UNIVERSITI PUTRA MALAYSIA

ACTINOMYCETES AS PLANT GROWTH PROMOTER OF RICE PLANTS AND BIOCONTROL AGENT AGAINST BACTERIAL LEAF STREAK DISEASE

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FP 2019 28

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By

ERNEEZA MOHD HATA

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

January 2019

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This thesis is dedicated to

MY HUSBAND

MY SON

MY MOTHER AND FATHER

MY FATHER AND MOTHER IN LAW'S SOULS

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

ACTINOMYCETES AS PLANT GROWTH PROMOTER OF RICE PLANTS AND BIOCONTROL AGENT AGAINST BACTERIAL LEAF STREAK DISEASE

By

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January 2019

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Bacterial leaf streak (BLS) disease caused by *Xanthomonas oryzae* pv. *oryzicola (Xoc)* is one of the most important rice bacterial diseases. Genotypic and phenotypic similarity of this pathogen to the causal agent of bacterial leaf blight (BLB), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), has driven an interest on BLS study. Bacterial leaf streak control measures are focusing on the development of resistant varieties and application of copper-based fungicide. Actinomycetes possess a remarkable potential as biological agent. This study was conducted with the following objectives; 1) to characterize the causal pathogen of BLS disease, *Xoc* and to differentiate it from other *Xanthomonas oryzae* pathovar, *Xoo*, 2) to characterize and determine the potential of actinomycetes as biocontrol agent against *Xoc* and as plant growth promoter and 3) to evaluate the efficacy of actinomycetes treatment on rice growth promotion and BLS suppression through the induction of defense-related enzymes.

Five *Xoc* isolates were discovered in this study and successfully differentiated with *Xoo*. BLS symptoms were pronounced during early infection stage, yellow small streak lesions can be observed in rice leaves. *Xoc* can be detected and differentiated with *Xoo* by multiplex PCR method. Molecular characterization by gyrase subunit B gene amplification had successfully identified all five isolates as *Xanthomonas oryzae* pv. *oryzicola* (accession number in GeneBank database (MH560793-MH560797). All isolates developed hypersensitivity reaction on *Nicotiana tabacum*. Isolate TKC1 is the most virulent isolate, produced 6.78 cm lesion length in pathogenicity assay compared to other four *Xoc* isolates.

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Out of 20 *Xoc* antagonistic actinomycetes, 60% were belonged to various species of *Streptomyces*. Rhizospheric actinomycetes, SS8 demonstrated the highest ($p \leq 0.05$) antagonistic activity with inhibition value of 17.67 mm, followed by TKSC3 (14.00 mm). Isolate SS8 and TKSC3 were identified as *Streptomyces* sp SW4-2S and *Streptomyces shenzhenensis*, respectively by 16s rRNA amplification. Both isolates also possess potentials as hydrolytic enzyme producer and plant growth promoter based on *in vitro* assessment.

In vitro assessment with 12 hours seed bacterization with single or consortium treatment of TKSC3 and SS8 isolates revealed significant ($p \leq 0.05$) improvement in rice seed germination and seedling vigor performance. TKSC3 and SS8 were both root colonizing and endophytic streptomycetes with average population ranged from 0.66 to 6.52 x 10^3 CFU/g in seedling roots at 10 days after seed bacterization treatment. Consortium treatment (TKSC3+SS8) exhibited the highest values in plant growth parameters, total chlorophyl, soil and leaf nutrient contents in glasshouse experiment. Consortium treatment (TKSC3+SS8) also demonstrated the highest BLS disease suppression efficiency at 81.02% with the lowest AUDPC value of 95.79. Single and consortium treatments of actinomycetes successfully suppressed BLS disease by enhancing defense-related enzymes accumulation in the rice plant. Peroxidase, polyphenol oxidase, phenyl ammonia lyase and β,1-3 glucanase enzymes activity were increased in actinomycete-treated plants compared to untreated plants which started at 2 days post inoculation. This study confirmed that actinomycetes possess huge potentials as plant growth promoter and biocontrol agent against *Xoc* pathogen.

