Evaluation of Diazotrophic Bacteria as Biofertilizers

By

Medhin Hadish Kifle

BSc (University of Asmara, Eritrea)

MSc (University of KwaZulu Natal, South Africa)

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Science (SAEES)

College of Agriculture, Engineering and Science (CAES)

University of KwaZulu-Natal

Pietermaritzburg, South Africa

November 2013

THESIS SUMMARY

Inoculation with diazotrophic bacteria is well documented as a means to enhance growth and increase yields of various crops, especially when used as an alternative or a supplement to the use of nitrogenous fertilizers and agrochemicals for sustainable agriculture. Nitrogen is the most limiting nutrient for increasing crop productivity, and the use of chemical sources of N fertilizers is expensive, and may contribute to environmental pollution. Therefore, there is a need to identify diazotrophic inoculants as an alternative or supplement to N-fertilizers for sustainable agriculture. The search for effective diazotrophic bacterial strains for formulation as biofertilizers has been going on for over 40 years and a number of inoculant biofertilizers have been developed and are commercially available.

In the current study, 195 free-living diazotrophic bacteria were isolated from soils collected from the rhizosphere and leaves of different crops in different areas within the KwaZulu-Natal Province, Republic of South Africa. Ninety five of the isolates were selected for further screening because they were able to grow on N-free media using different carbon sources. Isolates that were very slow to grow on N-free media were discarded. Of these, 95 isolates were screened *in vitro* for growth promotion traits tests including tests for ammonia production and acetylene reduction. The best 20 isolates that were also able to reduce acetylene into ethylene were selected for growth-promotion trials on maize under greenhouse conditions. Of the 20 isolates, ten isolates enhanced ($P = 0.001$) growth of maize above the Un-inoculated Control. Molecular tests were conducted to identify the ten most promising isolates selected in the *in vitro* study. In the greenhouse study, these diazotrophic isolates were screened for their ability to enhance various growth parameters of maize (*Zea mays* L.), following various inoculation techniques (drenching, seed treatment, foliar spray and combination of these). Inoculations with the five best diazotrophic isolates by various methods of application increased dry weight and leaf chlorophyll content ($P < 0.001$, $P = 0.001$), respectively, compared to the Untreated Control. Although, all methods of application of diazotrophic inoculants used in this study resulted in increased dry weight and leaf chlorophyll content, combined methods of application (seed treatment + drenching) and sole application (seed treatment) were significantly more ($P < 0.05$) efficient. The best five most promising isolates were identified for growth promotion of maize under greenhouse conditions. They were also assessed for their effects on germination of wheat

in vitro and were further tested in combination with various levels of nitrogenous fertilizer for growth-promotion of wheat (*Triticum aestivum* L.). These five isolates were also investigated for their potential to enhance growth and yields of maize and wheat crops in field trials, when combined with a low dose of nitrogenous fertilizer. These isolates were further studied for their contribution for enhancing plant growth through nitrogen fixation by predicting N content in leaves using a chlorophyll content meter (CCM-200) and correlated to extractable chlorophyll level at $R^2 = 0.96$.

In this study, relative to the Un-inoculated Control, the best five isolates enhanced growth of maize and wheat when combined with a 33% N-fertilizer levels for a number of growth parameters: increased chlorophyll levels and heights of maize, shoot dry weight of maize and wheat; and enhanced root and shoot development of these crops in both greenhouse and field conditions. The best contributions of diazotrophic bacteria was achieved by Isolate LB5 + 0% NPK (41%), V9 + 65% NPK (28.9%), Isolate L1 + 50% NPK (25%), Isolate L1 + 25%NPK (22%) and LB5 + 75% NPK (15%) undergreenhouse conditions. At 30 or 60 DAP, isolates with 33%N-fertilizer caused relatively higher dry weight than the 100%NPK. Inoculation of Isolate StB5 without 33N% fertilizer cuased significant ($P<0.005$) increases in stover dry weight.
In field studies, inoculation of diazotrophic bacteria alone or with 33%N-fertilizer resulted in relatively greater increases of dry weight, stover dry weight, number of spikes and yield at different growth stages higher than the Un-inoculated or Unfertilized Control. However, the increases were not statistically significant. The use of microbial inoculants in combination with low doses of nitrogenous fertilizers can enhance crop production without compromising yields. The isolates obtained in this study can effectively fix nitrogen and enhance plant growth. The use of microbial inoculants can contribute to the integrated production of cereal crops with reduced nitrogenous fertilizer inputs, as a key component of sustainable agriculture.

Key words: Biological nitrogen fixation, plant growth-promotion; diazotrophs, N-fertilizer

DECLARATION

I, Medhin Hadish Kifle, declare that the research reported in this thesis, except where otherwise indicated, and is my original work. This thesis has not been submitted for any degree or examination at any other university. This thesis does not contain other persons' data, pictures, graphs or other.

This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then their words have been re-written but the general information attributed to them has been referenced.

Signed:……..
Medhin H. Kifle

Signed:……..
Prof. Mark D. Laing

ACKNOWLDEGEMENTS

I wish to acknowledge the contributions of my supervisor Professor Mark D. Laing. I sincerely thank him for his supervision throughout the study. I wish to thank him for his guidance, constructive and stimulating ideas, professional help, financial support and encouragements.

Dr Mike Morris for his constructive ideas and suggestions throughout the study;

Dr Sackey Yobo for encouragement during my study;

Professor H. Shimelis for his assistance in data analysis and for encouragement;

Matthew and Heather for their assistance with the use of MALDI Biotyper;

Dr Gubba for encouragement during my study;

Mr Ian Doidge for assistance with the field trials;

Mrs. Susan Van der Merwe for her technical assistance with the greenhouse and field trials and for her love and encouragement;

Dr Reannah Otanga for her love and encouragement;

Mr Richard Burgdorf for his laboratory skills;

Dr Iona Basdew for editing my literature review;

Professor Ignatius Nsahlai for proof reading the thesis;

Mr Matthew Erasmus for technical assistance with the greenhouse and field experiments;

Miss Diane Fowlds for technical assistance;

Mr Kim Govender for technical assistance;

My Eritrean brothers, Dr Michael Bairu and his family, Dr Habteab Ghebrehiwet and his family, Dr Eyob Kidane and his family and Dr Daniel Teclu and Dr Samson Tesfay and his family, for their support and encouragement during my studies;

The Plant Pathology postgraduate students for their love, support and encouragement;

I am grateful to the Hadish Kifle family for their love, support, prayers and encouragement during my study;

My special and deepest appreciation goes to my husband, Woldeab Kibreab Ghebremariam, for his encouragement, love, unending patience and believing in me.

Finally I wish to acknowledge the Almighty God for granting me strength and courage to face all the challenges that I came across during this study.

DEDICATION

This thesis is dedicated to
my husband, Woldeab K. Ghebremariam
our children, Amanuel, Ariam and Betiel,
and all the Hadish Kifle family
for
their love, encouragement and support.

TABLE OF CONTENTS

PROLOGUE

By 2050 the global human population is projected to increase by 50%, and the global grain demand is projected to double (Alexandratos, 1999). Poor soil fertility is one of the major constraints for crop production (Ouédraogo *et al.*, 2001). Millions of people in the world are fed by modern agriculture, benefiting from increased yields resulting from greater inputs of fertilizer, pesticides and other technologies (Cassman, 1999). However, ensuring sustainability of agriculture, enhancing crop growth and improving crop yields, all without compromising environmental integrity or human health are major challenges (Tilman *et al.*, 2002). Moreover, continuous use of agrochemicals may impact negatively on the environment (Poudel *et al.*, 2001; Wilson and Tisdell, 2001)**.** The high cost of fertilizers also inflates the cost of crop production. The use of microorganisms in agriculture has therefore been identified as a cheaper and more environmentally friendly alternative or supplementary mechanism to improve crop production and reduce production costs (Parr *et al.*, 1994; Wu *et al.*, 2005; Berg, 2009).

The first major groups of biofertilizers identified were *Rhizobium* spp., that fix nitrogen from the atmosphere in root nodules on legumes. They have been used commercially as inoculants for legumes for over 100 years (Boonkerd and Singleton, 2002). Research in the field of biofertilizers has resulted in the development of different kinds of microbial inoculants or biofertilizers including nitrogen fixing bacteria, phosphate solubilizing microorganisms, vesicular–arbuscular mycorrhizae (VAM) and plant growth promoting rhizobacteria (PGPR). Several free-living bacteria genera have been reported to enhance plant growth, subsequently increasing yields of crops (Kloepper *et al.*, 1989; Glick, 1995; Kennedy *et al.*, 2004; Lucy *et al.*, 2004). Improvements in growth parameters resulting from the use of microbial inoculants, combined with reduced rates of chemical fertilizers, have been also reported in previous research (Chen, 2006; Jilani *et al.*, 2007; Adesemoye *et al.*, 2009; Kumar *et al.*, 2009). Research on the use of microbial inoculants to enhance growth and increase yields of crops has been the focus of many studies (Okon and Vanderleyden, 1997; Dobbelaere *et al.*, 2001; Riggs *et al.*, 2001; Matiru and Dakora, 2004; Mehnaz *et al.*, 2010). Typically these beneficial microorganisms have been isolated from the rhizosphere of plants and formulated into microbial inoculants.

The aim of the current study were to isolate diazotrophic bacteria from the rhizosphere and leaves of different cereal crops, and evaluate their potential to promot plant growth The specific objectives were to:

- Isolate and identifiy diazotrophic bacteria from the rhizosphere and leaves of wheat and maize;
- Screen these bacteria *in vitro* as plant growth-promoters;
- Select the most efficient bacterial strains for use as bio-inoculants;
- Evaluate the effect of diazotrophic bacteria as biofertilizers on the growth of maize and wheat in both the greenhouse and field;
- Evaluate effective inoculation techniques and simplest methods of application to be adopted by small-scale farmers;
- Determine the optimum dose of nitrogenous fertilizer to be used in combination with biofertilizer inoculation, aiming to integrate the application of chemical fertilizers and biofertilizers with an optimal yield; and
- Investigate the effects of combining bacterial inoculants with reduced levels of N fertilizer, and co-inoculation of these bacterial isolates with a strain of *Trichoderma harzianum* (Eco-T).

The referencing system used in this thesis is based on the specific style used in the journal Crop Science.

The thesis is in the form of discrete research chapters, each following the format of a stand-alone research paper. This is the dominant thesis format adopted by the University of KwaZulu-Natal because it facilities the publishing of research out of theses far more readily than the older monograph form of thesis. As such, there is some unavoidable repetition of references and some introductory information between chapters.

References

Adesemoye A., Torbert H., Kloepper J. (2009) Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology 58:921-929.

Alexandratos N. (1999) World food and agriculture: outlook for the medium and longer term. Proceedings of the National Academy of Sciences 96:5908-5914.

Berg G. (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11-18.

Boonkerd, N. and P. Singleton. (2002) Production of rhizobium biofertilizer. In: Biotechnology of Biofertilizers. Kannaiyan, S. (Ed), Narosa Publishing House, New Delhi: 122-128.

Cassman K.G. (1999) Ecological intensification of cereal production systems: Yield potential, soil quality, and precision agriculture. Proceedings of the National Academy of Sciences 96:5952-5959.

Chen J. (2006) The combined use of chemical and organic fertilizers and/or biofertilizer for crop growth and soil fertility, International Workshop on Sustained Management of the soil-rhizosphere system for efficient crop production and fertilizer use. Bangkok, Thailand. pp. 20.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Vanderleyden J., Dutto P., Labandera-Gonzalez C., Caballero-Mellado J., Aguirre J.F., Kapulnik Y. (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Functional Plant Biology 28:871-879.

Glick B.R. (1995) The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41:109-117.

Jilani G., Akram A., Ali R.M., Hafeez F.Y., Shamsi I.H., Chaudhry A.N., Chaudhry A.G. (2007) Enhancing crop growth, nutrients availability, economics and beneficial rhizosphere microflora through organic and biofertilizers. Annals of Microbiology 57:177-184.

Kennedy I.R., Choudhury A., Kecskés M.L. (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7:39-44.

Kumar S., Pandey P., Maheshwari D. (2009) Reduction in dose of chemical fertilizers and growth enhancement of sesame (*Sesamum indicum* L.) with application of rhizospheric competent *Pseudomonas aeruginosa* LES4. European Journal of Soil Biology 45:334-340.

Lucy M., Reed E., Glick B.R. (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86:1-25.

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

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

Okon Y., Vanderleyden J. (1997) Root-associated *Azospirillum* species can stimulate plants. ASM News-American Society for Microbiology 63:366-370.

Ouédraogo E., Mando A., Zombré N. (2001) Use of compost to improve soil properties and crop productivity under low input agricultural system in West Africa. Agriculture, Ecosystems andEnvironment 84:259-266.

Parr J.F., Hornick S., Kaufman D. (1994) Use of microbial inoculants and organic fertilizers in agricultural production ASPAC Food and Fertilizer Technology Centerpublishers. Pp.1-15.

Poudel D., Horwath W., Mitchell J., Temple S. (2001) Impacts of cropping systems on soil nitrogen storage and loss. Agricultural Systems 68:253-268.

Riggs P.J., Chelius M.K., Iniguez A.L., Kaeppler S.M., Triplett E.W. (2001) Enhanced maize productivity by inoculation with diazotrophic bacteria. Functional Plant Biology 28:829-836.

Tilman D., Cassman K.G., Matson P.A., Naylor R., Polasky S. (2002) Agricultural sustainability and intensive production practices. Nature 418:671-677.

Wilson C., Tisdell C. (2001) Why farmers continue to use pesticides despite environmental, health and sustainability costs. Ecological Economics 39:449-462.

Wu S., Cao Z., Li Z., Cheung K., Wong M. (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155-166.

CHAPTER ONE
LITERATURE REVIEW

1.1 Introduction

In the developing world, maize (*Zea mays* L.), wheat (*Triticum aestivum* L) and rice (*Oryza sativa* L) are the most important staple crops and require relatively large nitrogen inputs for their production. Over 80% of our atmosphere is Nitrogen (N_2), which cannot be used by plants unless converted into nitrate or nitrite either chemically by the Haber–Bosch process, or by Biological Nitrogen Fixation (BNF). Most of the nitrogenous fertilizer is produced by industrial N fixation. It requires approximately 18.5 Mcal of fossil energy to produce one kg of N-fertilizer (Da Silva *et al.*, 1978). Each unit of N fertilizer produced requires two units of petroleum (Hamdi, 1982; Wagner, 1997). This is expensive, especially for farmers in the less developed countries, and is not sustainable because petroleum is a non-renewable resource. This is a major problem in southern Africa (Mafongoya *et al.*, 2007; Mtambanengwe and Mapfumo, 2008), Central Africa (Mafuka *et al.*, 2007) and the entire sub-Saharan region (Kimetu *et al.*, 2004), a region in which soil nutrient reserves are being depleted because of continued nutrient mining by intensified cropping without adequate replenishment of nutrients that have been removed. One method of increasing crop yields is the use of chemical fertilizers that are expensive and may pollute the environment (Bhattacharjee *et al.*, 2008a). However, Africa has the lowest fertilizer use in the world because artificial fertilizers are neither available nor affordable to small-scale farmers in the region (Boddey *et al.*, 1995a; Kimetu *et al.*, 2004). Improving agricultural productivity in Africa requires building up and maintenance of soil fertility, despite the low incomes of smallholder farmers (Mafongoya *et al.*, 2007). This has led to interest in biofertilization with an emphasis on BNF (Peoples *et al.*, 1995a; Wagner, 1997). Biological nitrogen fixation uses microbes to convert atmospheric nitrogen into a plant-usable form, offering a cost effective and eco-friendly source of N fertilization. While BNF may generate only a fraction of total crop N requirements for commercial farmers (Kennedy *et al.*, 2004a), it may provide substantial inputs of N for resource poor farmers as a long term, sustainable option.

1.2 Biofertilizers

Biofertilizers are based on microorganisms that promote plant growth by increasing the supply or availability of primary nutrients to the host plant. When these microbes are applied to seed, plant surfaces, or soil, they colonize the rhizosphere or interior of the plant (Khalid *et al.*, 2004a). Beneficial rhizosphere bacteria, collectively called plant growth promoting rhizobacteria (PGPR), are the main constituents of biofertilizers. They may be more cost effective than chemical fertilizers (Kloepper *et al.*, 1989; Ahmad *et al.*, 2006a; Ahmad *et al.*, 2008). Use of microbial biofertilizers may reduce the need to use chemical fertilizers, which is crucial for small scale farmers (Rai, 2006). However, biofertilizers are dependent upon physical, environmental, nutritional and biological factors (Wani *et al.*, 1995). PGPRs exert their positive effects on plant growth both, directly and indirectly (Giller and Cadisch, 1995). Members of the genera *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, *Bacillus, Paenibacillus,* and some members of the Enterobacteriaceae are effective as biocontrol and biofertilization agents in agriculture (Siddiqui, 2006). These beneficial bacteria enhance emergence, colonize roots, stimulate growth and enhance yield (Niranjan Raj *et al.*, 2006).

1.2.1 Direct Growth Promotion

Biofertilizers promote plant growth and health by nitrogen fixation (Vessey, 2003), synthesizing phytohormones (Klee *et al.*, 1987; Frankenberger and Arshad, 1995; Dobbelaere *et al.*, 2003; Rodrigues *et al.*, 2008), solubilization of inorganic phosphate and mineralization of organic phosphate (making phosphorous available to plants) (Glick, 1995; Rodríguez and Fraga, 1999; Khalid *et al.*, 2004b), and as microbial iron transport agents by secreting sideropheres that solubilize and sequester Fe from the soil and provide it to plant cells. (Kloepper *et al.*, 1980).

1.2.1.1 Biological Nitrogen Fixation (BNF)

The global use of N-fertilizers increases annually (Vance, 2001). However, an estimated 58 Tg N of ((Vitousek, *et al.*, 2013)). In agricultural systems, BNF takes place as a result of symbiotic relationships involving legumes and *Rhizobium* spp. (Peoples *et al.*, 1995b), or by non-symbiotic associations between free-living diazotrophs and plant roots (Peoples and Craswell, 1992). The latter are various species of symbiotic nitrogen-fixing bacteria that have been studied for their ability to successfully colonize roots, stems and leaves of non-

leguminous plants such as rice, sugarcane, wheat and maize (Bhattacharjee *et al.*, 2008b). Several nitrogen-fixing bacteria, e.g., *Acetobacter diazotrophicus* Gillis et al., *Herbaspirillum seropedicae* Baldani et al., *Azoarcus* spp. and *Azotobacter* strains (Steenhoudt and Vanderleyden, 2000) have been shown to colonize graminaceous plants (rice, wheat, maize) and exert plant growth promoting effects in their non-leguminous hosts via nitrogen fixation and phytohormonal stimulation of root development and root activity (Rothballer *et al.*, 2009). Khalid *et al*. (2004b) confirmed the potential of associative diazotrophic bacteria to promote the growth of many cereals and grasses. Their capacity to fix atmospheric nitrogen (N_2) makes them a viable option to generate BNF, which is economically attractive. Although many genera and species of N_2-fixing bacteria are isolated from rhizosphere of various cereals, mainly members of the genera of *Azospirillum*, *Azotobacter* and *Herbaspirillum* have been widely shown to increase yield of cereals under field conditions.

Rhizobium inoculants are used for leguminous crops (Peoples and Craswell, 1992), whereas *Azotobacter* may be used with crops like wheat, maize, mustard, cotton, potato and vegetable crops (Martinez Toledo *et al.*, 1988; Rai and Gaur, 1988). *Azospirillum* inoculants have been recommended for sorghum, millet, maize, sugarcane and wheat (Kapulnik *et al.*, 1981; Venkateswarlu and Rao, 1983; Mertens and Hess, 1984; Rai and Gaur, 1988; Dobereiner *et al.*, 1995). In the field, increasing plant production through enhanced BNF needs the establishment of effective N_2-fixing systems (Boddey and Dobereiner, 1988; Ishizuka, 1992).

1.2.1.2 Phytohormones

A phytohormone is an organic compound which is produced naturally in plants and it is active in small amounts in controlling growth and other functions (Letham, 1969). There are three types of phytohormones: auxins, gibberellins, and cytokinins. The production of phytohormones by plant-growth promoting rhizobacteria is considered to be an important mechanism by which these bacteria promote plant growth. All three types of hormones involve several stages of plant growth and development, such as cell elongation, cell division and tissue differentiation (Letham, 1969; Costacurta and Vanderleyden, 1995). Symbiotic bacteria *Rhizobium* and *Bradyrhizobium* synthesize indole-3-acetic acid (IAA) via indole-3-pyruvic acid (IPA). IAA is naturally occurring auxin with broad physiological effects. Many bacteria are able to produce IAA, including bacteria that are phytopathogenic, as well as those that are plant-growth promoting (Lambrecht *et al.*, 2000). Some PGPR may stimulate

root proliferation by IAA biosynthesis, therefore they may enhance uptake of soil minerals and nutrients by the host plant (Lambrecht *et al.*, 2000).

1.2.1.3 Phosphate Solubilization

After nitrogen, phosphorus is the major plant growth-limiting nutrient despite, its abundance in soil in both inorganic and organic forms. Phosphate is poorly accessible to plant because of its high reactivity with aluminum, iron and calcium, which leads to its precipitation (Gyaneshwar *et al.*, 2002). Group of heterotrophic microorganisms such as Bacillus and Pseudomonas are known to have the ability to solubilize inorganic P from insoluble sources (Wani, et al., 2007). They dissociate the phosphates from soil complex through several mechanisms, such as the production of organic acids which dissolve or chelate inorganic phosphate, or the production of phosphatases and phytases, which dissociate phosphorus from organic complexes (Vikram *et al.*, 2007). They increase the availability of soil phosphate, promoting plant uptake of this element (Rodríguez and Fraga, 1999). They also release phosphates by secretion of acids and phosphatases that solubilize and mineralize phosphates and make them available to plants (Kim *et al.*, 2010). This group includes the following bacteria: *Bacillus megaterium* de Bary, *B. circulans* Jordan, *B. subtilis* (Ehrenberg) Cohn, *Pseudomonas striata* Chester, and *P. rathonis* Miligula. Root growth is regulated by phosphorus availability and in early stages of plant growth, it benefits the plant by stimulating the production of deeper and more abundant roots (Henry *et al.*, 2010).

1.2.1.4 Siderophores

Siderophores (iron carriers) are defined as relatively low molecular weight molecules that have a high specificity for chelating or binding iron. Siderophores are produced by many microorganisms, including bacteria, yeast, and fungi, to extract iron from the environment (Neilands, 1995). Bacteria living in the soil or water must have a mechanism to solubilize iron precipitates in order to assimilate iron from the environment. Plant growth promoting rhizobacteria have been associated with improved plant growth through a direct effect on the plant, through antagonism against phytopathogenic microorganisms by production of siderophores, which are high affinity Fe^{3+} chelators, that enhances the microbial acquisition of Eron (Fe) in irone deficient environment (Scher and Baker, 1982) and cyanide (Loper and Buyer, 1991; Flaishman *et al.*, 1996; Howard, 1999).

1.2.2 Indirect Growth Promotion

1.2.2.1 Biocontrol of Phytopathogens

Diazotrophs are able to decrease or prevent the deleterious effects of pathogenic microorganisms through antibiotic production, suppressing pathogens or combinations of them (Dobbelaere *et al.*, 2003). Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides the front-line defense for roots against attack by pathogens (Weller, 1988).

1.2.2.2 Antibiotic Production

Plant growth promoting rhizobacteria (PGPR) are indigenous to soil and the plant rhizosphere and play a major role in the biocontrol of plant pathogens (Dowling and O'Gara, 1994). The production of antibiotic substances by some strains has been recognized as a major factor in the suppression of many root pathogens. Associative and endophytic nitrogen-fixing bacteria often produce antibiotic substances to promote plant growth and control phytopathogens (Bally and Elmerich, 2007). Many of these PGPR have the ability to produce disease-suppressive antibiotic such as phenazine-1-carboxylic, 2,4-diacetylphloroglucinol, streptomycin, pyoluteorin and pyrrolnitrin (Rai and Gaur, 1988).

1.2.2.3 Induced Systemic Resistance (ISR)

Biocontrol can also be mediated by activation of induced systemic resistance (ISR) responses in plants, and by modification of hormonal levels in the plant tissues (Bowen and Rovira, 1999). ISR occurs naturally as a result of colonization of the roots by beneficial soil-borne microorganisms, such as plant-growth promoting rhizobacteria and mycorrhizal fungi. Different beneficial microbe-associated molecular patterns are recognized by the plant, which results in a mild, but effective activation of the plant immune responses in plant tissues.

1.3 Field Crops

Cereals such as wheat, rice, and maize are the major cereals that sustain humanity (Fischer *et al.*, 2007). These crops need 20 to 40 kg soil N ha^{-1} per crop to satisfy the N requirements for each tonne of grain produced (Peoples and Craswell, 1992). In the developing world, maize is the most important staple crop, and nitrogen is the most important input required for maize production (Nziguheba *et al.*, 2005). As a staple food, maize has a large and stable market and is the most important agricultural product in South Africa. On the basis of area and volume of production, it remains the most important dry-land crop, globally. However, yields

in Africa are extremely low, at about 10% of potential, around 700 kg of maize per hectare against a potential seven tonnes per hectare in Central America (Gladwin *et al.*, 2001).

Wheat is generally grown in three production regions in South Africa, i.e., winter/spring-planted wheat in the summer rainfall region, winter-planted wheat types under dry land conditions, and spring wheat types grown under irrigation in the summer rainfall region (Hatting *et al.*, 2000). Wheat requires 50% of its total nitrogen by mid to late tillering. For example, winter wheat would ideally produce two to three tillers which support most of the yield by early spring (Holmes *et al.*, 2006). The other 50% of their total nitrogen requirement needs to be applied early enough to supply the high demand of these growing tillers (Blankenau *et al.*, 2002).

Farmers often lack irrigation, and Africa has the lowest global fertilizer usage (on average, less than five kg ha^{-1}) (Borlaug and Dowswell, 1995) because farmers are simply unable to afford inputs used by their developed world counterparts. The possibility of using BNF on cereals and other non-legume crops has been proposed (Boddey *et al.*, 1995b; Dobereiner *et al.*, 1995). Studies on sorghum, maize and wheat inoculated with *Azospirillum* have revealed a BNF contribution of five kg N ha^{-1} yr^{-1} (Okon and Labandera-Gonzalez, 1994). Unkovich and Baldock (2008) pointed out that the contribution of N by free living soil bacteria for crop growth in Australia is probably <10 kg ha^{-1} yr^{-1}. Because the contribution of N by free living soil bacteria for crop growth is so small, researchers in developed countries have suggested that the ability of PGPR to fix N is no longer an important criterion for classification of a bacterium as a biofertilizers (Peoples *et al.*, 2002; Boyle *et al.*, 2008). However, food shortages and malnutrition are still widespread problems in the developing world and it is therefore important to use whatever potential there is to increase the output of low external input agriculture. To make the cultivation of cereals sustainable and less dependent on nitrogen fertilizer , it is important to use PGPRs that can biologically fix nitrogen and produce growth enhancing substances (for example, indole-3-acetic acid and siderophores) (Table 1.3). These may contribute to enhanced cereal yield (Mtambanengwe and Mapfumo, 2008).

1.4 Biological Nitrogen Fixation (BNF) by Non-symbiotic Diazotrophs

The only biological reaction counterbalancing the loss of N from soils or ecosystems is BNF (Hurek and Reinhold-Hurek, 2003). Activity of non-symbiotic nitrogen-fixing bacteria is low in systems with crop residues containing high levels of plant available nitrogen because the nitrogenase enzyme activation slows down if sufficient fixed nitrogen is available in the soil environment (Reference fix). Moreover, the reduction of atmospheric nitrogen (N_2) to ammonia (NH_3) by the nitrogenase enzyme consumes large amounts of energy (Kim and Rees, 1994) and depends on adenosine 5 ' - triphosphate (ATP), Mg'+, and a source of low potential electrons (Watt *et al.*, 1975).

It was observed that non-leguminous plants like rice, sugarcane, wheat and maize form an extended niche for various species of N_2-fixing bacteria (Bhattacharjee *et al.*, 2008b). These bacteria thrive within the plant, successfully colonizing roots, stems and leaves. Free - living diazotrophs that has been repeatedly detected in association with plant roots include *Acetobacter diazotrophicus* Gillis, *Herbaspirillum seropedicae* (Leifson) Ding and Yokota, *Azoarcus* spp. and *Azotobacter* spp**.** (Steenhoudt and Vanderleyden, 2000**).** Some of these diazotrophic bacteria have been called endophytes because of their occurrence mainly within plant tissue (James *et al.*, 1997). Endophytic diazotrophs have been isolated from several grasses in which significant BNF has been demonstrated, particularly Brazilian sugarcane varieties, but also rice, maize, and sorghum (Boddey and Dobereiner, 1995). They have been linked with high level of N-fixation, particularly in sugarcane where the bacteria are found in large numbers (Boddey *et al.*, 1991; Dobereiner *et al.*, 1995). BNF by some diazotrophic bacteria such as *Azotobacter*, *Clostridium, Azospirillum*, *Herbaspirillum* and *Burkholderia* can substitute for urea-N (Kennedy *et al.*, 2004a). *Clostridium* spp. was the first gram positive, strictly anaerobic archaebacterium that was shown to be capable of nitrogen-fixation (Dixon and Wheeler, 1983).

1.4.1 Free Living N_2-fixing Bacteria

Free living nitrogen fixers represent a range of microorganisms including bacteria living on plant residues (saprophytes), bacteria which live entirely within plants (endophytes) and bacteria living in close association with the plant root (rhizobacteria). Free-living nitrogen-fixing bacteria reside in the rhizosphere of certain plants (including many grasses) and fix nitrogen in nutrient-rich plant rhizospheres. In the free-living system, plants gain benefit

when bacteria die and release nitrogen to the environment (Bentley and Carpenter, 1984), or when bacteria are loosely associated with roots of plants (James, 2000).

1.4.1.1 The Genus *Azotobacter*

Azotobacter is a gram negative bacterium that is usually motile, oval or spherical in shape, and forms thick walled and elongated cysts 1.4-2.0 µm in diameter (Socolofsky and Wyss, 1961). It prefers aerobic condition but can grow under low oxygen pressure and fixes N, at a rate of at least 10 mg N_2 per gram of carbohydrates consumed (Drozd and Postgate, 1970). It is able to grow at a pH range of 4.8 - 8.5 and fixes N at optimum pH of 7.0 - 7.5 (Dilworth *et al.*, 1988).The species *Azotobacter vinelandii* Lipman and *Azotobacter chroococcum* Beijerinck are free-living, aerobic heterotrophic diazotrophs that depend on an adequate supply of reduced carbon compounds such as sugars for energy (Kennedy *et al.*, 2004a). These bacteria have been reported to stimulate crop yield and this led to the artificial inoculation ('Azotobacterin') of crops in Russia in the 1950s. Inoculation with *Azotobacter* can increase rice yield up to 0.9 t ha^{-1} and N accumulation up to 15 kg ha^{-1} (Yanni and El-Fattah, 1999). Similarly, inoculum of *A. chroococcum* was effective in enhancing the vegetative growth of maize (Nieto and Frankenberger, 1991). It has also been reported that wheat yields increased up to 30% with *Azotobacter* inoculation (Kloepper *et al.*, 1991). However, it is not clear whether these beneficial effects were due to nitrogen fixation or to the production of growth substances by the bacteria (Stewart, 1969).

1.4.2 N2-fixation Associated Bacteria

In the rhizosphere of grasses, many N_2-fixing microorganisms are present. Some are strongly associated with plants they inhabit and respond strongly to the availability of plants nutrients. Nitrogen-fixing plant growth promotion rhizobacteria (PGPR) include the following species: *Azotobacter paspali* Döbereiner (Approved Lists, 1980), *Azospirillum lipoferum* Beijerinck Tarrand *et al*., *Azospirillum brasilense* Tarrand et al. and *Azotobacter amazonense* which have been studied for more than 50 years, though their contribution of fixed nitrogen to crop plants are controversial (Giller and Cadisch, 1995). For example, Bashan *et al*. (1989) demonstrated that the contribution of a Nif- strain of A*zospirillum brasilense* to the improvement of tomato seedling growth was not through nitrogen fixation. However, Boddey and Knowles (1987) suggested that when some of these bacteria associated with specific hosts such as sugarcane and *Panicum* sp., nitrogen fixation can become quite

significant. There are numerous N_2-fixing bacteria taxa such as: *Acetobacter diazotrophicus* Gillis *et al*. and *Herbaspirillum* spp. that are associated with sugarcane, sorghum and maize, and are considered to enhance crop yields (Triplett, 1996; James *et al.*, 1997).

1.4.2.1 The Genus *Azospirillum*

Bacteria of the genus *Azospirillum* (a subclass of proteobacteria) have been known for many years as PGPR (Okon and Labandera-Gonzalez, 1994; Steenhoudt and Vanderleyden, 2000). *Azospirillum* species are aerobic heterotrophs that convert atmospheric nitrogen into ammonium under microaerobic conditions at low oxygen levels, through the action of a nitrogenase complex (Roper and Ladha, 1995; Steenhoudt and Vanderleyden, 2000). They grow extensively in the rhizosphere of graminaceous plants (Kennedy and Tchan, 1992) and penetrate the root to grow endophytically (James *et al.*, 2000). They are also capable of producing antifungal and antibacterial compounds, growth regulators and siderophores (Pandey and Kumar, 1990). Irrespective of their form of application and their mode of action on plants, the genus *Azospirillum* can provide bio-fertilizer strains. Okon and Labandera-Gonzalez (1994) argued that the term biofertilizer is not appropriate for *Azospirillum* spp. because their application does not replace the application of nitrogen fertilizers. They have the ability to colonize the root cortex of plants, especially grass family (Gramineae), and act as plant growth promoting agents, mostly via phytohormonal stimulation of root development and activity (Rothballer *et al.*, 2009). The beneficial effect of *Azospirillum* on several crops could be resulted from both nitrogen fixation and its stimulating effects on root development (Döbereiner, 1987). Examples of *Azospirillum* inculants in different countries (Table 1.1).

Table 1.1 Use of biofertilizer inoculants, mainly *Azospirillum* spp. in different countries

Countries	Crops	Biofertilizers inoculants	References
Israel	wheat, sorghum	*Azospirillum brasilense*	Sarig *et al*. (1984); Avivi and Feldman (1982); Inbal and Feldman (1982)
Egypt	wheat, maize	*Azospirillum brasilense*	Hegazi *et al*. (1981); Hegazi *et al*. (1983)
India	sorghum, wheat, pearl millet (*Pennisetum americanum*), barley	*Azospirillum brasilense*	Pal and Malik (1981); Rai and Gaur (1982); Rao *et al*.(1985); Subba Rao *et al*.(1985)
Britain	maize, wheat	*Azospirillum brasilense*	O'Hara *et al*. (1981); Lethbridge and Davidson (1983)
Belgium	Wheat	*Azospirillum brasilense*	Reynders and Vlassak (1982)
Germany	spring wheat	*Azospirillum lipoferum*	Mertens and Hess (1984)
Australia	Digitgrass	*Azospirillum brasilense*	Schank *et al*. (1981)
USA	sorghum, *Pennisetum* sp.	*Azospirillum brasilense*	Smith *et al*. (1984); Pacovsky *et al*. (1985)
Brazil	maize, sorghum and wheat	*Azospirillum brasilense*	Lin *et al*. (1983); Rennie *et al*. (1983)

1.4.3 Endophytic N2-fixing Bacteria

Endophytic diazotrophs, such as *Acetobacter*, *Azoarcus*, and *Herbaspirillum*, reside in graminaceous plant within plant tissues, and may fix nitrogen (Table 1.2). These endophytic bacteria live within plant tissues without causing visible damage to the host plant and may promote plant growth directly or indirectly. For example, they can establish themselves inter-cellularly in the root system of non-legumes (especially cereals) and fix nitrogen endophytically (Cocking, 2003). Inoculating different crops and grasses, such as sugarcane in Brazil (Boddey *et al.*, 2003), wetland rice in Asia (Ladha and Reddy, 2003), and cereal fields in Canada (Rennie and Thomas, 1987) with these endophytic bacteria, has resulted in improved crop production without artificial nitrogen input.

Diazotrophic endophytic bacteria fall into two groups: *facultative* and *obligate* (Baldani *et al.*, 1997). Facultative endophytes are those that survive in the soil or on plant surfaces and are able to colonize the interior of some plants (Cocking, 2003). For example: endophytic *Azospirillum* strains are facultative endophytes (Baldani *et al.*, 1997), entering host plants via seeds or wounds at lateral root junctions (James and Olivares, 2010). Obligate endophytes are those that survive poorly in the soil and appear to have a requirement for living within a host plant (Baldani *et al.*, 1997). For example: *Herbaspirillum* spp., *Acetobacter diazotrophicus* Gillis *et al*. and *Burkholderia* spp. usually live inside plants, within their xylem vessels and in intercellular spaces (James and Olivares, 2010).

.

1.4.3.1 The Genus *Herbaspirillum*

The genus *Herbaspirillum* were initially thought to be a new *Azospirillum* species but later it was shown to have no close relatedness with *Azospirillum* spp. (Baldani *et al.*, 1986a). *Herbaspirillum* spp. are endophytes that colonize sugarcane, rice, maize, sorghum and other cereals (James *et al.*, 2000). In the late 1980s some Brazilian varieties of sugarcane were shown to be able to obtain significant contributions from endophytic diazotrophs that infected the interior of plants (Baldani *et al.*, 1986a). *Herbaspirillum seropedicae* (Leifson) Ding and Yokota was first isolated in Rio de Janeiro, Brazil (Baldani *et al.*, 1986a), and it has the Nif-N gene which is necessary for nitrogenase activity (Klassen *et al.*, 1999). Boddey *et al.* (2003) discovered other endophytic diazotrophs including *Gluconacetobacter diazotrophicus* Corrig. (Gillis et al.) Yamada *et al*., *Herbaspirillum seropedicae* (Leifson) Ding and Yokota, *H. rubrisubalbicans* (Christopher and Edgerton) Baldani *et al*. and *Burkholderia* spp. within

sugarcane. However, it was not clear which endophyte is responsible for the measured BNF. Motobu *et al*. (2006) suspected that *G. diazotrophicus* was the dominant contributor of BNF to sugarcane. Kennedy *et al*. (1997) noted that the endophytes made a significant contributor to the nitrogen economy of sugarcane. In other studies, inoculation of cereals with N_2 fixing bacteria such as *H. seropedicae*, increased plant growth and grain yield (Divan Baldani *et al.*, 2000). However, the total increase in N content in the inoculated plants may not be only through N_2 fixation by the microorganism but it may be through increased development of the root system, which promoted water absorption and mineral uptake, leading to a yield increase (Okon, 1985).

Table 1.2 Examples of endophytic diazotrophs and their host crops

PGP	**Relationship to the host**	**Host crops**	**References**
Azoarcus sp.	Endophytic	kallar grass	Hurek *et al*. (2002)
		sorghum	Stein *et al*. (1997)
		rice	Egener *et al*. (1999)
Burkholderia sp.	Endophytic	rice	Baldani *et al*. (2000)
Gluconacetobacter diazotrophicus	Endophytic	sorghum	Isopi *et al*. (1995)
		sugarcane	Boddey *et al*. (2001)
			Sevilla *et al*. (2001)
Herbaspirillum sp.	Endophytic	Rice	James *et al*. (2002)
		Sorghum	James *et al*. (1997)
		Sugarcane	Pimentel *et al*. (1991)

Table 1.3 Biology, and potential role of some diazotrophs promoting crop production

Diazotrophs	Condition	Habitat	Energy source	Mechanisms of effect	References
Azotobacter chroococcum	Aerobic	Rhizosphere	Organics in soil	BNF	Kennedy and Tchan (1992)
Clostridium spp.	Anaerobic	Soil saprophyte	Organics in soil	BNF	Kennedy and Tchan (1992)
Azospirillum spp.	Microaerobic	Rhizosphere, mildly endophytic	in roots, stems and leaves organics in soil, root exudates and plant tissue	BNF, PGP	Reinhold and Hurek (1988) Mirza *et al*. (2000) Okon and Kapulnik (1986)
H. seropedicae	Microaerobic	Endophytic, rhizosphere	Root exudates	BNF, PGP	Baldani *et al*. (1986b)
Azoarcus sp.	Microaerobic	Endophytic	Root exudates	BNF	Hurek et al. (1994) Reinhold-Hurek *et al*. (1993)
B. vietnamiensis		Rhizosphere, endophytic	Organics in soil and root exudates	BNF, PGP	Baldani et al. (1997)
R. leguminosarum bv. *trifolii*		Endophytic in roots	Root exudates	PGP	Yanni *et al*. (1997) Yanni *et al*. (2001)
R. etli bv. *Phaseoli*		Endophytic in roots	Root exudates	PGP	Gutie´rrez-Zamora and Martı´nez-Romero (2001)
A. caulinodans	Microaerobic	Endophytic in roots	Root exudates	PGP	Anyia, *et al*. (2004)
A. diazotrophicus	Microaerobic	Endophytic in roots, stems and leaves	Root exudates and plant tissue	BNF	Baldani *et al*. (1997) Boddey *et al*. (1991)

Biological nitrogen fixation (BNF); plant growth promotion (PGP)

1.5 Factors Affecting Nitrogen Fixation

1.5.1 Energy Source

Biological nitrogen fixation depends on the availability of carbohydrates. In general, large quantities of carbohydrate are required for high rates of nitrogen fixation because there is intense competition between nitrogen-fixing and non-nitrogen-fixing forms (Stewart, 1969). Symbiotic nitrogen-fixing bacteria receive energy directly from the host legume but free-living bacteria have to compete for their sources of energy within the soil (Chen *et al.*, 1993).

