UNIVERSITI PUTRA MALAYSIA

INFLUENCE OF RHIZOBACTERIA ON NITROGEN FIXATION, NITROGEN REMOBILISATION AND PLANT GROWTH PROMOTION IN MAIZE (ZEA MAYS L.)

KUAN KHING BOON

FP 2015 7
INFLUENCE OF RHIZOBACTERIA ON NITROGEN FIXATION, NITROGEN REMOBILISATION AND PLANT GROWTH PROMOTION IN MAIZE (ZE A MAYS L.)

By

KUAN KHING BOON

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

April 2015
All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

INFLUENCE OF RHIZOBACTERIA ON NITROGEN FIXATION, NITROGEN REMOBILISATION AND PLANT GROWTH PROMOTION IN MAIZE (ZEA MAYS L.)

By

KUAN KHING BOON

April 2015

Chairman: Professor Zulkifli Hj. Shamsuddin, PhD

Faculty: Agriculture

Currently, there has been a renewed interest in plant growth promoting rhizobacteria (PGPR) as biofertiliser in sustainable agriculture. High yielding maize varieties are widely available to growers but their yields depend heavily on high nitrogen (N) nutrient inputs. Following that, a substantial amount of unused applied fertiliser-N would be leached and/or volatilised to the atmosphere and raise environmental concerns. Alternatively, we hypothesised that N remobilisation in plant can be manipulated using PGPR to increase the grain yield, based on an understanding that the plant N remobilisation is directly correlated to its plant senescence. Thus, a series of laboratory and glasshouse studies were conducted at Universiti Putra Malaysia (UPM) with the following objectives; (i) to isolate, characterise and identify effective indigenous PGPR, (ii) to determine the effects of PGPR inoculation on N uptake, plant growth and ear yield of maize and (iii) to determine the amount of N$_2$ fixed by PGPR and their influence on N remobilisation in maize over time (D$_{50}$ and D$_{65}$). PGPR were isolated from Cash Crop Teaching and Research Field, UPM and Paddy Field Rehabilitation Project, Sik, Kedah using Tryptic Soy Agar (TSA) and streaked on N-free semisolid malate medium (Nfb) and Pikovskaya agar. Indole-3-acetic acid (IAA) production was evaluated using colorimetric test. Biochemical tests of 57 PGPR isolates showed that 10 PGPR of varied Gram stains were positive for multiple traits namely N$_2$ fixation, phosphate solubilisation and IAA production of up to 13 µg mL$^{-1}$. These PGPR were inoculated on maize seedlings under in-vitro condition and the four effective PGPR were identified using 16S rDNA gene sequencing as Klebsiella sp. Br1, Klebsiella pneumoniae Fr1, Bacillus pumilus S1r1 and Acinetobacter sp. S3r2. N$_2$ fixation of PGPR in association with maize was determined using $^{15}$N isotope dilution technique in a glasshouse experiment with two harvests, namely prior to anthesis (D$_{50}$) and ear (D$_{65}$) harvests. The treatments were an uninoculated control, a reference PGPR (Bacillus subtilis UPMB10) and four indigenous PGPR (Br1, Fr1, S1r1 and S3r2). PGPR inoculation had increased bacterial populations in the non-rhizosphere (4.8x10$^7$ cfu g$^{-1}$, 5.9x10$^7$ cfu g$^{-1}$), rhizosphere (1.5x10$^8$ cfu g$^{-1}$, 6.3x10$^8$ cfu g$^{-1}$) and root-endosphere (3.0x10$^4$ cfu cm$^{-1}$, 7.3x10$^4$ cfu cm$^{-1}$) of maize under in-vitro and glasshouse conditions, respectively. PGPR inoculation also increased the chlorophyll content (20.2%, 11.5%), total N uptake (58.6%, 69.6%),...
plant height (31.0%, 20.3%), dry weight of top (51.3%, 33.8%) and root (56.0%, 43.5%) of maize under these two conditions. Ear yield of PGPR inoculated maize increased up to 30.9% under glasshouse condition. The results of $^{15}$N isotope dilution study showed PGPR inoculation namely by *Bacillus pumilus* S1r1 had the highest N$_2$ fixing capacity of 30.5% Ndfa (N derived from atmosphere) (262 mg N$_2$ fixed plant$^{-1}$) and 25.5% Ndfa (304 mg N$_2$ fixed plant$^{-1}$) of the total N requirement of maize top, which was equivalent to 14.0 kg N ha$^{-1}$ and 16.2 kg N ha$^{-1}$ at D$_{50}$ and D$_{65}$, from an extrapolated 53333 plants ha$^{-1}$, respectively. The older plants contributed more N$_2$ fixed per plant although the rate of N$_2$ fixation has peaked prior to anthesis, due to continuous N$_2$ fixation throughout plant maturity. Leaves (old, ear and young), tassel and stalk served successively as N sinks and sources towards ear formation. N remobilisation and plant senescence in maize was delayed by PGPR inoculation, as suggested by the significant interactions (p<0.05) found between PGPR and time of harvests in N uptake and at. % $^{15}$N$_e$ parameters of tassel, respectively. Moreover, the phenomenon was also supported by the significantly lower (p<0.05) N uptake in the inoculated tassels of maize treated with PGPR namely *B. pumilus* S1r1, *K. pneumoniae* Fr1, *B. subtilis* UPMB10 and *Acinetobacter* sp. S1r1 at D$_{65}$ harvest. This study provided evidence that *Bacillus pumilus* S1r1 inoculation can biologically fix atmospheric N$_2$ and provides an alternative way besides plant breeding to manipulate N remobilisation in maize plant for higher ear yield at reduced fertiliser-N rate.
PENGARUH RHIZOBAKTERIA TERHADAP PENGIKATAN NITROGEN, REMOBLISASI NITROGEN DAN PENINGKATAN TUMBESARAN POKOK JAGUNG (ZEA MAYS L.)

