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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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

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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 (4.8×10^7 cfu g^{-1}, 5.9×10^7 cfu g^{-1}), rhizosphere (1.5×10^8 cfu g^{-1}, 6.3×10^8 cfu g^{-1}) and root-endosphere (3.0×10^4 cfu cm^{-1}, 7.3×10^4 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.

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.)

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. 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 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 (4.8×10^7 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 ^{15}N isotop menunjukkan bahawa inokulasi dengan PGPR seperti *Bacillus pumilus* S1r1 umumnya mempunyai kapasiti pengikatan N_2 yang terbanyak iaitu 30.5% Ndfa (N berasal dari atmosfera) (262 mg N_2 diikat $pokok^{-1}$) dan 25.5% Ndfa (304 mg N_2 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 D_{50} dan D_{65}, daripada pengekstrapolasian 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 penuaian di parameter pengambilan N dan % $^{15}N_e$ 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 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.

Members of the Thesis Examination Committee were as follows:

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Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

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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)
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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 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 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 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_{50} and D_{65}) under glasshouse condition.

REFERENCES

Amir, H.G., Shamsuddin, Z.H., Halimi, M.S., Ramlan, M.F. and Marziah, M. 2003. N_2 fixation, nutrient accumulation and plant growth promotion by rhizobacteria in association with oil palm seedlings. *Pakistan J. Biol. Sc.* 6:1269-1272.

Aquilanti, L., Favilli, F. and Clementi, F. 2004. Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. *Soil Biol. Biochem.* 36: 1475-1483.

Arruda, L., Beneduzi, A., Martins, A., Lisboa, B., Lopes, C., Bertolo, F., Passaglia, L. M. P. and Vargas, L. K. 2013. Screening of rhizobacteria from maize (*Zea mays L.*) in Rio Grande do Sul State (South Brazil) and analysis of their potential to improve plant growth. *Appl. Soil Ecol.* 63: 15-22.

Baldani, V.L.D. and Döbereiner, J. 1980. Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* 12: 433-439.

Barker, A.V. and Bryson, G.M. 2007. Nitrogen. In: Barker, A.V. and Pilbeam, D.J. (Eds.), *Handbook of Plant Nutrition*. Florida, USA: CRC Press.

Bashan, Y., Puente, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin, G., Ferrera-Cerrato, R. and Pedrin, S., 1995. Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. *Appl. Environ. Microbiol*. 61: 1938–1945.

Beegle, D.B., Piekielek, W., Lingenfelter, D. and Fox, R. 2008. The early season chlorophyll meter test for corn. Agronomy Facts 53. Pennsylvania State Univ., University Park.

Benizri, E., Baudoin, E. and Guckert, A. 2001. Root colonization by inoculated plant growth rhizobacteria. *Biocontrol Sci. Techn.* 11: 557-574.

Berry, W. L. and Knight, S. 1997. Plant culture in hydroponics. In Langham, R. W. and Tibbitts, T. W. (Eds.). *Plant growth chamber handbook*. North Central Regional Research Publication N0. 340. Iowa AHEES Spec. Rep. No. 99.

Boddey, R.M. 1987. Methods for quantification of nitrogen fixation associated with gramineae. *CRC Crit. Rev. Plant Sci*. 6: 209-266.

Boddey, R.M., Oliveira, O.C., Urquiga, S., Reis, V.M. Olivares, F.L., Baldani, V.L.D. and Döbereiner, J. 1995. Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. *Plant Soil* 174: 195-209.

Boddey, R.M., Urquiaga, S., Alves, B.J.R and Reis, V. 2003. Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. *Plant Soil* 252:139-149.

Bray, R.H. and Kurtz, L.T. 1945. Determination of total, organic and available form of phosphorus in soils. *Soil Sci.* 59: 39-45.

Bremner, J.M. 1996. Nitrogen total. In Spark, D.L. (Ed.). *Methods of soil analysis, Part 3: Chemical Methods.* Wisconsin, USA. Soil Science Society of America.

Bürgmann, H., Stefan, M., Micheal, B., Franco, W. and Josef, Z. 2005. Effects of model root exudates on structure and activity of a soil diazotroph community. *Environ Microbiol.* 7 (11): 1711-1724.

Campbell, C.R. and Plank, C.O. 2000. Reference sufficiency ranges: Corn. In Campbell, C.R. (Ed.). *Reference sufficiency ranges for plant analysis in the southern region of the United States.* Southern Cooperative Series Bulletin. Revised 2011.

