

## **ACKNOWLEDGEMENTS**

Thank God for His mercy that I am able to finish this dissertation.

First and foremost, I am heartily thankful to my supervisor, Dr. Geok Yuan Annie Tan and Dr. Normaniza Osman for their guidance, supervision and support.

I would also like to thank to Aaron Teo, Chew Teng, Mei Sim and Mohd. Hafiz for their help in some laboratory procedures and Pei Eik who helped and guided me to construct dendograms.

I would also like to thank my mother for her endless prayers.

My heartfelt gratitude also goes to my beloved wife, Nor Kamila bt Kamaruzaman for her patience, love and support.

# UNIVERSITI MALAYA

## ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **ALAZHAR BIN HASSIN**

(I.C/Passport No: **691020105113**)

Registration/Matric No: **SGF 060018**

Name of Degree: **MASTER OF BIOTECHNOLOGY**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

**DIVERSITY OF NITROGEN FIXING BACTERIA ASSOCIATED WITH SLOPE GRASS  
AXONOPUS COMPRESSUS**

Field of Study: **SOIL MICROORGANISM**

I do solemnly and sincerely declare that:

- (1) I am the sole author/writer of this Work;
- (2) This Work is original;
- (3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
- (4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
- (5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
- (6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature

Date

Subscribed and solemnly declared before,

Witness's Signature

Date

Name: **GEOK YUAN ANNIE TAN (DR.)**  
Designation: **SENIOR LECTURER**

## ABSTRACT

Vegetation cover, especially grasses, is proven to have a significant contribution for slope stabilization. Nitrogen fixing bacteria supply some amounts of nitrogen (N) required by slope grasses for proper growth and development. In this study, three slopes were chosen based on their soil strengths namely slope A (130-140 kPa), B (80-100 kPa) and C (50-70 kPa). This study showed that the more stable slopes will also have a lower soil saturation level. There was a positive relationship between sizes of bacterial populations with soil saturation level ( $r^2 = 0.60$ ,  $p < 0.05$ ). Similar correlations were also observed between soil shear strength with soil saturation levels ( $r^2 = 0.58$ ,  $p < 0.05$ ) as well. Culturable nitrogen-free living bacteria were isolated and enumerated from roots of *Axonopus compressus*, a slope grass using Burk's nitrogen-free medium. The diversity of free-living nitrogen fixing bacteria was initially determined by the REP-PCR and ERIC-PCR fingerprinting method. Results indicated that REP-PCR give better variable. Hence, the method was used throughout the study. Dendograms were constructed from REP-PCR profiles of a total of 31 strains. The cluster analysis indicated that the diversity of nitrogen-fixing bacteria on the grass roots was quite high and closely related among the population. The information about the presence of nitrogen-free fixing bacteria will greatly assist future management of vegetation to stabilise slopes.

## ABSTRAK

Litupan tumbuhan, terutamanya rumput, telah dibuktikan mempunyai kesan yang signifikan ke atas kestabilan cerun. Bakteria pengikat nitrogen membekalkan sebahagian daripada nitrogen (N) yang diperlukan oleh rumput cerun untuk pembiakan dan pertumbuhan yang sempurna. Dalam kajian ini, tiga cerun telah dipilih berdasarkan perbezaan kekuatan ikatan tanah dan dinamakan sebagai cerun A (130-140 kPa), B (80-100 kPa) dan C (50-70 kPa). Kajian ini telah mengukuhkan lagi kenyataan bahawa cerun yang lebih stabil mempunyai aras ketepuan tanah yang lebih rendah. Terdapat hubungkait yang positif di antara saiz populasi bakteria dengan aras ketepuan tanah ( $r^2 = 0.60$ ,  $p < 0.05$ ). Hubungkait yang sama juga dapat diperhatikan antara kekuatan ikatan tanah dengan aras ketepuan tanah ( $r^2 = 0.58$ ,  $p < 0.05$ ). Bakteria pengikat nitrogen bebas yang boleh dikultur telah diasingkan daripada akar rumput *Axonopus compressus* menggunakan media bebas nitrogen Burk's. Pada awalnya, kepelbagaian bakteria pengikat nitrogen bebas ditentukan dengan menggunakan kaedah REP-PCR dan ERIC-PCR Keputusan telah menunjukkan bahawa kaedah REP-PCR memberikan hasil yang lebih baik. Justeru itu, kaedah tersebut telah digunakan dalam kajian selanjutnya. Dendogram telah dihasilkan dari profil REP-PCR yang terhasil daripada sejumlah 31 strain. Analisis kluster menunjukkan bahawa kepelbagaian bakteria pengikat nitrogen pada akar rumput adalah agak tinggi dan mempunyai hubungan yang hampir dalam kalangan populasi tersebut. Maklumat berkaitan kehadiran bakteria pengikat nitrogen bebas adalah amat berguna dalam pengurusan tumbuhan untuk kestabilan cerun.