Oleh

KUAN KHING BOON

April 2015

Pengerusi: Profesor Zulkifli Hj. Shamsuddin, PhD

Fakulti: Pertanian

Buat masa ini, minat terhadap rhizobakteria penggalak pertumbuhan tanaman (PGPR) sebagai biobaja dalam pertanian lestari makin mendapat perhatian. Varieti jagung yang berhasil tinggi boleh didapati dengan mudah oleh para petani tetapi hasil tuaian amat bergantung pada input nitrogen (N) yang tinggi. Berikut itu, kebanyakan baja N yang digunakan akan melarutlesap dan/atau meruapkan ke atmosfera lalu menimbulkan kebimbangan alam sekitar. Sebagai alternatif, kami membuat hipotesis bahawa remobilisasi N dalam tumbuhan boleh dimanipulasikan menggunakan PGPR untuk meningkatkan hasil bijirin, berdasarkan pemahaman bahawa remobilisasi N adalah berkaitan secara langsung dengan kesenesenan tumbuhan. Oleh itu, suatu siri kajian di makmal dan rumah kaca telah dijalankan di Universiti Putra Malaysia (UPM) dengan objektif-objektif berikut; (i) untuk mengasingkan, mencirikan dan mengenal pasti PGPR tempatan yang berkesan, (ii) untuk menentukan kesan inokulasi PGPR terhadap pengambilan N, tumbesaran pokok dan hasil tongkol pada jagung dan (iii) untuk menentukan jumlah N$_2$ yang diikat oleh PGPR dan pengaruh terhadap remobilisasi N dalam jagung secara berkala (D$_{50}$ dan D$_{65}$). PGPR telah diasingkan dari Kawasan Pengajaran dan Penyelidikan Tanaman Kontan, UPM dan Projek Baikpulih Sawah Padi, Sik, Kedah menggunakan Triptik Soya Agar (TSA) dan dicoretkan pada medium bebas-N separuh pepejal malate (Nfb) dan Pikovskaya agar. Penghasilan indole-3-asetik asid (IAA) telah dinilai menggunakan ujian meter warna. Hasil ujikaji biokimia terhadap 57 strain PGPR menunjukkan bahawa 10 PGPR yang terdiri daripada pelbagai Gram adalah positif dalam pelbagai ciri-ciri seperti pengikatan N$_2$, pelarutan fosfat dan penghasilan IAA sebanyak 13 µg mL$^{-1}$. Anak-anak benih jagung telah diinokulasi dengan PGPR di bawah keadaan in-vitro dan empat PGPR yang berkesan telah dikenal pasti sebagai Klebsiella sp. Br1, Klebsiella pneumoniae FR1, Bacillus pumilus S1r1 dan Acinetobacter sp. S3r2 menggunakan 16S rDNA turutan gen. Pengikatan N$_2$ oleh PGPR secara sekutuan dengan jagung telah ditentukan dengan menggunakan teknik pencairan $^{15}$N isotop di dalam eksperimen rumah kaca dengan dua tuaian, iaitu penuaian sebelum antesis (D$_{50}$) dan hasil tongkol (D$_{65}$). Rawatan-rawatan adalah satu kawalan tanpa inokulasi, satu PGPR rujukan (Bacillus subtilis UPMB10) dan empat PGPR tempatan (Br1, FR1, S1r1 dan S3r2). PGPR inokulasi telah meningkatkan populasi bakteria di
