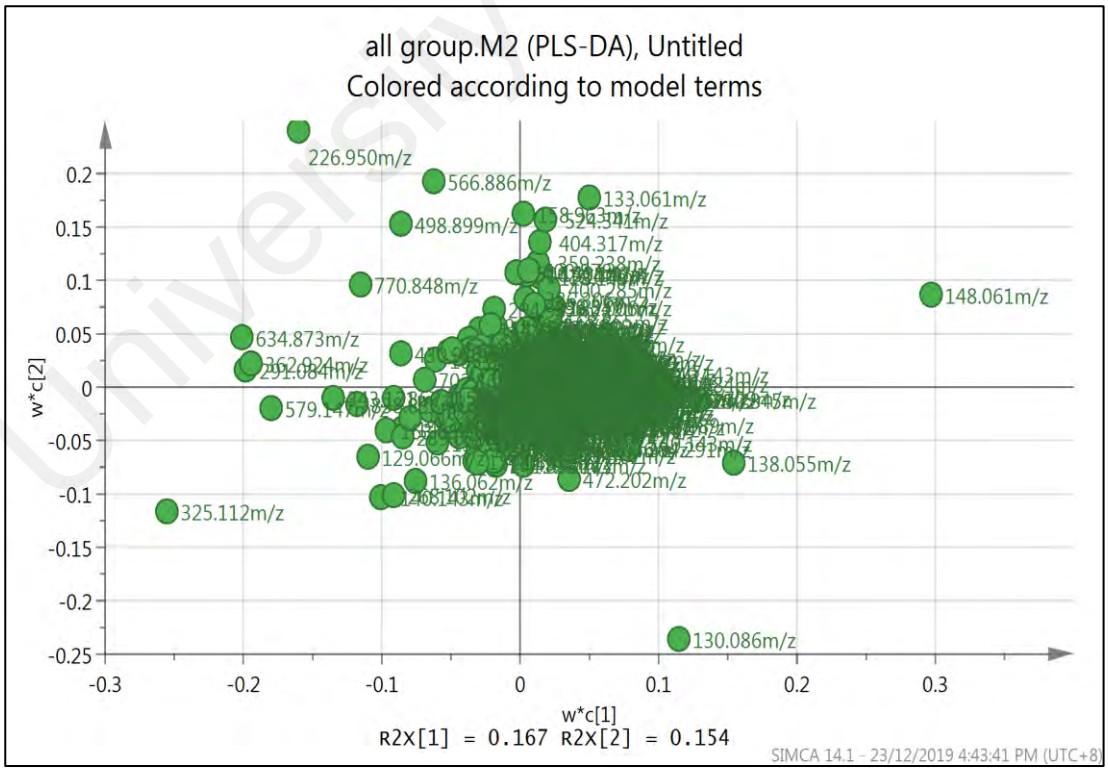


Figure 4.3: PLS-DA loading plot corresponds to the score plot of the PLS-DA model.

