

UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF STREPTOMYCES AND THEIR BIOCONTROL ACTIVITIES AGAINST RICE BLAST PATHOGEN *Pyricularia oryzae*

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By

HAYMAN KAKA KHAN AWLA

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

May 2016



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DEDICTION

I Would Like To Dedicate My Thesis To

My Beloved Parents

My Lovely Wife

My Dearly Loved Two Kids

Ahmad

Tlova



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

CHARACTERISATION OF STREPTOMYCES AND THEIR BIOCONTROL ACTIVITIES AGAINST RICE BLAST PATHOGEN *Pyricularia oryzae*

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May 2016

Chairman : Associate Professor Wong Mui Yun, PhD Faculty : Agriculture

Blast disease caused by the fungus Pyricularia oryzae is the most significant disease affecting rice cultivation and causing serious yield losses. To reduce the usage of chemicals, an alternative method for a sustainable control of the disease is necessary. Streptomyces have been known to produce antimicrobial compounds and are potential biocontrol agents. This study was carried out to isolate, identify and characterise Streptomyces isolates for in vitro screening against P. oryzae, to identify the antimicrobial compounds produced by selected Streptomyces isolates and their mechanisms of biocontrol, and to evaluate the efficacy of selected Streptomyces isolate against P. oryzae in the glasshouse. A total of 54 Streptomyces isolates were obtained from four sites in peninsula Malaysia including healthy and infected paddy fields. All the isolates were tested against P. oryzae by dual culture test on Potato Dextrose Agar (PDA) and 14 isolates showed growth inhibition of P. oryzae. The best two isolates with more than 80% Percentage of inhibition of radial growth (PIRG %) were selected for subsequent experiments. The isolates were identified as Streptomyces sp. based on the morphological, physiological and chemical characteristics and confirmed with 16S rRNA sequence analysis that was compared with related bacteria in GenBank database using MEGA 6.1. Isolates UPMRS4 (Streptomyces sp.) and UPM28 (Streptomyces zaomyceticus) were found to be the most effective Streptomyces against the pathogen with PIRG showing 98.3% and 86.3%, respectively. For extraction of bioactive compounds from UPMRS4, eight different solvents were used and the crude extract obtained from ethyl acetate was found to give the highest PIRG (88%). Bioactive volatile compounds from the ethyl acetate crude extract were identified using gas chromatography-mass spectrometry (GC-MS). Twenty-two volatile compounds were identified as major compounds in the isolated UPMRS4 that were possibly responsible for the antifungal activity. Four nonvolatile compounds from the same crude extract were identified using Liquid chromatography-Mass spectrometry MS (LC-MS MS) and the main compounds were N-Acetyl-D, L-phenylalanine, amicoumacin, fungichromin and rapamycin. For the glasshouse study, UPMRS4 was used as a seed coating for in vivo study with four treatments. The results showed significant disease suppression and enhancement of yield attributes compared to the untreated control. UPMRS4 isolate was able to reduce 80% of disease severity compared with other treatments and increase shoot height (15.13%), shoot dry weight (45.7%), leaf surface area (44.6%),

root length (48.9%), root dry weight (63.2%), number of tillers (42.2%), yield (36.9%), panicle length (15.4%) and the number of spikelets/panicle (29.3%) compared to the control plants at three months after inoculation. Defense-related gene expression in rice leaf samples with seed coating application in both inoculated and uninoculated with *P. oryzae* was evaluated using quantitative real-time PCR. Chitinase (*Cht-1*), β -1, 3glucanase, *OsPRla, Oswrky45* and *OsJAMYB* genes were selected for the study during the three days. Rice plants inoculated with *Streptomyces* sp. UPMRS4 isolate demonstrated a higher abundance of defence gene expression compared with the nontreated controls. The outcomes indicate that UPMRS4 induced rice defence by enhancing the expression levels of OsPR1a, *Cht-1*, *Gns1*, *Oswrky45* (SA) and *OsJAMYB* (JA).



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PENCIRIAN ASINGAN STREPTOMYCES DAN AKTIVITI BIOKAWALANNYA MELAWAN PATOGEN BLASTA PADI *Pyricularia oryzae*