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

ACTINOMYCETES SEBAGAI PENGGALAK PERTUMBUHAN POKOK PADI DAN AGEN BIOKAWALAN TERHADAP PENYAKIT JALUR DAUN BAKTERIA

Oleh

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Penyakit Jalur Daun Bakteria (BLS) yang disebabkan oleh *Xanthomonas oryzae* pv. *oryzicola (Xoc)* adalah antara penyakit padi yang penting yang disebabkan oleh bakteria. Persamaan genotip dan fenotip antara patogen ini dengan agen penyebab penyakit hawar daun bakteria (BLB), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) telah mendorong minat dalam kajian penyakit BLS. Langkah kawalan untuk penyakit BLS lebih difokuskan kepada pembangunan varieti rintang dan penggunaan fungisid berasaskan kuprum. *Actinomycetes* memiliki potensi yang luar biasa sebagai agen biologi. Oleh itu kajian ini dijalankan berdasarkan objektif berikut; 1) untuk mencirikan patogen punca penyakit BLS, dan membezakan antara *Xoc* dan *Xoo*, 2) untuk mencirikan dan menentukan potensi *actinomycetes* sebagai agen biokawalan terhadap *Xoc* dan sebagai penggalak pertumbuhan padi dan 3) untuk menilai keberkesanan rawatan *actinomycetes* ke atas pertumbuhan padi dan pengawalan penyakit BLS melalui induksi rintangan enzim.

Lima isolat *Xoc* telah ditemui dalam kajian ini dan berjaya dibezakan dengan *Xoo*. Simptom-simptom BLS jelas kelihatan ketika peringkat awal jangkitan, lesi jalur kecil berwarna kuning dapat dilihat pada daun pokok padi. *Xoc* dapat dikesan dan dibezakan dengan *Xoo* melalui kaedah PCR multipleks. Kaedah pencirian molekular melalui amplifikasi gen gyrase subunit B berjaya mengenalpasti kesemua lima isolat sebagai *Xanthomonas oryzae* pv. *oryzicola* (nombor kemasukan data GenBank (MH560793-MH560797). Kesemua isolat menunjukkan reaksi hipersensitif terhadap *Nicotiana tabacum*. Isolat TKC1 merupakan isolat yang paling virulen, menghasilkan 6.78 cm panjang lesi berbanding empat isolat lain dalam ujian kepatogenan.

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Daripada dua puluh actinomycetes antagonis *Xoc,* 60% daripadanya adalah daripada pelbagai spesis *Streptomyces*. Actinomycetes daripada rizosfera, SS8 menunjukkan aktiviti antagonis yang tinggi ($p<0.05$) dengan nilai perencatan sebanyak 17.67 mm, diikuti dengan TKSC3 (14.00 mm). Isolat SS8 dan TKSC3 masing-masing dikenalpasti sebagai sebagai *Streptomyces* sp SW4-2S dan *Streptomyces shenzhenensis* melalui amplifikasi 16s rRNA. Kedua-dua isolat menunjukkan potensi sebagai pengeluar enzim hidrolisis dan penggalak pertumbuhan pokok berdasarkan penilaian *in vitro*.

Penilaian *in vitro* menunjukkan pembakteriaan biji benih selama 12 jam melalui rawatan tunggal atau konsortium daripada isolat TKSC3 dan SS8 memberi penambahbaikan yang signifikan ($p<0.05$) terhadap percambahan biji benih dan indeks pertumbuhan. Kedua-dua TKSC3 dan SS8 berupaya mengkoloni pada akar dan merupakan *streptomycetes* bersifat endofit dengan purata populasi antara 0.66 ke 6.52 x 10^3 CFU/g dalam akar pada anak benih yang telah 10 hari dirawat dengan cara pambakteriaan biji benih. Rawatan konsortium (TKSC3+SS8) menunjukkan nilai yang tinggi untuk parameter pertumbuhan pokok, jumlah klorofil, kandungan nutrien tanah dan daun melalui eksperimen rumah kaca. Rawatan konsortium (TKSC3+SS8) juga menunjukkan keberkesanan kawalan penyakit BLS yang paling tinggi pada 81.02% dengan nilai AUDPC yang terendah iaitu 95.79. Rawatan tunggal dan konsortium *actinomycetes* berjaya mengawal penyakit BLS dengan mempertingkatkan pengumpulan enzim berkaitan pertahanan pada pokok padi. Enzim *peroxidase, polyphenol oxidase, phenyl ammonia lyase* dan *β,1-3 glucanase* telah dipertingkatkan dalam pokok yang dirawat dengan *actinomycetes* dibandingkan dengan pokok yang tidak dirawat bermula 2 hari selepas inokulasi patogen. Kajian ini mengesahkan *actinomycetes* mempunyai potensi yang besar sebagai penggalak pertumbuhan pokok dan agen kawalan biologi terhadap patogen *Xoc.*

