EFFECT OF SALT-TOLERANT PLANT GROWTH-PROMOTING RHIZOBACTERIA INOCULATION ON CROP GROWTH, BIOCHEMICAL PROPERTIES AND YIELD OF RICE

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By

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

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Soil salinity causes huge negative impacts on the global agricultural sector and is currently a crucial issue in wetland rice production (*Oryza sativa* L). Due to the unsatisfactory results of conventional salinity mitigation practices, the use of indigenous salt-tolerant plant growth-promoting rhizobacteria (PGPR) on existing rice cultivar could be a new alternative. A series of experiments were conducted under laboratory and glasshouse conditions to fulfill the following objectives; i) to screen for potential salt-tolerant PGPR from coastal salt-affected rice cultivation areas; ii) to select suitable rice varieties for an optimum response towards inoculation of salt-tolerant PGPR; iii) to elucidate the mechanism of salt-tolerant PGPR inoculation on improving crop growth, biochemical properties, and yield of rice. In experiment 1, a total of 44 strains were isolated and screened based on their qualitative salinity tolerance and plant growth-promoting properties. These isolates were subjected to quantitative screening at five levels of NaCl concentrations (0, 0.5, 1, 1.5, and 2M) and the results showed that three isolates labeled as UPMRB9, UPMRE6, and UPMRG1 were able to grow on the highest NaCl-amended media and maintained a relatively high bacterial population. Isolate UPMRB9 produced the highest amount of exopolysaccharides (31.5 g L^{-1}) and absorb the highest amount of sodium (24.8 mg L^{-1}) on 1.5M of NaCl-amended media. UPMRE6 and UPMRB9 produced the highest floc yield (22.97 g L^{-1}) and biofilm (OD 1.37), respectively, both on 1M of NaCl-amended media. The characteristics of UPMRB9 as a salt-tolerant isolate were supported by the Scanning Electron Microscope (SEM) observation which showed higher EPS, floc yield, and biofilm formation when exposed to salt stress condition. The beneficial characterization studies also revealed UPMRB9 as the highest Indole Acetic Acid (IAA) producer. Besides, UPMRE6 recorded the highest phosphate and potassium solubilizations in all NaCl-amended media. These three potential isolates namely UPMRB9, UPMRE6, and UPMRG1 were identified using 16s rRNA gene sequence technique, and the blast results revealed 99% similarity with *Bacillus*

tequilensis, Bacillus aryabhattai, and *Providencia stuartii*, respectively. The characterization of EPS extracted from the bacterial isolates showed the presence of hydroxyl, carboxyl, amino, sulfhydryl, and phosphate functional groups which have shown a strong binding affinity to toxic Na^+. In experiment 2, a glasshouse study was conducted involving seven rice varieties that were exposed to four different levels of salinity (0, 4, 8, 12 dSm^{-1}) at the seedling stage. Based on the morpho-physiological and biochemical respond towards salt stress, three rice varieties namely BRRI dhan67, Putra-1, and MR297 were identified as salt-tolerant, moderately salt-tolerant, and salt-susceptible varieties, respectively, and were selected for further study. In experiment 3, the selected isolates (UPMRB9, UPMRE6, and UPMRG1) were inoculated to the salt-responsive rice varieties (BRRI dhan67, Putra-1, and MR297) at the seedling stage under 8 dSm^{-1} of salinity. Results showed that inoculation of UPMRB9 to BRRI dhan67 produced significantly highest seedling dry matter (1.50 g) due to the increased chlorophyll and relative water content, and reduced electrolyte leakage and the ratio of Na/K in the inoculated plants. In experiment 4, the plant inoculation test was extended up to yield stage involving 2 strains of UPMRB9 and UPMRE6. Results showed that inoculation of UPMRB9 to the rice variety Putra-1 responded for the highest rate of photosynthesis (10.52 µmol CO_2 $s^{-2}m^{-1}$), proline content (10.48 µmol g^{-1} FW), total soluble sugar content (6.29 mg g^{-1} FW), superoxide dismutase (SOD) production (89.70 unit mg^{-1} FW) and uptake of phosphorous (4.64 g/plant). The higher accumulation of osmoprotectants like proline and total soluble sugar in Putra-1 rice markedly improved the regulation of antioxidant enzymes and thus improved plant resistance to salinity which eventually enhanced plant photosynthesis. Besides, BRRI dhan67 treated with UPMRB9 highly responded towards augmenting stomatal conductance, malondialdehyde (MDA) content, catalase (CAT) production, higher uptake of K and Ca, along with increasing the yield parameters of 1000 grain weight (27.33g), filled grains (79%) and grains per plant (27.66g). In this case, UPMRB9 assisted uptaking higher amounts of K and Ca, which helped towards achieving maximum grains of BRRI dhan67 through reducing the toxicity of Na^+. Considering all these positive traits, it can be concluded that the locally-isolated salt-tolerant bacterial strains could contribute significantly towards improving the physiological and biochemical characters and grain yield components of rice plants under salt stress conditions. These promising PGPR strains can be a potential biofertilizer inoculant to mitigate the problems raised due to the global climate change for coastal rice cultivation. This study has successfully demonstrated the possible salt-tolerance mechanism of PGPR and response of their inoculation on different local rice varieties under salt stress conditions especially under the Malaysian rice cultivation scenario which has not been studied in detail previously.