Cappuccino, J. and Sherman, N. 2010. Microbiology: A laboratory manual. 9th International Edition. USA. Pearson Benjamin Cummings

Chalk, P.M. and Ladha, J.K. 1999. Estimation of legume symbiotic dependence: An evaluation of techniques based on ^{15}N dilution. *Soil Biol. Biochem.* 31: 1901-1917.

Chelius, M.K. and Triplett, E.W. 2001. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41: 252-263.

Chen, G., Hoglong, Z. and Zhang, Y. 2003. Soil microbial activities and carbon and nitrogen fixation. *Res. Microbiol.* 157: 393– 398.

Choudhury, A.T.M.A. and Khanif, Y.M. 2001. Evaluation of the effects of nitrogen and magnesium fertilization on rice yield and fertilizer nitrogen efficiency using 15N tracer technique. *J. Plant Nutr.* 24: 855-871.

Cliquet, J.B., Deléens, E., Bousser, A., Martin, M., Lescure, J.C., Piroul J.L., Mariotti, A. and Morot-Gaudry, J.F. 1990a. Estimation of the carbon and nitrogen allocation during stalk elongation by ^{13}C and ^{15}N tracing in *Zea mays* L. *Plant Physiol.* 92: 78-87.

Cliquet, J.B., Deléens, E. and Mariotti, A. 1990b. C and N mobilization from stalk and leaves during kernel filling by ^{13}C and ^{15}N tracing in *Zea mays* L. *Plant Physiol.* 94: 1547-1553.

Compant, S., Clément, C. and Sessitsch, A. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42: 669-678.

Coombs, J.T. and Franco, C.M.M. 2003. Isolation and identification of actinobacteria from surface sterilized wheat roots. *Appl. Environ. Microbiol.* 69: 5603-5608.

Danso, S.K.A. 1988. The use of ^{15}N enriched fertilisers for estimating nitrogen fixation in grain and pasture legumes. In Beck, B.K. and Materon, L.A. (Eds.). *Nitrogen fixation by legumes in Mediterranean agriculture*.

Danso, S.K.A., Hardarson, G. and Zapata, F. 1993. Misconceptions and practical problems in the use of ^{15}N soil enrichment techniques for estimating N_2 fixation. *Plant Soil* 152:25–52.

Department of Agriculture Malaysia. 2012. Vegetables and cash crops statistic. Malaysia.

Diaz, C., Lemaître, T., Christ, A., Azzopardi, M., Kato, Y., Sato, F., Morot-Gaudry, J.F., Le Dily, F. and Masclaux-Daubresse, C. 2008. Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in *Arabidopsis* under low nitrogen nutrition. *Plant Physiol.* 147: 1437-1449.

Dierolf, T., Fairhurst, T. and Mutert, E. 2001. Soil fertility kit: A toolkit for acid, upland soil fertility management in Southeast Asia. Potash & Phosphate Institute (PPI). Singapore.

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

Edgar, R.C. 2004a. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5:113.

Edgar, R.C. 2004b. Muscle: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–1797.

Egamberdiyeva, D. 2007. The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Appl. Soil Ecol.* 36: 184-189.

El-Azeem, S., Mehana, T.A. and Shabayek, A.A., 2007. Some plant growth promoting traits of rhizobacteria isolated from Suez Canal region, Egypt. *African Crop Sci. Proceed.* 8: 1517–1525.

Gallais, A., Coque, M., Le Gouis, J., Prioul, J.L., Hirel, B. and Quilléré, I. 2007. Estimating the proportion of nitrogen remobilization and of postsilking nitrogen uptake allocated to maize kernels by nitogen-15 labeling. *Crop Sci*. 47: 685-693.

Gallais, A., Coque, M., Quilléré, I., Prioul, J.L., and Hirel, B. 2006. Modelling postsilking nitrogen fluxes in maize (*Zea mays*) using 15N-labelling field experiments. *New Phytol*. 172: 696-707.

Gastal, F. and Lemaire, G. 2002. N uptake and distribution in crops: an agronomical and ecophysiological perspective. *J. Exp. Bot.* 53 (370): 789-799.

Glickmann, E. and Dessaux, Y. 1995. A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* 61: 793-796.

Greenwood, D.J., Lemaire, G., Gosse, G., Cruz, P., Draycott, A. and Neeteson, J.J. 1990. Decline in percentage N of C3 and C4 crops with increasing plant mass. *Ann. Bot.* 66: 425 – 436.

Hall, B.G. 2013. Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol.* 5: 1229-1235.

Halvorson, A.D., Follett, R.F., Bartolo, M.E. and Schweissing, F.C. 2002. Nitrogen fertilizer use efficiency of furrow-irrigated onion and corn. *Agron. J.* 94: 442-449.

