UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF ANTAGONISTIC RHIZOBACTERIA AND BIOPRIMING FOR THE CONTROL OF Fusarium proliferatum, THE CAUSAL PATHOGEN OF BAKANAE DISEASE IN RICE

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FP 2015 22

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By

NURUL WAHIDA BINTI RAMLI

Thesis Submitted to the School Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

January 2015

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Bakanae disease is one of the important diseases of rice. Major rice growing areas in Malaysia affected with Bakanae disease showed a significant decrease on yield and quality. Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means in managing soil borne plant diseases. Beneficial bacteria and fungi applied as seed treatments provide unique opportunities for protection against soil-borne fungal pathogens and they are potential alternative to fungicides. The objectives of this study are, therefore, to isolate, characterize and identify the antagonistic microorganisms in rice and to evaluate their potential as bio-inoculants against Bakanae disease in rice. From the total of 315 microorganisms isolated from 25 different samples of rhizosphere soil and rice roots, 71% were bacteria, 27% fungi and 2% actinomycetes. The isolated microbial organisms were subsequently tested for their antagonistic activity *in vitro* against *Fusarium proliferatum*. In dual culture testing, 12 bacterial isolates exhibited significantly high antagonistic activity in the range of 53 to 84%. The highest antagonistic activities were based on their percentage inhibition in radial growth of *F. proliferatum* (84%), which was exhibited by isolate B7. Secondary screening was carried out for detection of microbial compounds, siderophore production, chitinase activity, indole-acetic acid production and phosphate solubilizing activity for the potential isolates. Isolate B7 was able to produce siderophore, chitinase, and IAA, and had P solubilizing activity. Molecular identification identified B7 as *Bacillus cereus*. Based on their colonization and establishment in rice seedlings, *B. cereus* (B7) were selected for further *in vivo* screening for its efficacy in controlling Bakanae disease on rice in the glasshouse. Seed treatment with *B. cereus* on rice seedlings pre-inoculated with *F. proliferatum* suppressed the Bakanae incidence compared to the control. Inoculation of seedlings with *B. cereus* reduced Bakanae incidence by 51%. Based on this disease reduction from this research, *B. cereus* showed potential to be used as bio-inoculant for controlling Bakanae disease on rice.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

CHARACTERIZATION OF ANTAGONISTIC RHIZOBACTERIA AND BIO-PRIMING FOR THE CONTROL OF *Fusarium proliferatum* CAUSING BAKANAE DISEASE IN RICE

By

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January 2015

Chairman : Associate. Professor Zainal Abidin Mior Ahmad, PhD

Faculty : Agriculture

Rice is the staple food of Malaysians and mainly planted for domestic consumption. Diseases are estimated to cause annual yield and quality losses of 8 to 10 percent. Production costs are also increased by the use of chemicals and cultural methods of disease control. Bakanae disease is one of the important diseases of rice. Major rice growing areas in Malaysia affected with Bakanae disease showed a significant decrease on yield and quality. Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means in managing soil borne plant diseases. Beneficial bacteria and fungi applied as seed treatments provide unique opportunities for protection against soil-borne fungal pathogens and they are potential alternative to fungicides. The objectives of this study are, therefore, to isolate, characterize and identify the antagonistic microorganisms in rice and to evaluate their potential as bio-inoculants against Bakanae disease in rice. From the total of 315 microorganisms isolated from 25 different samples of rhizosphere soil and rice roots, 71% were bacteria, 27% fungi and 2% actinomycetes. The isolated microbial organisms were subsequently tested for their antagonistic activity *in vitro* against *Fusarium proliferatum*. In dual culture testing, 12 bacterial isolates exhibited significantly high antagonistic activity in the range of 53 to 84%. The highest antagonistic activities were based on their percentage inhibition in radial growth of *F. proliferatum* (84%), which was exhibited by isolate B7. Secondary screening was carried out for detection of microbial compounds, siderophore production, chitinase activity, indole-acetic acid production and phosphate solubilizing activity for the potential isolates. Isolate B7 was able to produce siderophore, chitinase, and IAA,

