UNIVERSITI PUTRA MALAYSIA

ROLES OF PLANT GROWTH PROMOTING RHIZOBACTERIA IN SUPPRESSION OF GANODERMA BASAL STEM ROT IN OIL PALM

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By

WAHEEDA PARVIN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfilment of the Requirements for the Degree of Doctor of Philosophy

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To

My dearest father Prof. Md. Abdul Wahab,
My loveliest mother Aleya Begum
&
My beloved husband Dr. Md. Mahbubur Rahman
ROLES OF PLANT GROWTH PROMOTING RHIZOBACTERIA IN SUPPRESSION OF GANODERMA BASAL STEM ROT IN OIL PALM

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October 2014

Chairman: Assoc. Professor Wong Mui Yun, PhD

Faculty: Agriculture

The Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* is a major economic concern and it is a predominant disease of oil palm in Southeast Asia including Malaysia. Until now there is no effective control measure available for this disease. A sustainable control measure is using bio-control agents such as Plant Growth Promoting Rhizobacteria (PGPR). Two PGPR, *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 isolated from oil palm rhizosphere were studied for their potential to be used as biocontrol agents. The objectives of this study were (i) to determine the mechanisms of plant growth promotion and pathogen suppression by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3, (ii) to identify and quantify the antibiotics produced by selected PGPR and to determine their effects on *G. boninense* mycelial growth *in vitro*, and (iii) to evaluate the effects of selected antibiotic application on the development of BSR disease and on the expression of defense related genes during *Ganoderma*-oil palm interaction. Experiments were conducted to detect phytohormones, antibiotics, siderophores, and volatile substance, hydrogen cyanide (HCN) produced by these two bacterial strains. Various antibiotics produced were identified and quantified using High Performance Liquid Chromatography (HPLC). *In vitro* bioassay was carried out to determine the effect of antibiotics and volatiles produced on *G. boninense*. Subsequent experiments were conducted in the glasshouse with the selected antibiotic to evaluate BSR disease development and to detect pathogenesis-related (PR) genes induced during *Ganoderma*-oil palm interaction at the intervals of 0, 2, 4, 6, and 8 weeks after inoculation. The results showed that *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 produced phytohormones indole-3-acetic acid (IAA), salicylic acid (SA) and zeatin. But only *P. aeruginosa* UPMP3 produced volatile substance HCN. The results revealed that *P. aeruginosa* UPMP3 produced various antibiotics: 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine (PHZ), pyocyanin (PYO), phenazine -1- carboxylic acid (PCA), pyoluteorin, phenazine-1-carboxamide (PCN) and pyrrolnitrin, while *B. cepacia* UPMB3 produced pyocyanin, pyoluteorin and pyrrolnitrin. For *in vitro* bioassay using antibiotics and volatile substances, it was observed that the inhibition of *Ganoderma*
mycelial radial growth caused by \textit{P. aeruginosa} UPMP3 and \textit{B. cepacia} UPMB3 were 94.21% and 21.38% for antibiotics, respectively and 51.16% and 8.89% for volatile substances, respectively after 7 days of incubation. \textit{P. aeruginosa} UPMP3 was more effective than \textit{B. cepacia} UPMB3 in suppressing \textit{Ganoderma in vitro}. Based on these results, \textit{P. aeruginosa} UPMP3 was selected for further studies. Three antibiotics namely PHZ, PCA and PYO were extracted from \textit{P. aeruginosa} UPMP3 due to the availability of these standards and quantified using (HPLC). Standards for PHZ, PCA and PYO were completely separated with retention times of 41.590, 39.740 and 34.863 min, respectively. At 250 nm wave length, the bacterial strain produced a maximum concentration of PHZ (1.36 µg/mL) and PCA (9.62 µg/mL). At 262 nm the maximum concentration of PYO was 15.48 µg/mL. For \textit{in vitro} bioassay, PHZ was more effective than PCA and PYO in suppressing \textit{Ganoderma} at concentration of 1mg/mL. The inhibition percentages were 100%, 78.61% and 91.87%, respectively. For glasshouse study, 5 treatments were used: T1, Negative control; T2, Positive control; T3, Phenazine (1mg/mL); T4, Phenazine (2mg/mL); T5, Hexaconazole (0.048 mg/mL). Plants in T4 showed the highest disease reduction of 52.76% compared to T5 (25.27%). Two putative pathogenesis-related (PR) genes including chitinase and \(\beta\)-1, 3-glucanase were differentially expressed in oil palm roots with different treatments. Chitinase was expressed constantly throughout the sampling intervals while \(\beta\)-1, 3-glucanase expressed at later intervals. The results of this study showed that the antibiotic phenazine has strong antimicrobial activity against \textit{G. boninense in vitro} and was comparable to chemical fungicide in suppressing BSR in glasshouse conditions. The antibiotic phenazine extracted from \textit{P. aeruginosa} UPMP3 could be potentially developed as a commercial formulation to suppress BSR disease of oil palm to reduce the application of harmful pesticides, thus limiting their hazardous effects on the environment.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Doktor Falsafah

