Efficacy of selected rhizobacteria for growth promotion of maize and suppression of *Fusarium* root and crown rot in South Africa

by

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DECLARATION

I, the undersigned, declare that the MSc thesis entitled: “Efficacy of selected Rhizobacteria for growth promotion of maize and suppression of *Fusarium* root and crown rot in South Africa” submitted for the MSc degree in Micorbiology at the University of Pretoria is my own work and has not previously been submitted by me for any degree at this or any tertiary institution.

…….. ……

Gerhardus Breedt Date

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To the Lord who gave me the wisdom and strength to grow in tough times as said in Psalm 28 verse 7: “The LORD is my strength and my shield; my heart trusts in him”

Contents

Preface

Zea mays L. commonly known as “maize” is a grain crop within the family of *Poaceae* (Paliwal, 2000). According to Miracle (1965), maize was introduced to South Africa (R.S.A.) by the Portuguese in the 17th century from South America. Since the introduction, maize production has developed into one of the largest agricultural branches in South Africa and rated as the most important grain crop in R.S.A. (Du Plessis, 2003).

According to the FAO (2014) South African maize yield per hectare has decreased from an average of 4.81tha^{-1} in 2011 to 4.19tha^{-1} in 2013. On the other hand the areas planted in the Western and Central regions of Africa have increased significantly from 3.2 million hectares to 8.9 million hectares since 1961 to 2005. This devotion of land to maize production increased the total yield per country but the average yield is still 1.3tha^{-1} compared to the production potential of 8.6tha^{-1} (FAO, 2004). Various environmental, cultural and nutritional factors e.g. adapted cultivars, plant population, crop management, fertilisation, weed, disease and insect control etc. could explain the low yields observed in these maize producing areas (ARC, 2008). These limiting factors are dependent on each other and as the human population increases, more pressure is being put on the soil to increase production which results in the slow exhaustion of soil fertility (Shepard & Soule, 1998).

The discovery of mineral fertilizers led to the replacement of biological fertilizers. According to Oikarinen (1996) these mineral fertilizers are inefficient, negatively affect the environment and lower the product quality. The slow exhaustion of soil fertility and the negative impact mineral fertilisers have on the environment has created the need to study biosystems in order to promote sound farming practices. The advances in modern science created a niche to better understand biosystems and the impact chemical fertilisers have on these systems. In turn the better the understanding of the negative impacts on these biosystems the better it will promote the environmentally friendly biological fertiliser concept.

The term rhizosphere refers to the soil around the root system that the plant influences biologically through secreting various substances into this soil

compartment. Rhizobacteria are bacteria classified as living in the rhizosphere soil compartment and if beneficial to the plant, the rhizobacteria are termed Plant Growth Promoting Rhizobacteria (PGPR).

Various studies have been conducted to utilise the PGPR-plant interaction to promote plant growth and thereby increase farming efficiency and improve food security. The current study evaluated selected PGPR isolates from the University of Pretoria's culture collection for their efficacy as growth enhancing agents in maize. The selected PGPR isolates were first screened for their growth promoting capabilities under greenhouse conditions. From these screening trials the best isolates were identified and re-evaluated in the greenhouse at various dosages, nutrient levels and different formulations to ensure optimal performance before testing under field conditions.

After the greenhouse trials the best performing isolates were extensively evaluated for three seasons from 2010 to 2013 in field trials conducted in the South Western area of the Limpopo Province, South Africa. During the first season remarkable effects were obtained with the seed inoculated isolates S1 and S2-08 resulting in a $1.12 tha^{-1}$ grain increase over that of the control. However, these strains were identified as *Bacillus cereus* and they could be potential human pathogens. These strains tested positive for the human diahoreal toxin and were subsequently excluded from the study and replaced with other PGPR strains that showed potential as plant growth stimulants in various other research trials at the University of Pretoria.

During the successive years the overall best performing rhizobacterial strain in all soil types was T-19, identified as *Lysinibacillus sphearicus*. From this study it was evident that different soil types had a profound effect on the ability of the rhizobacteria to promote plant growth. In the field trials it was observed that the higher the clay content the lower the PGPR stimulatory effect on the plant and *vice versa.*

In the laboratory, isolate T-19 tested positive for indole-3-acetic acid (IAA) production and limited nitrogen fixation, while the commercial product Brus® tested positive for

all the modes of action that were evaluated. This then raised the question as to why the *Lysinibacillus sphearicus* strain (T19) with its limited mode of action consistently outperformed inoculants with a broader spectrum of action when evaluated under field conditions.

In the current study PGPR strains were identified that had demonstrated plant growth enhancement capabilities, not only under greenhouse conditions, but also under field conditions. These strains appear to have commercial potential as biofertilisers. This is becoming increasingly important in the context of the global emphasis on more environmentally friendly agricultural practices. By demonstrating significant increases of maize yield in the field after seed treatment with selected PGPR strains, the current study provided additional evidence for the potential of PGPR application in the context of a more sustainable agricultural system.

Chapter 1

General introduction

1.1 Background

Wheat (*Triticum aestivum*) and maize (*Zea mays*) are the two most important crops worldwide and sustainable agricultural systems for these crops are urgently required (FAO, 2001). In both wheat and maize production areas, agricultural intensification has placed an enormous amount of pressure on the soil, leading to its degradation (FAO, 2001). The use of advantageous rhizobacteria to alter the rhizosphere may serve as a substitute to synthetic fertilizers improving the assimilation of chemical nutrients and water usage by the plant (de Freitas, 2000).

Hiltner first defined the term “rhizosphere” in 1904. He stated that “the rhizosphere is the soil compartment influenced by the root”. The rhizosphere “deposits” have been described by Spaepen *et al*. (2009) as the sum of carbon transferred from the roots to the soil including macromolecule secretions like enzymes, root exudates, mucilage and dead cell lysates. This site around the roots influenced by the root’s “deposits” can be described as the most fertile areas in the soil for microbial growth.

It has long been hypothesized that various bacterial species, when applied to the plant rhizosphere can promote health, efficiency and yield of crop plants. These biological entities, which are generally indigenous to the rhizosphere of plants, are commonly termed Plant Growth Promoting Rhizobacteria (PGPR). Plant Growth Promoting Rhizobacteria may include representatives of diverse genera, with *Pseudomonas* and *Bacillus* species among the most researched (Thomashaw, 1996). The health of an agricultural rhizospheric ecosystem is directly dependent on its micro-organisms and amendment with PGPR can help enhance the crop plants to attain higher yields. These positive influences on plant health are attributed to PGPR improving soil fertility and the rhizospheric environment through processes such as the biological control of plant pathogens, seed establishment and nutrient cycling (Jeffries *et al*., 2003).

The natural roles of rhizospheric microorganisms are being marginalized by conventional farming practices in the agricultural sector (Mader *et al.,* 2002). Various biotic and abiotic factors such as soil type, pH and microflora have a large impact on PGPR efficiency (van Veen *et al.,* 1997). Soil is an irregular environment and *in vivo* experiments are very difficult to replicate (Bashan, 1998).

Various experiments have shown that PGPR can affect the plants' physiology directly by producing and releasing secondary metabolites (plant growth regulators/phytohormones/ biologically active substances), or indirectly by reducing or preventing harmful effects of pathogenic rhizosphere organisms and/or facilitating the accessibility and uptake of nutrients to the plant (Rosas *et al*., 2008). According to Zahir *et al*. (2004), PGPR selection is the most significant criterion when it comes to maximizing the benefits this venture may hold in promoting more sustainable agriculture. PGPR can promote plant growth by means of the following mechanisms: (i) production of plant growth regulators that can influence plant growth (Glick, 1995), (ii) nitrogen provided to the plant via asymbiotic nitrogen fixation mechanisms (Boddey & Dobereiner, 1995), (iii) antagonistic symbiosis (niche exclusion) by means of siderophore production that limit pathogen growth in the rhizosphere (Scher & Baker, 1982), (vi) biocontrol of pathogens by means of antibiotic production, (v) cyanide production (Shanahan *et al*., 1992. ,Flaishman *et al*., 1996), and (vi) facilitating mineral/nutrient accessibility (Gaur, 1990).

1.2 Aim

Extensive research has been done on Rhizobacteria as plant growth stimulants, with very promising results. This technology can be adapted and applied to the farming sector to reduce fertilization costs, enhance crop yield per hectare and protect crops against certain diseases. The aim of the current study was to evaluate PGPR inoculants on a basis that could ultimately lead to its application to maize production in South Africa. South African farmers have little knowledge of the benefits of PGPR technology as its' importance in the agricultural sector has not been extensively evaluated. With the promising results obtained in this study, the benefits to farmers can be enormous as it would help this sector to reduce costly fertilizer expenses.

Furthermore it could contribute significantly to food security and creation of a more sustainable agricultural sector.

1.3 General objectives

- To evaluate rhizobacterial isolates from the University of Pretoria's PGPR culture collection for enhancement of maize growth and yield under greenhouse and field conditions.
- To determine the biocontrol effectiveness of the selected rhizobacterial isolates against *Fusarium* spp.
- To determine the modes of action (MOA) of the most effective plant growth promoting isolates.

