UNIVERSITI PUTRA MALAYSIA

APPLICATION OF PHYLLOSPHERE BACTERIAL ANTAGONIST AGAINST RICE SHEATH BLIGHT

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FP 2015 87

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By

SHAMIMA AKTER

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

January 2015

DEDICATION

To

My affectionate parents with gratitude

and

My beloved husband for his sacrifices and understanding

Abstract of thesis presented to the Senate of Universiti Putra Malaysia, in fulfillment of the requirements for the degree of Doctor of Philosophy

APPLICATION OF PHYLLOSPHERE BACTERIAL ANTAGONIST AGAINST RICE SHEATH BLIGHT

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January 2015

Chairman : Associate Professor Jugah B. Kadir, PhD

Faculty : Agriculture

Antagonistic bacteria originating from the rhizosphere are being used in sheath blight management. However, little is known about the potential of bacteria inhabiting the phyllosphere. Hence, a study was initiated with the aim of assessing the effective bacterial antagonists against the disease. A total of 325 bacterial isolates obtained from 100 rice plant samples collected from different locations of Malaysia and Bangladesh were preliminarily screened. Out of the 325 bacterial isolates, 14 were selected based on their ability to inhibit the growth of *R. solani*. In dual culture tests isolates KMB25, TMB33, PMB38, UMB20 and BMB42 showed 68.44, 60.89, 60.22, 50.00 and 48.22% inhibitions, respectively. In extracellular metabolites tests most of these isolates showed comparatively higher percentages of growth inhibition of the fungus than in dual culture tests. Selected isolates were negative to indole, methyl red, Voges Proskaeur, and starch hydrolysis but positive to catalase, urease, and nitrate reduction tests. Isolates KMB25, TMB33 and PMB38 were positive to gelatin liquefaction, while isolates UMB20 and BMB42 were negative to the test. Biolog identified *P. fluorescens* (UMB20), *P. aeruginosa* (KMB25, TMB33 and PMB38) and *P. asplenii* (BMB42) with the similarity index ranging from 0.52 to 0.70. The identities of the selected three bacterial isolates UMB20, KMB25 and BMB42 were further confirmed through 16S rDNA gene sequencing. According to the GenBank database of NCBL, UMB20 and BMB42 were identified as *P. fluorescens* and KMB25 as *P. aeruginosa.* Fungal growth inhibition ranging from 86.85 to 93.15% was obtained by these strains in volatile and 100% was in diffusible metabolites test. Among the 3 strains, UMB20 and BMB42 produced indole 3-acetic acid and chitinase, but not protease. All of them produced cellulase, siderophore, HCN, and ammonia and were able to solubilize phosphate. Strains UMB20 and BMB42 were preserved in peat and talc as single strains or in mixtures. The peat formulation was found to be more suitable than talc to retain longer shelf life of individuals and strain mixtures with sufficient viable cells. At 4°C of storage condition peat formulations were better than room temperature (28±2°C) condition. In bioefficacy tests of peat based bacterial formulations under glass house conditions, the strain mixture and UMB20 alone significantly reduced the disease severity in terms of area under disease progress curve (AUDPC) compared to the untreated control. Percent reduction of AUDPC was 32.79, 32.58 and 21.19 for strain mixture (UMB20+BMB42), UMB20 and BMB42, respectively. Significantly lowest disease progression rate (0.01unit/day) was found in the strain mixture applied plants. In addition to disease suppression, the strain mixture enhanced the plant height, percentage of effective tillers per hill, and percentage of fertile spikelets per panicle. Effects of all the treatments on flag leaf area, total number of tillers and number of effective tillers were insignificant. Significantly highest weight of 100-grain (1.65 g) was obtained from the strain mixture applied plants. The *Pseudomonas* bacteria isolated from rice plants had the potential to inhibit the fungal growth *in vitro* and *in vivo* and possessed most of the plant growth promoting characteristics. They have the potential to be utilized as biocontrol agents for management of sheath blight in rice.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