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ACKNOWLEDGEMENTS

All praises to Allah S.W.T the Almighty, for giving me the blessing, chance, strength and endurance to complete this study. Your blessing and compassionate has made my journey through this far.

First and foremost, I would like to thank Universiti Putra Malaysia for giving me this oppurtunity to pursue my doctoral degree. Also not to forget Ministry of Higher Education (MOHE) for granted a research grant under LRGS (Food Security: Enhancing sustainable rice production) that funded most of research materials in this study.

I would like to express my sincere gratitude to my supervisor Dr. Kamaruzaman Sijam for his time, generous guiddance, patience, encouragement and support throughtout this journey. I also would like to extend my gratitude and appreciation to my committee members, Dr. Mohd Termizi Yusof and Dr. Dzarifah Mohamed Dzulperi for your generous support, help and encouragement. I do appreciate all the kindness given towards me and may Allah grant all your wishes.

My appreciation also goes to all staff and ex-staff in Faculty of Agriculture especially Ms. Junaina Jaafar, Mr. Johari Sarikat, Ms. Asmalina Abu Bakar, Mr. Mohamed Nazri Abdul Rahman, Ms. Nor Azlina Razak, Ms. Roslina Nor and Ms. Noorizajune Abu Bakar. I also acknowledge every supports, advices and wisdom given by all of my friends and labmates; Dr. Nor Aisyah Azman, Nor Fatin Ideris, Dr. Tavga Sulaiman, Latifah Musanif, Hasmaniza Zainul Abidin, Juliesafarina Mohd Janjang, Dr. Norhayu Asib, Dr. Anis Syahirah Mokhtar, Zatil Aqmar Shukri, Iszuanie Ilyas, Azza, Hazlina and all those friends who i could not list here.

To my father and mother, Haji Mohd Hata Mohd Fadzil and Hajjah Asmah Sabirin, without your blessing and doa', i might not be able to stand were I am now. The word "Thank You" is undervalued compared to what you have done. I would also like to acknowledge for the strengh given to me by my siblings; Kak Yong Rosmaliza, Abg Haris, Yana, Jaja, Hafiz, Daus, Rahim and Eina.

Special thanks were saved for the last, to the very special man in my heart, my beloved husband, Edi Edham Othman for his tremendous patience, support and sacrifices. All your valuable time and sacrifices that contributed to this journey were overwhelming and the word thank you is never enough to repay all of your kindness. Also to my little hero, the greatest gift from Allah, Eijaz Yusuff Edi Edham, who empower me to complete my PhD journey.

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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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TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiv
LIST OF FIGURES		xvi
LIST OF ABBREVIATIONS		xix
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEWS**	3
	2.1 Rice Industry in Malaysia	3
	2.2 Major Constraint of Rice Production in Malaysia	5
	2.3 Bacterial Diseases of Rice	6
	2.4 Bacterial Leaf Streak (BLS) Disease	6
	2.4.1 Distribution and Epidemiology	6
	2.4.2 Disease Symptoms	7
	2.4.3 *Xanthomonas oryzae* pv. *oryzicola*	8
	2.4.4 Control Strategies	9
	2.5 Actinomycetes	10
	2.5.1 Characteristics and Diversity of Actinomycetes	10
	2.5.2 Actinomycetes as Plant Growth Promoter (PGP) and Biocontrol Agent	11
	2.6 Mechanisms Involved in Plant Growth Promotion by PGPR	12
	2.6.1 Biological Nitrogen Fixation	12
	2.6.2 Phosphate Solubilisation	13
	2.6.3 Siderophore Production	13
	2.6.4 Phytohormone Production	14
	2.7 Mechanisms Involved in Antagonistic Activity by Biocontrol Agent	14
	2.7.1 Antibiosis	14
	2.7.2 Lysis Mechanism	15
	2.7.3 Induction of Host Resistance	15
3	**IDENTIFICATION AND CHARACTERIZATION OF *Xanthomonas oryzae* pv. *oryzicola* ISOLATES OBTAINED FROM RICE PLANTS**	17
	3.1 Introduction	17
	3.2 Materials and Methods	18
	3.2.1 Samples Collections	18
	3.2.2 Isolation and Morphological Characterization	19
	3.2.3 Hypersensitivity Reaction on *Nicotiana tabacum*	20