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Chapter 2

Literature review: A general overview of PGPR as biological control and plant growth stimulating agents

2.1 Introduction

Wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) are the most vital cereals worldwide (Rosas *et al*., 2008) and the main goal of the agricultural sector is production of safe, affordable fresh produce of the highest quality for an ever growing human population. With these high standards set by consumerism, keeping in mind economic profitability, the farmers also have to deal with economical and/or environmental constraints. In the Southern African region maize is the largest crop produced and also the largest source of carbohydrates. South Africa is the main producer of maize on the African continent. On 2.5 million hectares, RSA produces approximately 10-12 million tons (Figure 1) of maize per annum (Syngenta, 2013). The Free State, North West and Mpumalanga provinces are the leading maize production areas with an estimate yield of 4.05 million tons, 2.33 million tons and 2.19 million tons, respectively, for the 2010/2011 production season (SAGL, 2011).

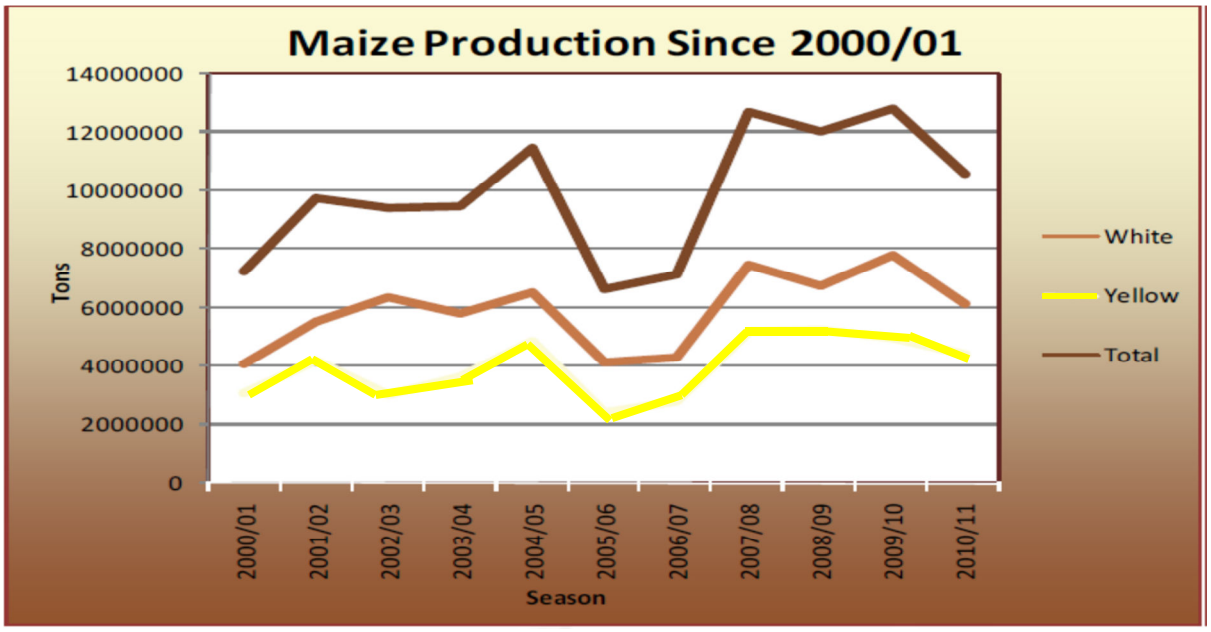

Figure 1: South African national annual maize yield estimates (SAGL, 2011)

With mounting problems associated with synthetic agrochemicals, such as environmental and human health problems, resistance to pests, the need for alternative agricultural practices that can alleviate the deterioration of valuable agricultural land, has increased (Avis *et al*., 2008). The use of PGPR and Biological Control Agents (BCA) as inoculants constitutes a biological alternative to synthetic agrochemicals in sustainable production systems (Rosas *et al*. 2008). Exploring the effect of plant / micro-organisms interaction on plant nutrition and soil fertility has attracted much national and international interest. This interest is primarily due to the negative impact fertilizers has on the environment, public health and the high cost of fertilizers worldwide (Adesemoye *et al*., 2009).

As an alternative to conventional agricultural production systems the solution may be to modify plant growth in a desired direction by using the beneficial interactions between Rhizobacteria and plants. The objective is to modify the rhizosphere in order to increase production and health of the plant by supplementing/replacing the resident microflora with beneficial microorganisms (Zahir *et al*., 2004). This initiative ensures protection of agricultural lands, environmental protection and consumer health by increasing the effectiveness of various agro-chemicals.

Antoun & Pre'vost (2005) and Avis *et al*. (2008) stated that various soil micro-organisms have proven beneficial to improve overall plant health and have been incorporated into a wide variety of productions systems as part of either production management, pest management or both. By using plant growth promoting rhizobacteria (PGPR) to assist the farmers, fertilization costs can be reduced while limiting or reversing the extent of the negative effect of synthetic agrochemicals on the environment. According to Di Cello *et al*. (1997) PGPR are promising entities to improve agricultural performance and boost food production under low soil fertility.

Various mechanisms exist (Figure 2) by which PGPR influence the growth and development of the plant. Literature suggests that growth promotion is the result of multiple plant growth stimulating mechanisms (Martinez-Viveros *et al*. 2010). According to Glick *et al*. (2007), PGPR have either an indirect or a direct effect on the plant. Consistent with this hypothesis the indirect effect occurs when PGPR influence plant growth by acting as a biological control agent (BCA) that limits the

damage done by plant pathogens. On the other hand the direct effect occur when PGPR influence plant growth by synthesizing hormones that enhance plant growth or facilitate the uptake of nutrients. Growth enhancing effects may be attributed to mechanisms such as the production of plant growth promoting hormones in the rhizosphere and other plant growth promoting activities (Arshad & Frankenberg, 1993).

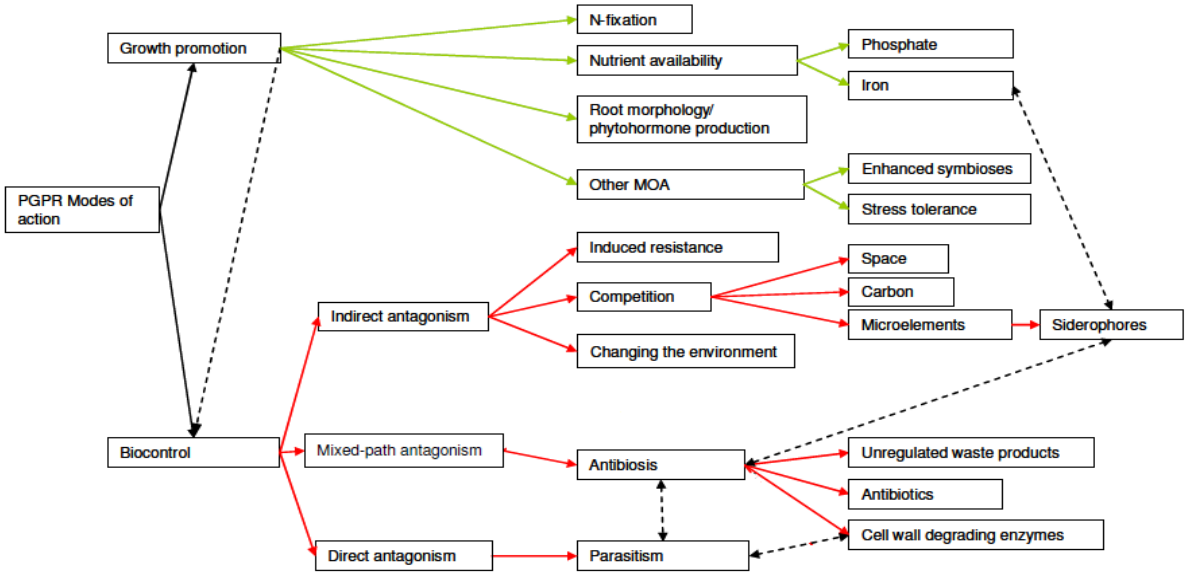

Figure 2. Diagram summarizing different modes of action of PGPR (adapted from Pretorius, 2012).

The exact mechanisms by which plant growth promoting rhizobacteria (PGPR) enhance plant growth are complex and not entirely unravelled, but it is proposed by various researchers to comprise of the following direct or indirect mechanisms.

- PGPR production or substitution of plant growth regulators such as Indole-Acetic Acid, Gibberelic Acid, Cytokinins or Ethylene (Glick, 1995).
- Asymbiotic PGPR nitrogen fixation that provide nitrogen that the plant can utilize (Boddey & Dobereiner, 1995).
- Antagonistic actions against phytopathogenic micro-organisms as a result of the production of antibiotics (Shanahan *et al*., 1992), cyanide (Flaishman *et al*., 1996) and/or siderophores (Scher & Baker, 1982) that indirectly enhance plant growth by suppressing phytopathogenic organisms.
- Solubilisation of mineral phosphates and other difficult-to-access nutrients, increasing the availability of the nutrients to the plant (Gaur, 1990; De Freitas *et al*., 1997).

