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RAPID DETECTION OF Ganoderma-INFECTED OIL PALMS BY MICROWAVE ERGOSTEROL EXTRACTION

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By

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RAPID DETECTION OF *Ganoderma*-INFECTED OIL PALMS BY MICROWAVE ERGOSTEROL EXTRACTION

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Chairman : Associate Professor Zainal Abidin Mior Ahmad, PhD
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Basal stem rot (BSR) is a devastating disease to oil palm in Malaysia. Detection of BSR was based on foliar symptoms and presence of *Ganoderma* fruiting body. Enzyme-Linked Immunosorbent Assays-Polyclonal Antibody (ELISA-PAB) and Polymerase Chain Reaction (PCR) has been proposed as an early detection method for the disease. However, these techniques are complex, time consuming and have accuracy limitations. In plantations, census needs to be carried out regularly at 6 months interval. Therefore, a robust, rapid and reliable early detection method is highly desirable for large scale BSR monitoring. An ergosterol method was developed, whereby it correlated well with the degree of infection in oil palm. This current study was designed to develop a simpler, rapid and efficient ergosterol detection method with utility in the field using microwave assisted extracted (MAE) method. Pure *Ganoderma* mycelium (isolated from Gua Musang Felda) was used for optimizing the extraction method with different microwave settings based on microwave power level, temperature and combination of microwave power level and temperature available on the microwave model (Sharp Jet Convectional Grill, model TTAG A437 with capacity 1.5 cu. ft) with three different exposure time (10, 20 and 30 seconds). The microwave setting with highest ergosterol concentration extracted which is the combination of 70ºC and medium high (MH) power with 30 seconds exposure time was used for the subsequent experiment involving *Ganoderma* inoculated germinataed oil palm seeds, seedlings and infected field palms for validation of the protocol and also development of semi-quantitative thin layer chromatography (TLC) method. The extraction procedure involves extracting a small amount of *Ganoderma* mycelium and *Ganoderma*-infected oil palm stem tissues (0.4g and 1.0g respectively) suspended in low volumes of solvent followed by irradiation in a conventional microwave oven with the best combination of 70°C and MH power for 30 seconds, resulting in simultaneous extraction and saponification. Ergosterol was detected in all infected samples and not detected in healthy samples by thin layer chromatography

and high performance liquid chromatography with diode array detection. Healthy samples was describe as palms apparently normal and free from disease compared to infected palms that showed appearance of foliar symptoms and fruiting body at the base of palm trunk or on the trunk of the fallen palm and also at palm stump. The method was particularly effective in extracting high yields of ergosterol (22.65 µg g^{-1}) from infected oil palm and enables rapid analysis of field samples on site allowing infected oil palms to be treated or culled very rapidly. Positive relationship was observed between ergosterol content and inoculation period, where ergosterol concentration increase directly with the increase of inoculation period from day 3 to week 28 (1.04µg g^{-1} and 28.22µg g^{-1} respectively) in the inoculated oil palm seedlings. In germinated seeds, ergosterol concentration also increase with the increase in the inoculation period from 6 hours to 168 hours after inoculation (0.96 µg g^{-1} and 8.24 µg g^{-1} respectively) which means the *G. boninense* mycelial mass colonizing the roots of the palms also increased. Ergosterol was detected as early as day three after inoculation in diseased oil palm seedlings. These results indicated the early establishment of *G. boninense* in the root tissues. This present study did not wash or surface sterilizes the roots before extraction of ergosterol, therefore ergosterol can be detected earlier on the root tissues once the fungus attached to the root tissue surface. The result also showed that external disease severity can be observed as early as week eight after inoculation. As disease progresses, palms show a pale appearance, retarded growth and finally death. Besides that, TLC analysis also showed a good correlation with the high performance liquid chromatography (HPLC) quantification where the intensity of the spot produce by TLC analysis showed high intensity of spot for higher ergosterol concentration quantified by HPLC and vice versa. Therefore, a semi-quantitative TLC analysis can be applied for handling large amount of samples during field survey. Based on the present work, it can be concluded that MAE is an efficient method to be used in ergosterol detection where ergosterol acts as biomarker for detection of BSR disease. Besides, TLC analysis can be used for detection of ergosterol in field palms, as it is easier and can be carried out on site and suitable for large field survey during the census.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAEDAH PANTAS MENGESAN KELAPA SAWIT YANG DIJANGKITI DENGAN *Ganoderma* MENGGUNAKAN KAEDAH EKTRAK GELOMBANG KETUHAR