1.5.2 Oxygen

Aerobes such as *Azotobacter* require oxygen for metabolism. The oxygen levels have a significant effect on the efficiency with which aerobes fix nitrogen (Stewart, 1969). *Azospirillum* is a microaerobic organism which requires low oxygen levels for the expression of nitrogenase activity (Tarrand *et al.*, 1978) and nitrogen-fixation occurs in microaerobic, nitrogen-limited conditions (Eckert *et al.*, 2001). Nitrogen-fixation is inhibited by oxygen because dinitrogenase reductase is rapidly and irreversibly inactivated by oxygen. Nitrogen-fixation efficiency is greatly increased at a low partial pressure of oxygen (Parker and Scutt, 1960).

1.5.3 Combined Nitrogen fertilization

Combined nitrogen sources inhibit nitrogen fixation. The inhibition appears when nitrogen is reduced to ammonia and the presence of free ammonia represses nitrogenase activity. However, the degree and type of inhibition depend on the level of supplied combined nitrogen (Stewart, 1969).

1.5.4 Iron, Molybdenum and Hydrogen-ion Concentration

Iron and molybdenum are the only metals present in the nitrogenase complex (Stewart, 1969). Optimum nitrogen fixation occurs when 0.02 to 0.05 ppmof iron and 0.2 ppm of molybdenum are supplied. For example, addition of molybdenum to tropical soils has often markedly increased nitrogen fixation (Stewart, 1969). The nitrogenase enzyme operates best over a fairly narrow pH range of 7.0 and fixation falls off markedly above and below 6.5 to

7.0 (Stewart, 1969). Yet most of the agricultural soils in the southern hemisphere have a pH of 4.0-6.0 (Sanchez, 2002).

1.6 Nitrogen-fixation Detection

1.6.1 Total N-balance Method

This method measures whether the plant or soil system accumulates N over time from N_2 fixation inputs. Nitrogen losses from the system through ammonia volatilization, denitrification and leaching, may result in an underestimate of the fixed N in the system (Herridge *et al.*, 2008).

1.6.2 The Nitrogen Difference Method

The nitrogen difference method is adequate for active nitrogen fixers. This method can be used in soils of limited N supply (Herridge *et al.*, 2008) but it will not detect increases of less than about 1% in the total nitrogen, even when uniform samples can be taken (Chalk and Smith, 1994). This method and N balance has been largely replaced by the ^{15}N and Ureide Methods (Herridge *et al.*, 2008).

1.6.3 The Stable Isotope (^{15}N) Method

The most definitive measurements of BNF make use of the stable, heavy isotope, ^{15}N, and requires access to a mass spectrometer (Sprent, 1979). In this method, incorporation of $^{15}N_2$ (labeled dinitrogen) into plant or microbial cells is measured. Exposure of samples to about 10% $^{15}N_2$, in a balance of argon or helium to eliminate competition from $^{14}N_2$ is needed. Following incubation, samples can be digested and the ^{15}N content of the materials can be determined using a mass spectrometer. Detection of ^{15}N in tissues or cells provides definitive proof of BNF and allows for a very accurate quantification of the amount of nitrogen-fixation that has occurred (Lima *et al.*, 1987; Danso, 1995; Boddey *et al.*, 2001). This method is accurate but is time consuming and expensive, both in terms of the equipment needed and the cost of the isotope itself (Robinson, 2001). It is technically challenging and requires substantial inputs of labour. Moreover, errors in quantifying the N fluxes can introduce uncertainties into the final estimates of N_2-fixation (Chalk *et al.*, 1994).

1.6.4 The Acetylene Reduction Assays

The nitrogenase enzyme is capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4) and is universally responsible for biological N_2-fixation. Both gases can be detected and quantified using gas chromatography (Hardy *et al.*, 1973).This test provides a sensitive measure of nitrogenase activity and is useful for detecting the N_2 fixation activities of bacterial cultures or plant residues that may be harbouring N_2-fixing bacteria (Herridge *et al.*, 2008). Enclosing the particular agent in a gas-tight vessel and periodically removing and injecting into the gas chromatograph to evaluate ethylene (C_2H_4) may disturb the N_2-fixing species, which may result in a decline in activity, especially in the *Rhizobium*/legume symbiosis (Bergersen, 1970; Dixon and Wheeler, 1983; Minchin *et al.*, 1983; Minchin *et al.*, 1994; Vessey, 1994). However, this method is far simpler and faster than other methods.

1.7 Application of Biofertilizers

Some rhizospheric bacteria have been developed as biofertilizers and biopesticides to minimize excessive use of inorganic fertilizers as well as to protect the environment and plant health (Kennedy *et al.*, 2004b; Ahmad *et al.*, 2006b; Banerjee *et al.*, 2006). In many countries several PGPR formulations are currently available as commercial products for agricultural production (Alarcón and Ferrera-Cerrato, 2000; Lucy *et al.*, 2004; Wu *et al.*, 2005; Nakayan *et al.*, 2009). *Bacillus subtilis* is one of the first widely sold PGPR strain marketed by Gustafson, Inc. as Kodiak in the USA (Alarcón and Ferrera-Cerrato, 2000; Harman *et al.*, 2010). In China microbial agents made up of different strains of *Bacillus* (*B. brevis* Migula,, *B. cereus* Frankland and Frankland, *B. coagulans* Hammer, *B. firmus* Werner, *B. licheniformis* (Weigmann) Chester *and B. sphericus* Meyer and Neideand *B. subtilis* (Ehrenberg) Cohn have been commercially available since 1980s (Chen *et al.*, 1996; Zhang *et al.*, 1996).

Various strains of *A. brasilense*. and *A. lipoferum* have also been used to inoculate cultivars of different species of plants (Okon and Labandera-Gonzalez, 1994). For example in sorghum, inoculated maize and wheat with *Azospirillum* have revealed a contribution of 5 kg N ha^{-1} yr^{-1}. In India the Gujarat State Fertilizers Company (GSFC) has commercialized two biofertilizers namely: Sadar *Azotobacter* and Sadat *Azospirillum* for cereals, cash crops and vegetables since 1984. Three years later a phosphate solubilizing biofertilizer (Sadar phosphate) was commercialized. Since 1995 a new N-fixing biofertilizer (Sadar Super culture) for sugarcane was introduced to the market. In Egypt, there are groups of commercial

products called Cerealin that contain different species of bacteria, depending on the crop. Inoculation of citrus trees with Cerealin (containing *Azospirillum brasilense*) increased yields of Washington navel orange (Shamseldin *et al.*, 2010). Similarly, Cerealin (containing *Bacillus polymyxa* Ash et al. and *Azotobacter*) increased turfgrass height, turf density, fresh and dry weights, and total chlorophylls and carotenoids (Monem *et al.*, 2001). Another commercial biofertilizer called Nemales, containing *Serratia* spp., has been shown to increase growth and crude protein content in wheat (Banerjee *et al.*, 2006). In the Philippines, a biofertilizer product called 'BIO-N' is available in the market and used for the production of rice and corn, and has reduced use of chemical fertilizer by 30-50% (Monsalud, 2008). In Indonesia there are 41 commercial biofertilizers in use (Husen *et al.*, 2007). There have been many reports worldwide on the continuous research on the effects of biofertilizers, which include laboratory, greenhouse and field experiments over the years (Okon and Labandera-Gonzalez, 1994). Biofertilizers have emerged as an important component of an integrated nutrient supply system and hold the promise of improving crop yields through environmentally better nutrient supplies. However, the application of microbial fertilizers in practice has not achieved consistent results.

1.8 Limitations of Biofertilizers

Biofertilizers are dependent upon physical, environmental, nutritional and biological factors.

Factors such as high soil temperature or low soil moisture (Rao, 1982), extreme soil acidity or alkalinity (Stamford *et al.*, 2007), and low phosphorous and molybdenum availability (Egamberdiyeva, 2007) can all negatively affect the performance of microbial inoculants. Furthermore, poor quality control in the production process can result in ineffective strains being sold as soil inoculants (Martínez-Viveros *et al.*, 2010), together with insufficient concentrations of microorganisms and high level of contaminants (Kannaiyan, 2003). Further problems can be associated with its incorrect transportation and storage conditions that affect the viability of the inoculants (Odame, 1997). Moreover, the presence of high native populations (Thiyagarajan *et al.*, 2003; Martínez-Viveros *et al.*, 2010) or the presence of bacteriophages, may result in a poor survival of the microbial biofertilizer inoculants as they compete with indigenous bacteria for available growth substrates (Martínez-Viveros *et al.*, 2010).

1.9 Future prespects of biofertilizer

The air in our atmosphere is made up of Nitrogen gas (N_2). This gas is of no use to most organisms and can only be beneficial to plant growth if it is first converted to ammonium and/or nitrate. This can either be done through industrial processes, in the manufacture of chemical fertilizers, or through biological nitrogen fixation. Plant growth enhancements and yield increases following inoculation of non-legumes with *Azospirillum brasilense* were initially attributed to biological nitrogen fixation by some researchers. However, it is always difficult to ascertain that a PGPR promotes plant growth by using only a single mode of action. One of the generally accepted concepts is also that beneficial PGPR are effective only when they successfully colonize and persist in the plant rhizosphere (Bloemberg and Lugtenberg, 2001). Studies by Biswas *et al*. (2000) and Riggs *et al*. (2001) reported that improvements in growth parameters of various crops as a result of bacterial inoculations at reduced levels of nitrogenous fertilizers. Therefore, isolating and screening suitable microbial inoculants may enhance nitrogen fertilizer efficiency, leading to enhanced crop production at lower doses of fertilizers. Finally, some questions needs answering, Are there effective N_2-fixing bacterial in the soil rhizosphere, root and leaves? Is nitrogen fixed by N_2-fixing microorganisms enough to promote plant growth?

References

Ahmad F., Ahmad I., Aqil F., Ahmed Wani A., Sousche Y.S. (2006a) Plant growth promoting potential of free-living diazotrophs and other rhizobacteria isolated from Northern Indian soil. Biotechnology Journal 1:1112-1123.

Ahmad F., Ahmad I., Aqil F., Wani A.A., Sousche Y.S. (2006b) Plant growth promoting potential of free-living diazotrophs and other rhizobacteria isolated from Northern Indian soil. Biotechnology Journal 1:1112-1123.

Ahmad F., Ahmad I., Khan M. (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163:173-181.

Alarcón A., Ferrera-Cerrato R. (2000) Biofertilizers: importance and management in agriculture. Agricultura Técnica en México 26:191-203.

Anyia A.O., Archambault D.J., Slaski J.J. (2004) Growth promoting effects of the diazotroph *Azorhizobium caulinodans* on Canadian wheat cultivars. Fourth International Crop Science Congress, Environmental Technologies, Alberta Research Council, Vegreville, Alberta, T9C 1T4, Canada. http://www.cropscience.org.au/ics2004/

Avivi Y., Feldman M. (1982) The response of wheat to bacteria of the genus *Azospirillum*. Israeli Journal of Botany 31:237-246.

Baldani J., Baldani V.L.D., Seldin L., Döbereiner J. (1986a) Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. International Journal of Systematic Bacteriology 36:86-93.

Baldani J.I., Caruso L., Baldani V.L.D., Goi S.R., Döbereiner J. (1997) Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry 29:911-922.

Baldani V.L.D., Alvarez M.A.B., Baldani J., Döbereiner J. (1986b) Establishment of inoculated *Azospirillum* spp. in the rhizosphere and in roots of field grown wheat and sorghum. Plant and Soil 90:35-46.

Baldani, V.D., J.I. Baldani, J. Döbereiner. 2000. Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. Biology and Fertility of Soils 30: 485-491

Bally R., Elmerich C. (2007) Biocontrol of plant diseases by associative and endophytic nitrogen-fixing bacteria. Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations 5:171-190.

Banerjee M.R., Yesmin L., Vessey J.K. (2006) Plant-growth-promoting rhizobacteria as biofertilizers and biopesticides. In: Handbook of Microbial Biofertilizers. Rai, MK. (Ed.), Food Production Press, USA:137-181.

Bentley B.L., Carpenter E.J. (1984) Direct transfer of newly-fixed nitrogen from free-living epiphyllous microorganisms to their host plant. Oecologia 63:52-56.

Bergersen F. (1970) The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Australian Journal of Biological Sciences 23:1015-1026.

Bhattacharjee R., Singh A., Mukhopadhyay S. (2008a) Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. Applied Microbiology and Biotechnology 80:199-209. DOI: 10.1007/s00253-008-1567-2.

Bhattacharjee R.B., Singh A., Mukhopadhyay S. (2008b) Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. Applied Microbiology and Biotechnology 80:199-209.

Biswas J.C., Ladha J.K. Dazzo F.B. (2000). *Rhizobia* inoculation improves nutrient uptake and growth of lowland rice. Soil Science Society of America Journal 64**,** 1644–1650.

Blankenau K., Olfs H.W., Kuhlmann H. (2002) Strategies to improve the use efficiency of mineral fertilizer nitrogen applied to winter wheat. Journal of Agronomy and Crop Science 188:146-154.

Bloemberg, G.V., Lugtenberg, B.J.J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion in Plant Biology 4, 343-350.

Boddey R., Oliveira O.C., Urquiaga S., Reis V., Olivares F.L., Baldani V., Döbereiner J. (1995a) Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. Plant and Soil 174:195-209.

Boddey R., Urquiaga S., Reis V., Döbereiner J. (1991) Biological nitrogen fixation associated with sugar cane. Plant and Soil 137:111-117.

Boddey R.M., Dobereiner J. (1988) Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. Plant and Soil 108:53-65.

Boddey R.M., Dobereiner J. (1995) Nitrogen fixation associated with grasses and cereals: recent progress and perspectives for the future. Nutrient Cycling in Agroecosystems 42:241-250.

Boddey R.M., Knowles R. (1987) Methods for quantification of nitrogen fixation associated with Gramineae. Critical Reviews in Plant Sciences 6:209-266.

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

Boddey R.M., Polidoro J.C., Resende A.S., Alves B.J.R., Urquiaga S. (2001) Use of the ^{15}N natural abundance technique for the quantification of the contribution of N_2 fixation to sugar cane and other grasses. Functional Plant Biology 28:889-895.

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

Borlaug N.E., Dowswell C.R. (1995) Mobilising science and technology to get agriculture moving in Africa. Development Policy Review 13:115-129.

Bowen G., Rovira A. (1999) The rhizosphere and its management to improve plant growth. Advances in Agronomy 66:1-102.

Boyle S.A., Yarwood R.R., Bottomley P.J., Myrold D.D. (2008) Bacterial and fungal contributions to soil nitrogen cycling under Douglas fir and red alder at two sites in Oregon. Soil Biology and Biochemistry 40:443-451.

Bradstreet R.B. (1965) The Kjeldahl Method for Organic Nitrogen. Academic Press, Inc., New York.

Bremner JM, Mulvarey CS. (1982) Methods of Soil Analysis. In: Chemical and Microbiological Properties Madison. Page AL, Miller RH, Keeny DR (Eds.). American Society of Agronomy. Pp. 595–622.

Chalk P., Smith C. (1994) ^{15} N isotope dilution methodology for evaluating the dynamics of biologically fixed N in legume-non-legume associations. Biology and Fertility of Soils 17:80-84.

Chalk P., Wood J., Crawford D., Smith C. (1994) Estimating gross nitrogen mineralization and immobilization rates in anaerobic and aerobic soil suspensions. Soil Science Society of America Journal 58:1652-1660.

Chen Y., Mei R., Liu L., Kloepper J. (1996) The use of yield increasing bacteria (YIB) as plant growth-promoting rhizobacteria in Chinese agriculture. In, Management of Soil Borne Diseases. Utkhede, R.S. and Gupta, V.K. (Eds.). Kalyani Publishers, New Delhi, India. Pp. 165–176.

Chen Y.P., Lopez-de-Victoria G., Lovell C.R. (1993) Utilization of aromatic compounds as carbon and energy sources during growth and N_2-fixation by free-living nitrogen fixing bacteria. Archives of Microbiology 159:207-212.

Cocking E.C. (2003) Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant and Soil 252:169-175..

Costacurta A., Vanderleyden J. (1995) Synthesis of phytohormones by plant-associated bacteria. Critical Reviews in Microbiology 21:1-18.

Da Silva J.G., Serra G.E., Moreira J.R., Concalves J.C., Goldemberg J. (1978) Energy balance for ethyl alcohol production from crops. Science 201:903-906.

Danso S.K.A. (1995) Assessment of biological nitrogen fixation. Nutrient Cycling in Agroecosystems 42:33-41.

Dilworth M.J., Eady R.R., Eldridge M.E. (1988) The vanadium nitrogenase of *Azotobacter chroococcum*. Reduction of acetylene and ethylene to ethane. Biochemical Journal 249:745-751.

Divan Baldani V., Baldani J.I., Döbereiner J. (2000) Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. Biology and Fertility of Soils 30:485-491.

Dixon R.O.D., Wheeler C.T. (1983) Biochemical, physiological and environmental aspects of symbiotic nitrogen fixation. In, Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications. Gordon, J.C. and Wheeler, C.T. (Eds.), Springer, Netherlands. pp. 107-171.

Dobbelaere S., Vanderleyden J., Okon Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22:107-149.

Döbereiner J. (1987) Nitrogen-Fixing Bacteria in Nonleguminous Crop Plants. Science Technology, Madison and Springer, Verlag, Berlin.

Dobereiner J., Urquiaga S., Boddey R.M. (1995) Alternatives for nitrogen nutrition of crops in tropical agriculture. Nutrient Cycling in Agroecosystems 42:339-346.

Dowling, D.N., F. O'Gara. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. Trends in Biotechnology 12: 133-141.

Drozd J., Postgate J. (1970) Effects of oxygen on acetylene reduction, cytochrome content and respiratory activity of *Azotobacter chroococcum*. Journal of General Microbiology 63:63-73.

Eckert B., Weber O.B., Kirchhof G., Halbritter A., Stoffels M., Hartmann A. (2001) *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass *Miscanthus*. International Journal of Systematic and Evolutionary Microbiology 51:17-26.

Egamberdiyeva D. (2007) The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Applied Soil Ecology 36:184-189.

Egener T., Hurek T., Reinhold-Hurek B. (1999) Endophytic expression of nif genes of *Azoarcus* sp. strain BH72 in rice roots. Molecular Plant-Microbe Interactions 12:813-819.

Fischer S.E., Fischer S.I., Magris S., Mori G.B. (2007) Isolation and characterization of bacteria from the rhizosphere of wheat. World Journal of Microbiology and Biotechnology 23:895-903.

Flaishman M.A., Eyal Z., Zilberstein A., Voisard C., Haas D. (1996) Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. MPMI-Molecular Plant Microbe Interactions 9:642-645.

Frankenberger W.T., Arshad M. (1995) Phytohormones in Soils: Microbial Production and Function. CRC Handbooks, Boca Raton, Florida, USA.

Giller K., Cadisch G. (1995) Future benefits from biological nitrogen fixation: an ecological approach to agriculture. Plant and Soil 174:255-277.

Gladwin C.H., Thomson A.M., Peterson J.S., Anderson A.S. (2001) Addressing food security in Africa via multiple livelihood strategies of women farmers. Food Policy 26:177-207.

Glick B.R. (1995) The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41:109-117.

Gutierrez-Zamora M., Martınez-Romero E. (2001) Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). Journal of Biotechnology 91:117-126.

Gyaneshwar P., Naresh Kumar G., Parekh L., Poole P. (2002) Role of soil microorganisms in improving P nutrition of plants. Plant and Soil 245:83-93.

Hamdi Y. (1982) Application of Nitrogen-fixing Systems in Soil Improvement and Management. Food & Agriculture Organization of the UN (FAO), Rome.

Hardy R.W.F., Burns R.C., Holsten R.D. (1973) Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5:47-81. Harman G.E., Obregón M.A., Samuels G.J., Lorito M. (2010) Changing models for commercialization and implementation of biocontrol in the developing and the developed world. Plant Disease 94:928-939.

Hatting J.L., Poprawski T.J., Miller R.M. (2000) Prevalences of fungal pathogens and other natural enemies of cereal aphids (Homoptera:Aphididae) in wheat under dryland and irrigatedconditions in South Africa. BioControl 45:179-199.

Hegazi N., Khawas H., Monib M. (1981) Inoculation of wheat with *Azospirillum* under Egyptian conditions. In, Current Perspectives in Nitrogen Fixation. Gibson, A.H. and Newton, W.E. (Eds.), Australian Academy of Science, Canberra, p. 493.

Hegazi N.A., Monib M., Amer H.A., Shokr E.S. (1983) Response of maize plants to inoculation with azospirilla and (or) straw amendment in Egypt. Canadian Journal of Microbiology 29:888-894.

Henry A., Chaves N.F., Kleinman P.J.A., Lynch J.P. (2010) Will nutrient-efficient genotypes mine the soil? Effects of genetic differences in root architecture in common bean *Phaseolus vulgaris* L.) on soil phosphorus depletion in a low-input agro-ecosystem in Central America. Field Crops Research 115:67-78.

Herridge D.F., Peoples M.B., Boddey R.M. (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311:1-18.

Holmes J., Engel R., Miller P. (2006) Cropping sequence effect of pea and pea management on spring wheat in the Northern Great Plains. Agronomy Journal 98:1610-1619.

Howard D.H. (1999) Acquisition, transport, and storage of iron by pathogenic fungi. Clinical Microbiology Reviews 12:394-404.

Hurek T., Handley L.L., Reinhold-Hurek B., Piché Y. (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Molecular Plant-Microbe Interactions 15:233-242.

Hurek T., Reinhold-Hurek B. (2003) *Azoarcus sp*. strain BH72 as a model for nitrogen-fixing grass endophytes. Journal of Biotechnology 106:169-178.

Hurek T., Reinhold-Hurek B., Van Montagu M., Kellenberger E. (1994) Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. Journal of Bacteriology 176:1913-1923.

Husen E., Simanungkalit R., Saraswati R., Irawan. (2007) Characterization and quality assessment of Indonesian commercial biofertilizers. Indonesian Journal of Agricultural Science 8:31-38.

Inbal E., Feldman M. (1982) The response of a hormonal mutant of common wheat to bacteria of the genus *Azospirillum*. Israeli Journal of Botany31:257-264.

Isopi R., Fabbri P., Del Gallo M., Puppi G. (1995) Dual inoculation of *Sorghum bicol*or (L.) Moench ssp. *bicolor* with vesicular arbuscular mycorrhizas and *Acetobacter diazotrophicus*. Symbiosis 18:43-45.

James E. (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crops Research 65:197-209.

James E., Olivares F., Baldani J., Döbereiner J. (1997) *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue *Sorghum bicolor* L. Moench. Journal of Experimental Botany 48:785-798.

James E.K., Gyaneshwar P., Barraquio W.L., Mathan N., Ladha J.K. (2000) Endophytic diazotrophs associated with rice. In, The Quest for Nitrogen Fixation in Rice. Ladha, J.K., Reddy, P.N. (Eds.), International Rice Research Institute, Makati City, Philippines pp.119-140.

James E.K., Gyaneshwar P., Mathan N., Barraquio W.L., Reddy P.M., Iannetta P.P.M., Olivares F.L., Ladha J.K. (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Molecular Plant-Microbe Interactions 15:894-906.

James K.E., Olivares L.F. (2010) Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Critical Reviews in Plant Sciences 17: 77-119.

Kannaiyan S. (2003) Inoculant production in developing countries-problems, potentials and success. Developments inPlant and Soil Sciences 99:187-198.

Kapulnik Y., Kigel J., Okon Y., Nur I., Henis Y. (1981) Effect of *Azospirillum* inoculation on some growth parameters and N-content of wheat, sorghum and panicum. Plant and Soil 61:65-70.

Kennedy I.R., Choudhury A., Kecskés M.L. (2004a) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. (2004b) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kennedy I.R., Pereg-Gerk L.L., Wood C., Deaker R., Gilchrist K., Katupitiya S. (1997) Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. Plant and Soil 194:65-79. Kennedy I.R., Tchan Y.T. (1992) Biological nitrogen fixation in non-leguminous field crops: Recent advances. Plant and Soil 141:93-118.

Khalid A., Arshad M., Zahir Z.A. (2004b) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology 96:473-480. Kim J., Rees D.C. (1994) Nitrogenase and biological nitrogen fixation. Biochemistry 33:389-397.

Kim S.S., Pham M.A., Kim K.W., Son M.H., Lee K.J. (2010) Effects of microbial fermentation of soybean on growth performances, phosphorus availability, and antioxidant activity in diets for juvenile olive flounder (*Paralichthys olivaceus*). Food Science and Biotechnology 19:1605-1610.

Kimetu J.M., Mugendi D.N., Palm C.A., Mutuo P.K., Gachengo C.N., Bationo A., Nandwa S., Kungu J.B. (2004) Nitrogen fertilizer equivalencies of organics of differing quality and optimum combination with inorganic nitrogen source in Central Kenya. Nutrient Cycling in Agroecosystems 68:127-135..

Klassen G., Pedrosa F.O., Souza E.M., Yates M.G., Rigo L.U. (1999) Sequencing and functional analysis of the nif ENX or f1 or f2 gene cluster of *Herbaspirillum seropedicae*. FEMS Microbiology Letters 181:165-170.

Klee H., Horsch R., Rogers S. (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. Annual Review of Plant Physiology 38:467-486.

Kloepper J.W., Leong J., Teintze M., Schroth M.N. (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886.

Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7:39-44.

Kloepper J.W., Zablotowicz R.M., Tipping E.M., Lifshitz R. (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. In,The Rhizosphere and Plant Growth.

Keister, D.L. and Cregan, P.B. (Eds.), Kluwer Academic Publishers, Netherlands. Pp. 315-326.

Ladha J.K., Reddy P.M. (2003) Nitrogen fixation in rice systems: state of knowledge and future prospects. Plant and Soil 252:151-167.

Lambrecht M., Okon Y., Broek A.V., Vanderleyden J. (2000) Indole-3-acetic acid: a reciprocal signalling molecule in bacteria–plant interactions. Trends in Microbiology 8:298-300.

Letham D.S. (1969) Cytokinins and their relation to other phytohormones. Bioscience 19:309-316.

Lethbridge G., Davidson M. (1983) Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ^{15}N isotope dilution. Soil Biology and Biochemistry 15:365-374.

Lima E., Boddey R.M., Döbereiner J. (1987) Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N aided nitrogen balance. Soil Biology and Biochemistry 19:165-170.

Lin W., Okon Y., Hardy R.W.F. (1983) Enhanced mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Applied and Environmental Microbiology 45:1775-1779.

Loper J.E., Buyer J.S. (1991) Siderophores in microbial interactions on plant surfaces. Molecular Plant-Microbe Interaction 4:5-13.

Lucy M., Reed E., Glick B.R. (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86:1-25.

Mafongoya P.L., Bationo A., Kihara J., Waswa B.S. (2007) Appropriate technologies to replenish soil fertility in southern Africa. In, Advances in Integrated Soil Fertility Management in sub-Saharan Africa: Challenges and Opportunities. Bationo A., Waswa B., Kihara J. and Kimetu J. (Eds.), Springer, Netherlands. pp. 29-43.

Mafuka M.M., M N., Nkasa C., Ibwenzi K., K T. (2007) Effect of combining organic leafy biomass and inorganic fertilizer on tomato yields and nematodes control in Arenosols in Kinshasa area. In, Advances in Integrated Soil Fertility Management in sub-Saharan Africa: Challenges and Opportunities. Bationo A., Waswa B., Kihara J. and Kimetu J. (Eds.), Springer, Netherlands. pp. 359-364.

Martínez-Viveros O., Jorquera M., Crowley D., Gajardo G., Mora M. (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. Journal of Soil Science and Plant Nutrition 10:293-319.

Martinez Toledo M., Gonzalez-Lopez J., Rubia T., Moreno J., Ramos-Cormenzana A. (1988) Effect of inoculation with *Azotobacter chroococcum* on nitrogenase activity of *Zeamays* roots grown in agricultural soils under aseptic and non-sterile conditions. Biology and Fertility of Soils 6:170-173.

Mertens T., Hess D. (1984) Yield increases in spring wheat (*Triticum aestivum* L.) inoculated with *Azospirillum lipoferum* under greenhouse and field conditions of a temperate region. Plant and Soil 82:87-99.

Minchin F., Witty J., Sheehy J., Müller M. (1983) A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. Journal of Experimental Botany 34:641-649.

Minchin F.R., Witty J.F., Mytton L.R. (1994) Reply to 'Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay' by J.K. Vessey. Plant and Soil 158:163-167.

Mirza M.S., Rasul G., Mehnaz S., Ladha J.K., So R.B., Ali S., Malik K.A. (2000) Beneficial effects of inoculated nitrogen-fixing bacteria on rice. Biology and Fertility of Soils 31:191-204.

Monem M.A.S.A., Khalifa H.E., Beider M., El Ghandour I.A., Galal Y.G.M. (2001) Using biofertilizers for maize production: response and economic return under different irrigation treatments. Journal of Sustainable Agriculture 19:41-48.

Monsalud R.G. (2008) Harnessing Microbial Resources for Sustainable Crop Production: The Philippine Experience. Journal of The Faculty of Agriculture Shinsh University 4:29-33.

Motobu M., Amer S., Koyama Y., Hikosaka K., Sameshima T., Yamada M., Nakamura K., Koge K., Kang C.B., Hayasidani H. (2006) Protective effects of sugar cane extract on endotoxic shock in mice. Phytotherapy Research 20:359-363.

Mtambanengwe F., Mapfumo P. (2008) Smallholder farmer management impacts on particulate and labile carbon fractions of granitic sandy soils in Zimbabwe. Nutrient Cycling in Agroecosystems 81:1-15.

Nakayan P., Shen F., Hung M., Young C. (2009) Harnessing Microbial Resources for Sustainable Crop Production. Asian Journal of Food and Agro-Industry Especial Issue, S66-S68.

Neilands J. (1995) Siderophores: structure and function of microbial iron transport compounds. Journal of Biological Chemistry 270:26723-26726.

Nieto K., Frankenberger W. (1991) Influence of adenine, isopentyl alcohol and *Azotobacter chroococcum* on the vegetative growth of *Zea mays*. Plant and Soil 135:213-221.

Niranjan Raj S., Shetty H., Reddy M. (2006) Plant growth promoting rhizobacteria: potential green alternative for plant productivity: In, PGPR: Biocontrol and Biofertilization. Siddiqui,Z. (Ed.). Springer, Netherlands. pp. 197-216.

Nziguheba G., Merckx R., Palm C.A. (2005) Carbon and nitrogen dynamics in a phosphorus-deficient soil amended with organic residues and fertilizers in western Kenya. Biology and Fertility of Soils 41:240-248.

O'Hara G., Davey M., Lucas J. (1981) Effect of inoculation of *Zea mays* with *Azospirillum brasilense* strains under temperate conditions. Canadian Journal of Microbiology 27:871-877.

Odame H. (1997) Biofertilizer in Kenya: Research, production and extension dilemmas. Biotechnology and Development Monitor 30:20-23.

Okon Y. (1985) *Azospirillum* as a potential inoculant for agriculture. Trends in Biotechnology 3:223-228.

Okon Y., Kapulnik Y. (1986) Development and function of *Azospirillum*-inoculated roots. Plant and Soil 90:3-16.

Okon Y., Labandera-Gonzalez C.A. (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry 26:1591-1601.

Pacovsky R., Paul E., Bethlenfalvay G. (1985) Nutrition of sorghum plants fertilized with nitrogen or inoculated with *Azospirillum brasilense*. Plant and Soil 85:145-148.

Pal U., Malik H. (1981) Contribution of *Azospirillum brasilense* to the nitrogen needs of grain sorghum (*Sorghum bicolor* (L) Moench) in humid sub-tropics. Plant and Soil 63:501-504.

Pandey A., Kumar S. (1990) Inhibitory effects of *Azotobacter chroococcum* and *Azospirillum brasilense* on a range of rhizosphere fungi. Indian Journal of Experimental Biology 28:52-54.

Parker C., Scutt P. (1960) The effect of oxygen on nitrogen fixation by *Azotobacter*. Biochimica et Biophysica Acta 38:230-238.

Peoples M., Boddey R., Herridge D., Leigh G. (2002) Quantification of nitrogen fixation. In, Nitrogen Fixation at the Millennium. Leigh, G.J. (Ed.). Elsevier, Amsterdam. pp 357–389.

Peoples M.B., Craswell E.T. (1992) Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. Plant and Soil 141:13-39.

Peoples M.B., Herridge D.F., Ladha J.K. (1995b) Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? Plant and Soil 174:3-28.

Pimentel J., Olivares F., Pitard R., Urquiaga S., Akiba F., Döbereiner J. (1991) Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. Plant and Soil 137:61-65.

Rai M.K. (2006) Handbook of microbial biofertilizers CRC Handbooks, Boca Raton, Florida, USA.

Rai S., Gaur A. (1982) Nitrogen fixation by *Azospirillum* spp. and effect of *Azospirillum lipoferum* on the yield and N-uptake of wheat crop. Plant and Soil 69:233-238.

Rai S., Gaur A. (1988) Characterization of *Azotobacter* spp. and effect of *Azotobacter* and *Azospirillum* as inoculant on the yield and N-uptake of wheat crop. Plant and Soil 109:131-134.

Rao N. (1982) Biofertilizers. Interdisciplinary Science Reviews 7:220-229.

Rao N.S.S., Tilak K.V.B.R., Singh C. (1985) Effect of combined inoculation of *Azospirillum brasilense* and vesicular-arbuscular mycorrhiza on pearl millet (*Pennisetum americanum*). Plant and Soil 84:283-286.

Reinhold-Hurek B., Hurek T., Gillis M., Hoste B., Vancanneyt M., Kersters K., De Ley J. (1993) *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. International Journal of Systematic Bacteriology 43:574-584.

Reinhold B., Hurek T. (1988) Location of diazotrophs in the root interior with special attention to the kallar grass association. Plant and Soil 110:259-268.

Rennie R., deFreitas J., Ruschel A., Vose P. (1983) ^{15}N isotope dilution to quantify dinitrogen (N_2) fixation associated with Canadian and Brazilian wheat. Canadian Journal of Botany 61:1667-1671.

Rennie R., Thomas J. (1987) ^{15}N-Determined effect of inoculation with N_2 fixing bacteria on nitrogen assimilation in Western Canadian wheats. Plant and Soil 100:213-223.

Reynders L., Vlassak K. (1982) Use of *Azospirillum brasilense* as a biofertilizer in intensive wheat cropping. Plant and Soil 66:217-223.

Riggs, P.J., Chelius, M.K., Iniguez, A.L., Kaeppler, S.M. and Triplett, E.W. (2001). Enhanced maize productivity by inoculation with diazotrophic bacteria. Australian Journal of Plant Physiology 28, 829–836.

Robinson D. (2001) ^{15}N as an integrator of the nitrogen cycle. Trends in Ecology and Evolution 16:153-162.

Rodrigues E.P., Rodrigues L.S., de Oliveira A.L.M., Baldani V.L.D., Teixeira K.R.S., Urquiaga S., Reis V.M. (2008) *Azospirillum amazonense* inoculation: effects on growth, yield and N 2 fixation of rice (*Oryza sativa* L.). Plant and Soil 302:249-261.

Rodríguez H., Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17:319-339.

Roper M.M., Ladha J. (1995) Biological N_2 fixation by heterotrophic and phototrophic bacteria in association with straw. Plant and Soil 174:211-224.

Rothballer M., Schmid M., Hartmann A. (2009) Diazotrophic bacterial endophytes in Gramineae and other plants: Prokaryotic symbionts in plants. Microbiology Monographs 8:273-302.

Sáez-Plaza, P., Michałowski, T., Navas, M.J., Asuero, A.G., Wybraniec, S. 2013. An overview of the Kjeldahl method of nitrogen determination. Part I. Early history, chemistry of the procedure, and titrimetric finish. Critical Reviews in Analytical Chemistry 43: 178-223.

Sanchez, P.A. 2002. Soil fertility and hunger in Africa. Science(Washington) 295: 2019-2020.

Sarig S., Kapulnik Y., Nur I., Okon Y. (1984) Response of non-irrigated *Sorghum bicolor* to *Azospirillum* inoculation. Experimental Agriculture 20:59-66.

Schank S., Weier K., MacRae I. (1981) Plant yield and nitrogen content of a digitgrass in response to *Azospirillum* inoculation. Applied and Environmental Microbiology 41:342-345.

Scher F.M., Baker R. (1982) Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. Phytopathology 72:1567-1573.

Sevilla M., Burris R.H., Gunapala N., Kennedy C. (2001) Comparison of benefit to sugarcane plant growth and 15N2 incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and nif mutant strains. Molecular Plant-Microbe Interactions 14:358-366.

Shamseldin A., El-Sheikh M.H., Hassan H., SS K. (2010) Microbial bio-fertilization approaches to improve yield and quality of washington navel orange and reducing the survival of nematode in the soil. Journal of American Science 6:264-271

Siddiqui Z. (2006) PGPR: Prospective biocontrol agents of plant pathogens PGPR: biocontrol and biofertilization. In, PGPR: Biocontrol and Biofertilization. Siddiqui,Z. (Ed.).Springer, Netherlands. pp. 111-142.

Smith R.L., Schank S., Milam J., Baltensperger A. (1984) Responses of *Sorghum* and *Pennisetum* species to the N_2-fixing bacterium *Azospirillum brasilense*. Applied and Environmental Microbiology 47:1331-1336.

Socolofsky M., Wyss O. (1961) Cysts of *Azotobacter*. Journal of Bacteriology 81:946-954.

Sprent J.I. (1979) The Biology of Nitrogen-fixing Organisms. McGraw-Hill Book Co.(UK) Ltd., London.

Stamford N., Santos P., Santos C., Freitas A., Dias S., Lira M. (2007) Agronomic effectiveness of biofertilizers with phosphate rock, sulphur and *Acidithiobacillus* for yam bean grown on a Brazilian tableland acidic soil. Bioresource Technology 98:1311-1318.

Steenhoudt O., Vanderleyden J. (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiology Reviews 24:487-506.

Stein T., Hayen-Schneg N., Fendrik I. (1997) Contribution of BNF by *Azoarcus* sp. BH72 in *Sorghum vulgare*. Soil Biology and Biochemistry 29:969-971.

Stewart W. (1969) Biological and ecological aspects of nitrogen fixation by free-living micro-organisms. Proceedings of the Royal Society of London Series B: Biological Sciences 172:367-388.

Subba Rao N., Tilak K., Singh C. (1985) Synergistic effect of vesicular-arbuscular mycorrhizas and *Azospirillum brasilense* on the growth of barley in pots. Soil Biology and Biochemistry 17:119-121.

Tarrand J.J., Krieg N.R., Döbereiner J. (1978) A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Canadian Journal of Microbiology 24:967-980.

Thiyagarajan T., Backiyavathy M., Savithri P. (2003) Nutrient management for pulses-A Review. Agricultural Reviews-Agricultural Research Communications Center, India 24:40-48.

Triplett E.W. (1996) Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. Plant and Soil 186:29-38.

Unkovich M., Baldock J. (2008) Measurement of asymbiotic N_2fixation in Australian agriculture. Soil Biology and Biochemistry 40:2915-2921.

Vasane S.R., Kothari R. (2006) Optimization of secondary hardening process of banana plantlets (*Musa paradisiaca* L. var. Grand Nain). Indian Journal of Biotechnology 5:394-399.

Venkateswarlu B., Rao A. (1983) Response of pearlmillet to inoculation with different strains of *Azospirillum brasilense*. Plant and Soil 74:379-386.

Vessey J.K. (1994) Measurement of nitrogenase activity in legume root nodules: In defense of the acetylene reduction assay. Plant and Soil 158:151-162. Vessey J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.