For the plant to attain nutrients from the soil, a good root system is a prerequisite. The root system supports the plant, not only as a dynamic anchorage structure, but also by extracting nutrients from the soil. Superior adventitious rooting and improved number and length of lateral roots are the subject of various research projects (Molina-Favero *et al*., 2007). In research conducted by Vessey (2003) and Zhang *et al*. (2007), they independently found a variety of root morphological changes as a result of PGPR activity that increased the root surface area which resulted in an increased nutrient uptake from the soil. According to Gyaneshwar *et al*. (2002) only a portion of fertilizer applied to plants is utilized due to factors limiting the uptake of nutrients. Examples of this are phosphorus and nitrogen. Phosphorus precipitates in the soil, directly limiting its uptake by the plant. Nitrogen on the other hand is considered as one of the most expensive nutrients and approximately 65% of nitrogen in fertilizer is lost due to runoff, erosion, gaseous emission and leaching (Bhattacharjee *et al.,* 2008).

Growth promotion can be direct or indirect, but trying to separate the individual growth promotion effects can be complicated. This dilemma was stated by Kloepper (1993) who suggested that the indirect and direct growth promotion assessment should be viewed as two sides of the same coin. Strains of rhizobacteria selected for biological control commonly exhibit plant growth enhancing abilities in the absence of a pathogen. Various approaches to screen for rhizobacteria are used to exploit the preferred stimulatory effect on the host and these includes 1) promotion of root/shoot growth under gnotobiotic conditions; 2) *in vitro* production of plant growth regulators/ biologically active substances and the 3) evaluation of Aminocyclopropane-1-carboxylate (ACC) deaminase activity of the Rhizobacteria (Zahir *et al.,* 2004). The exact mechanisms by which plant growth promoting rhizobacteria (PGPR) enhance plant growth are complex and not entirely unravelled but each corresponding study provides more insight into this complexity.

In this review, the various effects PGPR mechanisms have on soil fertility and plant health will be reviewed in order to establish PGPR applicability in the agricultural sector to address the mounting problems associated with the deterioration of agricultural soils.

2.2 Mode of action: Direct mechanisms

2.2.1 Nitrogen fixation

2.2.1.1 Biological nitrogen fixation (BNF)

Biological nitrogen fixation (BNF) by soil bacteria is considered one of the main mechanisms whereby plants can benefit in a symbiotic/asymbiotic association with rhizosphere micro-organisms (Glick, 1995). These BNF bacteria can convert unreactive atmospheric nitrogen to ammonium (NH_3) that can be utilized by plants. Atmospheric nitrogen (N_2) is converted by the nitrogenase enzyme to ammonium according to the following reaction $N_2+8H+8\bar{e}+16ATP \rightarrow Nitrogenase \rightarrow 2NH_3+H_2+16ADP+ 16Pi$ (Bhattacharjee *et al.,* 2008). One of the benefits that these micro-organisms offer plants is the fixation of atmospheric nitrogen in return for fixed carbon released from root exudates (Glick, 1995).

The valuable symbiosis between nitrogen fixing bacteria and plants is well known worldwide, particularly between *Rhizobia* and leguminous plants. A number of researchers, amongst others Malik *et al*. (1997) and Antoun *et al*. (1998) showed that free-living bacteria and rhizobial strains can also enhance the growth of cereal plants directly by nitrogen fixation. The symbiotic relationship between nitrogen fixing bacteria (NFB) and plants provide an alternative for the excessive use of fertilizer which in turn limits ground water contamination and greenhouse gas emissions (N_2O). This symbiosis has also been observed in various non-leguminous plants belonging to the *Poaceae* family e.g. rice, maize and wheat (Malik *et al*., 1997.; Vedder-weiss *et al*., 1999; Van Dommelen *et al*., 2009).

Malik *et al.* (1997) showed that *Azospirillum* strain N4 is a major nitrogen contributer when the *Azospirillum* inoculated rice is compared to the non-inoculated control. Dazzo *et al*. (2000) used the 15 N Natural abundance technique and found that there was an increase in nitrogen content of rice inoculated with BNF bacteria. Rodriguez *et al*. (2008) showed that *Azospirillum amazonense* inoculated rice supplied up to 27% of the plant assimilated nitrogen and that most of the isolates they screened had low nitrogenase activity. An overview of literature on BNF bacteria and their role in nitrogen fixation was undertaken by Zahir *et al.* (2004). In this review they reported that various studies have shown that the inoculation of cereal grains with

BNF bacteria resulted in a remarkable increase in the utilization and economy of nitrogen.

Research conducted by Dobbelaere *et al*. (2003) suggested the ability of bacteria to fix nitrogen may give them a competitive advantage by enabling bacteria to attain high population densities. Nitrogen produced by these large populations may be sufficient to increase the availability of soil nitrogen to the point where the plants benefit from these levels. Research has also shown that some ammonia/NO_3 producing rhizospheric bacteria can induce the plant to increase uptake of nitrogen by inducing gene expression of nitrogen associated genes through the production of ammonia/ NO_3 (Dobbelaere *et al*., 2003).

2.2.2 Solubilisation and uptake of nutrients

Even when there is an ample supply of nutrients in the soil for optimum plant growth, the plant may still show deficiency due to non-availability of these nutrients. Rhizospheric micro-organisms may solubilize these non-available nutrients, for example, iron and phosphorus, thereby making them readily available for absorption by plants (Glick, 1995). The rhizospheric relationship between fungi and bacteria can affect the phosphorus cycle and enhance the nutrient supply to the plant (Toro *et al*. 1997). PGPR may improve the solubility of nutrients by releasing organic acids, sugar acids and CO_2, thus creating acidic conditions that can increase solubility of inorganic phosphates (Pietr *et al*., 1990).

Leong (1986) indicated that the mobility of iron (Fe^{2+}) can be increased by complexing with PGPR produced siderophores. This was supported by Wang *et al*. (1993) where they stated that various plants can make use of these bacterial-iron-siderophore-complexes to acquire iron from the soil. According to Zahir *et al*. (2004) further studies in this field are needed to determine the quantitative conditions for microbial siderophore production and the effect they may have on the plant.

2.2.2.1 Phosphate

Phosphorus is one of the major limiting nutrients in crop production and rock phosphates make up the bulk of phosphate in the soil. This type of phosphorus is highly insoluble. The major reason for limited phosphate in the soil is phosphorus'

high reactivity to metal complexes, e.g. iron and aluminium that precipitates or adsorbs between 75-90% of the phosphorus in the soil (Igual *et al.,* 2001).

Apatit, oxyapatit and hydroxyapatite are the main forms of phosphate that occur in the stratum rock layer. The main characteristic of these kinds of phosphate (P) is that they are insoluble and represent the largest pool of this element in the soil. In the agricultural sectors, large amounts of soluble inorganic P are applied but a large portion also becomes immobilized resulting in low utilization efficiency (Rodriguez & Fraga, 1999).

There are two mechanisms whereby micro-organisms solubilise insoluble mineral phosphate. The first is via acidification of the soil rhizosphere by micro-organisms that release organic acids such as citrate and gluconate. This mechanism also acts as a chelating agent for iron and aluminium that is associated with phosphate availability. The second pathway is the release of the enzyme phosphatase that mobilizes organic phosphates by dephosphorylating the phosphoanhydride and phosphor-ester bonds through hydrolysis. Some of the most effective phosphate solubilizers are *Rhizobium* spp*., Bacillus* spp. and *Pseudomonas* spp. These phosphate solubilizers are not regarded as growth promoters by increasing the plants phosphate levels but indirectly affect the soil phosphate levels to the benefit of the plant (Rodriguez & Fraga, 1999).

2.2.2.2 Iron

Various bacteria have the ability to efficiently utilize iron-oxides from the soil. These iron-oxides are mostly unavailable to the plant but bacteria have developed a mechanism to absorb the soil iron by secreting iron chelating siderophores. These low molecular weight siderophores are ferric iron-specific and bind to iron as chelating agents. The secreted siderophores chelate iron from the environment and increase the availability of ferrous iron (Fe^{2+}) to the plant and microorganisms. This mechanism is thought to play a role in competition between microorganisms in the rhizosphere for iron (Spaepen *et al.,* 2009). The theory implies that the plant may utilize the iron from the microorganism's siderophore production if the soil iron is low but according to Marschner & Romheld (1994) some of these reports are questionable.

2.2.3 Production of plant growth regulators (PGR) and plant growth promoting substances (PGPS)

The main characteristic of plant growth regulators is that they are organic compounds that affect the plant physiological system (growth, development, cell and tissue differentiation) in low concentrations. This mechanism of plant growth enhancement is suggested as the most credible and various studies have shown improvements in plant growth/development in response to inoculation. There are five known plant growth regulators, namely, gibberellins, cytokinins, ethylene, auxins and abscisic acid. Gomez-Roldan *et al.* (2008) found a new phytohormone known as strigolactone that inhibits branching of the shoots and plays a role in mycorrhizal symbiosis initiation. Other phytohormones that have been identified are polyamines, nitric oxide, peptide hormones brassinolides, jasmonic and salicyclic acid.