APLIKASI BAKTERIA ANTAGONIS FILOSFERA TERHADAP PENYAKIT HAWAR SELUDANG PADI

Oleh

SHAMIMA AKTER

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Bakteria antagonistik berasal dari rizosfera telah digunakan dalam pengurusan hawar seludang. Walau bagaimanapun, sedikit yang diketahui mengenai potensi bakteria yang mendiami filosfera. Oleh itu, satu kajian telah dimulakan dengan tujuan untuk menilai bakteria antagonis yang berkesan terhadap penyakit itu. Sejumlah 325 pencilan bakteria yang diperolehi daripada 100 sampel tanaman padi yang diambil dari lokasi yang berlainan di Malaysia dan Bangladesh yang pada awalnya telah disaring. Daripada 325 pencilan bakteria, 14 telah dipilih berdasarkan keupayaan mereka untuk menghalang pertumbuhan *R. solani*. Dalam ujian dua pencilan kultur, KMB25, TMB33, PMB38, UMB20 dan BMB42 telah menunjukkan 68,44, 60,89, 60,22, 50.00 dan 48.22% perencatan, masing-masing. Dalam ujian metabolik ekstrasel, kebanyakan pencilan ini menunjukkan peratusan yang agak tinggi di dalam perencatan pertumbuhan kulat daripada dalam ujian dua kultur. Pencilan terpilih adalah negatif untuk indol, metil merah, voges proskaeur, dan hidrolisis kanji tetapi positif untuk katalase, urease, dan ujian penurunan nitrat. Pencilan KMB25, TMB33 dan PMB38 pula positif kepada pencairan gelatin, manakala pencilan UMB20 dan BMB42 adalah negatif untuk ujian ini. Sistem biolog telah mengenal pasti *P. fluorescens* (UMB20), *P. aeruginosa* (KMB25, TMB33 dan PMB38) dan *P. asplenii* (BMB42) dengan indeks persamaan antara 0.52 – 0.70. Identiti tiga pencilan bakteria yang dipilih UMB20, KMB25 dan BMB42 disahkan lagi melalui penjujukan gen 16S rDNA. Menurut pangkalan data gen bank UMB20 dan BMB42 telah dikenal pasti sebagai *P. fluorescens* dan KMB25 sebagai *P. aeruginosa*. Perencatan pertumbuhan kulat yang terdiri daripada 86.85 – 93.15% telah diperolehi oleh strain ini dalam bentuk meruap dan 100% adalah dalam ujian peresapan metabolit. Di antara 3 jenis strain, UMB20 dan BMB42 menghasilkan indole 3-asetik asid dan kitinase, tetapi tidak protease. Kesemua mereka menghasilkan selulase, siderophore, HCN, dan ammonia dan mampu mencairkan fosfat. Strain UMB20 dan BMB42 adalah dipelihara dalam tanah gambut dan talkum sebagai jenis tunggal atau campuran. Formulasi gambut pula didapati lebih sesuai daripada talkum untuk mengekalkan jangka masa hidup bagi strain individu dan campuran dengan sel-sel hidup yang mencukupi. Penyimpanan gambut di dalam suhu 4°C keadaan sejuk adalah lebih baik daripada suhu bilik (28±2°C). Di dalam ujian bioefikasi gambut

berasaskan formulasi bakteria di bawah keadaan rumah kaca, campuran strain dan UMB20 sahaja keterukan penyakit ini berkurangan dengan signifikan dari segi kawasan di bawah lengkung kemajuan penyakit (AUDPC) berbanding dengan kawalan yang tidak dirawat. Peratus pengurangan AUDPC adalah 32.79, 32.58 dan 21.19 masing-masing untuk campuran strain (UMB20 + BMB42), UMB20 dan BMB42. Kadar perkembangan penyakit terendah (0.01unit / hari) sangat signifikan untuk campuran strain kepad tanaman yang digunakan. Selain kawalan penyakit, campuran strain telah meningkatkan ketinggian tumbuhan, peratusan anak padi yang efektif bagi setiap bukit, dan peratus spikelet yang subur bagi setiap penikel. Kesan semua rawatan kepada kawasan keluasan daun, jumlah anak padi dan bilangan anak padi juga tidak ketara. Berat tertinggi yang signifikan bagi 100-bijian (1.65 g) telah diperolehi daripada campuran strain kepada tanaman yang digunakan. Bakteria *Pseudomonas* yang dipencilkan daripada tanaman padi mempunyai potensi untuk menghalang pertumbuhan kulat *in vitro* dan *in vivo* dan mempunyai kebanyakan ciri-ciri penggalak pertumbuhan tanaman. Ia juga mempunyai potensi untuk digunakan sebagai agen kawalan biologi untuk pengurusan hawar seludang untuk padi.