and had P solubilizing activity. B7 was identified by the Biolog® identification system as *Bacillus cereus*. Subsequently, molecular characterization also confirmed B7 as *Bacillus cereus*. Based on their colonization and establishment in rice seedlings, *B. cereus* (B7) were selected for further *in vivo* screening for its efficacy in controlling Bakanae disease on rice in the glasshouse. Seed treatment with *B. cereus* on rice seedlings pre-inoculated with *F. proliferatum* suppressed the Bakanae incidence compared to the control. Inoculation of seedlings with *B. cereus* reduced Bakanae incidence by 51%. Based on this disease reduction from this research, *B. cereus* showed potential to be used as bio-inoculant for controlling Bakanae disease on rice.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN TERHADAP RIZOSBAKTERIA ANTAGONISTIK DAN RAWATAN BENIH BAGI PENGAWALAN TERHADAP *Fusarium proliferatum*, PATOGEN PENYEBAB PENYAKIT BAKANAE PADA PADI

By

NURUL WAHIDA BINTI RAMLI

Januari 2015

Pengerusi : Professor Madya Zainal Abidin Mior Ahmad, PhD

Fakulti : Pertanian

Padi adalah makanan ruji rakyat Malaysia dan kebanyakannya ditanam untuk kegunaan domestik. Penyakit dianggarkan menyebabkan penurunan hasil tahunan dan kerugian kualiti antara 8 hingga 10 peratus. Kos pengeluaran juga meningkat dengan penggunaan bahan kimia dan kaedah kawalan penyakit secara kultur. Penyakit Bakanae adalah salah satu penyakit padi yang penting. Kawasan utama penanaman padi di Malaysia yang dikesan menghidapi penyakit Bakanae menunjukkan penurunan yang sekata dari segi pengeluaran dan kualiti. Kawalan biologi patogen tumbuhan menggunakan mikroorganisma antagonis adalah satu cara yang tidak menggunakan bahan kimia dan berpotensi untuk digunakan dalam pengurusan penyakit tumbuhan keluaran tanah. Bakteria dan kulat berfaedah digunakan sebagai rawatan benih boleh memberikan peluang yang unik sebagai perlindungan daripada kulat patogen tanah dan sebagai alternatif kepada racun kulat. Oleh itu, objektif kajian ini adalah untuk memencilkan, mencirikan, dan mengenalpasti mikroorganisma antagonis dari padi dan seterusnya menilai potensi mikroorganisma ini sebagai bio-inokulan terhadap penyakit Bakanae padi. Sebanyak 315 mikroorganisma telah dipencilkan daripada 25 sampel tanah dan akar padi dimana, 71% adalah bakteria, 27% kulat dan aktinomisit 2%. Mikrob yang telah dipencilkan kemudiannya disaring secara *in vitro* untuk menguji aktiviti antagonistik terhadap *Fusarium proliferatum*. Dalam ujian saringan kultur berpasangan, 12 pencilan bakteria telah menunjukkan aktiviti antagonistic yang tinggi dalam julat 53 hingga 84%. Aktiviti antagonistik yang paling tinggi berdasarkan peratus rencatan pertumbuhan radial *F. proliferatum* (84%) ditunjukkan oleh isolat B7. Saringan kedua telah dijalankan untuk mengesan kompaun mikroorganisma, penghasilan “siderophore”, aktiviti “chitinase, penghasilan asid indol-