## TABLE OF CONTENTS

Title	Page
<b>Acknowledgement</b> .....	i
<b>Original Literary Work Declaration</b> .....	ii
<b>Abstract</b> .....	iii
<b>Abstrak</b> .....	iv
<b>Table of Contents</b> .....	v-vii
<b>List of Figures</b> .....	viii-ix
<b>List of Tables</b> .....	x
<b>List of Appendices</b> .....	xi
<b>List of Abbreviations</b> .....	xii-xiii
<b>1.0 CHAPTER 1: INTRODUCTION</b> .....	1
1.1 Introduction.....	1-3
1.2 Objectives of study.....	3
<b>2.0 CHAPTER 2: LITERATURE REVIEW</b> .....	4
2.1 Nitrogen fixation.....	4
2.2 Biological nitrogen fixation .....	5
2.2.1 Mechanism of biological nitrogen reduction.....	5-7
2.2.2 Regulation of nitrogen fixation.....	7
2.3 Nitrogen fixing bacteria.....	7
2.3.1 Symbiotic.....	8-9
2.3.2 Free living (non-symbiotic) .....	9
2.4 Plants and bacteria.....	11
2.4.1 Nitrogen fixing bacteria and plants.....	11
2.4.2 The importance of biological nitrogen fixation.....	12

2.5	Erosion.....	13
2.5.1	Vegetation cover.....	13-14
2.5.2	Hydrological role of vegetation.....	16
2.5.3	Mechanical role of vegetation.....	16-17
2.6	Microflora and slope stability.....	17-18
2.7	Characteristics of slope grass, <i>Axonopus compressus</i> .....	18
<b>3.0</b>	<b>CHAPTER 3: MATERIALS AND METHODS.....</b>	<b>20</b>
3.1	Description of the sites.....	20
3.2	Soil treatment.....	22
3.3	Measurements.....	22
3.3.1	Soil water profile.....	22
3.3.2	Shear strength.....	24
3.4	Isolation and purification medium.....	25
3.5	Isolation of nitrogen fixing bacteria.....	25
3.6	Purification and preservation of isolates.....	25-26
3.7	Enumeration of bacteria.....	26
3.8	Colonial characterisation of bacterial strains.....	26
3.9	Molecular characterisation of bacterial strains.....	26
3.5.1	DNA extraction.....	26
3.5.2	Agarose gel electrophoresis.....	27
3.5.3	Amplification of DNA fragments using REP primers.....	27
3.5.4	Amplification of DNA fragments using ERIC primers.....	27-28
3.10	PCR fingerprint analysis.....	28
<b>4.0</b>	<b>CHAPTER 4: RESULTS.....</b>	<b>29</b>
4.1	Profile of slope soil.....	29
4.1.1	Soil water profiles.....	29
4.1.2	Shear strength.....	29
4.1.3	Correlation between shear strength and soil profiles.....	32

4.2	Isolation of nitrogen fixing bacteria.....	35
4.2.1	Gram staining.....	35
4.2.2	Colonial and cultural characters.....	37
4.2.3	Enumeration of bacteria (cfu/g) .....	45
4.2.4	Correlation between mean number of cfu/g and soil profile.....	46
4.3	Molecular characterisation.....	48
4.3.1	DNA extraction.....	48
4.3.2	DNA fingerprinting profile.....	49
4.3.3	Fingerprint analysis and dendogram.....	53
<b>5.0</b>	<b>CHAPTER 5: DISCUSSIONS.....</b>	<b>56</b>
5.1	Soil profile.....	56-57
5.2	Culturable bacterial populations.....	57-59
5.3	DNA fingerprinting as a measure of diversity.....	59-62
<b>6.0</b>	<b>CHAPTER 6: CONCLUSION.....</b>	<b>64</b>
6.0	Conclusion.....	64-65
	<b>REFERENCES.....</b>	<b>66-73</b>
	<b>APPENDICES.....</b>	<b>74-81</b>