PERANAN RIZOBAKTERIA PENGGALAK TUMBESARAN TUMBUHAN DALAM PERENCATAN REPUT PANGKAL BATANG KELAPA SAWIT

Oleh

WAHEEDA PARVIN

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Penyakit reput pangkal batang disebabkan oleh *Ganoderma boninense* merupakan masalah ekonomi utama dan merupakan penyakit dominan kelapa sawit di Asia Tenggara termasuk Malaysia. Setakat ini tiada cara kawalan yang berkesan untuk penyakit ini. Satu kaedah lestari adalah menggunakan agen kawalan biologi seperti rizobakteria penggalak tumbesaran tumbuhan (PGPR) yang merupakan suatu pendekatan mesra alam. Dua PGPR iaitu *Pseudomonas aeruginosa* UPMP3 dan *Burkholderia cepacia* UPMB3 dari pemencilan rizosfera kelapa sawit telah dikaji potensinya sebagai agen kawalan biologi. Objektif kajian ini ialah (i) menentukan mekanisma penggalak tumbesaran tumbuhan dan perencatan patogen oleh *Pseudomonas aeruginosa* UPMP3 dan *Burkholderia cepacia* UPMB3, (ii) mengenalpasti jumlah antibiotik yang dihasilkan serta menilaikan khasiat antibiotik tersebut ke atas tumbesaran miselia *G. boninense* secara *in vitro* dan (iii) menentukan khasiat penggunaan antibiotic terhadap perkembangan penyakit BSR dan ekspresi gen perencaatan dalam interaksi *G. boninense*-kelapa sawit. Eksperimen dijalankan untuk mengenalpasti fitohormon, antibiotik, siderofor dan bahan merup seperti hidrogensianida (HCN), yang dihasilkan oleh dua strain bakteria tersebut. Pelbagai antibiotik yang dihasilkan telah dikenalpasti menggunakan 'High Performance Liquid Chromatography' (HPLC). Bioasai *in vitro* dijalankan untuk menentukan khasiat antibiotik dan bahan merup yang dihasilkan terhadap *G. boninense*. Eksperimen seterusnya dijalankan di rumah kaca dengan menggunakan antibiotic tertentu bagi menilaikan perkembangan penyakit pangkal batang reput dan mengesankan gen patogenesis ketika interaksi *Ganoderma*-kelapa sawit dalam tempoh 0, 2, 4, 6 and 8 minggu selepas inokulasi. Hasil kajian menunjukkan *P. aeruginosa* UPMP3 dan *B. cepacia* UPMB3 menghasilkan fitohormon asid indol-3-asetik (IAA), asid salisik (SA) dan zeatin. Tetapi, hanya *P. aeruginosa* UPMP3 menghasilkan bahan merup HCN. Keputusan ini menunjukkan *P. aeruginosa* UPMP3 mampu menghasilkan pelbagai antibiotik: 2,4-diasetilfloroglusinol (2,4-DAPG), fenazin (PHZ), fikosianin (PYO), asid fenazin -1-
karbosilik (PCA), pioluteorin, fenazin-1-carboxamide (PCN) dan pirolnitrin manakala 
dan B. cepacia UPMB3 menghasilkan fikosianin, pioluteorin, dan pirolnitrin. Bagi 
