EFFICACY OF MICROBIAL APPROACHES IN CONTROLLING *Ganoderma boninense* Pat AND THEIR EFFECT ON SOIL MICROBIAL DIVERSITY

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THESIS SUBMITTED IN FULFILLMENT
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ABSTRACT

Basal Stem Rot (BSR) is the most destructive disease of oil palm (*Elaeis guineensis* Jacq.) which caused by bracket fungus; *Ganoderma boninense*. With no remedy to date, this disease has jeopardized the industry of oil palm critically. Several approaches including cultural, chemical, mechanical and biological measures have been reported in attempt to control this disease, but unfortunately, none have given satisfactory result. Therefore, a more reliable approach that could manage this disease, besides a continuous and sustainable oil palm production is worth to be further investigated. Introduction of microbes in disease management have been used for decades. An astute observation of low incidence of disease due to *Ganoderma* pathogen in natural ecosystem suggests that a healthy natural ecosystem can hinder the incidence of *Ganoderma* pathogen outbreak. In this respect, study on the potential of microbes in suppressing the growth of *Ganoderma boninense* and its effect to the soil microbial diversity were investigated in this project. Three microbes-based products which are Living Soil Microbes (LSM), Agrinos (AGN) and Agriorganica (AO) were tested in this study. LSM contains multi-strain of *Bacillus* spp. and *Trichoderma* spp., whereas AGN consist of *Bacillus* spp., *Pseudomonas* spp. and *Aspergillus* sp. Meanwhile, AO comprises of three living food microbes which are *Lactobacillus, Nattobacillus* and *Saccharomyces cerevisiae* developed from Japanese fermentation and brewing technology. To examine the ability of the treatments in reducing BSR disease incidence, assessments in nursery and field trial were conducted. The results from both trials showed that LSM, AGN and AO were able to reduce the colonization of *G. boninense* based on the reduction of ergosterol content compared to untreated control. Based on the nursery results in prevention of *Ganoderma* colonization trial, after two months interval, LSM and AO showed greater reduction of ergosterol content with 1.991 µg g^{-1} and 1.994 µg g^{-1} respectively, however LSM recorded the lowest Disease Incidence (DI) with only 30%. After four months interval, LSM and AGN showed greater reduction in ergosterol content with 2.261 µg g^{-1} and 2.214 µg g^{-1} respectively, however, AGN and AO had the lower DI with 60%. Meanwhile, based on the results in suppression of *Ganoderma* colonization trial, after two months of interval, all treatments successfully reduced the ergosterol content and DI within the time frame. After four months interval, all treatments showed an increment in DI, however, LSM had the lowest ergosterol content which is 2.388 µg g^{-1}. Meanwhile, assessment in the field trial showed that both LSM and AO had significantly reduced the amount of ergosterol to 0.663 µg g^{-1} and 1.817 µg g^{-1}. Both LSM and AO also successfully reduced the DI down to 12% and 24% respectively. The effect of the application of microbial products to the soil microbial community was evaluated based on the isolation using Colony Forming Unit (CFU) and later identification using Biolog and molecular techniques. The numbers of CFU for bacteria and yeast after application of these microbes remain unchanged, which

were varied between 10^4 to 10^6 cfu/g and 10^2 to 10^5 cfu/g of soil respectively. However, the number of CFU for fungi was increased up to 10^4 cfu/g of soil. Identification based on Biolog and sequence homology technique shows that new species arose and overcome the predominated species after application of these microbes. The new identified species were *Enterobacter* spp., *Microbacterium* spp., *Burkholderia* spp., *Yarrowia* spp., *Paecilomyces* spp., *Neosertoya* spp. etc. *In vitro* antagonistic assay of microbial treatments showed that only LSM has the activity against *G. boninense* (Percentage Inhibition of Radial Growth, PIRG=70%). Observation under Scanning Electron Microscopy (SEM) showed that *Ganoderma* mycelium was highly disrupted and lysed after exposure to LSM treatment. Potential bioactive component produced by LSM during this interaction which inhibiting the growth of *G. boninense* was further investigated via Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Liquid Chromatography Mass Spectrometry (LCMS). Several potential antimicrobes compounds were detected including Pyrene-1,6-dione, 12-Deoxyaklanonic acid, N-Methyl-a-aminoisobutyric acid, 4-O-8',5"-5'-Dehydrotriferulic acid, Halstoctacosanolide A, N-acetyl-leu-leu-tyr-amide, Methenamine, 12-oxo-10Z-dodecenoic acid, Gly-Met-OH and Lovastatin. These metabolites may probably contribute on the antagonistic effect of LSM microbes against *G. boninense*. This study showed that the use of biological control agents (BCAs) give promising results in controlling *G. boninense* under different level and environment. The use of BCAs could offer an alternatives for the use of fungicide and it may well be worth to be looked in more detail into the potential of BCAs for its future prospects in agriculture.