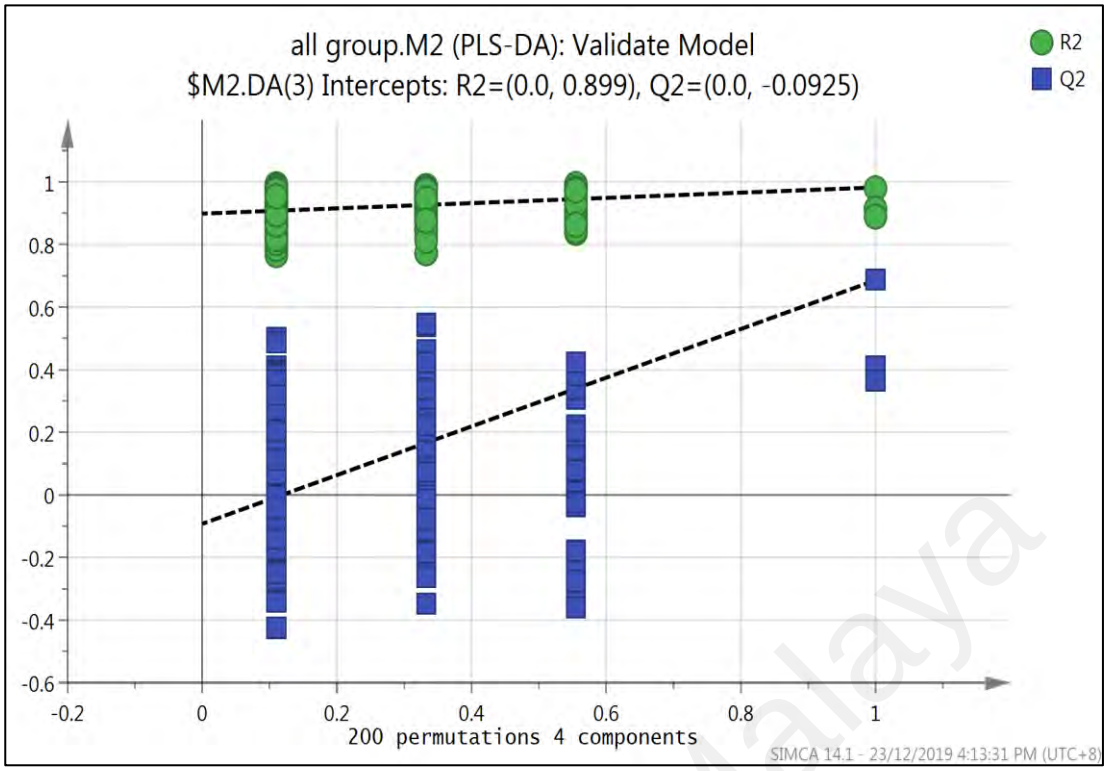


Figure 4.4: Permutation test to validate PLS-DA model.

4.4 Profiling Potential Metabolites

Prior to ANOVA test, list of VIP values had been identified. Figure 4.5 shows the VIP values more than 2.00. The sum of squares of all VIP's is equal to the number of terms in the model. Hence, the average VIP is equal to 1. Larger VIP-values indicate "important" X-variables, and lower values indicate "unimportant" X-variables. The VIP plot is sorted from high to low, and shows confidence intervals for the VIP values, normally at the 95% level. Among all potential biomarker metabolites, those with VIP more than 2.00 and p-value of ANOVA less than 0.05 are shown in Table 4.4. The post-hoc Sig. Comparison column shows the comparisons between different levels that are significant given the p-value threshold. From the potential biomarkers, 12 has been putatively identified using metlin and their general informations were tabulated as in Table 4.5.