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3.2.4 Pathogenicity Assay of *X.oryzae* pv. *oryzicola* 20
3.2.5 Biochemical Characterization 21
3.2.5.1 Gram Characterization 21
3.2.5.2 Starch Hydrolysis 21
3.2.5.3 Growth on 0.2% Casamino Acid 21
3.2.5.4 Growth on L-alanine as Carbon Source 21
3.2.5.5 Growth on 0.001% Copper Nitrate 22
3.2.5.6 Acetoin Production 22
3.2.6 Molecular Identification through Insertion Sequence Element IS1113 Amplification and *gyrB* Housekeeping Gene Amplification 22
3.2.6.1 DNA Extraction 22
3.2.6.2 Insertion Sequence Element IS1113 Amplification 23
3.2.6.3 DNA gyrase Subunit B Gene Amplification 23
3.2.6.4 PCR Product Fractionation 24
3.2.6.5 PCR Product Purification and Sequence Identification 24
3.2.6.6 Phylogenetic Analyses 24
3.2.7 Rapid Identification of *Xanthomonas oryzae* Pathovar using Multiplex Sequencing Method 24
3.3 Results and Discussion 25
3.3.1 Isolation and Morphological Characterization of Causal Pathogen 25
3.3.2 Hypersensity Reaction on *Nicotiana tabacum* 27
3.3.3 Pathogenicity Assay of *X.oryzae* pv. *oryzicola* 29
3.3.4 Biochemical Characterization 31
3.3.5 Molecular Identification through Insertion Sequence Element IS1113 Amplification and *gyrB* Housekeeping Gene Amplification 33
3.3.6 Rapid Identification of *X. oryzae* pathovar Using Multiplex Sequencing Method 36
3.4 Conclusion 37

4 CHARACTERIZATION AND EFFICACY OF ACTINOMYCETES AS BIOCONTROLL AGENT AGAINST *Xanthomonas oryzae* pv. *oryzicola* AND PLANT GROWTH PROMOTER OF RICE PLANT 38
4.1 Introduction 38
4.2 Materials and Methods 38
4.2.1 Isolation of Actinomycetes 38
4.2.2 Screening of Actinomycetes Antibacterial Activity against *X.oryzae* pv. *oryzicola* 40
4.2.3 Identification of Actinomycetes Using Molecular Method 41
4.2.3.1 DNA Extraction 41
4.2.3.2 16s rRNA amplification 42
4.2.3.3 PCR Product Fractionation 42

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4.2.3.4 PCR Product Purification and Sequence Identification 42
4.2.4 *In vitro* Assessment of Plant Growth-Promoting Activities by Antagonistic Actinomycetes 42
4.2.4.1 Phosphate Solubilization 42
4.2.4.2 Siderophore Production 43
4.2.4.3 Phytohormone (Indole Acetic Acid, IAA) Production 43
4.2.4.4 Nitrogen Fixation 43
4.2.4.5 Hyrogen Cyanide Production 44
4.2.5 *In vitro* Assessment of Hydrolytic Enzyme Production by Antagonistic Actinomycetes 44
4.2.5.1 Amylase Production 45
4.2.5.2 Protease Production 45
4.2.5.3 Lipase Production 45
4.2.5.4 Cellulase Production 45
4.2.6 Statistical Analysis 45
4.3 Results and Discussion 45
4.3.1 Actinomycetes Population and Isolation 45
4.3.2 Screening of Actinomycetes Antibacterial Activity against *X. oryzae* pv. *Oryzicola* 47
4.3.3 Identification of Actinomycetes using Molecular Method 50
4.3.4 *In Vitro* Assessment of Plant Growth-Promoting Activities by Antagonistic Actinomycetes 52
4.3.5 *In Vitro* Assessment of Hydrolytic Enzyme Production by Antagonistic Actinomycetes 57
4.4 Conclusion 60