Oleh

MUNIROH BINTI MD SAAD

Januari 2015

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Reput pangkal batang (BSR) adalah penyakit yang merbahaya dalam industri kelapa sawit di Malaysia. Pengesanan penyakit BSR kelapa sawit yang disebabkan oleh *Ganoderma* adalah berdasarkan simtom pada daun dan pengeluaran jasad berbuah. Enzyme-Linked Immunosorbent Assays-Polyclonal Antibody (ELISA-PAB) dan polymerase chain reaction (PCR) telah dicadangkan sebagai kaedah pengesanan awal penyakit ini. Walaubagaimanapun, teknik-teknik ini adalah rumit, memerlukan masa yang lama dan mempunyai had-had ketepatan. Di ladang, bancian perlu dijalankan secara berkala dalam tempoh setiap 6 bulan. Oleh itu, kaedah pengesanan awal yang mudah, pantas dan berkesan adalah sangat wajar diaplikasikan untuk pemantauan penyakit BSR pada skala besar. Satu kaedah pengesanan ergosterol telah dibangunkan yang berkait rapat dengan tahap jangkitan *Ganoderma* pada kelapa sawit. Kajian semasa ini telah direka bertujuan untuk membangunkan kaedah pengesanan ergosterol yang lebih ringkas, cepat, dan efisien dengan kemudahan di ladang menggunakan kaedah ekstrak ketuhar gelombang mikro (MAE). Miselium tulen *Ganoderma* telah digunakan untuk mengoptimumkan kaedah pengekstrakan dengan menggunakan tetapan ketuhar gelombang mikro yang berbeza berdasarkan kuasa dan suhu gelombang mikro dan juga kombinasi kuasa dan suhu ketuhar gelombang mikro yang terdapat pada ketuhar gelombang mikro model (Sharp Jet Convectional Grill, model TTAG A437 dengan saiz 1.5 cu. ft) . Tetapan ketuhar gelombang mikro yang menghasilkan kepekatan ergosterol tertinggi iaitu kombinasi 70ºC dan kuasa sederhana rendah, dipanaskan selama 30 saat telah digunakan untuk eksperimen berikutnya yang melibatkan inokulasi *Ganoderma* pada benih bercambah, anak benih kelapa sawit, dan tisu kelapa sawit untuk mengesahkan protocol dan juga pembangunan kaedah separa- kuantitatif thin layer chromatography (TLC). Prosedur pengekstrakan ini melibatkan pengekstrakan miselium