Harrington, J.T., Mexal, J.G. and Fisher, J.T. 1994. Volume displacement provides a quick and accurate way to quantify new root production. *Tree Planter's Notes* 45 (3): 121-124.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: A review. *Ann. Microbiol.* 60: 579-598.

Hoagland, D.R. and Arnon, D.I. 1950. The water culture method for growing plants without soil. *Calif. AES Circular* 347.

Hoben, H.J. and Somasegaran, P. 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Appl. Environ. Microbiol.* 44(5):1246–1247.]

Hodge, A., Robinson, D. and Fitter, A. 2000 Are microorganisms more effective than plants at competing for nitrogen? *Trends Plant Sci*. 5: 304-308.

Holt, J.G., Kreig, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T., 1994. Bergey's Manual of Determinative Bacteriology (9th Edition). Williams and Wilkins, Baltimore, USA.

IndexMundi. Jun 2014. Retrieved from http://www.indexmundi.com/commodities/?commodity=urea&months=120&currency=myr.

International Atomic Energy Agency (IAEA). 2001. Manual on "use of isotope and radiation methods in soil and water management and crop nutrition". IAEA-TCS-14.

James, E.K. 2000. Nitrogen fixation in endophytic and associative symbiosis. *Field Crop Res*. 65: 197-209.

Kalagudi, G.M. and Shenoy, V.V. 1998. Induction of root nodules and genetic variability for paranodulation in rainfed upland rice. *Curr. Sci*. 75: 1056-1058.

Kang, S.M., Joo, G.J., Hamayun, M., Na, C.I., Shin, D.H., Kim, H.K., Hong, J.K. and Lee, I.J. 2009. Gibberellin production and phosphate solubilization by newly isolated strain of *Acinetobacter calcoaceticus* and its effect on plant growth. *Biotechnol. Lett*. 31:277-281.

Kang, S.M., Khan, A.L., Hamayun, M., Shinwari, Z.K., Kim, Y.H., Joo, G.J and Lee, I.J. 2012. *Acinetobacter calcoaceticus* ameliorated plant growth and influenced gibberellins and functional biochemicals. *Pak. J. Bot.* 44(1): 365-372.

Khalid, A., Arshad, M., and Zahir, Z.A., 2004. Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. *J. Appl. Microbiol*. 96, 473–480.

Kichey, T., Hirel, B., Heumez, E., Dubois, F. and Le Gouis, J. 2007. In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crop. Res.* 102: 22–32.

Koh, M.C., Lim, C.H., Karichiappan, K., Chua, S.B., Chew, S.T. and Phang, S.T.W. 1998. Random amplified polymorphic DNA fingerprints for identification of red meat species. *Meat Sci.* 48 (3/4): 275-285.

Kovtunovych, G., Lar, O., Kamalova, S., Kordyum, V., Kleiner, D., and Kozyrovska, N. 1999. Correlation between pectate lyase activity and ability of diazotrophic *Klebsiella oxytoca* VN13 to penetrate into plant tissue. *Plant Soil* 215: 1-6.

Kuklinsky-Sobral, J., Araújo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A. and Azevedo, J.L. 2004. Isolation and characterization of soybean-associated bacteria and their plant growth promotion. *Environ. Microbiol*. 6 (12): 1244-1251.

Lawrie, A.C. and Wheeler, C.T. 1974. The effects of flowering and fruit formation on the supply of photosynthetic assimilates to the nodules of *Pisum sativum* L. in relation to the fixation of nitrogen. *New Phytol*. 60: 817–821.

Lemaire, G., Jeuffroy, M.H. and Gastal, F., 2008. Diagnosis tool for plant and crop N status in vegetative stage: Theory and practices for crop N management. *Europ. J. Agronomy* 28: 614-624.

Leong, C.O. 2005. *Manual Teknologi Penanaman Jagung Manis*, Serdang, MARDI.

Leggett, J. E. and Frere, M. H. 1971. Growth and nutrient uptake by soybean plants in nutrient solutions of graded concentrations. *Plant Physiol*. 48: 457-460.

Lugtenberg, B. and Kamilova, F. 2009. Plant-Growth-Promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63:541–56.

Maathuis, F. 2009. Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* 12 (3): 250–258.

Malaysian Agricultural Research and Development Institute (MARDI, *Institut Penyelidikan dan Kemajuan Pertanian Malaysia*). 2006. *Anggaran kos pengeluaran dan pendapatan bagi tanaman kontan*. *Kementerian Pertanian dan Industri Asas Tani*. Malaysia.

Marques, A.P.G.C., Pires, C., Moreira, H., Rangel, A.O.S.S. and Castro, P.M.L. 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biol. Biochem.* 42: 1229-1235.