Vikram A., Hamzehzarghani H., Alagawadi A., Krishnaraj P., Chandrashekar B. (2007) Production of plant growth promoting substances by phosphate solubilizing bacteria isolated from vertisols. Journal of Plant Sciences 2:326-333.

Vitousek, P.M., D.N. Menge, S.C. Reed and C.C. Cleveland. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philosophical Transactions of the Royal Society B: Biological Sciences 368: 1-9.

Wagner G. (1997) *Azolla*: A review of its biology and utilization. The Botanical Review 63:1-26. DOI: 10.1007/bf02857915.

Wani S., Rupela O., Lee K. (1995) Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes. Plant and Soil 174:29-49.

Wani P.A, Khan, M.S., Zaidi, A. 2007. Co-inoculation of nitrogen fixing and phosphate solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea. Acta Agronomica Hungarica 55:315-323.

Watt G.D., Bulen W.A., Burns A., Hadfield K.L.M. (1975) Stoichiometry, ATP/2e values, and energy requirements for reactions catalyzed by nitrogenase from *Azotobacter vinelandii*. Biochemistry 14:4266-4272.

Weller D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26:379-407.

Wu S.C., Cao Z.H., Li Z.G., Cheung K.C., 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.

Yanni Y., El-Fattah F. (1999) Towards integrated biofertilization management with free living and associative dinitrogen fixers for enhancing rice performance in the Nile delta. Symbiosis 27:319-331.

Yanni Y.G., Rizk R., Corich V., Squartini A., Ninke K., Philip-Hollingsworth S., Orgambide G., De Bruijn F., Stoltzfus J., Buckley D. (1997) Natural endophytic association between *Rhizobium leguminosarum* bv. *trifolii* and rice roots and assessment of its potential to promote rice growth. Plant and Soil 194:99-114.

Yanni Y.G., Rizk R.Y., El-Fattah F.K.A., Squartini A., Corich V., Giacomini A., de Bruijn F., Rademaker J., Maya-Flores J., Ostrom P. (2001) The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots. Functional Plant Biology 28:845-870.

Zhang S., Xu W., Yan Z., Mei R. (1996) Research and commercialization of yield-increasing bacteria (YIB) in China. In, Advances in Biological Control of Plant Diseases. Tang, W.H., Cook, R.J. and Rovira, A.D. (Eds.), China Agricultural University Press, Beijing, China. Pp. 47-53.

CHAPTER TWO
ISOLATION AND *IN VITRO* SCREENING OF DIAZOTROPHIC BACTERIA FOR PLANT GROWTH PROMOTION

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg,

South Africa

Abstract

Diazotrophic bacteria were isolated from rhizosphere soil, roots and leaves of maize collected from Cedara, Greytown and Ukulinga, KwaZulu-Natal, South Africa. The ability of these bacteria to fix nitrogen was confirmed by their ability to grow on a semi-solid nitrogen-free media, an ammonia production test and nitrogenase activity using the Acetylene Reduction Assay (ARA). Bacteria which grew on N-free media with a carbon source (sucrose, D-mannitol or malate) and tested positive for ammonia production were then further tested using the ARA. Ethylene (C_2H_4) production was quantified and ranged from 0 to 73 nmoles of $C_2H_4\,h^{-1}$ culture^{-1}. Isolates that produced ≥40 n mole of $C_2H_4\,h^{-1}$ culture^{-1} were re-screened on maize plants, and 50% of them caused significant ($P < 0.001$) increases of stomatal conductance, dry weight and chlorophyll content index of maize leaves. The rest of these isolates caused no significant ($P > 0.05$) increases in dry matter, stomatal conductance and chlorophyll level compared to an untreated and unfertilized control. Furthermore, the untreated and unfertilized control had the lowest measured parameters, and the untreated and 100% NPK fertilized control had the highest stomatal conductance, chlorophyll level and dry weight. The best eleven isolates were identified, using partial 16s rRNA sequence analysis. Isolates StB5, A3, A6, B1 and A61 showed a 99% similarity with *Pseudomonas* spp., Isolate V9 and A5 showed 97% similarity with *Burkholderia ambifaria,* Isolate L1 94% similarity with *Enterobacter* spp., Isolate V16 97% similarity with *Bacillus megaterium,* Isolate A2 100% similarity with *Klebsiella* spp., and Isolate LB5 100% similarity with *Pantoea* spp. The identification of these isolates was confirmed by MALDI TOF biotype classification. Isolates StB5, A3, A6, B1 and A61 were identified as *Pseudomonas nitroreducens* at score values of 1.98, 1.90, 1.96, 2.03 and 1.88, respectively. Isolates V9 (2.46) and A5 (1.86) were identified as *Burkholderia ambifaria*, Isolate L1 (2.33) as *Enterobacter cloacae,* Isolate V16 (1.72) as

Bacillus megaterium, A2 (2.24) as *Klebsiella variicola* and Isolate LB5 (2.27) as *Pantoea ananatis*.

Key words: diazotrophs, nitrogen fixation, ARA, stomatal conductance, chlorophyll level, MALDI TOF

2.1 Introduction

Biological nitrogen fixation (BNF) is the conversion of atmospheric N_2 to ammonium, a form of N that can be utilized by plants. This is done by certain bacteria (diazotrophs), which contain nitrogenase, the enzyme complex that catalyzes the conversion of N from the gaseous to the combined form. Diazotrophic bacterial are able to grow without external sources of fixed nitrogen but they are dependent on an adequate supply of reduced carbon compounds such as sugars for energy (Bashan *et al.*, 2004). They appear to be physiologically adapted for utilization of specific substrates or classes of substrates (Bagwell and Lovell, 2000). Selective media (N-free semi-solid), which simulate their soil environment, have been used to isolate several bacteria from root rhizosphere. They have been called diazotrophs (Döbereiner, 1988). In many studies, acetylene reduction assay (ARA) is a test that has been used to measure the nitrogenase activity by these diazotrophic bacteria because it is cheap and simple (Boddey and Dobereiner, 1995; Boddey and Knowles, 1987).

Diazotrophs are either free-living, or symbiotic between legumes and rhizobia (Vessey *et al*., 2005). The free-living diazotrophs grow in soils (Döbereiner, 1992a), rhizosphere soils (Martin *et al*., 1989; Döbereiner, 1992a; Dobbelaere *et al*., 2003; Vessey, 2003), the rhizoplane (Bagwell and Lovell, 2000; Vessey, 2003) or can be found within plant tissues (endophytic) (Olivares *et al*., 1996a; Palus *et al*., 1996; Reinhold-Hurek and Hurek, 1998; Roesch *et al*., 2008). Diazotrophic bacterial species and strains belonging to genera such as *Acetobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Derxia, Enterobacter, Burkholderia, Herbaspirillum* and *Pseudomonas* have all been isolated from the rhizosphere of various crops (Glick, 1995; Barraquio *et al*., 1997; James *et al*., 2000; Dobbelaere *et al*., 2003). *Azospirillum* spp. are considered to be rhizospheric (growing close to or on root surfaces) (Kennedy and Tchan, 1992). Some *Azospirillum* strains can also be endophytic, being found within the roots of some graminaceae (Cocking, 2009). *Azotobacter* are colonists of the rhizoplane (Kennedy *et al*., 1997). Genera such as *Herbaspirillum*, *Ideonella* and *Klebsiella* appeared to be rare in soil but dominant in the interior of plants (Roesch *et al*., 2008). Their ability to colonize different ecological niches, together with their

competitive advantages under conditions of inadequate carbon substrates and N-deficiency situations, make them economically important in agriculture and may be used as biofertilizer inoculants for improving crop yields (Urquiaga *et al*., 1992).

Rhizospheric diazotrophs are competitive with the soil micro-flora for C substrates and release fixed N to the plant only after their death (Rao *et al*., 1998; Dobbelaere *et al*., 2003). Genera of *Azospirillum* and *Azotobacter* have been widely studied. For example: when corn seeds were inoculated with *Azospirillum brasilense* corrig. Tarrand *et al*.**, s**hoot dry weight of corn increased by 20 to 30% (Lin *et al*., 1983). In another experiments by Bashan *et al*. (1989), inoculation of several plants with *A. brasilense* resulted in increases in plant dry weight and yield. Sarig *et al*. (1988) also reported that same species caused a 15-18% increase in grain yield of sorghum and that it increased yields of cereal and forage grasses (Okon, 1985). Many researchers believe that the positive effect of these bacterial species on non-leguminous plant yields may not only be due to nitrogen fixation but also from stimulating plant growth by producing active compounds, such as phytohormones and vitamins (Kapulnik *et al.*, 1981; Okon, 1985; Boddey *et al.*, 1986; Caballero-Mellado *et al.*, 1992; Dobbelaere *et al.*, 2003).

Herbaspirillum seropedicae Baldani *et al*., *H. rubrisubalbicans* Christopher and Edgerton Baldani *et al*. and *Acetobacter diazotrophicus* Gillis *et al*. are recognized diazotrophic plant endophytes. They colonize roots, stems, and leaves of various graminaceous plants (Baldani *et al*., 1986; Urquiaga *et al*., 1992; Dong *et al*., 1994; Olivares *et al*., 1996a) and are able to fix nitrogen. It is believed that some of these endophytic diazotrophic bacteria contribute substantial amounts of N to certain graminaceous crops (Barraquio *et al*., 1997; Boddey *et al*., 1991). As they are uniformly distributed within plant tissues in a protective environment (Urquiaga *et al*., 1992), they can fix N in plants and transfer the fixed N products to their hosts. Brazilian varieties of sugarcane are capable of obtaining over 60% of their nitrogen from BNF (Boddey *et al*., 1995b). Döbereiner (1992b) and Boddey *et al*. (2003) also suggested that *Herbaspirillum* spp. may be responsible for replacing N fertilizer by BNF in Brazilian varieties of sugarcane. Similarly, Fujii *et al*. (1987) reported that inoculation of rice with endophytic diazotrophic bacteria such as *Klebsiella oxytoca* (Flugge) Lautrop and *Enterobacter cloacae* (Jordan) Hormaeche increased dry weight and fixed N of inoculated rice plants.

The aims of this study were to isolate nitrogen fixing bacteria using N-free semi-solid media with different carbon sources, evaluate them for nitrogenase activities, screen them for any beneficial effects on plant parameters, and to identify them to the species level.

2.2 Methods and Materials

2.2.1 Bacterial Isolation

Diazotrophic bacteria were isolated from soil rhizospheres, roots and leaves of maize plants collected from Cedara (Agricultural research collage, Hawick), Greytown, and Ukulinga (University of KawaZulu –Natal Research Farm, Pietermaritzburg), (KwaZulu-Natal, Republic of South Africa). Roots and leaves were surface sterilized with 3.5% sodium hypochlorite for five minutes and subsequently rinsed three times with sterile distilled water, using a modified protocol of Kloepper *et al*. (1991). Roots and leaves were cut into pieces and grounded with 10 mℓ of distilled water. A modified protocol of Döbereiner (1988) (N-free semi-solid media) was used to isolate rhizospheric, rhizoplane and endophytic diazotrophs. Pure cultures of diazotrophic bacteria were then isolated by serial dilution, and plated onto an N-Free (NF) media containing of either 20g $\ell^{-1}$of mannitol, sucrose or malate as the carbon source; 0.2 g ℓ^{-1} K_2HPO_4; 0.2 g ℓ^{-1}NaCl; 0.2 g ℓ^{-1} ; $MgSO_4.7H_2O$; 0.1 g $\ell^{-1;}$ K_2SO_4; 5.0 g ℓ^{-1} $CaCO_3$; 20 g ℓ^{-1} agar (Merck) for a solid agar medium, or with 5 g of agar per liter for a semi-solid medium. These bacteria were incubated at 30°C for 4 days.

Soil samples were collected from the rhizosphere of maize and wheat from different sites by uprooting the root system and placing them in plastic bags for transport to the laboratory. They were stored at 4 C for subsequent analysis. Excess soil was shaken off and the soil adhering to the plant roots was collected from each soil sample. Ten grams of each soil sample were transferred to a 250 m-Erlenmeyer flask containing 90 mℓ sterile distilled water and shaken at 150 rpm in an orbital shaker incubator for 30 minutes. Plates with NF medium Mannitol as a Carbon source for diazotrophic bacteria were inoculated with 0.1 mℓ of suspensions obtained from the above dilution procedure (3 replicates per dilution). The pH was adjusted to 6.5 using 98% Sulfuric acid and 50% Sodium hydroxide. After five days of incubation, colonies were transferred onto a fresh N-free media, and after 2 days were streaked out onto Tryptone Soy Agar (TSA) (Merck) plates. Bacterial isolates were selected by size and shape of colony and by their ability to grow on N-free media. These colonies were sub-cultured onto TSA and incubated at 30°C, purified and stored in 15% glycerol at -80°C.

2.2.2 Ammonia Production Test

Ammonia production was analyzed using the qualitative method of Ahmad *et al*. (2008). Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10mℓ peptone water in each tube and incubated for 48–72 hrs at 28 ± 2^0C. Nessler's reagent (0.5 mℓ) was added in each tube. Development of a brown to yellow colour was a positive test for ammonia production.

2.2.3 Acetylene Reduction Assay (ARA)

The basis for the assay is the fact that nitrogenase, the enzyme complex in diazotrophic microorganisms that reduces nitrogen to ammonia, also reduces acetylene to ethylene. Ninety three bacterial diazotrophs were isolated from soil, roots and leaves using an N-free semi-liquid medium, mannitol, sucrose and malate were used as carbon sources. Isolation of pure cultures was obtained after several transfer onto N-free agar media incubated at 28 ± 2^0C for 5-7 days. One mℓof pure culture grown in Tryptone Soy Broth (TSB) for 24 hrs were inoculated onto 10 mℓ of nitrogen-free semi-liquid medium, with 0.5% mannitol as a carbon source, solidified by 0.3% gellan gum in 20ml serum bottles and closed with a red rubber septum (SIGMA-ALDRICH, William Freeman and Co., Ltd.) and incubated for 72 hrs at 28^0 C. Bottles that showed bacterial growth were assayed for acetylene reduction. Ten percent of the atmosphere in the bottles was replaced with acetylene (C_2H_2), whereas bottles without acetylene were used as the control. After 2hrs, at 24^0 C, 0.25 mℓ gaseous samples from each bottle were removed and analyzed for ethylene with a Hewlett-Packard 5830 A gas chromatograph fitted with a 2m - 2.1mm, 80 - 100 mesh, Poropak R column. Oven temperature was adjusted to 70^0 C. Injection and flame-ionization detector temperatures were adjusted to 150^0 C. Nitrogen carrier gas flow rate was adjusted to 50mℓ min^{-1}.

2.2.4 Source of Seeds

Seeds of white maize of the cultivar, Mac's Medium Pearl, (an open pollinated variety) were bought from McDonalds Seeds®[1] and were used throughout the experiment.

[1]MacDonald's Seeds (Ltd).P.O. Box 40, Mkondeni, 3212, Pietermaritzburg, Republic of South Africa

2.2.5 Bacterial Inoculation

2.2.5.1 Inoculum Preparation

Bacterial isolates were grown in 100 mℓ Erlenmeyer flasks each containing a 25 mℓ Tryptic Soy Broth (TSB) (Merck) for 3 d at 28 ± 2°C in a shaker at 150 rpm. Flasks were inoculated with bacteria previously grown in TSA for 48 hrs. After 3 d bacteria were harvested by centrifugation using a Beckman J2-HS Centrifuge[2] at 9000 rpm for 15 mints. The broth was decanted and bacterial pellets were re-suspended in sterile distilled water. Bacterial cells were then counted using a plate dilution technique on TSA plates, and adjusted to a concentration of 10^8 colony forming unit (c.u). $m\ell^{-1}$ of water.

2.2.5.2 Seed Treatment

Twenty out of ninety bacterial isolates which produced relatively high C_2H_4 levels were selected for further greenhouse screening for N-fixation and growth promotion. Maize seeds were treated with bacterial isolates at a concentration of $2.4x10^8$ cfu and dried at room temperature overnight. Treated maize seeds were planted on pine bark artificial growing medium (Table 2.1).

[2]Beckman Coulter Inc. 4300 N Harbour Boulevard, Box 3100, Fullerton, California, 92834-300., USA

Table 2.1 Composted Pine Bark Growing Medium Analysis (KwaZulu-Natal Agriculture and Environmental Affairs[3])

Sample	Pine bark
C%	12.39
S%	0.11
N%	0.43
Ca%	0.61
Mg%	0.10
K%	0.28
P%	0.18
Moisture %	25.07
Na mg kg^{-1}	405.0
Zn mg kg^{-1}	73.0
Cu mg kg^{-1}	16.7
Mn mg kg^{-1}	802
Fe mg kg^{-1}	12293
Al mg kg^{-1}	7120

2.2.6 Measurements of Stomatal Conductance and Chlorophyll Index

Greenhouse measurements of stomatal conductance and leaf chlorophyll were made on six to eight weeks old seedlings. Leaf stomatal conductance was measured with a portable meter (SC-1 Leaf Porometer, Decagon Devices, Inc.)[4]. Measurements of stomatal conductance were made between 9:00am- 3:00pm on sunny days on 8 to 10 leaves of each maize plant on five plants per treatment. Chlorophyll was measured using a portable, handheld device called chlorophyll meter[5] (that estimates the chlorophyll content of leaves). Measurements were made on 8 to 10 leaves on each of fifteen maize plants per treatment.

[3]KZN Agriculture and Environmental Affairs, Private Bag X9059, Pietermaritzburg, 3200, Republic of South Africa

[4]SC-1 Leaf Porometer, Decagon Devices, Inc., 2365 NE Hopkins Court, Pullman, WA 99163 – USA

[5]CCM-200 Plus, Opti-Science Inc., 8 Winn Avenue, Hudson, NH, USA, 03051.

2.2.7 Bacterial Identification

2.2.7.1 DNA Extraction and 16S rRNA Sequence Analysis

One mℓ of 24 hrs bacterial culture was centrifuged at 14,000 rcf (relative centrifugal force (rcf) for 5 mins. The pellet was suspended in 25µℓ of (10 mM) Tris and one mℓ of buffer was added and incubated at 60^0C for 1h. One ml of a second buffer (CTAB) was added and gently mixed. Then the bacterial-buffer suspension were divided into two in 1.5 ml tubes, and 500 µℓ of chloroform-iso-amyl alcohol was added and mixed gently, and the resultant mixture was centrifuged at 14,000 rcf for 10 mints. By avoiding the layer of impurities, 900 µℓ of clear supernatant was removed as the sample. To this was added 600 µℓ of propan-2-ol and refrigerated at -20^0C for 1h. It was centrifuged for 15 mints at 4,000 rcf and the supernatant was discarded. The pellet were washed with 50 µℓ of 70% ethanol solution and dried in a laminar flow with lid of the tube being left open for 30 mints. The pellet was then suspended in 50 µℓ of 10mM Tris (pH 8) or 0.5 X TE buffer. At this point the DNA purity and quality were checked on the Nanodrop UV spectrophotometer equipment (Nanodrop 1000, Inqaba Biotech)[6] and a 5 µl sample was run on 0.8% agarose gel (SeakemLE Agarose, Whitehead Scientific (Pty) Ltd www.whitesci.co.za)) stained with SYBRSafe Nucleic Acid Stain (Invitrogen), with a GeneRuler 1 kb DNA Ladder Plus molecular weight marker (Thermo-Fisher)[7] to confirm the presence, size and quality of genomic DNA. Once the purity of the DNA was checked, it was sent to the Central Analytic Facility, Stellenbosch University for sequencing and BLAST identification. The BLAST identifications were then confirmed by Matrix Assisted Laser Desorption Ionization-Time of Flight (Maldi-TOF) classification (Brucker Daltonik Maldi-TOF Biotyper (www.bruker.com)).

2.2.7.2 Bruker Daltonik MALDI Biotyper Classification

Bacterial cultures were sub-cultured on 10 % TSA for 24 hrs at 30^0C. A single bacterial colony were taken and placed into a 2 ml Eppendorf tube with 300 µℓ of ultra-pure water, and 900 µℓ of pure ethanol were added, mixed and the suspension was centrifuged at 14,000 rcf for 2 mints. A small pellet of bacterial cells was visible at the bottom of the tube. The liquid was removed, and the pellet was briefly re-spun followed by the removal of residual ethanol. It was then re-suspended in 10 µℓ of 70% formic acid, and 10 µℓ of

[6] Inqaba Biotec, P.O.Box 1435, Hatfield 0028, Pretoria, South Africa

[7] Thermo Fisher Scientific Inc., 81 Wyman street, Waltham, MA 02454, US

acetonitrile was added, and the sample was vortexed briefly. The mixture was centrifuged for 2 min at 14,000 rcf, and the supernatant transferred into a clean micro-tube. The sample to be analyzed was warmed to room temperature, and a 1 µℓ sample was spotted onto a steel target plate (Bruker Daltonics Inc., Billerica, MA, USA) and gently mixed with 2 µℓ of matrix solution.

2.2.8 Experimental Design

Maize plants treated with bacterial isolates were watered every other day with 500 mℓ nutrient solution containing: 0.11mℓ ℓ^{-1} H_3PO_4; 0.13 gℓ^{-1} KOH; 0.14 g ℓ^{-1} K_2SO_4; 0.74 gℓ^{-1} $CaCl_2.2H_2O$; 0.10 g ℓ^{-1} $MgSO_4.7H_2O$ and 0.02 g ℓ^{-1} of micronutrients (MICROPLEX®). There were two controls: one was untreated and supplemented with a complete fertilizer solution (NPK soluble fertilizer [3:1:3(38)] at a rate of 1g ℓ^{-1}); and the second was untreated and unfertilized (Control-none). Plants were supplied 500 mℓ of water or hydroponic fertilizer every second day. Each treatment consisted of three pots with a top diameter of 200 mm that held 2kg of composted pine bark. Each pot was seeded with five seeds. Pots with each of the five isolates were watered daily with an equal amount of a nutrient solution of hydroponics soluble fertilizer containing in g ℓ^{-1} of water NPK, [3:1:3 (38) Complet®], 0.25, micronutrients (Microplex), 0.02 (Ocean Agriculture, Mulder's Drift, South Africa)7, with phosphorus and potassium levels adjusted to the full amounts recommended for each crop. The Un-treated Control and not fertilized (control) was watered with tap water and the Fully Fertilized Control (100% NPK) with a solution of NPK, [3:1:3 (38) Complete®) at a rate of 1g ℓ^{-1} w/v).The seedlings were thinned to three plants per pot.

The experiment was arranged in a randomized complete block design (RCBD), replicated three times. Two months after planting chlorophyll and stomatal conductance were measured and plants were harvested and dry weight was taken after the biomass was dried in an oven for 72 hrs at 70°C.

2.2.9 Statistical Analysis

Experiments were repeated twice, unless otherwise stated. Data was analyzed using GenStat® Executable release 14th Edition Statistical Analysis Software. Significant differences between treatments were determined using Duncan Multiple Range Test at 5% siginificant level.

2.3 Results

2.3.1 Isolation and Preliminary Screening of Bacterial Diazotrophs

There were differences between diazotrophic isolates, in their ability to grow on semi-solid N-free medium, and using D-mannitol, D-malate or sucrose as carbon sources (Table 2.2). All these isolates were able to grow well on the N-free semi-liquid medium when sucrose were used as the carbon source (Table 2.2), and generated ammonia. About 20% of the bacterial isolates grew well on N-free media with D-mannitol, sucrose or malate as a growth substrate. Approximately, 80% of the bacterial isolates showed slow growth on N-free medium with D-mannitol or malate after 5-7d of incubation period at 28 ± 2^0C. Growth rate on preliminary screening of bacterial diazotrophs are presented in Table 2.2.

2.3.2 Preliminary Screening of Bacterial Diazotrophs for Nitrogenase Activity

The diazotrophic nature of all the recovered isolates was determined by ARA. Ethylene was quantified by gas chromatography (GC) and the results were expressed in nano moles of C_2H_4 produced h^{-1} $culture^{-1}$. All the isolates exhibited nitrogenase activity, but the level of activity varied with different isolates (Table 2.3). Approximately 17% of the isolates produced very little C_2H_4, no more than the Control; 66% of the isolates produced significantly ($P < 0.001$). Higher C_2H_4 compared to the Control (N-free semi-liquid medium). Only 20% of the isolates produced highly significant levels of C_2H_4 (40-73 nmoles of C_2H_4 $h^{-1}culture^{-1}$) ($P < 0.001$).

Table 2.2 Growth of bacterial isolates on N-free media with three different carbon source and screening for NH_3 production

Isolates	mannitol	sucrose	Malate	NH_3 test	Treatments	mannitol	sucrose	malate	NH_3 test
Bt10	slow	well	Slow	+	Mr55	slow	well	well	+
Bt14	slow	well	Slow	+	V13	well	well	slow	+
Bt3	slow	well	Slow	+	V4	well	well	slow	+
M11	slow	well	Slow	+	V6	well	well	slow	+
SB1	slow	well	Slow	+	V7	well	well	slow	+
Mr25	slow	well	Slow	+	LB9	slow	well	well	+
Mr53	slow	well	Slow	+	Mr23	slow	well	slow	+
Mr150	slow	well	Slow	+	Mr19	slow	well	slow	+
Mr55	slow	well	slow	+	x	slow	well	well	+
Mr121	slow	well	well	+	Mr148	slow	well	slow	+
Mr2	slow	well	slow	+	Mr17	slow	well	slow	+
Mr20	slow	well	slow	+	D6	well	well	slow	+
Mr8	slow	well	well	+	M9	well	well	slow	+
Mr37	slow	well	well	+	Mr9	slow	well	well	+
Mr141	slow	well	well	+	V14	well	well	slow	+
Mr35	slow	well	well	+	V3	well	well	slow	+
Mr63	slow	well	well	+	Mr34	slow	well	slow	+
V15	well	well	slow	+	Mr22	slow	well	slow	+
StB3	well	well	slow	+	Bt7	slow	well	well	+
E9	well	well	slow	+	Mr54	slow	well	slow	+
LB2	well	well	slow	+	Mr27	slow	well	well	+
Mr37	well	well	slow	+	V17	slow	well	well	+
V18	well	well	slow	+	Bt4	slow	well	well	+
Bt2	well	well	slow	+	Bt12	slow	well	slow	+

(+) = positive test for ammonia production; slow = poor bacterial growth media; fast = well growth on the media with different carbon sources

Bacterial growth were considered slow when the mass doubling time was longer than 10-12 hrs and there were few number of visible colonies on plate against incubation time

Table 2.2 Continued Growth of bacterial isolates on N-free media with three different carbon source and screening for NH_3 production

Isolates	Mannitol	sucrose	malate	NH_3 test	Treatments	mannitol	sucrose	malate	NH_3 test
M12	well	well	slow	+	G3	slow	well	well	+
StB12	slow	well	well	+	V11	slow	well	well	+
V1	slow	well	slow	+	StB8	slow	well	slow	+
Br2	well	well	slow	+	Mr131	well	well	slow	+
Bt1	well	well	slow	+	Bt13	well	well	well	+
Mr16	well	well	slow	+	Mr105	well	well	well	+
RB1	well	well	slow	+	StB1	well	well	well	+
Bt15	well	well	slow	+	StB13	well	well	well	+
Mr6	slow	well	well	+	Bt5	well	well	well	+
RB6	well	well	slow	+	V20	well	well	well	+
V12	slow	well	well	+	V8	well	well	well	+
Mr13	slow	well	slow	+	A61	well	well	well	+
RB2	slow	well	well	+	A2	well	well	well	+
StB7	slow	well	well	+	B1	well	well	well	+
LB7	slow	well	well	+	A6	well	well	well	+
Mr7	slow	well	slow	+	A5	well	well	well	+
Bt9	well	well	slow	+	A3	well	well	well	+
Bt6	well	well	slow	+	LB5	well	well	well	+
Bt8	slow	well	well	+	L1	well	well	well	+
V2	well	well	slow	+	StB5	well	well	well	+
StB17	slow	well	slow	+	V16	well	well	well	+
V5	slow	well	slow	+	V9	well	well	well	+

.

Table 2.3 Nitrogenase activity measured (by the Acetylene Reduction Assay (ARA) method of the bacterial isolates

Isolates	nmol of C_2H_4 h^{-1} $culture^{-1}$		Isolates	nmol of C_2H_4 h^{-1} $culture^{-1}$		Isolates	nmol of C_2H_4 h^{-1} $culture^{-1}$	
Broth	0.32	a	M12	21.86	cdefghijklmn	Bt12	29.01	ghijklmnop
Bt14	4.81	ab	StB12	21.91	cdefghijklmn	Bt7	29.46	ghijklmnop
M11	6.2	ab	V1	21.91	cdefghijklmn	StB8	29.87	ghijklmnop
Mr2	6.24	ab	Br2	21.96	cdefghijklmn	G3	31.78	hijklmnopq
SB1	6.46	ab	Bt1	21.96	cdefghijklmn	V3	31.99	ijklmnopq
Mr63	6.51	ab	RB1	21.96	cdefghijklmn	Mr27	32.27	ijklmnopq
Bt10	7.29	abc	Bt15	22.01	cdefghijklmn	V14	32.39	ijklmnopqr
Bt3	7.32	abc	RB6	22.01	cdefghijklmn	Mr57	32.77	jklmnopqr
Mr141	7.61	abc	V12	22.01	cdefghijklmn	Mr34	33.04	klmnopqr
Mr20	7.90	abc	Mr148	22.02	cdefghijklmn	Bt4	33.26	klmnopqr
Mr25	9.10	abcd	RB2	22.06	cdefghijklmn	Bt5	33.49	lmnopqr
Mr8	9.76	abcde	StB7	22.06	cdefghijklmn	V20	33.58	lmnopqr
StB3	10.46	abcde	LB7	22.11	cdefghijklmn	Bt11	33.69	lmnopqr
V15	10.96	abcde	Bt9	22.15	cdefghijklmn	Mr21	33.94	mnopqrs
Mr53	12.26	abcdef	Bt6	22.20	cdefghijklmn	StB13	35.09	nopqrst
Mr35	12.29	abcdef	Bt8	22.20	cdefghijklmn	V10	35.33	nopqrst
E9	12.66	abcdef	V2	22.20	cdefghijklmn	Mr150	35.54	nopqrstu
Mr38	15.40	bcdefg	StB17	22.30	cdefghijklmn	Mr131	37.13	nopqrstu
Mr58	15.96	bcdefg	V6	22.40	cdefghijklmn	StB1	37.23	nopqrstu
Mr6	16.44	bcdefgh	M9	22.41	cdefghijklmn	Mr37	38.82	opqrstuv
V5	17.02	bcdefghi	V7	22.45	cdefghijklmn	A2	42.00	pqrstuv
Mr16	17.22	bcdefghi	LB9	22.55	cdefghijklmn	A3	45.10	qrstuv
V4	17.25	bcdefghij	x	22.80	cdefghijklmn	B1	45.84	qrstuv
Mr19	17.32	bcdefghij	D6	24.19	defghijklmno	A61	46.69	qrstuv
Mr13	17.38	bcdefghij	Mr17	24.19	defghijklmno	A5	47.28	rstuv
Mr7	17.53	bcdefghij	Mr22	24.73	efghijklmno	LB5	48.36	stuv
Mr121	17.76	bcdefghijk	Mr9	25.09	efghijklmno	L1	48.75	tuv
Mr55	18.22	bcdefghijkl	V18	25.20	efghijklmno	A6	49.88	uv
LB2	18.75	bcdefghijklm	V17	27.48	fghijklmnop	StB5	52.37	v
V13	19.05	bcdefghijklm	V19	28.40	ghijklmnop	V16	65.15	w
Bt2	21.86	cdefghijklmn	V11	28.82	ghijklmnop	V9	73.20	w
CV%	30.4							
DMRT	12.035							
Sed	6.101							
F-test	9.662							
P-value	<0.001							

Means with the same letter in the same column are not significantly different at $P \leq 0.05$

2.3.3 Secondary Screening of Diazotrophic Bacteria for Nitrogen Fixation, Using Plant Growth Parameters

Maize plants were inoculated with 20 different nitrogen fixing bacterial isolates. Stomatal conductance, chlorophyll content index of the leaves and dry weight of each plant were measured. Of the twenty diazotrophic isolates, 50% of the induced high siginificant increases ($P < 0.001$) in the maize leaf chlorophyll level, stomatal conductance and dry weight, relative to the Untreated and Unfertilized Control (Table 2.4). The rest of these isolates had no effect ($P < 0.05$) on dry matter, stomatal conductance and chlorophyll level compared to the Untreated and Unfertilized Control. Plants of the Untreated and Unfertilized Control (Control-none) showed the lowest stomatal conductance, chlorophyll level and dry weight. As expected, plants of the 100%NPK fertilized (NPK) Control had the highest stomatal conductance, chlorophyll level and dry weight (Table 2.4). Based on chlorophyll level measurements, Isolate StB5 contributed 59%, Isolate V9 56.7%, Isolate V16 56.5%, Isolate L1 53.3%, LB5 52.2%, Isolate A3 51.8%, Isolate A5 51.6%, Isolate A6 49.3%, Isolate B1 47.8%, and Isolate A2 46.8% to the index of the chlorophyll levels of maize plants.

Table 2.4 *In vivo* screening of diazotrophic bacteria for nitrogen fixation

Bacterial Isolates	Chlorophyll content index (CCI)		Dry weight(g)		Stomatal conductance (m mol m^{-2} s^{-1})	
Control	4.45	a	2.77	a	54.2 (1.733)	a
StB8	5.08	ab	3.63	ab	121.6 (2.085)	abc
Mr131	5.24	abc	3.62	ab	122.5 (2.086)	abc
Bt13	5.38	abcd	3.63	ab	122.5 (2.087)	abc
Mr105	5.59	abcde	4.13	abcd	142.0 (2.152)	abcd
StB1	5.83	abcde	5.17	bcd	137.7 (2.127)	abc
StB13	5.95	abcdef	3.79	abc	130.3 (2.115)	abc
Bt5	6.08	abcdef	3.72	abc	147.3 (2.165)	abcd
V20	6.11	abcdef	5.85	cd	147.6 (2.169)	abcd
V8	6.21	abcdefg	5.79	cd	134.6 (2.129)	abc
A61	6.28	bcdefg	6.23	d	206.5 (2.295)	cde
A2	6.37	bcdefg	5.84	cd	198.3 (2.274)	cde
B1	6.52	bcdefg	3.80	abc	244.1 (2.383)	de
A6	6.71	bcdefg	3.41	ab	218.1 (2.335)	cde
A5	7.03	cdefg	2.42	a	188.8 (2.253)	bcde
A3	7.06	cdefg	5.26	bcd	219.3 (2.341)	cde
LB5	7.11	defg	5.51	bcd	203.4 (2.278)	cde
L1	7.26	efg	5.85	cd	206.2 (2.281)	cde
V16	7.70	fg	5.99	d	265.0 (2.423)	e
V9	7.72	fg	2.80	a	253.3 (2.404)	e
StB5	8.03	g	3.48	ab	244.7 (2.386)	de
NPK	13.62	h	9.90	e	517.5 (2.712)	f
CV%	14		23.90		23.20(4.2)	
DMRT	1.544		1.833		90.41(0.198)	
SED	0.765		0.908		43.00(0.093)	
P	<0.001		<0.001		<0.001	

Means with the same letter in the same column are not significantly different at $P < 0.05$; values in parenthesis are transformed data using log base 10 for the stomatal conductance

2.3.4 Identification of Diazotrophic Isolates

Comparative analyses of nucleotide sequences of amplified 16S rRNA fragments, using a BLAST approach, revealed that Isolates StB5, A3, A6, B1 and A61 exhibited sequence similarities of 100% with *Pseudomonas* spp. Isolate V9 and A5 showed a 97% similarity with *Burkholderia ambifaria*. Isolate L1 had a 94% similarity with *Enterobacter* spp.; Isolate V16 had a 97% similarity with *Bacillus megaterium;* Isolate A2 had a 100% similarity with *Klebsiella spp*.; and Isolate LB5 had a 100% similarity with *Pantoea* spp. (Table 2.5).

With this system, a score of ≥2.000 indicates species level identification, a score of 1.700 to 1.999 indicates identification to the genus level, and a score of <1.700 is interpreted as no identification. Isolates StB5, A3, A6, B1 and A61 were identified as *Pseudomonas nitroreducens* with MALDI-

TOF scores of 1.98, 1.90, 1.96, 2.03 and 1.88, respectively. Isolates V9 (2.46) and A5 (1.86) were identified as *Burkholderia ambifaria*, Isolate L1 (2.33) as *Enterobacter cloacae*, Isolate V16 (1.72) as *Bacillus megaterium*, A2 (2.24) as *Klebsiella variicola* and Isolate LB5 (2.27) as *Pantoea ananatis*. An independent identification of these isolates, based on 16S rRNA gene and Maldi-TOF Biotyper, confirmed their identity.

Table 2.5 Affiliation of the isolates in the GenBank and the identification of the closest type strain based on the 16S rRNA gene sequencing and Bruker Daltonik MALDI-TOF Biotyper classification

Isolates	**16S rRNA similarities (highest match)**	**BrukerMALDI Biotype (highest score)**
V16	*Bacillus megaterium* Strain As-30 (97%)	*Bacillus megaterium* (1.722)
A5	*Burkholderia* sp. IBP-VNS127 (99%)	*Burkholderia ambifaria* (1.867)
V9	*Burkholderia ambifaria* (99%)	*Burkholderia ambifaria* (2.462)
L1	*Enterobacter cloacae* Strain G35-1(98%)	*Enterobacter cloacae* (2.327)
A2	*Klebsiella variicola* (99%)	*Klebsiella variicola* (2.243)
LB5	*Pantoea ananatis* (97%)	*Pantoea ananatis* (2.268)
A3	*Pseudomonas nitroreducens* Strain R5-791 (99%)	*Pseudomonas nitroreducens* (1.901)
A6	*Pseudomonas nitroreducens*. (99%)	*Pseudomonas nitroreducens* (1.96)
B1	*Pseudomonas nitroreducens*. (99%)	*Pseudomonas nitroreducens* (2.034)
StB5	*Pseudomonas nitroreducens* Strain R5-791 (99%)	*Pseudomonas nitroreducens* (1.989)
A61	*Pseudomonas nitroreducens*. (99%)	*Pseudomonas nitroreducens* (1.882)

2.4 Discussion

Isolation and screening for potential diazotrophic bacteria are crucial steps in research on biofertilizers, in order to discover efficient nitrogen fixing bacteria. There is a need to develop simple, inexpensive and quick procedures with repeatable and reliable results (Ahmad *et al*., 2006; Döbereiner, 1988). For instance, an in *vitro* screening procedure (growth on N-free semi-solid media, ARA and the ammonia production test), the combination of which provides rapid, repeatable results. Bacterial isolates were selected based on their growth behavior in a nitrogen-free semi-solid medium typified by ammonia production analyses of liquid cultures, which confirmed their capacity to fix N_2 in pure culture. All these isolates were able to grow well on an

N-free semi-liquid medium when sucrose was used as carbon source but grew slowly on D-mannitol and malate.

Single colonies that grew well and produced ammonia in N-free liquid medium were then tested in an acetylene reduction assay. Acetylene reduction values ranged from 4.81 to73.0 nmoles of ethylene produced h^{-1} $culture^{-1}$. It is difficult to compare the nitrogenase activity of bacterial strains studied in this work with the results obtained by others, mainly due to the different methods used and the different ways of expressing the levels of nitrogen fixation. These results on nitrogenase activity were in agreement with the results of Różycki *et al*. (1999) who reported similar nitrogenase activity of diazotrophic bacteria, most of which belonged to the genera *Pseudomonas* and *Bacillus*. However, it is difficult to extrapolate data from acetylene reduction assays to the actual dinitrogen fixation because this assay only measures nitrogenase activity and reveals no information on whether the fixed N can be incorporate into plants (Boddey *et al*., 1995a).

In this study, 50% of the tested isolates induced 50% to 60% increases in dry weight, stomatal conductance and chlorophyll content index compared to untreated and unfertilized maize plants. These increases were due to the inoculation of these diazotrophic isolates and strongly support our hypothesis that inoculation with diazotrophic bacteria may be beneficial in enhancing plant growth. However, water stress produced quite large reductions in the content of chlorophyll and stomatal conductance rate. Therefore, water stress in plants should be avoided by daily watering on the previous day prior to measuring. Additionally, stomatal conductance recovery was affected by direct sunlight. This suggests that the recovery should be done during the middle portion of the day, between 09:00 to 15:00.