Ganoderma dan tisu batang kelapa sawit yang dijangkiti *Ganoderma* dalam kuantiti yang sedikit (0.4g dan 1.0g masing-masing). Tisu tersebut direndam di dalam metanol dengan aruhan sinaran di dalam ketuhar gelombang mikro konvensional dengan kombinasi 70°C dan kuasa sederhana tinggi selama 30 saat, menghasilkan pengekstrakan dan saponifikasi berlaku secara serentak. Ergosterol dikesan dalam semua sampel yang dijangkiti dan tidak dikesan pada sampel yang tidak dijangkiti dengan menggunakan teknik kromatografi TLC dan high performance liquid chromatography (HPLC) diikuti pengesanan pelbagai diod. Sampel yang tidak dijangkiti penyakit adalah sampel yang kelihatan normal dan bebas dari penyakit, berbanding kelapa sawit yang dijangkiti penyakit yang menunjukkan gejala pada daun dan pembentukan jasad berbuah pada dasar batang kelapa sawit atau pada batang kelapa sawit yang tumbang dan juga pada tunggul kelapa sawit. Kaedah ini berkesan dalam mengekstrak ergosterol dengan kepekatan yang tinggi (22.65 µg g^{-1}) daripada tisu kelapa sawit yang dijangkiti dan ini membolehkan sampel ladang dianalisis lebih pantas dan membenarkan kelapa sawit yang dijangkiti penyakit dirawat dengan cepat. Hubunagan yang positif dapat dilihat antara kandungan ergosteerol dan masa inokulasi, di mana kepekatan ergosterol meningkat selari dengan peningkatan masa inokulasi daripada hari ke tiga hingga minggu ke lapan selepas inokulasi (masing-masing 1.04µg g^{-1} dan 28.22µg g^{-1}) pada anak benih kelapa sawit. Pada anak benih bercambah kelapa sawit, kepekatan ergosterol juga meningkat selari dengan peningkatan masa inokulasi daripada enam jam hingga 168 jam selepas inokulasi (maing-masing 0.96 µg g^{-1} dan 8.24 µg g^{-1}) bermaksud jumlah miselium *G. boninense* yang meliputi akar sawit juga meningkat. Ergosterol dapat dikesan seawal hari ke tiga selepas di inokulasi pada benih kelapa sawit. Keputusan ini menunjukkan kehadiran awal *G. boninense* pada tisu akar. Di dalam kajian ini, tisu akar tidak dicuci atau di steril sebelum di ekstrak, jadi ergosterol dapat dikesan lebih awal pada tisu akar setelah kulat melekat pada permukaan tisu akar. Hasil kajian ini juga menunjukkan skala tahap penyakit luaran dapat dilihat seawal lapan minggu selepas inokulasi. Semakin penyakit merebak, kelapa sawit menunjukkan gejala pucat, pertumbuhan terbantu, dan akhirnya mati. Selain itu, analisis TLC juga menunjukkan korelasi yang baik dengan pengiraan HPLC di mana keamatan tompok yang dihasilkan oleh analisis TLC menunjukkan keamatan paling tinggi bagi pengiraan HPLC yang paling tinggi dan sebaliknya. Oleh itu, analisis separa-kuantitatif TLC boleh digunakan untuk mengendalikan jumlah sampel yang besar dalam kajian lapangan

Berdasarkan kajian ini, kesimpulan yang boleh dibuat adalah, kaedah pengekstrakan menggunakan ketuhar gelombang mikro (MAE) adalah efisien dan boleh digunakan dalam pengesanan ergosterol di mana ergosterol berperanan sebagai penanda untuk pengesanan awal penyakit BSR. Selain itu, analisis TLC boleh digunakan untuk mengesan ergosterol di dalam tisu kelapa sawit di ladang kerana ia lebih mudah dan boleh dilaksanakan di-situ dan sesuai untuk kajian lapangan yang berskala besar.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master in Science. The members of the supervisory committee were as follows:

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LIST OF ABBREVIATIONS

AE	Alkaline Extraction
bp	Base Pair
BSR	Basal Stem Rot
C	Carbon
CRD	Complete Randomize Design
DNA	Deoxyribonucleic acid
DS	Disease Severity
FW	Fresh Weight
GIS	Geographical Information System
GSM	*Ganoderma* Selective Media
Ha	Hectare
HPLC	High Performance Liquid Chromatography
hrs	Hours
IPP	Isopentenyl Diphosphate
ITS	Internal Transcribe Spacer
LSD	Least Significant Different
MAE	Microwave Assisted Extraction
MEA	Malt Extract Agar
MEB	Malt Extract Broth
MH	Medium High
Min	minute
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAE	Non-alkaline Extraction
nm	Nanometer
PCR	Polymerase Chain Reaction
PIRG	Percentage of Radial Growth
RAPDs	Random Amplified Polymorphic DNA
Rf	Retention Value
RNA	Ribonucleic Acid
RWB	Rubber Wood Block
SE	Standard error
Sec	Second
SEM	Scanning Electron Microscopy
SFE	Supercritical Fluid Extraction
TLC	Thin Layer Chromatography
USE	Ultrasonicator Extraction Method
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis*) is a monocotyledon in the family Arecaceae (formerly Palmae) within the subfamily Cocosoideae (Corley and Tinker, 2003). It is a major crop that grows in the tropical areas, especially in Southeast Asia. Palm oil is used worldwide for the production of food, cosmetics, pharmaceuticals, biodiesel and oleo chemical industry, where it is being used for making soaps and detergents (Kalam and Masjuki, 2002; Corley and Tinker, 2003; Turner *et al*., 2008). Oil palm industry contributes to the Malaysian economy and also in the development of country's rural areas (Chin, 2008). In Malaysia, cultivation of oil palm has increased year by year with 1.5 million hectares (ha) in the 1985 to 5.39 million ha in 2014 (MPOB, 2014).