Rhizobacteria are considered as the main external source of plant growth regulators (Patten & Glick, 1996.; Arshad & Frankenberger, 1998). Barea *et al*. (1976) found that production of PGR is inconsistent between PGPR isolates. Among the 50 isolates evaluated, 86% produced auxins, 58% gibberellins and 90% manufactured kinetin-like compounds. A range of other experiments support the microbial production of PGR, for example Mansour *et al*. (1994). The results of Noel *et al.* (1996) indicate a link between PGPR plant growth regulator production that affect the IAA and cytokinine levels in canola and lettuce. Table 2.1 lists Rhizobacteria that are known to produce plant growth regulators (Zahir *et al*., 2004).

Plant growth promoting rhizobacteria can also be harmful to the plant when the concentrations of certain compounds produced by the PGPR are exceeded. Examples of situations that can be harmful to the plant's physiology include i) high levels of siderophore-mediated antagonism for iron, high levels of ii) ethylene, iii) Indole-3-acetic acid (IAA), iv) Hydrogen cyanide (HCN) and v) certain unidentified phytotoxins (Zahir *et al*., 2004). The results of Barazani & Friedman (1999) indicated that plant growth inhibition can be caused by elevated levels of auxin. These non-pathogenic micro-organisms were collectively termed Deleterious Rhizospheric Micro-Organisms (DRMO's).

Table 2.1: Plant growth regulator production by PGPR (Zahir *et al.,* 2004)

In vitro production of plant growth regulators by Rhizobacteria

PGPR (if identified)	Plant growth regulator	Reference
Arthrobacter mysorens 7,	Indole-3-acetic acid, ethylene	Pishchik *et al*. (2002)
Flavobacterium sp. L30		
Klebsiella CIAM880		
Azobacter beijernickii	Cytokinine-like substances	
A. beijernickii	Auxin, gibberellin-like substances	Nieto and Frankenberger (1989)
A. chroococcum	Gibberellin-like substances, gibberellic acid, indole-3-acetic acid	Azcon and Barea (1975)
A. chroococcum	Gibberellin-like substances	Brown and Burlingham (1968)
A. chroococcum	Gibberellin-like substances	Martinez-Toledo et al. (1988)
A. chroococcum	*t*-Zeatin, isopentyl adenine, ribosylzeatin, dihydrozeatin riboside	Salmeron *et al.* (1990)
A. chroococcum	Indole-3-acetic acid	Nicto and Frankenberger (1989)
A. paspali	Cytokinin-like substance,Indole-3-acetic acid, gibberellin -like substance	Muller *et al*. (1989)
A. vinelandii	Cytokinine-like substance	Barea and Brown (1974)
A. vinelandii	*t*-Zeatin, isopentyl adenosine	Nieto and Frankenberger (1989)
A. vinelandii	Indole-3-acetic acid	Taller and Wong (1989)
A. vinelandii	Indole-3-acetic acid, gibberellin like substance	Lee *et al*. (1970)
Azobacter sp.	Indole-3-acetic acid, gibberellin like substance	Gonzalez-Lopez *et al*. (1986)
Azobacter sp.	Indole-3-acetic acid	Mahmoud *et al*. (1984)
A. brasilense	Cytokinine-like substance, gibberellin-like substance	Zahir *et al*. (1998a, b, 2000)
A. brasilense	Isopentyl adenine, isopentyl adenosine, zeatin	Khalid *et al.* (2001)
A. brasilense	Gibberellin, gibberelic acid, *iso*-gibberelic acid	Horemans *et al*. (1986)
A. brasilense	Indole-3-acetic acid	Janzen *et al*. (1992)
A. lipoferum	Indole-3-acetic acid	Martin *et al*. (1989)
A. lipoferum	Gibberellin, gibberelic acid, *iso*-gibberelic acid	Martin *et al.* (1989)
Azospirillum sp.	Gibberellin-like substance	Bottini *et al*. (1989)
Azospirillum sp.	Gibberellic acid	Hubbel *et al*. (1979)
Azospirillum sp.	Indole-3-acetic acid	Lucangeli and Bottini (1997)
Azospirillum sp.	Indole-3-acetic acid	Dobbelaere *et al*. (2001)
Aeromonas sp.	Ethylene	Lambrecht *et al*. (2000)
Azospirillum sp.	Ethylene	Billington *et al.* (1979)
Bacillus licheniformis	Ethylene	Strzelezyk *et al*. (1994)

B. licheniformis	Physiologically active gibberellins	Fukuda *et al*. (1989)
B. pumilus	Physiologically active gibberellins	Gutierrez-Manero *et al*. (2001)
B. subtilis	Ethylene	Gutierrez-Manero *et al*. (2001)
B. mycoides	Ethylene	Billington *et al*. (1979)

2.2.3.1 Auxins

A broad spectrum of biological cellular processes which range from cell polarity, endocytosis, cell elongation, embryogenesis, differential growth etc. are regulated by the plant hormone, auxin (Sauer *et al.,* 2013). The most abundant documented auxin is Indole-3-acetic acid. This compound is made by the plant from the aromatic precursor, tryptophan. Various bacteria have been reported to have the ability to produce IAA with 80% of rhizospheric bacteria estimated to have this ability (Patten & Glick, 1996). Auxins affect the plant in various ways which can include increased root hair formation and apical dominance with a tropistic response (Spaepen *et al*., 2007).

Experiments with IAA mutant PGPR isolates have shown that the isolates had the ability to manipulate plant growth by inducing increased root hair formation or in some cases decreasing root length (Malhotra & Sivestave, 2009). They also showed that PGPR have the potential to manipulate plant growth but this IAA dose response mechanism should be studied further in order to establish the specific effect of PGPR excreted IAA on plant growth.

2.2.3.2 Cytokinins

The inter-relationship between cytokinins and IAA dose is closely regulated by the plant physiology. If cytokinin concentration is increased, the plant favours shoot development and if IAA is decreased then root development is stimulated. Cytokinine derivatives of aminourines are a broad chemical group of which kinetin and zeatin are the most well-known. These chemical compounds, identified as cell division inducers are produced in the root tip and physiologically active seeds. After cytokinin production, the synthesized chemical is transported through the xylem to the shoots

where it can regulate various plant physiological processes like leaf expansion, cell division and senescenic delay (Spaepen *et al*., 2007).

As in the case of auxins, cytokinins are widely produced by rhizospheric bacteria and the cytokinin compound produced by bacteria is identical to that which is found in the plant (Frankenberger & Arshad, 1995). Isopentenyl pyrophosphate and 5'-adenosine monophosphate are the precursors for the production of cytokinins and it is theorized that the bacteria's contribution to the auxin and cytokinin levels in the soil directly influence the growth and the development of the plant (Spaepen *et al*., 2007).

2.2.3.3 Gibberellins (GAs)

One hundred or more chemical compounds belong to the Gibberellin family. They are physiologically involved in cell division and elongation of the plant cells. These compounds also influence almost all life stages of a plant from seed germination to fruit set. Not much is known about GAs due to the immense amount of different GA compounds and the difficulty to distinguish between precursor and biologically active GA compounds (Yamaguchi, 2008). However, they are synthesized from melvonic acid and are classified as a tetra-cyclid diterpenoic acid. The precise mechanism for PGPR-GAs mediated mechanisms are not known but it is theorized that there is a direct correlation between root colonization density and root hair densities (Spaepen *et al*., 2007). The strongest evidence of the effect of GAs' was documented by Lucangeli & Bottini (1996) with the reversal of maize dwarfism by the inoculation with *Azospirillum lipoferum* in maize GA biosynthesis mutants. Results obtained by Lucangeli & Bottini (1996) were supported by Boiero *et al.* (2007) which indicated a significant shoot growth in rice and maize dwarf mutants when treated with *Azospirillum* spp. that excreted gibberellin like substances.

2.2.3.4 Ethylene (ET)

In the methionine cycle ethylene (ET) is produced from methionine by the enzyme S-adenosylmethionine synthetase and through a stepwise process finally converted by ACC oxidase to ET, cyanide and CO_2. This hormone was first thought to only be involved in fruit ripening and was named accordingly as a ripening hormone. However, various other effects of ET on plants have been discovered e.g. seed

germination, abscission, senescence of flowers and leaves. The phytohormone also plays a part in plant-pathogen interactions and cell expansions (Spaepen *et al.,* 2007).

High concentrations of ET affect plant physiology by inhibiting root, shoot and axillary bud growth, cell division and DNA synthesis and vice versa (Burg, 1973). This observation was supported by Bhattacharyya & Jha (2011) who observed that high endogenous levels of ethylene significantly decreased overall plant growth and crop performance. According to Glick (2007) it is thought that PGPR that express the enzyme 1-aminocyclopropane-1-carboxylate deaminase (AcdS) can stimulate plant growth by degrading one of the ET intermediate molecules, 1-aminocyclopropane-1-carboxylate (ACC). The plant growth stimulating effect attributed to ACC deaminase production by PGPR was observed in studies conducted by Zahir *et al*. (2008) and Zafar-ul-Hye (2014). Their results indicated the overall growth parameters of maize development and mineral fertilizer use efficiency was significantly increased when inoculated with ACC deaminase producing PGPR compared to the untreated control. The Rhizobacteria therefore act as a sink to degrade plant produced ET and in turn decrease the stress on the plant (Spaepen *et al*., 2007).

