CHARACTERIZATION AND EVALUATION OF THE BIOLOGICAL CONTROL ACTIVITY OF *Stenotrophomonas maltophilia* AGAINST RICE BLAST DISEASE CAUSED BY *Pyricularia oryzae*

By

AZADEH BADRI FARIMAN

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

January 2020

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DEDICATION

Specially dedicated to,

My dear supervisor, Prof. Dr. Wong Mui Yun; My dearly loved late Father, Nasser; My beloved Mother, Fatemeh; and dear sister, Shabnam for their invaluable love, understanding, tolerance, sacrifice and moral support.

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

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January 2020

Chairman : Professor Wong Mui Yun, PhD
Institute : Tropical Agriculture and Food Security

Blast is a widespread and damaging disease of cultivated rice caused by the fungus *Pyricularia oryzae* leading to serious yield losses. *Stenotrophomonas maltophilia* was shown to produce antimicrobial compounds and has the ability to act as a biological control agent. This study was conducted with three objectives, 1) to isolate, identify and characterise *S. maltophilia* isolates for *in vitro* screening against *P. oryzae*, 2) to identify the antimicrobial compounds produced by selected *S. maltophilia* isolate, and 3) to determine the efficacy of the selected isolate against *P. oryzae* and to evaluate the expression of defense-related genes in rice during the pathogen-host interaction in glasshouse trial. Root and rhizosphere samplings were conducted in Selangor, Penang, Perak and Kedah states. The emerged bacteria isolates were identified as *S. maltophilia* based on morphological method using *Xanthomonas maltophilia*-selective agar medium and molecular method using polymerase chain reaction (PCR). A total number of 40 colonies were isolated from healthy rice roots and rhizospheres. PCR amplified products were sequenced and compared with related bacteria in the GenBank database. A phylogenetic analysis was conducted using MEGA6 program. Bacteria isolates were confirmed as S. *maltophilia* and were placed under *S. maltophilia* cluster. Isolates were subjected to dual culture and culture filtrate tests to determine their antagonistic activities. In dual culture test, eleven isolates showed percent inhibition of radial growth (PIRG) more than 55% and in culture filtrate test, four isolates showed PIRG more than 85%. All four isolates showed the optimum growth temperature of 30-40°C. These four isolates were used for hydrolytic enzymes and secondary metabolites production test *in vitro*. All of the tested isolates produced protease, chitinase, cellulase, pectinase and lipase. They also produced indole-3-acetic acid (IAA), ammonia and, siderophore. Bioactive compounds produced by *S. maltophilia* isolate UPMKH2 were identified using Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MSMS). Two antimicrobial compounds Maculosin and L, L-Cyclo (leucylprolyl) were identified. For *in vivo* trial, rice plants

were inoculated with *P. oryzae* conidia suspension and *S. maltophilia* isolate UPMKH2 was applied using both seed treatment and foliar spray. The results showed significant disease suppression, increased plant growth parameters and enhancement of yield-related attributes in plants treated with UPMKH2 in both methods compared to the untreated control with the highest suppression of disease at 55.58 %. Defense-related gene expression in rice leaf samples with seed treatment application inoculated with *P. oryzae* was evaluated using RT-qPCR and the result showed that *S. maltophilia* UPMKH2 was able to increase the expression of target genes, β-1, 3- glucanase (*Gns1*) and chitinase (*Cht-1*), compared with the non-treated control. *S. maltophilia* demonstrated the ability to suppress mycelial growth of *P. oryzae* with optimal growth at 30-40°C and produced plant growth-promoting compounds *in vitro*, and high efficacy to reduce blast disease *in vivo*. Thus, *S. maltophilia* has great potential to be used as a biological control agent against rice blast disease in the field and also could be a good candidate to address in global warming issue in rice production in the future.