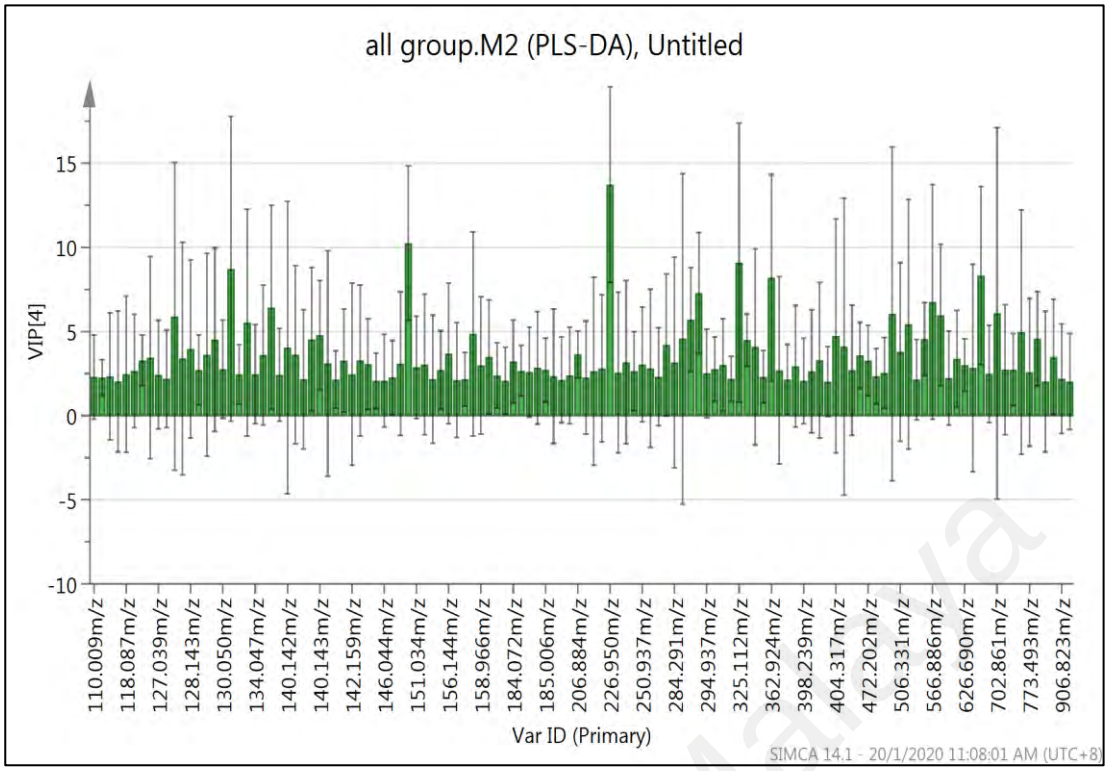


Figure 4.5: VIP values more than 2.00 identified from ANOVA test.

Table 4.4: List of potential biomarker metabolites.

No.	Peaks (mz/rt)	f.value	p.value	-log10(p)	FDR	Tukey's HSD
1	0.27min 634.873m/z	5.9394	0.019664	1.7063	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
2	1.25min 250.937m/z	6.194	0.017583	1.7549	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
3	1.62min 185.006m/z	4.6337	0.036827	1.4338	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
4	1.69min 212.102m/z	4.4051	0.041542	1.3815	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
5	1.73min 129.066m/z	7.6414	0.009812	2.0082	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
6	1.75min 148.061m/z	26.494	0.000166	3.7807	0.086076	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
7	1.78min 132.066m/z	33.361	7.16E-05	4.1448	0.045865	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
8	1.78min 295.111m/z	7.4893	0.010393	1.9833	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
9	1.80min 184.072m/z	8.9966	0.006077	2.2163	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
10	1.85min 343.121m/z	24.355	0.000224	3.6498	0.086076	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
11	1.95min 472.202m/z	5.5489	0.023489	1.6291	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
12	1.96min 130.086m/z	12.791	0.002024	2.6939	0.46265	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
13	14.56min 156.144m/z	8.2236	0.007931	2.1007	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
14	17.41min 153.138m/z	5.2277	0.027353	1.563	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
15	2.47min 146.044m/z	19.38	0.000501	3.3005	0.16024	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
16	2.56min 321.960m/z	4.74	0.034863	1.4576	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3
17	8.35min 579.147m/z	4.7313	0.035018	1.4557	0.65139	2-1; 3-1; 4-1; 3-2; 4-2; 4-3

Table 4.5: List of putatively identified metabolites.

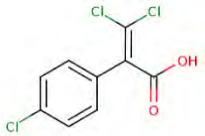
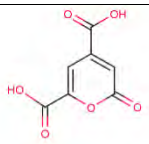
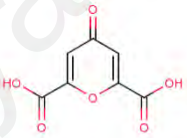
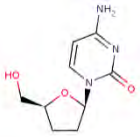
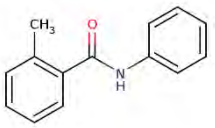
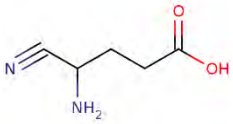
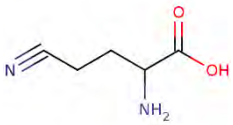
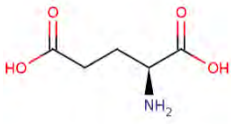
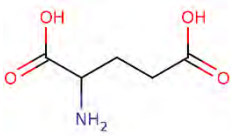
No.	m/z	Exact mass	Name	Formula	Structure
1	250.937	249.9355	2-(4'-Chlorophenyl)-3,3-dichloropropenoate	C9H5Cl3O2	
2	185.006	184.0008	2-Pyrone-4,6-dicarboxylate	C7H4O6	
	185.006	184.0008	Chelidonic acid	C7H4O6	
3	212.102	211.0957	Zalcitabine	C9H13N3O3	
	212.102	211.0997	Mebenil	C14H13NO	
	129.066	128.0586	4-Amino-4-cyanobutanoic acid	C5H8N2O2	
	129.066	128.0586	2-Amino-4-cyanobutanoic acid	C5H8N2O2	
5	148.061	147.0532	L-Glutamate	C5H9NO4	
	148.061	147.0532	Glutamate	C5H9NO4	

Table 4.5, continued.