About 50% of these isolates were identified as *Pseudomonas* spp. The predominance of this genus both in the soil and in the root zone may be due to low nutritional requirements, its capacity to utilize numerous complex organic substrates (Krotzky and Werner, 1987) and its tolerance to low pH (Eckford *et al*., 2002). Nitrogenase active members of this genus have also been isolated by other researchers (Vermeiren *et al*., 1999; Mano and Morisaki, 2008;). One of the selected isolates was identified as *Pantoea ananatis* which had been isolated from the leaves

and roots of healthy plants, yet it was capable of nitrogen fixing and caused no apparently harmful effects to treated plants.

The genus *Burkholderia* is well known because it has strains that cause diseases in humans (Bevivino *et al*., 1994; Miralles *et al*., 2004; Chiarini *et al*., 2006; Mendes *et al*., 2007; Jacobs *et al*., 2008). In this study two nitrogen fixing *Burkholderia* spp. were isolated from maize rhizospheres and showed no harmful effect on treated plants. In another studies, Estrada *et al*. (2002) isolated a strain of endophytic, N_2-fixing *Burkholderia* sp. associated with maize in Mexico. Perin *et al*. (2006) also recovered N_2-fixing *Burkholderia* from the rhizosphere of maize and from surface-sterilized leaves of sugarcane cultivated in Rio de Janeiro, Brazil. Similarly, Reis *et al*. (2004), in their ecological survey of nitrogen-fixing bacteria, isolated the genus *Burkholderia* from the rhizosphere and interior of sugarcane and maize plants in Brazil, Mexico and South Africa. *Burkholderia heleia* sp. nov., a N_2-fixing bacterium was also isolated by Aizawa *et al*. (2010) from an aquatic plant in Vietnam.

One of the selected diazotrophic isolate in this study was identified as *Bacillus megaterium* de Bary. El-Komy (2005) also isolated *B. megaterium* strains and reported that some strains were powerful phosphate solubilizers and nitrogen fixers on the roots of wheat plants. Wu *et al*. (2005) also reported on the ability of *B. megaterium* to solubilize phosphate. Foster (1964) and Brown (1974) reported on tthe use of "azotobacterin" (*Azotobacter chroococcum*) and "phosphobacterin'' (*B. megaterium)* inoculations in the Soviet Union, and that yield increases of 10% to 20% were reported under a wide variety of practical agricultural conditions; one diazotrophic isolate was identified as *E. cloacae* and another as *Klebsiella variicola*. They are prominent diazotrophs that are often found associated with maize, as endophytes, or on roots and in the rhizosphere soil. Berge *et al*. (1991) reported that an *E. cloacae was* the most abundant diazotrophic bacterium in the rhizosphere of maize-growing soils in France. The selected diazotrophic bacteria showed promise by enhancing plant growth under greenhouse conditions. However, this needs further research to confirm these results under realistic agricultural conditions. Therefore, the selected diazotrophic isolates might be potentially beneficial and should be tested more in greenhouses and field conditions with maize and wheat to confirm their application as a commercial biofertilizer.

2.5 References

Ahmad F., Ahmad I., Aqil F., Ahmed Wani A., Sousche Y.S. (2006) Plant growth promoting potential of free-living diazotrophs and other rhizobacteria isolated from Northern Indian soil. Biotechnology Journal 1:1112-1123.

Ahmad F., Ahmad I., Khan M. (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163:173-181.

Aizawa T., Ve N.B., Nakajima M., Sunairi M. (2010) *Burkholderia heleia* sp. *nov*., a nitrogen-fixing bacterium isolated from an aquatic plant, *Eleocharis dulcis*, that grows in highly acidic swamps in actual acid sulfate soil areas of Vietnam. International Journal of Systematic and Evolutionary Microbiology 60:1152-1157.

Bagwell C., Lovell C. (2000) Microdiversity of culturable diazotrophs from the rhizoplanes of the salt marsh grasses *Spartina alterniflora* and *Juncus roemerianus*. Microbial Ecology 39:128-136.

Baldani J., Baldani V.L.D., Seldin L., Döbereiner J. (1986) Characterization of *Herbaspirillum seropedicae* gen. *nov*., sp. *nov*., a root-associated nitrogen-fixing bacterium. International Journal of Systematic Bacteriology 36:86-93.

Barraquio W., Revilla L., Ladha J. (1997) Isolation of endophytic diazotrophic bacteria from wetland rice. Plant and Soil 194:15-24.

Bashan Y., Ream Y., Levanony H., Sade A. (1989) Nonspecific responses in plant growth, yield, and root colonization of non-cereal crop plants to inoculation with *Azospirillum brasilense* Cd. Canadian Journal of Botany 67:1317-1324.

Bashan Y., Holguin G., De-Bashan L.E. (2004) Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Canadian Journal of Microbiology 50:521-577.

Berge O., Heulin T., Balandreau J. (1991) Diversity of diazotroph populations in the rhizosphere of maize (*Zea mays* L.) growing on different French soils. Biology and Fertility of Soils 11:210-215.

Bevivino A., Tabacchioni S., Chiarini L., Carusi M.V., Del Gallo M., Visca P. (1994) Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. Microbiology 140:1069-1077.

Boddey R., Oliveira O.C., Urquiaga S., Reis V., Olivares F.L., Baldani V., Döbereiner J. (1995a) Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. Plant and Soil 174:195-209.

Boddey R., Urquiaga S., Reis V., Döbereiner J. (1991) Biological nitrogen fixation associated with sugar cane. Plant and Soil 137:111-117.

Boddey R.M., Baldani V.L.D., Baldani J.I., Döbereiner J. (1986) Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field-grown wheat. Plant and Soil 95:109-121.

Boddey R.M., Dobereiner J. (1995) Nitrogen fixation associated with grasses and cereals: recent progress and perspectives for the future. Nutrient Cycling in Agroecosystems 42:241-250.

Boddey R.M., Knowles R. (1987) Methods for quantification of nitrogen fixation associated with Gramineae. Critical Reviews in Plant Sciences 6:209-266.

Boddey R.M., Oliveira O.C., Urquiaga S., Reis V.M., Olivares F.L., Baldani V.L.D., Döbereiner J. (1995b) Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant and Soil 174:195-209. DOI: 10.1007/bf00032247.

Boddey R.M., Urquiaga S., Alves B.J.R., Reis V. (2003) Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. Plant and Soil 252:139-149. DOI: 10.1023/a:1024152126541.Brown M.E. (1974) Seed and root bacterization. Annual Review of Phytopathology 12:181-197.

Caballero-Mellado J., Carcano-Montiel M., Mascarua-Esparza M. (1992) Field inoculation of wheat (*Triticum aestivum*) with *Azospirillum brasilense* under temperate climate. Symbiosis13: 243-253.

Chiarini L., Bevivino A., Dalmastri C., Tabacchioni S., Visca P. (2006) *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends in Microbiology 14:277-286.

Cocking E.C. (2009) The challenge of establishing symbiotic nitrogen fixation in cereals. Nitrogen Fixation in Crop Production. Agronomy Monograph 52:35-63.

Dobbelaere S., Vanderleyden J., Okon Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22:107-149.

Döbereiner J. (1988) Isolation and identification of root associated diazotrophs. Plant and Soil 110:207-212.

Döbereiner J. (1992a) History and new perspectives of diazotrophs in association with non-leguminous plants. Symbiosis 13:1-13.

Döbereiner J. (1992b) Recent changes in concepts of plant bacteria interactions: endophytic N2 fixing bacteria. Ciência e Cultura 44:310-313.

Dong Z., Canny M.J., McCully M.E., Roboredo M.R., Cabadilla C.F., Ortega E., Rodes R. (1994) A nitrogen-fixing endophyte of sugarcane stems (A new role for the apoplast). Plant Physiology 105:1139-1147.

Eckford R., Cook F.D., Saul D., Aislabie J., Foght J. (2002) Free-living heterotrophic nitrogen-fixing bacteria isolated from fuel-contaminated Antarctic soils. Applied and Environmental Microbiology 68:5181-5185.

El-Komy H.M.A. (2005) Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. Food Technology and Biotechnology 43:19-27.

Estrada P., Mavingui P., Cournoyer B., Fontaine F., Balandreau J., Caballero-Mellado J. (2002) An N_2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico. Canadian Journal of Microbiology 48:285-294.

Foster J.W. (1964) General biology in the Soviet Union, 1964. BioScience 14:15-20.

Fujii T., Huang Y.D., Higashitani A., Nishimura Y., Iyama S., Hirota Y., Yoneyama T., Dixon R. (1987) Effect of inoculation with *Klebsiella oxytoca* and *Enterobacter cloacae* on dinitrogen fixation by rice-bacteria associations. Plant and Soil 103:221-226.

Glick B.R. (1995) The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41:109-117.

Jacobs J.L., Fasi A.C., Ramette A., Smith J.J., Hammerschmidt R., Sundin G.W. (2008) Identification and onion pathogenicity of *Burkholderia cepacia* complex isolates from the onion rhizosphere and onion field soil. Applied and Environmental Microbiology 74:3121-3129.

James E.K., Gyaneshwar P., Barraquio W.L., Mathan N., Ladha J.K. (2000) Endophytic diazotrophs associated with rice. The quest for nitrogen fixation in rice. International Rice Research Institute, Makati City, Philippines:119-140.

Kapulnik Y., Kigel J., Okon Y., Nur I., Henis Y. (1981) Effect of *Azospirillum* inoculation on some growth parameters and N-content of wheat, sorghum and panicum. Plant and Soil 61:65-70.

Kennedy I.R., Pereg-Gerk L.L., Wood C., Deaker R., Gilchrist K., Katupitiya S. (1997) Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. Plant and Soil 194:65-79.

Kennedy I.R., Tchan Y.T. (1992) Biological nitrogen fixation in non-leguminous field crops: Recent advances. Plant and Soil 141:93-118.

Kloepper J.W., Zablotowicz R.M., Tipping E.M., Lifshitz R. (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. In: D. L. Keister and P. B. Cregan (ed.), The rhizosphere and plant growth. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 315-326.

Krotzky A., Werner D. (1987) Nitrogen fixation in *Pseudomonas stutzeri*. Archives of Microbiology 147:48-57.

Lin W., Okon Y., Hardy R.W.F. (1983) Enhanced mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Applied and Environmental Microbiology 45:1775-1779.

Mano H., Morisaki H. (2008) Endophytic bacteria in the rice plant. Microbes and Environments 23:109-117.

Martin P., Glatzle A., Kolb W., Omay H., Schmidt W. (1989) N_2-fixing bacteria in the rhizosphere: Quantification and hormonal effects on root development. Zeitschrift für Pflanzenernährung und Bodenkunde 152:237-245.

Mendes R., Pizzirani-Kleiner A.A., Araujo W.L., Raaijmakers J.M. (2007) Diversity of cultivated endophytic bacteria from sugarcane: genetic and biochemical characterization of *Burkholderia cepacia* complex isolates. Applied and Environmental Microbiology 73:7259-7267.

Miralles I.S., Maciel M.C.A., Angelo M.R.F., Gondini M.M., Frota L.H.F., Reis C.M.F., Hofer E. (2004) *Burkholderia pseudomallei*: a case report of a human infection in Ceará, Brazil. Revista do Instituto de Medicina Tropical de São Paulo 46:51-54.

Okon Y. (1985) *Azospirillum* as a potential inoculant for agriculture. Trends in Biotechnology 3:223-228.

Olivares F.L., Baldani V.L.D., Reis V.M., Baldani J.I., Döbereiner J. (1996a) Occurrence of the endophytic diazotrophs and *Herbaspirillum spp*. in roots, stems, and leaves, predominantly of Gramineae. Biology and Fertility of Soils 21:197-200. DOI: 10.1007/bf00335935.

Palus J.A., Borneman J., Ludden P.W., Triplett E.W. (1996) A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L. and *Zea luxurians* Iltis and Doebley. Plant and Soil 186:135-142.

Perin L., Martínez-Aguilar L., Paredes-Valdez G., Baldani J., Estrada-de Los Santos P., Reis V., Caballero-Mellado J. (2006) *Burkholderia silvatlantica* sp. *nov*., a diazotrophic bacterium associated with sugar cane and maize. International Journal of Systematic and Evolutionary Microbiology 56:1931-1937.

Rao V., Ramakrishnan B., Adhya T., Kanungo P., Nayak D. (1998) Review: Current status and future prospects of associative nitrogen fixation in rice. World Journal of Microbiology and Biotechnology 14:621-633.

Reinhold-Hurek B., Hurek T. (1998) Life in grasses: diazotrophic endophytes. Trends in Microbiology 6:139-144.

Reis V., Estrada-De los Santos P., Tenorio-Salgado S., Vogel J., Stoffels M., Guyon S., Mavingui P., Baldani V., Schmid M., Baldani J. (2004) *Burkholderia tropica* sp. *nov*., a novel nitrogen-fixing, plant-associated bacterium. International Journal of Systematic and Evolutionary Microbiology 54:2155-2162.

Roesch L.F.W., Camargo F.A.O., Bento F.M., Triplett E.W. (2008) Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. Plant and Soil 302:91-104.

Różycki H., Dahm H., Strzelczyk E., Li C.Y. (1999) Diazotrophic bacteria in root-free soil and in the root zone of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.). Applied Soil Ecology 12:239-250.

Sarig S., Blum A., Okon Y. (1988) Improvement of the water status and yield of field-grown grain sorghum (*Sorghum bicolor*) by inoculation with *Azospirillum brasilense*. The Journal of Agricultural Science 110:271-277.

Urquiaga S., Cruz K.H.S., Boddey R.M. (1992) Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. Soil Science Society of America Journal 56:105-114.

Vermeiren H., Willems A., Schoofs G., De Mot R., Keijers V., Hai W., Vanderleyden J. (1999) The rice inoculant strain *Alcaligenes faecalis* A15 is a nitrogen-fixing *Pseudomonas stutzeri*. Systematic and Applied Microbiology 22:215-224.

Vessey J., Pawlowski K., Bergman B. (2005) Root-based N_2-fixing symbioses: legumes, actinorhizal plants, *Parasponia sp*. and Cycads. Plant and Soil 274:51-78.

Vessey J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.

Wu S., Cao Z., Li Z., Cheung K., Wong M. (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155-166.

CHAPTER THREE

EFFECT OF DIFFERENT METHODS OF APPLICATION OF DIAZOTROPHIC INOCULANTS ON MAIZE GROWTH:A GREENHOUSE STUDY

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

Diazotrophs are living microorganisms capable of fixing atmospheric nitrogen in the soil and thereby increasing crop yields, minimizing fertilizer costs, and improving agricultural sustainability. In this study, the effectiveness of different methods of application of five diazotrophic bacteria onto maize (*Zea mays L.*) was studied under greenhouse condition at the University of KwaZulu-Natal, Pietermaritzburg South Africa. Methods of applications of diazotrophic inoculants included: seed treatment, drench, foliar spray, seed treatment + drench, seed treatment + foliar spray, foliar spray + drench and seed treatment + foliar spray +drench. Diazotrophic bacteria found within rhizosphere soils, roots and stems of field grown maize were previously isolated and identified as: *Bacillus megaterium* (Isolate V16), *Burkholderia ambiferia* (Isolate V9), *Enterobacter cloacae* (Isolate L1), *Pantoea ananatis* (Isolate LB5), and *Pseudomonas nitroreducens* (Isolate StB5). Inoculation of five diazotrophic isolates by the different methods of application significantlly increased dry weight and leaf chlorophyll content ($P < 0.001$, $P = 0.001$). Overall, all methods of applications of the diazotrophic inoculants used in this study resulted in measureable increases in dry weight and leaf chlorophyll content, combined methods of application (seed treatment + drenching) and sole application (seed treatment) were significantly ($P < 0.001$) efficient and effective.

Key words: diazotrophic bacteria, application methods, leaf chlorophyll level and growth promotion

3.1 Introduction

Maize, wheat, and rice are the major cereal grains that sustain humanity (Fischer *et al.*, 2007). In the developing world, maize is the most important staple crop, and nitrogen is the most important input required for maize production (Nziguheba *et al.*, 2005). In the developing world, maize production averages around only 700 kg of maize per hectare compared with the yield potential of seven tonnes per hectare in the USA (Gladwin *et al.*, 2001). In part this is because farmers in Africa use the least fertilizer in the world because they cannot afford the inputs used by their developed world counterparts (Borlaug and Dowswell, 1995). The routine application of high levels of chemical N-fertilizers may induce a series of negative consequences on the soil ecology and from the runoff of N into water systems (Acosta-Martinez and Tabatabai, 2000; Adesemoye and Kloepper, 2009). To reduce the dependence on N fertilizers in agriculture, the use of nitrogen-fixing bacteria may be an alternative agricultural practice.

Plant-growth-promoting rhizobacteria (PGPR) have been identified as having the potential to provide nutrients in sustainable systems in crop production (Saharan and Nehra, 2011). Whilst rhizobia are well established as nitrogen fixing in symbiosis with legumes, free-living, root-associated diazotrophic bacteria can provide a source of biologically fixed N for cereal crops (Rao *et al.*, 1998). It is also well documented that inoculation with diazotrophic bacteria can increase soil fertility and enhance plant productivity (Hayat *et al.*, 2010). These diazotrophic bacteria include isolates of many soil bacteria, including the following genera: *Enterobacter*, *Pseudomonas*, *Pantoea*, *Burkholderia*, *Klebsiella*, *Azospirillum*, *Azotobacter* and *Bacillus.* These are all PGPR and are now being widely researched for use to enhance plant productivity (Fuentes-Ramirez and Caballero-Mellado, 2006; Steenhoudt and Vanderleyden, 2006; Caballero-Mellado *et al.*, 2007; Mirza *et al.*, 2007; Yachana, 2012). For example, Yanni and El-Fattah (1999) reported that selected strains of *Azotobacter***,** *Pseudomonas* and *Azospirillum* increased the yield of rice by 20% to 55%, and a strain of diazotrophic *Burkholderia* increased the biomass of a rice crop by 69% (Kennedy *et al.*, 2004). Some endophytic diazotrophs have been also discovered in crops such as sugarcane, which were able to fix 60%–80% of the annual plant N requirement (Dobereiner *et al.*, 1993; Boddey *et al.*, 1995). *Bacillus megaterium* deBary has been characterized as a PGPR. Many researchers believed that the ability of this strain to consistently enhance the growth of maize and other crop species under field conditions was due

to phosphate solubilization (Brown, 1974; De Freitas *et al.*, 1997; Rodríguez and Fraga, 1999; Wu *et al.*, 2005). However, a nitrogen fixing strain, *B. megaterium* C4, originally isolated from a maize rhizosphere, was found to have a colonization pattern similar to those of many Gram-negative diazotrophs, such as *Azospirillum brasilense* Tarrand *et al*. (Liu *et al.*, 2006). Similarly Raju *et al.* (1972) isolated a strain of *Enterobacter cloacae*, which was an N_2-fixing, PGPR in the rhizosphere of a maize plant. Berge *et al*. (1991) found that strains of *Enterobacter cloacae* (Jordan) Hormaeche and Edwards 1960, *Klebsiella* spp., and *Pseudomonas* spp. were the most abundant diazotrophs in maize roots. In many cases, the PGPR diazotrophs also express biocontrol activity against plant diseases (Hinton and Bacon, 1995). There is a problem in this field of PGPRs because some of the plant associated PGPR genera such as: *Burkholderia, Enterobacter, Pantoea* and *Pseudomonas* may also be opportunistic pathogens on humans (Berg, *et al*., 2005, Tyler and Triplett, 2008).

As it is very useful to isolate and identify bacterial strains with plant growth-promoting capabilities, optimizing methods of application of these strains to specific plant organs is needed. Bressan and Borges (2004) reported that a foliar spray treatment was effective to inoculate bacterial endophytes which successful migrated inside stems of maize plants. Similarly, foliar application of PGPR strains of *Azotobacter*, *Azospirillum* and *Beijerinckia* was reported by Sudhakar *et al*. (2000) to be an effective method of application resulting in an increased fruit and leaf yield of mulberry (*Morus* spp.). In another study, strawberry plants were inoculated with *Bacillus* M3, *Pseudomonas* BA-8 or *Bacillus* OSU-142, either by root inoculation technique or foliar sprays. Both methods resulted in increased yields, growth and P, Fe, Cu and Zn content in the strawberry plants and increased soil P, Fe, Zn, K, and Mg availability (Esitken *et al.*, 2010).

The objective of this work was to compare methods of application of five strains of diazotrophic inoculants onto maize, aiming to optimize their plant growth promoting performance on maize plant. These bacterial isolates, *Bacillus megaterium* (V16), *Pseudomonas* spp. (StB5), *Enterobacter cloacae* (L1), *Burkholderia ambiferia*. (V9), and *Pantoea ananatis* (LB5), had been isolated and screened previously (unpublished data in chapter two), to confirm their diazotrophic activities.

3.2 Methods and Materials

3.2.1 Inoculum Preparation

Diazotrophic bacteria were isolated from soil rhizospheres, roots and leaves of maize plants collected from Cedara (Agricultural research collage, Hawick), Greytown, and Ukulinga (University of KawaZulu –Natal Research Farm, Pietermaritzburg), (KwaZulu-Natal, Republic of South Africa). Roots and leaves were surface sterilized with 3.5% sodium hypochlorite for five minutes and subsequently rinsed three times with sterile distilled water, using a modified protocol of Kloepper *et al*. (1991). Roots and leaves were cut into pieces and grounded with 10 mℓ of distilled water. A modified protocol of Döbereiner (1988) (N-free semi-solid media) was used to isolate rhizospheric, rhizoplane and endophytic diazotrophs.

Bacterial inocula were prepared by streaking each bacterial strain onto N-free agar (mannitol as a carbon source). After colonies grew, 10 mℓ of sterile distilled water was introduced into each petri dish before hockey stick agitation of these colonies to create bacteria suspensions. Cell numbers were then adjusted to a required concentration for each application method using a Neubauer improved haemocytometer[8].

3.2.2 Source of Seed

Seeds of white maize of the cultivar, Mac's Medium Pearl, (an open pollinated variety) were bought from McDonalds Seeds®[9].

3.2.3 Application Methods

The bacterial strains were inoculated onto maize plant using seven different application methods:

i. a seed treatment
ii. a drench
iii. a foliar spray
iv. seed treatment + drench
v. seed treatment + foliar spraying
vi. drench + foliar spraying
vii. seed treatment + drench + foliar spray.

[8]Neubauer improved cell counting chamber, Hirschmann Laborgerate GMbH and Co. KG, HauptstraBe 7-15, 74246 Eberstadt, Germany

[9]MacDonald's Seeds (Ltd), P.O. Box 40, Mkondeni, 3212, Pietermaritzburg, Republic of South Africa

Maize seeds were sterilized prior to treatment or planting by dipping them in 95% ethanol and then transferring them to 1% sodium hypochlorite for 4 min, followed by rinsing in sterilized distilled water eight times.

3.2.3.1 Seed Treatment

Surface sterilized maize seeds were coated with a suspension of the bacterial inoculants (10^{10} colony forming units (CFU) $m\ell^{-1}$) and an adhesive (2% gum arabic) and allowed to air-dry overnight. Bacterial cell counts were approximately 10^8 CFU per seed. Seeds treated with sterile distilled water amended with gum arabic served as a Non-Treated Control.

3.2.3.2 Drenching

After emergence of the seedlings grown in pots with a top diameter of 200 mm that held 2kg of composted pine bark, a bacterial suspension at a concentration of 10^6 CFU ml^{-1} (5 $m\ell$ $plant^{-1}$ and followed by 5 $m\ell$ $plant^{-1}$ a week later) was drenched (Kifle and Laing, 2011). The Un-treated Control received no bacterial inoculations.

3.2.3.3 Foliar Spray

Bacterial suspensions from 48 hour old plated cultures were adjusted to 10^7 CFU $m\ell^{-1}$ and mixed with 0.01% aqueous Break-thru® (polyether-polymethylsiloxane-copolymer)[10] (www.agricare.co.za) as adjuvant and sprayed onto 6 weeks old maize leaves grown in pots with a top diameter of 200 mm that held 2kg of composted pine bark using 2ℓ hand-held sprayers. After emergence of seedlings (three per pot), plants were sprayed twice to run-off at a rate of 5 $m\ell$ $plant^{-1}$ at a concentration of 10^6 CFU $m\ell^{-1}$. Two sprays were applied, a week apart. As a Control treatment, plants were sprayed with 5 $m\ell$ of Break-thru® at 0.01% in water, on the same dates as the bacterial treatments.

3.2.4 Growth Medium and Application of Nutrient Solution

Maize plants were grown in 75 mm diameter plastic pots containing composted pine bark in a fan-and-pad controlled environment tunnel (Controlled Environment Facility, University of KwaZulu-Natal, Pietermaritzburg, South Africa). Plants were hand watered (250 ml pot^{-1}) every

[10]Western farm service, Inc. P.O.Box 1168, Fresno, California 93715

three days supplemented with nutrient solution: (0.14 g ℓ^{-1} K_2SO_4, 0.13 g ℓ^{-1} KOH, 0.1 g ℓ^{-1} $MgSO_4$, 0.74 g ℓ^{-1} $CaCl_2.2H_2O$, 0.11 mℓ ℓ^{-1} H_3PO_4 to make up 100 % PK fertilizer and 0.02 g ℓ^{-1} of Microplex®[1] to provide micronutrients. Eighteen pots were used as a Positive Control, and were therefore watered with a 100% NPK soluble fertilizer [3:1:3 (38)][11]. As a Low Nitrogen Control, another 18 pots were watered with 100% PK nutrient solution and 0.02 g ℓ^{-1} of Microplex®. As a Zero Fertilizer Control, another 18 pots were watered with only water (Untreated Control). When plants reached the six leaf stage, leaf chlorophyll content was measured using chlorophyll meter[12] and the fresh biomass was harvested and then placed in brown paper bags and dried at 70°C in the oven for 72 hours. Dry samples were weighed for shoot dry weight determination.

3.2.5 Experimental Design

The experimental design was in 5 x 7 factorial design (five bacterial isolates, 7 application methods), with three control treatments, using three replications (5 bacterial isolates x 7 application methods x 3 replicates) and 3 controls x 6 pots x 3 replicates, arranged in the greenhouse in a randomized complete blocks design. Each treatment consisted of six pots with a top diameter of 75 mm filled with composted pine bark. Each pot was planted with five seeds, which were thinned to three plants per pot after germination.

Experimental Analysis

Factorial analysis of variance was performed using the General Linear Model of ANOVA, of Genstat® 14th edition. An F value for main treatment effects and their interaction were considered significant at $P \leq 0.05$ level. Treatment means were separated using DMRT test at the 5% probability level.

[11]Ocean Agriculture (Pty) Ltd, P.O. Box 741, Muldersdrift, 1747, South Africa
[12] CCM-200 Plus, Opti-Science Inc., 8 Winn Avenue, Hudson, NH, USA, 03051.

3.3 Results

3.3.1 Single Method of Application of Five Selected Diazotrophic Bacterial Inoculants on Maize Growth under Greenhouse Conditions

Five selected diazotrophic bacterial isolates increased ($P < 0.001$) dry weight and leaf chlorophyll content of maize when they were applied by single methods of application, seed treatment, drench or foliar spray (Table 3.1), when compared with the Untreated Control. Seeds treated with these five diazotrophic inoculants had increased leaf chlorophyll content by 31.7% to 65.0% and dry weight by 123.4% to 291.4%. When applied by drenching, these inoculants increased dry weight by 53.9% to 59.7% and leaf chlorophyll content by 134% to 171% (Table 3.2). When these diazotrophic bacteria were applied in a foliar spray they increased the leaf chlorophyll of maize plants by 59.2% to 72.6% and increased dry weight by 121% to 165%.

Among individual diazotrophic treatments, Isolate L1 (*Enterobacter cloacae*) increased leaf chlorophyll content, and Isolate StB5 (*Pseudomonas* spp.) and Isolate V16 *(Bacillus megaterium*) increased dry weight, when they were applied as seed treatments. When applied as a drench, Isolate LB5 (*Pantoea ananatis*) induced a higher leaf chlorophyll content and Isolate StB5 (*Pseudomonas* spp.) increased maize dry weight. When applied by foliar spray, Isolate L1 (*Enterobacter cloacae*) contributed to higher leaf chlorophyll content and Isolate V16 (*B. megaterium*) to greater dry weight.

Table 3.1 The effect of single methods of application of five diazotrophic bacterial inoculants on the growth of maize

Treatments	Seed treatment (St)				Drench (Dr)				Foliar spray (Fs)			
	CCI		Dry weight (g)		CCI		Dry weight (g)		CCI		Dry weight (g)	
Control	6.41	a	1.73	a	2.97	a	1.523	a	3.09	a	1.643	a
100%PK	7.21	ab	5.68	ab	5.59	a	4.56	a	4.7	a	4.36	a
StB5	14.53	c	22.23	c	12.24	b	12.38	b	10.77	b	9.65	b
V16	14.10	c	22.23	bc	11.55	b	11.803	b	10.79	b	11.783	b
V9	13.53	bc	12.69	bc	12.51	b	10.71	b	11.65	b	10.017	b
LB5	14.27	c	13.97	c	11.47	b	12.047	b	12.4	b	10.79	b
L1	14.78	c	14.61	bc	12.71	b	11.667	b	13.21	b	11.597	b
100%NPK	22.74	d	21.89	c	21.28	c	13.757	b	18.19	c	15.34	c
CV%	15.70		25.6		8.1		13.0		9.7		11.4	
LSD	3.689		5.905		1.594		2.226		1.801		1.88	
SED	1.72		2.753		0.743		1.038		0.839		0.876	
P	<0.001		<0.001		<0.001		<.001		<0.001		<.001	

Means with the same letter in the same column are not significantly different at $P \leq 0.05$;

100%PK= plants were un-inoculated and fertilized with 100% potassium and phosphorous plus a micronutrient solution (Microplex®);

100%NPK= plants were un-inoculated but fertilized with 100% NPK [3:1:3 (38)]®and Microplex®;

Control= plants un-inoculated and no fertilizer application;

CCI=Chlorophyll Content Index;

DW= Dry weight

Table 3.2 Comparing the performance of five strains of diazotrophic bacteria in enhancing maize growth, relative to an Unfertilized Control

Treatments	Seed treatments (St)				Drench				Foliar spray			
	CCI	%CCI Equivalent	DW(g)	% over Control	CCI	%CCI Equivalent	DW (g)	%over Control	CCI	% CCI Equivalent	DW (g)	% over Control
Control	7.21	31.71	5.68	-	5.59	26.27	4.56	-	4.70	25.84	4.36	-
StB5	14.53	63.89	22.23	291.37	12.24	57.52	12.38	171.49	10.77	59.21	9.65	121.33
V16	14.10	62.01	22.23	291.37	11.55	54.28	11.80	158.84	10.79	59.32	11.783	170.25
V9	13.53	59.50	12.69	123.42	12.51	58.79	10.71	134.87	11.65	64.05	10.017	129.75
LB5	14.27	62.75	13.97	145.95	11.47	53.90	12.05	164.19	12.40	68.17	10.79	147.48
L1	14.78	64.99	14.61	157.22	12.71	59.73	11.67	155.86	13.21	72.62	11.597	165.99
100%NPK	22.74	100	21.89	385.38	21.28	100	13.757	301.69	18.19	100	15.34	351.83

Means with the same letter in the same column are not significantly different at $P \leq 0.05$;

100%PK= plants were un-inoculated and fertilized with 100% potassium and phosphorous and a micronutrient solution (Microplex®);

100%NPK= plants were un-inoculated but fertilized with 100% NPK [3:1:3 (38)]®and Microplex®;

Control= plants un-inoculated and no fertilizer application;

CCI=Chlorophyll Content Index;

DW= Dry weight

3.3.2 Combined Application Methods of Different Diazotrophic Bacterial Strains on Maize Growth

A comparison of dry weights and leaf chlorophyll content when diazotrophic isolates were applied by seed treatment + drench confirms that diazotrophic isolates had growth-promoting abilities (Table 3.3). The mean dry weight of plants treated with the diazotrophic isolates was 117%-202% higher than the Untreated Control plants (Table 3.4), and there was a 34.3% - 40.64% increase in leaf chlorophyll content of maize plant (Table 3.5). When these selected diazotrophs were applied by seed treatment + foliar spraying dry weight increased by 154% - 194% (Table 3.4) over the Untreated Control, and leaf chlorophyll content increased by 65.7%-71.96% (Table 3.5). Combining foliar spray +drench increased leaf chlorophyll content by 55.86% - 60.37% (Table 3.5) and dry weight by 42%-114% (Table 3.4) over the Untreated Control. Combining all three application methods, seed treatments + drench + foliar spray, increased leaf chlorophyll content by 58.05%-66% (Table 3.5) and increased dry weight by 82% - 102% (Table 3.4) over the Untreated Control.

Table 3.3 Effect of combined methods of application of five selected diazotrophic bacterial inoculants on the growth of maize

	Seed Treat (St) + Drench (Dr)				Seed Treat (St)+ Foliar spray (Fs)				Fs + Dr				St + Dr + Fs			
Treatments	**CCI**		**Dry weight (g)**		**CCI**		**Dry weight (g)**		**CCI**		**Dry weight (g)**		**CCI**		**Dry weight (g)**	
Control	2.95	a	4.93	a	2.91	a	1.73	a	3.88	a	2.36	a	3.09	a	2.4	a
PK	5.64	a	5.36	ab	4.66	a	4.24	a	7.11	a	6.55	ab	5.34	a	6.09	b
StB5	15.97	b	14.88	bc	14.4	b	10.8	b	12.86	b	12.64	bc	12.83	b	12.35	c
V16	15.09	b	16.17	c	13.89	b	11.44	b	12.78	b	14.02	bc	12.99	b	11.76	c
V9	14.43	b	15.71	bc	13.97	b	11.34	b	12.80	b	9.36	abc	13.91	b	11.29	c
LB5	14.85	b	12.05	b	13.15	b	12.39	b	11.90	b	10.27	abc	13.31	b	11.1	c
L1	15.28	b	11.67	b	13.45	b	12.47	b	12.35	b	10.91	bc	12.22	b	11.99	c
NPK	24.31	c	19.81	c	20.01	c	18.01	c	21.30	c	15.36	c	21.05	c	16.04	d
CV%	8.3		24.6		10.4		14.5		9.0		25.7		7.6		11.2	
LSD	1.978		5.998		2.197		2.624		1.874		4.575		0.735		2.036	
SED	0.922		2.796		1.024		1.224		0.874		2.133		1.577		0.949	
P	<0.001		<0.001		<0.001		<.001		<0.001		<0.001		<0.001		<0.001	

Means with the same letter in the same column are not significantly different at $P \leq 0.05$

100%PK= plants were un-inoculated and fertilized with 100% potassium and phosphorous and micronutrients

100%NPK= plants were un-inoculated but fertilized with 100% NPK [3:1:3 (38)] ® and micronutrients

Control= plants un-inoculated and no fertilizer application

CCI=Chlorophyll Content Index;

DW= Dry weight

Table 3.4 The effect on maize growth of five isolates of diazotrophic bacteria with multiple application techniques

Bacteria	Seed Treatment (St) + Drench (Dr)		Seed Treatment (St) + Foliar spray (Fs)		Drench + Foliar spray		Seed treat + Drench + Foliar	
	Dry weight (g)	% over Control	Dry weight(g)	% over Control	Dry weight (g)	% over Control	Dry weight (g)	% over Control
Control	5.36	-	4.24	-	6.55	-	6.09	-
StB5	14.88	177.61	10.80	154.72	12.64	92.98	12.35	102.79
V16	16.17	201.68	11.44	169.81	14.02	114.05	11.76	93.1
V9	15.71	193.10	11.34	167.45	9.36	42.90	11.29	85.39
LB5	12.05	124.76	12.39	192.22	10.27	56.79	11.10	82.27
L1	11.67	117.67	12.47	194.10	10.91	66.57	11.99	96.88

Table 3.5 The Effect of diazotrophic bacterial isolates on leaf chlorophyll content with multiple application methods

Bacteria	St + Dr		St +FS		Dr + Fs		St + Dr + Fs	
	CCI	%CCI Equivalent	CCI	%CCI Equivalent	CCI	%CCI Equivalent	CCI	%CCI Equivalent
StB5	15.97	65.69	14.40	71.96	12.86	60.38	12.83	60.95
V16	15.09	62.07	13.89	69.42	12.78	60.00	12.99	61.71
V9	14.43	59.36	13.97	69.82	12.80	60.09	13.91	66.08
LB5	14.85	61.09	13.15	65.72	11.90	55.87	13.31	63.23
L1	15.28	62.85	13.45	67.22	12.35	57.98	12.22	58.05
100% NPK	24.31	100	20.01	100	21.30	100	21.05	100

ST = seed treatment

Dr = drenching

FS = foliar spray

100%NPK= plants were un-inoculated but fertilized with 100% nitrogen, potassium and phosphorous [3:1:3 (38)] ® and micronutrients

CCI=Chlorophyll Content Index;

DW= Dry weight (g)

3.3.3 Factorial Analysis of the Application of Five Diazotrophic Bacterial Strains Using Different Methods of Application

Inoculation of five diazotrophic bacterial isolates using three different methods of application, and their combinations, increased leaf chlorophyll content and dry weight of maize the various combinations used (Table 3.5). Of these application techniques, seed treatment alone and seed treatment + drench treatment had significant ($P < 0.001$) effects on dry weight and leaf chlorophyll content. On the other hand, foliar sprays or drench treatments alone had no effect on leaf chlorophyll content. There was a significant interaction between the bacterial strains and the different methods of application on leaf chlorophyll content and dry weight, ($P = 0.007$ and $P = 0.024$, respectively).Isolates all performed similarly across all treatment metods but siginificantlly better than control or PK. However NPK was still best.

Table 3.6 Analysis of the effect of different methods of application of five diazotrophic inoculants on maize growth

Source	level	Dry weight		CCI	
Main Effect					
Bacterial Isolates		P<0.001		P<0.001	
	Control	2.33	a	3.61	a
	PK	5.26	b	5.75	b
	V16	12.01	c	12.88	c
	LB5	12.60	c	13.11	c
	StB5	12.75	c	13.37	c
	V9	13.09	c	13.26	c
	L1	13.53	c	13.37	c
	NPK	16.08	d	21.27	d
Methods of application		P<0.001		P<0.001	
	St	12.71	b	13.41	c
	Fs	9.40	a	10.62	a
	Dr	9.81	a	11.22	ab
	St + Fs	10.30	a	11.92	b
	St + Dr	13.93	b	13.62	c
	Dr + Fs	10.18	a	11.93	b
	St +Fs + Dr	10.38	a	11.82	b
M. application × B. isolates		P=0.024		P=0.007	

Means with the same letter in the same column are not significantly different at $P \leq 0.05$

3.4 Discussion

In the present investigation, diazotrophic isolates were evaluated for their effects on growth of maize. Plant growth promotion by the diazotrophic strains using different methods of inoculation sometimes resulted in increases of dry weight and leaf chlorophyll content. Isolate L1 (*Enterobacter cloacae)* performed the best in increasing leaf chlorophyll content when applied by either seed treatment, drenching or foliar spray inoculation. Isolate V16 *(Bacillus megaterium*) induced a greater dry weight when inoculated by foliar spray than the other diazotrophic isolates. This is in agreement with Sudhakar *et al*. (2000), who reported that foliar sprays of nitrogen fixing bacteria on mulberry were the best inoculation method.

In this study, interestingly, the strain of *B. megaterium* (V16) expressed growth promotion effects on maize through N-fixation, even though phosphate solubilizing is widely reported as the key route that this bacterial species uses to promote plant growth. For example, Raja *et al*. (2006) reported that an isolate of *B. megaterium* enhanced plant growth by solubilizing phosphate but it failed to fix nitrogen.

There was significant interaction between the five diazotrophs and their methods of application, either by seed treatment alone or the combination of seed treatment + drench, in terms of measured leaf chlorophyll contents or plant dry matter. Seed treatment as a sole application, or in a combination of seed treatment + drench, induced higher leaf chlorophyll content and dry weight. Given its efficacy as a solo treatment, and that seed treatment was the simplest and most convenient method of application, this method of application can be recommended to farmers as the best method of application of diazotrophs for plant growth promotion.

3.5 References

Acosta-Martinez V., Tabatabai M. (2000) Enzyme activities in a limed agricultural soil. Biology and Fertility of Soils 31:85-91.

Adesemoye A.O., Kloepper J.W. (2009) Plant–microbes interactions in enhanced fertilizer-use efficiency. Applied Microbiology and Biotechnology 85:1-12.

Berg G., L. Eberl, Hartmann A. (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environmental Microbiology 7: 1673-1685.

Berge O., Heulin T., Balandreau J. (1991) Diversity of diazotroph populations in the

rhizosphereof maize (*Zea mays* L.) growing on different French soils. Biology and Fertility of Soils 11:210-215.

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

Borlaug N.E., Dowswell C.R. (1995) Mobilising science and technology to get agriculture moving in Africa. Development Policy Review 13:115-129.