bukan-rhizosfera \((4.8 \times 10^7 \text{ cfu g}^{-1}, 5.9 \times 10^7 \text{ cfu g}^{-1})\), rhizosfera \((1.5 \times 10^8 \text{ cfu g}^{-1}, 6.3 \times 10^8 \text{ cfu g}^{-1})\) dan endosfera-akar \((3.0 \times 10^4 \text{ cfu cm}^{-1}, 7.3 \times 10^4 \text{ cfu cm}^{-1})\) pada pokok jagung di bawah keadaan in-vitro dan rumah kaca masing-masing. PGPR inokulasi juga meningkatkan kandungan klorofil \((20.2\%, 11.5\%)\), jumlah pengambilan \(N\) \((58.6\%, 69.6\%)\), ketinggian pokok \((31.0\%, 20.3\%)\), berat kering pucuk \((51.3\%, 33.8\%)\) dan akar \((56.0\%, 43.5\%)\) pada pokok jagung di bawah kedua-dua keadaan. Hasil tongkol jagung yang diinokulasi dengan PGPR juga meningkat sebanyak \(30.9\%\) di dalam rumah kaca. Hasil kajian pencairan \(^{15}\text{N}\) isotop menunjukkan bahawa inokulasi dengan PGPR seperti \(Bacillus pumilus\) S1r1 umumnya mempunyai kapasiti pengikatan \(N_2\) yang terbanyak iaitu \(30.5\%\) \(N_{dfa}\) \((262 \text{ mg N} _2\text{ diikat pokok}^{-1})\) dan \(25.5\%\) \(N_{dfa}\) \((304 \text{ mg N} _2\text{ diikat pokok}^{-1})\) daripada jumlah keperluan \(N\) di pucuk jagung, yang bersamaan dengan \(14.0 \text{ kg N ha}^{-1}\) dan \(16.2 \text{ kg N ha}^{-1}\) di \(D_{50}\) dan \(D_{65}\), daripada pengekstrapolasion \(53333\) pokok ha\(^{-1}\), masing-masing. Pokok-pokok yang lebih tua telah menyumbangkan jumlah \(N_2\) diikat per pokok yang lebih banyak walaupun kadar pengikatan \(N_2\) telah memuncak sebelum antesis, kerana pengikatan \(N_2\) berlaku sepanjang tempoh kematangan pokok. Daun-daun (lama, tongkol dan muda), jambak bunga jantan dan tangkai berfungsi sebagai singki dan sumber \(N\) secara berturutan sehingga penghasilan tongkol. Remobilisasi \(N\) dan kesenesenan pokok jagung telah ditangguhkan melalui inokulasi PGPR, seperti yang dicadangkan pada interaksi-interaksi yang signifikan \((p <0.05)\) di antara PGPR dan masa penuaan di parameter pengambilan \(N\) dan \(\% \^{15}\text{N}_e\) di jambak bunga jantan masing-masing. Bahkan, fenomena ini juga disokong oleh penurunan pengambilan \(N\) yang signifikan \((p <0.05)\) pada jambak bunga jantan jagung yang diinokulasi dengan PGPR seperti \(B. pumilus\) S1r1, \(K. pneumoniae\) FR1, \(B. subtilis\) UPMB10 dan \(Acinetobacter\) sp. S1r1 pada tuaian \(D_{65}\). Kajian ini membuktikan bahawa inokulasi dengan \(Bacillus pumilus\) S1r1 boleh mengikat \(N_2\) dari atmosfera secara biologi dan menyediakan suatu alternatif selain pembibakbakan tanaman untuk memanipulasi remobilisasi \(N\) dalam pokok jagung demi hasil tongkol yang lebih tinggi di kadar baja \(N\) yang rendah.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere appreciation and thanks to Professor Dr. Zulkifli Hj. Shamsuddin, Associate Professor Dr. Radziah Othman and Dr. Khairuddin Abd. Rahim for their useful advice, guidance and tolerance throughout the whole planning and execution of this study. My heartfelt gratitude is extended to the late Associate Professor Dr. Anuar Abd. Rahim for his guidance in the statistical analyses.