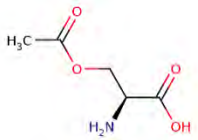
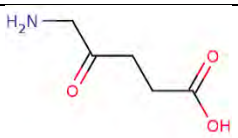
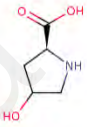
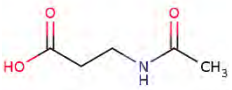
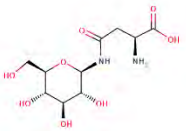
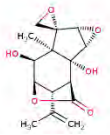
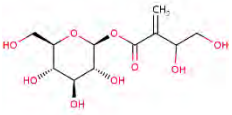
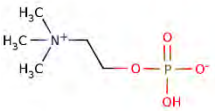
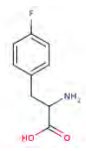
	148.061	147.0532	O-Acetyl-L-serine	C5H9NO4	
6	132.066	131.0582	5-Aminolevulinate	C5H9NO3	
	132.066	131.0582	L-Allohydroxyproline	C5H9NO3	
	132.066	131.0582	N-Acetyl-beta-alanine	C5H9NO3	
7	295.111	294.1063	N-Glycosyl-L-asparagine	C10H18N2O8	
	295.111	294.1103	Tutin	C15H18O6	
	295.111	294.0951	Tuliposide B	C11H18O9	
	184.072	184.0739	Choline phosphate	C5H15NO4P	
	184.072	183.0696	p-Fluorophenylalanine	C9H10FNO2	

Table 4.5, continued.

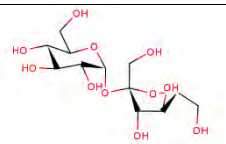
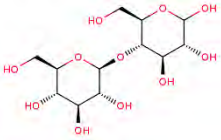
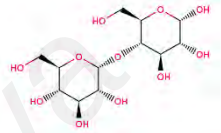
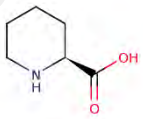
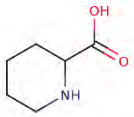
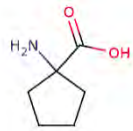
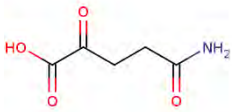
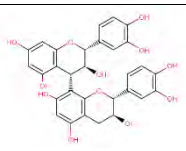
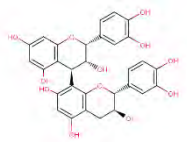
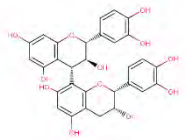
9	343.121	342.1162	Sucrose	C12H22O11	
	343.121	342.1162	Cellobiose	C12H22O11	
	343.121	342.1162	Maltose	C12H22O11	
10	130.086	129.079	L-Pipecolate	C6H11NO2	
	130.086	129.079	DL-Pipecolate	C6H11NO2	
	130.086	129.079	Cycloleucine	C6H11NO2	
11	146.044	145.0375	2-Oxoglutaramate	C5H7NO4	
	146.044	145.0375	4-Oxoglutaramate	C5H7NO4	
12	579.147	546.1526	Procyanidin B3	C30H26O10	

Table 4.5, continued.