ACKNOWLEDGEMENTS

All praises to Almighty Sobhan Allahutala for His endless blessings, kindness, guidance, strength for successfully completion of my degree. May His name is glorified and merciful.

At the outset, I would like to express my deep sense of gratitude to Associate Professor Dr. Jugah B Kadir, the Chairman of my supervisory committee for his meticulous guidance, motivation and help throughout my research and preparation of this thesis. This work would never see the light of the day without his planning, ethical and moral support in my research and constant encouragement. His ceaseless and propitious academic help is exemplary. I have learnt many things from him during my work. His concern in everything including my illness and bad time gave me inner strength to go on.

I fall short words to express my sincere respects, gratefulness to my supervisory committee member Dr. Abdul Shukor Juraimi, Professor and Dean, Faculty of Agriculture for his scholastic and valuable advice, financial help and moral support in all phases of my study.

I profusely thank another member Dr. Halimi Mohd Saud, Associate Professor and Deputy Dean, Faculty of Agriculture for his affectionate feelings, logistic support from his laboratory, and cordial assistance in my academic all through.

I am thankful to all staff members of Plant Protection Department, Faculty of Agriculture, UPM for their cordial help and assistance to conduct the research in the laboratory as well as in glass house. I acknowledge the help from my colleagues and friends, Dr. Abdul Latif, Yeasmin, Mahmud, Fahim, Amirul, Salha and Bibi.

I am expressing my heartfelt gratitude to the Government of the People's Republic of Bangladesh for granting me the scholarship under NATP program to pursue the PhD degree. My deepest gratitude to my institute Bangladesh Rice Research Institute (BRRI) for the approval my deputation. I would like to thank all of my colleagues at BRRI for their helps in different ways.

I am indebted to my parents, Md. Azaher Ali and Mrs. Ashful Begum. I respectfully acknowledge their love, blessings and good wishes. I am grateful to all of my teachers from primary to university levels. I am thankful to all of my sisters, brothers, neighbors, relatives for their love and encouragement to pursue the higher studies.

Specially, I like to thank Md. Solaiman Hossain, my beloved husband for his love, tremendous patience, sacrifices and continuous moral support. Without his sacrifices and better understanding I could not be able to complete my degree.

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

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LIST OF ABBREVIATIONS

%	Percent
PDI	Percent Disease Incidence
%RLH	Percent Relative Lesion Height
µg	Microgram
µL	Microliter
2, 4- DAPG	2, 4-Diacetylphloroglucinol
AG	Anastomosis Group
ANOVA	Analysis of Variance
AUDPC	Area Under Disease Progress Curve
bp	Base pairs
CAS	Chromo Azurol S
cfu	Colony Forming Units
CMC	Carboxymethyl cellulose
CRD	Completely randomized design
CTAB	Cetyl Trimethyl-ammonium bromide
DMAB	*p*-dimethylaminobenzaldehyde
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
GL	Gelatin Liquefaction
GN	Gram-Negative
ha	Hectare
HCN	Hydrogen Cyanide
HDTMA	Hexadecyletrimethyllammonium
HPLC	High Performance Liquid Chromatography
HR	Hypersensitivity reaction
IAA	Indole acetic acid
IAR	Intrinsic Antibiotic Resistance
ISR	Induced Systemic Resistance
KBA	Kings B Agar
KBB	Kings B Broth
kg	Kilogram
LBB	Luria-Bertani Broth
LSD	Least Significant Difference
L-Trp	L-Tryptophan
M	Molar
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
MP	Muriate of Potash
MR	Methyl red
N	Normal
NA	Nutrient Agar
NB	Nutrient Broth
NBRIP	National Botanical Research Institute`s Phosphate

nm	Nanometer
NPK	Nitrogen Phosphorus Penta oxide Potassium oxide
NR	Nitrate Reduction
^{o}C	Degree Celsius
p.	Page
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria
PIRG	Percent Inhibition of Radial Growth
PO	Peroxidase
pp.	Pages
ppm	Parts per million
psi	Pound per square inch
Rf	Retention Factor
RH	Relative Humidity
rpm	Rotation per minute
SA	Salicylic acid
SAS	Statistical Analysis System
SDS	Sodium dodecyl sulphate
ShB	Sheath blight
SM	Succinic Medium
SMA	Skim Milk Agar
sp.	species (singular)
spp.	species (plural)
TAE	Tris - Acetate - EDTA
TSB	Trypticase Soya Broth
TSP	Triple Super Phosphate
UV	Ultra Violet
v/v	Volume per volume
VP	Voges Proskeaur
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) is the most widely cultivated food crop in the world and is the staple food for more than half of the world's population (Zeigler and Barclay, 2008). The major rice producer countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil, and Japan (IRRI, 2008; FAO, 2013). In Malaysia, rice is the third most important crop next to rubber and oil palm. Selangor, Kedah, Penang and Perak are the main rice growing states in Peninsular Malaysia. Currently, rice self-sufficiency level is around 73% with an average yield of 3.7 t ha^{-1} which is below the potential level (Akinbile *et al*., 2011).