5 EFFECT OF STREPTOMYCETES ON GROWTH PROMOTION OF RICE PLANT, BACTERIALL LEAF STREAK SUPPRESSION AND INDUCTION OF DEFENSE RELATED ENZYMES 61
5.1 Introduction 61
5.2 Materials and Methods 62
5.2.1 Effect of Streptomycetes Treatment on Rice Seedlings(*In Vitro* Assessment) 62
5.2.1.1 Streptomycetes Inoculum Preparation 62
5.2.1.2 Treatment Preparation and Delivery Method 62
5.2.1.3 Germination Test and Seed Vigor Performance 63
5.2.1.4 Streptomycetes Enumeration in Rhizoplane and Endosphore 63
5.2.2 Effect of Streptomycete Treatments on Early Rice Growth Under Greenhouse Conditions 64
5.2.2.1 Treatment Preparation and Delivery Method 64

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5.2.2.2 Plant Growth Assessment 64
5.2.2.3 Total Chlorophyll Content 64
5.2.2.4 Soil and Tissue Analysis 65
5.2.3 Effect of Streptomycetes Treatment on BLS Disease Suppression and Induction Of Defense Related Enzymes 65
5.2.3.1 Treatment Preparation and Delivery Method 65
5.2.3.2 *Xanthomonas oryzae* pv. *oryzicola* Inoculation 66
5.2.3.3 Disease Assessment 66
5.2.3.4 Induction of Defense Enzymes by Streptomycetes Strains in Rice Against *Xoc* Pathogen 67
5.2.4 Statistical Analysis 70
5.3 Results and Discussion 70
5.3.1 Effect of Streptomycetes on Rice Seedling Promotion (*In Vitro* Assessment) 70
5.3.2 Effect of Streptomycete Treatments on Rice Growth Promotion Under Greenhouse Conditions 75
5.3.3 Effect of Streptomycetes Treatment on BLS Disease Suppression and Induction Of Defense Related Enzymes 77
5.3.3.1 Effect of Streptomycetes Treatment on BLS Disease 77
5.3.3.2 Induction of Defense Enzymes By Streptomycete Treatments in Rice Against *Xoc* Pathogen 81
5.4 Conclusion 86

6 SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 87
6.1 Summary 87
6.2 Conclusions 90
6.3 Recommendations for Future Research 90

REFERENCES 91
APPENDICES 110
BIODATA OF STUDENT 136
LIST OF PUBLICATIONS 137

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LIST OF TABLES

Table		Page
2.1	Differentiation in Biochemical Test of Pathovars of *Xanthomonas oryzae*.	9
2.2	Taxanomy and Classification of Actinomycetales	10
3.1	Total Rice Plants Collected from Different Sampling Location with Related Information	18
3.2	List of Specific Primers for Two Pathovars Of *X. oryzae* (*Xoc* And *Xoo*) based on Lang et al. (2010) Multiplex Method.	25
3.3	Number of *Xoc* Isolates Obtained Based on Morphological Characteristic in PSA Medium.	27
3.4	Hypersensitivity Response of the 23 Potential *Xoc* Isolates and New Designated Code	28
3.5	Lesion Length Produced by Suspected *Xoc* Isolates in This Study in Pathogenicity Assay	30
3.6	Comparison of Biochemical Characterizations of Suspected *Xoc* Isolates	32
3.7	Molecular Identification based on *gyrB* Gene Amplification	34
3.8	Molecular Identification and Accession Numbers of Pathogen Isolates	36
4.1	Details of Sampling Locations for Actinomycetes Isolation	39
4.2	Sample Preparation for Actinomycetes Isolation for Each Types of Samples.	40
4.3	Actinomycetes Population in Soil, Rhizosphere, Roots, and Leaves from Different Locations, Rice Varieties and Plant Growth Stages.	48
4.4	Rhizospheric and Endophytic Classification and Distribution af Actinomycetes Isolates from Different Locations and Isolation Sources	49
4.5	16s rRNA Molecular Identification af Active Antagonistic Actinomycetes against *Xoc*	50