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

PENCIRIAN DAN PENILAIAN AKTIVITI KAWALAN BIOLOGI *Stenotrophomonas maltophilia* TERHADAP PENYAKIT KARAH PADI YANG DISEBABAKAN OLEH *Pyricularia oryzae*

Oleh

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Karah padi yang disebabkan oleh kulat *Pyricularia oryzae* adalah penyakit yang merosakkan tanaman beras secara meluas serta menyebabkan kerugian hasil yang serius. Didapati bahawa *Stenotrophomonas maltophilia* mempunyai kemampuan untuk bertindak sebagai agen kawalan biologi dan menghasilkan antibiotik serta sebatian yang dapat menggalakkan pertumbuhan tumbuhan. Kajian ini dijalankan dengan tiga objektif, 1) untuk mengasing, mengenalpasti dan mencirikan isolat *S. maltophilia* daripada sawah padi untuk pemeriksaan secara *in vitro* terhadap *P. oryzae*, 2) untuk mengenalpasti sebatian antimikrob yang dihasilkan oleh isolat *S. maltophilia* yang dipilih, serta 3) untuk menentukan keberkesanan isolat *S. maltophilia* yang dipilih terhadap *P. oryzae* dalam kajian kaca rumah. Penyampelan akar dan rizosfera telah dilakukan di negeri Selangor, Pulau Pinang, Perak dan Kedah. Bakteria yang diperolehi telah dikenalpasti sebagai *S. maltophilia* berdasarkan kaedah morfologi menggunakan media khas *Xanthomonas maltophilia* dan kaedah molekul menggunakan PCR. Sejumlah 40 koloni telah diasingkan daripada akar dan rizosfera pokok padi yang sihat. Bagi pengenalpastian secara molekul, jujukan yang diperolehi daripada PCR telah dibandingkan dengan jujukan bakteria yang berkaitan dalam pangkalan data GenBank dan analisis filogenetik telah dilakukan menggunakan program MEGA6. Dalam analisis filogenetik, isolat-isolat yang diperoleh tergolong dalam kluster *S. maltophilia*. Isolat-isolat telah digunakan dalam ujian dwi-kultur dan ujian kultur filtrat untuk menentukan aktiviti antagonistik mereka. Dalam ujian dwi-kultur, 11 isolat menunjukkan PIRG lebih daripada 55% dan dalam ujian filtrat kultur, empat isolat menunjukkan PIRG lebih daripada 85%. Keempat-empat isolat menunjukkan suhu pertumbuhan optimum 30-40°C. Keempat-empat isolat ini digunakan dalam ujian penghasilan enzim hidrolitik dan ujian metabolit sekunder. Semua isolat berkebolehan dalam penghasilan enzim ‘protease’, ‘chitinase’, selulase, ‘pectinase’ dan ‘lipase’. Isolat-isolat ini juga menghasilkan IAA, ammonia dan ‘siderophore’ apabila diuji secara *in vitro*. Sebatian bioaktif yang dihasilkan oleh *S.*

maltophilia isolat UPMKH2 telah diekstrak dengan menggunakan etil asetat dan dikenalpasti menggunakan kromatografi cecair-MS/MS (LC-MS/MS). Dua sebatian antimikrob 'Maculosin' dan 'L, L-Cyclo' (leucylprolyl) telah dikenalpasti. Dalam eksperimen secara *in vivo*, tanaman padi telah dirawat dengan larutan konidia *P. oryzae* dan *S. maltophilia* isolat UPMKH2 digunakan sebagai rawatan benih dan semburan pada daun. Eksperimen secara *in vivo* menunjukkan pengurangan penyakit yang signifikan, peningkatan faktor pertumbuhan tumbuhan dan peningkatan komponen berkaitan hasil tanaman pada tanaman yang dirawat sebagai rawatan benih dan semburan pada daun berbanding dengan tanaman yang tidak dirawat, dengan pengurangan tertinggi penyakit sebanyak 55.58%. Untuk kajian ekspresi gen, RNA diekstrak diikuti dengan kaedah 'RT-qPCR'. Ekspresi gen yang berkaitan dengan pertahanan dalam sampel daun padi dengan aplikasi rawatan benih yang diinokulat dengan *P. oryzae* telah dinilai. Didapati bahawa *S. maltophilia* isolat UPMKH2 mampu meningkatkan ekspresi gen sasaran, 'β-1, 3 glucanase' (*Gns1*) dan 'chitinase' (*Cht-1*), berbanding dengan tumbuhan yang dirawat dengan patogen sahaja (kawalan). *S. maltophilia* menunjukkan keupayaan untuk menahan pertumbuhan *P. oryzae* dengan pertumbuhan optima pada suhu 30-40°C dan menghasilkan faktor penggalak pertumbuhan tanaman secara *in vitro*. *S. maltophilia* juga terbukti berkesan dalam mengawal penyakit karah padi secara *in vivo*. Oleh itu, *S. maltophilia* berpotensi besar untuk digunakan sebagai agen kawalan biologi terhadap penyakit karah padi di ladang juga boleh menjadi calon yang baik untuk digunakan dalam menangani isu iklim panas global dalam pengeluaran padi di masa depan.