Yield potential of rice under farmer's field conditions has never been achieved due to be encountered by different biotic and abiotic stresses. Among the biotic stresses, diseases play important roles in reducing the yield. Rice is confronted with more than 70 diseases caused by fungi, bacteria, viruses or nematodes (Manidipa *et al.*, 2013). Out of these, sheath blight caused by *Rhizoctonia solani* AG-1 IA is the most important disease in tropical and subtropical rice growing countries of the world with the incidence of more than 90% (Mathivavan and Shanmugaiah, 2011) incurring yield losses up to 50% annually (Zheng *et al.*, 2013).

To combat the disease, different management practices including host resistance, cultural practices, and chemical and biological control measures are being used. The disease cannot be satisfactorily managed through host resistance alone because of the low level of inherent resistance to this pathogen. Unfortunately, no resistant rice cultivar against the pathogen is available elsewhere (Ou, 1985; Suudi *et al.*, 2013). However, cultural practices have contributed to the management of the disease to some extent. Currently, management of this disease is depended on chemical fungicides. Widespread and indiscriminate use of chemical fungicides has been a serious concern of public health and scrutiny due to hazardous effects on the environment, non-target organisms and possible carcinogenicity of some chemicals (Heydari, 2007; Heydari *et al.*, 2007). Other problems include, development of new resistant races of pathogens (Houssein *et al.*, 2010), plant phytotoxicity (Foster and Hausbeck, 2010) and high cost of chemicals. Increasing awareness of these fungicide-related problems has emphasized the need for non-chemical and eco-friendly disease control methods. In this context, biological control of sheath blight is viewed as a viable and cost effective method with sustainable yield potential.

Biological control using bio-agents is an excellent alternative to chemicals and has been proven successful for controlling plant diseases in many countries. Various fungi from the genus *Aspergillus, Gliocladium, Paecilomyces, and Trichoderma* and bacteria from the genus *Bacillus, Pseudomonas, Serratia, Erwinia, Rhizobium* and *Paenibacillus* are good examples. Among them, bacteria under the genus *Pseudomonas* especially fluorescent pseudomonads are important candidates for biological control and have been successfully used in sheath blight management across the world. The beneficial effects of *P. fluorescens* have been attributed to the production of antibiotics, metabolites, phytohormones, siderophores, lytic enzymes, and hydrogen cyanide (HCN); and to phosphate solubilization, and induction of systemic resistance against different pathogens (Podile and Kishore, 2006)

Pseudomonas is known as an aggressive colonizer in both rhizosphere and phyllosphere. A great majority of antagonistic bacteria have been isolated from the rhizosphere, identified and used as biocontrol agents (Manjula *et al*., 2002; Sessitsch *et al*., 2004), and a few from the phyllosphere. The cells of microrganism inhabiting the phyllosphere are often exposed to various physical and chemical stresses due to fluctuation of environmental conditions. Thus, they possess the additional advantages in terms of survivability and virulence under stress conditions compared to the rhizoshpere. Hence, there is a possibility to explore for more potential bacteria from this habitat. Until now, no indigenous phylloplane bacteria against *Rhizoctonia solani* on rice have been documented in Malaysia. Therefore, this study was designed to isolate antagonistic bacteria from rice plant samples collected from different rice growing regions of Peninsular Malaysia. It was hypothesized that these bacterial antagonists will be the effective biocontrol agents against *R. solani* causing sheath blight in rice.

To accomplish this, laboratory and glasshouse experiments were conducted with the following major objectives-

1) To characterize and identify the antagonistic bacteria isolated from rice plants
2) To determine the modes of action deployed in biocontrol of sheath blight and plant growth promoting activities by selected bacterial strains
3) To determine the efficacy of selected bioformulated bacterial strains against sheath blight under glasshouse conditions

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