ISOLATION OF POTENTIAL ENDOPHYTIC BACTERIA OF OIL PALM AGAINST *GANODERMA BONINENSE*

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ABSTRACT

Fifteen culturable endophytic bacteria were isolated from symptomless roots of oil palm sampled from oil palm plantation site located at the School of Sustainable Agriculture, Sandakan campus of Universiti Malaysia Sabah. Characterizations of the bacterial isolates were carried out based on morphological characteristics, including colony colour, elevation, margin and the appearance displayed on nutrient agar and potato dextrose agar media. Dual culture test was carried out on PDA media to select isolate with antagonistic character against *Ganoderma boninense*. Results revealed degrees of inhibition toward mycelial growth of *G. boninense* vary accordingly to the bacterial isolates. Among the fifthteen isolated endophytic bacteria, only two bacterial isolates (P_{15} and P_{18}) showed potential inhibitory effect against *G. boninense* with their percentage inhibitions of radial growth (PIRG) recorded were more than 50% in both dual culture and culture filtrate tests. There was significantly difference in inhibitory effect ($P<0.05$) for dual culture test and in culture filtrate test. The isolated P_{15} and P_{18} were further investigated in greenhouse trial. Results from *in planta* greenhouse trial further suggested that isolate P_{15} and P_{18} were capable to increase vegetative growth of oil palm seedlings while providing early protection against *G. boninense* infection. They play a role in keeping the *G. boninense* population below threshold for Basal Stem Rot (BSR) initiation by restricting its entry and movement in the seedlings.

MENGESAN BAKTERIA ENDOFIT DARIPADA KELAPA SAWIT

YANG BERKESAN MELAWAN *GANODERMA BONINESE*

ABSTRAK

Lima belas bakteria endofitik telah diasingkan daripada akar kelapa sawit yang sihat daripada ladang kelapa sawit yang terletak di Sekolah Pertanian Lestari, kampus Sandakan, Universiti Malaysia Sabah. Pencirian bakteria telah dijalankan berdasarkan ciri-ciri morfologi, termasuk warna koloni, permukaan koloni dan corak koloni yang dipaparkan pada agar nutrien dan media agar PDA. Ujian dwi-kultur telah dijalankan pada media PDA untuk memilih bakteria endofitik dengan watak antagonis terhadap *Ganoderma boninense*. Keputusan menunjukkan darjah perencatan terhadap pertumbuhan mycelial *G. boninense* adalah berbeza antara bakteria. Antara lima belas bakteria endofitik yang diasingkan, hanya dua bakteria (P_{15} dan P_{18}) menunjukkan potensi perencatan terhadap *G. boninense* dengan peratusan perencatan pertumbuhan mycelial (PIRG) yang lebih daripada 50% dalam dua kedua-dua ujian dwi-kultur dan ujian turasan kultur. Terdapat perbezaan secara ketara ($P <0.05$) dalam kedua-dua ujian yang dijalankan. Seterusnya, bakteria endofitik telah diuji terhadap anak benih sawit. Keputusan dari rumah hijau (*in planta*) mencadangkan bahawa endofitik bakteria P_{15} dan P_{18} mampu untuk meningkatkan pertumbuhan vegetatif benih kelapa sawit di samping memberikan perlindungan awal terhadap jangkitan *G. boninense*. Bakteria ini memainkan peranan dalam mencegah *G. boninense* berkembang dengan menyekat kemasukan dan pergerakan dalam benih.

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

$t\ ha^{-1}$	Tonnes per hectare
^oC	Celsius
%	Percentage
β	Beta
γ	Gamma
Fe^{3+}	Iron ion
kg	kilogram
m	meter
mL	mililiter
µL	microliter
mins	minutes
cm	centimeter
nm	nanometer
rpm	Revolutions per minute
v/v	Volume to Volume
ACC	1-aminocyclopropane-1-carboxylase
BRI	Biotechnology Research Institute
BSR	Basal Stem Rot
cfu	Colony forming unit
CO_2	Carbon dioxide
$CONH_2$	Carboxamide group
COOH	Carboxyl group
CRD	Complete Randomized Design
DDR	2,3-deepoxy-2,3-didehydrorhizoxin
DNA	DeoxyriboNucleic Acid
DSI	Disease Severity Index
G+C	Guanine-plus-Cytosine
HCN	Hydrogen Cyanide
HSD	Honestly Significant Different
IAA	Indole-3-acetic acid
ISR	Induced Systemic Resistance
LPS	Lipoplysaccharides
MPOB	Malaysia Palm Oil Board
NA	Nutrient Agar
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
PDA	Potato Dextrose Agar
PGPB	Plant Growth Promoter Bacterium
PGPR	Plant Growth Promoting Rhizobacteria
PIRG	The percentage inhibition of radial growth
PLT	Pyoluteorin
PR	Pathogenesis-related
PRN	Pyrrolnitrin
PVC	Polyvinyl Chloride
PVD	Pyoverdine
rDNA	Ribosomal DeoxyriboNucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SA	Salicylic Acid

LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

SAR	Systemic Acquired Resistance
SDW	Sterile Distilled Water
SPSS	Statistical Package for the Social Science
SSA	School of Sustainable Agriculture
TCBS	Thiosulphate citrate bile salt sucrose (II)
UMSKS	University Malaysia of Sabah Campus Sandakan
UV	Ultra violet

LIST OF FORMULAE

Formula **Page**

CHAPTER 1

INTRODUCTION

1.1 Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the most crucial species in the genus of *Elaeis* which belongs to the family of *Palmae*. Oil palm is indigenous to West Africa where it distribute in large range of area from Sierra Leona, Liberia, the Ivory Coast, Ghana and Cameroon to the equatorial regions of the Republics of Congo and Zaire. The first introduction of African oil palm seedlings were planted in the Botanic Garden in Bogor in 1848 (Teoh, 2002). The first commercial oil palm plantation was established in Sumatra, Indonesia. In 1911, the oil palm seeds were brought by Fauconnier and planted in Rantau Panjang Estate in Selangor, Malaysia (Teoh, 2002).

Today, oil palm is the most important plantation crop in Malaysia. It produces with an average yield of approximately 4 t ha^{-1} annually. Oil palm industry contributed substantially to the Malaysian's economy. The oil palm exportation reached about RM31 billion increased by 8.54% or RM2.44 billion in 2006 compared to 2005 (Najmie, 2011). According to Basiron (2007), the areas of oil palm plantation had increased from 54,000 hectares in 1960 to 4.05 million hectares in 2005. This represents a compound annual growth of 10.06%. While, the production from oil palm industry increased from 94,000 tones in 1960 to 15 million tones in 2005, reflecting a compound annual growth of 11.93% per year (Basiron, 2007).

In Malaysia, the oil palm is blessed by being largely disease free, but suffering from one major disease known as the Basal Stem Rot (BSR) caused by *Ganorderma boninense*. It is the major disease in oil palm plantation with no known effective cure at present. The economic life span of oil palm is 25-30 years. BSR can kill more than

80% of stands by the time they are half-way through normal economic life (Abdul Razak *et al.,* 2004). Palms infected with *G. boninense* produce yield 21% less than healthy palms at the same age (Nazzeb *et al.*, 2000). Singh (1990) found that heavily infected field yielded 26% less at 11 years after planting and 46% less at 15 years by which time incidence was 67%.

There is currently no effective cure for *G. boninense* infection in an existing stand. Field control of BSR by contact chemicals are not very successful (Soepena *et al.,* 2000) even with those *in vitro* screened effective against the fungus (Khairudin, 1990). Control by physical methods such as clean clearing and tree surgery had transient effects though there is testify that BSR can be eliminated if all the disease inoculum is removed before planting or replanting the crop. Preventive and ameliorative treatments such as biological control are commonly carried out in the oil palm plantation. Therefore, biological control can be a cure for the BSR disease and can be merely to arrest the disease spread by inoculation with a biological control agent that could guard from the plant inside out (endophytes).

Endophytes are microorganisms that live within the plants cells. The functions of endophytic microoganisms should be preferable to other biological control agents as they are colonizers internally and therefore more able to compete in vascular systems, depriving *Ganoderma* from both nutrients and space for its proliferation. Astute observations of the low incidence of disease due to pathogenic *Ganoderma* species in some natural stands, suggest that the disease is most likely kept under control by some biological means. Due to these observations, recent control measures to overcome the BSR incidence are now focused on the use of biological control agents. For example, saprophytes can be used to compete against *Ganoderma boninense* to reduce its opportunity for colonizing oil palm roots. Several promising antagonists such as *Trichoderma* (Shukla and Uniyal, 1989; Wijesekera *et al.*, 1996; Sariah, 2003) and *Penicillium* (Dharmaputra *et al.*, 1989) have shown *in vitro* antagonistic activity against *G. boninense.*

1.2 Justification of Study

Endopyhtes are organisms inhabiting plant organs that at some time in their life cycle can colonize the internal plant tissue without causing apparent harm to the host. Screening the potential endophytes in the oil palm roots and re-introducing them to the infected oil palm can theoretically leads to enhance suppression of BSR disease. The use of endophytic bacteria are preferred to other biological control agents as they are internal colonizers, with better ability to compete within the vascular system and limiting *G. boninense* for both nutrient and space during its proliferation. Besides that, the growth of the plant by endophytes can be stimulated through the promoting of nitrogen fixation, production of pyhtohormones and by enhancing available ability of minerals. Once the selection of the most potential endophytic bacteria, it can be inoculated into the roots of the oil palm seedlings for the manipulation of the indigenous bacterial communities in order to enhance the suppression of soil-borne pathogen and *Ganoderma* as well. Endophytes create least residue to the environment as compared to chemical control methods. Endophytes can be the important biological control agents which they are environmental friendly and there is no negative effect on both oil palm trees and the soil. Besides of suppressing of BSR disease incidence, the endophytes can promote the growth of the oil palm as well. The endophytes can be incorporated into the bio-fertilizers and it will be important and economical supplements for the oil palm trees.