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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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LIST OF ABBREVIATIONS

ANOVA	Analysis Of Variance
AUDPC	Area under disease progress curve
BCA	Biological control agent
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
CRD	Completely Randomized Design
CTAB	Cetyltrimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agriculture organization of the united nations
IAA	Indole 3-acetic acid
IRRI	International rice research institute
ISR	Induced systemic resistance
LSD	Least significant difference
Min	Minute
MVOCs	Microbial volatile organic compounds
NA	Nutrient agar
NaOAc	Sodium acetate
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PSB	Phosphate-solubilising bacteria
P-R	Pathogenesis-Related

PGPB	Plant Growth-Promoting Bacteria
PGPR	Plant growth promoting rhizobacteria
PIRG	Percentage inhibition of radial growth
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
Rpm	Rotation per minute
SAR	Systemic-acquired resistance
SAS	Statistical Analysis System
Spp	Species
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
Tris-HCl	Hydroxymethyl-hydrochloride
TBE	Tris base, boric acid, EDTA
VOC	Volatile organic compounds
XMSM	*Xanthomonas maltophilia*-selective agar medium
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa* L.) is being consumed as the main energy source in most world regions (Greenland, 1997; Hayasaka, *et al.,* 2008). Rice provides 30 to 50% of the daily intake of calories (Jena *et al*., 2018). After wheat, rice is the second essential staple food largely in Asia (Naureen *et al*., 2009; Rajamoorthy *et al*., 2015; Dahare *et al*., 2020). Rice is widely cultivated in South Asia, Thailand, China, Korea and Japan (Zhao *et al*., 2020). In Malaysia, rice is grown on 671679 ha, which is 8.5% of total agricultural land (7.839 million ha) (Shamshiri *et al*., 2018).

Rice greatly suffers from blast disease which is caused by *Magnaporthe oryzae* (anamorph, *Pyricularia oryzae* Cavara) (Couch and Kohn, 2002; Jagadeesh and MK, 2018). Kihoro *et al.* (2013) have reported that blast disease can cause 60 to 100% yield losses. Generally, the blast pathogen targets all aboveground parts of the rice plant. In 2001, Ishiguro has described the penetration and infection process of its conidia on the surface of rice leaves. Apart from leaf blast, panicle blast also results in rice yield losses (Silva *et al*., 2009). In 2005, the blast disease affected approximately 4,033 ha of rice cultivation in Malaysia (Malaysian Quarantine Inspection Services, Department of Agriculture, unpublished data). Besides, the panicle blast caused yield losses of 50-70% in the affected area (Zakaria and Misman, 2018). Routinely, pesticides are used to control the disease. However, its use is undesirable because of its high cost and detrimental effects on the environment (Manandhar *et al.* 1998). Furthermore, the spread of disease can be very rapid and using resistant varieties is at best only for short term (Baldwin *et al*., 2004). Baesd on Anusha *et al*. (2019) Plants can be protected from fungal pathogens by the naturally occurring antagonistic bacteria.