	579.147	578.1424	Procyanidin B1	C30H26O12	
	579.147	578.1424	Procyanidin B4	C30H26O12	

CHAPTER 5: DISCUSSION

5.1 Soil Physico-chemical Properties

This research carried out revealed that the physicochemical properties of soil such as soil TC, texture and pH between Ultisol and Oxisol soil were significantly different. The results suggested that these soil properties might affect the distribution of metabolites between the different types of soil orders. Soils affect plant activities by supplying organic matter and play a vital role in weathering rocks and minerals. Many plant species have a distinct ecological amplitude that shows restriction to specific soil types. In the numerous interactions between plants and soil, microorganisms also play a key role (Lambers et al., 2009). The abundance of microorganisms present in the soil is critical for decomposing organic residues and recycling soil nutrients (Hoorman, 2010). Soil organisms decomposed the organic matter and make some of the nutrients available for plants and secreted glue-like substances that bound soil particles together which improved soil structure. The improved soil structure allowed the root growth and movement of air and water through the soil (Shen & Yang, 2008).

This research reported that the TC content in Oxisol soil was significantly higher compared to Ultisol soil. The TC content is the sum of organic, inorganic and elemental carbon. Carbon is the main element present in soil organic matter which makes up about 58% by weight (Bianchi et al., 2008). As part of soil TC, organic carbon is a vital component of productive agriculture which measures carbon content within the soil organic matter (Schumacher, 2002). The amount and type of organic matter in soils played a critical role in the extent to which a plant was affected by certain diseases. In some cases, organic matter may increase the incidence and severity of a disease caused by fungi as well as other microbes. Previous research found that organic matter and nutrients in coffee industrial residue influenced Cassava Root Rot disease caused by soil-borne fungus *Fusarium solani*. The incorporation of organic matter in the soil generated the effect of volatile compounds that killed the fungus in the soil and resulted in the suppression of the soil-borne pathogens (Silva et al., 2017).

Another research found that the soil organic matter influenced the severity of hazelnut Cytospora Canker disease caused by a fungus known as *Cytospora corylicola*. The research also found a strong negative correlation between soil organic matter and the disease severity index (Lamichhane et al., 2014).

This research also reported that soil pH in Oxisol soil was more acidic compared to Ultisol soil that was less acidic. Soils acidify naturally as they weathered over millions of years. The acidity of any soil varies according to the type of rock it came from, the length of time it had weathered and the local climate. As a result, some soils could be naturally very acidic while others were more alkaline (Fageria & Nascente, 2014). Soil pH was another important factor that influenced the development of soil-borne diseases. One of the examples was the Clubroot disease which is caused by the obligate parasite *Plasmodiophora brassicae Woronin*. This disease is one of the most serious soil-borne diseases of cruciferous crops and a major problem in highly acidic soil. The research has found that the resting spore germination of the *P. brassicae* was higher at low pH values than at high pH values (Rashid et al., 2013). Another research found that the pH of medium affecting the growth of *G. boninense*, the same pathogen studied in this research. Abundant mycelial growth was found to be in the pH range of 3.7 to 5 and as the acidity of the medium decreased its growth became poor (Nawawi & Ho, 1990). Recently, another research illustrated the impacts of soils with different chemical compositions where the soil pH was reported to be associated with about 50% of disease severity index and necrotic bole tissues due to *Ganoderma* sp. (Goh et al., 2017). These findings were on par with this research that revealed the same result where both soil orders were found to be acidic. This factor could be one of the reasons *Ganoderma* sp. infection to happen as the acidic pH environment is favorable for the soil-borne fungus to live.

Even though there is no significant difference between soil EC of Ultisol and Oxisol soil, the mean value of soil EC in Oxisol soil was reported to be slightly higher compared to Ultisol soil. The distribution of water within soils played a crucial role in governing fungal

development and activity (Ritz & Young, 2004). The EC of soil was influenced by the concentration and composition of dissolved salts. Salts increased the ability of a solution to conduct an electric current, so a high EC value indicated a high salinity level (Slinger & Tenison, 2007). Little was known about how salinity stress in the plant might affect its susceptibility to pathogens. One previous research elucidated the effects of water salinity and *Fusarium oxysporum f. sp. lycopersici* on tomato growth. The research reported that the increase in salinity stress to the inoculated tomato plants enhanced the severity of Fusarium wilt disease and resulted in a significant increase in the leaf damage index recorded from 35 to 62 days post-planting (Daami et al., 2009).