Special appreciation to Mr. Dzulkifli Duaji, Department of Land Management, UPM for his continuous assistance throughout this study, to Mrs. Latiffah Noordin, Agrotechnology and Biosciences Division, Malaysian Nuclear Agency (Nuklear Malaysia) for her technical assistance in $^{15}$N analyses and Dr. Sheikh Hasna Habib, Department of Agriculture Technology, UPM for her guidance in bacterial identification. Not forgetting, my gratitude to all lecturers, supporting staffs, colleagues and friends at Faculty of Agriculture, UPM for their invaluable advice and support to this study.

Thankfully acknowledge the financial assistance of Graduate Research Fellowship from UPM and MyMaster-MyBrain15 from the Ministry of Education Malaysia (formerly known as Ministry of Higher Education Malaysia) for making this graduate study possible.

My heartfelt thanks to Ms. Lim Lee Ging for her continuous support and sharing my ups and downs throughout the study duration. Lastly, I would like to thank my family, friends and everyone who contributed and supported me in various ways to the completion of this study either directly or indirectly.
I certify that a Thesis Examination Committee has met on 10 April 2015 to conduct the final examination of Kuan Khing Boon on his thesis entitled "Influence of Rhizobacteria on Nitrogen Fixation, Nitrogen Remobilisation and Plant Growth Promotion in Maize (Zea mays L.)" 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.

Members of the Thesis Examination Committee were as follows:

**Wan Noordin Wan Daud, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

**Halimi Mohd Saud, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Aminuddin Hussin, PhD**
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

**Amir Hamzah Ahmad Ghazali, PhD**
Associate Professor
School of Biological Sciences
Universiti Sains Malaysia
Malaysia
(External Examiner)

---

**Zulkarnain Zainal, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 May 2015
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:

Zulkifli Haji Shamsuddin, PhD  
Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

Radziah Othman, PhD  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

Khairuddin Abdul Rahim, PhD  
Director  
Division of Agrotechnology and Biosciences  
Malaysian Nuclear Agency (Nuklear Malaysia)  
(Member)

BUJANG KIM HUAT, PhD  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
Declaration by Graduate Student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) for communication, including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: ___________________________________   Date: __________________

Name and Matric No.: Kuan Khing Boon (GS 29126)
Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: __________________________
Name of Chairman of Supervisory Committee: __________________________

Signature: __________________________
Name of Member of Supervisory Committee: __________________________

Signature: __________________________
Name of Member of Supervisory Committee: __________________________
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>APPROVAL</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>DECLARATION</td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td></td>
<td>LIST OF PLATES</td>
<td>xvii</td>
</tr>
<tr>
<td></td>
<td>LIST OF ABBREVIATIONS</td>
<td>xviii</td>
</tr>
<tr>
<td>1</td>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td><strong>LITERATURE REVIEW</strong></td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Maize Cultivation in Malaysia</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>Plant Growth-Promoting Rhizobacteria (PGPR)</td>
<td>4</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Mechanisms of Plant Growth Promotion</td>
<td>6</td>
</tr>
<tr>
<td>2.2.1.1</td>
<td>Biological Nitrogen Fixation (BNF)</td>
<td>8</td>
</tr>
<tr>
<td>2.2.1.2</td>
<td>Phosphate Solubilisation</td>
<td>9</td>
</tr>
<tr>
<td>2.2.1.3</td>
<td>Indole-3-Acetic Acid (IAA) Production</td>
<td>10</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Plant Growth Promotion by Rhizobacteria (Bacillus spp., Klebsiella spp. and Acinetobacter spp.)</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>15N Isotope Dilution Technique</td>
<td>12</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Estimation of Associative BNF by PGPR</td>
<td>13</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Estimation of Plant N Remobilisation</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td><strong>ISOLATION, CHARACTERISATION AND IDENTIFICATION OF RHIZOBACTERIA FROM MAIZE ROOTS</strong></td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and Methods</td>
<td>19</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Soil and Root Sampling</td>
<td>19</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Soil pH Determination</td>
<td>19</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Isolation of Rhizospheric and Endophytic PGPR from Maize Roots</td>
<td>19</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Qualitative Nitrogen (N₂) Fixation Determination</td>
<td>20</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Phosphate Solubilisation Determination</td>
<td>20</td>
</tr>
<tr>
<td>3.2.6</td>
<td>IAA Production</td>
<td>20</td>
</tr>
<tr>
<td>3.2.7</td>
<td>PGPR Culture Characterisation</td>
<td>21</td>
</tr>
</tbody>
</table>
3.2.8  Gram Stain and KOH Test  
3.2.9  Bacterial Growth Curve  
3.2.10  PGPR Identification Using PCR-Based  
       16S rDNA Gene Sequencing  
3.2.10.1  PGPR Strains  
3.2.10.2  Preparation of Total Cell DNA  
3.2.10.3  Quantitation of Total DNA  
3.2.10.4  PCR Amplification of DNA  
3.2.10.5  Agarose Gel Electrophoresis  
3.2.10.6  PCR Product Purification  
3.2.10.7  16S rDNA Gene Sequencing  
3.2.10.8  Phylogenetic Analysis  