asetik dan aktiviti pelarutan fosfat oleh isolat yang berpotensi. Isolat B7 telah dikenalpasti dapat menghasilkan "siderophore", "chitinase" dan asid indol-asetik,dan mampu menjalankan aktiviti melarutkan fosfat. Isolat B7 telah dikenalpasti melalui sistem pengenalan BIOLOG® sebagai *Bacillus cereus*. Selain itu, pencirian melalui kaedah molekular juga telah mengesahkan identiti B7 sebagai *B. cereus*. Berdasarkan pengkolonian dan penghasilan mereka dalam benih padi, *B. cereus* telah dipilih untuk dinilai keupayaan mereka dalam mengawal penyakit. Bakanae pada padi di rumah kaca. Rawatan benih dengan *B. cereus* pada benih padi yang telah diinokulat dengan *F. proliferatum* menunjukkan bakteria ini dapat mengawal *F. proliferatum* berbanding dengan kawalan. Benih padi yang telah diinokulat dengan *B. cereus* menunjukkan kadar pengurangan penyakit Bakanae sebanyak 51%. Berdasarkan pengurangan penyakit dalam penyelidikan ini, *B. cereus* menunjukkan potensi untuk digunakan sebagai bio-inokulan untuk mengawal penyakit Bakanae pada padi.

ACKNOWLEDGEMENTS

All praises and thanks to almighty Allah SWT, the most merciful, for his blessings and the strength to complete this study.

I would like to express my sincere gratitude to my supervisor and committee member Associate Professor Dr. Zainal Abidin Mior Ahmad and Dr. Nusaibah Syd Ali for their valuable advice, support, encouragement, patience and understanding that made this study possible. Not forgetting, my former supervisor, Prof. Dr. Sariah Meon for her constructive comments during the on-going research work and the preparation of this thesis.

Special thanks are extended to all staffs of the Institute of Tropical Agriculture (ITA), UPM for their assistance in laboratory work and not to forget to all Bioscience Institute staff (Microscopy Unit) for their kind assistance in SEM analysis. A special acknowledgment is given to my labmate, Muniroh Md. Saad for her help and moral support towards the completion of this thesis.

Finally, a million thanks to my husband, father, mother, brothers and sisters for their love, support and inspiration throughout the period of my study in UPM and my entire life.

I certify that an Examination Committee has met on 28 January 2015 to conduct the final examination of Nurul Wahida bt. Ramli on her thesis entitled "Characterization of Antagonistic Rhizobacteria and Bio-Priming for the Control of *Fusarium proliferatum*, Causing Bakanae Disease in Rice" 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 Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master in Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

Ab	Absorbance
ASM	Actinomycete Selective Media
ANOVA	Analysis of Variance
AUDPC	Area under Disease Progress Curve
bp	Base pair
cfu	Colony Forming unit
cm	Centimeter
CRD	Completely Randomized Design
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DI	Disease incidence
DR	Disease reduction
DS	Disease severity
ER	Epidemic rate
G	Gram
Kb	Kilo-base pair
Kg	Kilogram
LSD	Least Significant Different
m	Meter
µL	Micro liter
µm	Micrometer
mg	Miligram
mL	Milimeter
mm	Milimolar
ng	Nanogram

NA	Nutrient agar
NB	Nutrient broth
%	Percent
PIRG	Percent inhibition of Radial Growth
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
SEM	ScanningElectron Microscopy
Spp	species
SDW	Sterile Distilled Water
v/v	Volume /volume
IF	Inoculated Fluid
IAA	Indole Acetic Acid
CAS	Chrome Azurol S
Min	Minute
h	Hour

CHAPTER 1

INTRODUCTION

Rice (*Oryzae sativa* L.), a member of family Poaceae is commonly grown in tropical and subtropical regions of the world (Ezuka and Kaku, 2000). Almost 90% of the world's rice is grown in Asia and constitutes a staple food for 2.7 billion people worldwide (Salim *et al*., 2003). Crawford and Lee (2003) reported that between 1961 and 2002, per capita consumption of rice increased by 40%. They also explained the rice consumption is highest in Asia, where average per capita consumption is greater than 80 kg/person/year. In Malaysia, the total consumption of rice has increased from 2.7 million tonnes in 1985 to 4 million tonnes in 2009 due to the increase in population (Fatimah *et al*., 2011).