5.2 Extraction Method

The research found that the solvent combination of chloroform, methanol and water resulted in lower number of peaks detected compared to the solvent combination of methanol and water. Extraction is the first step to separate the desired natural products from the raw materials. The selection of the solvent is crucial for solvent extraction. Many solvents, including methanol, ethanol, acetone, and water, have been used for extracting bioactive compounds from the plant material. Due to the variety of bioactive compounds contained in plant materials and their differing solubility properties in different solvents, the optimal solvent for extraction depends on the plant materials and the compounds that are to be isolated (Truong et al., 2019). Most study reported that methanol was the best solvent for extracting bioactive compounds from the plant since it resulted in the highest extraction yield and the highest content of phenolics, alkaloids, flavonoids, and terpenoids. This could be because the plant material contains high levels of polar compounds that are soluble in solvents with a high polarity such as water and methanol (Zhang et al., 2018).

The polarity, from least polar to most polar, among the three solvents used in this research, is as follows: Chloroform < Methanol < Water. Mostly methanol is used for extracting various polar compounds, but a certain group of non-polar compounds is soluble in methanol if not

readily soluble. Therefore, methanol is commonly used for the extraction of bioactive compounds (Altemimi et al., 2017). Water is called the "universal solvent" because it can dissolve more substances than any other liquid. It is water's chemical composition and physical attributes that make it such an excellent solvent. Water molecules have a polar arrangement of oxygen (negative charge) and hydrogen (positive charge). This allows the water molecule to become attracted to many other different types of molecules (Sharp, 2001).

The other parameter needed for extracting more secondary metabolites in plants is the additional step of homogenization method using ultrasonication bath. Ultrasonication bath facilitates the extraction process of metabolites from the plant by generating high shear forces and microbubbles that enhances surface erosion, fragmentation and mass transfer. This will result in high yield of extracted metabolites and fast rate of extraction (Awad et al., 2012). Some researchers have reported on the combination of aqueous methanol and ultrasonication bath for better metabolomic analysis in the plant sample which is on par with the findings of this study (Annegowda et al., 2012; Awad et al., 2012).

5.3 Overview on PCA and PLS-DA Models

Classification and discriminant analysis are an important area of multivariate data analysis. Multivariate data analysis methods can utilize the information in highly multicollinear data for exploring within-class similarity and between-class diversity. This is of relevance in the area of spectrometry and 'omics' data analysis such metabolomics where the number of variables can be very large and the correlation among them substantial. In PCA the scores plot shows correlations between observations. When PCA results in clustering of observations, it is sometimes worth trying to further resolve such groupings by means of PLS-DA where a dummy matrix of three Y-variables expressing class identity of the biological samples was created. In PLS-DA, the number of model classes must not be too high. Experience shows that PLS-DA is useful with 2-4 classes, but when the number of classes exceeds four, discrimination results may become incomprehensible and difficult to overview.

Prior to the data analysis, the 12 biological samples were log-transformed in order to obtain a more normal distribution of the data. From Figure 4.1, the PCA modelling yielded a two-component model with no outliers as all observations situated inside the ellipse. The first component explains 19% (Q2X = 0.19) of the variation and the second component 17% (R2X = 0.17). The PCA score plot shows groups thus it is advisable to understand the differences between the groups by doing a PLS-DA. PLS-DA was then performed, which gave a four-component model with R2X[1] = 0.17 and R2X[2] = 0.15. The ellipse for each group represented Hotelling's T2 95% confidence interval. In PLS-DA, score plots of the t/t-type are interesting because one wants to overview the class discriminating ability of a developed model.

