MOLECULAR CHARACTERIZATION OF *Pantoea* spp. THAT CAUSE BACTERIAL LEAF BLIGHT DISEASE IN PADDY PLANTS

By

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ABSTRACT

MOLECULAR CHARACTERIZATION OF *Pantoea* spp. THAT CAUSE BACTERIAL LEAF BLIGHT DISEASE IN PADDY PLANTS

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Plant diseases due to phytopathogenic bacteria are the major cause of economic losses in the sector of agriculture. Recently, an outbreak of bacterial leaf blight (BLB) disease occurred in the paddy fields of Sekinchan, Malaysia in October to December 2016. The outbreak was the worst in the last 30 years whereby over 4,400 ha of paddy field was affected, leading to 50 to 70% yields loss and with a loss exceeded RM 5 million. During the outbreak, bacteria were isolated from the diseased paddy plants and soil samples. The putative causative agents were identified to be *Pantoea* spp. Members of the genus *Pantoea* belong to the family of *Enterobacteriaceae*, they are Gram negative rod-shaped bacteria with smooth yellow colonies. On the other hand, BLB is a vascular disease resulting in systemic infection that produces tannish-grey to white lesions along the vein. In this study, five strains of *Pantoea* isolates were characterized. Among them, four strains were isolated from the diseased paddy plants and soil samples from Sekinchan and one strain was isolated from soil sample of UTAR, Kampar campus. Virulence study of five *Pantoea* isolates on rice cultivar was verified and their biochemical characteristics were further investigated.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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HAW XUE QI

APPROVAL SHEET

This final year project report entitled "**MOLECULAR CHARACTERIZATION OF *Pantoea* spp. THAT CAUSE BACTERIAL LEAF BLIGHT DISEASE IN PADDY PLANTS"** was prepared by HAW XUE QI and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **HAW XUE QI** (ID No: **15ADB06974**) has completed this final year project entitled "**MOLECULAR CHARACTERIZATION OF *Pantoea* spp. THAT CAUSE BACTERIAL LEAF BLIGHT DISEASE IN PADDY PLANTS**" under the supervision of Dr. Loh Pek Chin from the Department of Biomedical Science, Faculty of Science.

I understand that University will upload softcopy of my final year project in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(HAW XUE QI)

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LIST OF ABBREVIATIONS

°C	degree Celsius
µL	microliter
µm	micrometer
Bcc	*Burkholdera cepacia* complex
BLASTn	nucleotide-nucleotide Basic Local Alignment Search Tool
BLB	bacterial leaf blight
bp	base pair
cfu	colony forming unit
cm	centimetre
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Exo-SAP	exonuclease-shrimp alkaline phosphatase
g	gram
h	hour/hours
HR	highly resistant
HS	highly susceptible
L	liter
LP	lipopeptide
M	molar
MEGA	Molecular Evolutionary Genetics Analysis
min	minute/minutes

mL	milliliter
MLSA	multilocus sequence analysis
MLST	multilocus sequence typing
MR	MARDI
MR	moderately resistant
MS	moderately susceptible
NA	nutrient agar
NB	nutrient broth
NCBI	National Center for Biotechnology Information
OF	oxidative fermentative
PCR	polymerase chain reaction
PSA	peptone sucrose agar
R	resistant
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolution per minute
s	second/seconds
S	susceptible
SIM	sulfide-indole-motility
spp.	species
Tris-HCl	Tris hydrochloride
v/v	volume per volume
w/v	weight per volume
YDC	yeast dextrose chalk

CHAPTER 1

INTRODUCTION

Rice is the most important staple food crop with global production of 759.6 million tons (Food and Agriculture Organization of the United Nations, 2018). Rice consumption accounts for 90% of the world population particularly in Asia with China, India and Indonesia contribute 30.85%, 20.12% and 8.21% of total global rice production respectively (USDA, 2012). The rice crop is cultivated worldwide as it can adapt to different environmental conditions. In addition, rice is not only utilized as a foodstuff, but it is also an important ingredient for production of medicines and cosmetics, as well as involved in crafts and religious ceremonies (Plants of the World online, 2017).