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4.6	Plant Growth-Promoting Activities by Antagonistic Actinomycetes	56
4.7	Production of Hydrolytic Enzyme by Active Antagonistic Actinomycetes	58
5.1	*In vitro* assessment on Rice Seed Germination and Seedlings Quality.	63
5.2	Treatments of *In Vivo* Assessment on BLS Disease Suppression and Induction of Defense-Related Enzyme	66
5.3	Formula for Disease Score, Disease Severity, Area Under Disease Progress Curve (AUDPC) and Disease Suppression Efficiency	67
5.4	Effect of Seed Bacterization Period and Types of Treatments on Seed Germination and Seedling Growth Performance *In Vitro*	71
5.5	Actinomycetes Population on Rhizoplane and Endosphere From Root Seedlings at Day 10 after Seed Bacterization Treatment	74
5.6	Effect of Streptomycete-Seed Bacterization on Growth of Rice Seedlings after 30 Days Transplanting Under Greenhouse Conditions	75
5.7	Total Chlorophyll and Nutrient Profile in Leaf and Soil From Rice Plant Grown under Greehouse Conditions (30 Days After Transplanting)	76
5.8	Effect of Different Treatment on *Xoc* Inoculated Plant	81

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LIST OF FIGURES

Figure		Page
2.1	Rice Production and Total Imports of Rice in Malaysia for 2012-2017 (MOA 2017)	3
2.2	Hectareage Percentage of Paddy Varieties for Overall Granary Area in Peninsular Malaysia on 2014 (DOA 2015)	4
2.3	Average Yield of Paddy and Rice by All Seasons in Malaysia, 2010-2017 (MOA 2017)	5
2.4	Distribution Map of *Xanthomonas oryzae* pv. *oryzicola* Worldwide (EPPO, 2017)	7
2.5	Symptoms of BLS Disease in Rice Cultivation Area	8
2.6	Comparison of Induced Resistance in Plants, which Lead to Similar Phyenotypic Reponses	16
3.1	Sampling Location of BLS Disease in this Study	18
3.2	Symptoms of BLS Disease and BLB Disease Observed in Rice Cultivation Areas in Kuala Muda, Kedah And Cherang Ruku, Kelantan	19
3.3	Isolation and Morphological Identification of *Xoc* on PSA Media.	26
3.4	Hypersensitivity Reactions on *Nicotiana tabacum* cv. *Xanthi.*	28
3.5	Pathogenicity Test on MR219 Variety Observed within 10 DPI (Days of Postinoculation)	30
3.6	Agarose Gel Electrophoresis of PCR Products Amplified by TXT/TXT4R Primer Pair for IS1113 Insertion Sequence Element	33
3.7	Agarose Gel Electrophoresis of PCR Products Amplified by *gyrb* Primer Pair for DNA gyrase Subunit B Characterization	34
3.8	gyrB Gene Amplification Characterized and Differentiated *X. oryzae pv. oryzicola* from other *Xanthomonas* Strains	35