2.2.3.5 Abscisic acid (ABA)

Abscisic acid is stress related and causes the plant to induce fruit ripening, stomatal closure, inhibits seed germination and earlier flower formation. It also has a protective response to adverse conditions such as drought, metal toxicity, cold, heat and salt stress. Abscisic acid is synthesized from dimethylallyl diphosphate and isopentyl diphosphate and is produced by all plant organs (Spaepen *et al*., 2007). *Azospirillum brasilense* and *Bradyrhizobium japonica* have been shown to produce ABA (Boiero *et al.,* 2007 & Cohen *et al.,* 2008) and according to Boiero *et al.* (*2007*), PGPR isolates that excrete ABA may increase plant growth as it inhibits cytokinin production under stress conditions. A study conducted by Porcel *et al.* (2014) found that when ABA mutant tomato plants were inoculated with the PGPR *Bacillus megaterium*, growth inhibition was observed while the wild type tomato plants showed growth stimulation. The results indicated that the growth inhibition observed in the ABA mutants could be contributed to ethylene buildup in the plant. The study

concluded that internal plant ABA levels may be essential for the growth promoting effect of PGPR by maintaining low levels of ethylene within the plant.

2.3 Biocontrol activity of PGPR

Biological substitutes for pesticides currently face many constraints. The task is to firstly find, then develop and implement biological control agents. This is not a straight forward task with the majority of attempts having an unsuccessful track record, although PGPR shows great potential as found in a study conducted by Shahzaman (2014) where *Fusarium oxysporum* mycelial growth was inhibited by 62.82% of *Pseudomonas* spp. tested. These biological substitutes in most cases must be optimized and improved before being fully attuned to pesticides.

The prospects for new biological control microbes are infinite. Renwick *et al.* (1991) concluded that the most effective biocontrol agents that occur naturally are mixtures of these PGPR antagonists rather than a high number of a single antagonistic species. This combination of antagonists may also have a broader range of bio-control activity (Duffy & Weller, 1995). The efficacy and activity range of various microbes could be genetically fused to form a broad range, highly proficient micro-organism that could substitute and offer a more biologically friendly alternative to pesticides. Sadly until GMO's have unlimited use, the only PGPR or BCA agents available will be determined by natural selection and not biotechnology (Campbell, 1994).

Fundamental and realistic screening methods must be used with environmental parameters to produce a successful biocontrol micro-organism for the control of phytopathogens (Campbell, 1994). Variations in experimental results can be due to various biotic and abiotic factors which influence the PGPR and/or BCA agents. Egamberdiyeva (2007) reported that soil types can influence bacterial growth as they found that nitrogen, phosphate and potassium assimilation levels differed between two different soil types. This variation between soil types necessitates the need for *in vivo* trials to determine the effectiveness of micro-organisms (Chanway & Holl, 1993).

The inconsistency in the performance of PGPR under *in vivo* conditions can be attributed to environmental and biotic factors that may affect the microbial growth.

Environmental factors can include climatic conditions, soil characteristics or the composition/activity of the native micro-flora of the soil (Ahmad *et al.,* 2008). Woeng *et al.* (2000) described biocontrol as a mechanism of multiple traits that is dependent on various factors. These factors include whether the PGPR inoculant can 1) establish itself in the rhizosphere and 2) outcompete the resident microbial population whilst 3) protecting the host plant against site and timely pathogen infections.

According to Subba Rao (1993), the formation of lateral roots is a favourable area for colonization by pathogenic bacteria which can be controlled by the diverse mechanisms of PGPR such as rivalry for substrate and niche exclusion. It has been suggested by Pal & Mc Spadden (2006) that the various mechanisms for biocontrol activity, e.g. parasitism, competition, induced resistance, can be grouped into three categories according to their mechanism for biocontrol activity and the influences in the disease cycle and environment. These categories are indirect antagonism where the pathogen is indirectly affected, direct antagonism where the pathogen is directly affected by targeting the pathogen and lastly mixed path antagonism where the pathogen is directly affected but not specifically targeted.

2.3.1 Indirect antagonism

2.3.1.1 Induced systemic resistance

Induced systemic resistance is a plant response to various stress factors that induce resistance by activating two pathways, namely 1) induced systemic resistance activated by jasmonic acid, ethylene and 2) systemic acquired resistance activated by salicylic acid. Both these pathways induce defence gene expression, cell wall strengthening, phytoalexin accumulation in the cells, increased chitinase and peroxidase activity and the production of biopolymers e.g. lignin, glycoproteins to protect the cell walls (Larkin *et al.,*1998; Walters 2009).

Tuzun and Kloepper (1995) stated that induced system resistance can result in the priming of the plant defence that is induced by minute quantities of an inducing agent. This could be beneficial to the plant by increasing the response time in order to effectively defend itself against pathogen attack. Woeng *et al*. (2000) has shown

that *Fusarium udum* and *Pseudomonas* spp. can be controlled in pigeon peas by using *Pseudomonas aeruginosa* and *Bacillus cereus* to induce systemic resistance. A study conducted by Athinuwat *et al.* (2014) showed the potential of *Bacillus subtilis*, *Pseudomonas flourenscens*, *B. amyloliquefaciens* and *P. pabuli* as effective antibiosis agents when used to significantly suppress stalk rot and bacterial leaf streak of maize caused by *Acidovorax avenae* subsp. a*venae* and Pectobacterium zeae (=*Erwinia chrysanthemi* pv*. zeae)* when applied as a seed treatment and foliar spray. The significant disease suppression was attributed to the increased levels of salicyclic acid accumulation which attained significant levels five days after treating the PGPR inoculated maize with the abovementioned pathogens. This type of resistance may hold various benefits due to its broad spectrum of activity without significantly affecting plant growth. PGPR induced resistance and can therefore be seen as a viable solution to increase disease control efficiency.

2.3.1.2 Induced systemic tolerance

PGPR are responsible for induced enhanced tolerance to abiotic stresses on the plant. Studies conducted on *Arabidopsis* have shown that the drought tolerance gene can be upregulated when induced by PGPR. It has also been shown that the PGPR can affect the sodium balance in saline soils and in this manner decrease the stresses on the plant caused by saline conditions (Yang *et al*., 2007).

2.3.1.3 Competition

Competition is a type of indirect antagonism that is resource related where the PGPR suppress pathogens by means of resource exclusion e.g. nutrients. The result is a constant state of nutrient limitation and starvation for other micro-organisms. This form of antagonism is directly linked to the biomass of the antagonist. The antagonist must be in sufficient quantities at the right time to out-compete the pathogen for resources, thus limiting pathogen establishment on the plant (Brussaard *et al.* 2007). These antagonists need to have a versatile metabolism to utilize a wide variety of nutrients and scavenge nutrients to exclude pathogens. The biocontrol agent must also be able to colonize and survive in the plants' environment to offer constant protection (Brussaard *et al.,* 2007; Raaijmakers *et al.,* 2008).

The most documented example of competition is the production of siderophores by PGPR. Siderophores bind to the iron in the rhizosphere to form ferric-siderophore complexes. These complexes limit fungal and bacterial uptake of iron in the rhizosphere and can only be utilized by PGPR that induced the formation of the complexes. These complexes are then recognized by specific outer membrane receptors and imported into the cell. This limits the uptake of iron by other fungal and bacterial species that need this nutrient for growth and ATP and DNA precursor production under anaerobic conditions (Rosas, 2007).

2.3.2 Direct antagonism

2.3.2.1 Parasitism

Parasitism is where the antagonist parasitizes the pathogen directly causing its destruction and ultimately death. The most well-known example of this form of biocontrol is from the genus *Trichoderma* that parasitizes other fungal species (Howell, 2003). Research indicates that *Trichoderma* spp. not only parasitize other fungal species but also act as a direct biocontrol agent via different mechanisms which include 1) the secretion of a wide range of antibiotics, 2) out-competing various phytopathogens for soil nutrients, 3) degrading enzymes e.g. pectinases needed for *Botryts cinerea* to infect hosts and 3) inducing localised and systemic resistance of the plant to plant pathogens (Elad, 1996; Zimand *et al.,* 1996 and Harman *et al.,* 2004).

According to Leveau & Preston (2008), certain bacteria with mycophage capabilities, e.g. *Bacillus, Pseudomonas* and *Paenibacillus,* have also been shown to parasitize fungi by means of three mechanisms, namely; 1) necrotrophy, 2) endocellular biotrophy and 3) extracellular biotrophy. Necrotrophs kill fungal pathogens by permealising and lysing the fungal cells while extracellular biotrophs live in symbiosis with the fungus and utilize the fungal exudates as nutrients. Endocellular biotrophs on the other hand live inside the fungal hyphae and directly absorb the needed nutrients from the hosts' cytoplasm. Biotrophs can convert to necrotrophs and ultimately cause death to the fungal host if there is a significant biotroph population increase.