Keywords: basal stem rot, microbial approach, *Ganoderma boninense*, soil microbes, antagonist

ABSTRAK

KEBERKESANAN PENGGUNAAN MIKROB DALAM MENGAWAL Ganoderma boniense Pat DAN KESANNYA TERHADAP KEPELBAGAIAN MIKROB DALAM TANAH

Reput Pangkal Batang (RPB) ialah penyakit paling memudaratkan pokok kelapa sawit (Elaeis guineensis Jacq.) yang mana disebabkan oleh sejenis kulat; Ganoderma boninense. Penyakit ini telah merugikan industri kelapa sawit dengan teruk dan sehingga kini, tiada langkah yang mampu memulihkan jangkitan ini. Beberapa pendekatan termasuk langkah kultura, kimia, mekanikal dan biologi telah dilaporkan dalam percubaan untuk mengawal penyakit ini, tetapi malangnya, tiada yang mampu memberikan hasil memuaskan. Oleh itu, satu pendekatan yang lebih teliti mungkin mampu mengawal penyakit ini, di samping pengeluaran hasil kelapa sawit yang berterusan dan mapan adalah wajar untuk dikaji. Penggunaan mikrob dalam pengurusan penyakit telah digunakan selama berdekad lamanya. Satu kajian mendapati bahawa kadar penyakit disebabkan oleh patogen Ganoderma dalam ekosistem semulajadi adalah rendah dan ini menandakan bahawa ekosistem semulajadi mampu menghalang insiden wabak patogen Ganoderma. Oleh itu, kajian terhadap potensi mikrob dalam mengurangkan penyakit RPB dan kesannya ke atas kepelbagaian mikrob dalam tanah telah dijalankan. Tiga produk berasaskan mikrob iaitu Living Soil Microbes (LSM), Agrinos (AGN) dan Agriorganica (AO) telah diuji dalam kajian ini. LSM mengandungi pelbagai strain Bacillus spp. dan Trichoderma spp., manakala AGN terdiri daripada Bacillus spp., Pseudomonas spp. dan Aspergillus sp. Sementara itu, AO terdiri daripada 3 mikrob hidup makanan iaitu Lactobacillus, Nattobacillus dan Saccharomyces cerevisiae yang dihasilkan daripada teknologi penapaian Jepun. Untuk menguji keupayaan rawatan-rawatan tersebut dalam mengurangkan kadar penyakit RPB, percubaan di nurseri dan ladang telah dijalankan. Keputusan daripada kedua-dua percubaan menunjukkan bahawa LSM, AGN dan AO boleh mengurangkan insiden penyakit RPB berdasarkan pengurangan kandungan ergosterol berbanding dengan pokok yang tidak dirawat. Berdasarkan dengan keputusan di dalam percubaan pencegahan jangkitan Ganoderma di nurseri, selepas selang dua bulan, LSM dan AO menunjukkan penurunan kandungan ergosterol dengan masing-masing mencatatkan 1.991 µg g^{-1} dan 1.994 µg g^{-1}, namun LSM mencatatkan kejadian jangkitan (KJ) terendah dengan hanya 30% jangkitan. Selepas selang empat bulan, LSM dan AGN menunjukkan kandungan ergosterol terendah dengan masing-masing mencatatkan 2.261 µg g^{-1} dan 2.214 µg g^{-1}, namun, AGN dan AO mencatatkan KJ yang lebih rendah iaitu 60% jangkitan. Manakala berdasarkan dengan keputusan di dalam percubaan pengurangan jangkitan Ganoderma, selepas selang dua bulan, semua rawatan menunjukkan penurunan kandungan ergosterol dan KJ yang memberansangkan. Selepas selang