Rice or its scientific name, *Oryza sativa* is a member of *Poaceae* family and is widely cultivated rice species in Asia. Besides O. *sativa*, an African cultigen named O. *glaberrima* is also widely cultivated but is limited in West Africa (Namara, 2015). There is a number of rice varieties used for paddy plantation in Malaysia as shown Figure 1.1. In general, the rice varieties can be classified into two groups, which are the short-duration varieties and long-duration varieties. The maturity of short-duration varieties take about 105-120 days and these varieties are designated by MARDI as MR 1, MR 7, MR 10, MR 84, MR 103, MR 123, MR 167, MR 219, MR 220, MR 221, MR 222 and MRQ74. Besides, a 120-days variety will spend 60 days in the vegetative phase and, 30 days in the each reproductive phase and ripening phase when planted in

tropical area. In contrast, the long-duration varieties require at least 150 days which is longer period than short-duration varieties for maturation (Ricepedia, n d.).

Figure 1.1: Different rice varieties used for paddy plantation (Adapted from Rice Knowledge Bank, n.d.).

Unfortunately, *Oryza sativa* is susceptible to bacterial leaf blight (BLB) disease which is caused by *Pantoea* spp. (Lee et al., 2010; Mondal el al., 2011). According to Swings et al. (1990), BLB disease is the most devastating paddy disease. BLB disease can be manifested by either leaf blight on mature plant or presented by kresek symptoms during the seedling stage (Agropedia, 2008; Mondal et al., 2011).

In October to December 2016, BLB disease outbreak that caused severe loss to the paddy farmers in Sekinchan area has been reported (Figure 1.2). Apparently, this is the worst rice disease outbreak in the Sekinchan area in the past 30 years, where 90% of the 4,440 hectares of paddy fields were infected, causing yield loss between 50%-70%. The most affected rice cultivar was MR

284, indicated that this cultivar was highly vulnerable to BLB. A total of RM 5 million is estimated to be lost during this outbreak. Therefore, this outbreak was a major constraint to the rice production in Malaysia and the livelihood of rice-growing farmers of Sekinchan (Lai and Chan, 2016). Farmers had tried various ways to improve the condition through the application of chemical pesticides and organic treatment as well as planting a more resistance cultivars such as MR 219, MR 220, MR 263 and MR 297. However, the chemical pesticides showed less effectiveness in the control of BLB disease. Besides, the resistance cultivars were also not resilient varieties, instead they were susceptible to BLB disease (Muthiah, 2013; Lai and Chan, 2016).

Figure 1.2: Paddy field in Sabak Bernam infected with BLB disease (Adapted from Lai and Chan, 2016).

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CHAPTER 2

LITERATURE REVIEW

2.1 Origin of cultivated rice

Rice or *Oryza sativa* belongs to *Poaceae* family is widely cultivated rice species in tropical and subtropical regions of Asia. Besides *O. sativa*, an African cultigen named *O. glaberrima* is also widely cultivated but is limited in West Africa (Namara, 2015). According to Khush (1997), *O. sativa* has five wild relatives and they are under the genus of *Oryza* as shown in Figure 2.1. Among these, *O. rufipogon* is closely related to *O. sativa* and is believed to be the progenitor of *O. sativa*. *O. sativa* can be divided into two ecotypes which are indica and japonica. Furthermore, *O. rufipogon* can be categorized as perennial and annual, which is normally based on the habitat preference and life-history traits (Morishima, 1992). Perennial population of *O. rufipogon* generally showed higher evolutionary potential, thus the perennial type can be the possible progenitor of *O. sativa* (Oka, 1974). However, according to Chang (1976), annual type of *O. rufipogon* is also a potential progenitor for *O. sativa* due to its high seed productivity.