2.3.3 Mixed path antagonism

2.3.3.1 Antibiosis

Antibiosis is a form of biocontrol which excludes other fungal and bacterial species by colonizing niches and excreting allele-chemicals, creating unfavourable conditions for other organisms. The biocontrol agent can produce various chemicals, e.g. lytic enzymes, antibiotics, volatile agents and waste products, which can inhibit pathogen growth or cause the destruction of the pathogen (Larkin *et al.,* 1998; Compant *et al.,* 2005). Antibiotic production is one of the most well documented and understood mechanisms of biocontrol. *Pseudomonas* spp. are one of the most well-known antibiotic producers and have been shown to effectively control *Gaeumannomyces graminis* that causes take all disease in wheat (Raaijmakers & Weller, 1998). Antibiotic producing *Bacillus subtilis* excrete bacilysin and iturin that has been shown to control the cucumber pathogen *Fusarium oxysporum* (Handelsman & Stabb, 1996; Rosas 2007; Chung *et al.,* 2008). A wide range of antifungal compounds have been identified by Duffy *et al.* (2004) known as Diacetylphloroglucinol (DAPG). *Pseudomonas fluorescens* secretes these compounds and has been shown to effectively control seedling and soil borne fungal pathogens. Lytic enzymes, e.g. chitinase, glucanase, cellulase, amylase and proteases, have been shown to play an important role in biocontrol. Antagonists can produce lytic enzymes that degrade and lyse microbial cell walls and this in turn provides nutrients for the antagonist (Chet *et al.,* 1990). Volatile compounds on the other hand inhibit fungal growth and spore germination. There are various volatile compounds produced by antagonists, e.g. amines, alkenes aldehydes, alcohols, sulphides, ketones, ammonia and hydrogen cyanide, that act as a fungistasic agent (Arora *et al.,* 2007; Kai *et al.,* 2007; Vesperman *et al.,* 2007; Zou *et al.,* 2007).

2.4 Fusarium species infecting maize

Root rot pathogen complex affecting maize production

Maize is grown throughout the world as the staple diet for millions of people because of its ease of cultivation and high yield per hectare (Asiedu, 1989). In field and storage, maize is targeted by various pests and pathogens. Insects are ranked as the most important factor in maize losses (Gwinner *et al.,* 1996). The second most important constraint is fungi (Ominski *et al*., 1994). Various fungal species attack

maize and *Fusarium* spp. are the most widespread pathogens of this host and can reduce yield, estimated at 10% to 30% (Bottalico, 1998). Pathogens associated with maize root systems can be divided into two categories according to Hugo (1995) based on their isolation frequencies. The first category is root pathogens which include *Exserohilum pedicellatum, Macrophomina phaseolina* and various *Fusarium* spp. The second category is the root colonizers that include species such as *Phoma* spp*., Curvularia* spp*.* and *Fusarium chlamydosporum.* Classification into these categories was based on the time of fungal association with the plant. Root pathogens generally associate with the plant early in the growing season while colonizers only associate at a later maturing stage.

According to Hugo (1995), *Fusarium* spp. are the most commonly isolated root rot fungi in maize production areas but their distribution may vary according to different localities. Maize root rot pathogens do not have the ability to kill the plant and only sometimes show above ground symptoms however, they are of importance for the following three reasons. Firstly, the root damage results in decreased levels of nutrient and water absorption. Secondly, the plants are unable to utilize all the soil moisture in situations of drought and thirdly, root rot weakens the roots that support the plant resulting in lodged plants (Moolman, 1992).

“Root rot in maize is described as a complex of fungi that in space and time result in disease development”. The reason for this statement by Hugo (1995) is that different fungal species are associated with the maize root system throughout the year. This association of different species make it very complex to establish the primary disease causing pathogen. Studying different localities and the pathogens involved in this root rot complex can make it possible to deduce the spectrum of fungi involved in this complex.

Major shortcomings are encountered when trying to quantify the losses caused by root rot on yield. Severe losses have been reported but taking into consideration the complexity of this root rot complex makes in-field quantification attributed exclusively to a primary pathogen very difficult (Hugo,1995). Various *Fusarium* spp. are linked with maize diseases, for example, *F. proliferatum, Gibberella verticillioides, F. graminearum, F. anthophilum* (Scott, 1993; Munkvold & Desjardins, 1997). Of all

these *Fusarium* spp., the most commonly isolated from diseased maize worldwide is *G. verticillioides*. *Fusarium* stalk rot is caused by *Fusarium graminearum* and *Fusarium* stalk rot by *Gibberella verticillioides* (syn. G. fujikuroi) (CIMMYT, 2004). These two pathogens affect a broad range of hosts. Symptoms caused on maize by both *Fusarium graminearum* and *F. moniliforme* are very similar to that of *Stenocarpella* or *Cephalosporium*. The only way to differentiate the diseases from each other is by microscopic spore and fruiting body examination (Bell *et al*., 2009).

Both *F. graminearum* and *Gibberella verticillioides* are endophytes that have a long term association with the plant and symptoms can range from severe to asymptomatic (Bell *et al*., 2009). According to Munkvold & Desjardins (1997) symptomless infections are ignored because of “no-visible” damage. According to Fandohan *et al*. (2003) infection by *Fusarium* spp. can occur at all life stages of maize development but must follow one of four pathways. The first pathway is by infecting the seed and by systemic vascular movement infects the whole plant to the newly developing kernels. In the second pathway the pathogen gains access to the plant vascular system through infecting the roots. The third pathway is by conidial infection of the stalk and the cob via water and air dissemination of conidia. The last pathway is through wounds caused by vectors such as insects where the inoculum is deposited into the host plant.

2.4.1. Fusarium graminearum

The asexual conidia of *Fusarium graminearum* are hyaline, curved and have 3 to 5 septate about 4-6 X 10-30um in diameter. Chlamydospores are seldom produced in diseased lesions but the perfect stage of *Fusarium graminearum* develops within infected lesions. The perithecia are black and include 8 hyline ascospores with up to 3 septa. The pathogen overwinters as perithecia and ascospores that only mature in the current growing season and are then the primary source of inoculum. Ascospores formed by the perithecia are wind/rain dispersed and can infect the stem, roots and leaves of the plant. Conidia in lesions can initiate the secondary life cycle and these spores are then disseminated by rain/wind (Bell *et al.,* 2009). *Fusarium graminearum* is widely distributed in most of the prominent maize growing areas in the world as can be seen in Figure 3.

Figure 3: Countries where *Fusarium graminearum* has been reported are highlighted in yellow (Bell *et al*., 2009)

2.4.2 Gibberella verticillioides

Both macro-conidia and micro-conidia are formed throughout the life cycle of *Gibberella verticillioides*. The macro-conidia are hyaline and curved with 3 to 5 septa that measure 2-5 X 15-60µm. The micro-conidia are prolifically produced and usually occur in a chain. *Gibberella verticillioides* can also be seed borne and can be common in the field when the infected seeds are used as the planting material. *Gibberella verticillioides* is widespread and occurs in most of the dominant maize production areas of the world (Figure 4). The foremost form of dissemination of *G. verticillioides* is by the production of conidia. The micro- and macro-conidia produced by the mycelia in infected crop residues are wind/rain splash disseminated to adjacent plants where they can infect (Bell *et al*., 2009).

Figure 4: Countries where *Gibberella verticillioides* occur are highlighted in yellow (Bell *et al*., 2009)

2.4.3 Health implications associated with Fusarium spp.

Various *Fusarium* spp. produce mycotoxins (Bottalico, 1998) and according to Prelusky *et al.* (1994) all three of the above *Fusarium* spp. can produce the toxins deoxynivalenol (DON), fumonisin and zearalenone. According to Prelusky *et al.* (1994), the DON toxin has been shown to cause weight reduction in swine and the zealarone toxin to cause reproductive problems which include infertility and swine estrogenic syndrome. Fumonisins have also been linked to liver cancer in rats (Gelderblom *et al.,* 1988) and human esophegeal cancer (IARC, 1993).

According to Bottalico (1998), there is a worldwide concern of the presence of mycotoxins in foods and feeds. It is estimated by Charmley *et al*. (1995) that 25% of the world's food crops are affected by mycotoxins. Mycotoxins can be grouped into three groups; 1) trichothecenes, 2) zearalenones, and 3) fumonisins and according to Battalico (1998) the toxins listed in bold in Table 2.2 are of most concern.

Table 2.2: Mycotoxin production by *Fusarium* species from cereals

***Fusarium* specie**	**Mycotoxins**
F. acuminatum	**T2**, **HT2, DAS, MAS, MON**, NEO
F. avenaceum	**MON**
F. chlamydosporum	**MON**
F. crookwellense	**NIV, FUS, ZEN,** ZOH
F. culmorum	**DON, ZEN, NIV, FUS,** ZOH, AcDON
F. equiseti	**DAS, ZEN, ZOH,** NIV, DAcNIV, MAS, FUS
F. graminearum	**DON, ZEN, NIV, FUS, AcDON,** DAcDON, DAcNIV
F. heterosporum	**ZEN, ZOH**
F. moniliforme	**FB1**
F. oxysporum	**MON**
F. poae	**DAS**, **MAS, NIV, FUS,** T2, HT2, NEO
F. proliferatum	**FB1, BEA, MON, FUP**
F. sambucinum	**DAS,** T2, NEO, ZEN, MAS
F. semitectum	**ZEN**
F. sporotrichioides	**T2, HT2, NEO,** MAS, DAS
F. subglutinans	**BEA, MON,** FUP
F. tricinctum	**MON**
F. venenotum	**DAS**

List of abbreviations Ac-DON = Mono-acetyldeoxynivalenols (3-AcDON, 15-AcDON); Ac- NIV = Mono-acetylnivalenol (15-AcNIV); BEA = Beauvericin; DAc-DON = Di-acetyldeoxynivalenol (3,15-AcDON); DAcNIV = Diacetylnivalenol (4,15-AcNIV); DAS = Diacetoxyscirpenol; DON= Deoxynivalenol (Vomitoxin); FB1= Fumonisin B1; FUP = Fusaproliferin; FUS = Fusarenone-X (4-Acetyl-NIV); HT2 = HT-2 toxin; MAS = Monoacetoxyscirpenol; MON = Moniliformin; NEO =Neosolaniol; NIV = Nivalenol; T2 =T-2 toxin; ZEN = Zearalenone; ZOH = Zearalenols (a and b isomers).