Bressan W., Borges M.T. (2004) Delivery methods for introducing endophytic bacteria into maize. BioControl 49:315-322.

Brown M.E. (1974) Seed and root bacterization. Annual Review of Phytopathology 12:181-197.

Caballero-Mellado J., Onofre-Lemus J., Estrada-de los Santos P., Martínez-Aguilar L. (2007) The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Applied and Environmental Microbiology 73:5308-5319.

De Freitas J., Banerjee M., Germida J. (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biology and Fertility of Soils 24:358-364.

Dobereiner J., Reis V., Paula M., Olivares F. (1993) Endophytic diazotrophs in sugar cane, cereals and tuber plants. Current Plant Science and Biotechnology in Agriculture 17:671.

Esitken A., Yildiz H.E., Ercisli S., Figen Donmez M., Turan M., Gunes A. (2010) Effects of plant growth promoting bacteria (PGPB) on yield, growth and nutrient contents of organically grown strawberry. Scientia Horticulturae 124:62-66.

Fischer S.E., Fischer S.I., Magris S., Mori G.B. (2007) Isolation and characterization of bacteria from the rhizosphere of wheat. World Journal of Microbiology and Biotechnology 23:895-903. Fuentes-Ramirez L, Caballero-Mellado J (2006) Bacterial biofertilizers. In: Siddiqui ZA (Ed) PGPR: Biocontrol and Biofertilization. Springer-Verlag, Heidelberg, pp 143-172.

Fuentes-Ramirez L, Caballero-Mellado J (2006) Bacterial biofertilizers. In: Siddiqui ZA (Ed) PGPR: Biocontrol and Biofertilization.Springer-Verlag, Heidelberg, pp 143-172.

Gladwin C.H., Thomson A.M., Peterson J.S., Anderson A.S. (2001) Addressing food security in Africa via multiple livelihood strategies of women farmers. Food Policy 26:177-207.

Hayat R., Ali S., Amara U., Khalid R., Ahmed I. (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60:579-598.

Hinton D.M., Bacon C.W. (1995) *Enterobacter cloacae* is an endophytic symbiont of corn. Mycopathologia 129:117-125.

Yachana J.H.A., Subramanian R.B. (2012) Isolation of root associated bacteria from the local variety of rice GJ-17. World Research Journal of Geoinformatics 1: 21-26.

Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kifle M., Laing M. (2011) Determination of optimum dose and frequency of application of free-living diazotrophs (FLD) on lettuce. African Journal of Agricultural Research 6:671-675.

Liu X., Zhao H., Chen S. (2006) Colonization of maize and rice plants by strain *Bacillus megaterium* C4. Current Microbiology 52:186-190.

Mirza B.S., Mirza M.S., Bano A., Malik K.A. (2007) Co-inoculation of chickpea with *Rhizobium* isolates from roots and nodules and phytohormone-producing *Enterobacter* strains. Australian Journal of Experimental Agriculture 47:1008-1015.

Nziguheba G., Merckx R., Palm C.A. (2005) Carbon and nitrogen dynamics in a phosphorus-deficient soil amended with organic residues and fertilizers in western Kenya. Biology and Fertility of Soils 41:240-248.

Raja P., Uma S., Gopal H., Govindarajan K. (2006) Impact of bio-inoculants consortium on rice root exudates, biological nitrogen fixation and plant growth. Journal of Biological Sciences 6:815-823.

Raju P., Evans H.J., Seidler R.J. (1972) An asymbiotic nitrogen-fixing bacterium from the root environment of corn. Proceedings of the National Academy of Sciences 69:3474-3478.

Rao V., Ramakrishnan B., Adhya T., Kanungo P., Nayak D. (1998) Review: Current status and future prospects of associative nitrogen fixation in rice. World Journal of Microbiology and Biotechnology 14:621-633.

Rodríguez H., Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17:319-339.

Saharan B., Nehra V. 2011. Plant growth promoting rhizobacteria: a critical review. Life Science and Medical Research 21: 1-30.

Steenhoudt O., Vanderleyden J. (2006) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiology Reviews 24:487-506.

Sudhakar P., Chattopadhyay G., Gangwar S., Ghosh J. (2000) Effect of foliar application of *Azotobacter, Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). Journal of Agricultural Science 134:227-234.

Tyler H.L., Triplett, E.W. (2008) Plants as a habitat for beneficial and/or human pathogenic bacteria. Annual Review of Phytopathology 46: 53-73.

Wu S., Cao Z., Li Z., Cheung K., Wong M. (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155-166.

Yachana J.H.A., Subramanian R.B. (2012) Isolation of root associated bacteria from the local variety of rice GJ-17. World Research Journal of Geoinformatics 1: 21-26.

Yanni Y., El-Fattah F. (1999) Towards integrated biofertilization management with free living and associative dinitrogen fixers for enhancing rice performance in the Nile delta. Symbiosis 27:319-331.

CHAPTER FOUR

EFFECTS OF SELECTED DIAZOTROPHS ON GROWTH AND YIELD OF MAIZE

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg,

South Africa

Abstract

Greenhouse and field experiments were conducted at Ukulinga Farm, University of KwaZulu-Natal, Pietermaritzburg South Africa in the 2010/2011 and 2011\2012 growing seasons to study the effect of N-fixing bacterial isolates on the growth and yield of maize. Eight nitrogen fixing bacterial isolates including: *Bacillus megaterium* (V16), *Pseudomonas* spp. (StB5, A2, A6 and A61), *Burkholderia ambifaria* (V9), *Enterobacter cloacae* (L1) and *Pantoea ananatis* (LB5) were used. These were used as inoculants on maize plants aiming to stimulate plant growth, maintain or increase yield while reducing the need for N fertilizer. *Trichoderma harzianum* (Eco-T®)[13] was used as a positive control for a germination test in the laboratory. All the diazotrophic bacteria and Eco-T®, increased germination by 25-54.3 %. Seeds treated with microbial Isolates StB5, V16 and Eco-T® increased ($P = 0.003$) shoot length, and isolate StB5, V16, L1, V9, A2 and Eco-T® increased ($P < 0.001$) root length and seed vigor index of maize. Under greenhouses conditions bacterial inoculations caused only small increases ($P > 0.05$) in leaf chlorophyll content. However, when these bacterial isolates were integrated with 33%N fertilizer, the chlorophyll content and dry weigh increased ($P < 0.05$) compared to the Un-inoculated and Unfertilized Control. In the field, in year 2010/2011, plants treated with selected diazotrophic bacteria, with or without 33% N-fertilizer, had no effect ($P > 0.05$) on germination, grain yield, dry weight and plant height at (30, 60 or 90 DAP) and leaf chlorophyll content both at 30 and 60 DAP compared to the Un-inoculated and Unfertilized Control and 100% NPK. Germination increased ($P < 0.001$) by 19.9 - 135%. In year 2011/2012, plant dry weight at 30, 60 or 90 DAP was increased by 66%, 50% and 70% ($P < 0.001$) with *Bacillus megaterium* (V16), and 51%, 45% and 18% ($P < 0.001$) with StB5 (*Pseudomonas nitroreducens*), respectively. Compared to the Un-inoculated and Un-fertilized

[13]Plant Health Products (Pty) Ltd, P.O.Box 207, Nottingham Road, KwaZulu-Natal, South Africa

Control, seed treatment with *B. megaterium* (V16) and *P. nitroreducens* (StB5) increased ($P < 0.001$) the grain yield by 46.1% and 41%, respectively. Plant height and leaf chlorophyll content also increased ($P < 0.001$) by the inoculation of the selected diazotrophic bacteria.

Key words: diazotrophic bacteria, seedling growth, seed vigor chlorophyll level, maize, germination

4.1 Introduction

Maize is the most important staple crop in the developing world. As a staple food, maize has a large market and is the most important agricultural product in South Africa. However, poor soil fertility, draught and disease are measure problems to crop production (Lynch, 2007). A source of nitrogen is necessary for high yields for all agricultural and horticultural crops. Therefore, use of diazotrophic bacteria as bio-inoculants for cereals might eventually be a standard agronomic practice on most crops.

Microorganisms that promote plant growth either by nitrogen fixation or other mechanisms belong to a range of genera: e.g., *Azospirillum, Azotobacter, Bacillus, Pseudomonas* and *Serratia* (Bashan *et al.*, 2004). Most plant growth promoting rhizobacteria (PGPR) are N-fixing bacteria (diazotrophs) (Table 4.1). Their ability to fix nitrogen probably makes the organisms better adapted to live in the rhizosphere. A widely studied diazotrophic bacterium, *Azospirillum brasilense* Tarrand *et al*., was once believed that it has beneficial effects on non-legumes via biological nitrogen fixation (BNF). However, multiple inoculation experiments done by Dobbelaere *et al*. (2003) failed to show a substantial contribution of BNF to plant growth in most cases. This is indicating that *A. brasilense* promotes plant growth not only through N-fixation but also through other mechanisms such as phytohormones (Spaepen *et al.*, 2009).

Increases in growth and yield of agronomically important crops in response to inoculation with diazotrophic bacteria have been reported by Kennedy *et al*. (2004b), Okon and Labandera-Gonzalez (1994) and Bashan *et al*.(2004). Strains of *Bacillus spp., Pseudomonas spp., Enterobacter spp., Burkholderia spp.* and *Pantoea* spp. can affect seed germination, seedling growth and yield (de Freitas, 2000; Mar Vázquez *et al.*, 2000; Liu *et al.*, 2006). Inoculation of plants with *Enterobacter* spp. can also result in significant increases in various growth parameters, such as increases in plant biomass, nutrient uptake, N content, plant

height, leaf size and root length of cereals (Bashan, *et al.*, 2004) and could also be used as biocontrol agent (Duponnois and Mateille, 1999).

The effect of diazotrophic bacteria on growth and yield of cereals has been studied by many researchers. However, the effect of diazotrophic bacteria on growth parameters from germination to yield were not evaluated simultaneously. The main objective of this study was to evaluate if selected diazotrophic bacterial strains could affect seed germination and certain economically important agronomic performances of maize (*Zea mays* L.) grown under greenhouse and field conditions, and assess how these bacterial strains would perform with or without 33% N-fertilizer.

Table 4.1 Example of diazotrophs in promoting plant growth based on their ability to fix N_2

PGPR	Host plant	References
Bacillus polymyxa	Wheat	Omar *et al.*, 1996
Burkholderia species	in rice	Divan Baldani *et al.*, 2000
Azotobacter species	maize and wheat	Pandey *et al.*, 1998; Mrkovacki and Milic, 2001
Azospirillum species	maize, rice and wheat	Boddey *et al.*, 1986; Garcia de Salomone and Dobereiner, 1996; Malik *et al.*, 1997
Azoarcus species	kallar grass, sorghum and rice	Stein *et al.*, 1997; Egener *et al.*, 1999; Hurek *et al.*, 2002
Anabaena and *Nostoc*	rice and wheat	Obreht *et al.*, 1993; Hashem, 2001
Gluconacetobacter diazotrophicus	sorghum and sugarcane	Isopi *et al.*, 1995; Sevilla *et al.*, 2001; Boddey *et al.*, 2003
Herbaspirillum species	rice, sorghum and sugarcane	Boddey *et al.*, 1995; James *et al.*, 1997; James *et al.*, 2002
Bacillus spp.	sugar beet, peanut, potato, bean, sorghum, and wheat	Çakmakçi *et al.*, 2006; López-Bucio *et al.*, 2007; Ortíz-Castro *et al.*, 2008
Pseudomonas fluorescens-putida	potato, sugar beet and radish	Kloepper *et al.*, 1980
Pseudomonas putida and *P. fluorescens*	canola, wheat and potato	Frommel *et al.*, 1993; Shaharoona *et al.*, 2007
Pantoea ananatis	dune grass	Taulé *et al.*, 2012
Burkholderia cepacia	Maize	Bevivino *et al.*, 1994

4.2 Methods and Materials

4.2.1 Performance of Diazotrophic Bacteria Using Germination Bioassay

Ninety five bacteria naturally present in the rhizosphere or on roots and leaves of maize were isolated from three different sites (Cedara, Greytown and Ukulinga farm), South Africa. These were screened for N-fixing ability and growth promoting characteristics (data not printed in Chapter 4). Eight of the 93 isolates with N-fixing ability (V16, V9, StB5, LB5, L1, A2, A3, A6 and V61) were evaluated for growth of maize using a paper towel method following the procedures described by Nezarat and Gholami (2009). These diazotrophic isolates were identified as *Bacillus megaterium* (V16)), *Pseudomonas* spp., (StB5, A2, A3, A6 and A61), *Burkholderia ambifaria* (V9), *Pantoea ananatis* (LB5) *and Enterobacter cloacae* (L1) using 16r DNA sequencing, and a Bruker Daltonik MALDI Biotyper classification. Twenty five maize seeds were surface sterilized with 1% sodium hypochlorite for 5 min washed 5 times with sterilized distilled-water, coated with a suspension of the bacterial inocula (10^8 colony forming units (CFU)) plus an adhesive (2%) gum arabic), before air-drying overnight. A mean cell count was 10^6 CFU per seed. Seeds treated with sterile distilled-water amended with gum arabic served as the Un-treated Control, and seeds treated with 2×10^9 conidia g^{-1} of *Trichoderma harzianum* Eco-T® were used as a Positive control. *Trichoderma harzianum* Eco-T® is a registered, formulated biocontrol product, effective for plant growth promotion was provided by Plant Health Products (Pty) Ltd (Yobo, *et al*., 2004). Each treatment was replicated three times. Seeds were germinated in a growth chamber at 28^0C. After five days, the number of germinated seeds was counted, and root and shoot length of individual seedling was measured to determine the vigor index with the following formula: Seeds vigor index = [(mean root length + mean shoot length) X germination %] (Abdul Baki and Anderson, 1973).

4.2.2 Seed Source

Seeds of white maize of the cultivar, Mac's Medium Pearl, (an open pollinated variety) were bought from McDonalds Seeds®[14]; surface sterilized with 1% sodium hypochlorite for 5 min and washed 5 times with sterilized distilled water.

[14]McDonalds Seed Company (Pty) Ltd., Pietermaritzburg, Republic of South Africa

4.2.3 Seed Treatment

Maize seeds (*Zea mays*) were treated with the bacterial inoculums (10^8 colony forming units (CFU)) and adhesive (2% gum arabic) suspension and allowed to air-dry overnight. Cell count was10^6 CFU per seed. Seeds treated with sterile distilled-water amended with gum arabic served as a Un-treated control. Seeds were planted in 75 mm pots using composed pine back as a growing medium. Temperatures varied between 26-28 °C under greenhouse conditions. Eight weeks later, leaf chlorophyll content was measured at the 6-foliate stage using a hand held chlorophyll content meter (CCM-200 plus)[15]. Dry weight was obtained by harvesting the total biomass of the maize plants after they were oven-dried for 72 h at 70°C.

4.2.4 Fertilizer Application

Pots were hand watered every three days (250 mℓ pot^{-1}) supplemented with reduced N-fertilizer 33% N (calcium nitrate (48))[16] at a rate of 0.33 $g\ell^{-1}$) or soluble fertilizer was applied at a rate of 0.224 $g\ell^{-1}$ KH_2PO_4, 0.149 $g\ell^{-1}$ K_2SO_4, 0.324 $g\ell^{-1}$ KCl, 0.203 $g\ell^{-1}$ $MgSO_4$, to make up 100% PK fertilizer. Nine pots that served as the positive control were watered with 100% NPK soluble fertilizer [3:1:3(38)] at a rate of 1$g\ell^{-1}$and another nine pots were watered with 33%N and 100% PK. In the field, lime ammonium nitrate (LAN)[1], Super phosphate[17] and Potash were used as sources of normal amount recommended for N, P and K fertilizers for the growth of maize.

4.2.5 Field Experiments

Field experiment was conducted at Ukulinga, a research farm of the University of KwaZulu–Natal, Pietermaritzburg, South Africa (29° 24′ E; 30° 24′ S).Soils at the site are classified as Westleigh forms (Soil Classification Working Group 1991) with clay content of 55%. The experimental treatments were arranged in a split plot design with three replicates. Each plot was 8.7m long x 3.75m wide, consist of six rows with a 0.75m inter row spacing. Plots were irrigated when there was no rain to ensure that no water deficit occurred during the crop growth cycle. Crops were fully protected against weeds and pest in the two experimental seasons. Total leaf

[15]Optic-science, 8 Winn Avenue, Hudson. NH 03051. USA
[16] Ocean Agriculture (Pty) Ltd. P.O. Box 741, Muldersdrift, 1747, South Africa
[17]Omnia Fertilizer Group (Pty) Ltd. P.O.Box 69888, Bryanston,2021, South Africa

chlorophyll assessments were performed for all plots at the 6 and 8 foliate-stage. Chlorophyll readings were taken on the midpoint of the youngest fully expanded leaf and on the ear leaf. Ten leaves were measured at random in the plot and leaf chlorophyll content was calculated for each plot. Plant height was measured by randomly selecting ten plants from each plot and measuring the distance from the ground to the stem tip. Samples were oven-dried at 70 ^{0}C for three days and dry weights recorded. Full doses of potassium (K) and phosphorus (P) fertilizers were applied according to soil test recommendation from the soil testing laboratory at Cedara, South Africa. Experiment plots were hand-planted on 24th of November 2010 and the second season on 10th of November 2011. Germinated seedlings were counted 14 days after planting (DAP) and the germination percentage calculated.

4.2.6 Experimental Design

The experimental split plot design was in factorial combination of two factors: Bacterial isolates x 33%N- fertilizer, with the main plots arranged in a randomized complete block. Each treatment was replicated three times. In the main plot, five bacterial isolates were evaluated: Isolates V16 (*B. megaterium*), StB5 (*B. ambifaria*), V9 (*P. nitroreducens*), LB5 (*P. ananatis*), L1 (*E. cloacae*). In the split plot, two levels of N-fertilizer (0%N, 33%N) were tested.

4.2.7 Experimental Analysis

One way ANOVA was used to analyze the data from greenhouses and factorial analysis was performed using Genstat® 14th edition for the field data.100% NPK was not part of the factorial ANOVA applied to the other treatments F values for main treatment effects and their interaction were considered significant at $P \leq 0.05$ level. When a particular factor or an interaction of factors significantly influenced a variable, means were separated using Duncan's multiple range tests at 5% probability level.

4.3 Results

4.3.1 Germination of Maize Inoculated With N-Fixing Bacteria and Eco-T®

All the eight selected diazotrophic bacterial isolates and Eco-T® increased (at least at $P<0.05$) germination by 25-54.3% (Table 4.2). Compared to the Un-treated control (Table 4.2), treating maize seeds with these bacterial isolates and Eco-T® significantly promoted ($P < 0.05$) germination. Seeds treated with bacterial Isolates StB5, V16 or Eco-T® significantly increased ($P = 0.003$) shoot length under greenhouse conditions. Moreover, seed treatment with bacterial isolates StB5, V16, L1, V9, and A2, and Eco-T® increased ($P < 0.001$) root length and enhanced the seed vigor index of maize (Table 4.2 and Figure 4.1).

Table 4.2 Effect of N-fixing bacterial isolates on the growth of maize seedlings

Treatment	Germination %		Shoot length (cm)		Root length(cm)		Seed Vigor Index
Control	34.87	a	7.83	a	2.67	a	366.14
A2	59.56	b	10.17	ab	13.00	bc	1380.01
A61	65.15	bc	9.33	ab	6.67	ab	1042.40
LB5	71.18	bc	8.33	a	7.33	ab	1114.68
StB5	71.85	bc	21.83	c	16.00	c	2718.09
A6	77.74	bc	9.00	ab	7.17	ab	1257.06
V16	79.24	bc	17.50	bc	19.17	c	2905.73
L1	79.54	bc	15.33	abc	15.67	c	2465.74
V9	82.70	bc	15.67	abc	17.33	c	2729.10
Eco-T®	89.20	c	22.33	c	18.00	c	3597.44
CV%	19.80		33.30		30.70		
Lsd	24.11		7.845		6.485		
Sed	11.48		3.734		3.087		
P	0.01		0.003		<.001		

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$ Control = Un-treated control

4.3.2 Effect of Selected N-Fixing Bacteria and 33%N-Fertilizer on Growth of Maize

Eight of the selected diazotrophic bacterial isolates were evaluated for their effect on the growth of maize under greenhouse conditions. In addition to this, these bacterial isolates were tested with reduced level of N-fertilizer (33%N). Control treatments included un-inoculated with no fertilizer (control + 0%N) and un-inoculated with reduced fertilizer (Control + 33%N). Without fertilizer, no significant increases in leaf chlorophyll content were observed. However, when these bacterial isolates were combined with 33% N fertilizer leaf chlorophyll content siginficanlty higher than un-inoculated with no fertilizer (control + 0%N) ($P = 0.02$) (Table 4.3). When some of these bacterial isolates combined with 33%N fertilizer showed numberical increases in dry weight compared to Un-inoculated and 33% N-fertilizer (Table 4.3).

Table 4.3 The effect of selected N-fixing bacteria with and without N-fertilizer on the growth of maize in greenhouse study

N%-Fertilzer	Isolates	Chlorophyll (CCI)		Dry weight (g)	
0	Control	3.75[0.57]	a	1.67[0.22]	a
	StB5	3.58[0.55]	a	7.38[0.85]	defg
	V9	4.29[0.63]	a	6.31[0.81]	cdef
	LB5	4.32[0.64]	a	4.97[0.69]	bcd
	A3	4.39[0.64]	a	3.82[0.58]	b
	V16	4.48[0.65]	a	5.75[0.76]	bcde
	A2	4.52[0.65]	a	4.40[0.64]	bc
	A6	4.59[0.66]	a	3.77[0.57]	b
	L1	4.68[0.66]	a	4.84[0.68]	bcd
	A61	4.84[0.68]	a	4.60[0.66]	bcd
33	Control	8.76[0.94]	c	10.93[1.03]	ghi
	StB5	10.12[1.00]	c	9.66[0.98]	fgh
	V9	8.78[0.94]	c	8.68[0.93]	efgh
	LB5	8.62[0.93]	bc	9.68[0.97]	fgh
	A3	8.37[0.92]	bc	10.58[1.02]	ghi
	V16	10.36[1.02]	c	16.00[1.19]	i
	A2	7.88[0.90]	bc	11.02[1.02]	ghi
	A6	6.50[0.80]	b	13.55[1.12]	hi
	L1	8.73[0.93]	c	9.93[0.99]	fgh
	A61	8.21[0.91]	bc	11.66[1.06]	hi
N%-Fertilizer X Isolates		P=0.02[0.025]		P=0.004[0<001]	
	CV%	15.80[8.7]		28.5[12]	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$, values in parenthesis are transformed data using log base 10

4.3.3 Growth Parameters of Maize under Field Conditions

Seed treatment with five N-fixing bacterial isolates under field conditions improved germination levels. In the season of 2010-2011, application of bacterial isolates V16, StB5, V9, LB5 and L1 resulted in increases in seed germination levels (18.4%-38.5% (Table 4.4). In the season of 2011-2012, application of the isolates V16, StB5, V9, LB5 and L1 increased germination by 17, 37, 7,

27 and 22%, respectively; when these bacterial isolates were combined with 33%N, germination increased by 51, 51, 48, 26 and 37%, respectively.

Table 4.4 The effect of N-fixing bacteria on the germination of maize in the field

Treatments		Field grown maize Germination (%)			
N%-Fertilizer	**Isolates**	**2010-2011**		**2011-2012**	
0	Control	35.3	a	37.3	a
	V16	55.5	abc	54.7	bc
	StB5	56	abc	74.6	ef
	V9	69.5	c	59	cd
	L1	58.9	abc	64.7	cde
	LB5	61.5	bc	44.6	ab
33	Control	43.1	ab	82.7	fg
	V16	55.5	abc	54.7	bc
	StB5	53.7	abc	88	fg
	V9	57.8	abc	85.3	fg
	L1	67.2	bc	74	def
	LB5	68.9	c	63	cde
	NPK	66.7		95	
N-fertilizer X Isolates		P=0.056		P<0.001	
	CV%	15.80[8.7]		28.5[12]	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

4.3.4 Effects of Nitrogen Fixing Bacterial Isolates on the Dry Weight of Maize (2010-2011 season)

The effects of five selected diazotrophic bacterial isolates with or without 33%N- fertilizer on grain yield and dry weight of maize in the field are presented in Table 4.5 for the 2010-2011 seasons. Analysis of variance showed no significant differences among treatment combinations for dry weight at 30 or 90 Days After Planting (DAP) (Tables 4.5). However, variation among these treatments means were found for dry weight at 60 DAP (Tables 4.5). Plants treated with selected diazotrophic bacteria with or without 33%N-fertilizer and plant fertilized with 100%NPK had no significant differences on dry weight at 30, 60 or 90 DAP. These selected diazotrophic bacterial isolates, with or without 33%N-fertilizer, did not increase maize grain yield (Table 4.5).

Table 4.5 Maize growth parameter in the (2010-2011) season

Treatments		Dry weight in 2010/2011 season							
Fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**		**Yield (kg plant^{-1})**	
	Control	18.85	a	142.8	abc	796.3	a	3.61a	
0	L1	13.99	a	148.7	abc	588.8	a	4.23ab	
	V16	14.03	a	129.2	ab	686.6	a	4.51abc	
	LB5	15.73	a	124.3	a	783.4	a	4.39abc	
	V9	17.12	a	145.7	abc	731.6	a	4.59abc	
	StB5	17.55	a	145.3	abc	726.5	a	4.44abc	
33	Control	17.01	a	159.9	c	730..4	a	4.99bc	
	L1	18.26	a	159.3	c	769.0	a	4.48abc	
	V16	19.49	a	155.3	bc	721.2	a	4.21ab	
	LB5	17.48	a	152.8	bc	681.8	a	3.74a	
	V9	15.91	a	160.8	c	666.6	a	4.21ab	
	StB5	17.23	a	160.9	c	720.0	a	4.34ab	
	NPk	18.56		159.4		719.1			
N-fertilizer		F=3.01	P=0.10	F=14.34	P=0.001	F=0.03	P=0.873	F=0	P=0.995
isolates		F=0.13	P=0.97	F=1.65	P=0.205	F=77	P=0.56	F=0.28	P=0.917
N x isoaltes		F=1.25	P=0.326	F=0.46	P=0.764	F=0.26	P=0.89	F=2.59	P=0.055
CV%		18		9.4		20.9		13.3	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

- Control = untreated and unfertilized DAP = Days After Planting

4.3.5 Effects of Nitrogen Fixing Bacterial Isolates on the Dry Weight of Maize in the (2011-2012) Season

Results showed that there were numerical increases in dry weight because of the inoculation of the N_2-fixing bacterial isolates, with or without added N-fertilizer (Table 4.6). Increases in plant biomass at 30, 60 and 90 DAP were 20.3%, 72.2% and 46.2% with Isolate StB5; 13.3%, 81.9% and 41.1% with Isolate L1; 3.5%,73.6%, 37.1% with Isolate LB5, 10.5%,71.5%, 49.3% with Isolate V9; 11.2%, 93% and 27.9 with Isolate V16 under field conditions. Increases in plant biomass (dry weight) were observed due to bacterial inoculation and 33% N-fertilizer application. Increased in dry weight at 30, 60 and 90 DAP were 32.2%, 115.9% and 65.3% with Isolate StB5 + 33%N, 18.2%,106.9% and 82.4%; with Isolate L1+33N%, 16.8%, 101.2% and 35.3%; 39.9%, 124.2% and 49.1% with V9, and 32.9%, 116.6% and 59.3% with V16. Seed treatment with V16 and StB5 numerically increased the grain yield of field-grown maize by 46.1% and 41%.

Table 4.6 Maize growth parameters in the (2011/2012) Season

Treatments		**Dry weight (g plant^{-1}) in Season 2011-2012**							
N-fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**		**Yield (kg plot-1)**	
0	Control	14.3	a	56.5	a	363.3	a	2.95	a
	LB5	14.8	a	98.1	b	498	abc	3.02	ab
	V9	15.8	a	96.9	b	542.3	abc	3	ab
	V16	15.9	a	109.3	bcd	464.7	ab	3.02	ab
	L1	16.2	a	102.8	bc	512.7	abc	2.96	a
	StB5	17.2	ab	97.3	b	531	abc	3.3	abc
33	Control	26.3	bc	117.0	bcd	565	bc	4.06	bc
	LB5	16.7	ab	113.7	bcd	491.7	abc	3.94	abc
	V9	20.0	ab	126.7	d	541.7	abc	3.55	abc
	V16	19.0	ab	122.4	cd	578.7	bc	4.31	c
	L1	16.9	ab	116.9	bcd	662.7	cd	3.96	abc
	SB5	18.9	ab	122.0	cd	600.7	bcd	4.16	c
100%	NPK	34.4		131		771.3		5.27	
N-fertilizer X Isolates		P<0.001		P<0.001		P<0.001		P<0.001	
CV		21.4		6.3		10.5		0.9168	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

4.3.6 Effects of Selected N-Fixing Bacterial Isolates in the (2010-2011) Season on Maize Growth

In the 2010-2011 season inoculation of bacterial isolates with or without 33%N fertilizer caused no significant effect on maize height at 30, 60 or 90 DAP (Table 4.7).

Table 4.7 Effect of selected N-fixing bacterial isolates on the maize height in the 2010-2011 seasons

Treatments		**Height (cm) maize plant^{-1} in 2010-2011 season**					
N%-fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**	
0	Control	44.91	a	70.59	a	176.5	ab
	L1	46.37	a	68.18	a	173.3	ab
	V16	46.82	a	62.85	a	154.2	a
	LB5	48.94	a	69.19	a	159.5	a
	V9	49.81		66.06	a	171.3	ab
	StB5	59.3		67.15	a	179.2	ab
33	Control	56.36		68.89	a	176.8	ab
	L1	46.26		69.89	a	165.3	ab
	V16	47.82		67.25	a	186.7	b
	LB5	46.21	a	63.38	a	168	ab
	V9+	44.69		68.27	a	178.2	ab
	StB5	47.41		69.63	a	186	b
	NPK	57.52	a	72.85	a	176.7	ab
N-fertilizer		P=0.198		P=0.677		P=0.05	
Isolates		P=0.538		P=0.827		P=0.139	
N-fertilizer x isolates		P=0.629		P=0.694		P=0.116	
	CV%	17.1		8.7		7.4	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

-

4.3.7 Effects of Selected N-Fixing Bacterial Isolates in the 2011-2012 Season on growth of Maize

Maize height was significantly ($P < 0.001$) higher when seeds were treated with N-fixing bacteria (isolates StB5, L1, V9 and V16) after 30, 60 or 90 DAP (Table 4.8). At 30 DAP, maize plants from seeds treated with bacterial isolates and added N-fertilizer (33% N) had similar height as the control (un-treated and fertilized with 33%N). Plant heights at 30 or 60 DAP inoculated with Isolate LB5 showed no significant increases ($P < 0.05$) compared to untreated

and 0%N. At 90 DAP; plants height treated with Isolate V16 was scored equivalent plant dry weight to plants fertilized with 100% NPK.

Table 4.8 Effect of bacterial isolates on the growth of maize in the season 2011-2012

		Maize height (cm plant^{-1}) in season 2011/2012					
N%-Fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**	
0	Control	11.03	a	47.67	a	102.7	a
	LB5	17.53	ab	51.2	ab	150.7	b
	StB5	24.2	bc	63.27	cd	157.3	bc
	L1	24.47	bc	57.9	bc	158.7	bcd
	V16	27.0	c	66.67	cd	152.3	b
	V9	28.6	c	64.07	cd	159	bcd
33	Control	41.37	de	82.43	f	172	cde
	LB5	36.57	d	71.5	de	162.7	bcd
	StB5	39.83	de	83.83	f	173.7	cde
	L1	36.27	d	78.8	ef	174	cde
	V16	40.17	de	81.5	f	181.7	ef
	V9	39.83	de	83.17	f	176.7	de
100%	NPK	45.33		87.33		195	
N%-Fertilizer X Isolates	P	P<0.001		P<0.001		P<0.001	
	CV%	14.1		7.2		5.9	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

4.3.8 Effects of Selected N-Fixing Bacterial Isolates on Chlorophyll Content

In the 2010-2011 season, inoculation of selected diazotrophic with or without N-fertilizer resulted in no significant increases on the chlorophyll content both at 30 or 60 DAP (Table 4.9). In the 2011-2012 growing season, inoculation of selected isolates (Isolate V16) combined with 33% N-fertilizer caused relatively higher chlorophyll content at 60 DAP compared to untreated and fertilized control (Table 4.10). At 30DAP, Plants treated with isolates (StB5, V16 and V9) shoed significantly (P<0.001) higher chlorophyll content.

Table 4.9 Effect of Bacterial isolates on the leaf chlorophyll content in year 2010/2011

Treatments		**Chlorophyll (CCI) 2010/2011**			
N fertilizer	**Bacterial isolates**	**30DAP**		**60DAP**	
0	Control	39.95	a	69.63	a
	LB5	48.94	a	79.19	a
	L1	46.37	a	80.16	a
	StB5	59.30	a	77.15	a
	V9	45.90	a	76.49	a
	V16	47.92	a	75.55	a
33	Control	60.42	a	79.91	a
	LB5	46.21	a	73.38	a
	L1	48.98	a	78.41	a
	StB5	47.41	a	78.61	a
	V9	44.69	a	81.03	a
	V16	47.82	a	77.25	a
100%	NPK	57.52		85.11	
N-fertilizer		P=0.652	F=0.21	F=1.02	P=0.324
Treatments		P=0.591	F=0.76	F=0.66	P=0.657
N-fertilizer x Treatments		P=0.042	F=0.79	F=1.69	P=0.179
		CV%=16.1		CV%=6.7	

- Means with the same letter in the same column are not significantly different at $P \leq 0.05$

Table 4.10 Effect of Bacterial isolates on the leaf chlorophyll content in year 2011/2012

Treatments		Chlorophyll level (CCI) 2011/2012 season			
N%-Fertilizer	**Isolates**	**30DAP**		**60DAP**	
0	Control	21.22	a	18.17	a
	LB5	30.68	ab	42.93	abc
	L1	31.28	ab	37.87	abc
	StB5	34.95	b	30.93	ab
	V9	37.70	b	36.77	abc
	V16	39.63	b	41.60	abc
33	Control	39.65	b	45.28	abc
	LB5	35.45	b	54.13	bc
	L1	38.05	b	60.74	c
	StB5	37.32	b	53.60	bc
	V9	35.77	b	56.90	bc
	V16	40.63	b	59.92	c
100	NPK	38.60		57.98	
N-fertilizer		P<.001 F=22.63		P<0.001 F=46.57	
Treatments		P=0.001 F=6.07		P=0.013 F=3.75	
N-fertilizer x Treatments		P<0.001 F=7		P=0.54 F=0.742	
		CV%=9.4		CV%=20	

4.4 Discussion

Maize seeds treated with diazotrophic bacterial isolates including: *Bacillus megaterium* (V16), *Burkholderia ambiferia* (V9), *Enterobacter cloacae* (L1), *Pantoea ananatis* (LB5), *Pseudomonas s*p. (StB5, A2, A6 and A61)and formulated products of *Trichoderma harzianum* (Eco-T®) significantly increased levels of germination and vigor index. Shoot length was promoted by Isolates StB5, V16 and by Eco-T®. Root length was enhanced by Isolate StB5, V16, L1, V9 and by Eco-T®. Increases in germination, root and shoot length and seed vigor index, in response to these isolates may be associated with their ability to fix nitrogen and to produce growth promoting substances. These results are consistent with the findings of Lugtenberg and Kamilova (2009) who reported stimulation of the growth of tomato (*Lycopersicon esculent* L.), pepper (*Capsicum Annum* L.) and mung bean (*Vigna radiata* L.) plants when inoculated with *Enterobacter cloacae* CAL3. Similarly, Zakria *et al.* (2008) had reported that nitrogen fixing *Enterobacter* spp. Strain 35 stimulated the growth of *Brassica oleracea*. Lifshitz *et al*. (1987) also found that inoculation of canola (*Brassica campestris* L.) seed with a strain of *Pseudomonas putida* (Trevisan) Migula increased root length significantly. In another study by Hameeda *et al*. (2008) maize seeds inoculated with a strain of *Pseudomonas* spp. increased germination by 20–40%.

Experiments were carried out under greenhouse conditions using eight selected diazotrophic bacteria to determine their ability to fix nitrogen and enhance plant growth, with 33%N-fertilizer. Inoculation of the isolates without N-fertilizer showed no significant increase of chlorophyll content compared to un-treated control. However, maize dry weight was numerically increased. These increases of dry weight may be due to other plant growth promotion characters of the selected diazotrophic bacteria. This result indicated that growth and metabolic activity of soil microorganisms were limited by the availability of nutrients. Consequently, application of reduced N-fertilizer is needed if these diazotrophs are to be used effectively especially by commercial farmers.

Further experiments were carried out in the field for two seasons (2010/2011 and 2011/2012). Selected diazotrophic bacterial inoculation and 33% N-fertilizer application affected positively

the growth parameters investigated; especially In 2011/2012 season demonstrated the importance of evaluating potential growth promoting bacteria under a variety of experimental condition and plant growth stages. In the 2010/2011 season, growth parameters were generally showed relatively higher levels of germination as a result of these diazotrophic bacterial strains, with or without added N-fertilizer. However, germination was not significantly higher than the Unfertilized and Un-inoculated Control. The most important result was that there were no significant differences between plants fertilized with 100% NPK fertilizer and plants treated with selected diazotrophic bacteria with or without 33% N-fertilizer in plant height, dry weight and leaf chlorophyll level at 30, 60 or 90 DAP. However, yield increases were significantly higher in plants fertilized with 100%NPK compared to plants treated with selected diazotrophic bacteria with or without 33%N-fertilizer.

In the 2011/2012 season, plant height at 30, 60 or 90DAP and leaf chlorophyll content at 30 and 60 DAP showed relative increases as a result of the inoculation with selected diazotrophic bacterial isolates. Except at early stage (30 DAP), dry weight response to all inoculants at 60 clearly showed the beneficial role of these diazotrophic bacteria. Although the result showed no significant increases as result of inoculation of the selected diazotrophic bacteria, the growth parameters results were numerically higher. Similar results were reported by Ridge and Rovira (1968) in (Kloepper *et al.*, 1989), that wheat yield increased up to 43% with *Bacillus* inoculations. The enhancing effect of seed inoculation with diazotrophic bacteria on shoot dry weight and yield of maize has been reported by many researchers (Garcia de Salamone *et al.*, 1996; Dobbelaere *et al.*, 2002; Kennedy *et al.*, 2004a; Wu *et al.*, 2005; Liu *et al.*, 2006; Perin *et al.*, 2006; Shaharoona *et al.*, 2006; Gutierrez-Miceli *et al.*, 2008; Oliveira *et al.*, 2009). Such an improvement may be attributed to nitrogen-fixing and phosphate solubilizing capacity of bacteria, as well as the ability of these microorganisms to produce growth promoting substances (Kloepper *et al.*, 1991; Rodríguez and Fraga, 1999; Kloepper *et al.*, 2004).

In conclusion, Isolates V16 (*Bacillus megaterium*), StB5 (*Pseudomonas nitroreducens*), V9 (*Burkholderia ambifaria*), L1 (*Enterobacter cloacae*) and LB5 (*Pantoea ananatis)* may have plant growth promoting action, together with the ability to fix nitrogen.

4.5 References

Abdul- Baki A., Anderson, J.D. (1973) Vigor determination in soybean seed by multiple criteria. Crop Science 13: 630-633.

Bashan Y., Holguin G., L.E. D.-B. (2004) *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Canadian Journal of Microbiology 50:521-577.

Bevivino A., Tabacchioni S., Chiarini L., Carusi M.V., Del Gallo M., Visca P. (1994) Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. Microbiology 140:1069-1077.

Boddey R.M., Baldani V.L.D., Baldani J.I., Döbereiner J. (1986) Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field-grown wheat. Plant and Soil 95:109-121.

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

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

Çakmakçi R., Dönmez F., Aydın A., Şahin F. (2006b) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biology and Biochemistry 38:1482-1487. de Freitas J.R. (2000) Yield and N assimilation of winter wheat (*Triticum aestivum* L., var. Norstar) inoculated with rhizobacteria. Pedobiologia 44:97-104.

Divan Baldani V.L., Baldani J.I., Döbereiner J. (2000) Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. Biology and Fertility of Soils 30:485-491.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Okon Y., Vanderleyden J. (2002) Effect of inoculation with wild type *Azospirillum brasilense* and *A. irakense* strains on development and nitrogen uptake of spring wheat and grain maize. Biology and Fertility of Soils 36:284-297.

Dobbelaere S., Vanderleyden J., Okon Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22:107-149.