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3.9	Multiplex Polymerase Chain Reaction Primers Differentiated *Xoc* from *Xoo*	37
4.1	Sampling Location of Actinomycetes in this Study	39
4.2	Determination of Antibacterial Activity by Chloroform Vapour Method	41
4.3	Positive Reactions of Antibacterial Activity against *Xoc* by 20 Antagonistic Actinomycetes.	46
4.4	*In Vitro* Antibacterial Activities of Active Actinomycetes against *Xoc* Pathogen Isolate, TKC1.	46
4.5	Agarose Gel Electrophoresis of PCR Products Amplified By 8F/1492R Primer Pair for 16s rRNA Amplification	51
4.6	Phosphate Solubilization on Pikovskaya Media	52
4.7	Siderophore Production on Chrome Azurol S Agar Media (CAS)	53
4.8	IAA Production on Media Supplemented with L-Tryptophan and Salkowski Reagent as Indicator	54
4.9	Nitrogen fixation activity on nitrogen free medium	54
4.10	Colour Observation of Filter Papers Impregnated with Picric Acid Solution Placed on the Petri Dish Lid.	55
4.11	Positive Reaction of each Hydrolytic Enzyme Assay Conducted in This Study.	57
4.12	Classification of Different Strains of Actinomycetes Based on Hydrolytic Enzyme Production and Plant Growth-Promoting Assessment.	59
5.1	The Index Value with Corresponding Levels of Severity for Bacterial Leaf Streak (BLS) Disease Based on Standard Evaluation System (SES) by IRRI	66
5.2	Seedling Growth Observation in Paper Towel Assay	72
5.3	Light Microscopy Image of Untreated and Treated Seedling Roots	73

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5.4	Effect of Streptomycetes Treatment (by Seed Bacterization and Soil Drench) upon *Xoc* Pathogen Challenged on Disease Severity	78
5.5	BLS Symptom at 10 DPI	79
5.6	*Xoc*-Inoculated Plant with Seed Bacterization and Soil Drench Treatment of Streptomycetes and Pathogen Control Treatment at 20 DPI	80
5.7	Total Protein of Each Tissue Extracted from Leaf Samples Collected from Different Treatment and Interval Time	82
5.8	Defense Enzymes (POX, PPO, PAL, GLU) Activity in Leaves.	83

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LIST OF ABBREVIATIONS

BLS	Bacterial leaf streak
Xoc	*Xanthomonas oryzae* pv. *oryzicola*
BLB	Bacterial leaf blight
Xoo	*Xanthomonas oryzae* pv. *oryae*
ISR	Induced systemic resistance
PGPR	Plant growth-promoting bacteria
DNA	Deoxyribonucleic acid
ETP	Economic Transformation Programme
NKEA	National Key Economic Areas
EPP	Entry Point Projects
kg/ha	Kilogram per hectare
Xap	*Xanthomonas axonopodis* pv. *punicae* (Xap)
N_2	Nitrogen
ATP	Adenosine triphosphate
P	Phosphorus
IAA	Indole-3-acetic acid
GA	Gibberellins
CK	Cytokinins
ABA	Abscisic acid
ET	Ethylene
SAR	Systemic acquired resistance
PR	Pathogenisis-related
PAL	Phenylalanine ammonia lyase
POX	Peroxidase
PPO	Polyphenol oxidase
PSA	Peptone sucrose agar
cm	Centimeter
°C	Degree celcius
nm	nanometer
CFU/mL	Colony forming unit per milimeter
mL	Milimeter
KOH	Potassium hydroxide
PSB	Peptone sucrose broth
µL	microliter
DPI	days post inoculation
PCR	Plymerase chain reaction
TAE	Tris-acetic-EDTA
HR	Hypersensitivity reactions
gyrB	Gyrase unit B
AIA	Actinomycetes isolation agar
ISP-2	Yeast Malt Agar
CAS	Chrome Azurol S
$FeCl_3$	Iron III chloride
$HCLO_4$	Acid perchloric
M	Molar
IAA/mL	Indole-3-acetic acid per milimeter
NF	Nitrogen free

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TSA	Tryptone soy agar
HCN	Hydrogen cyanide
CMC	Carboxymethyl cellulose
CRD	Completely randomised design
ANOVA	Analysis of variance
CFU/g	Colony forming unit per gram
mm	Milimeter
µg/mL	Microgram per milimeter
NC	Negative control
PC	Positive control
cm	centimeter
rpm	Rotation per minute
V	Volt
NPK	Nitrogen phosphorus potassium
RCBD	Randomized block design
v/v	Volume per volume
mg/g	Miligram per gram
AUDPC	Area under disease progress
GLU	β-1, 3-glucanase
mM	Mili Molar
dai	Days after inoculation
µmol min^{-1} mg $protein^{-1}$	Micromol per minute per miligram protein
nmol min^{-1} mg $protein^{-1}$	nanomol per minute per miligram protein