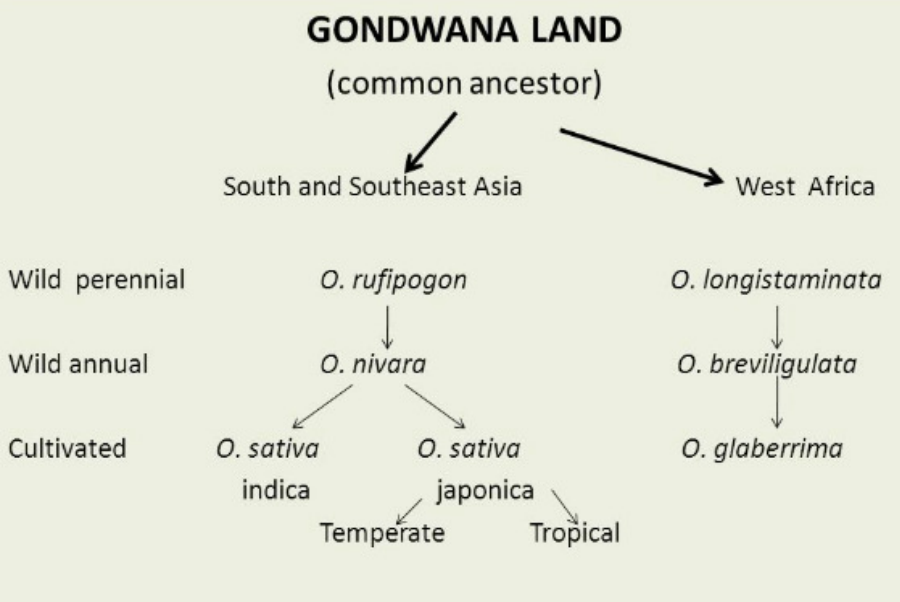

Figure 2.1: The evolutionary pathway of cultivated rice species (Adapted from Namara, 2015).

2.2 Bacterial leaf blight (BLB) disease

The BLB disease in paddy plants is mainly due to two phytopathogen bacteria such as infections of *Xanthomonas* spp. and *Pantoea* spp. The disease can be manifested by either leaf blight on mature plant or presented by kresek symptoms during the seedling stage. During the leaf blight phase, the pathogen enters the plant through wounds or natural openings. The pathogen then replicates in the xylem tissues (Tabei, 1997) and grow vigorously throughout the vascular system (Ezuka and Kaku, 2000).

2.3 Pathogens that caused bacterial leaf blight disease in paddy plants

2.3.1 Bacterial leaf blight disease of paddy plants caused by *Xanthomonas oryzae pv. Oryzae*

Rice is one of the most important food crops around the world especially of the Asia countries. However, this food crop is susceptible to many diseases including bacterial leaf blight (BLB) disease. BLB disease caused by *Xanthomonas oryzae pv. Oryzae* is one of the oldest known diseases and was initially reported in Fukuoka prefecture Kyushu Island, Japan in 1884 to 1885 and then reported in other growing countries (Ezuka and Kaku, 2000). In Asia, BLB caused by *Xanthomonas* has been an important threat to rice production (Swings et al., 1990). In 1951, BLB was first reported in India. In Philipines, the susceptible rice crops experienced yield loss of 22.5% in wet season and 7.2% in dry seasons, while the resistant rice crops encountered lesser yield loss of about 9.5% and 1.8% respectively (Savary et al., 2000). In the year 1982 to 1994, there was an incidence of BLB outbreak being reported in the rice fields of Peninsular Malaysia. The loss is estimated to be about RM 50 million (Saad and Habibuddin, 2010). In February 2014, another BLB outbreak caused by *Xanthomonas* was being reported in the rice fields of Padang Besar with the estimated yield loss of 60,000 metric ton (Utusan Online, 2014).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Apparatus and consumables

All the apparatus and consumables used are as shown in Appendix A.

3.2 Preparation of culture media

All media were sterilized by autoclaving at 121°C for 15 minutes before use.

