



UNIVERSITI PUTRA MALAYSIA

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

KUAN KHING BOON

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MAIZE (*ZEA MAYS L.*)**

By

KUAN KHING BOON

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
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April 2015

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

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April 2015

Chairman: Professor Zulkifli Hj. Shamsuddin, PhD

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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 N₂ fixed by PGPR and their influence on N remobilisation in maize over time (D₅₀ and D₆₅). 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 N₂ 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. N₂ fixation of PGPR in association with maize was determined using ¹⁵N isotope dilution technique in a glasshouse experiment with two harvests, namely prior to anthesis (D₅₀) and ear (D₆₅) 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 (4.8×10⁷ cfu g⁻¹, 5.9×10⁷ cfu g⁻¹), rhizosphere (1.5×10⁸ cfu g⁻¹, 6.3×10⁸ cfu g⁻¹) and root-endosphere (3.0×10⁴ cfu cm⁻¹, 7.3×10⁴ 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) ($262 \text{ mg N}_2 \text{ fixed plant}^{-1}$) and 25.5% Ndfa ($304 \text{ mg N}_2 \text{ fixed plant}^{-1}$) of the total N requirement of maize top, which was equivalent to $14.0 \text{ kg N ha}^{-1}$ and $16.2 \text{ kg N ha}^{-1}$ 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. % $^{15}\text{N}_e$ 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.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGARUH RHIZOBAKTERIA TERHADAP PENGIKATAN NITROGEN, REMOBILISASI NITROGEN DAN PENINGKATAN TUMBESARAN POKOK JAGUNG (*ZEA MAYS L.*)

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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. Berikutan 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 kesenesehan 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 $13 \mu\text{g mL}^{-1}$. 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 (4.8×10^7 cfu g⁻¹, 5.9×10^7 cfu g⁻¹), rhizosfera (1.5×10^8 cfu g⁻¹, 6.3×10^8 cfu g⁻¹) dan endosfera-akar (3.0×10^4 cfu cm⁻¹, 7.3×10^4 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 ¹⁵N isotop menunjukkan bahawa inokulasi dengan PGPR seperti *Bacillus pumilus* S1r1 umumnya mempunyai kapasiti pengikatan N₂ yang terbanyak iaitu 30.5% Ndfa (N berasal dari atmosfera) (262 mg N₂ diikat pokok⁻¹) dan 25.5% Ndfa (304 mg N₂ diikat pokok⁻¹) daripada jumlah keperluan N di pucuk jagung, yang bersamaan dengan 14.0 kg N ha⁻¹ dan 16.2 kg N ha⁻¹ di D₅₀ dan D₆₅, daripada pengekstrapolasi 53333 pokok ha⁻¹, masing-masing. Pokok-pokok yang lebih tua telah menyumbangkan jumlah N₂ diikat per pokok yang lebih banyak walaupun kadar pengikatan N₂ telah memuncak sebelum antesis, kerana pengikatan N₂ 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 ditanggihkan melalui inokulasi PGPR, seperti yang dicadangkan pada interaksi-interaksi yang signifikan ($p < 0.05$) di antara PGPR dan masa penuaian di parameter pengambilan N dan % ¹⁵N_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 D₆₅. Kajian ini membuktikan bahawa inokulasi dengan *Bacillus pumilus* S1r1 boleh mengikat N₂ dari atmosfera secara biologi dan menyediakan suatu alternatif selain pembiakbakaan tanaman untuk memanipulasi remobilisasi N dalam pokok jagung demi hasil tongkol yang lebih tinggi di kadar baja N yang rendah.

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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.

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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 |

| | |
|--|---|
| MARDI | Malaysian Agricultural Research and Development Institute |
| MOP | Muriate of Potash |
| MUSCLE | Multiple Sequence Comparison by Log-Expectation |
| N | Generation numbers |
| N ₂ fixed plant ⁻¹ | Total amount of N ₂ fixed per plant |
| N/A | Not available |
| NA | Nutrient agar |
| NCBI | National Center for Biotechnology Information |
| ND | Not determined |
| Nfb | N-free semi-solid malate medium |
| NHI | Nitrogen harvest index |
| NRE | Nitrogen remobilisation efficiency |
| NUE | Nitrogen use efficiency |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PGP | Plant growth promotion |
| PGPR | Plant growth-promoting rhizobacteria |
| RH | Relative humidity |
| rpm | Revolutions per minute |
| SAS | Statistical Analysis System |
| SEM | Standard error of the mean |
| SPAD | Soil Plant Analysis Development |
| TAE | Tris-Acetate EDTA |
| TPC | Total plate count |
| TSA, TSB | Tryptic Soy Agar/Broth |

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 N₂ 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 Döbereiner, 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 N₂ 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 N₂ fixed by PGPR and their influence on N remobilization in maize over time (D₅₀ and D₆₅) under glasshouse condition.



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