kajian bioasai secara in vitro dengan menggunakan antibiotik dan bahan meruap, tahap 
kerencatan pertumbuhan miselium Ganoderma oleh P. aeruginosa UPMP3 dan B. 
cepacia UPMB3 adalah masing-masing 94.21% dan 21.38% bagi antibiotik dan 51.16% 
dan 8.89% masing-masing bagi bahan meruap, selepas 7 hari tempoh inkubasi. P. 
aeruginosa UPMP3 didapati lebih berkesan berbanding B. cepacia UPMB3 bagi 
merencat pertumbuhan Ganoderma secara in vitro. Berdasarkan keputusan ini, P. 
aeruginosa UPMP3 dipilih bagi kajian seterusnya. Tiga antibiotik iaitu PHZ, PCA dan 
PYO telah diekstrak daripada P. aeruginosa UPMP3 dan penentuan jumlah dilakukan 
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dan PCA (9.62 µg/mL). Pada 262 nm, kepekatan maksima dicapai oleh PYO adalah 15.48 
µg/mL. Untuk bioasai in vitro, didapati fenazin lebih efektif berbanding PCA dan PYO 
untuk merencat Ganoderma pada kepekatan 1 mg/mL. Peratusan perencatan adalah 
masing masing 100%, 78.61% dan 91.87%. Untuk kajian rumah kaca, 5 rawatan telah 
digunakan: T1, Kawalan negatif; T2, Kawalan positif; T3, Phenazine (1mg/mL); T4, 
Phenazine (2mg/mL); T5, Hexaconazole (0.048 mg/mL). Pokok dalam rawatan T4 
menunjukkan perencatan penyakit yang tertinggi (52.76%) berbanding T5 (25.27%). 
Dua gen putative patogenesis iaitu khitinase dan β-1, 3-gluankanase telah 
menunjukkan ekspresi yang berbeza dalam akar pokok kelapa sawit dalam rawatan yang berbeza. 
Ekspresi khitinase adalah tetap sepanjang tempoh eksperimen manakala ekspresi β-1, 3-
gluukanase berlaku lebih lambat. Keputusan kajian ini menunjukkan bahawa antibiotik 
fenazin mempunyai aktiviti antimikrob yang tinggi terhadap Ganoderma in vitro 
dan menunjukkan persamaan dengan racun kulat dalam perencatan BSR di bawah keadaan 
rumah kaca. Antibiotik fenazin yang diekstrak daripada P. aeruginosa UPMP3 
mempunyai potensi untuk dibangunkan sebagai formulasi komersial untuk mengawal 
penyakit reput pangkal batang kelapa sawit bagi mengurangkan penggunaan racun 
perosak yang berbahaya dan seterusnya mengurangkan kesan berbahaya terhadap alam 
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I certify that a Thesis Examination Committee has met on 28 October 2014 to conduct the final examination of Waheeda Parvin on her thesis entitled "Roles of Plant Growth Promoting Rhizobacteria in Suppression of Ganoderma Basal Stem Rot in Oil Palm" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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### LIST OF ABBREVIATIONS