2.5 Effect of PGPR inoculation on maize

In vitro trials conducted by Javed *et al.* (1998) using five inoculants identified as *Pseudomonas* spp. showed consistent enhancement of maize growth and yield. Grain yield increased up to 18.9% while combined mass, cob length, 1000-grain and straw mass was significantly enhanced by 20.8%, 11.6%, 17.2% and 27.1 %, respectively. Thirty days after inoculation with rhizobacteria, Noumavo *et al.* (2013) found a significant increase in root and shoot development of maize under greenhouse conditions when maize seed were inoculated with *Pseudomonans fluorescens* and *P. putida*. Almaghrabi *et al.* (2014) reported similar greenhouse results that indicated a significant increase of maize seed germination, root length and root weight 15 days after inoculation with *Bacillus subtilis* or *Pseudomonas fluorescens.* Under field conditions Zahir *et al*. (1998) inoculated maize seeds with a combination of four isolates (two *Azotobacter* spp. and two *Pseudomonas* spp.) in the field after application of NPK fertiliser at 150-100 kg/ha. Collective inoculation of the isolates considerably increased grain yield by 19.8%, cob weight, cob length,

1000 grain weight, plant height, nitrogen content in the straw and grain by 21.3%, 20.6%, 9.6%, 8.5%, 18% and 19.8% respectively compared to the uninoculated control plants.

Vedderweiss *et al*. (1999) reported that maize seed inoculated with *Azospirillum* spp. at a concentration of 10^6 cfu/ml enhanced fresh root and shoot weight of seedlings while Stancheva *et al*. (1992) reported that inoculation of maize seed with *Azospirillum brasilense* strain 1774 in a blend with 100kg N/ha fertilizer gave the same result as 200kg N/ha of non-inoculated plants. This model of increased nitrogen utilization by the plant is supported by Dobbelaere *et al*. (2001). Their soil analysis showed that AZOGREEN-m® inoculated maize showed a higher nitrogen content within the plant but a lower nitrogen soil content to that of the control. According to the results of Yazdani *et al.* (2009) the combination of PGPR with mineral fertilizers could reduce phosphate application by 50% without affecting the yield. A list of root exudates affecting the rhizosphere can be seen in Table 2.3.

Table 2.3: Root exudates affecting the rhizosphere (Jones *et al.* 2004)

Phenolics	Chelating nutrients with little solubility (e.g. Fe)
	Source of nutrients
	Increase microbial growth
	Inducing or inhibiting rhizobial Nod genes
	Signal attracting microbes
	Controlling pathogens
Phytosiderophores and amino acids	Souce of nutrients
	Chelating nutrients with little solubility (e.g. Fe)
	Signal attracting microbes
Organic acids	Source of nutrients
	Signal attracting microbes
	Chelating nutrients with little solubility (e.g. Fe)
	Inducing or inhibiting rhizobial Nod genes
Purines	Source of nutrients
Vitamins	Increasing the growth of plants and microbes
	Source of nutrients
Enzymes	Enhancing P solubility from organic molecules
	Increasing the mineralization rate of organic products
Sugars	Source of nutrients
	Increasing microbial growth
Root cells	Controlling cell cycling and gene expression by product signalling
	Enhancing microbial growth
	Producing chemoattractants
	Producing protein and mucilage
	Production of molecules to increase the rhizosphere immunity

The valuable effects of PGPR inoculation have been well documented There are however often variable results especially in field trials (Zahir *et al*., 2004). There is a great need for a sustainable agricultural system, creating a niche for farmers to utilize the soil more efficiently while cutting high input costs and further evaluation and refinement of PGPR under field conditions may be a viable solution for a sustainable system.

2.6 Agricultural applications of PGPR

Inoculation with PGPR may hold great promise as a potential agrochemical replacement for fertilizers and pesticides. Until now there is enough data to support the use of biological microbe applications in the agricultural sector (Reddy *et al.* 2014). This was supported by Chen *et al*. (1994) with an overview of the yield increasing bacteria (YIB) project conducted in China. The implemented YIB project covered 28 provinces of over 3.3 million hectares. The isolates evaluated in these projects were *B. cereus, B. firmus* and *B. lichniformis.* Results from wheat trials indicated an 8.5% to 16% increase in yield while maize yield increases ranged between 6% and 11%. Similar results were found by Gholami (2009) on maize inoculated with six different strains of *Azospirillum* spp. and *Pseudomonas* spp., resulting in a significant increase in seed germination, seed vigor, plant height, dry weight and most importantly yield. The findings of Gholami (2009) was supported by a study conducted by Mirshekari *et al.* (2012) that showed the complimentary effect of a mixture of *Azospirillum lipoferum* and *Azobacter chroococcum* increasing overall plant health and yield of maize. A conventional tillage study conducted by Agrawal & Pathak (2010) also indicated the potential of PGPR as a supplement to increase mineral fertilizer efficiency. Wheat was inoculated with the phosphate solubilizing PGPR *Pseudomonas fluorescens* which increased yield by 36% compared to the control, but at 50% of the control phosphate dose. Various other researchers have reported beneficial effects of PGPR inoculation on numerous other crops as indicated in Table 4 (Zahir *et al*., 2004).

2.7 Conclusion

According to literature PGPRs can be classified as biopesticides and biofertilizers with most of the tested isolates having overlapping applications. The large volumes of literature illustrate the tremendous potential of PGPR application in agriculture but most of the modes of actions are evaluated on an individual basis. The current research conducted focuses mainly on the relative contribution of individual PGPR mechanisms instead of the synergistic effect of all the contributing modes of action. Future research into this synergistic effect may in turn contribute to overcome the inconsistency hurdle as observed in infield performance of PGPR.

Table 4: Biological control agents in Agriculture (Zahir *et al., 2004*)

Biological control of certain diseases, pathogens and insects in different crops by PGPR

Crop	Disease/ pathogen/ insect	PGPR	Reference
Barley	Powdery mildew	*B. subtilis*	Schinbeck *et al.* (1980)
Beans	Halo blight	*Ps. fluorescens strain 97*	Alstrom (1991)
	Sclerotinia rolfsii	*Ps. Cepacia*	Fridlender *et al.* (1993)
Carnation	*Fusarium* wilt	*Pseudomonas sp. (WCS417r)*	Van Peer *et al.*(1991)
Cotton	Damping off	*Ps. fluorescens*	Howell and Stipanovich (1979;1980
	Meloidogyne incognita, M. arenaria	*B. subtilis*	Sikora (1998)
	Rhizoctonia solani	*Ps. Cepacia*	Fridlender et al. (1993)
	Helicoverpa armigera	*Ps. Gladioli*	Qingwen *et al. (*1998)
Cucumber	*Cucumber antracnose*	*Ps. praida (89B-27), Sernatia marcescens (90-166)*	Wei *et al.* (1991, 1996)
	Pythium ultimum	*Ps. Cepacia*	
	Bacterial wilt	*Ps. putida (89B-27), s. Marcescens (90-166)*	Kloepper *et al.* (1993)
	Bacterial angular leaf spot	*Ps. putida (89B-27), s. Marcescens (90-166), Flavomonas oryzihabitans INR-5, Bacillus pumilus (INR-7)*	Kloepper *et al.* (1993)
	Fusarium wilt	*Ps. putida (89B-27), s. Marcescens (90-166)*	Liu *et al.*(1995b)
	Cucumber mosaic virus	*Ps. putida (89B-27), s. Marcescens (90-166)*	Raupach *et al.* (1996)
	Striped cucumber beetle	*Ps. putida (89B-27), s. Marcescens (90-166), Flavomonas oryzihabitans INR-5, Bacillus pumilus (INR-7)*	Zehnder *et al. (1997a)*
	Spotted cucumber beetle	*Ps. putida (89B-27), s. Marcescens (90-166), Flavomonas oryzihabitans INR-5, Bacillus pumilus (INR-7)*	Zehnder *et al. (1997a)*
	Fusarium wilt	*Miture of Paenibacillus sp. 300, Streptomyces sp. 385*	Singh *et al*. (1999)
Green gram	*Aspergillus* sp., *Curvularia* sp., *Fusarium oxysporum, Rhizoctonia solani*	*Pseudomonas sp. (WCS417r)*	Sindhu *et al.* (1999)
Maize	Corn ear worm	*Ps. Maltophila*	Bong and Sikorowski (1991)
	Fusarium moniliformes	*Ps. cepacia strain 526 and 406*	Hebbar *et al.* (1992)
Mung bean	Root rot, Root knot	*Ps. aeruginosa, B. subtilis*	Siddiqui *et al.* (2001)
Rice	Rice sheath blight	*Streptomyces spp. and Bacillus cereus in combination with Ps. Fluorescens strain Pf1 and Fp7*	Sung and Chung (1997)

2.8 References

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Chapter 3

Screening of Rhizobacterial isolates for growth promotion of maize in the greenhouse.