Oleh

HAYMAN KAKA KHAN AWLA

Mei 2016

Pengerusi : Profesor Madya Wong Mui Yun, PhD Fakulti : Pertanian

Penyakit blasta yang disebabkan oleh fungus Pyricularia oryzae merupakan penyakit paling utama yang menjejaskan penanaman padi dan mendatangkan kerugian hasil yang serius. Untuk mengurangkan penggunaan bahan kimia, kaedah alternatif untuk memperoleh kawalan yang mampan terhadap penyakit ini adalah perlu. Streptomyces telah diketahui dapat menghasilkan sebatian antimikrob dan berpotensi sebagai agen biokawalan. Kajian ini dijalankan untuk mengasing, mengenal pasti dan membuat pencirian terhadap asingan Streptomyces bagi penyaringan secara in vitro terhadap P. oryzae, untuk mengenal pasti sebatian antimikrob yang dihasilkan oleh asingan Streptomyces terpilih dan mekanisme biokawalannya, dan untuk menilai efikasi asingan Streptomyces terpilih melawan P. oryzae dalam rumah kaca. Sejumlah 54 asingan Streptomyces telah diperolehi dari empat tapak kajian di semenanjung Malaysia termasuklah padi sihat dan terjangkit. Kesemua asingan telah diuji terhadap P. orvzae secara ujian dwi kultur ke atas Agar Kentang Dektrosa (PDA) dan 14 asingan menunjukkan perencatan tumbesaran terhadap P. oryzae. Dua asingan terbaik dengan lebih daripada 80% peratus tumbesaran jejarian inhibition of radialgrowth (PIRG %) telah dipilih bagi eksperimen selanjutnya. Asingan tersebut telah dikenal pasti sebagai Streptomyces sp. berdasarkan ciri-ciri morfologi, fisiologi dan kimia serta disahkan dengan analisis jujukan 16S rRNA yang dibandingkan dengan bakteria berkait dalam pangkalan data GenBank menggunakan MEGA 6.1. Asingan UPMRS4 (Streptomyces sp.) dan UPM28 (Streptomyces zaomyceticus) telah dikenal pasti sebagai asingan Streptomyces yang paling efektif terhadap patogen tersebut dengan peratusan PIRG 98.3% dan 86.3%, masing-masingnya. Bagi pengekstrakan sebatian bioaktif dari UPMRS4, lapan pelarut berbeza telah digunakan dan ekstrak mentah yang diperolehi daripada etil asetat telah didapati memberikan peratusan PIRG tertinggi (88%). Sebatian bioaktif meruap daripada ekstrak mentah etil asetat telah dikenal pasti menggunakan kromatografi gas-spektrometri jisim (GC-MS). Dua puluh dua sebatian meruap telah dikenal pasti sebagai sebatian utama dalam UPMRS4 yang diasingkan, yang berkemungkinan bertanggung jawab terhadap aktiviti antifungus. Empat sebatian tidak meruap daripada ekstrak mentah yang sama telah dikenal pasti menggunakan kromatografi cecair-spektrometri jisim MS (LC-MS/MS) dan sebatian utama adalah N-Asetil-D, L-fenilalanine, amicoumacin, fungikromin and rapamycin. Bagi kajian rumah kaca, UPMRS4 telah digunakan sebagai penyalut benih bagi dua kajian in vivo dengan empat rawatan. Hasil menunjukkan menunjukkan perencatan penyakit yang ketara dan peningkatan sifat-sifat hasil berbanding sampel kawalan yang tidak dirawat. Asingan UPMRS4 telah berjaya mengurangi 80% keterukan penyakit berbanding dengan rawatan lain dan meningkatkan ketinggian pucuk (15.1%), berat kering pucuk (45.7%), luas permukaan daun (44.6%), panjang akar (48.9%), berat kering akar (63.2%), bilangan anak gagan (42.2%), hasil (36.9%), panjang panikel (15.4%) dan bilangan spikelet/panikel (29.3%) berbanding tumbuhan kawalan pada tempoh tiga bulan selepas inokulasi. Ekspresi gen terkait-pertahanan dalam sampel daun padi yang menggunakan salutan benih dalam kedua-dua P. oryzae yang terinokulasi dan tidak terinokulasi telah diuji menggunakan analisis kuantitatif PCR masa-nyata. Gen kitinase (Cht-1), β -1, 3glukanase, OsPRla, Oswrky45 dan OsJAMYB telah dipilih untuk dikaji selama tiga hari. Pokok padi yang terinokulasi dengan asingan Streptomyces sp. UPMRS4 menunjukkan kelimpahan ekspresi gen pertahanan yang lebih tinggi berbanding sampel kawalan yang tidak dirawat. Dapatan kajian menunjukkan UPMRS4 mengaruh sistem pertahanan padi dengan meningkatkan aras ekspresi OsPR1a, Cht-1, Gns1, Oswrky45 (SA) dan OsJAMYB (JA).

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LIST OF ABBREVIATIONS

Avr	Pathogen avirulence
Bar	A unit of pressure
BCAs	Biocontrol agents
CFU	Colony Forming Unit
cfu/ml	Colony forming unit per milliliter
CRD	Completely randomized design
DHP	Di-potassium hydrogen phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNTPs	Deoxynucleoside triphosphates
DW	
EDTA	Dry weight
	Ethylenediaminetetra acetic acid
Fwd_name	Forward name
Fwd_seq	Forward sequence
GC-MS	Gas chromatography mass spectrometry
GL	Gelatin liquefaction
На	Hectare
HPLC	High performance liquid chromatography
HR	Hypersensitivity reaction
IAA	Indole 3-acetic acid
ISR	Induced systemic resistance
ITS	Internal transcribed spacer regions
Kb	Kilo base
Μ	Molar
M. WT.	Molecular weight
MeOH	Methanol
Mg	Milligram
MIC	Minimum inhibitory concentration
Mm	Milli molar
NaCO ₃	Sodium carbonate
NaOH	
	Sodium hydroxide
NCBI	National centre of biotechnology information
Ng	Nanogram
Nm	Nanometer
UPMP.o	Pyricularia oryzea
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Ph	Logarithm of hydrogen ion activity
PSB	phosphate solubilizing bacteria
PSM	phosphate solubilizing microorganisms
QTLs	Quantitative Trait loci
R	Resistance
rDNA	Ribosomal deoxyribonucleic acid
Rev name	Reverse name
Rev seq	Reverse sequence
RNA	Ribonucleic acid