| Ab       | Absorbance                              |
| ACC     | 1-aminocyclopropane-1-carboxylic acid   |
| AHL     | N-Acyl homoserine lactones              |
| AMF     | Arbuscular mycorrhizal fungi            |
| ANOVA   | Analysis of Variance                    |
| AUDPC   | Area Under Disease Progress Curve       |
| bp      | Base pair(s)                            |
| BSR     | Basal Stem Rot                          |
| CA      | Casamino Acid                            |
| CAS     | Chrome azurol sulphonate                |
| cDNA    | Complementary Deoxyribonucleic acid     |
| CFU     | Colony forming units                    |
| CRD     | Completely Randomized Design            |
| C_t     | Cycle number                            |
| DAPG    | Diacetylphloroglucinol                  |
| DI      | Disease incidence                       |
| DMSO    | Dimethyl sulfoxide                      |
| DNA     | Deoxyribonucleic acid                   |
| dNTP    | deoxyribose nucleotide triphosphate    |
| DR      | Disease reduction                       |
| DS      | Disease severity                        |
| DW      | Dry weight                              |
| ER      | Epidemic rate                           |
| FW      | Fresh weight                            |
| HCl     | Hydrochloric acid                       |
| HCN     | Hydrogen cyanide                        |
| HDTMA   | Hexadecyltrimethyl ammonium bromide     |
| HPLC    | High performance liquid chromatography  |
| IAA     | Indole-3-acetic acid                    |
| ISR     | Induced systemic resistance             |
| Kb      | Kilo- base pair                         |
| KB      | King’s broth                            |
| LSD     | Least significant difference            |
| L-tryp  | L-tryptophan                            |
| MEA     | Malt extract agar                       |
| MPOB    | Malaysian Palm Oil Board                |
| mRNA    | Messenger RNA                           |
| NA      | Nutrient agar                           |
| NB      | Nutrient Broth                          |
| NPR1    | Nonexpressor of PR Genes1              |
| OD      | Optical density                         |
| PCA     | Phenazine-1-carboxylic acid             |
| PCN     | Phenazine-1-carboxamide                 |

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<td>PDA</td>
<td>Patato Dextrose Agar</td>
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<tr>
<td>PGPR</td>
<td>Plant Growth Promoting Rhizobacteria</td>
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<tr>
<td>PHZ</td>
<td>Phenazine</td>
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<tr>
<td>PIRG</td>
<td>Percentage Inhibition of Radial Growth</td>
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<td>PPM</td>
<td>Pigment production medium</td>
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<tr>
<td>PR</td>
<td>Pathogenesis –related</td>
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<tr>
<td>PYO</td>
<td>Pyocyanin</td>
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<tr>
<td>Plt</td>
<td>Pyoluteorin</td>
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<tr>
<td>PRN</td>
<td>Pyrrolnitrin</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>rpm</td>
<td>Rotation per minute</td>
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<tr>
<td>RT PCR</td>
<td>Reverse transcription Polymerase Chain Reaction</td>
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<tr>
<td>RWB</td>
<td>Rubber Wood Block</td>
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<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>sp.</td>
<td>Species (singular)</td>
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<tr>
<td>Spp.</td>
<td>Species (plural)</td>
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<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA buffer</td>
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<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>wpi</td>
<td>Week post inoculation</td>
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<td>Z</td>
<td>Zeatin</td>
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CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is the most crucial species in the genus *Elaeis* which belongs to the family Arecaceae (former name Palmae). It is one of the most important crops in the world and a major source of oils and fats. Although oil palm originated from West Africa and South America, it becomes popular in South Asia especially Malaysia and Indonesia. Currently, Malaysia is the world 2nd largest producer and exporter of palm oil (Bivi *et al.*, 2010; Halimah *et al.*, 2013). Palm oil has recently become the world’s leading edible vegetable oil, with Europe and China at present the major markets (Mayes *et al.*, 2008). Based on the prediction of the trends in the use of edible vegetable oils with an increasing world population, Corley (2009) postulated that the demand for edible vegetable oil will rise 250 million tons per year. High demand for the edible vegetables oil has pressured on the oil palm industry in Malaysia to improve the status of oil palm production in order to fulfil this requirement (Corley, 2009). According to Gustone (2011), the palm oil production in Malaysia increased from only 1.3 million tons in 1975, through 4.1 million tons in 1985, 7.8 million tons in 1995, 17.8 million tons in 2009/10 up to 18.8 million tons in 2012/13 (Source: OilWorld2013). Besides, the favourable climate, comparatively low labour costs, and the liberal policies of Government attract the oil palm developers to expand this crop in South East Asia (Colchester *et al.*, 2006).