1.3 Objectives

The objectives of this study are:

1. To isolate the potential endophytic bacteria in the roots of symptomless palms.
2. To evaluate the efficiency of endophytic bacteria in suppression of *G.boninense in vitro* (culture plate).
3. To evaluate the efficiency of endophytic bacteria in suppression of *G.boninense in planta* (nursery).
4. To determine the effects of endophytic bacteria on seedling growth of oil palm.

1.4 Hypotheses

The hypotheses for this study are:

H_{01}: There is no significant effect of the endophytic bacteria on *Ganoderma boninense in vitro* and *in planta* and the growth of the oil palms seedlings.

H_{a1}: There is significant effect of the endophytic bacteria on *Ganoderma boninense in vivo* and *in planta* and the growth of the oil palms seedlings.

H_{02}: There are is significant effect of the endophytic bacteria on the growth of the oil palms seedlings after the application of treatments.

H_{a2}: There is significant effect of the endophytic bacteria on the growth of the oil palms seedlings after the application of treatments.

CHAPTER 2

LITERATURE REVIEWS

2.1 Oil Palm (*Elaeis guinensis* Jacq.)

Elaeis guineensis Jacq. which is commonly known as oil palm is the most important species in the genus *Elaeis* (family: *Palmae*). The second species is *Elaeis oleifera Cortes* which is found in South and Central America and is known as the American oil palm. Oil palm was the major commodity inside the Malaysian agriculture sector. Commercial cultivation of the oil palm started in 1917 and has expanded tremendously in recent years because it is suitable to be planted in most lands in Malaysia.

Malaysia is presently the world's leading exporter of oil palm having a 60% market share and oil palm was second only to soybean as the major source of vegetable oil. The total oil palm planted area in the country increased by 4.3% to 4.48 million hectares in 2008. The expansion in planted area occurred mainly in Sabah and Sarawak with a combined growth of 7% compared to 2% in Peninsular Malaysia. Sabah remained the largest oil palm planted state, accounting for 1.33 million hectares or 30% of the total planted area in the country. However, a soil fungus pathogen, *Ganoderma boninense*, which causes basal stem rot in oil palms trunk, ruins thousands of hectares of plantations in Southeast Asia every year. The disease causes infected palm trunks to fracture at the base, thus causing direct loss of oil palm trees.

2.2 Basal Stem Rot (BSR) Disease

Ganoderma boninense is the causal agent of basal stem rot (BSR) disease which is the major disease in oil palm plantation in Malaysia (Singh, 1990). Previously, BSR was only found on older plants, but recently the disease was observed on five-year-old or younger plants. According to Singh (1990), the BSR was found to infect the palms as young as 12-24 months after planting in the estate and 4-5 years after planting had been reported was the most increased incidence of the infection. Beside the very old palms, palms between 7-15 years old are also infected. The BSR disease is presently in most oil palm plantation particularly in the coastal marine clay areas (Khairudin, 1990), peat, localized inland soils (Ariffin *et al.*, 1993; Rao, 1990) and replanted areas (Singh, 1990).

The *G. boninense* problems had been known for decades, but the search for solution was considerably hampered by a natural constraint. The disease does not cause external symptoms until it is too far advanced and at a stage when trees cannot respond to treatment anymore. Primary infection of palms by *Ganoderma* species has been considered to occur by contact of living palm roots with colonized debris within the soil (Idris *et al.,* 2002). Secondary spread of inoculums has been assumed to be by contact of living palm roots with each other (De Oliviera *et al.*, 2005).

2.2.1 Infection of *Ganoderma boninense*

Rees *et al.* (2009) reported a detailed demonstration of the reproducible infection of intact roots of oil palm with *G. boninense*. Infection showed penetration, followed by rapid longitudinal progression of hyphae and colonisation of the lower stem (bole) of oil palm. In newly colonised tissue, *G. boninense* behaved as a hemibiotroph with numerous, wide, intracellular hyphae occupying entire host cells that possessed intact cell walls and contained discernible cytoplasm and organelles. In the bole this phase coincided with a complete depletion of previously abundant starch grains in advance of invasion. Subsequently, in the roots and colonised stem bases, widespread necrotrophic, enzymatic attack of all layers of the host cell walls occurred. Hyphae were intra, intercellular and intramural and associated with host cell wall degradation, which was often at a distance from hyphae, resulting in cavities within cell walls.