Bakanae disease is caused by one or more *Fusarium* species and is also found as a complex of disease symptoms including seedling blight, root and crown rot, stunting and abnormal elongation (Desjardins *et al*., 2000). Besides, Bakanae is one of the three major disease of rice reported including blast and brown spot. The disease reaches up to 20% in epidemic cases and automatically caused the worldwide crop losses (Cumagun *et al.,* 2011). Bakanae disease has been spreading in recent years and now found from newer parts of Asia where Bakanae was not formerly documented. For example, over the last five years Bakanae has been found to become a major disease in Pakistan (Bhalli *et al*., 2001). After that, in Bangladesh Bakanae was reported been increasing at an alarming rate from year to year (Mainul *et al*., 2011). In Malaysia, the first incidence of Bakanae disease was observed to be serious in the year 1985 during the second rice planting season in Kedah, Kelantan and Perak (Zainudin *et al*., 2008a).

There are several methods developed to control plant diseases including chemical, cultural and biological techniques (Saremi *et al*., 2011). The best method used for controlling the disease is the cultivation of resistant varieties. However, this method is not common against Bakanae disease because of the reduction in the genetic potential of the host due to the high genetic variation of the pathogen (Iqbal *et al*., 2011). It is difficult to control the seed-borne diseases caused by fungi because the fungal hyphae develop and become dormant inside the seed (Javaid and Anjum, 2006). If infected seed is sown in non-infested soil, the pathogen may become established in that soil (Neergard, 1986).

The most common management practice for Bakanae is seed treatment using fungicides such as thiram, thiophanate-methyl, or benomyl which is effective. However, resistance of the fungal pathogen to the fungicides has been reported (Rosales and Mew, 1997) and these fungicides also cannot prevent Bakanae infection after transplanting (Bhalli *et al.,* 2001). Besides that, treatments are expensive and the pesticides pollute the environment (Kazempour and Elahinia, 2007). Currently, the possible non-chemical method for plant disease control is the addition of

antagonistic microorganisms to the soil and one of the biological control of soil borne plant pathogens (Elad *et al*., 1980). The integration of biological control and other strategies promised a greater level of protection with sustained rice yields and also very eco-friendly and cost effective strategy (Manandhar and Yami *et al*., 2008).

The most ideal biocontrol agent for the management of foliar infections and soil borne pathogens may be the ones that survive in both the rhizosphere and phyllosphere (Kazempour and Elahinia, 2007). There is increasing interest in using microorganisms to control soil-borne plant pathogens. In the recent years, the search for options to chemical control of plant pathogens has increased due to the development of fungicide resistance (Zhou *et al.* 1994; Bhalli *et al*. 2001) in pathogens besides the increased health concerns for the producer and the consumer (Reddy *et al*., 2007). Plant growth promoting rhizobacteria (PGPR) are components of biocontrol agents (BCA) which are easy to handle, grow rapidly, and colonize the rhizosphere aggressively (Weller, 1988). The PGPR colonize the root systems through seed bacterization and shows antagonistic activity against phytopathogens (Nautiyal, 1997).

Antagonistic bacteria have drawn attention worldwide because of their ability to produce siderophores, antimicrobial compounds, enzymes and phytohormones (Wilson *et al*.,1992). The use of *Talaromyces* species as a biological control agent against Bakanae disease has been documented (Tateishi *et al*., 2006). The superior colonization of the *Talaromyces* sp and it mycoparasitic nature allow it to utilize control against the pathogen on emerging coleoptiles, husks and roots at an early stage of growth (Kato *et al*.2012). *Trichoderma* species isolated from rhizosphere soil have been successfully used for biological control against rice seedborne pathogens (Watanabe *et al*., 2005).

This research was undertaken with the following objectives:

1. To isolate, characterize and identify rhizobacteria from the rhizosphere zone of rice plant and to determine the antagonistic traits against *Fusarium proliferatum.*
2. To evaluate the bio-inoculant potential of B7 as seed treatment in glass house experiment for the control of Bakanae disease of rice.

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