empat bulan, semua rawatan menunjukan peningkatan KJ yang mendadak, namun LSM menunjukkan kandungan ergosterol terendah iaitu 2.388 µg g^{-1}. Manakala, berdasarkan dengan penilaian di ladang, LSM dan AO menunjukkan pengurangan kandungan ergosterol yang ketara dengan masing-masing mencatatkan 0.663 µg g^{-1} dan 1.817 µg g^{-1}. Kedua-dua rawatan LSM dan AO juga mencatatkan KJ terendah dengan masing-masing mencatat 12% dan 24% jangkitan. Kesan aplikasi rawatan mikrob ke atas kepelbagaian mikrob dalam tanah dinilai berdasarkan kaedah unit pembentukan koloni (UPK) dan seterusnya dikenalpasti menggunakan kaedah Biolog dan teknik molekular. Bilangan koloni bakteria dan yis yang terasing selepas aplikasi rawatan mikrob tidak berubah dengan bacaan masing-masing pada 10^4 sehingga 10^6 koloni/g dan 10^2 sehingga 10^5 koloni/g tanah. Walau bagaimanapun, jumlah bacaan koloni bagi kulat telah meningkat sehingga 10^4 koloni/g tanah. Penegenalpastian mikrob berdasarkan teknik Biolog dan jujukan homologi menunjukkan bahawa terdapat spesis baru yang muncul dan mengatasi spesis predominan selepas pengaplikasian rawatan mikrob ini. Antara spesis-spesis baru yang dikenal pasti adalah Enterobacter spp., Microbacterium spp., Burkholderia spp., Yarrowia spp., Paecilomyces spp., Neosertoya spp. dan lain-lain. Pencerakinan antagonis setiap rawatan secara in vitro menunjukkan bahawa hanya LSM mempunyai aktiviti dalam melawan Ganoderma (Peratus Perencatan Pertumbuhan Radial, PIRG =70%). Pemerhatian di bawah Mikroskop Pengimbas Elektron (MPE) menunjukan mycelium Ganoderma mengalami gangguan dan pemecahan selepas dirawat dengan LSM. Sebarang komponen bioaktif berpotensi yang dihasilkan oleh LSM semasa interaksi perencatan pertumbuhan G. boninense selanjutnya dikaji menggunakan Fasa Berbalik-Kromatografi Cecair Berprestasi Tinggi (FB-KCBT) dan Kromatografi Cecair-Spektrometri Jisim (KCSJ). Beberapa kompoun berpotensi antimikrobial yang dikesan termasuk Pyrene-1,6-dione, 12-Deoxyaklanonic acid, N-Methyl-a-aminoisobutyric acid, 4-O-8',5''-5'-Dehydrotriferulic acid, Halstoctacosanolide A, N-acetyl-leu-leu-tyr-amide, Methenamine, 12-oxo-10Z-dodecenoic acid, Gly-Met-OH dan Lovastatin. Komponen-komponen ini berkemungkinan terlibat ke atas kesan antagonis mikrob LSM terhadap G. boninense. Kajian ini menunjukan bahawa penggunaan agen pengawal biocontrol (APB) memberikan keputusan yang memberangsangkan dalam mengawal G. boninense di bawah tahap dan persekitaran yang berbeza. Penggunaan APB boleh memberikan alternatif terhadap penggunaan racun kimia dan potensi APB wajar diselidiki lebih mendalam terhadap penggunaannya di masa hadapan dan prospek di dalam pertanian.

***Kata kunci**: reput pangkal batang, pendekatan mikrob, Ganoderma boninense, mikrob tanah, antagonis*

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LIST OF SYMBOLS AND ABBREVIATIONS

±	plus-minus
+	plus
-	minus
=	equals to
≥	more or equal to
/	divide by
%	percentage
^{o}C	degree Celcius
α	alpha
β	beta
µg	microgram
g	gram
kg	kilogram
L	litre
m	meter
µl	microlitre
µM	micromolar
pM	picomolar
mg	milligram
ml	millilitre
mm	millimeter
µg mL^{-1}	microgram per millilitre
mg mL^{-1}	milligram per millilitre
U	unit
pH	power of hydrogen
m/z	mass to charge ratio
rpm	revolution per minute
h	hour
min	minute

CFU	Colony forming unit
PIRG	Percentage Inhibition of Radial Growth
SPSS	Statistical Analysis for Social Science
UV	ultraviolet
LC	liquid chromatography
LC/MS	liquid chromatography-mass spectrometry
ESI	electrospray ionization
Qtof	quadrupole-tile of flight
®	registered trademark
MPOB	Malaysian Palm Oil Board
PORIM	Palm Oil Research Institute of Malaysia

CHAPTER 1

INTRODUCTION

1.1 Background

Oil palm (*Elaeis guineensis* Jacq.) is known as a truly "golden crop of Malaysia" since it generated profitable export earnings for the country and truly nature's gifts for alleviating poverty in Malaysia (Basiron, 2007). Area of oil palm cultivation has grown rapidly from just under 60,000 ha in the 1960s to approximately 5 million ha in 2013 (MPOB, 2013). With the phenomenal expansion of oil palm area, palm oil production has rose significantly with an increase from 91,793 tonnes in 1960 to 19.2 million tonnes in 2013, or by almost 198 times within 52 years. The fast growth of this industry has made Malaysia to become the world's second largest palm oil producer, accounting for 37 percent of the world's palm oil production.

However, the oil palm industry is being jeopardized by Basal Stem Rot (BSR) which is caused by *Ganoderma boninense*. Infection of this disease can cause numerous yield losses and ultimately result in the destruction of basal tissues hence death of diseases palms. With no known remedy, BSR remained the most significant constraint to sustainable oil palm production in the Southeast Asian countries, especially in Malaysia and Indonesia. Many efforts have been attempted to eliminate this disease, but to date, no promising remedy is reported (Bivi *et al*., 2010; Susanto *et al*., 2005). Recently, an astute observation by Bivi *et al*. (2010) showed that low incidence of BSR disease due to pathogenic *Ganoderma* species in some natural stands has given rise to assumption that this disease is most likely kept under control by some biological means. Therefore, recent control measures to overcome this disease are now focused on the use of biological control agents (BCA). Several promising antagonist BCAs such as *Trichoderma* spp. (Sariah, 2005; Susanto *et al*., 2005), *Penicillium* spp. (Dharmaputra *et al.,* 1989), *Burkholderia* spp. (Sapak *et al*., 2008), *Bacillus* spp. (Suryanto *et al*., 2012), and *Pseudomonas* spp. (Bivi *et al*., 2010) have shown high efficacy in controlling the growth and infection of *Ganoderma boninense* in nursery and field conditions. Several