3.3  Results  
3.3.1  Soil pH and PGPR  
3.3.2  N\textsubscript{2} Fixation  
3.3.3  Phosphate Solubilisation  
3.3.4  IAA Production  
3.3.5  PGPR Cultural Characteristics  
3.3.6  Gram Stain and KOH Test  
3.3.7  Bacterial Growth  
3.3.8  PGPR Identification  
3.3.8.1  Quantitation of Total DNA  
3.3.8.2  Identification and Phylogenetic Tree of PGPR Strains  

3.4  Discussion  
3.5  Conclusion  

4  INFLUENCE OF RHIZOBACTERIA ON PLANT GROWTH AND N UPTAKE OF MAIZE UNDER IN-VITRO CONDITIONS  
4.1  Introduction  
4.2  Materials and Methods  
4.2.1  Plant Experimental Designs  
4.2.2  Nutrient Solution Concentration Study  
4.2.2.1  Seed Preparation  
4.2.2.2  \textit{In vitro} Establishment of Maize Seedlings  
4.2.2.3  Determination of Leaf Chlorophyll (SPAD value)  
4.2.2.4  Plant Height, Root Length and Dry Weight  
4.2.3  PGPR Inoculation Study  
4.2.3.1  Seed Surface Sterilisation  
4.2.3.2  Preparation of Planting Media  
4.2.3.3  Preparation and Inoculation of PGPR  
4.2.3.4  Estimation of PGPR Populations
5 INFLUENCE OF RHIZOBACTERIA ON NITROGEN FIXATION AND NITROGEN FLUXES IN MAIZE AT VEGETATIVE AND EAR HARVESTS UNDER GLASSHOUSE CONDITION

5.1 Introduction

5.2 Materials and Methods

5.2.1 Pot Preparation

5.2.2 Experimental Design and Treatments

5.2.3 Preparation of Inoculum

5.2.4 Preparation of $^{15}$N Labelled Fertiliser

5.2.5 Determination of Leaf Chlorophyll (SPAD value)

5.2.6 Determination of Plant Height, Girth and Root Volume

5.2.7 Estimation of Total Microbial Population

5.2.8 Total N Analysis and Estimation of N$_2$ Fixation ($^{15}$N Isotope Dilution Method)

5.2.9 Statistical Analysis

5.3 Results

5.3.1 Total Bacterial Population

5.3.2 Leaf Chlorophyll Content (SPAD value)

5.3.3 N Concentration and Distribution

5.3.4 Total N Uptake and Distribution
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.5</td>
<td>$^{15}$N Isotope Dilution Analysis and Distribution</td>
<td>68</td>
</tr>
<tr>
<td>5.3.6</td>
<td>$N_2$ Fixation by PGPR and Distribution</td>
<td>70</td>
</tr>
<tr>
<td>5.3.7</td>
<td>Plant Height and Girth</td>
<td>74</td>
</tr>
<tr>
<td>5.3.8</td>
<td>Dry Matter Yield and Distribution of Vegetative Growth</td>
<td>75</td>
</tr>
<tr>
<td>5.3.9</td>
<td>Root Volume</td>
<td>78</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>79</td>
</tr>
<tr>
<td>5.5</td>
<td>Conclusion</td>
<td>86</td>
</tr>
</tbody>
</table>