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

INOKULASI GARAM-TOLERAN RIZOBAKTERIA PENGGALAK TUMBESARAN TANAMAN PADA PERTUMBUHAN FISIOLOGI, SIFAT BIOKIMIA DAN HASIL PADI.

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Saliniti tanah menyebabkan kesan negatif yang ketara terhadap sektor pertanian global, dan ini merupakan isu yang penting dalam pengeluaran padi sawah (*Oryza sativa* L). Kaedah mengatasi masalah saliniti secara konvensional selalunya memberikan hasil yang tidak memberangsangkan, dan penggunaan Rizobakteria Penggalak Tumbesaran Tanaman (PGPR) yang tahan masin serta wujud secara semulajadi berpotensi sebagai alternatif baru. Satu siri kajian telah dijalankan di peringkat makmal dan rumah kaca untuk mencapai objektif berikut; i) untuk memilih dan mengenalpasti PGPR yang tahan masin dan berpotensi dari kawasan penanaman padi di pesisir pantai yang terjejas dengan masalah saliniti; ii) untuk memilih pelbagai padi yang sesuai dan memberikan respon optimum terhadap inokulasi PGPR tahan masin; dan iii) untuk mengenalpasi mekanisma inokulasi PGPR tahan masin dalam menggalakkan pertumbuhan pokok, ciri biokimia dan hasil padi. Dalam ujikaji 1, sejumlah 44 bakteria telah diasingkan dan dipilih berdasarkan ciri-ciri tahan masin dan menggalakkan pertumbuhan pokok secara kualitatif. Bakteria-bakteria ini telah didedahkan pada lima tahap kemasinan (0, 0.5, 1, 1.5 dan 2M) menggunakan NaCl dan hasil menunjukkan tiga bakteria dilabel sebagai UPMRB9, UPMRE6 dan UPMRG1 boleh hidup pada tahap kemasinan tertinggi iaitu 2M NaCl disampling mengekalkan populasi bakteria yang tinggi. Bakteria UPMRB9 menghasilkan jumlah eksopolisakarida yang tertinggi (31.5 gL^{-1}) dan menyerap garam pada kadar yang tertinggi pada media yang diubah dengan 1.5 M NaCl. Bakteria UPMRE6 dan UPMRB9 menghasilkan flokulasi (22.9 gL^{-1}) dan *biofilm* (OD 1.37) yang tertinggi pada kepekatan 1M NaCl. Ciri-ciri UPMRB9 sebagai bakteria yang tahan masin ini disokong oleh hasil pengimejan menggunakan *Scanning Electron Microscope* (SEM) yang menunjukkan penghasilan EPS, flokulasi dan biofilm apabila terdedah kepada keadaan tinggi garam. Kajian ciri-ciri berfaedah bakteria juga menunjukkan UPMRB9 sebagai penghasil *Indole-3-Acetic Acid* (IAA) yang tertinggi. Selain itu, bakteria UPMRE6 menunjukkan solubilisasi fosforus dan kalium yang tertinggi pada semua