Endophytes are microorganisms mostly fungi and bacteria that live inside plant tissues without producing disease symptoms (Petrini, 1991; Azevedo and Araujo, 2007; Barros *et al.,* 2010). Endophytes are unexplored components of biodiversity, particularly in the tropics. Ferreira *et al*. (2008) have reported that endophytic bacteria are potential candidates for biocontrol agents because they share an ecological niche analogous to phytopathogens and they can be transmitted through seeds. Endophytic bacteria have been isolated from different plants such as cabbage, sugar cane, sunflower, maize, soybean, beet, wheat and rice (Santos *et al.,* 2018). It is essential to determine the microbial abundance and to understand the effects of diverse environmental conditions on microbial diversity in order to utilise endophytes as biocontrol agents (Andreote *et al*., 2009).

In general, a considerable number of bacterial species from the plant rhizosphere have the ability to exert useful effects on plant development. Hence, the utilisation of plant growth-promoting rhizobacteria (PGPR) as biocontrol agents for agricultural development is preferred (Kloeppe *et al*., 1999; Sharifi-Noori *et al*., 2015). Bio-

antagonistic bacteria from *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas* and *Enterobacter* genera are among well-known biocontrol agents and they can be largely found in the rhizosphere of most cereals (Naureen *et al.*, 2005). For example, *Pseudomonas fluorescens* strain RB04 appeared to be highly potential in controlling *P. oryzae (*Shyamala and Sivakumaar, 2012). Tendulkar *et al.* (2007) reported the fungicidal activity of lipopeptide secreted by *Bacillus lichenformis* BC98 against rice blast. Besides, biocontrol activity of PGPR against blast disease was reported by Sharifi-Noori *et al.* (2015). Modes of action of these antagonistic bacteria include parasitism through production of extracellular enzymes, and stimulation of plant resistance mechanisms (Whipps, 2001; Shyamala and Sivakumaar, 2012), competition for iron acquisition through production of siderophores and inhibition of the pathogen by antimicrobial compounds (Duffy and Defago, 1999; Shyamala and Sivakumaar, 2012). As biological control is a vital component of integrated disease management, identification of rhizobacteria with biocontrol efficacy against blast pathogen and assessment of these antagonists for application in field conditions are imperative (Shyamala and Sivakumaar, 2012).

Stenotrophomonas maltophilia is typically found in soil (Singer and Debette, 1993; Khobragade *et al*., 2018) and plants (McInroy and Kloepper, 1994; Wilson and Lindow, 1994; Berg *et al*., 1996; Etesami and Alikhani, 2016). These bacteria with their potential for bioremediation have been the center of much consideration. (Blake *et al.*, 1993; Ghosh and Saha, 2013). *S. maltophilia* was characterised as a rhizosphere bacterial species of potential agronomic importance (Mazzola *et al*., 1995; Kobayashi *et al*., 1995; Messiha *et al*., 2007; Elhalag *et al.*, 2016; Singh and Jha, 2017). For instance, *S. maltophilia* strain 34S1 was recognised as a biocontrol agent for summer patch disease of Kentucky bluegrass caused by *Magnaporthe poae* (Kobayashi *et al*., 1995). In addition, the potential of *S. maltophilia* PD4560 for the control of potato wilt disease against *Ralstonia solanacearum* was highlighted (Elhalag *et al.*, 2016). The biocontrol mechanisms of *S. maltophilia* include production of anti-microbial compounds, activities of extracellular chemicals such as chitinase and protease and colonisation of plants' rhizosphere (Kobayashi *et al*., 2002). However, to date, biocontrol potential of *S. maltophilia* against *P. oryzae* on rice has not been explored in Malaysia.

Hypothesis:

Stenotrophomonas maltophilia isolated from rice plant could be effective in suppressing blast disease and defense genes in rice can be induced by *S. maltophilia.*

Thus, this study was carried out with the following objectives:

1. To isolate, identify and characterise *S. maltophilia* isolates using morphological and molecular methods and their antagonism activity against *P. oryzae in vitro.*
2. To determine the antimicrobial compounds produced by selected *S. maltophilia* isolates and mechanisms of biocontrol.
3. To determine the efficacy of selected *S. maltophilia* isolate in controlling *P. oryzae* and evaluation of defense gene expression in rice during pathogen-host interaction under glasshouse conditions.

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