6 GENERAL DISCUSSION AND CONCLUSION 87

REFERENCES 90
APPENDICES 99
BIODATA OF STUDENT 113
LIST OF PUBLICATIONS 114
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Some recent PGPR strains, plant sources and/or targets and their mechanisms of plant growth promotions (PGP).</td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Conventional methods to measure biological nitrogen fixation (BNF) of PGPR.</td>
<td>13</td>
</tr>
<tr>
<td>3.1</td>
<td>PGPR strains for identification using 16S rDNA sequence.</td>
<td>22</td>
</tr>
<tr>
<td>3.2</td>
<td>Characteristics of selected nitrogen fixing PGPR isolated from UPM Serdang and Sik, Kedah.</td>
<td>27</td>
</tr>
<tr>
<td>3.3</td>
<td>Summary of NCBI BLAST results for 16S rDNA sequences from PGPR strains.</td>
<td>31</td>
</tr>
<tr>
<td>4.1</td>
<td>PGPR populations at harvest (14 DAT).</td>
<td>45</td>
</tr>
<tr>
<td>5.1</td>
<td>Chemical properties of Serdang series soil (<em>Typic Paleudult</em>, 0-15 cm depth).</td>
<td>57</td>
</tr>
<tr>
<td>5.2</td>
<td>Total bacterial population at ear harvest, D_{65}.</td>
<td>62</td>
</tr>
<tr>
<td>5.3</td>
<td>Distribution of N concentration (%) in the different plant parts of maize inoculated with PGPR strains at D_{50} (before anthesis) and D_{65} (ear harvest).</td>
<td>65</td>
</tr>
<tr>
<td>5.4</td>
<td>Distribution of total N uptake (mg plant^{-1}) in the plant top and in the different plant parts of maize inoculated with PGPR strains at D_{50} (before anthesis) and D_{65} (ear harvest).</td>
<td>67</td>
</tr>
<tr>
<td>5.5</td>
<td>Distribution %^{15}N atom excess (at. % ^{15}N_e) in the different plant parts and the mean weighted atom excess (WAE) for the plant top of maize inoculated with PGPR strains at D_{50} (before anthesis) and D_{65} (ear harvest).</td>
<td>69</td>
</tr>
<tr>
<td>5.6</td>
<td>Estimates of proportions of N2 derived from atmosphere and amounts of N2 fixed (in parenthesis and bold, mg N2 fixed plant^{-1}) in the plant top and in the different plant parts of maize inoculated with PGPR strains at D_{50} (before anthesis) and D_{65} (ear harvest).</td>
<td>72</td>
</tr>
<tr>
<td>5.7</td>
<td>Plant dry matter yield in the whole plant and in the different plant parts of maize inoculated with PGPR strains at D_{50} (before anthesis) and D_{65} (ear harvest).</td>
<td>77</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The main pools (boxes) and fluxes between pools (arrows) of nitrogen (N) in terrestrial ecosystems, excluding both animals and inputs via nitrogen fixation.</td>
</tr>
<tr>
<td>3.1</td>
<td>Number of isolated PGPR from UPM and Sik fields at respective soil pH range.</td>
</tr>
<tr>
<td>3.2</td>
<td>Bacterial growth curves of Fr1 strain.</td>
</tr>
<tr>
<td>3.3</td>
<td>Red-gel stained 1% agarose gel displaying amplified DNA products under UV-transilluminator.</td>
</tr>
<tr>
<td>3.4</td>
<td>Phylogenetic tree based on partial 16S rDNA sequences of UPMB10, Fr1, S1r1 and S3r2.</td>
</tr>
<tr>
<td>4.1</td>
<td>Chlorophyll content (SPAD value) of maize seedlings grown under different concentrations of N-free Hoagland’s nutrient solution.</td>
</tr>
<tr>
<td>4.2</td>
<td>Plant height and root length of maize seedlings grown under different concentrations of N-free Hoagland’s nutrient solution.</td>
</tr>
<tr>
<td>4.3</td>
<td>Dry weights of maize shoot and root grown under different concentrations of N-free Hoagland’s nutrient solution.</td>
</tr>
<tr>
<td>4.4</td>
<td>Effects of PGPR inoculations on leaf chlorophyll content (SPAD values) of maize seedlings.</td>
</tr>
<tr>
<td>4.5</td>
<td>Effects of PGPR inoculation on plant N concentration of maize seedlings.</td>
</tr>
<tr>
<td>4.6</td>
<td>Effects of PGPR inoculations on total N uptake of maize seedlings.</td>
</tr>
<tr>
<td>4.7</td>
<td>Effects of PGPR inoculation on plant height of maize seedlings.</td>
</tr>
<tr>
<td>4.8</td>
<td>Effects of PGPR inoculation on root length of maize seedlings.</td>
</tr>
<tr>
<td>4.9</td>
<td>Effects of PGPR inoculation on dry weight of maize top.</td>
</tr>
<tr>
<td>4.10</td>
<td>Effects of PGPR inoculation on dry weight of maize root.</td>
</tr>
<tr>
<td>5.1</td>
<td>Leaf chlorophyll content (SPAD values) of maize plants inoculated with PGPR at 4th, 5th, 6th and 7th WAP.</td>
</tr>
</tbody>
</table>
5.2 Percentages of N derived from the atmosphere (% Ndfa), fertiliser (% Ndff) and soil (% Ndfs) in plant top of maize inoculated with PGPR strains at D50 and D65 harvests.