## LIST OF FIGURES

<b>No.</b>	<b>Title</b>	<b>Page</b>
Figure 2.1	Summary of the biological nitrogen fixation mechanism.....	6
Figure 3.1	The sampling site which have three different range of soil shear strength.....	19
Figure 3.2	Soil sampling for water profiles and bacterial identification was obtained by using soil-coring apparatus.....	23
Figure 3.3	A vane tester used to measure soil shear strength.....	24
Figure 4.1	Soil water content and field capacity of the studied slopes.....	30
Figure 4.2	Shear strength value (kPa) at 30 cm of soil depth.....	31
Figure 4.3	Correlation between shear strength and SWC.....	33
Figure 4.4	Correlation between shear strength and saturation level of the soils.....	34
Figure 4.5	Selected strains isolated from slope type A.....	42
Figure 4.6	Selected strains isolated from slope type B.....	43
Figure 4.7	Selected strains isolated from slope type C.....	44
Figure 4.8	Correlation between mean numbers of cfu/g and SWC.....	46
Figure 4.9	Correlation between mean number of cfu/g and saturation level of soil.....	47
Figure 4.10	Agarose gel electrophoresis (0.8%) of the product from DNA extraction.....	48
Figure 4.11	Agarose gel electrophoresis (0.8%) of five DNA sample from different strain.....	49
Figure 4.12(a)	REP- PCR profile of strains from slope type A.....	50
Figure 4.12(b)	REP- PCR profile of strains from slope type B.....	51
Figure 4.12(c)	REP- PCR profile of strains from slope type C.....	52
Figure 4.13	Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type A.....	53
Figure 4.14	Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type B.....	54



Figure 4.15	Dendrogram obtained from cluster analysis of REP-PCR profile of strains from slope type C.....	55
Figure 4.16	Dendrogram obtained from all of 31 strains.....	63

## LIST OF TABLES

<b>No.</b>	<b>Title</b>	<b>Page</b>
Table 2.1	Some of nitrogen-fixing organisms.....	10
Table 2.2	Vegetation species for erosion control.....	15
Table 2.3	Characteristics of <i>Axonopus compressus</i> (Sw.) P. Beauv.....	19
Table 4.1	Soil water content (%) and field capacity (in parentheses) of the slopes studied.....	30
Table 4.2	Shear strength of the three soil slopes.....	31
Table 4.3	Soil water content (%) and shear strength (kPa) of the slopes studied.....	33
Table 4.4	Shear strength (kPa) and saturation level (%) of the slopes studied.....	34
Table 4.5	Colonies grown on N-free medium.....	36
Table 4.6	Distribution of Gram-positive and Gram-negative n-free fixing bacteria in the slopes as a comparison.....	37
Table 4.7	The observation of 22 Gram-positive diazotrophs.....	38
Table 4.8	The observation of 51 Gram-negative diazotrophs .....	39-41
Table 4.9	Mean number of cfu/g from triplicate plates of isolate medium	45
Table 4.10	Mean number of cfu/g ( $\times 10^5$ ) and SWC (%) of the slopes studied.....	46
Table 4.11	Mean number of cfu/g ( $\times 10^5$ ) and level of soil saturation (%) of the slopes studied.....	47

## LIST OF APPENDICES

<b>No.</b>	<b>Title</b>	<b>Page</b>
Appendix 1	SWC determination of the soil samples.....	74
Appendix 2(a)	Soil profile of slope type A.....	75
Appendix 2(b)	Soil profile of slope type B.....	76
Appendix 2(c)	Soil profile of slope type C.....	77
Appendix 3	A total of 77 plates are shown in figure (a), (b) and (c).....	78-80
Appendix 4	Bacterial strains in which genomic DNA were successfully extracted.....	81

## LIST OF ABBREVIATIONS

%	percent sign
° C	degree Celsius
ATP	Adenosine triphosphate
bp	base pair
Ca	Calcium
cm	centimeter
Cfu	Colony forming unit
DNA	Deoxyribonucleic acid
ddNTP	Dideoxynucleoside triphosphate
ERIC	Enterobacterial repetitive intergeneric concensus
<i>et al.</i>	<i>et alia</i>
EtOH	ethanol
FC	Field capacity
g	gram
<i>i. e.</i>	<i>id est</i>
kPa	kiloPascal
m	meter
M	molar
Mbp	Megabasepairs
MgSO <sub>4</sub>	Magnesium sulphate
min	minute
ml	milliliter
Max	Maximum
Mg	Magnesium
MgSO <sub>4</sub>	Magnesium sulphate

mRNA	Messenger ribonucleic acid
N	Nitrogen
NaCl	Sodium chloride
NaOH	Natrium hydroxide
No.	Number
PCR	Polymerase chain reaction
RC	Rojo Congo red medium
REP	Repetitive extragenic palindromic
RNA	Ribonucleic acid
sp	species
SWC	Soil water content
TBE buffer	Tris-Borate-EDTA buffer
TE buffer	Tris EDTA buffer
UV	ultraviolet
μl	micro liter