tahap kemasinan. Tiga bakteria ini iaitu UPMRB9, UPMRE6 dan UPMRG1 telah dikenalpasti menggunakan teknik *gene sequence* 16S rRNA dan hasil menunjukkan 99% similariti dengan *Bacillus tequilensis, Bacillus aryabhattai,* and *Providencia stuartii.* Kajian ciri-ciri EPS yang diekstrak daripada bakteria-bakteria ini menunjukkan adanya kumpulan berfungsi hidroksil, karboksil, amino, sulfhydryl dan fosfat, yang telah ditunjukkan mempunyai tarikan yang kuat dengan Na^+ ion yang toksik. Dalam ujikaji 2, kajian rumah kaca telah dijalankan melibatkan tujuh variati padi yang telah didedahkan pada empat tahap saliniti yang berbeza (0, 4, 8, 12 dSm^{-1}) pada peringkat anak benih. Berdasarkan kepada respon morfo-fisiologi dan biokimia terhadap saliniti, tiga variati padi iaitu BRRI dham67, Putra-1 dan MR297 telah dikenalpasti sebagai varieti toleran, sederhana toleran dan tidak toleran dan telah dipilih untuk kajian yang selanjutnya. Dalam ujikaji 3, ketiga-tiga bakteria (UPMRB9, UPMRE6 dan UPMRG1) telah diinokulasi kepada tiga variati padi berbeza tahap toleran pada saliniti (BRRI dhan67, Putra-1, and MR297) untuk mengkaji kesan pada peringkat anak benih dalam keadaan bergaram (8 dSm^{-1}). Hasil menunjukkan inokulasi UPMRB9 pada BRRI dhan67 telah menghasilkan berat kering anak benih paling tinggi (1.50 g) dan ini disebabkan kerana peningkatan kandungan klorofil dan kandungan relatif air dan pengurangan kebocoran elektrolit dan nisbah Na/K dalam tisu tumbuhan. Dalam ujikaji 4, kajian rumah kaca telah dipanjangkan sehingga tahap hasil padi melibatkan dua bakteria iaitu UPMRB9 dan UPMRE6. Hasil menunjukkan inokulasi UPMRB9 pada variati padi Putra-1 memberikan respon tertinggi melibatkan kadar fotosintesis (10.52 µmol CO_2 $s^{-2}m^{-1}$), kandungan prolin (10.48 µmol g^{-1} FW), jumlah kandungan gula terlarut (6.29 µmol g^{-1} FW), penghasilan *superoxide dismute* (SOD) (88.70 unit g^{-1} FW) dan penyerapan fosforus (4.64 g $plant^{-1}$). Pengumpulan *osmoprotectant* seperti proline dan kandungan gula terlarut dalam varieti padi Putra-1 telah meningkatkan regulasi enzim antioksida secara ketara dan meningkatkan tahap ketahanan pokok pada saliniti yang seterusnya akan meningkatkan kadar fotosintesis. Selain itu, varieti BRRI dhan67 yang diinokulasi dengan UPMRB9 telah memberikan respon yang baik dalam memperbaiki *stomatal conductance*, kandungan malondialdehyde (MDA), penghasilan catalase (CAT), penyerapan K dan Ca, di samping meningkatkan hasil padi iaitu berat 1000 gwt (27.33g), bijian terisi (79%) dan bijian/pokok (27.66g). Bakteria UPMRB9 telah membantu penyerapan K dan Ca yang mempengaruhi pencapaian hasil bijirin paling tinggi untuk varieti BRRI dhan67 melalui pengurangan kesan toksik Na^+. Mengambilkira semua kesan dan ciri positif ini, boleh dirumuskan bahawa bakteria tempatan yang tahan masin boleh menyumbang secara signifikan untuk menambahbaik ciri fisiologi dan biokimia dan seterusnya meningkatkan hasil pokok padi yang ditanam pada tanah masin. Bakteria-bakteria ini berpotensi sebagai biobaja untuk mengatasi masalah dalam penanaman padi yang timbul disebabkan oleh perubahan iklim global. Kajian ini telah berhasil menunjukkan mekanisma tahan masin oleh PGPR dan respon terhadap inokulasi ke atas varieti padi berbeza yang ditanam pada keadaan tinggi garam terutamanya terhadap senario penanaman padi di Malaysia yang masih tidak dikaji secara mendalam.

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I certify that an Examination Committee has met on date of viva voce to conduct the final examination of Rakiba Shultana on her PhD thesis entitled "Salt-tolerant PGPR inoculation on rice: a study on physiological growth, biochemical properties and yield "in accordance with the Universities and University College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15th March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BRRI	Bangladesh Rice Research Institute
CFU	Colony Forming Unit
CMC	Carboxy Methyl Cellulose
EL	Electrolyte Leakage
EPS	Exopolysaccharide
IAA	Indole Acetic Acid
MARDI	Malaysian Agricultural Research and Development Institute
PCR	Polymeric Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
RNA	Ribonucleic acid
RWC	Relative Water Content
TSB	Tryptic Soy Broth
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
CAT	Catalase
POD	Peroxidase
MDA	Malondialdehyde
RNA	Ribonucleic acid
EC	Electrical conductivity
EDTA	Ethylene-diamine-tetra acetic acid
TSS	Total Soluble Sugar
NaCl	Sodium Chloride
ATP	Adenosine Try phosphate