The greatest threat to sustainable oil palm production in South East Asia is from *Ganoderma* diseases, caused by the white rot fungus *Ganoderma boninense*. Basal stem rot (BSR) infection of oil palm by *Ganoderma* in Malaysia was first recorded in 1931. It can kill more than 80% of stands by the time they are halfway through normal economic life that constitutes a major threat to sustainable oil palm production in South East Asia including Malaysia (Mazliham *et al.*, 2007). Since *Ganoderma* has caused severe losses of oil palm production, controlling it is an important factor. Although many control measures have been developed, until now there is no effective control measure for this disease. The available technique of disease control is fungicidal treatment, though often applied ineffectively. *In vitro* studies by Idris *et al.* (2002) claimed that numerous fungicides were strongly inhibitory towards growth of *Ganoderma*. This phenomenon is probably due to the fact that *Ganoderma* has various resting stages such as melanised mycelium, basidiospores and pseudosclerotia that are more resistant to fungicides.

Therefore, alternative control measures are focused on the use of biocontrol agents, including Plant Growth Promoting Rhizobacteria (PGPR). The use of PGPR as biocontrol agents of soil borne plant pathogens, as an alternative or complementary strategy to physical and chemical disease management, has been investigated for over 70 years (Weller, 2007). PGPR are indigenous to soil and the plant rhizosphere plays a major role in the biocontrol of plant pathogens. They can suppress a broad spectrum of bacterial, fungal, viral and nematode diseases. The use of PGPR has become a common practice in many regions of the world. Recent progress in our understanding of their diversity, colonizing ability, and mechanism of action, formulation and application should facilitate their development as reliable biocontrol agents against plant pathogens.
There are several PGPR inoculants currently commercialized that seem to promote growth through suppression of plant disease (bioprotectants), improved nutrients acquisition (biofertilizers), or phytohormone production (biostimulants). Bacteria in the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are the biological control agents predominantly studied and increasingly marketed. They suppress plant disease through at least one mechanism, production of antibiotics or siderophores and induction of systemic resistance. Endophytic PGPR such as species of *Serratia*, *Pseudomonas*, *Burkholderia* and *Bacillus* have been shown to be used as biological control agent against several fungal and bacterial disease agents (Soylu et al., 2005). PGPR provide different mechanisms for suppressing plant diseases. They include competition for nutrients and space, antibiosis by producing antibiotics and production of siderophores which limits the availability of iron necessary for the growth of pathogens. Other important mechanisms include production of lytic enzymes such as chitLQDVHV DQG 3 glucanases which degrade chitin and glucan present in the cell wall of fungi. Certain PGPR trigger a phenomenon known as induced systemic resistance (ISR) phenotypically similar to systemic acquired resistance (SAR). Some PGPR are particularly suitable to be used as biocontrol agents because they can produce large amounts of secondary metabolites to protect plants from phytopathogens and stimulate plant growth.