Oil palm is subjected to numerous devastating diseases such as basal stem rot (BSR), vascular wilt, spear rot-bud rot, sudden wither and red ring (Corley and Tinker, 2003). However, BSR is the major disease encountered by Malaysian palms which is caused by *Ganoderma* species (Idris *et al*., 2011). Several attempts have been made to control BSR using various control methods; however until now none of the methods gave good control of *G. boninense* infection in established oil palm plantation due to late detection of *Ganoderma*. In plantation, census needs to be done regularly, practically at 3-6 months interval. To date, a practicable early detection method of the disease is still lacking. Basal stem rot is detected based on foliar associated symptoms and production of basidiomata at the base of infected stem. However, by the time visible symptoms appear, the palms are already at a serious stage and usually half of the basal tissues have been killed by the fungus (Idris, 2009). Enzyme-linked immunosorbent assays-polyclonal antibody (Idris and Rafidah, 2008) as well as PCR based techniques involving specific *Ganoderma* primers (Bridge *et al*., 2000; Utomo and Niepold, 2000; Yamoaka, *et al.,* 2000) has been proposed as early detection method of the disease. However these methods are complicated and time consuming for early detection of the disease in oil palm fields. Moreover, there are some limitations with PCR technique which require to be addressed before applying for detection of *Ganoderma* (Paterson, 2007a; Paterson *et al*. 2008; Paterson and Lima, 2009).

Ergosterol is a primary sterol in cell membranes of filamentous fungi and is either absent or presence in a minor component in the majority of higher plant (Madonna *et al*., 2001). First data published on the use of ergosterol analysis as a diagnostic method to detect BSR support the view that ergosterol have the utility for the detection of BSR in oil palm (Mohd Aswad *et al*., 2011). Ergosterol is specific to fungi and indicates live fungal biomass. Therefore, Parkinson and Coleman (1991) reported that ergosterol assay is commonly considered to be the most promising tools for detection and quantification of

fungal biomass. Parsi and Gorecki, (2006) also reported that the detection of ergosterol as fungal biomarker could be considered to be the method of choice

Previous study used non-alkaline extraction (NAE), alkaline extraction (AE), and ultrasonicator extraction (USE) methods for extraction of ergosterol. The organic solvent based method (classical) typically required large samples, large reagent volume, labor intensive, time consuming and also reported that AE and USE give low ergosterol concentration when compared to NAE method (Mohd Aswad *et al*., 2011). Therefore, more reliable extraction methods are required for the extraction of ergosterol. Young (1995) has developed microwave assisted extraction (MAE) method for extraction of ergosterol which required smaller samples and reagent volume, more economical in term of chemicals used, and using convectional equipment (Domestic Microwave). Microwave assisted extraction is therefore more convenient than other methods in terms of time for sample preparation, cost and sample size. In addition, more samples can be extracted at one time and a suitable, simple, rapid, and reliable extraction method for ergosterol detection in field palms during census.

Thus, present study is undertaken to apply ergosterol as bio indicator of *Ganoderma* infection grown in field palms with simple extraction method. The aims of this study are (I) to optimize a simple and rapid ergosterol extraction method from *Ganoderma* infected tissue using MAE, (ii) to establish the relationship between *Ganoderma*, ergosterol concentration, and BSR development; and to validate the efficiency of MAE method for extraction of ergosterol and test the sensitivity of TLC analysis for detection of ergosterol using artificially inoculated germinated seeds and seedlings; and infected field palm samples.

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