IAA	Indole Acetic Acid
BINA	Bangladesh Institute of Nuclear Agriculture
SES	Standard Evaluation Score
ANOVA	Analysis of Variance
OD	Optical Density
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
DAI	Days After Incubation
CAS	Chrome Azurol Sulfate
FTIR	Fourier Transform Infrared
UPLC	Ultra Performance Liquid Chromatography
RWC	Relative Water Content
TDM	Total Dry Matter
TCA	Trichloro Acetic Acid
TBA	Thiobarbituric Acid
FW	Fresh Weight
NCBI	National Centre for Biotechnology Information
ACN	Acetonitrile

CHAPTER 1

INTRODUCTION

1.1 Research Background

Rice is one of the highly consumed grain crops and it is estimated that nearly 50% of the global citizen depends totally or partially on it, especially in Asia (FAOSTAT, 2017). The rice cropping systems are gradually become unprotected due to the direct consequences of climate change. The huge portions of rice-cultivation land are situated in the coastline risky regions of South and Southeast Asia, which fulfills more than 65% of global rice demand (Masutomi *et al*., 2009). Climate change leads to the rise of seawater level which causes flood and triggers the intrusion of saltwater into the inland areas. It is stated that more than 50% of arable land will be threatened by 2050 due to the effect of soil salinization which is the consequence of climate change, illogical irrigation practices, excess use of chemical fertilizers, and lack of proper drainage systems (Ciftci *et al*., 2010: Djanaguiraman *et al.*, 2013; Chandrasekaran *et al*., 2014; Rubin *et al*., 2017).

In Malaysia, approximately 13% of the total landmass is occupied by the coastal areas, with an area of 4.43 million ha. Among the coastal areas, the west coast of Peninsular Malaysia is the most advanced in socioeconomically, with 57% of its coastline utilized for agricultural activities (Tajul Baharuddin and Mohd Masirin, 2017). However, salinity is the major constraints for agricultural production in coastal granary areas due to the seawater intrusion (Herman, *et al*., 2015). As a third most important crop, rice production in Malaysia is lower than its consumption and thus resulting in rice importation which gave the rice self- sufficiency level is around 75% with an average yield of 4.5 t ha^{-1} $season^{-1}$. To encounter the ever-increasing demand, rice production in Malaysia needs to increase to approximately 7 t ha^{-1} $season^{-1}$ (Masciarelli *et al.*, 2014; Tan *et al*., 2015). Moreover, it is also projected that around 0.1 million ha of Malaysian rice-growing areas would be affected by salinity by 2056 (Selamat and Ismail, 2008). The consequence of global warming is expected to increase the seawater level which may cause a dramatic effect on rice production especially in coastal areas (Hakim *et al.*, 2014). Therefore, sustainable and smart approaches are needed to mitigate and overcome the salt-stress issues hampering the rice cultivation in coastal granary areas to ensure the highest food security level in Malaysia.

1.2 Problem Statement

In Malaysia, most of the agricultural land in the coastal plains are generally not suitable for plant growth due to salinity problems (Hashim, 2003). The seawater intrusion is the most significant factor attributing to these salinity problems. Other less significant factors might also cause salinity problems such as tidal inundation, groundwater seepage, and over-drainage of adjacent areas. Several studies have shown that plants such as oil palm, rice, and turfgrass were affected by salinity in coastal areas of Malaysia and climate change is expected to worsen these existing environmental problems (Rao, 1982; Kimi, 1991; Uddin *et al.*, 2012). Groundwater for agricultural use in the coastal areas can be affected by sea-level rise in the 21st century due to climate change (IPCC, 2001). This can possess a serious threat especially when considering the huge amount of water requirements for agriculture. Even so, there is very limited information on the effect of seawater intrusion into the coastal agricultural areas in Malaysia affecting rice plants and what's more the possible solution for overcoming the aforementioned problems.

Physical removal of salts or chemical amendments is not only expensive but also has an adverse ecological impact and it is not possible to cover massive areas for soil renovation purposes. The best approach is to develop salt-tolerant rice cultivars specifically for this region. Although several research results showed some extent of the salt-tolerance potentiality of some rice varieties like MR211, MR232, MR263 and salt-tolerant line SS1-14 (Hakim *et al.*, 2014; Atabaki *et al.*, 2018; Ma *et al.*, 2018) but still those varieties are not recommended as salt-tolerant rice in Malaysia. Several salt-resistant rice varieties have been developed in many countries through traditional breeding and genetic transformations, but in many cases, the field performance of these varieties in actual saline areas have failed to reach a satisfactory level. Thus, these vast areas remain fallow and unproductive year-round.