The production of phytohormones by PGPR is now considered to be one of the most important mechanisms by which many rhizobacteria promote plant growth. The phytohormone producing ability is widely distributed among bacteria associated with soil and plants. Studies have demonstrated that the PGPR can stimulate plant growth through the production of auxins, gibberellins and cytokinins or by regulating the ethylene in the plant (Spaepen et al., 2008). Siderophores are low molecular weight compounds that are produced by bacteria and fungi as iron (Fe) chelating agents. Various studies have isolated siderophores producing bacteria belonging to the *Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Streptomyces* (Kuffner et al., 2008) genera from the rhizosphere. Volatiles play an important role in suppression of *Ganoderma* and inhibit sclerotial activity, limiting ascospore production, and reducing disease levels. *Pseudomonas* spp. produces secondary metabolites, also capable of producing organic volatiles such as HCN, benzothiazole, cyclohexanol, dimethyl trisulfide, and nonanal completely inhibit mycelial growth or sclerotia formation, which suggest their potential role in biological control. The production of antibiotics by PGPR is considered one of the most powerful biocontrol mechanisms for combating phytopathogens. It constitutes a wide and heterogeneous group of low molecular weight chemical organic compounds. Under laboratory conditions many different types of antibiotics produced by PGPR have shown to be effective against phytopathogenic agents (Raaijmakers et al., 2002).

Plants react to pathogen attack by the activation of a variety of defense mechanism that culminate in a number of physical and biochemical changes in the host plant. Infection of plants by potentially pathogenic microorganism has been shown to result in the accumulation of a novel class of proteins termed ‘pathogenesis related protein’ or PR proteins. Several classes of PR proteins have been shown to correspond to the KGUROWLF HQ\PHV FKLWQDVH 3DXFDQDVH %RWK FKLWQDVH DQG glucanase are known to be introduced during fungal infection (Sekeli et al., 2003).
Chitinase plays an important role in protecting plants against potentially pathogenic organisms. β-1, 3-glucanase or chitinase activities are able to inhibit fungi by attacking the glucans and chitin that make up fungal cell walls. The use of PGPR is preferable to other biological control agents as they are internal colonizers.

The roles of PGPR in protecting plants against pathogens have been mentioned by several authors. Some of rhizobacteria from the genera *Pseudomonas* and *Burkholderia* might have the potential to control *G. boninense*, as they were mostly found in healthy roots from symptomless palms. Zaiton et al. (2006) tested 863 bacterial isolates. Among them only 256 isolates gave PIRG > 50%. Therefore, 60 isolates from this category were selected for further screening test based on culture filtrate test. Preliminary screening in vitro showed the genera *Pseudomonas* and *Burkholderia* might have the potential to control *G. boninense*, and also produce secondary metabolites inhibitory to its growth. The results of the in vitro screening supported this speculation as the bacteria with the highest PIRG in the dual culture and culture filtrate tests were mostly *Burkholderia* and *Pseudomonas*. The isolates of *Pseudomonas aeruginosa* (P3) and *Burkholderia cepacia* (B3) had very high PIRG in the dual culture (75.95% and 70.80%, respectively) and culture filtrate tests (85.00% and 88.43%, respectively).

Thus, *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 were tested against *G. boninense* in oil palm seedlings at glasshouse test and found that these two bacteria increased plant growth and were effective in suppressing BSR (disease reduction 76.27% and 42.20%, respectively) (Zaiton et al., 2008).

On the basis of these reports this two bacteria were selected for this study. However, the mechanism in which *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 associate themselves with the pathogen *Ganoderma* and the host plant is not known yet. Besides, the ability of these strains to produce antifungal metabolites including antibiotics, siderophores, and volatiles and their efficiency in suppression of *Ganoderma* causing BSR incidence in oil palm has not been reported. Therefore, the present study was undertaken to investigate the antimicrobial activities of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 against *Ganoderma* causing BSR disease. The effect of PGPR in suppressing *Ganoderma in vitro* was investigated considering several approaches comprising detection of phytohormones, antibiotics, siderophores and volatile substances, and in glasshouse trial where vegetative growth, disease incidence and gene expression were assessed.

The specific objectives of this study were:

1. To determine the mechanisms of plant growth promotion and pathogen suppression produced by *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3.

2. To identify and quantify the antibiotics produced by selected PGPR and to determine their effects on *Ganoderma boninense* mycelial growth in vitro.

3. To evaluate the effects of selected antibiotic application on the development of BSR disease and on the expression of defense related genes during *Ganoderma*-oil palm interaction.
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