3.3 Citrate basal medium

Basal medium was prepared by mixing 5 g sodium chloride, 2 g sodium citrate, 15 g agar, 1 g ammonium dihydrogen phosphate, 1 g dipotassium phosphate, 0.2 g magnesium sulphate and 0.08 g bromothymol blue with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.2 MacConkey agar

MacConkey agar was prepared by mixing 50 g MacConkey agar powder with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.3 Nutrient agar

Nutrient agar was prepared by mixing 28 g nutrient agar powder with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.4 Nutrient broth

Nutrient broth was prepared by mixing 8 g nutrient broth powder with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.5 Oxidative fermentative (OF) medium

OF medium was prepared by mixing 9.8 g OF medium powder with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.6 Sulfide-indole-motility (SIM) medium

SIM medium was prepared by mixing 30 g SIM medium powder with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.3.7 Yeast dextrose chalk (YDC) agar

YDC agar was prepared by mixing 10 g dextrose, 5 g yeast extract, 10 g calcium carbonate and 10 g agar with dH_2O to a final volume of 1 L. The mixture was stirred to dissolve completely prior to autoclaving.

3.4 Preparation of sterilized reagents

All reagents were sterilized by autoclaving at 121°C for 15 minutes before use.

3.4.1 Crystal violet, 0.1% w/v

Crystal violet solution (0.1% w/v) was prepared by dissolving 0.2 g crystal violet powder in dH_2O to a final volume of 200 mL and then stirred overnight in a 250 mL amber reagent bottle. The solution was filter sterilized prior to use.

3.4.2 Glucose solution, 10% w/v

Glucose solution (10% w/v) was prepared by mixing 1 g glucose powder with dH_2O to a final volume of 10 mL.

3.4.3 Glycerol solution, 65% v/v

Glycerol solution (65% v/v) was prepared by mixing 162.5 g glycerol, 20 mL 1 M magnesium sulphate and 5 mL 1 M Tris-HCl (pH 8) with dH_2O to a final volume of 200 mL.

3.4.4 Saline solution, 0.85% w/v

Saline solution (0.85% w/v) was prepared by mixing 8.5 g sodium chloride powder with dH_2O to a final volume of 1 L.

3.4.5 Tris-acetate-EDTA buffer

Tris-acetate-EDTA (TAE) buffer was prepared by dissolving 242 g Tris base, 37.2 g EDTA and 57.1 mL glacial acetic acid in dH_2O to a final volume of 1 L. The buffer was diluted 50 times before use.

3.6 List of bacterial strains that used as positive and negative controls in biochemical tests

Table 3.2: List of bacterial strains used in this study

Bacterial strains used in biochemical tests	Source
Enterobacter aerogenes	Laboratory glycerol stock
Enterococcus faecalis	Laboratory glycerol stock
Escherichia coli	Laboratory glycerol stock
Klebsiella pneumonia	Laboratory glycerol stock
Pseudomonas aeruginosa	Laboratory glycerol stock

Salmonella typhimurium	Laboratory glycerol stock
Staphylococcus epidermidis	Laboratory glycerol stock

(Singh, unpublished)

3.7 Phenotypic observation of *Pantoea* strains

3.7.1 Preparation of master plates

Pantoea strains used were retrieved from glycerol stocks keep at -80ºC refrigerator prepared by seniors under the supervision of Dr. Loh Pek Chin (Table 3.1) (Loh et al., unpublished). Master plates were prepared by sub-culturing each strain on nutrient agar (NA) and incubated at 30°C for 24 h. The colony morphology of each strain was examined and recorded.

3.7.2 Preparation of bacterial smear

An inoculating loop was flame sterilized and a drop of 0.85% saline solution was placed to the centre of a clean slide. The inoculating loop was then re-sterilized and once cooled, a single isolated colony was picked and mixed evenly with the saline solution on the slide. The smear was spread evenly covering an area of about 15-20 mm diameter on a slide and left aside to air-dry. Once dried, the smear was heat-fixed by passing the slide quickly through the flame 2-3 times. The smear was now ready for gram staining (Gram Staining of Bacteria from Environmental Sources, 2017).

3.7.3 Gram staining

Gram staining procedure was carried out as described by Jonit et al. (2016). Bacterial smear was first treated with 0.1% crystal violet for 1 min and followed by water rinse. Bacteria then treated with Lugol iodine solution for 1 min, rinsed and decolorized with 95% ethanol for 30 s. After that, decolorized bacteria was water-rinsed again and counter-stained with safranin for 1 min. The stained smear was examined under oil immersion at 1000x magnification.