Therefore, the development of an eco-friendly approach to enhance plant growth under abiotic stresses has received more attention in the current modern agricultural systems (Viscardi *et al*, 2016). Taking into consideration this scenario, attention should be given to enhance the yield of salt-affected rice cultivation areas by taking advantage of the salt-tolerant plant growth-promoting rhizobacteria (PGPR). A clear understanding of PGPR mechanisms could help towards efficient utilization of the beneficial microorganisms for the enhancement of plant growth under salt stress soil conditions (Pan *et al.*, 2019). To date, insufficient research findings are available on the combined application of region-specific salt-tolerant PGPR with different salt-responsive rice varieties under saline conditions. With the proper approach, PGPR could be an eco-friendly and economically viable solution to enhance rice production potential in the saline regions (Jha *et al.*, 2012).

1.3 Significance of the study

The effective reclamation of the saline soils is difficult and complex due to frequent inundation and tidal flooding. The policy is to develop ecologically sound, compatible, and environmentally friendly measures that will be adopted in this region for increasing rice production. The introduction of salt-tolerant rice cultivars could be one of the potential approaches but without proper soil management, it will not produce the expected result. Hence, the utilization of osmotolerance mechanisms of salt-tolerant PGPR can be proposed for rice plants cultivated on saline soils. Understanding the PGPR mechanisms of salt tolerance and the complex plant-microbe interactions are expected to contribute to the utilization of saline prone areas for increasing crop productivity.

PGPR could enhance plant growth by increasing the availability of major nutrients such as nitrogen, phosphorus, potassium, zinc and, iron, and production of plant growth-promoting hormones (Jha *et al.*, 2012; Gururani *et al.*, 2013). Moreover, to counter the salinity effect, PGPR has also been proven to produce various salt-tolerant mechanisms such as exopolysaccharide production, bacterial flocculation or aggregation, and biofilm formation, which enhances colonization on plant root surfaces (Hong *et al*., 2017). In addition to that, the cementing properties of EPS will bind with several cations such as Na^+ and therefore reduces the ion accessibility to plants (Upadhyay *et al.*, 2011; Qureshi and Sabri, 2012a; Chen *et al.*, 2013). Previously, it was reported by several researchers that various genera of bacteria including *Bacillus, Pseudomonas, Paenibacillus, Burkholderia, Rhizobium, Azospirillum, Pantoea, Microbacterium, Enterobacter, Methylobacterium, Variovorax,* and *Achromobacter* have successfully increased host plants tolerance towards salt stress (Yang *et al.*, 2009; Grover *et al.*, 2011; Bharti *et al.*, 2013). Furthermore, PGPR can stimulate plant antioxidant enzyme production such as superoxide dismutase (SOD), peroxidase (POD), or catalase (CAT), which can detoxify the detrimental action of reactive oxygen species (ROS) in plants under salt stress (Han and Lee, 2005a). Thus, the mechanism of antioxidant enzyme in plants is very important as a plant's defense mechanism under salt stress conditions (Sarkar *et al*, 2018). Besides, the production of various compatible solutes by salt-tolerant PGPR namely proline and total soluble sugar provides osmo-protection in plants. PGPR-induced antioxidant enzymes and osmoprotectants help to initiate various chemical changes in plants such as alteration of total protein, the IAA concentration, total sugar, and ethylene contents (Yang *et al*., 2009).

The improvement of crop salinity tolerance through genetic engineering is crucial but it is always a lengthy and pricey process. Alternatively, PGPR inoculation could be a more sustainable option that can be attained in a shorter time. Several reports showed the imperious role of PGPR in mitigating salinity stress on multiple crops including rice (Singh and Jha, 2016). Nevertheless, extensive information is still needed involving the native bacterial population for the dissemination of indigenous bacteria in the rhizosphere of specific crops (Chahboune *et al.*, 2011). Since awareness is increasing extensively on sustainable agricultural practices, searching for region specific microbial strains is important as an eco-friendly approach towards crop production in targeted areas (Deepa *et al*., 2010). Therefore, it is hypothesized that with the proper practice of salt-tolerant PGPR application, it would be able to reduce the salt stress effect on rice plants grown on salt-affected areas.

1.4 Objectives

1. to screen for potential salt-tolerant PGPR from coastal salt-affected rice cultivation areas.
2. to select suitable rice varieties for an optimum response towards inoculation of salt-tolerant PGPR
3. to elucidate the mechanism of salt-tolerant PGPR inoculation on improving crop growth, biochemical properties, and yield of rice.

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