Masclaux-Daubresse, C., Daniele-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L. and Suzuki, A. 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* 105: 1141-1157.

Masclaux-Daubresse, C., Reisdorf-Cren, M. and Orsel, M. 2008. Leaf nitrogen remobilisation for plant development and grain filling. *Plant Biology* 10: 23-36.

Matiru, V.N. and Dakora, F.D. 2004. Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. *Afr. J. Biotechnol* 3 (1):1-7.

McGeehan, S.L. and Naylor, D.V. 1988. Automated instrumental analysis of carbon and nitrogen in plant and soil samples. *Commun. Soil Sci. Plan.* 19 (4): 493-505.

Mehnaz, S., Kowalik, T., Reynolds, B., and Lazarovitz, G. 2010. Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions. *Soil Biol. Biochem.* 42: 1848–1856.

Ministry of Agriculture and Agro-Based Industry Malaysia. 2014 Agrofood Statistics 2013. Malaysia.

Mirza, M.S., Rasul, G., Mehnaz, S., Ladha, J.K., So, R.B., Ali, S. and Malik, K.A. 2000. Beneficial effects of inoculated nitrogen-fixing bacteria on rice. In Ladha JK, Reddy PM (Eds). *The quest for nitrogen fixation in rice*. International Rice Research Institute, Los Banõs, Philippines.

Montañez, A., Abreu, C., Gill, P. R., Hardarson, G., and Siracdi, M. 2009. Biological nitrogen fixation in maize (*Zea mays L.*) by 15N isotope-dilution and identification of associated culturable diazotrophs. *Biol. Fertil. Soils* 45: 253-263.

Munier-Jolain, N.G., Ney, B. and Duthion, C. 1996. Termination of seed growth in relation to nitrogen content of vegetative parts in soybean plants. *Eur. J. Agron.* 5: 219–225.

Nor, H.M.S., Faridah, H., Sharizan, A. and Sebrina, S.S. 2012. *Jagung manis hybrid baru* Hibrimas. *Buletin Teknologi MARDI*.

Park, M., Kim, C., Yang, J., Lee, H., Shin, W., Kim, S. and Sa, T. 2005. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiol. Res.* 160: 127-133.

Patten, C.L. and Glick, B.R., 1996. Bacterial biosynthesis of indole-acetic acid. *Can. J. Microbiol.* 42, 207–220.

Peoples, M.B. and Dalling, M.J. 1988. Proteolysis and amino acid metabolism. In: Nooden, L.D. and Leopold, A.C. (Eds.), *Senescence and aging in plants*. New York. Academic press.

Perrig, D., Boiero, M.L., Masciarelli, O.A., Penna, C., Ruiz, O.A., Cassán, F.D., and Luna, M.V. 2007. Plant-growth-promoting compounds produced by two agronomically strains of *Azospirillum brasilense*, and implications for inoculant formulation. *Appl. Microbiol. Biotechnol.* 75, 1143–1150.

Pikovskaya, R.I. 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiologia* 17: 362-370.

Piromyou, P., Buranabanyat, B., Tantasawat, P., Tittabutr, P., Boonkerd, N. and Teaumroong, N. 2011. Effect of plant growth promoting rhizobacteria (PGPR) inoculation on microbial community structure in rhizosphere of forage corn cultivated in Thailand. *Eur. J. Soil Biol.* 47: 44-54.

Plénet, D. and Lemaire, G. 2000. Relationships between dynamics of nitrogen uptake and dry matter accumulation in maize crops. Determination of critical N concentration. *Plant Soil* 216: 65 – 82.

Powers, E. M. 1995. Efficacy of the Ryu nonstaining KOH technique for rapidly determining Gram reactions for food-borne and waterborne bacteria and yeasts. *Appl. Environ. Microbiol.* 61 (10): 3756-3758.

Richards, L.A., and Fireman, M. 1943. Pressure-plate apparatus for measuring moisture sorption and transmission by soils. *Soil Sci*. 56: 395–404.

Rodriguez, H. and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* 17: 319-339.

Roesch, L.F.W., Passaglia, L.M.P., Bento, F.M., Triplett, E.W. and Camargo, F.A.O. 2007. Diversity of diazotrophic endophytic bacteria associated with maize plants. *Diversidade de bactérias diazotróficas endofíticas associadas a plantas de milho. R. Bras. Ci. Solo.* 31, 1367–1380.