5.3 Plant height of maize plants inoculated with PGPR at 4th, 5th, 6th and 7th WAP.

5.4 Plant girth of maize plants inoculated with PGPR at 4th, 5th, 6th and 7th WAP.

5.5 Effects of PGPR inoculation on root volume of maize at ear harvest, D65.
**LIST OF PLATES**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Qualitative N\textsubscript{2} fixation determination of PGPR on Nfb plate.</td>
<td>28</td>
</tr>
<tr>
<td>3.2</td>
<td>Phosphate solubilisation abilities of PGPR on Pikovskaya plates.</td>
<td>28</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

% Ndfa, % Ndff, % Ndfs  Percentage of Nitrogen derived from atmosphere, fertiliser and soil, respectively.

1/3 N  One third of MARDI recommended fertiliser-N rate

14 DAT  14 days after transplanting

ACC deaminase  1-aminocyclopropane-1-carboxylate deaminase

ANOVA  Analysis of Variance

ARA  Acetylene Reduction Assay

at. % $^{15}N_e$  Percentage of $^{15}N$ atom excess

BLAST  Basic Local Alignment Search Tool

BNF  Biological Nitrogen Fixation

bp  Base pair

CEC  Cation Exchange Capacity

CIRP  Christmas Island Rock Phosphate

cfu  Colony forming unit

D$_{50}$, D$_{65}$  Days after planting (50 and 65), representing before anthesis and at ear harvests, respectively.

DAP  Days after planting

DMRT  Duncan’s Multiple Range Test

Dunnett’s test  Dunnett’s Multiple Comparison Test

EDTA  Ethylenediaminetetraacetic acid

g  gravity

GT  Generation time

HS  Hoagland’s solution

IAA  Indole-3-acetic acid

IAEA  International Atomic Energy Agency
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARDI</td>
<td>Malaysian Agricultural Research and Development Institute</td>
</tr>
<tr>
<td>MOP</td>
<td>Muriate of Potash</td>
</tr>
<tr>
<td>MUSCLE</td>
<td>Multiple Sequence Comparison by Log-Expectation</td>
</tr>
<tr>
<td>N</td>
<td>Generation numbers</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt; fixed plant&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>Total amount of N&lt;sub&gt;2&lt;/sub&gt; fixed per plant</td>
</tr>
<tr>
<td>N/A</td>
<td>Not available</td>
</tr>
<tr>
<td>NA</td>
<td>Nutrient agar</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ND</td>
<td>Not determined</td>
</tr>
<tr>
<td>Nfb</td>
<td>N-free semi-solid malate medium</td>
</tr>
<tr>
<td>NHI</td>
<td>Nitrogen harvest index</td>
</tr>
<tr>
<td>NRE</td>
<td>Nitrogen remobilisation efficiency</td>
</tr>
<tr>
<td>NUE</td>
<td>Nitrogen use efficiency</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGP</td>
<td>Plant growth promotion</td>
</tr>
<tr>
<td>PGPR</td>
<td>Plant growth-promoting rhizobacteria</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPAD</td>
<td>Soil Plant Analysis Development</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate EDTA</td>
</tr>
<tr>
<td>TPC</td>
<td>Total plate count</td>
</tr>
<tr>
<td>TSA, TSB</td>
<td>Tryptic Soy Agar/Broth</td>
</tr>
</tbody>
</table>
WAE  Weighted atom excess
WAP  Week after planting
CHAPTER 1