As shown by the Figure 4.2, the four classes of biological samples are clearly separated and displayed the relationships between all 12 variables at the same time. Variables contributing similar information are grouped: that is, they are correlated. CU2 and CU3 are examples of variables that are positively correlated. When the numerical value of one variable increase or decrease, the numerical value of the other variable tends to change in the same way. The variables IU1 and IO1 are inversely correlated, meaning that when IU1 increases, IO1 decreases, and vice versa. These variables are negatively or inversely correlated as they are positioned on opposite sides of the plot origin, in diagonally opposed quadrants. Furthermore, the distance to the origin also conveys information. The further away from the plot origin a variable lies, the stronger impact that variable has on the model. This means, for instance, that the variables CU1 separate the groups from the others. Moreover, the model interpretation suggests that biological samples like CO2 and IO2 share some metabolites. Biological samples close to each other have similar properties whereas those far from each other are dissimilar concerning metabolites distribution profiles. Biological samples of IU1, IU2 and IU3 are located together in the lower right-hand corner, this representing a group of to the center (origin) of the plane, which indicates that they have average properties.

From Figure 4.3, the R2 value is 0.899 while the Q2 value is -0.0925. This model fits the criteria for validity, in which all blue Q2-values to the left are lower than the original points to the right and the blue regression line of the Q2-points intersects the vertical axis at below zero. The R2-values always show some degree of optimism. All green R2-values to the left are lower than the original point to the right, this is also an indication for the validity of the original model. The large R2 (close to 1) is a necessary condition for a good model, but it is not enough. Poor models may occur (models that cannot predict) even with a large R2. A poor R2 arise when there is poor reproducibility (much noise) in the training data set, meanwhile a poor Q2 arise when the data have much noise, or when the model is dominated by a few scattered outliers.

5.4 Profiling Potential Metabolites

Biomarker discovery is the critical step for metabolomics studies. Selection of the informative metabolites is of great importance for metabolic pathways analysis and biological interpretation. In PLS-DA model, potential biomarker candidates were selected based on values of VIP of all variables. The VIP value of each variable in the model was calculated to indicate the most appropriate combination of metabolites which can produce an effective prediction power and indirectly reflects the correlation of metabolites with plant disease. A higher VIP value represents a stronger contribution to classification between groups. From all 17 significant m/z values (representing metabolites present in biological samples grown in Ultisol soil), six m/z values were identified to increase significantly in metabolite peak intensity. Their respective m/z values are 212.102, 148.061, 184.072, 472.202, 130.086 and 321.960. As for biological samples from Oxisol soil, 11 m/z values increased significantly, namely 185.006, 159.066, 295.111, 184.072, 343.121, 472.202, 130.086, 153.138, 146.044, 321.960 and 579.147. The rest m/z values reported decreasing significantly in metabolites intensity (refer to appendix C for the increase and decrease of the peak intensity of putatively identified metabolites where the bar plots on the left show the original values (mean +/-sd) and the box and whisker plots on the right summarize the normalized values). Two m/z values

of biological samples from Oxisol soil (184.072 and 146.0440) were recorded to increase significantly in *Ganoderma*-infected oil palm rachis and at the same time absent in its healthy samples. The plant metabolites were then putatively identified as choline phosphate or p-fluorophenylalanine and 2-oxoglutaramate or 4-oxoglutaramate. Another interesting finding includes, the m/z values of biological samples from Oxisol soil increased significantly and at the same time absent in biological samples from Ultisol soil, namely 129.066, 132.066 and 146.044. This indicates that there are metabolites responsible for the stress mechanism present in plants grown on the different types of soil orders. The three plant metabolites were putatively identified as 4-Amino-4-cyanobutanoic acid or 2-Amino-4-cyanobutanoic acid, 5-Aminolevulinate or L-Allohydroxyproline or N-Acetyl-beta-alanine and 2-oxoglutaramate or 4-oxoglutaramate.

5-Aminolevulinic acid (ALA) is a common precursor of tetrapyrroles as well as a crucial growth regulator in higher plants. ALA has been proven to be effective in improving photosynthesis and alleviating the adverse effects of various stresses in higher plants. ALA also increased plant tolerance to low-temperature stress, but the physiological and biochemical mechanisms that underlie its effects are not fully understood. A research suggested that the up-regulation of antioxidant enzyme activities, nutrient contents, and hormone accumulation with the application of ALA increases tolerance to low-temperature stress. Chlorophyll biosynthesis pathway enhanced by exogenous ALA also improved the tolerance of plant under salinity (Wu et al., 2018).