Duponnois R., Mateille T. (1999) Beneficial effects of *Enterobacter cloacae* and *Pseudomonas mendocina* for biocontrol of *Meloidogyne incognita* with the endospore-forming bacterium *Pasteuria penetrans*. Nematology 1:95-101.

Egener T., Hurek T., Reinhold-Hurek B. (1999) Endophytic expression of nif genes of *Azoarcus* sp. strain BH72 in rice roots. Molecular Plant-Microbe Interactions 12:813-819.

Frommel M., Nowak J., Lazarovits G. (1993) Treatment of potato tubers with a growth promoting *Pseudomonas* sp.: Plant growth responses and bacterium distribution in the rhizosphere. Plant and Soil 150:51-60.

Garcia de Salamone I., Döbereiner J., Urquiaga S., Boddey R. (1996) Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by the 15 N isotope dilution technique. Biology and Fertility of Soils 23:249-256.

Garcia de Salomone I., Döbereiner J. (1996) Maize genotype effects on the response to *Azospirillum* inoculation. Biology and Fertility of Soils 21:193-196.

Gutierrez-Miceli F., Moguel-Zamudio B., Abud-Archila M., Gutierrez-Oliva V., Dendooven L. (2008) Sheep manure vermin-compost supplemented with a native diazotrophic bacteria and mycorrhizas for maize cultivation. Bioresource Technology 99:7020-7026.

Hameeda B., Harini G., Rupela O., Wani S., Reddy G. (2008) Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiological Research 163:234-242.

Hashem M.A. (2001) Problems and prospects of cyanobacterial biofertilizer for rice cultivation. Functional Plant Biology 28:881-888.

Hurek T., Handley L.L., Reinhold-Hurek B., PichÃ Y. (2002) *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Molecular Plant-microbe Interactions 15:233-242.

Isopi R., Fabbri P., Del Gallo M., Puppi G. (1995) Dual inoculation of *Sorghum bicolor* (L.) Moench ssp. *bicolor* with vesicular arbuscular mycorrhizas and *Acetobacter diazotrophicus*. Symbiosis 18:43-55.

James E.K., Gyaneshwar P., Mathan N., Barraquio W.L., Reddy P.M., Iannetta P.P.M., Olivares F.L., Ladha J.K. (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. Molecular Plant-microbe Interactions 15:894-906.

James E.K., Olivares F.L., Baldani J.I., Döbereiner J. (1997) *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue *Sorghum bicolor* L. Moench. Journal of Experimental Botany 48:785-798.

Kennedy I.R., Choudhury A., Kecskés M.L. (2004a) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. (2004b) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.Kloepper J.W., Leong J., Teintze M., Schroth M.N. (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886.Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7:39-44.

Kloepper J.W., Ryu C.M., Zhang S. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Kloepper J.W., Zablotowicz R.M., Tipping E.M., Lifshitz R. (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. In, The Rhizosphere and Plant Growth. Keister, D.L. and Cregan, P.B. (Eds.). Kluwer Academic Publishers. The Netherlands. Pp. 315-326.

Lifshitz R., Kloepper J.W., Kozlowski M., Simonson C., Carlson J., Tipping E.M., Zaleska I. (1987) Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. Canadian Journal of Microbiology 33:390-395.

Liu X., Zhao H., Chen S. (2006) Colonization of maize and rice plants by strain *Bacillus megaterium* C4. Current Microbiology 52:186-190. DOI: 10.1007/s00284-005-0162-3.

López-Bucio J., Campos-Cuevas J.C., Hernández-Calderón E., Velásquez-Becerra C., Farías-Rodríguez R., Macías-Rodríguez L.I., Valencia-Cantero E. (2007) *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin-and ethylene-independent signaling mechanism in *Arabidopsis thaliana*. Molecular Plant-Microbe Interactions 20:207-217.

Lugtenberg B., Kamilova F. (2009) Plant-growth-promoting rhizobacteria. Annual Review of Microbiology 63:541-556.

Lynch, P.J. (2007). Roots of the second green revolution. *Australian Journal of Botany* **55**, 493-512.

Malik K.A., Bilal R., Mehnaz S., Rasul G., Mirza M.S., Ali S. (1997b) Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant and Soil 194:37-44.

Mar Vázquez M., César S., Azcón R., Barea J.M. (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Applied Soil Ecology 15:261-272.

Mrkovacki N., Milic V. (2001) Use of *Azotobacter chroococcum* as potentially useful in agricultural application. Annals of Microbiology 51:145-158.

Obreht Z., Kerby N.W., Gantar M., Rowell P. (1993) Effects of root-associated N_2-fixing cyanobacteria on the growth and nitrogen content of wheat (*Triticum vulgare* L.) seedlings. Biology and Fertility of Soils 15:68-72.

Okon Y., Labandera-Gonzalez C.A. (1994) Agronomic applications of A*zospirillum*: An evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry 26:1591-1601. Oliveira C., Alves V., Marriel I., Gomes E., Scotti M., Carneiro N., Guimaraes C., Schaffert R., Sá N. (2009) Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biology and Biochemistry 41:1782-1787.

Omar M.N.A., Hamouda A.M., Mahrous N.M. (1996) Evaluation the efficiency of inoculation some diazotrophs on yield and protein content of three wheat cultivars under graded levels of nitrogen fertilization. Annals of Agricultural Science Ain-Shams University 41 (2): 579 – 590.

Ortíz-Castro R., Valencia-Cantero E., López-Bucio J. (2008) Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. Plant Signaling and Behavior 3:263-265.

Pandey A., Sharma E., Palni L.M.S. (1998) Influence of bacterial inoculation on maize in upland farming systems of the Sikkim Himalaya. Soil Biology and Biochemistry 30:379-384.

Perin L., Martínez-Aguilar L., Castro-González R., Estrada-de Los Santos P., Cabellos-Avelar T., Guedes H., Reis V., Caballero-Mellado J. (2006) Diazotrophic *Burkholderia* species

associated with field-grown maize and sugarcane. Applied and Environmental Microbiology 72:3103-3110.

Rodríguez H., Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17:319-339.

Sevilla M., Burris R.H., Gunapala N., Kennedy C. (2001) Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and nif mutant strains. Molecular Plant-Microbe Interactions 14:358-366.

Shaharoona B., Arshad M., Zahir Z.A., Khalid A. (2006) Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. Soil Biology and Biochemistry 38:2971-2975.

Shaharoona B., Jamro G., Zahir Z., Arshad M., Memon K. (2007) Effectiveness of various *Pseudomonas* spp. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.). Journal of Microbiology and Biotechnology 17:1300-1307.

Spaepen S., Vanderleyden J., Okon Y. (2009) Plant growth-promoting actions of rhizobacteria. Advances in Botanical Research 51:283-320.

Stein T., Hayen-Schneg N., Fendrik I. (1997) Contribution of BNF by *Azoarcus* sp. BH72 in *Sorghum vulgare*. Soil Biology and Biochemistry 29:969-971.

Taulé C., Mareque C., Barlocco C., Hackembruch F., Reis V.M., Sicardi M., Battistoni F. (2012) The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum* L.), and the identification and characterization of part of the associated diazotrophic bacterial community. Plant and Soil 356:35-49.

Wu S., Cao Z., Li Z., Cheung K., Wong M. (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155-166.

Yobo, K., M. Laing, C. Hunter and M. Morris. 2004. Biological control of *Rhizoctonia solani* by two *Trichoderma* species isolated from South African composted soil. South African Journal of Plant and Soil 21: 139-144.

Zakria M., Ohsako A., Saeki Y., Yamamoto A., Akao S. (2008) Colonization and growth promotion characteristics of *Enterobacter* sp. and *Herbaspirillum* sp. on *Brassica oleracea*. Soil Science and Plant Nutrition 54:507-516.

CHAPTER FIVE
MAIZE RESPONSE TO INOCULATION OF DIAZOTROPHS AT VARIOUS LEVELS OF NITROGEN FERTILIZATION: GREENHOUSE STUDY

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg,

South Africa

Abstract

Maize response to inoculation with three diazotrophic isolates V16 (*Bacillus megaterium*), L1 (*Enterobacter* spp.) and V9 (*Burkholderia* spp.) was studied when combined with five levels of N fertilization (0%, 25%, 50%, 75 and 100%) under greenhouse condition. Seed inoculation with Isolate V16, L1 and V9 significantly affected dry weight, chlorophyll content and plant height. Inoculation with Isolate L1 (*Enterobacter* spp.) increased dry weight, plant height and chlorophyll content by 12.8%, 34.4% and 32.1%, respectively, compared to the untreated and unfertilized control. Isolate L1 at 25% of the recommended N level increased dry weight, plant height and chlorophyll content by 11.87, 2.01 and 43.42% compared to the Uninoculated + 25%N. Inoculation with Isolate V9 (*Burkholderia* spp.) increased dry weight by 17%, chlorophyll content by 16.5% and plant height by 53.68%, compared to untreated and unfertilized control. This diazotrophic strain plus 25% of the recommended rate of N increased dry weight by 11.3%, chlorophyll content by 20.88% and plant height by 18.63% than the control of untreated and fertilized with 25% of recommended N level. Isolate V16 increased dry weight, plant height and chlorophyll content by 30.87%, 71.05% and 35.27%, respectively, compared to the untreated and unfertilized control. At 25% of the recommended N level, Isolate V16 enhanced dry weight, plant height and chlorophyll content by 22.14, 42.63 and 33.13%, respectively, compared to the untreated and 25% of recommended N level.. Diazotrophic treatments in response to concurrent N applications can be able to be determined by chlorophyll content meter (CCM) reading. The result showed that when the levels of N-fertilizer increased, the chlorophyll content also increased. Correlation analysis indicated that 98% of the variation in N application levels was predicted by CCM readings. Extractable total chlorophyll, chlorophyll a and chlorophyll b were also linearly correlated with the chlorophyll content meter readings (**y** =

0.070**x** – 3.199, $\mathbf{r}^2$ = 0.80; **y** = 0.119**x** – 2.355, $\mathbf{r}^2$ = 0.81; **y** = 0.066**x** – 4.012, $\mathbf{r}^2$ = 0.79), respectively.

Key words: Chlorophyll content index, diazotrophic bacterial isolates, maize growth and CCM-200

5.1 Introduction

The rising concern regarding nitrogen fertilizer production has been highlighted on a global stage by increases in global oil prices. Replacement of chemical fertilizers with biofertilizers is an attractive goal for sustainable agriculture. Nitrogen is the macro-nutrient that most frequently limits the growth and productivity of non-leguminous plants (Schepers *et al.*, 1992) and it is the most limiting factor in maize production (McCarty and Meisinger, 1997). A number of diazotrophic bacteria were previously found to interact with plants either in the rhizosphere or endophytic. Given the ability of diazotrophs to fix N, some strain may relieve N-deficiencies where there is inadequate application of N fertilizers. The genera *Bacillus*, *Burkholderia* and *Enterobacter* are known to penetrate the roots of cereals and grow intercellular as root endophytes as well as growing in the rhizosphere (Reinhold-Hurek and Hurek, 1998; Wakelin and Ryder, 2004).

Numerous *Bacillus* strains express plant growth promoting (PGP) activities. Besides having PGP properties, some strains can also fix nitrogen. When strains of *Bacillus* sp. were inoculated onto *Pinus contorta* Dougl seedlings, they contributed 4% of seedling foliar nitrogen (Chanway and Holl, 1991). In another study, *Bacillus* M3 alone or in combination with *Bacillus* OSU-142 increased yield, growth and nutrition of raspberry (*Rubus idaeus* L.) plants grown under organic growing conditions (Orhan *et al.*, 2006). *Burkholderia* is a genus rich in plant-associated nitrogen-fixers (Caballero-Mellado *et al.*, 2004). Many N_2-fixing isolates *Burkholderia* have been recovered from the rhizosphere, or as endophytes, from sugarcane (*Saccharum officinarum* L.), maize (*Zea mays* L.) and teosinte (*Zea diploperennis* H.) plants in Brazil, Mexico and South Africa (Estrada *et al.*, 2002; Reis *et al.*, 2004).Some are novel *Burkholderia* species (Perin *et al.*, 2006). When rice was inoculated with *B. vietnamiensis* in field trials, it increased grain yields up to 0.8 t ha^{-1} and fixed 25-30kg N ha^{-1}(Tran Van *et al.*, 2000). *B.vietnamiensis* can fix 19% of the

rice plant N from the atmosphere (Baldani *et al.*, 2000). Montañez *et al*. (2012) showed that maize (*Zea mays* L.) can also establish beneficial associations with various nitrogen fixing and plant growth promoting bacteria (PGPB). Raju *et al*. (1972) isolated nitrogen fixing *Enterobacter cloacae* from maize plants. Nelson and Craft (1991) showed that *Enterobacter cloacae* also controlled *Sclerotinia homoeocarpa* F.T.Benn. It has been used as biocontrol agent for the control of postharvest diseases of fruits and vegetables, and as a pre-plant seed treatment for suppression of damping-off (Hinton and Bacon, 1995).

The use of diazotrophic bacteria as biofertilizers for agriculture has been the focus of numerous studies. Inoculation of sugarcane with diazotrophic endophytes resulted in increases in production of up to 35% (Boddey *et al.*, 2003), and between 7.1% and 31.9% of dry mass increase (de Oliveira *et al.*, 2006). However, there are significant challenges to predict the level of N supplied by these diazotrophic bacterial strains to plants. The standard methods for determining plant N status involve extractions and spectrophotometric determinations. Typically, a sample must be detached, ground up in a solvent and assayed in a spectrophotometer. However, these methods are destructive and time consuming (Smith *et al.*, 1998). Bullock and Anderson (1998) showed that leaf N content is highly correlated with leaf chlorophyll (CHL) concentration. Cate and Perkins (2003) also showed chlorophyll concentrations correlate positively with leaf N. This relationship should make it possible to use leaf chlorophyll content to estimate crop N status (Daughtry *et al.*, 2000). Development of portable chlorophyll meters (Opti-Sciences, Inc. Hudson, USA), that take instantaneous measurements of chlorophyll without leaf destruction, has emerged as tool to indirectly assess plant N status (Waskom *et al.*, 1996). It is a hand held device, which relies on transmittance and absorbance of light to assess the leaf chlorophyll content of plants (Pal *et al.*, 2012).

The objectives of this study were to evaluate the potential of different diazotrophic inoculants for maize growth at different rates of N fertilization.

5.1.1 Methods and MaterialSource of inoculum and preparation

Bacterial isolates were isolated from the soil rhizosphere, root or leaves of different plants using standard isolation procedures and selected through an *in vitro* studies and greenhouse study (Chapter 2) and were also assessed for their effects on germination of wheat *in vitro* (Chapter 3) and maize growth in field (chapter 4).

5.1.2 Source of Seeds

Seeds of white maize of the cultivar, Mac's Medium Pearl, (an open pollinated variety) were bought from McDonalds Seeds®[18].

5.1.3 Seed Inoculation

Maize seeds were inoculated prior to planting by coating seeds with a bacterial suspension in gum arabic. The seed was treated with different diazotrophic bacterial isolates suspension amended with 2% gum Arabic. Cell numbers were adjusted to 10^8 cfu ml^{-1} using distilled water. Cell number per seed was verified after inoculation by suspending seeds in water and plating various dilutions on nutrient agar plates. Seeds were planted within 24-48 h after inoculation.

5.1.4 Fertilizer

Limestone Ammonium Nitrate (LAN)[19] was used for N- fertilizer source with levels of, 100%N (400kg ha^{-1}), 75%N (300kg ha^{-1}), 50%N (200kg ha^{-1}), 25%N (100kgha^{-1}) and 0%N (0kgha^{-1}). Super phosphate[20] and Potash[21] were used as sources of P and K, respectively. Full amount of P and K were used as recommended by local Fertilizer Advisory Center, Cedara, Pietermaritzburg, South Africa.

[18]McDonalds Seed Company (Pty) Ltd., Pietermaritzburg, South Africa
[19] Sasol Nitro a division of Sasol chemical industries Ltd. P.O.Box 5486, Johannesburg 2000, Republic of South Africa
[20]Omnia Fertilizer Group (Pty) Ltd. P.O.Box 69888, Bryanston,2021, South Africa
[21]Omnia Fertilizer Group (Pty) Ltd. P.O.Box 69888, Bryanston,2021, South Africa

5.1.5 Chlorophyll Content Meter (CCM) Readings

We used a hand-held CCM-200 chlorophyll content meter (Opti-Sciences, Inc. Hudson, USA)[22]. The CCM-200 Plus, has a 0.71-cm^2 measurement area, and calculates a chlorophyll content index (CCI) based on absorbance measurements at 660 and 940 nm. The claimed accuracy of the CCM-200 is ±1.0 CCI units. For scientists and farmers with limited direct access to laboratory analysis for N, the meter provides a cheap and convenient estimate of chlorophyll content per unit leaf area during vegetative growth. The CCI was sampled on five leaves from each branch segment with the CCM sensing head held as close as possible to the junction of the central vein and the next adjacent major vein without including major vein tissue under the sensor. Five non-overlapping measurements were taken on each leaf from homogeneous, healthy leaf tissue, and a mean value calculated from the measurements for each maize plant.

5.1.6 Chlorophyll Extraction

After the CCI had been sampled, five 6.4-mm diameter disks were punched from each leaf in the approximate locations of the CCI measurements. The disks were extracted in 80% (v/v) acetone at 4 °C in the dark. Transmittance of the extract was measured with a Spectronic-Unicam Genesis/8 spectrophotometer. 1.5 ml of each extract was then transferred to disposable polystyrene cuvettes. The spectrophotometer (range 200–1100 nm, spectral band width 5 nm, wave length accuracy ±1 nm, and wavelength setting repeatability of ±0.3 nm; model U-1100, Hitachi Ltd, Tokyo, Japan), was calibrated to zero absorbance using a blank of 80%(v/v) acetone. Absorbance of both blank and sample were measured at 645 and 663 nm. Total chlorophyll was calculated according to Wellburn (1994). The equation of Arnon (1949) modified by Porra (2002) was used to calculate the chlorophyll concentration: CHL_a (µg g^{-1} of fresh weight) = 12.25A663.2 – 2.79A646.8; CHL_b (µg g^{-1} of fresh weight) = 21.5A646 – 5.1A663; CHL_{a+b} (µg g^{-1} of fresh weight)=(1000A470 –1.82Ca – 85.02Cb)/198.

[22] Optic-Sciences (Pty) Ltd, 8 Winn Avenue. Hudson, NH 03051, USA

5.1.7 Statistical Analysis

Experiments were repeated twice and results were pooled and averaged. Data was analyzed using GenStat® 14th Executable release Statistical Analysis Software. A 4x5 factoral ANOVA was used to analized the data. Differences between treatments were distinguished using Duncan's Multiple Range test (DMRT) at 5% significance level

5.2 Results

5.2.1 Chlorophyll Readings of Maize Plants at Six Leave Stage

Mean chlorophyll meter reading for each treatment was expressed as a chlorophyll content index (CCI) of the mean reading for the different levels of N-fertilized treatment. N fertilizer increment was compared to the chlorophyll meter reading at the six leaf stage of the maize plants. When levels of N-fertilizer increased, the chlorophyll content also increased (Figure 5.1). Levels of N-fertilizer application were correlated 96%, 97% and 99% maize response in CCI, dry weight and height, respectively.

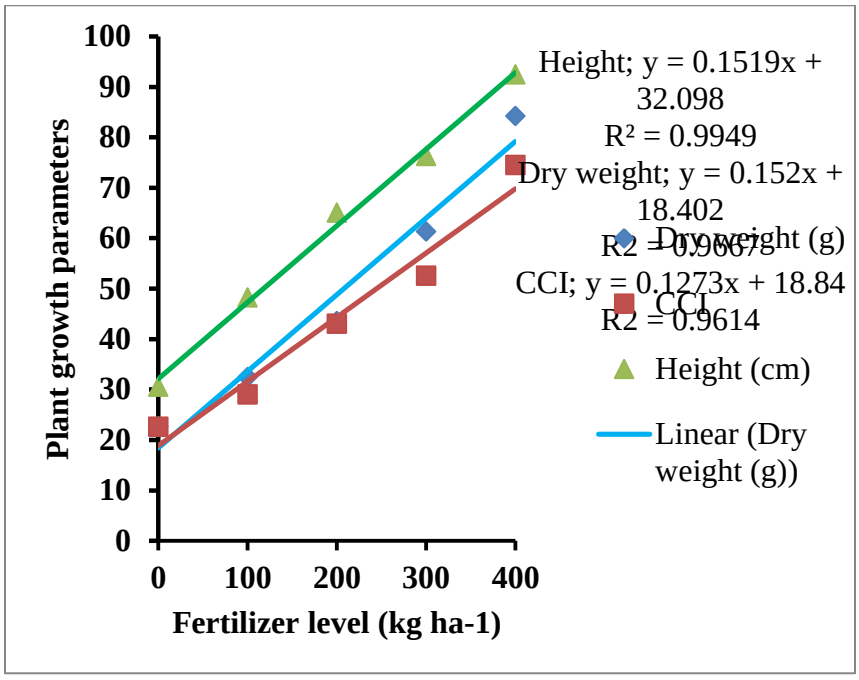

Figure 5.1 Relationship between N fertilizer levels and CCI, dry weight and height of maize inoculated with three different diazotrophs

5.2.2 Extractable chlorophyll (a) values versus chlorophyll content meter (CCM) readings

Extractable chlorophyll (a) values from maize leaves at the 6 leaf stage ranged from 27.61 to 122.35 (µg g^{-1} of fresh weight). The relationship between extractable chlorophyll (a) and CCI was significantly linear, with an r^2 indicating that 81% ($P < 0.001$) of the variation was explained by a linear model (Figure 5.2). The relationship of CCI and chlorophyll (a) demonstrated the accuracy of chlorophyll meter readings.

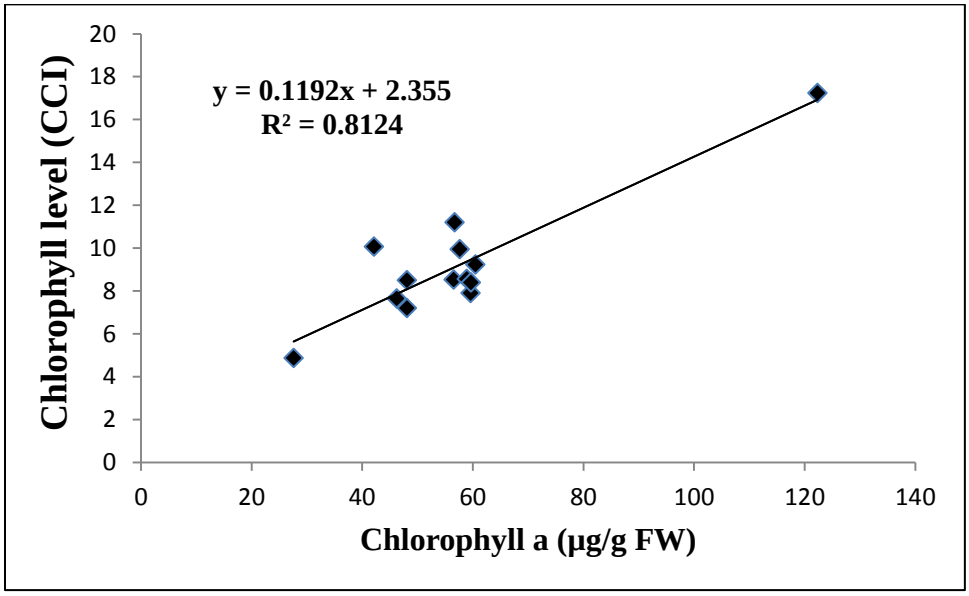

Figure 5.2 Relationship between chlorophyll a in the leaves of maize plants, and chlorophyll meter readings (CCI)

5.2.3 Extractable Chlorophyll (b) Values Verses Chlorophyll Content Meter (CCM) Readings

Extractable chlorophyll (b) values from maize leaves at the 6 leaf stage ranged from 33.74 to 199.43 (µg g^{-1} of fresh weight). The relationship between extractable chlorophyll (b) and CCI was significantly linear, with an r^2 indicating that 79% ($P < 0.001$) of the variation was explained by a linear model (Figure 5.3).

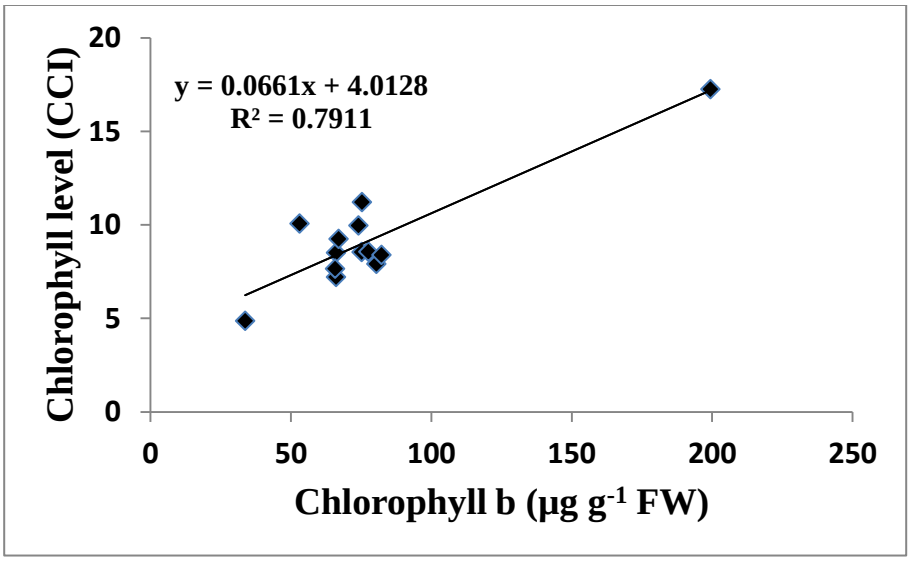

Figure 5.3 Relationship between chlorophyll (b) in the leaves of maize plants, and Chlorophyll meter readings (CCI) inoculated with different diazotrophic bacteria

5.2.4 Extractable Total Chlorophyll (a + b) Verses Chlorophyll Content Meter (CCM)

Extractable chlorophyll (a+b) values from maize leaves at the 6 leaf stage ranged from 39.03 to 198(µg g^{-1} of fresh weight). The relationship between extractable total chlorophyll (a + b) and CCI was significantly linear, with an r^2 indicating that 80% ($P < 0.001$) of the variation was explained by a linear model (Figure 5.4).

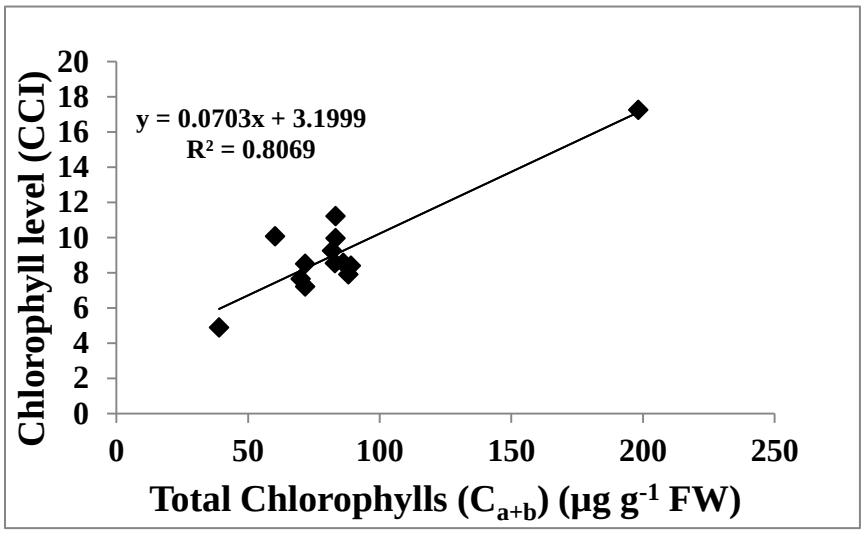

Figure 5.4 Relationship between chlorophyll (a+b) in the leaves of maize plants, and Chlorophyll meter readings (CCI) inoculated with different diazotrophic bacteria

5.2.5 Relationship between N-Fertilizer Application and Different Diazotrophs Inoculants on the Growth of Maize Plants

Three bacterial strains × five N-fertilizer level combinations were tested. Three of the diazotrophs showed no siginificant increases of dry weight, height and chlorophyll content when 0% N source was provided (Table 5) compared to the untreated and unfertilized control. Mean chlorophyll meter readings varied on plant dry weight treated with different diazotrophs and N-fertilizer levels. Plants inoculated with these diazotrophs plus 25%N- fertilizer showed no significant increases in dry weight, CCI compared to the untreated control + 25% N (Table 5.1). Significant increases in plant height were observed when isolates L1 and V16 combined with 25%N fertilizer. Plants treated with diazotrophic bacteria plus N-fertilizer recoreded numerical higher plant heights and dry weight than solo applications of N-fertilizer levels (Table 5.5).

Inoculation of maize seeds with *Enterobacter* sp., and *Bacillus megaterium* alone significantly increased dry weight ($P = 0.005$) and plant height ($P = 0.003$) (Table 5.1). Applications of N-fertilizer levels also significantly increased dry weight ($P < 0.001$) and plant height ($P < 0.001$). However, there was no interaction ($P = 0.687$ and $P=0.0653$) between applications of different N-fertilizer levels in combination with any of the diazotrophic inoculants. Inoculation of diazotrophic bacteria alone did not affect ($P = 0.159$) the chlorophyll content index (CCI).

Table 5.1 Effects of diazotrophs in combination with different levels of N–fertilizer on maize growth

Treatments		Growth parameters					
N-Fertilizer	**Isolates**	**Dry weight (g)**		**Chlorophyll (CCI)**		**Plant height (cm)**	
0	Control	22.61	a	22.57	a	30.50	a
	L1	25.51	ab	30.33	ab	40.30	b
	V9	26.59	ab	26.30	a	46.93	bc
	V16	29.59	abc	30.53	ab	52.17	cd
25	Control	32.43	bcd	28.97	ab	48.20	bc
	L1	36.28	cde	41.55	abcd	58.81	def
	V9	36.10	cde	35.02	abc	57.18	cde
	V16	39.61	def	41.32	abcd	64.17	ef
50	Control	43.49	efg	43.02	abcde	64.99	ef
	L1	46.02	fg	48.48	bcde	67.39	efg
	V9	44.63	fg	40.03	abcd	66.89	efg
	V16	50.45	g	50.10	bcde	68.42	fg
75	Control	61.27	h	52.50	cdef	76.26	gh
	L1	65.94	h	63.67	efg	77.00	gh
	V9	64.18	h	53.70	cdefg	77.40	gh
	V16	65.02	h	71.94	fg	77.00	gh
100	Control	84.17	ij	74.47	g	92.40	i
	L1	88.3	j	56.07	cdefg	84.40	hi
	V9	78.03	i	54.8	cdefg	92.73	i
	V16	87.86	j	59.63	defg	91.47	i
N-fertilized		F=327.51	P=<0.001	F=21.75	P<0.001	F=117.36	P=<0.001
Isolates		F=4.96	P=0.005	F=1.82	P=0.159	F5.41	p=0.003
N-fertilizer X Isolates		f=0.76	p=0.0687	F=0.79	P=0.653	F=1.92	P=0.063
		CV%=8.7		CV%=24		CV%=8.8	

Treatment means followed by the same letter are not significant at P > 0.05 probability level

5.3 Discussion

Maize is one of the most widely cultivated cereals in the world, and its production is highly dependent on chemically produced nitrogen fertilizers. In this study on the inoculation of three diazotrophs (*Enterobacter* sp., *Bacillus megaterium* and *Burkholderia* sp.) alone or combined with N-fertilizer at all levels confirmed that diazotrophs can make contribution to maize growth and that there are differences in their capacity to support N_2 fixation. There were increases in plant height as a function of the N-fertilization level. The integration of biological nitrogen fixation (BNF) into crop production strategies may improve the sustainability of agricultural systems. In addition to their ability to fix nitrogen, these diazotrophs may stimulate plant growth indirectly through a combination of mechanisms, such as the synthesis of phytohormones and vitamins, the inhibition of plant ethylene synthesis, the stimulation of nutrient uptake (solubilization of inorganic phosphate, as well as mineralization of organic phosphate), and improvement of stress resistance and control of pathogenic microorganisms (Berge *et al.*, 1990; Triplett, 1996). The screening of diazotrophs which have the ability to fix nitrogen and promote plant growth directly or indirectly is a key factor for the eventual reduced application of N-fertilizer to several important crops such as wheat, maize and other plants. Inoculation of maize with *Burkholderia* sp. (V9), *Enterobacter* sp. (L1) and *Bacillus megaterium* (V16) caused numeical increases in dry weight, cholorphyll content and plant height, but not statistically significant. The growth of *Sorghum bicolor* was positively influenced by the inoculation of *Enterobacter* sp. strain BB23 (Chiarini *et al.*, 1998). Similarly, *Bacillus* M3 alone or in combination with *Bacillus* OSU-142 increased yield, growth and nutrition of raspberry plant under organic growing conditions (Orhan *et al*., 2006). The effect of the inoculation of diazotrophs on cereal productivity may also depend on plant genotype, bacterial strain, and soil type (Baldani *et al.*, 1987) as well as environmental conditions (Bhattarai and Hess, 1993). In this experiment, we have demonstrated that inoculation of diazotrophic isolates had significant ($P<0.001$) effects on plant highet at 25%N fertilizer. This suggests that the use of diazotrophs as plant growth promoting bacteria will be most valuable for maize production in low N-input agriculture.

Our result showed a strong linear correlation between extractable total chlorophyll content and CCI values. A similar result was reported by Cate and Perkins (2003) in different plant species. van den Berg and Perkins (2004) reported that 64% of the variation in N was predicted by CCI in

leaves of sugar maple. Study by Pal *et al*.(2012) found more than 80% of the variation in N was predicted by the CCM-200 reading. This indicates that the CCM is an effective tool for the rapid and non-destructive estimation of chlorophyll content in maize leaves. Once general relationships are established for a particular crop species, it should be possible to use the CCM as a tool for a variety of management and research applications.

5.4 Reference

Arnon D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Betavulgaris*. Plant Physiology 24:1-15.

Baldani V.L.D., Baldani J.I., Döbereiner J. (1987) Inoculation of field grown wheat (*Triticum aestivum*) with *Azospirillum* spp. in Brazil. Biology and Fertility of Soils 4:37-40.

Baldani V.L.D., Baldani J.I., Döbereiner J. (2000) Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. Biology and Fertility of Soils 30:485-491.

Berge O., Fages J., Mulard D., Balandreau J. (1990) Effects of inoculation with *Bacillus circulans* and *Azospirillum lipoferum* on crop-yield in field grown maize. Symbiosis 9:259-266.

Bhattarai T., Hess D. (1993) Yield responses of Nepalese spring wheat (*Triticum aestivum* L.) cultivars to inoculation with *Azospirillum* spp. of Nepalese origin. Plant and Soil 151:67-76.

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

Bullock D., Anderson D. (1998) Evaluation of the Minolta SPAD-502 chlorophyll meter for nitrogen management in corn. Journal of Plant Nutrition 21:741-755.

Caballero-Mellado J., Martínez-Aguilar L., Paredes-Valdez G., Estrada-de los Santos P. (2004) *Burkholderia unamae* sp. *nov*., an N_2-fixing rhizospheric and endophytic species. International Journal of Systematic and Evolutionary Microbiology 54:1165-1172.

Cate T.M., Perkins T. (2003) Chlorophyll content monitoring in sugar maple (*Acer saccharum*). Tree Physiology 23:1077-1079.

Chanway C., Holl F. (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Canadian Journal of Botany 69:507-511.

Chiarini L., Bevivino A., Tabacchioni S., Dalmastri C. (1998) Inoculation of *Burkholderia cepacia*, *Pseudomonas fluorescens* and *Enterobacter* sp. on *Sorghum bicolor*: Root colonization and plant growth promotion of dual strain inocula. Soil Biology and Biochemistry 30:81-87.

Daughtry C., Walthall C., Kim M., De Colstoun E.B., McMurtrey J. (2000) Estimating corn leaf chlorophyll concentration from leaf and canopy reflectance. Remote Sensing of Environment 74:229-239.

de Oliveira A.L.M., de Canuto E.L., Urquiaga S., Reis V.M., Baldani J.I. (2006) Yield of micropropagated sugarcane varieties in different soil types following inoculation with diazotrophic bacteria. Plant and Soil 284:23-32.

Estrada P., Mavingui P., Cournoyer B., Fontaine F., Balandreau J., Caballero-Mellado J. (2002) A N2-fixing endophytic *Burkholderia* sp. associated with maize plants cultivated in Mexico. Canadian Journal of Microbiology 48:285-294.

Hinton D.M., Bacon C.W. (1995) *Enterobacter cloacae* is an endophytic symbiont of corn. Mycopathologia 129:117-125.

McCarty G., Meisinger J. (1997) Effects of N fertilizer treatments on biologically active N pools in soils under plow and no tillage. Biology and Fertility of Soils 24:406-412.

Montañez A., Blanco A.R., Barlocco C., Beracochea M., Sicardi M. (2012) Characterization of cultivable putative endophytic plant growth promoting bacteria associated with maize cultivars (*Zea mays* L.) and their inoculation effects in vitro. Applied Soil Ecology 58:21-28. Nelson E.B., Craft C. (1991) Introduction and establishment of strains of *Enterobacter cloacae* in golf course turf for the biological control of dollar spot. Plant Diseases 75:510-514.

Orhan E., Esitken A., Ercisli S., Turan M., Sahin F. (2006) Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Scientia Horticulturae 111:38-43.

Pal P.K., Singh R.D., Prasad R. (2012) Non-destructive estimation of chlorophyll and nitrogen content in leaf of *Rosa damascena* (Mill). Soil Science and Plant Nutrition 58:604-610.

Perin L., Martínez-Aguilar L., Castro-González R., Estrada-de Los Santos P., Cabellos-Avelar T., Guedes H., Reis V., Caballero-Mellado J. (2006) Diazotrophic *Burkholderia* species associated with field-grown maize and sugarcane. Applied and Environmental Microbiology 72:3103-3110.

Porra R.J. (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynthesis Research 73:149-156.

Raju P., Evans H.J., Seidler R.J. (1972) An asymbiotic nitrogen-fixing bacterium from the root environment of corn. Proceedings of the National Academy of Sciences 69:3474-3478.

Reinhold-Hurek B., Hurek T. (1998) Life in grasses: diazotrophic endophytes. Trends in Microbiology 6:139-144.

Reis V., Estrada-De los Santos P., Tenorio-Salgado S., Vogel J., Stoffels M., Guyon S., Mavingui P., Baldani V., Schmid M., Baldani J. (2004) *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. International Journal of Systematic and Evolutionary Microbiology 54:2155-2162.

Schepers J., Francis D., Vigil M., Below F. (1992) Comparison of corn leaf nitrogen concentration and chlorophyll meter readings. Communications in Soil Science and Plant Analysis 23:2173-2187.

Smith C., Munson A., Coyea M. (1998) Nitrogen and phosphorus release from humus and mineral soil under black spruce forests in central Quebec. Soil Biology and Biochemistry 30:1491-1500.

Tran Van V., Berge O., Ngo Ke S., Balandreau J., Heulin T. (2000) Repeated beneficial effects of rice inoculation with a strain of *Burkholderiavietnamiensis*on early and late yield components in low fertility sulphate acid soils of Vietnam. Plant and Soil 218:273-284.

Triplett E.W. (1996) Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. Plant Soil 186:29-38..

van den Berg A.K., Perkins T.D. (2004) Evaluation of a portable chlorophyll meter to estimate chlorophyll and nitrogen contents in sugar maple (*Acer saccharum* Marsh.) leaves. Forest Ecology and Management 200:113-117.

Wakelin S.A., Ryder M.H. (2004) Plant growth-promoting inoculants in Australian agriculture. Online. Crop Management.

Waskom R., Westfall D., Spellman D., Soltanpour P. (1996) Monitoring nitrogen status of corn with a portable chlorophyll meter. Communications in Soil Science and Plant Analysis 27:545-560.

Wellburn R. (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of Plant Physiology 144:307-313.