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CHAPTER 1

INTRODUCTION

Rice is the most widely grown crop in the world and also staple food of more than 50% of the world's population (Chauhan et al. 2017). However, rice production is vulnerable to various diseases and bacterial leaf streak (BLS) is one of the constraints. The disease is widely distributed in tropical and subtropical Asian countries including China, Thailand, Malaysia, India, Vietnam, Phillippines and Indonesia (Nino-Liu et al., 2006). *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) is known as the causal pathogen for BLS disease. *Xoc* is highly related to *Xanthomonas oryzae* pv. *oryzae* (*Xoc*), the causal agent of Bacterial Leaf Blight (BLB) disease on rice. Both are phenotypically and genotypically highly related and difficult to differentiate (Wonni et al., 2014).

Cultivation of resistant varieties is one of the strategies to overcome BLS disease emergence. However, consecutive years of cultivation lead to disease susceptibility (Ouchdouch et al., 2001). Chemical and antibiotic application has also become an option, but cost and effectiveness frequently become an issue. Apart from that, highly dependence on chemical inputs causes several negative impacts on the development of plant resistance and effects on the environment (Compant et al., 2005).

Biocontrol agent has received a major attention due to inexpensive, long lasting and safe alternative in controlling plant diseases (Aghighi et al., 2004). Biocontrol agents are beneficial microorganisms that suppress disease emergence through antibiosis, lysis mechanism, plant growth promotion and systemic resistance induction (Berg and Hallmann, 2006). Plant growth promoting activities such as indole acetic acid (IAA) production, siderophore production, phosphate solubilisation, hydrogen cyanide, ammonia production and nitrogen fixation characteristics indirectly help to combat disease development. Therefore, biological control through the use of plant growth-promoting bacteria is the highlight among the potential solutions in disease control (Nelson, 2004).

Actinomycetes especially *Streptomyces* species are widely recognized as industrially important microorganisms because of their ability to produce novel secondary metabolites. Actinomycetes present extensively in soil and rhizosphere as free living and root colonizing bacteria that enhance plant growth promotion, improve soil fertility and provide protection to the plant against pathogen (Anwar et al., 2016). According to Goodfellow and Simpson (1987), most actinomycetes in soil and rizosphere belong to the genus

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Streptomyces and 75% of biologically active compounds are produced by this genus.

Protection against pathogen in plant system can be achieved by plant growth-promoting bacteria (PGPR) application. Introduction of PGPR strains at early plant growth stage mediate biocontrol effect indirectly by eliciting induced systemic resistance (ISR) against various pathogens (Heil and Bostock, 2002). ISR develops as a result of plant growth-promoting rhizobacteria (PGPR) colonization in plant roots and is mediated by a jasmonate- or ethylene-sensitive pathway (Pieterse et al., 2014). Most PGPR-mediated ISR studies were related to *Bacillus* and *Pseudomonas* genus (Choudhary et al., 2007; Chithrashree et al., 2011).

To date, documentation and information on *Xoc* strains in Malaysia was very limited. Apart from that, no specific and efficient treatment has been done to overcome the disease. Moreover, dual potentials of actinomycetes as biocontrol agent for BLS and plant promoter to the rice plant has yet been explored. Therefore the objectives of this study were :

1. To identify and characterize causal pathogen of BLS disease, *Xoc,* and to differentiate it from other *X. oryzae* pathovar, *Xoo*.
2. To characterize and to determine the potential of actinomycetes as biocontrol agent against *Xoc* and as rice plant growth promoter.
3. To evaluate the effectiveness of actinomycetes treatment in early rice growth promotion and BLS suppression through the induction of defense-related enzymes

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Erneeza binti Mohd Hata was born on November 27th, 1981 in Selangor. She holds a degree in Bachelor of Science (Chemical Technology) and Master of Science (Food Science & Technology), both in Universiti Kebangsaan Malaysia. Her Master project was on assessment of microbial contamination levels in street-vended foods.

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