A third developmental stage was the formation of an extensive, melanized, tough mycelium, or pseudo-sclerotium, which surrounded roots and comprised many very thick-walled cells encasing more typical thin-walled hyphae. Macroscopic observation of and isolation from the bole of randomly felled, commercial palms provided confirmatory evidence that multiple infections originated in the roots before spreading into the base of long-established palms (Rees *et al.*, 2009).

2.2.2 Control of basal stem rot disease

Attempts to control this disease in the fields with fungicide have been made by various workers, but the results are inconclusive, though some systematic fungicides seem to be promising. The methods of fungicide application include soil drenching, trunk injection, or combination of these two methods (Erwinsyah, 2008). However, the results of fungicidal control of this disease in the field have been inconclusive (Idris *et al.,* 2002). This phenomenon is probably due to the fact that *Ganoderma* has various resting stages such as melanised mycelium, basidiospores and pseudosclerotia (Susanto *et al.*, 2005) that are more resistant to fungicides. On the other hand, once external symptoms appear the infection is already too severe, and a biocontrol agent cannot control the pathogen during this stage.

2.3 Endophytic Bacteria

Endophytes are the microorganisms that live within or at least during part of their life cycle inside a palnt. Cultivable endophytic communities can be isolated after surface sterilization of plant materials. They can generally be found within the cells, in the intercellular spaces and in the vascular system (Aravind *et al.*, 2009). The endophytes can also be found in various tissues such as tubers, fruit, stems, seeds and ovules. Zaiton (2006) discovered that among 1,323 microbial endophytes isolated from 3,600 oil palm roots, 65.23% were bacteria, 32.73% fungi and 2.04% actinomycetes. As cited in the extensive review of Kobayashi and Palumbo (2000), both gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. However, most of the endopyhtes isolated from xylem vessels (about 78 to 84% of the population) were Gram-negative (Gardner *et al.*, 1982). Ranges of 93.04% to 100% of the isolated endophytic bacteria from oil palms roots were Gram negative and only 6.95% are Gram positive (Zaiton, 2006; Rahamath *et al.*, 2010). There are different mechanisms by which the endophytes enter and then colonize the plants such as the entry through stomata, lenticels, wounds including broken trichomes, the emerge of lateral roots areas, germinating radicle, undifferentiated meristematic root tissue, the penetration of the junction or cracks between root hair and adjacent epidermal cells and the enzymatic processes involving degradation of cell wall bound polysaccharides (Quadt-Hallmann *et al.*, 1997).

The common bacterial species of endophytes reside in the plants are Fluorescent Pseudomonads, *Bacillus* spp., *Herbaspirillum* spp., *Serratia marcescens* and *Streptomyces* spp. (McLnroy *et al.*, 1995). Some of the endopyhtic bacteria from genera *Pseudomonas*, *Burkholderia* and *Serratia* are mostly found in healthy roots from symptomless palms. Study proved that in most plant the population densities of the endophytes are the greatest in the plant roots with the densities ranging from 10^4 to 10^6 colony forming unit (cfu) per g fresh weight in cotton and sweet corn roots (McLnroy *et al.*, 1995).

The population density of endophytes is highly variable by which it is mostly depending on the bacterial species, the host genotype as well as the host development stage, inoculum density and the environmental condition (Pillay *et al.*, 1997; Tan *et al.*, 2003). In oil palm, the population of endophytes varies due to the different palms age. Endophytes are more in mature (more than 11 years) followed by middle-aged (6-10 years) and young (1-5 years) palms (Zaiton, 2006). There is less significant different in the endophytes population in different types of soil such as coastal and peat soils. However, the inland soils there are high abundance of the endophytes with the genera of *Pseudomonas* and *Burkholderia* (Zaiton, 2006).

2.3.1 Fluorescent pseudomonads

Fluorescent pseudomonads are non-enteric, Gram-negative, aerobic, straight, or slightly curved rods, which are non-fermenting and mobile belonging to γ-proteobacteria (Galli *et al.*, 1992). They are pervasive bacteria which are inhabitants of soil, water and phyllosphere commonly but predominant in plant rhizosphere due to the exudation of organic acids, sugars and amino acids (Lugtenberg and Dekkers 1999). Fluorescent pseudomonads are the most promising group of plant growth-promoting rhizobacteria (PGPR) involved in biological control of plant disease. Saprophytic fluorescent pseudomonads are typical inhabitants of agricultural field soils and plant rhizosphere and are involved in several interactions with plants (Schroth *et al.*, 1992). They are capable of utilizing many plant exudates as nutrient (Lugtenberg *et al.*, 1999) and are known to possess important traits in bacterial fitness such as the

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