CHAPTER SIX

EVALUATION OF THE EFFECT OF DIAZOTROPHS ON WHEAT GROWTH UNDER GREENHOUSE AND FIELD CONDITIONS

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, South Africa

Abstract

The effect of diazotrophic bacteria on seed germination, seedling growth and yield of greenhouse and field grown winter wheat were evaluated in greenhouse trials, and field trials in 2011 and 2012. In these experiments five diazotrophic bacterial strains were used. These bacteria had been isolated previously: V16 (*Bacillus megaterium*), V9 (*Burkholderia ambiferia*), Stb5 (*Pseudomonas* sp.), L1 (*Enterobacter cloacae*) and LB5 (*Pantoea ananatis*). In laboratory studies, seed inoculation significantly enhanced seed germination and seedling vigour index. Their effect on the growth of winter wheat was measured in greenhouse trials where each bacterium isolate was combined with different levels of chemical fertilizers. Diazotrophic bacterial inoculation with a combination of different levels of NPK fertilizer significantly ($P < 0.001$) increased dry weight by 3.3% to 104%. Maximum dry weight of biomass (104%) was obtained when fertilizer was applied at 65% NPK (of optimum fertilization level) together with Isolate L1. In a field trial in 2011 plant dry weight, number of spikes, straw dry weight and yield were numerically ($P > 0.05$) higher than the untreated and unfertilized control. In the 2012 field trial plant dry weight and yield were significantly increased by the application of bacterial inoculations, especially with 33% N-fertilizer. Inoculation of wheat seeds with diazotrophic bacterial strains significantly increased dry weight at 30days after germination. However, by the end of both seasons, the combination of N-fertilizer application and diazotrophic bacterial inoculation did not have a significant effect ($P = 0.8$) on dry weight and yield of winter wheat.

Key words: diazotophic bacteria, seedling growth, chlorophyll content, germination, wheat

6.1 Introduction

Joshi and Bhatt (2011) recorded that wheat is a major staple food crop that sustains 35% of the world's population. South Africa is suitable for the cultivation of a large variety of crops, including wheat. Nitrogenous chemical fertilizers are essential in modern agriculture to enhance food production. However, a substantial proportion of these fertilizers are lost through gaseous emissions, denitrification and leaching of nitrates into ground water (Sekhon, 1995), which impacts negatively on the environment (Hagin and Lowengart, 1995; Rejesus and Hornbaker, 1999).

Bacteria in the rhizosphere of plants that exert beneficial effects to the plants are called plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1989). Plant growth promoting rhizobacteria promote growth directly by providing nutrients or enhancing nutrient uptake, and indirectly by suppressing plant pathogens (Vessey, 2003; Ahmad *et al.*, 2008). Use of microbial inoculants to enhance growth and increase yields of crops has attracted the interest of many researchers (Kloepper *et al.*, 1991; De Freitas *et al.*, 1997; Okon and Vanderleyden, 1997; Kennedy *et al.*, 2004; Nain *et al.*, 2010; Yasin *et al.*, 2012). Several free-living bacteria genera have been reported to enhance growth and increase yields of crops of agronomic importance. A significant increase in growth rates have been reported in sugarcane due to application of *Acetobacter diazotrophicus* Beijerinck (Boddey *et al.*, 1991). In another study, Boddey *et al*. (1995) showed that certain Brazilian cultivars of sugarcane obtained over 150 kg of N ha^{-1} $year^{-1}$ from biological nitrogen fixation (BNF). Similarly, when wheat and barley were treated with *Azospirillum* and *Pseudomonas* strains, increases in dry weight of plant between 16.8 and 78% were achieved with wheat, and between 54.5% and 68% with barley (Hegazi *et al.*, 1998). Application of *Azospirillum* strains can increase wheat yields under greenhouse and field conditions (Hegazi *et al.*, 1998; Dobbelaere *et al.*, 2002; Saubidet *et al.*, 2002; Khalid *et al.*, 2004b). When rice was inoculated with *A. lipoferum* (Beijerinck) Comb, increases in plant height, tiller number and yields of rice were observed (Elbeltagy *et al.*, 2001; Balandreau, 2002). Kennedy and Islam (2001) in a review of BNF noted that application of *Azotobacter* sp. may contribute up to 50% of wheat N nutrient requirements under greenhouse conditions, and can increase rice yields by more than 20% in the field. In another study, Tran Van *et al*. (2000) reported that application of *Burkholderia vietnamiensis* Gillis *et al.* increased rice yields by 13%-

22%. Choudhury and Kennedy (2004) found that another species of this genus increased rice biomass by 69% per plant; and documented increases in root and shoot length, grain weight and grain yield as a result of inoculation of *Herbaspirillum seropedicae* Baldani yield increases in the greenhouse were reported when this strain was applied to maize (*Zea mays* L.), sorghum (*Sorghum bicolor* ssp. Bicolor), sugarcane (*Saccharum officinarum* L.) and wheat (*Triticum aestivum* L.) (James, 2000).

However, the quantities of N fixed by BNF in cereal crops is relatively limited when compared to the application of fertilizer sources of N. Yet, resource-poor small-scale farmers cannot afford the cost of fertilizers, and they are the single biggest input cost for many commercial farmers. Use of nitrogen fixing (diazotrophic) bacteria has therefore been proposed as an alternative to nitrogenous fertilizers used in small scale farmers. Integration of chemical fertilizers together with biofertilizers, mainly with BNF, may attain sustainability, secure economic return and build up soil fertility (Hegazi *et al.*, 1998). Fuentes-Ramirez et al. (1993) have shown that a large numbers of *A. diazotrophicus* strains can be isolated from sugarcane grown under low doses of nitrogen fertilizer as compared to those grown with high doses. Likewise, Pedraza *et al*. (2009) reported that diazotrophic bacteria in combination with nitrogen fertilizers reduced the amount of nitrogen fertilizer that needs to be applied to plants. Dobbelaere *et al*. (2001) and Jilani *et al.* (2007) also found that the best results were obtained from diazotrophic inoculations combined with moderate nitrogen fertilizer applications. For positive responses to diazotrophic bacterial inoculations on crop productivity, plant genotype (Moutia *et al.*, 2010), bacterial strains and soil type (Jagnow, 1987; Baldani *et al.*, 2002b) and environmental conditions (Kızılkaya, 2008) all play important roles. The purpose of the current study was to evaluate previously screened, free-living diazotrophic bacterial strains that are capable of enhancing maize growth and increase yields, with or without starter N-fertilizer, as an option to enhance crop yields in low-input production of wheat.

6.2 Methods and Materials

6.2.1 Source of Inoculum

Diazotrophs were isolated from the rhizosphere of different plants using standard isolation procedures and selected through *in vitro* studies such as the acetylene reduction assay and ammonia production (Chapter 2). Isoaltes were selected based on the higher ARA result and growth promotion effect in greenhouses. Selected diazotrophic isolates were identified as *Bacillus megaterium* (V16)), *Pseudomonas* spp.,(StB5), *Burkholderia ambifaria* (V9), *Pantoea ananatis* (LB5) and *Enterobacter cloacae* (L1) using both 16r DNA sequencing, and Bruker Daltonik MALDI Biotyper classification (Bruker Daltonics Inc., Billerica, MA).

6.2.2 Source of Seeds

The wheat seed *Triticum aestivum* L. (PAN 3494) was supplied by Pannar Seed company (Pty) Ltd[23].

6.2.3 Inoculum Preparation

Bacteria cultures were inoculated into tryptic soy broth (TSB) and incubated for 48 hours at 28ºC in an orbital shaker incubator[24] at 150 (rpm). Cells were harvested by centrifuging at 10,000 rpm for 15 minutes at 4ºC (Beckman Coulter Avanti J-26 XPI High Speed Centrifuge)[25]. Cell numbers were then adjusted to 10^8 cfu $ml^{-1}$with sterile distilled water, and their viability was confirmed using a plate count method.

6.2.4 Seed Germination Bioassay

Twenty five wheat seeds were surface sterilized with 1% sodium hypochlorite for 5 minutes and washed 5 times with sterilized distilled-water and soaked into the bacterial inocula (10^8 colony forming units (CFU)) and adhesive (2% gum Arabic) suspension, then seeds were coated with 2 g of 2×10^9 conidia g^{-1} of *Trichoderma harzianum* (Eco-T®) and allowed to air-dry overnight. Cell count was 10^6 CFU per seed. Seeds were treated with 2 g of 2×10^9 conidia g^{-1} of Eco-T®.

[23] Pannar Seeds (Pty)Ltd. P.O.Box 19, Greytown 3250,South Africa
[24]Shalom Laboratory Supplies c.c. 132 Commercial Road, International Plaza, Durban 4001, P. O. Box 57030, Musgrave Road Durban 4062
[25] Beckman Coulter Inc. 4300 N Harbour Boulevard, Box 3100, Fullerton, California, 92834-300., USA.

Treatment of seed with sterile distilled-water amended with gum Arabic served as the Control. These seeds were germinated in a growth chamber at 28^0C. After five days, the number of germinated seeds was counted, and root and shoot length of individual seedling was measured to determine the vigor index with the following formula: Seeds vigor index= [(mean root length + mean shoot length) X germination %] (Abdul Baki and Anderson, 1973).

6.2.5 Field Site

Field trials were conducted at Ukulinga Research Farm, University of KwaZulu–Natal, Pietermaritzburg, South Africa (29° 24′ E; 30° 24′ S). Soils at this site are classified as having Westleigh forms (Soil Classification Working Group 1991). Plots were irrigated when there was no rain to ensure that no water deficit occurred during the crop growth cycle. Crops were fully protected against weeds and pests in the two experimental seasons.

6.2.6 Fertilizer

In the greenhouse experiment, pots were hand watered every three days (250 ml pot^{-1}) supplemented with soluble fertilizer, applied at a rate of 0.224 g L^{-1} KH_2PO_4, 0.149 g L^{-1} K_2SO_4, 0.324 g L^{-1} KCl, 0.203 g L^{-1} $MgSO_4$ to make up the 100%PK fertilizer solution. Nine pots representing the positive control were watered with 100% NPK soluble fertilizer [3:1:3(38)] [26] at a rate of 1 gℓ^{-1}.Another nine pots were watered with water only, and served as the Untreated and Unfertilized Control.

In the field trials, the entire field was fertilized with the full amount of P (superphosphate) and no potassium were used as recommended by local Fertilizer Advisory Centre, Cedara, Pietermaritzburg, of South Africa. Two sub plots were treated with either 33% of the normal amount of nitrogen (N) (as limestone ammonium nitrate (LAN)) recommended for the crop. The other was not fertilized with N. Two thirds of the fertilizer was applied at sowing and one third five weeks after sowing. These experiments were hand-planted on the 24th of June 2011season and on 20th of June 2012 season.

[26] Ocean Agriculture (Pty) Ltd. P.O. Box 741, Muldersdrift, 1747, South Africa

6.2.7 Experimental Design

In the field split plot design with two factors were used: Bacterial isolates x N-fertilizer were the primary treatments, arranged in a randomized complete block. Each treatment was replicated three times. In the main plot, five bacterial isolates were evaluated: Isolates V16 (*Bacillus megaterium*), StB5 (*Bacillus ambifaria*), V9 (*Pseudomonas* sp.), LB5 (*Pantoea ananatis*), L1 (*Enterobacter cloacae*). In the split plot, two levels of N-fertilizer (0% N and 33% N) were tested in combination with the five bacteria. Plots were 2m x 1m rectangles. Each plot had six rows spaced at 20 cm with a distance of 10 cm between plants. Both pre-emergence and post-emergence herbicides were used to control weeds. Five plants were sampled for shoot dry biomass measurements every 30 days for three months. These plants were harvested at the soil level, dried in an oven at 70ºC for 72 hours and weighed. Yield parameters such as number of spikes were recorded. Yield per plot was determined by threshing the spikes.

6.2.8 Statistical Analysis

Greenhouse data were subjected to analysis of variance (ANOVA) using GenStat Release 14., copyright 2011, VSN International Ltd. A factorial ANOVA was conducted to compare the main and interaction effects of isolates and N-fertilization levels for the field data. When a significant F-test was found in the ANOVA, treatment mean comparisons were performed using Duncan's Multiple Range Test at the 5% level of significance.

6.3 Results

6.3.1 Effect of Bacterial Seed Inoculation Enhancing Seed Germination and Seedling Vigour Index in the Laboratory

Five selected diazotrophic bacterial isolates and a formulated fungal strain, Eco-T® (*Trichoderma harzianum*), increased % germination (23 - 41.29%) and seedling vigour index. Seeds treated with these bacterial isolates and Eco-T® had better ($P<0.001$) germination and vigour index compared to Un-inoculated and Unfertilized Control (Table 6.1). Seed inoculations enhanced ($P < 0.001$) shoot length and root length, especially Isolate V16.

Table 6.1 Effect of diazotrophic bacteria on wheat growth using the paper towel method *in vitro*

Treatments	%Germination		Shoot length(mm)		Root length (mm)		Vigour Index	
Control	33.33	a	3.17	a	3.50	a	209	a
V9	56.32	b	12.83	b	15.50	b	1597	b
LB5	58.52	bc	13.33	b	15.33	b	1692	b
L1	58.16	bc	16.67	b	17.33	b	1935	bc
StB5	63.96	bcd	15.50	b	17.50	b	2134	bcd
Eco-T®	74.60	cd	16.33	b	21.33	b	2810	cd
V16	80.21	d	18.83	b	20.33	b	3121	d
CV%	8.7		22.2		20.6		17.8	
Lsd	9.428		5.459		5.807		610.98	
Sed	4.327		2.505		2.665		280.42	
P	<0.001		0.001		<0.001		<0.001	

Means followed by same letter are not significant at P≤0.005

6.3.2 Effect of Diazotrophic Bacteria, with or Without Different Levels of NPK Fertilizer, on Wheat Growth under Greenhouse Condition

Diazotrophic bacterial inoculation with different levels of NPK fertilizer caused significant increases in dry weight (P<0.001) (Table 6.2). The greatest increase in dry weight (41%) was obtained with Isolate LB5 when 0% NPK was applied. Significnat dry weight increases were recorded with Isolate V9 at 50%NPK and Isolate LB5 at 65%NPK compared to untreated and fertilized controls. The interaction effect of inoculum and levels of NPK fertilizer was not significant (P=0.349, Table 6.2).

Table 6.2 Effect of diazotrophs alone and in combination with five levels of NPK fertilizer on the growth of winter wheat under greenhouse condition

Treatments			
N-fertilizer	**Isolates**	**Dry weight (g)**	
0	Control	1.96	a
	StB5	2.29	a
	V9	2.713	a
	L1	3.633	a
	V16	4.627	ab
	LB5	6.923	bc
25	Control	7	bc
	StB5	7.01	bc
	V9	8.063	cde
	L1	9.75	cdefgh
	V16	8.627	cdef
	LB5	7.087	bc
50	Control	7.613	cd
	StB5	9.247	cdefg
	V9	9.403	cdefg
	L1	10.68	efgh
	V16	9.88	cdefgh
	LB5	10.63	efgh
65	Control	9.08	cdefg
	StB5	10.69	efgh
	V9	12.583	h
	L1	10.297	defgh
	V16	9.787	cdefgh
	LB5	12.073	gh
75	Control	9.843	cdefgh
	StB5	9.86	cdefgh
	V9	10.747	efgh
	L1	11.153	fgh
	V16	11.3	fgh
	LB5	11.747	gh
100	NPK	12.09	
N-fertilizer		F=67.64	P<0.001
Isolates		F=6.36	P<0.001
N-fertilizer X Isolates		F=1.13	P=0.349
		CV%= 17.8	

6.3.3 Trial One and Two- Effect of Diazotrophic Inoculants, in Combination with Reduced N-Fertilization, on Winter Wheat Growth in the Field in 2011

In the first trial, inoculation of five diazotrophic isolates with or without N-fertilizer caused no significant increases in dry weight at 30, 60 or 90 DAP (Table 6.4). Moreover, at 60 or 90 DAP, these isolates with or wthout N-fertilizer caused no significant increases in number of spikes and yield ($P < 0.005$) (Table 6.5). However, when these isoaltes where inoculated with or without N-fertilizer scored relative higher dry weight than the untreated and fertilized controls. At 30 or 60 DAP, isolates with 33%N-fertilizer caused relatively higher dry weight than the 100%NPK. Inoculation of Isolate StB5 without 33N% fertilizer cuased significant ($P<0.005$) increases in stover dry weight (Table 6.5). The interaction between the different diazotrophic inoculants and N-fertilizer applications was not significant ($P<0.001$) (Table 6.4).

In Trial Two, inoculation of diazotrophic bacteria alone or with 33%N-fertilizer resulted in relatively greater increases of dry weight, stover dry weight, number of spikes and yield at different growth stages higher than the Un-inoculated or Unfertilized Control (Table 6.6 and Table 6.7). However, the increases were not statistically significant.

.

Table 6.4 Effect of diazotrophs with or without 33% N-fertilizer on the wheat growth in Year 2011(Trial one)

Treatments		Dry weight (g) Trial one					
N-fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**	
0	Control	1.21	a	15.27	ab	56.78	a
	V16	1.237	a	16.38	abc	69.99	ab
	V9	1.537	abc	14.27	a	69.26	ab
	StB5	1.257	a	14.53	a	72.9	ab
	LB5	1.387	ab	17.88	abc	84.59	ab
	L1	1.527	abc	15.07	ab	67.67	ab
33	Control	1.723	abc	18.01	abc	78.18	ab
	V16	1.893	bc	19.53	abc	82.1	ab
	V9	1.74	abc	20.88	abc	70.83	ab
	StB5	1.93	bc	22.96	bc	70.54	ab
	LB5	2.01	c	23.7	c	74.56	ab
	L1	1.907	bc	24.13	c	96.79	b
	100% NPK	1.67		21.28		102.09	
N-fertilizer		F=22.57	P<0.001	F=8.96	P<.001	F=2.93	P=0.101
Isolates		F=0.52	P=0.758	F=0.79	P=0.569	F=1.18	P=0.349
N-fertilizer Xisolates		F=0.52	P=0.756	F=0.61	P=0.693	F=1.14	P=0.371
		CV%=19.5		CV%=22.2		CV%=20.3	

Table 6.5 Effect of diazotrophs, with or without 33% N fertilization, on different growth parameters of winter wheat in Year 2011

Treatments		**Experiment one**					
N-fertilizer	**Isolates**	**Stover dry weight (g)**		**Yield (g)**		**No of spike**	
0	Control	151.8	a	100.3	a	148.3	a
	V9	177.0	ab	121.2	ab	136.0	a
	V16	173.6	ab	112.0	ab	133.0	a
	L1	175.5	ab	131.5	ab	139.0	a
	LB5	202.2	ab	134.0	ab	144.3	a
	StB5	215.4	b	143.4	ab	147.3	a
33	Control	203.1	ab	145.7	ab	140.0	a
	V16	184.8	ab	114.2	ab	130.3	a
	LB5	165.7	ab	145.1	ab	138.0	a
	L1	191.0	ab	124.5	ab	152.3	a
	V9	191.6	ab	164.2	b	143.3	a
	StB5	209.2	b	158.1	b	164.3	a
100%	NPK	265.0		177.3		168.3	
Fertilizer_level		F=0.08	P=0.782	F=0.13	P=0.723	F=0.28	P=0.604
Treatments		F=1.92	P=0.138	F=2.17	P=0.095	F=1.02	P=0.431
Fertilizer_level XTreatments		F=0.95	P=0.471	F=1.15	P=0.362	F=0.46	P=0.804
		CV%=15.3		CV%=20.4		CV%=13.5	

Table 6.6 Effect of diazotrophs with or without 33% N-fertilizer on the wheat growth in Year 2011(Trial two)

Treatments		**Dry weight (g) Trial two**					
N-fertilizer	**Isolates**	**30DAP**		**60DAP**		**90DAP**	
0	Control	1.21	a	14.27	a	56.78	a
	V16	1.237	a	16.38	abc	69.99	ab
	StB5	1.257	a	14.53	a	84.59	ab
	LB5	1.387	ab	15.27	ab	72.9	ab
	L1	1.527	abc	15.07	ab	67.67	ab
	V9	1.537	abc	17.88	abc	70.83	ab
33	Control	2.01	c	18.01	abc	74.56	ab
	LB5	1.723	abc	23.7	c	78.18	ab
	V9	1.74	abc	20.88	abc	69.26	ab
	V16	1.893	bc	19.53	abc	82.1	ab
	L1	1.907	bc	24.13	c	96.79	b
	StB5	1.93	bc	22.96	bc	70.54	ab
	100%NPK	1.67		14.19		59.67	
N-fertilizer		23.57	<.001	18.96	<.001	2.93	0.101
Treatments		0.52	0.758	0.79	0.569	1.18	0.349
N-fertilizer X	Isolates	0.52	0.756	0.61	0.693	1.14	0.371
		CV%=19.5		CV%=22.2		CV%=20.3	

Table 6.7 Effect of diazotrophs, with or without 33% N fertilization, on different growth parameters of winter wheat in Year 2011

Treatments		Experiment two					
N-fertilizer	**Isolates**	**No. of spikes**		**Stover dry weight (g)**		**Yield (g)**	
0	Control	146	a	201.9	a	145.7	ab
	V9	126	a	193.9	a	121.2	ab
	V16	131	a	200.2	a	112	ab
	L1	127.3	a	197.1	a	131.5	ab
	LB5	139.7	a	203.6	a	100.3	a
	SB5	155.3	a	208.6	a	143.4	ab
33	Control	155	a	223.4	a	164.2	b
	V16	150.3	a	209.1	a	145.1	ab
	LB5	133	a	196.8	a	134	ab
	L1	152	a	224.4	a	124.5	ab
	V9	143.3	a	244.2	a	114.2	ab
	SB5	134	a	205.9	a	158.1	b
100%	NPK	141		214.1		138	
N_fertilizer		0.96	0.338	0.98	0.333	0.13	0.723
Treatments		0.1	0.99	0.44	0.817	2.17	0.095
N_fertilizer.Treatments		0.27	0.926	1.03	0.427	1.15	0.362
		CV%=15.2		CV%=24		CV%=20.4	

Means followed by same letter are not statistically significant at P<0.05

6.5 Year 2012 - Field Trials on the Effect of Diazotrophic Inoculants in Combination with Reduced N-Fertilizer on the Growth of Winter Wheat

In Trial Three, all the diazotrophic bacterial inoculum with or without 33% N-fertilizer, had no significant effect on dry weight at 30, 60 or 90 DAP ($P<0.005$) (Table 6.8). However, at 30 DAP, Isolates StB5 followed by Isolate L1 scored relatively higher dry weight than the untreated and 33%N-fertilized control (Table 6.8). Despite the increases were not signifnicant, all isolates with added fertilizer resulted in relatively higher yiled compared to untreated and 33%N-fertilized control (Table 6.9).

In Trial Four, at 30 of 60 DAP, Isolate StB5 without 33%N-fertilzier caused significant increase ($P<0.005$) in dry weight (Table 6.8). At 60 DAP, Isolate V9 without add N-fertilizer was also significantly ($P<0.005$) increased dry weight. Isolates StB5 and LB5 without N-fertilizer caused signifnicant increases in yield (Table 6.9). Whilst the main effects were significant, the interaction effect of inoculum and 33% N- fertilization were not statistically significant for dry weight at various growth stages, or for yield in both Trial Three or Trial Four (Table 6.8 and Table 6.9).

Table 6.8 Effect of diazotrophs with or without 33% N-fertilizer on the wheat growth in Year 2012 (Trial Three and Four)

Treatments		Dry weight (g) Trial Three						Dry weight (g) Trial Four					
N-fertilizer	Isolates	30DAP		60DAP		90DAP		30DAP		60DAP		90DAP	
0	control	0.43	ab	22.77	a	126.5	a	0.28	a	11.14	a	105.8	a
	V16	0.33	a	25.14	a	119.9	a	0.37	ab	18.25	abc	104.4	a
	StB5	0.39	a	21.22	a	114.9	a	0.51	bc	21.54	bc	104.2	a
	L1	0.39	ab	16.83	a	97.4	a	0.35	ab	18.53	abc	120.3	a
	V9	0.41	ab	18.91	a	104	a	0.26	a	21.91	bc	117.2	a
	LB5	0.47	abc	20.6	a	116	a	0.29	a	15.29	ab	99.4	a
33	control	0.47	abc	22.06	a	101.5	a	0.61	c	27.34	c	103.7	a
	V16	0.37	a	20.33	a	119.1	a	0.49	bc	25.96	c	119.3	a
	StB5	0.68	c	17.74	a	114.7	a	0.44	abc	26.81	c	84	a
	L1	0.62	bc	18.1	a	116.7	a	0.41	abc	25.29	c	113.8	a
	V9	0.44	ab	19.89	a	139.4	a	0.43	abc	22.65	bc	86.5	a
	LB5	0.52	abc	23	a	128.7	a	0.54	bc	19.75	abc	113.7	a
100	NPK	0.60		33.17		163		0.5		42.21		155.6	
N-fertilizer		F=8.25	P=0.009	F=0.13	P=0.727	F=0.54	P=0.471	F=17.3	P<.001	F=16.68	P<.001	F=0.39	P=0.537
Isolates		F=1.86	P=0.143	F=0.69	P=0.638	F=0.25	P=0.933	F=1.21	P=0.338	F=1.36	P=0.278	F=0.65	P=0.662
N-fertilizer X Isolates		F=1.48	P=0.235	F=0.32	P=0.894	F=0.8	P=0.561	F=2.64	P=0.051	F=1.58	P=0.207	F=0.86	P=0.522
		cv%=25.4		CV%=30		cv%=24.2		CV%=25.3		CV%=23.8		CV%=22.8	

Table 6.8 Effect of diazotrophic bacteria in combination of reduced N-fertilizer on the wheat yield in 2012

Treatments		**Yield (kg plot-1)**			
N%-Fertilizer	**Isolates**	**Trial Three**		**Trial Four**	
0	Ccontrol	0.18	a	0.23	a
	V16	0.33	ab	0.38	abc
	StB5	0.28	ab	0.41	bc
	L1	0.31	ab	0.3	abc
	V9	0.3	ab	0.36	abc
	LB5	0.28	ab	0.46	bcd
33	Control	0.38	ab	0.45	bcd
	V16	0.41	b	0.51	cd
	StB5	0.42	b	0.52	cd
	L1	0.42	b	0.6	d
	V9	0.43	b	0.49	cd
	LB5	0.4	b	0.47	bcd
	NPK	0.44		0.46	
N%-Fertilizer		F=25.26	P<0.001	F=1.67	P=0.210
Isolaes		F=0.25	P=0.94	F=0.31	P=0.0049
N%-Fertilizer X Isolates		F=2.53	P=0.06	F=0.65	P=0.664
		CV%=20.8			CV%=32.7

Means followed by same letter are not statistically significant at P<0.05

6.4 Discussion

During 2011 and 2012, greenhouse and field experiments were carried out to evaluate the response of winter wheat to inoculation with selected diazotrophic bacteria. Inoculation with selected diazotrophs significantly increased germination and seedling vigour. Inoculation generally affected root length and shoot length. These increases in the early growth of wheat seedlings inoculated with diazotrophic bacteria (*Bacillus megaterium*, *Pseudomonas* sp., *Burkholderia* sp., *Enterobacter cloacae* and *Pantoea ananatis*) suggest that beside their N-fixing ability, these organisms may also produce growth substances that would enhance the germination

and growth of wheat seedlings. Growth substance such as indole acetic acid (IAA) and gibberellins (GA) can be produced by both *Azotobacter* sp. and *Azotomonas* sp. which can enhance the growth of wheat (Pati *et al.*, 1995). Dobbelaere *et al*. (2003) and Karthikeyan *et al.* (2007) found that the seed of various crops, when inoculated with plant growth promoting rhizobacteria (PGPR), germinated faster.

In the greenhouse experiments, inoculation of wheat seeds with diazotrophic bacteria (*B. megaterium*, *Pseudomonas* sp., *Burkholderia* sp., *E. cloacae* and *P. ananatis*) with NPK fertilizer at 0%, 25%, 50%, 65% and 75% increased dry weight from 0.08% to 41% more than the Controls. Seeds inoculated with diazotrophic bacteria alone developed a greater dry weight by 2.7% - 41% relative to the control. This enhanced seedling growth may be attributed to several causes, such as: biological nitrogen fixation (BNF), production of plant growth hormones, siderophores and biological control of sub-lethal fungal pathogens. All isolates used in the current study reduced acetylene to ethylene, which is a characteristic of diazotrophic bacteria (Chapter 2). Stimulation of plant growth and yield increases in wheat as a result of inoculation with diazotrophs has been documented by others (Kloepper *et al.*, 1989; Boddey *et al.*, 1995; Hegazi *et al.*, 1998). Reports on increases in wheat dry biomass following inoculation with rhizobacteria are well documented (Ozturk *et al.*, 2003; Khalid *et al.*, 2004a; Salantur *et al.*, 2006; Shaharoona *et al.*, 2008).

In this study, at 25% NPK application rate, all of the inoculated treatments increased the dry weight by 0.08% to 22.7% over the Untreated plus 25% NPK fertilizer control. At 50% NPK fertilizer application level, the inoculatns increased dry weight; by 13.5% - 25.3%, over the Untreated plus 50% NPK fertilized treatment. At 65% NPK, the dry weight increase due to the bacterial inoculation was ranged 10-28.9% over the untreated pluse 65% NPK. At 75% NPK, the isolates effect on dry weight was ranged 0.1-15.7% over the untreated pluse 75% NPK control.

The possible reason for the lack of plant growth responses to inoculation with diazotrophs combined with high levels of N-fertilizer are probably associated with a lower nitrogenase activity of the diazotrophs. Nitrogenase activity of diazotrophs is strongly influenced by ambient ammonium levels (Burris *et al.*, 1991). Over application of chemical fertilizers have a negative effect on growth and activity of diazotrophs. Okon and Labandera-Gonzalez (1994) reviewed results obtained with different crops following inoculation with *Azospirillum* strains in several

countries over a period of twenty years. Maximum shoot dry weight in this study was recorded with Isolate LB5 (*Pantoea ananatis*) without added N-fertilization.

In Year 2011 two field experiments (Trials One and Two) were conducted and out of five diazotrophic bacterial isolates, StB5 alone influenced stover dry weight at 30 DAP. Çakmakçi *et al*. (2006) also showed that effects of PGPRs were greater at early growth stages of plants than later. Seed inoculation with diazotrophic bacterial isolates, with or without N-fertilizer, and at the highest N rates (100% NPK), did not influence plant growth parameters and yield production. This result suggests that the residual soil N content was already adequate for wheat production in 2011.

In Year 2012 two more field trials were conducted. In these trials seed inoculation with the selected diazotrophic bacteria, with or without N-fertilizer, enhanced wheat growth and increased yield. These results were achieved by reducing soil N to a minimum level by planting wheat and rice repeatedly in the field without any fertilizer application, prior to running Trials Three and Four. A second factor was that there was substantially more rainfall in Year 2012 than in 2011. It seems that bacterial strains have higher potential to enhanced crop growth and yield with reduced N-fertilizer and greater rainfall.

The best contributions of diazotrophic bacteria was achieved by Isolate LB5 + 0% NPK (41%), V9 + 65% NPK (28.9%), Isolate L1 + 50% NPK (25%), Isolate L1 + 25%NPK (22%) and LB5 + 75% NPK (15%) undergreenhouse conditions.

These results show the potential of an integrated management strategy that incorporates diazotrophs and reduced N-fertilizers a means to increase wheat yields. The selected diazotrophs could be used as biofertilizers for spring wheat in agricultural systems utilizing zero or low N inputs. Increases in biomass and yields of crops of agricultural importance after diazotrophic bacterial inoculations, in the presence of low doses of N fertilizer, have been recorded by others. Kennedy *et al*. (2004) recorded several studies in which significant increases in growth and yield of several crops were reported following inoculation with several free-living bacteria genera, in combination with low doses of nitrogen fertilizer. Increase in yields following seed inoculation with *Azospirillum* strains in combination with low doses of nitrogen have been reported by other

authors (Fuentes-Ramírez *et al.*, 1999; Shahaby *et al.*, 2000; Dobbelaere *et al.*, 2001; Baldani *et al.*, 2002a).

In conclusion, though the increases in plant growth parametes or yield was not statisitcailly significant, the effect of the inoculation diazotrophs, with or without N-fertilization scored relatvely higher plant growth parameters or yield under both greenhouse and field conditions compared to untreated controls.

References

Abdul-Baki, A., Anderson, J.D. (1973) Vigor determination in Soybean seed by multiple criteria. Crop Science 13: 630-633.

Ahmad F., Ahmad I., Khan M.S. (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163:173-181. DOI: http://dx.doi.org/10.1016/j.micres.2006.04.001

Balandreau J. (2002) The spermosphere model to select for plant growth promoting rhizobacteria. Biofertilisers in action. Rural Industries Research and Development Corporation, Canberra:55-63.

Baldani J., Oliveira A., Guimarães S., Baldani V., Reis Jr F., Silva L., Reis V., Teixeira K.d.S., Döbereiner J. (2002a) Biological nitrogen fixation (BNF) in non-leguminous plants: the role of endophytic diazotrophs. In, Nitrogen Fixation: From Molecules to Crop Productivity. Pedrosa, F.O., Hungria, M., Yates, F., Newton, W.E. (Eds). Kluwer Academic Publishers, The Netherlands, pp. 397-400.

Baldani J.I., Reis V.M., Baldani V.L., Döbereiner J. (2002b) Review: A brief story of nitrogen fixation in sugarcane—reasons for success in Brazil. Functional Plant Biology 29:417-423.

Boddey R., Urquiaga S., Reis V., Döbereiner J. (1991) Biological nitrogen fixation associated with sugar cane. Plant and Soil 137:111-117.

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

Burris R.H., Hartmann A., Zhang Y., Fu H. (1991) Control of nitrogenase in *Azospirillum* sp. Plant and Soil 137:127-134.

Çakmakçi R., Dönmez F., Aydın A., Şahin F. (2006) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biology and Biochemistry 38:1482-1487.

Choudhury A., Kennedy I. (2004) Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biology and Fertility of Soils 39:219-227.

De Freitas J., Banerjee M., Germida J. (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biology and Fertility of Soils 24:358-364.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Okon Y., Vanderleyden J. (2002) Effect of inoculation with wild type *Azospirillum brasilense* and *A. irakense* strains on development and nitrogen uptake of spring wheat and grain maize. Biology and Fertility of Soils 36:284-297.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Vanderleyden J., Dutto P., Labandera-Gonzalez C., Caballero-Mellado J., Aguirre J.F., Kapulnik Y. (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Functional Plant Biology 28:871-879.

Dobbelaere S., Vanderleyden J., Okon Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22:107-149.

Elbeltagy A., Nishioka K., Sato T., Suzuki H., Ye B., Hamada T., Isawa T., Mitsui H., Minamisawa K. (2001) Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Applied and Environmental Microbiology 67:5285-5293.

Fuentes-Ramirez L., Jimenez-Salgado T., Abarca-Ocampo I., Caballero-Mellado J. (1993) *Acetobacter diazotrophicus*, an indoleacetic acid producing bacterium isolated from sugarcane cultivars of Mexico. Plant and Soil 154:145-150.

Fuentes-Ramírez L.E., Caballero-Mellado J., Sepúlveda J., Martínez-Romero E. (1999) Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. FEMS Microbiology Ecology 29:117-128.

Hagin J., Lowengart A. (1995) Fertigation for minimizing environmental pollution by fertilizers. Nutrient Cycling in Agroecosystems 43:5-7.

Hegazi N.A., Fayez M., Amin G., Hamza M.A., Abbas M., Youssef H., Monib M. 1998. Diazotrophs associated with non-legumes grown in sandy soils. In, Developments in plant and soil sciences, Vol. 79. Proceedings of the 7th International Symposium on Nitrogen Fixation with Non-Legumes, 16–21 October 1996, Faisalabad, Pakistan. Malik, K.A., Mirza, M. and Ladha, J.K. (Eds). Kluwer Academic Publishers, Dordrecht. pp. 209–222.

Jagnow G. (1987) Inoculation of cereal crops and forage grasses with nitrogen-fixing rhizosphere bacteria: Possible causes of success and failure with regard to yield response–a review. Zeitschrift für Pflanzenernährung und Bodenkunde 150:361-368.

James E. (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crops Research 65:197-209.

Jilani G., Akram A., Ali R.M., Hafeez F.Y., Shamsi I.H., Chaudhry A.N., Chaudhry A.G. (2007) Enhancing crop growth, nutrients availability, economics and beneficial rhizosphere microflora through organic and biofertilizers. Annals of Microbiology 57:177-184.

Joshi P., Bhatt A. (2011) Diversity and function of plant growth promoting rhizobacteria associated with wheat rhizosphere in North Himalayan Regional and International Journal of Environmental Science 1:1135-1143.

Karthikeyan B., Jaleel C., Gopi R., Deiveekasundaram M. (2007) Alterations in seedling vigour and antioxidant enzyme activities in *Catharanthus roseus* under seed priming with native diazotrophs. Journal of Zhejiang University-Science B 8:453-457.

Kennedy I., Islam N. (2001) The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. Animal Production Science 41:447-457.

Kennedy I.R., Choudhury A., Kecskés M.L. (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Khalid A., Arshad M., Zahir Z. (2004a) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology 96:473-480.

Khalid A., Arshad M., Zahir Z. (2004b) Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology 96:473-480.

Kızılkaya R. (2008) Yield response and nitrogen concentrations of spring wheat (*Triticum aestivum*) inoculated with *Azotobacter chroococcum* strains. Ecological Engineering 33:150-156.

Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7:39-44.

Kloepper J.W., Zablotowicz R.M., Tipping E.M., Lifshitz R. (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. In, The Rhizosphere and Plant Growth. Keister, D.L and Cregan, P.B. (Eds.). Kluwer Academic pulishers, The Netherlands. Pp. 315-326.

Moutia J.-F.Y., Saumtally S., Spaepen S., Vanderleyden J. (2010) Plant growth promotion by *Azospirillum* sp. in sugarcane is influenced by genotype and drought stress. Plant and Soil 337:233-242.

Nain L., Rana A., Joshi M., Jadhav S.D., Kumar D., Shivay Y., Paul S., Prasanna R. (2010) Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. Plant and Soil 331:217-230.

Okon Y., Labandera-Gonzalez C.A. (1994) Agronomic applications of *azospirillum*: An evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry 26:1591-1601.

Okon Y., Vanderleyden J. (1997) Root-associated *Azospirillum* species can stimulate plants. ASM News-American Society for Microbiology 63:366-370.

Ozturk A., Caglar O., Sahin F. (2003) Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. Journal of Plant Nutrition and Soil Science 166:262-266.

Pati B., Sengupta S., Chandra A. (1995) Impact of selected phyllospheric diazotrophs on the growth of wheat seedlings and assay of the growth substances produced by the diazotrophs. Microbiological Research 150:121-127.

Pedraza R.O., Bellone C.H., Carrizo de Bellone S., Boa Sorte P.M.F., Teixeira K.R.d.S. (2009) *Azospirillum* inoculation and nitrogen fertilization effect on grain yield and on the diversity of endophytic bacteria in the phyllosphere of rice rainfed crop. European Journal of soil Biology 45:36-43.

Rejesus R.M., Hornbaker R.H. (1999) Economic and environmental evaluation of alternative pollution-reducing nitrogen management practices in central Illinois. Agriculture, Ecosystems and Environment 75:41-53.

Salantur A., Ozturk A., Akten S. (2006) Growth and yield response of spring wheat (*Triticum aestivum* L.) to inoculation with rhizobacteria. Plant Soil and Environment 52:111-118..

Saubidet M.I., Fatta N., Barneix A.J. (2002) The effect of inoculation with *Azospirillum brasilense* on growth and nitrogen utilization by wheat plants. Plant and Soil 245:215-222.

Sekhon G. (1995) Fertilizer-N use efficiency and nitrate pollution of groundwater in developing countries. Journal of Contaminant Hydrology 20:167-184.

Shahaby A., Fayez M., Omar M., Shehata H. (2000) Integration of diazotrophs inoculation with organic and inorganic fertilization to improve wheat and maize productivity in sandy soils. Egyptian Journal of Agricultural Research 78:499-519.

Shaharoona B., Naveed M., Arshad M., Zahir Z.A. (2008) Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). Applied Microbiology and Biotechnology 79:147-155.

Tran Van V., Berge O., Ngo Ke S., Balandreau J., 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 and Soil 218:273-284.

Vessey J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.

Yasin M., Ahmad K., Mussarat W., Tanveer A. (2012) Bio-fertilizers, substitution of synthetic fertilizers in cereals for leveraging agriculture.Crop and Environment 3:62-66.