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Rpm	Revolving per minute
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
Tris-HCl	hydroxymethyl-hydrochloride
TSB	trypticase soya broth
u/µl	Unit per microliter
units/ml	Units per milliliter
UPMRS	University Putra Malaysia RrhizoStreptomyces
v/v	Volume per volume
Ver.	Version
w/v	Weight per volume



CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most important crops and it is consumed as a staple as well as primary source of energy and protein (Zhang *et al.*, 2007). The major rice producer countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil, and Japan (IRRI, 2008; FAO, 2013). Rice provides nutrition for six billion people worldwide (Maclean *et al.*, 2002). Asia, is home to 60 % of the world's population and where 90 % of the world's rice is grown. More than 3 billion Asians get 35–75 % of their calories from rice and its products (Khush, 2005).

In Malaysia, rice is a major crop besides oil palm, rubber, and cocoa grown by the private and public sectors. Approximately 72% of Malaysia's rice is grown in eight granary areas that produce two crops annually. In Malaysia rice is mainly grown as wet paddy and only small parts in east Malaysia are under dry land paddy cultivation (Papademetriou, 2001).

The production of rice faces many threats such as pests and diseases. Rice blast disease, caused by the fungus *Pyricularia oryzae*, ranks among the most significant diseases that affect rice cultivation because it is widespread in most of the rice growing areas and seriously affects the quantity and quality of yield (Hayasaka *et al.*, 2008). The disease is found in more than 85 countries (Hajano *et al.* 2011) and under the right environmental conditions it can be very damaging (Scardaci *et al.*, 1997). Losses in yield and harvest of the world rice crop have averaged 10-30% annually (Tongen *et al.*, 2006), and this affects significantly the cost of rice production. The disease can infect all above ground parts of the rice plant. Direct and indirect rice yield losses are due to leaf and panicle blast respectively (Silva *et al.*, 2009).

There are many difficulties in finding a solution for rice blast disease, although some control methods have been identified to be effective such as fungicide application, managing planting time as well as resistant cultivar usage. However, applying fungicides is not a preferred option due to cost implications as well as the fact that it is not friendly to the environment. Furthermore, the spread of the disease can be very rapid and using resistant varieties is at best only for the short term. (Baldwin *et al.*, 2004).

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, actinobacteria is a phylum of Grampositive bacteria, which are important candidates for biological control and have been successfully used in rice diseases management worldwide. The beneficial effects of actinomycetales have been attributed to the production of antibiotics, antifungals, metabolites, siderophores, lytic enzymes, hydrogen cyanide (HCN), phosphate solubilization, and induction of systemic resistance against different pathogens (Podile and Kishore, 2006).

Streptomyces is a largest genus of rhizobacteria known to be a rare source of bioactive metabolites which are able to produce vital compounds for medicine and agriculture (Prabavathy *et al.*, 2006) after undergoing various biological processes. *Streptomyces* are able to successfully control soil-borne plant pathogenic fungi by hydrolyzing their cell walls. Actinobacteria are being used currently as agents for the biological control of major pathogenic microorganisms in plants (Keiser *et al.*, 2000; Shahidi *et al.*, 2004).

To our best knowledge, until now no indigenous *Streptomyces* species against *Pyricularia oryzae* on rice have been documented in Malaysia. Therefore, this study was designed to isolate antagonistic actinomycetales from rice plant rhizosphere samples collected from different rice growing regions of Peninsular Malaysia. It was hypothesized that these *Streptomyces* antagonists will be effective for biocontrol against *P. oryzae* causing blast disease in rice.

The objectives of this research are:

- 1) To isolate, identify and characterise *Streptomyces* isolates from healthy rice fields and their biocontrol activity against *P. oryzae*.
- 2) To identify the antimicrobial compounds produced by selected *Streptomyces* isolates and their mechanism of biocontrol.
- 3) To evaluate the efficacy of selected *Streptomyces* isolate against rice blast disease under glasshouse conditions.

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LIST OF PUBLICATIONS

- Awla, H. K., Kadir, J., Othman, R., Rashid, T. S., and Wong, M. Y. (2016). Bioactive Compounds Produced by Streptomyces sp. Isolate UPMRS4 and Antifungal Activity against Pyricularia oryzae. American Journal of Plant Sciences, 7(07), 1077.
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- Hayman kakakhan Awla, Jugah Kadir, Radziah Othman and, Wong Mui Yun Characterization of *Streptomyces* sp. and its antagonistic activity against *Pyricularia oryzae* causing rice blast. Poster presented in the 2nd International Conference on Crop Improvement (ICCI 2015). Engineering Auditorium, Faculty of Engineering, Universiti Putra Malaysia, 2-3 December 2015.