Sachdev, D.P., Chaudhari. H.G., Kasture, V.M., Dhavale, D.D. and Chopade, B.A. 2009. Isolation and characterization of indole acetic acid (IAA) producing *Klebsiella pneumonia* strains from rhizosphere of wheat (*Triticum aestivum*) and their effect on plant growth. *Indian J. Exp. Biol*. 47: 993-1000.

Salon, C., Munier-Jolain, N.G., Duc, G., Viosin, A., Grandgirard, D., Larmurec, A., Emery, R.J.N. and Ney, B. 2001. Grain legume seed filling in relation to nitrogen acquisition: A review and prospects with particular reference to pea. *Agronomie* 21: 539–552.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. New York, USA: Cold Spring Harbor Laboratory Press.

Schmidt, J. P., Dellinger, A. E. and Beegle, D. B. 2009. Nitrogen recommendations for corn: An on-the-go sensor compared with current recommendation methods. *Agron. J*. 101: 916-924.

Schröder, J.J., Neeteson, J.J., Oenema, O. and Struik, P.C. 2000. Does the crop or the soil indicate how to save nitrogen in maize production? Reviewing the state of the art. *Field Crop. Res.* 66: 151-164.

Song, O.R., Lee, S.J., Lee, Y.S., Lee, S.C., Kim, K.K. and Choi, Y.L., 2008. Solubilization of insoluble inorganic phosphate by *Burkholderia cepacia* DA23 isolated from cultivated soil. *Braz. J. Microbiol.* 39, 151–156.

Stell, R. G. D., Torrie, J. H. and Dickey, D. A. 1980. Principles and procedures of statistics: A biometrical approach. McGraw-Hill, New York.

Ta, C.T. and Weiland, R.T. 1992. Nitrogen partitioning in maize during ear development. *Crop Sci.* 32: 443-451.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011 MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Method. *Mol. Biol. Evol.* 28: 2731-2739.

Thomas, H. and Smart, C.M. 1993. Crops that stay green. *Ann. Appl. Biol.* 123:193-219.

Thomas, G.W. 1982. Exchangeable cations. In Page, A.L., Miller, R.H. and Keeny, D.R (Eds.). Methods of soil analyses. *Part 2. Chemical and microbiological properties-agronomy, monograph no. 9* (2nd Edition). Madison, USA. American Society of Agronomy.

Tran Vân, V., Berge, O., Ke, S.N., Balandreau, J. and Heulin, T. 2000. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. *Plant Soil* 218:273–284.

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.

Uhart, S.A. and Andrade, F.H. 1995. Nitrogen and carbon accumulation and remobilisation during grain filling in maize under different source/sink ratios. *Crop Sci.* 35: 183–190.

Urquiaga, S., Cruz, K.H.S. and Boddey, R.M. 1992. Contribution of nitrogen fixation to sugarcane: ^{15}N and nitrogen balance estimates. *Soil Sci. Soc. American J.* 56: 105-114.

Verma, S. C., Ladha, J. K., and Tripathi, A. K. 2001. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *J. Biotechnol*. 91: 127-141.

Vessey, J.K. 1994. Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay. *Plant Soil* 158: 151-162.

Wang, D.L. and Anderson, D.W. 1998. Direct measurement of organic carbon content in soils by Leco CR-12 carbon analyzer. *Commun Soil Sci. Plan.* 29 (1-2): 15-21.

Weisburg, W.G., Bams, S.M., Pelletier, D.A. and Lane, D.J., 1991. 16S ribosomal DNA amplication for phylogenetic study. *J. Bacteriol.*, 173, 697–703.

Wu, S.C., Cao, Z.H., Li, Z.G., Cheung, K.C. and Wong, M.H. 2005. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A greenhouse trial. *Geoderma* 125:155–166.

Zakry, F.A.A. 2008. Nitrogen fixation and vegetative growth of immature oil palm inoculated with *Bacillus sphaericus* (strain UPMB-10), Master Thesis, Universiti Putra Malaysia.

Zakry, F.A.A., Shamsuddin, Z.H., Rahim, K.A., Zakaria, Z.Z. and Rahim, A.A. 2012. Inoculation of *Bacillus sphaericus* UPMB-10 to young oil palm and measurement of its uptake of fixed nitrogen using the ^{15}N isotope dilution technique. *Microbes Environ.* 27(3): 257-262.

Zhang, P., Zheng, J., Pan, G., Zhang, X., Li, L. and Tippkötter, R. 2007. Changes in microbial community structure and function within particle size fractions of a paddy soil under different long-term fertilization treatments from the Tai lake region, China. *Colloids Surf., B* 58: 264–270.

Zhao, Y. 2010. Auxin biosynthesis and its role in plant development. *Annu. Rev. Plant Biol.* 61: 49-64.