3.8 Biochemical tests

The following biochemical tests were performed on pure and fresh (18-24 h) cultures of *Pantoea* strains. Positive and negative controls were added for each test (Table 3.2).

3.9.3 Determination of PCR products by agarose gel electrophoresis

Targeted PCR products were confirmed by electrophoresis on 2% w/v agarose gel. A volume of 2 µL each PCR product was analysed on agarose gel along with 2 µL of 1kb DNA ladder. Electrophoresis was performed at 80 V for 40 min. After that, the gel was stained with ethidium bromide (EtBr) for 5 min and destained with dH_2O for 2 min. The EtBr-stained products were then visualized under an ultraviolet transilluminator.

3.9.4 Purification of PCR products

Once the amplification of the targeted genes have been confirmed via agarose gel electrophoresis, the PCR products were purified to remove excess primers, nucleotides and polymerase present in the PCR mix. The purification of PCR

products was conducted using ExoSAP-IT™ PCR product cleanup kit according to the manufacturer's instructions. The concentration of the purified PCR products were assessed with nanospectrophotometer as before (Section 3.9.1) and the concentration should be greater than 10 ng/uL in order to be sent for DNA sequencing.

3.9.5 DNA sequencing

The purified PCR products together with forward and reverse primers in separated tubes were sent directly for DNA sequencing at First Base Laboratories Sdn Bhd Shah Alam Selangor, Malaysia.

3.9.6 DNA sequence data analysis via BLASTn

The DNA sequencing data of four housekeeping genes was first deposited in NCBI gene bank to determine the identity and accession numbers for the *Pantoea* strains. The DNA sequences obtained were then subjected to Basic Local Alignment Search Tool Nucleotide (BLASTn) analysis, an open access database, which available on National Center for Biotechnology Information (NCBI) website with URL: https://blast.ncbi.nlm. nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.

3.12 Long-term storage of bacterial strains

Two loopfuls of colonies for each strain were evenly suspended in 0.5 mL of nutrient broth. The suspension was then mixed with 0.5 mL of 65% v/v glycerol solution to make a final of 50% glycerol stock and stored at -80°C.

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CHAPTER 4

RESULTS

CHAPTER 5

DISCUSSION

CHAPTER 6

CONCLUSIONS

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APPENDIX A

APPARATUS AND CONSUMABLES

The lists of apparatus and consumables used in this project are as follow:

Table A1: List of apparatus and their respective manufacturers.

Apparatus	Manufacturer
Centrifuge machines	Stigma
Electrophoresis system	Major science
Fume hood	NJ global
Gel imaging system	Syngene bio imaging
Heatblock	Bioshake iQ
Incubator	Sanyo
Laminar air flow cabinet	Isocide™
Light microscope	Leica CME
Microcentrifuge machines	Thermoscientific
Nanospectrophotometer	IMPLEN, Thermoscientific
PCR thermocycler	Eppendorf,
Spectrophotometer	Biochrom
Votexer	Science Lab Asia

Table A2: List of consumables and their respective manufacturers.

Consumable	Manufacturer
1kB DNA ladder	Thermo Fisher Scientific, USA
95 % ethanol	Copens Scientific, Malaysia
Agar-agar powder	Merck, Germany
Agarose powder	1st Base, Malaysia
BactiDrop™ oxidase reagent	Thermo Fisher Scientific, USA
Basal medium	Merck, Germany
Crystal violet	LabChem, Malaysia
ExoSAP-IT™	Thermo Fisher Scientific, USA
Forward and reverse primers	Integrated DNA technologies, US
GF-1 Bacterial DNA extraction kit	Vivantis, Singapore
Gram stain iodine	R & M chemicals, UK
Hydrogen peroxide	R & M chemicals, UK
MacConkey agar	Merck, Germany
Nutrient agar	Merck, Germany
Nutrient broth	Merck, Germany
PCR Green MasterMix	ProMega, USA
PCR MyTaq™ DNA Polymerase	Bioline, Malysia
PCR purification kit	Favorgen, Taiwan
RNase	Invitrogen, US
Safranin	LabChem, Malaysia
SIM medium	HiMedia, USA
Tris hydrochloride	Thermo Fisher Scientific, USA