CHAPTER SEVEN

EFFECTS OF INOCULATION OF *BACILLUS MEGATERIUM* (V16) AND *TRICHODERMA HARZIANUM* (ECO-T®), SINGLY OR CO-INOCULATION AT REDUCED N-FERTILIZER RATES, ON PLANT GROWTH

M.H. Kifle and M.D. Laing

Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences

University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg,

South Africa

Abstract

The synergistic effects of *Bacillus megaterium* deBary (V16) and *Trichoderma harzianum* Rifai (Eco-T®) with or without N-fertilizer on wheat and maize growth were determined under greenhouse conditions. Single inoculations of V16 and Eco-T® without added N-fertilizer increased maize dry weight by 97% and 46%, respectively, above the Un-fertilized and Un-inoculated Control. However the increases in shoot dry weight of maize were not statistically significant. Inoculation of V16 and Eco-T® together with added N-fertilizer (33%N) increased maize dry weight by 300% above the Un-fertilized and Un-inoculated Control. Inoculation of Isolate V16 or Eco-T® with added N-fertilizer (33%N) increased maize dry weight by 17% and 23%, respectively, above the Un-inoculated plus 33% N-fertilizer treatment. Plants inoculated with V16 with Eco-T® plus 33%N-fertilizer significantly ($P<0.001$) increased maize dry weight by 51%, above the Un-inoculated plus 33%N-fertilizer treated plants. Inoculation of Isolates V16, Eco-T® and V16 + Eco-T® without added N-fertilizer increased wheat dry weight by 91%, 117% and 269%,respectively, above the Un-inoculated and Un-fertilized control. The maximum increase in chlorophyll content index (CCI) (12.87) was observed with plants fertilized with 100%NPK. The dual inoculation of diazotrophic bacteria with Eco-T®, with or without reduced N-fertilizer, consistently increased the growth of wheat and maize.

Key words: Diazotrophic bacterium, Eco-T®, wheat, maize, reduced fertilizer, plant growth promotion

7.1 Introduction

Crop production needs to be increased substantially to reduce hunger and food insecurity in Africa. Maize and wheat are staple food and the most widely grown crops in Africa by small scale farmers (Bruns and Abel, 2003; Ortiz-Monasterio *et al.*, 2007). These crops need low levels of soil nitrogen in order to grow and flourish (Cakmak, 2002; Raun *et al.*, 2002; Ladha *et al.*, 2005). However, nitrogen fertilization is a major limitation to crop productivity (Pang and Letey, 2000; Cassman *et al.*, 2002). Most farmers in Africa are poor and use insufficient amounts of mineral fertilizers, or do not use any. The reasons include lack of access to commercial fertilizers and high transport costs (Jayne *et al.*, 2003; Alene *et al.*, 2008). Soil microorganisms play a significant role in organic matter decomposition and release of plant nutrients (Kuzyakov *et al.*, 2000). Plant growth promoting rhizobacteria (PGPR) can stimulate plant growth by fixing atmospheric nitrogen (Canbolat *et al.*, 2006), solubilizing phosphorus (Rodriguez *et al.*, 2006) and iron (Ma *et al.*, 2009) and producing plant hormones such as auxins, gibberellins, cytokinins and ethylene (Bashan and de Bashan, 2005; Naserirad *et al.*, 2011; Saharan and Nehra, 2011a). Enhancement of plant growth and increases in crop yields caused by microbial inoculants has been reported by a number of authors (Dobbelaere *et al.*, 2003; Kennedy *et al.*, 2004; Khalid *et al.*, 2004; Kloepper *et al.*, 2004; Lucy *et al.*, 2004; Çakmakçi *et al.*, 2006; Berg, 2009). Studies on the positive effects of PGPR on seed germination, seedling growth and yield of maize have been reported (Shaharoona *et al.*, 2006; Cassán *et al.*, 2009; Gholami *et al.*, 2009). Use of microbial inoculants may result in the productive use of reduced doses of chemical fertilizers because PGPR are thought to be more efficient under nutrient-limited conditions (Shaharoona *et al.*, 2008; Kumar *et al.*, 2009).Use of microbial inoculants, combined with reduced doses of chemical fertilizers, has been reported by Riggs *et al*. (2001) and Dobbelaere *et al*. (2001).Use of multiple strains for optimum crop production was proposed by Vessey (2003). Dual or multi-inoculation with bacterial strains or bacteria in combination with fungi or arbuscular mycorrhizal fungi can yield better results than single inoculations (Lucy *et al.*, 2004; Artursson *et al.*, 2006; Han and Lee, 2006; Adesemoye *et al.*, 2009). The objectives of this study were to investigate the effects of single or dual inoculation of PGPR, and fungi with or without reduced levels of N fertilizer, on maize and wheat growth.

7.2 Methods and Materials

7.2.1 Source of Inoculum

The bacteria isolates used in this study were selected through an *in vitro* screening of 95 diazotrophic bacteria for plant growth promoting activities (Chapter 2). The twenty isolates that displayed the most growth promoting activities were selected for secondary screening for enhancement of seedling growth under greenhouse conditions. The diazotrophic bacterial strain (*Bacillus megaterium*) used in this study was previous studied (Chapter 2,3,4,5 and 6) for plant growth and yield increases, together with a commercial biocontrol agent a strain of *Trichoderma harzianum* Rifai sold as Eco-T® by Plant Health Products (Pty) Ltd, Pietermaritzburg, South Africa).

7.2.2 Preparation of Inoculum

Bacterial cultures were inoculated into tryptic soy broth and incubated for 48 hours at 28ºC in an orbital shaker incubator[27] at 150 rpm. Cells were harvested by centrifuging (Beckman Coulter Avanti J-26 XPI high speed centrifuge)[28] at 10,000 rpm for 15 minutes at 4ºC. Cell numbers were then adjusted to 10^8 cfu $m\ell^{-1}$ by a dilution method using sterile distilled water. Cell counts were done using a counting chamber and viability confirmed by plate count method. This procedure was repeated for each subsequent experiment.

7.2.3 Seed Source

The maize seed *Zea mays* L. (AY 106 YR) used in these studies was purchased from MacDonald Seed Company[29]. The wheat seed *Triticum aestivum* L. (PAN 3494) was supplied by Pannar Seed company (Pty) Ltd[30].

[27] Shalom Laboratory Suppliers c.c. 132, Commercial Road, International Plaza, Durban 4001, P.O.Box 57030, Musgrave Road, Durban 4062, South Africa
[28] Beckman Coulter Inc. 4300 N Harbour Boulevard P.O.Box 3100, Averton, California 92834-340, USA
[29] MacDonald's Seeds (Pty)Ltd. 2 Trek Road, Mkondeni, Pietermaritzburg, 3212, P.O.Box 40, South Africa
[30] Pannar Seeds (Pty)Ltd. P.O.Box 19, Greytown 3250,South Africa

7.2.4 Seed Treatments

Seeds were disinfected by soaking in 0.02% sodium hypochlorite for two minutes, then rinsing them five times in sterile distilled water. Seed inoculation was done by soaking the seeds in a bacterial suspension in 2% gum arabic for two hours to enhance adhesion of the cells onto the seed. For a Control treatment, the seeds were soaked in a suspension of 2% gum arabic in sterile distilled water. The seeds were then dried on a lamina flow bench overnight. This procedure was followed for all seed inoculations in all other experiments.

7.2.5 Fertilizers

Pots with each inoculated treatments were watered daily with an equal amount of a nutrient solution of soluble fertilizer applied at a rate of 0.224 g L^{-1} KH_2PO_4, 0.149 g L^{-1} K_2SO_4, 0.324 g L^{-1} KCl, 0.203 g $L^{-1}MgSO_4$ and micronutrient (Microplex)[31] at a rate of (0.02 gℓ^{-1}). For the Fully Fertilized Control a solution of NPK, [3:1:3 (38) Complete™] at a rate of 1g $\ell^{-1}$was used. The 33%N treatments all used 0.33 gℓ^{-1} (w/v) of the same fertilizer for the reduced fertilizer control, with phosphorus and potassium levels adjusted to the full amounts recommended for each crop. The Un-inoculated Control was watered with tap water.

7.2.6 Experimental Design

A randomized complete blocks design was used. Nine treatments were applied, consisting of the diazotrophic bacterium, and one fungal strain (Eco-T®), and a combination of the two, with or without 33%N-fertilizer, plus three controls (Untreated+0%N, Untreated + 33% N and Untreated +100% fully fertilized controls) were used. Each treatment consisted of three pots with a top diameter of 200 mm, filled with composted pine bark. Each pot was seeded with five seeds. The pots were kept in the greenhouse with a temperature range of 25-30°C. The seedlings were thinned to three and five plants per pot for maize and wheat, respectively, after germination. The chlorophyll levels in maize leaves were measured using a chlorophyll content meter (CCM-200 plus)[32] at the sixth to eighth leaf stage to give a chlorophyll content index (CCI). Plants from each pot were harvested at the shoot base after eight weeks and were then dried at 70°C in an oven for 72 hours and weighed to obtain shoot dry biomass.

[31] Ocean Agriculture (Pty) Ltd. P. O. Box 741, Mulders Drift, Republic of South Africa, 1747
[32] CCM-200 Plus, Opti-Science Inc., 8 Winn Avenue, Hudson, NH, USA, 03051

7.2.7 Experimental Analysis

Data were subjected to analysis of variance (ANOVA) using GenStat Release 14.1, copyright 2011, VSN International Ltd.Treatment mean separation was done using to Fisher's LSD test, at 5% level of significance.

7.3 Results

Inoculation of *B. megaterium* (Isolate V16) or Eco-T® applied singly showed significant ($P<0.001$) increases in wheat dry weight but no significant increases in maize dry weight, compared to the Untreated-control. Similarly, Isolate V16 applied alone significantly increased maize chlorophyll level ($P<0.001$) higher than the Untreated-control (Table 7.1). Dual inoculation of Isolate V16 and Eco-T® without added N-fertilizer increased maize dry weight equivalent to the plants of the 33%N-fertilized treatment. The inoculants applied singly or together, combined with 33%N-fertilizer, increased maize and wheat shoot dry weight over the Untreated plus 33%N.

Inoculation of Isolate V16 plus 33%N-fertilizer was the best treatment for enhanced maize chlorophyll levels (Table 7.1). Isolate V16 or Eco-T® applied singly significantly ($P<0.001$) increased shoot dry weight of wheat over the Untreated control. Dual application of these two inoculants significantly increased shoot dry weight of wheat over the Untreated control, or Isolate V16 or Eco-T® applied singly. In the presence of 33% N-fertilizer, Isolate V16 performed as well as the combination of Isolate V16 plus Eco-T®, in enhancing shoot dry weight of wheat (Table 7.1). Of all the treatments, plants fertilized with 100% NPK showed the highest shoot dry weight of wheat and maize, and the highest chlorophyll levels of maize. Dual inoculation of Isolate V16 and Eco-T® plus 33%N-fertilizer increased maize dry weight by 51% and wheat dry weight by 22% (Table 7.1). Inoculation of Isolate V16 plus 33%N-fertilizer increased maize chlorophyll level by 9% over the Untreated plus 33%N.

Table 7.1 Combined effect of inoculants with or without reduced N-fertilizer on maize and wheat growth under greenhouse conditions

	Maize				Wheat	
Treatments	**Dry weight (g)**		**CCI**		**Dry weight (g)**	
Control	9.59	a	2.03	a	1.85	a
Eco-T®	14.01	a	7.25	ab	4.02	b
V16	18.92	a	8.01	bc	3.54	b
V16+Eco-T	45.81	b	9.33	bc	6.84	c
33%N	46.58	b	12.3	bc	7.82	cd
V16+33%N	54.86	bc	13.4	c	8.69	de
Eco-T+33%N	57.61	bc	11.05	bc	8.38	d
V16+Eco-T+33%N	70.52	c	11.12	bc	9.53	e
NPK	93.6	d	20.87	d	12.17	f
CV%	14		16.7		5.1	
Lsd	11.09		3.062		0.6203	
Sed	5.23		1.444		0.2928	
P	<0.001		P<0.001		<0.001	

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Fisher's L.S.D. test

CCI: Chlorophyll Content Index

Treatments: diazotrophic bacterial Isolate (V16), a commercial BCA (Eco-T®), 33%N-fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Centre, Cedara, Pietermaritzburg, Republic of South Africa; Un-inoculated and Fully Fertilized Control (100%NPK) and Un-inoculated and Un-fertilized (Control)

7.4 Discussion

In many studies, the inoculation of diazotrophs applied singly can only partly meet the N demand of plants because cereals and other non-legumes usually require high N levels for optimum yields. An eco-friendly and cost effective strategy that combines the use of reduced applications of chemical N-fertilizer combined with plant growth promoting inoculants may be important for

sustainable agriculture. Use of microbial inoculants to enhance plant growth and increase yields of agricultural crops has been under investigation for several years. Use of combined bio-inoculants for enhancing plant growth and yield has also shown promising results. However, inconsistence of data has been reported (Bashan and Holguin, 1997; Mansfeld-Giese *et al.*, 2002; Lucy *et al.*, 2004). Co-inoculation of plant growth bacteria and fungi, combined with a reduced level of N-fertilizer, therefore, could provide an option for optimum crop production.

Several microorganisms are known to have beneficial effect on plant growth and plant nutrient accumulation. In this study, inoculation of *Bacillus megaterium* (V16) and Eco-T®, applied singly, caused no significant increases in dry weight of maize. However, co-inoculation of *Bacillus megaterium* and Eco-T®, without any added N-fertilizer, enhanced shoot chlorophyll level of maize and shoot dry weight of wheat above the Un-inoculated and Un-fertilized control, the bacterium or Eco-T® were applied singly. Eco-T® known to be a bio-control agent, also showed stimulation of plant growth in the absence of a pathogen. This enhanced leaf N and shoot dry weight was either due to the BNF activity of *Bacillus megaterium* and plant stimulation effect of Eco-T®, or due to the increased nutrient uptake by these plants because of the inoculants. Previous studies have shown that effectiveness of PGPR using multi strains inoculations (Yang *et al.*, 2009). The results of the present study agree with the results of Jisha and Alagawadi (1996), who showed that the co-inoculation of *Bacillus polymyxa* (Prazmowcoki) Mace and *T. harzianum* enhanced growth of sorghum, as compared to either organism applied singly under greenhouse conditions. Similar result reported by Yobo *et al.* (2011) showed combined inoculation of *T. atroviride* SYN 6 and *B. subtilis* B69 increased seedling dry biomass of beans by 43% in greenhouses. In another studies, combined inoculation of biocontrol agents and PGPR suppressed plant disease (Nakkeeran *et al.*, 2006) and improved yields and nutrient uptake (Rudresh *et al.*, 2005). Combining *Bacillus megaterium* de Bary and *Azotobacter chroococcum* Beijerinck increased crop yields in field trials by 10-20% (Saharan and Nehra, 2011b). Ahmad *et al*. (2006) also showed that the co-inoculation of *Vigna radiata* L. T44 with *Bradyrhizobium* (Kirchner) Jordan with other rhizosphere bacteria gave better results than those inoculated with *Bradyrhizobium* alone.

A single microbial inoculation at reduced chemical fertilizer levels enhanced chlorophyll level of maize and dry biomass of wheat and maize above that of plants treated with reduced chemical fertilizer or bacterial isolates alone. The shoot dry biomass obtained with the seed inoculation with V16 plus Eco-T® at reduced N-fertilizer performed better than the 33% N-fertilizer. Similar results were recorded for sugarcane (*Saccharum officinarum* L.) by co-inoculation of two bacteria, which enhanced crop biomass under N-limited condition (Muthukumarasamy *et al.*, 2006). Inoculation of wheat with *Azospirillum brasilense* increased yield and other yield components significantly under low fertilizer rates compared to higher rates under field conditions (Dobbelaere *et al.*, 2001). Application of PGPR in combination with a reduced level of inorganic fertilizer enhanced nitrogen and phosphorus uptake in tomatoes (Adesemoye *et al.*, 2009). In this study, inoculation of Isolate V16 and Eco-T® increased maize dry weight by 51% and 22%, and wheat dry weight, above the Untreated plus 33%N-fertilizer. These results suggest that co-inoculation could meet up to 51% and 22% nutrient requirements of the two crops, respectively, and co-inoculation could supplement reduced amounts of N fertilizer without compromising crop yields. Other studies have also demonstrated that inoculation of wheat with *Azotobacter* could reduce urea N requirements by 50% under greenhouse conditions (Soliman and Monem, 1995; Hegazi and Fayez, 2001; Hellal *et al.*, 2011; Saharan and Nehra, 2011b). The present study suggests that the use of combined inoculants together with reduced N-fertilizer applications may provide an important alternative for the integrated management of fertilizers for sustainable agriculture.

Reference

Adesemoye A., Torbert H., Kloepper J. (2009) Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology 58:921-929.

Ahmad F., Ahmad I., Aqil F., Ahmed Wani A., Sousche Y.S. (2006) Plant growth promoting potential of free living diazotrophs and other rhizobacteria isolated from Northern Indian soil. Biotechnology Journal 1:1112-1123.

Alene A.D., Manyong V., Omanya G., Mignouna H., Bokanga M., Odhiambo G. (2008) Smallholder market participation under transactions costs: maize supply and fertilizer demand in Kenya. Food Policy 33:318-328.

Artursson V., Finlay R.D., Jansson J.K. (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environmental Microbiology 8:1-10.

Bashan Y., Holguin G. (1997) *Azospirillum*-plant relationships: environmental and physiological advances (1990-1996). Canadian Journal of Microbiology 43:103-121.

Bashan Y., de Bashan L. (2005) Plant growth-promoting. Encyclopedia of soils in the Environment 1:103-115.

Berg G. (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84:11-18.

Bruns H.A., Abel C.A. (2003) Nitrogen fertility effects on Bt δ-endotoxin and nitrogen concentrations of maize during early growth. Agronomy Journal 95:207-211.

Cakmak I. (2002) Plant nutrition research: priorities to meet human needs for food in sustainable ways. Plant and Soil 247:3-24.

Çakmakçi R., Dönmez F., Aydın A., Şahin F. (2006) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biology and Biochemistry 38:1482-1487.

Canbolat M.Y., Bilen S., Çakmakçı R., Şahin F., Aydın A. (2006) Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biology and Fertility of Soils 42:350-357.

Cassán F., Perrig D., Sgroy V., Masciarelli O., Penna C., Luna V. (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). European Journal of Soil Biology 45:28-35.

Cassman K.G., Dobermann A., Walters D.T. (2002) Agroecosystems, nitrogen-use efficiency, and nitrogen management. AMBIO: A Journal of the Human Environment 31:132-140.

Dobbelaere S., Croonenborghs A., Thys A., Ptacek D., Vanderleyden J., Dutto P., Labandera-Gonzalez C., Caballero-Mellado J., Aguirre J.F., Kapulnik Y. (2001) Responses of agronomically important crops to inoculation with *Azospirillum*. Functional Plant Biology 28:871-879.

Dobbelaere S., Vanderleyden J., Okon Y. (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22:107-149.

Gholami A., Shahsavani S., Nezarat S. (2009) The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. International Journal of Biological and Life Sceinces 1:35-40.

Han H., Lee K. (2006) Effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of pepper and cucumber. Plant Soil and Environment 52:130.

Hegazi N., Fayez M. (2001) Biological nitrogen fixation to maximize productivity of intercropped legumes and non-legumes: Ten years of field experimentations in semi-arid deserts of Egypt. Archives of Agronomy and Soil Science 47:103-131.

Hellal F., Mahfouz S., Hassan F. (2011) Partial substitution of mineral nitrogen fertilizer by bio-fertilizer on (*Anethum graveolens* L.) plant. Agriculture and Biology Journal of North America 4:652-660.

Jayne T.S., Govereh J., Wanzala M., Demeke M. (2003) Fertilizer market development: a comparative analysis of Ethiopia, Kenya, and Zambia. Food Policy 28:293-316.

Jisha M., Alagawadi A. (1996) Nutrient uptake and yield of sorghum (*Sorghum bicolor* L. Moench) inoculated with phosphate solubilizing bacteria and cellulolytic fungus in a cotton stalk amended vertisol. Microbiological Research 151:213-217.

Kennedy I.R., Choudhury A., Kecskés M.L. (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Khalid A., Arshad M., Zahir Z. (2004) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology 96:473-480.

Kloepper J.W., Ryu C.-M., Zhang S. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Kumar S., Pandey P., Maheshwari D. (2009) Reduction in dose of chemical fertilizers and growth enhancement of sesame (*Sesamum indicum* L.) with application of rhizospheric competent *Pseudomonas aeruginosa* LES4. European Journal of Soil Biology 45:334-340.

Kuzyakov Y., Friedel J., Stahr K. (2000) Review of mechanisms and quantification of priming effects. Soil Biology and Biochemistry 32:1485-1498.

Ladha J.K., Pathak H., J Krupnik T., Six J., van Kessel C. (2005) Efficiency of fertilizer nitrogen in cereal production: retrospects and prospects. Advances in Agronomy 87:85-156.

Lucy M., Reed E., Glick B.R. (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek 86:1-25.

Mansfeld-Giese K., Larsen J., Bødker L. (2002) Bacterial populations associated with mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. FEMS microbiology ecology 41:133-140.

Ma Y., Rajkumar M., Freitas H. (2009) Improvement of plant growth and nickel uptake by nickel resistant-plant-growth promoting bacteria. Journal of Hazardous Materials 166:1154-1161.

Muthukumarasamy R., Govindarajan M., Vadivelu M., Revathi G. (2006) N-fertilizer saving by the inoculation of *Gluconacetobacter diazotrophicus* and *Herbaspirillum* sp. in micropropagated sugarcane plants. Microbiological Research 161:238-245.

Nakkeeran S., Fernando W.D., Siddiqui Z.A. (2006) Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases, In, Siddiqui, S.A (ed). PGPR: Biocontrol and Biofertilization. Springer, Dordrecht, The Netherands. pp. 257-296.

Naserirad H., Soleymanifard A., Naseri R. (2011) Effect of integrated application of bio-fertilizer on grain yield, yield components and associated traits of maizec. American-Eurasian Journal of Agriculture andEnvironmental Science, 10 (2):271-277.

Ortiz-Monasterio J., Palacios-Rojas N., Meng E., Pixley K., Trethowan R., Pena R. (2007) Enhancing the mineral and vitamin content of wheat and maize through plant breeding. Journal of Cereal Science 46:293-307.

Pang X., Letey J. (2000) Organic farming challenge of timing nitrogen availability to crop nitrogen requirements. Soil Science Society of America Journal 64:247-253.

Raun W.R., Solie J.B., Johnson G.V., Stone M.L., Mullen R.W., Freeman K.W., Thomason W.E., Lukina E.V. (2002) Improving nitrogen use efficiency in cereal grain production with optical sensing and variable rate application. Agronomy Journal 94:815-820.

Riggs P.J., Chelius M.K., Iniguez A.L., Kaeppler S.M., Triplett E.W. (2001) Enhanced maize productivity by inoculation with diazotrophic bacteria. Functional Plant Biology 28:829-836.

Rodriguez H., Fraga R., Gonzalez T., Bashan Y. (2006) Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant and Soil 287:15-21.

Rudresh D., Shivaprakash M., Prasad R. (2005) Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer aritenium* L.). Applied Soil Ecology 28:139-146.

Saharan B., Nehra V. (2011a) Plant growth promoting rhizobacteria: a critical review. Life Science and Medical Research 21:1-30.

Saharan B., Nehra V. (2011b) Plant growth promoting rhizobacteria: A critical review. Life Sciences and Medical Research 21:1-30.

Shaharoona B., Arshad M., Zahir Z.A., Khalid A. (2006) Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. Soil Biology and Biochemistry 38:2971-2975.

Shaharoona B., Naveed M., Arshad M., Zahir Z.A. (2008) Fertilizer-dependent efficiency of Pseudomonadsfor improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). Applied Microbiology and Biotechnology 79:147-155.

Soliman S., Monem M. (1995) Influence of N^{15} labelled urea and *Azotobacter* on corn and nitrogen budget as affected by organic matter. Egyptian Journal of Soil Science 35:415-426.

Vessey J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571-586.

Yang J., Kloepper J.W., Ryu C.-M. (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends in plant science 14:1-4.

Yobo K., Laing M., Hunter C. (2011) Effects of single and combined inoculations of selected *Trichoderma* and *Bacillus* isolates on growth of dry bean and biological control of *Rhizoctonia solani* damping-off. African Journal of Biotechnology 10:8746-8756.

Thesis Overview

Globally, there is increasing need to reduce the cost of fertilizer inputs in agricultural crop production. The search for replacements or supplement to fertilizers and agrochemicals has attracted the attention of many researchers in the last few decades. Eighty percent of our atmosphere is made up of nitrogen gas (N_2). This gas is of no use to most organisms and can only be beneficial to plant growth if it is first converted to ammonium and/ or nitrate. This can be done through an industrial process (the Haber-Bosch reaction) used in the manufacture of nitrogenous fertilizers but this requires electricity and indirectly contributes to climate change, via the burning of hydrocarbon. However, use of N-fertilizer inputs in developing countries need to increase each year, in order for production to increase. This will cost billions of US dollars and may be harmful to the environment and to human health (Saleque, *et al.*, 2004, Kitchen, *et al.*, 2010, Powell, *et al.*, 2010). Many soils in Africa are severely depleted of nitrogen, making it difficult for smallholder farmers to produce the yields needed to feed growing populations. Use of microbial inoculants to enhance crop production has therefore been proposed as more affordable and environmentally sound option for sustainable agriculture (Wu, *et al.*, 2005). Diazotrophic bacteria are known by their ability to convert N_2 into ammonia which can be used by plants. They provide their host plants with competitive benefits in a C-rich and N-poor environment, with the result that they promote plant growth (Dobbelaere, *et al.*, 2003).

Inoculation of seed of crops with diazotrophic bacteria has been documented to increase plant growth and yields (Dobbelaere, *et al.*, 2003, Vessey, 2003, Choudhury, *et al.*, 2004, Ahmad, *et al.*, 2008). These diazotrophic bacteria have been shown to influence plant growth and yields through mechanisms such as biological nitrogen fixation (BNF), phytohormone production and phosphate-solubilization (Dobbelaere, *et al.*, 2003). Bacteria widely investigated for plant growth promotion include genera such as *Azospirillum* (Vessey, 2003, Bashan, *et al.*, 2010), *Azotobacter* (Dobbelaere, *et al.*, 2003, Wu, *et al.*, 2005), *Bacillus* (Çakmakçi, *et al.*, 2006, Adesemoye, *et al.*, 2008), *Klebsiella* (Vessey, 2003, Compant, *et al.*, 2010)and *Pseudomonas* (Dey, *et al.*, 2004, Ahmad, *et al.*, 2008).

In vitro studies were conducted to determine the possible mechanisms of plant growth promotion exhibited by these isolates. Ninety five bacteria were selected by the ability to grow on N-free media using different carbon sources (sucrose, D-mannitol or malate). Secondly, they were

subjected to a test for ammonia production, and were then further tested using the acetylene reduction assay (ARA). C_2H_4 production was quantified and ranged 0 to 73 n moles of $C_2H_4\,h^{-1}$ $culture^{-1}$. Isolates which produced ≥40 n moles of $C_2H_4\,h^{-1}$ $culture^{-1}$ were re-screened on maize plants *in vivo* and eleven of them caused significant ($P<0.001$) increases of stomatal conductance, dry weight and chlorophyll content index of maize leaves. These isolates were then identified using partial 16s rRNA sequence analysis and MALDI TOF Biotype classification and they were identified as *Pseudomonas* spp., *Burkholderia ambifaria, Enterobacter* spp., *Bacillus megaterium, Klebsiella* spp. and *Pantoea* spp.

The five best diazotrophic bacterial isolates were investigated for their effectiveness for different methods of application onto maize (*Zea mays L.*) under greenhouse conditions. These methods of application were drenching, seed treatment, foliar spray and a combination of these. The five isolates were also assessed for their effects on the germination of wheat *in vitro,* and were tested in combination with various levels of nitrogenous fertilizer for growth-promotion of wheat (*Tritium aestivum* L.). These five isolates were also investigated for their potential to enhance growth and yields of maize and wheat crops in field trials, especially when combined with a low dose of nitrogenous fertilizer. These isolates were further studied for their ability to enhance plant growth through nitrogen fixation by predicting chlorophyll content using a chlorophyll content meter (CCM-200), and correlated with chemical analysis for chlorophyll content. A study was also conducted on the *in vitro* interaction of isolates of *Bacillus megaterium* and Eco-T®, a commercial biocontrol agent (BCA), (an isolate of *Trichoderma harzianum* Rifai), to determine the value of applying the two microbes together to enhance plant growth.

In this overview we report the findings of this study and the issues that need to be addressed in future research. The findings from this research were as follows:

- Combination of the most promising bacterial isolates from the *in vitro* studies and a low dose of nitrogenous fertilizer to enhanced growth of maize and wheat under greenhouse conditions.

- Inoculation of selected diazotrophic isolates applied as seed treatment or seed treatment plus drench resulted in increases in dry weight and leaf chlorophyll content of maize.

- Indirect estimation of N content using chlorophyll content meter (CCM-200) and extractable leaf chlorophyll content were highly correlated
- Seed inoculation of maize with some bacterial isolates, in combination with a low dose of nitrogenous fertilizer, increased shoot dry biomass and yields of maize relative to the Uninoculated Control.

Seed inoculation of wheat with some bacterial isolates in combination with a low dose of nitrogenous fertilizer caused significantly higher biomass and yield than the Uninoculated Control under field conditions. Isolation of diazotrophic bacteria were carried out on N-free media using various carbon sources (sucrose, D-mannitol or malate). Similar methods of isolation were reported by (Park, *et al.*, 2005, Picossi, *et al.*, 2005, Tejera, *et al.*, 2005). Then ninety five isolates were able to grow well on media when sucrose was provided as carbon source but grew slowly on D-mannitol and malate. Many isolates produced ammonia in liquid cultures, which confirmed their capacity to fix N_2 in pure culture. Isolates which are slow to grow on N-free media might indicate that the isolates require microaerobic conditions for fixing nitrogen (Li, *et al.*, 2008). For further selecting and screening of prospective strains, ARA was used as a test for diazotrophy. Out of the 93 strains from the primary selection process, only strains with an ARA activity of ≥40 nmol C_2H_4 h^{-1} $culture^{-1}$were studied further. It is difficult to compare the nitrogenase activity of bacterial strains studied in this work with the results obtained by others, mainly due to the different methods used and the different ways of expressing the levels of nitrogen fixation. The results of this study on nitrogenase activity were in agreement with the results of Różycki*et al*. (1999) who reported similar levels of nitrogenase activity of diazotrophic bacteria, most of which belonged to the genera *Pseudomonas* and *Bacillus*. The strains which exhibited ≥40 nmol C_2H_4 h^{-1} $culture^{-1}$ and enhanced maize growth in greenhouses conditions were identified to genus level using 16s rRNA sequencing and MALDI Biotyper classification. Using partial 16s rRNA sequence analysis, Isolates StB5, A3, A6, B1 and A61 showed a 99% similarity with *Pseudomonas* spp., Isolate V9 and A5 showed 97% similarity with *Burkholderia ambifaria*, Isolate L1 94% similarity with *Enterobacter* spp., Isolate V16 97% similarity with *Bacillus megaterium*, Isolate A2 100% similarity with *Klebsiella* spp., and Isolate LB5 100% similarity with *Pantoea* spp. The identification was confirmed by MALDI TOF Biotype classification. Isolates StB5, A3, A6, B1 and A61 were identified as *Pseudomonas*

nitroreducens at score values of 1.98, 1.90, 1.96, 2.03 and 1.88, respectively. Isolates V9 (2.46) and A5 (1.86) were identified as *Burkholderia ambifaria*, Isolate L1 (2.33) as *Enterobacter cloacae*, Isolate V16 (1.72) as *Bacillus megaterium*, A2 (2.24) as *Klebsiella variicola* and Isolate LB5 (2.27) as *Pantoea ananatis*. Both identification methods showed high correlations with known genera and species. Similar result was reported by Saffert, *et al*. (2011) who used a Bruker Biotyper to identify Gram-negative bacilli to the genus and species level correctly.*In-vitro* screening of diazotrophic bacteria for nitrogenase activity provided a quick and viable technique for the selection of effective diazotrophic bacterial strains for use in sustainable agriculture. However, some of the effective isolates selected were subsequently shown to be closely related to bacterial species known to be pathogenic to animals and humans. Therefore, there is still a need to identify simpler techniques that include identification of the isolates that can be used for screening of larger numbers of isolates *in vitro*. Reports on a lack of correlation between results obtained *in vitro* and under field conditions exist in the literature (Schroth and Becker 1990; Williams and Asher, 1996). However, in this study, the most promising isolates identified *in vitro* also worked well under greenhouses and field conditions. They enhanced seedling growth of maize and wheat under greenhouse and field conditions. Isolate *Bacillus megaterium* (V16), *Burkholderia ambiferia* (V9), *Enterobacter cloacae* (L1), *Pantoea ananatis* (LB5) and *Pseudomonas nitroreducens* (StB5) enhanced shoot dry biomass and yield in wheat and maize. This demonstrated that isolates that exhibited good nitrogenase activity *in vitro* also enhanced plant growth in greenhouses. Khalid *et al*. (2004) also demonstrated that there was a positive correlation between the *in vitro* indole-3-acetic acid production by rhizobacteria and the increases in host growth parameters. Further research is required to establish the exact mechanism responsible for the observed results, to determine whether these results were due to the synergistic effects by various growth enhancement mechanisms.

Finding appropriate application methods of inoculum and optimum concentrations is a key for the use of diazotrophs to enhance plant growth. Inoculation of Isolate StB5 (*Pseudomonas* spp.) increased maize dry weight when applied by seed treatment or drenching alone, and induced higher leaf chlorophyll content when applied by several combinations of application (seed treatment + drench, seed treatment + foliar spray or drench + foliar spray). Isolate V9 (*Burkholderia ambiferia*) induced increases in dry weight when applied by a combination of seed

treatment + drench + foliar spray. Seed treatment as a sole application or in a combination of drenching induced higher leaf chlorophyll content and dry weight. Foliar application of PGPR strains of *Azotobacter*, *Azospirillum* and *Beijerinckia* was reported by Sudhakar *et al*. (2000) to be an effective method of application resulting in an increased fruit and leaf yield of mulberry (*Morus* spp.). Given its efficacy as a solo treatment, and that seed treatment is the simplest and most convenient method of application, this method of application can be recommended to farmers as the best method of application of diazotrophs for plant growth promotion.

Diazotrophic treatments and concurrent N applications increased plant N levels which was determined by chlorophyll content meter (CCM) readings. The result showed that when the levels of N-fertilizer increased, the chlorophyll content also increased. Correlation analysis indicated that 98% of the variation in N application levels was predicted by CCM readings. Similar results were also reported by Cate and Perkins (2003) that chlorophyll concentrations correlate positively with leaf N. This relationship should make it possible to use leaf chlorophyll content to estimate crop N status (Daughtry *et al.*, 2000).

Inoculation of diazotrophs in combination with a 65% nitrogenous fertilizer in wheat resulted in a greater shoot biomass than the Fully Fertilized Control, whereas increasing fertilizer doses above these levels did not seem to have any significant effect on the biomass of wheat. Maximum dry weight (41%) was obtained when fertilizer was applied at 0%NPK along with one of the isolates as compared to the Un-inoculated and Unfertilized Control under greenhouse conditions. The dry weight increases from Isolate StB5 together with a 65%NPK fertilizer application rate, out yielded the fully fertilized (100%NPK) Control. This observation indicates that these bacterial isolates were more effective at low levels of nitrogenous fertilizer applications. Similar results were reported by Ozturk, *et al*. (2003) improvements in growth parameters of barley and wheat as a result of bacterial inoculations at reduced levels of nitrogenous fertilizers. These findings confirm that the use of suitable microbial inoculants may enhance nitrogen fertilizer efficiency, leading to enhanced crop production at lower doses of these fertilizers. Use of the best isolates found in this study may provide an important component of integrated mineral management for maize and wheat production.

Therere is a problem in this field of PGPRs because some of the plant-associated PGPR genera such as *Burkholderia, Enterobacter, Pantoea* and *Pseudomonas* may also be opportunistic pathogens on humans (Berg, *et al*., 2005, Tyler and Triplett, 2008). However, there is currently no direct link between rhizosphere isolates and those that are pathogenic to animals and humans. If the necessary precautions are taken to ensure the safety of personnel dealing with the inoculants, these isolate identified in this study could therefore be grown and formulated as inoculant biofertilizers for enhancing maize and wheat production.

In conculusion, inoculation of diazotrophs alone or combined with reduced level of N-fertilization may reduce the requirement of N-fertilization as a major boost to science. This biofertilization technology may also make N-fertiliation possible for small scale farmers at 35-65% of the level used by commercial farmers at a price of more than R1000 ha^{-1} for N-fertilizer versus R30 ha^{-1} for bacterial inoculants on seeds, the costs are dramatically different for maize and wheat production.

References

Adesemoye A., Obini M., Ugoji E. (2008). Comparison of plant growth-promotion with *Pseudomonas aeruginosa* and *Bacillus subtilis* in three vegetables. Brazilian Journal of Microbiology 39: 423-426.

Ahmad F., Ahmad I., Khan M. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. MicrobiologicalRresearch 163: 173-181.

Bashan Y., de-Bashan L.E. (2010). How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. Advances in Agronomy 108: 77-136.

Berg G., Eberl L., Hartmann A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environmental Microbiology 7: 1673-1685.

Çakmakçi R., Dönmez F., Aydın A., Şahin F. (2006). Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biology and Biochemistry 38: 1482-1487.

Cate T.M., Perkins T. (2003) Chlorophyll content monitoring in sugar maple (*Acer saccharum*). Tree Physiology 23:1077-1079.

Choudhury A., Kennedy I.(2004). Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biology and Fertility of Soils 39: 219-227.

Compant S., Clément C., Sessitsch A.(2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry 42: 669-678.

Daughtry C., Walthall C., Kim M., De Colstoun E.B., McMurtrey J. (2000) Estimating corn leaf chlorophyll concentration from leaf and canopy reflectance. Remote Sensing of Environment 74:229-239.

Dey R., Pal K., Bhatt D., Chauhan S. (2004). Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiological Research 159: 371-394.

Dobbelaere S., Vanderleyden J., Okon Y.(2003). Plant growth-promoting effects of diazotrophs in the rhizosphere. Critical Reviews in Plant Sciences 22: 107-149.

Khalid A., Arshad M., Zahir Z.A. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. Journal of Applied Microbiology96 (3), 473–480.

Kitchen N.R., Sudduth K.A., Drummond S.T., Scharf P.C., Palm, H.L. Roberts, D.F. Vories E.D. (2010). Ground-based canopy reflectance sensing for variable-rate nitrogen corn fertilization. Agronomy Journal 102: 71-84.

Li J.H., Wang E.T., Chen W.F., Chen W.X. (2008). Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang Province of China. Soil Biology and Biochemistry 40: 238-246.

Ozturk A., Caglar O., Sahin F. (2003). Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. Journal of Plant Nutrition and Soil Science 166: 262-266.

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

Picossi S., Montesinos M.L., Pernil R., Lichtlé C., Herrero A., Flores E.(2005). ABC-type neutral amino acid permease N-I is required for optimal diazotrophic growth and is repressed in the heterocysts of *Anabaena* sp. strain PCC 7120. Molecular Microbiology 57: 1582-1592.

Powell J., Gourley C., Rotz C., Weaver D.(2010). Nitrogen use efficiency: A potential performance indicator and policy tool for dairy farms. Environmental Science and Policy 13: 217-228.

Rodríguez H., Fraga R. (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17:319-339.

Saffert R.T., Cunningham S.A., Ihde S.M., Jobe K.E.M., Mandrekar J., Patel R. (2011). Comparison of Bruker Biotyper matrix-assisted laser desorption ionization–time of flight

mass spectrometer to BD Phoenix automated microbiology system for identification of gram-negative bacilli. Journal of Clinical Microbiology 49: 887-892.

Saleque M., Abedin M., Bhuiyan N., Zaman S., Panaullah G. (2004). Long-term effects of inorganic and organic fertilizer sources on yield and nutrient accumulation of lowland rice. Field Crops Research 86: 53-65.

Schroth M.N., Becker J.O. (1990). Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In Biological Control of Soilborne Plant Pathogens. Hornby, D., Editor. Wallingford: CAB International, pp. 389-414.

Sudhakar P., Chattopadhyay G., Gangwar S., Ghosh J. (2000) Effect of foliar application of *Azotobacter, Azospirillum* and *Beijerinckia* on leaf yield and quality of mulberry (*Morus alba*). Journal of Agricultural Science 134:227-234.

Tejera N., Lluch C., Martinez-Toledo M., Gonzalez-Lopez J. (2005). Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere. Plant and Soil 270: 223-232.

Tyler H.L., Triplett E.W. (2008). Plants as a habitat for beneficial and/or human pathogenic bacteria. Annual Review of Phytopathology 46: 53-73.

Vessey J.K. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255: 571-586.

Williams G.E., Asher M.J.C. (1996). Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochlioides* on sugar-beet seedlings. Crop Protection15, 479-486.

Wu S., Cao Z., Li Z., Cheung K.,Wong M. (2005). Effects of a biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125: 155-166.