Although underappreciated, the non-proteinogenic amino acid β-alanine has important roles in plant physiology and metabolism. This compound acts as a defense compound that enables plants to withstand various stresses and as a precursor to the other compounds which are involved in a variety of functions. Furthermore, the amino acid is converted into β-alanine betaine, which has additional protective functions such as salt tolerance and homoglutathione that are critical for nitrogen fixation (Parthasarathy et al., 2019).

Plant glutamate metabolism (GM) plays a pivotal role in amino acid metabolism and orchestrates crucial metabolic functions, with key roles in plant defense against pathogens. These functions concern three major areas: nitrogen transportation via the glutamine synthetase and glutamine-oxoglutarate aminotransferase cycle, cellular redox regulation and tricarboxylic acid cycle-dependent energy reprogramming. During interactions with pathogens, the host GM is markedly altered leading to either a metabolic state where cell viability is maintained or to an opposite metabolic state where the process of cell death is facilitated. Collectively, alterations in the host GM in response to different pathogenic scenarios appear to function in two opposing ways, either backing the ongoing defense strategy to ultimately shape an efficient resistance response or being exploited by the pathogen to promote and facilitate infection (Seifi et al., 2013).

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

Soil physicochemical properties analyzed using U-Mann Whitney revealed that the physico-chemical properties of Ultisol and Oxisol soil are different at pH and TC level but not for EC level. A chi-square test of independence revealed that there is significant association between soil order and texture where the Ultisol soil was classified into sandy clay while Oxisol soil was classified into clay. For biological sample analysis, the initial PCA model revealed weak groupings among the four categories of biological samples. It was found that the CU1 reflected the level of cultural disturbance in the biological samples. Furthermore, it was concluded that there was a difference between two main groups of metabolite profiles between healthy and *Ganoderma*-infected oil palm tree rachis samples. In the last modelling stage, PLS-DA was attempted. A strongly significant PLS model was acquired, indicating that the 12 biological variables indeed contained class separating information. The separation of the four classes was slightly superior compared with the previous PCA modelling attempts. This model also fits the criteria for validity in permutation test. All blue Q2-values to the left are lower than the original points to the right and the blue regression line of the Q2-points intersects the vertical axis at below zero. From the model, 17 potential biomarker panels with VIP more than 2.00 and p-value of ANOVA less than 0.05 were identified. Two m/z value of biological samples from Oxisol soil (184.072 and 146.0440) were recorded to increase significantly in *Ganoderma*-infected oil palm rachis and at the same time absent in its healthy samples. The plant metabolites were putatively identified as choline phosphate or p-fluorophenylalanine and 2-oxoglutaramate or 4-oxoglutaramate. Another interesting finding includes, three m/z value of biological samples from Oxisol soils increased significantly and at the same time absent in biological samples from Ultisol soil (129.066, 132.066 and 146.044). The three plant metabolites were putatively identified as 4-Amino-4-cyanobutanoic acid or 2-Amino-4-cyanobutanoic acid, 5-Aminolevulinate or L-Allohydroxyproline or N-Acetyl-beta-alanine and 2-oxoglutaramate or 4-oxoglutaramate. This indicates that there are

metabolites responsible for the stress mechanism present in plants grown on the different types of soil orders.

6.2 Suggestions for Improvement

One of the major bottle-neck in LC-MS based metabolomics investigations is metabolite identification. Future work must focus on metabolite identification using a suitable approach such as computer-assisted metabolite identification, ion annotation using peak intensity correlation across LC-MS runs in addition to correlations between extracted ion chromatograms and spectral libraries that provide high-resolution ESI-MS/MS spectra that are useful for spectral matching. To understand how plants are affected by a specific biotic or abiotic stress or combinations thereof, the choice of appropriate growth conditions requires more careful attention. It is suggested that the research is repeated using biological samples grown under controlled environment conditions in greenhouses. For scientists who study a given plant species under optimal growth conditions, a simple preliminary experiment exploring various light, temperature and nutrient conditions will provide proper environmental conditions for further experimentation. Another important issue is the nature and an appropriate number of biological, technical and analytical replicates. Biological replication is significantly more important than technical replication and it is suggested to involve more replicates to ensure the robustness of the metabolite data obtained.

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