UNIVERSITI PUTRA MALAYSIA

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

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FP 2014 52

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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

October 2014

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Specially dedicated

To

My dearest father Prof. Md. Abdul Wahab,

My loveliest mother Aleya Begum

&

My beloved husband Dr. Md. Mahbubur Rahman

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the Degree of Doctor of Philosophy

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 phytohomones, 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 *P. aeruginosa* UPMP3 and *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. *P. aeruginosa* UPMP3 was more effective than *B. cepacia* UPMB3 in suppressing *Ganoderma in vitro.* Based on these results, *P. aeruginosa* UPMP3 was selected for further studies. Three antibiotics namely PHZ, PCA and PYO were extracted from *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 *in vitro* bioassay, PHZ was more effective than PCA and PYO in suppressing *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 β-1, 3-glucanase were differentially expressed in oil palm roots with different treatments. Chitinase was expressed constantly throughout the sampling intervals while β-1, 3-glucanase expressed at later intervals. The results of this study showed that the antibiotic phenazine has strong antimicrobial activity against *G. boninense in vitro* and was comparable to chemical fungicide in suppressing BSR in glasshouse conditions. The antibiotic phenazine extracted from *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

Oktober 2014

Pengerusi: Profesor Madya Wong Mui Yun, PhD

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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 dan menentukan jumlah antibiotik yang dihasilkan serta menilai kesan antibiotik tersebut ke atas tumbesaran miselia *G. boninense* secara *in vitro* dan (iii) menentukan kesan penggunaan antibiotic terhadap perkembangan penyakit BSR dan ekspresi gen pertahanan dalam interaksi *G. boninense*-kelapa sawit. Eksperimen dijalankan untuk mengenalpasti fitohormon, antibiotik, siderofor dan bahan meruap 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 kesan antibiotik dan bahan meruap yang dihasilkan terhadap *G. boninense.* Eksperimen seterusnya dijalankan di rumah kaca dengan menggunakan antibiotic tertentu bagi menilai perkembangan penyakit pangkal batang reput dan mengesan 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 meruap 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 dengan HPLC. Standard untuk PHZ, PCA dan PYO telah dipisahkan sepenuhnya pada masa retensi masing-masing 41.590, 39.740 dan 34.863 min. Pada jarak gelombang 250 nm, strain bakteria telah menghasilkan kepekatan PHZ (1.36 µg/mL) yang tertinggi 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-glukanase 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-glukanase berlaku lebih lambat. Keputusan kajian ini menunjukkan bahawa antibiotik fenazin mempunyai aktiviti antimikrob yang tinggi terhadap *Ganoderma in vitro* dan menunjukkan persamaan dengan racan 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 seterusmya mengurangkam kesan berbahaya terhadap alam sekitar.

ACKNOWLEDGEMENTS

First of all, I am grateful to Almighty Allah the most merciful for blessing me with health, power and support throughout my PhD study.

I would like to take this opportunity to express my deepest gratitude and special appreciation to my supervisor, Assoc. Prof. Dr. Wong Mui Yun for her thoughtful advice, valuable guidance, continuous inspiration and cooperation in making the completion of this thesis a success. My sincere appreciation is also extended to Assoc. Prof. Dr. Radziah Othman and Assoc. Prof. Dr. Hawa Jaafar, members of my supervisory committee for their valuable advice, technical guidance, and cooperation to complete the thesis.

I would like to express my gratitude the Emerging Nations Science Foundation for offering me one year fellowship of my post graduate study. I also acknowledged South East Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) for my thesis grant. My special thanks to Dr. Wahed Baksha, the former Director of Bangladesh Forest Research Institute, and also the present Director, Dr. Mukhlesur Rahman and my all colleagues for their inspiration and continuous support to complete my study.

A heartfelt thanks to all the former and current staffs, members and lab mates of Plant Protection Department, UPM for their support, help, encouragement and good times. Special thanks to Dr. Qazi Shirin Akhter Jahan, for helping me during my HPLC studies. Grateful appreciation to Sime Darby Seeds and Agricultural Services Sdn Bhd. for providing the oil palm seeds and seedlings in good condition. I would like to express my all course teachers of UPM for their valuable assistance during my study. I wish to thanks all of my friends and staffs from different faculties of UPM for their lab support.

Lastly, I want to express my deepest gratitude to my dearest parents, for teaching me to believe in myself. Special thanks to my affectionate younger brothers Engr. Sharif Mahmud and Ashique Mahmud for their technical guidance, consistent support, love and encouragement. My sincere appreciation also goes to my respected mother in law, my late father in law, my brothers and sisters for their inspiration, prayer, care and love, accompanying me to go through all my happiness and sadness in my study and encouragement for my success.

Finally, to the one who is not only close to myself, but also close to my heart and mind, my pillar of strength and success at every step in my life, my beloved husband, Dr. Md. Mahbubur Rahman, thanks for all the endless love, support, and continuous encouragement during the good and hard times it took for me to finish this work. Thanks also for spending his valuable time in reading and editing my thesis. His patience and understanding were essential to complete my thesis and sacrificing a lot in the course of my study which could never be paid.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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

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

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 KDVUHFHQWO\EHFRPHWKHZRUOG¶VOHDGLQJHGLEOHYHJHWDEOHRLOZLWK(XUR 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 to18.8 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 half-way 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 -1, 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 accumulaWLRQRIDQRYHOFODVVRISURWHLQVWHUPHGµSDWKRJHQHVLVUHO or PR proteins. Several classes of PR proteins have been shown to correspond to the KGUROWLF HQ]PHV FKLWLQDVH DQG -1, 3-JOXFDQDVH %RWK FKLWLQDVH -1, 3- 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 organisP)RU H[DPSOH -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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