INTRODUCTION

In Malaysia, both field and sweet corn varieties of maize are in high demand as animal feed and for human consumptions, respectively, although only the latter is widely cultivated as cash crops due to its higher return on investment (Nor et al., 2012). The ever-increasing total planted area of maize was 9322 ha in 2012 and valued at RM 334.4 million (Ministry of Agriculture and Agro-Based Industry Malaysia, 2014). Maize production is expected to increase annually to meet the increasing local and foreign (Brunei and Singapore) demands (Nor et al., 2012). Current cultivated maize are of high yielding varieties but their nutrient requirements, mainly N, could be as high as 0.5 kg N ha\(^{-1}\) day\(^{-1}\) during the initial three weeks after emergence (Schröder et al., 2000). Moreover, it was reported that only 30-50% of fertiliser-N applied are absorbed by plants, while the balance raises various environmental concerns in soil, atmosphere and water bodies (Hodge et al., 2000; Halvorson et al., 2002). Therefore, any considerable solution to supplement and reduce present chemical fertiliser-N use is critical.

Studies have shown that plant growth promoting rhizobacteria (PGPR) isolated as free-living soil bacteria from plant rhizosphere can reduce chemical fertiliser-N use and increase plant growth and yield when associated with plant roots and/or other plant parts (Boddey et al., 2003). A number of bacteria such as Azospirillum (Montañez et al., 2009), Klebsiella (Arruda et al., 2013), Burkholderia (Chelius and Triplett 2001), Bacillus (Park et al., 2005) and Pseudomonas (Piromyou et al., 2011) have been identified as PGPR to maize plants through biological N\(_2\) fixation (BNF), phosphate solubilisation, phytohormones (e.g. auxin and cytokinin) production and biological control of soil pathogens. BNF by PGPR have been reported to contribute up to 70% or 30 kg N ha\(^{-1}\) in crops such as maize (Montañez et al., 2009), sugarcane (Boddey et al., 1995), rice (Baldani and Dobereiner, 1980) and oil palm (Zakry et al., 2012). In addition, N remobilisation in plant plays a crucial role in determining the N content in grain at harvest, as 50-90% of N in grain is remobilised from N of other plant parts (Kichey et al., 2007).

Undeniably, the ability of PGPR to significantly fix atmospheric N\(_2\) for plant growth promotion has cultivated much interest in studying these rhizobacteria for sustainable agriculture (Lugtenberg and Kamilova, 2009). Moreover, the rapid urbanisation with increasing environmental awareness in the country and limited availability of fertile land, would eventually innovate the local maize industry towards sustainable maize production. Currently, research is focused on the ability of rhizobacteria to fix N in association with non-leguminous crops such as maize (Wu et al., 2005), sugarcane (James, 2000) and oil palm (Zakry et al., 2012). Information on indigenous PGPR association with maize plant in regard to BNF and their influence on N remobilisation in maize is minimal and concerted effort is also needed for effective plant growth promotion (PGP) and N management. The genetic and biochemical characteristics of effective PGPR in association with maize and their abundance in soil rhizosphere are critical for better understanding of the establishment and field applications of these inocula.
Therefore, this study was conducted to achieve the following objectives:

i. To isolate, characterise and identify effective indigenous PGPR from maize roots for their plant growth promoting abilities.

ii. To determine the effects of PGPR inoculation on total N uptake, plant growth and ear yield of maize under *in-vitro* and glasshouse conditions.

iii. To estimate the amount of N$_2$ fixed by PGPR and their influence on N remobilization in maize over time (D$_{30}$ and D$_{65}$) under glasshouse condition.
REFERENCES


91


Difco Laboratories, 1994. Gram stain set and reagents. Technical information bulletin, T1 3328. Difco Laboratories, Detroit, USA.


Uchida, R. 2000. Recommended plant tissue nutrient levels for some vegetable, fruit, and ornamental foliage and flowering plants in Hawaii. In Silva, J. A. and Uchida, R. (Eds.). *Plant nutrient management in Hawaii’s soils, approaches for tropical and subtropical agriculture*. College of Tropical Agriculture and Human Resources, University of Hawaii, Manoa.