Abstract

There is a need to reduce input costs and increase yield to ensure farming sustainability in the present agricultural system. The valuable effects of PGPR inoculation on agricultural crops have been well documented. However, there is often variation in the reproducibility of results for growth promotion, especially in field trials due to biotic and abiotic factors. In this study, a preliminary screening of 30 PGPR isolates was conducted in the greenhouse using maize seedlings. Each inoculant treatment was applied to the pot as a soil drench at the time of planting the maize seed. The plants were grown for 30 days before harvesting. The best performing treatments were bacterial strains A-08, A-26, A-32, A-40 and S2-08 resulting in a 28.32%, 10.79%, 24.88%, 19.35% and 7.16% increase in total dry mass of seedlings respectively. However, differences between treatments were not statistically significant. This could be due to the duration of the trial being too short and/or nutrient levels, especially phosphate being too low, or a too high bacterial inoculum dose being used. The inoculants; A08, A26, A32, A40, S2-08, S1-08, S4-08, S6-08 and the commercial product, Bacup® were selected for follow-up evaluation in the greenhouse based on their performance in the preliminary screening trial and previous trials by other researchers in the Department of Microbiology and Plant Pathology at the University of Pretoria (unpublished). The follow-up evaluation was a replicate of the PGPR screening trial except that the soil used in the trial was amended to standard nutrient levels for maize production. From this trial the best performing inoculant was S2-08 resulting in an overall wet mass increase of 9.70%. Isolate S6-08 inhibited total plant wet weight by 26.04% compared to the control although differences between treatments were statistically non-significant. The control outperformed all the treatments when the shoot length data is considered but the mass data suggests that the treatments can influence plant growth in a positive manner.

3.1 Introduction

The valuable effects of PGPR inoculation have been well documented. However results are often not reproducible when evaluating the effects of biological agents, under field conditions, due to biotic and abiotic factors that influence reproducibility of trial results (Zahir *et al*., 2004). There is a great need to change the present agricultural system to a more sustainable one making it possible for farmers to utilize the soil more efficiently while reducing high input costs.

Plant Growth Promoting Rhizobacteria can affect plants either indirectly by minimizing factors that limit growth, for example, pathogens or by affecting nutrient cycling such as nitrogen fixation, nutrient solubilisation or the production of phytohormones (Barea *et al*., 2005). Vedderweiss *et al.* (1999) found that maize inoculated with *Azospirillum* spp. at a concentration of 10^6 cfu/ml increased seedling fresh root and shoot formation. Javed *et al*. (1998) not only looked at the effect of rhizobacteria on maize seedlings but also evaluated their effect on maize up to a hard dough stage. Javed *et al*. (1998) identified five *Pseudomonas* spp. that significantly increased the grain yield by 18.9%, while cob weight, cob length, 1000-grain and straw weight was enhanced up to 20.8%, 11.6%, 17.2% and 27.1 %, respectively while studies conducted by Gholami (2009) showed that PGPR could increase the overall health and vigor of maize while cutting 50% of mineral phosphate without significantly reducing the yield. A study conducted by Noumavo *et al.* (2013) found a significant increase in root and shoot maize development when maize seed were inoculated with *Pseudomonans fluorescens* and *P. putida* under greenhouse conditions. Almaghrabi *et al.* (2014) results supported that of Noumavo *et al.* (2013) which indicated a significant increase of maize seed germination, root length and weight when inoculated with *Bacillus subtilis* or *Pseudomonas fluorescens* after a 15 days growth period under greenhouse conditions. Research by Stancheva *et al.* (1992) showed that *Azospirillum brasilense* strain 1774 can increase nitrogen use efficiency of maize by 100kg/ha when compared to the un-inoculated control which received 200kg/N/ha. This finding was supported by Dobbelaere *et al*. (2001) where their data showed the plants had a higher nitrogen content but a lower soil nitrogen content of approximately 20kg/N/ha when compared to the uninoculated control soil nitrogen content of 110 kg/ha.

In order to address the mounting problems associated with synthetic chemicals as mentioned by Avis *et al.* (2008) in the literature study and the biological solution to this problem by Rosas *et al*. (2008), the objective of this trial was to identify the best growth promoting PGPR isolates from the University of Pretorias' culture collection on maize seedlings in a preliminary screening trial. From this preliminary screening trial the best performing PGPR isolates would again be evaluated in a follow-up trial (2^{nd} trial) in order to refine the selection and to ensure that the PGPR isolates selected are the best isolates for further evaluation.

3.2 Materials and methods

3.2.1 Preliminary screening trial (greenhouse trial one)

Maize seed was inoculated with 30 PGPR isolates obtained from the culture collection of the University of Pretoria. These isolates were screened for their ability to stimulate growth of maize seedlings under greenhouse conditions with temperatures ranging between 15°C and 35°C.

3.2.1.1 Inoculant preparation for Preliminary screening trial (greenhouse trial one)

Three litres of nutrient broth (NB) (Biolab, Wadeville, South Africa) was prepared by adding 16g NB powder per litre distilled water. The 1L broth was subsequently divided into 30X100ml units in 250ml Erlenmeyer flasks. The flasks were sealed with a double foil layer and autoclaved at 120°C for 20 min. The flasks were removed from the autoclave and left to cool to a comfortable handling temperature.

Sterile NB was aseptically inoculated with each individual bacterial isolate using a flamed inoculation loop in the laminar flow and labelled according to the culture code. One flask containing sterile NB was not inoculated and served as the control. After labelling, the flasks were incubated at room temperature on a rotary shaker for 48 h at 150rpm.

3.2.2.2 Viable counting

To verify the bacterial cell concentration within the NB, viable cell were counted. The inoculants were aseptically transferred from the 100ml NB to two sterile 50ml plastic tubes. The latter were then centrifuged (Hettich Universal 2S, 1300) for 10min at 8000rpm after which the supernatant was removed with a sterile 5ml pipette. The pellet was re-suspended in 20ml of ½ strength sterile Ringer's solution (Merck) using a 5ml pipette and re-centrifuged at 8000rpm for 10 min. This washing procedure of the pellet was repeated 3 times. After the final washing step the pellet was re-suspended in 150ml sterile ½ strength Ringer solution (Merck).

One millilitre of each inoculant was transferred to a sterile test tube and labelled accordingly. In successive steps, 1ml from the labelled test tube was transferred to one of the newly prepared test tubes and mixed by gently pipetting. This transfer procedure was repeated nine times until the inoculants were diluted to 10^{-9}. Finally 100ul of each test tube were aseptically transferred with a 100ul pipette to the solid nutrient agar plates (Merck). The bacteria were spread on the plate and labelled accordingly before being sealed with Parafilm. The plates were incubated at room temperature for 24 hours whereafter the colonies were counted.

3.2.2.3 Planting of preliminary screening trial (greenhouse trial one)

Untreated maize seed (cultivar ZM523 from Pioneer) was surface sterilized by flooding the seed with 70% ethanol for 3 min followed by a 1 min immersion in 3% sodium hypochloride solution. The seeds were then rinsed five successive times with sterile dH_2O. After surface sterilization the seed was primed by submerging them in sterile distilled water for 3 hours.

One hundred and fifty plastic pots with a volume of 5 litres were surface sterilized by submerging them in a 0.1% solution of hypochloride for 12 hours and subsequent rinsing with distilled water. A polyurethane mesh of 200mmX200mm was placed in the bottom of each pot before filling them with pasteurized sandy loam topsoil. The soil was pasteurized by steaming at 80^0C for three hours and allowing the soil to cool down to ambient temperature. Each pot was filled with 3.85kg of the pasteurized soil

and five sterile, primed, maize seeds were planted in a heptagon formation at a depth of 2.5cm below the soil surface in each pot.

Five pots per treatment were prepared and placed in a greenhouse in a completely randomized design (CRD). Thirty millilitres of the prepared 150ml bacterial suspension (section 3.2.2) was used to inoculate the pots as a soil drench by adding 30 ml to the soil with a 5 ml pipette. Sterile pipettes were used after each individual treatment was applied.

Each treatment (Table 3.1) consisted of a single PGPR strain and the control was only inoculated with ½ strength sterile Ringers solution. Each pot was labelled according to the treatment and watered every day with tap water. Greenhouse temperatures ranged from 15°C to 35°C. A week after germination the seedlings were thinned out to two plants per pot. Seedling selection was based on homogeneity of shoot length.

3.2.2.4 Harvesting and data collection: Preliminary screening trial (greenhouse trial one)

The plants were allowed to grow for 30 days before harvesting. The soil was removed by soaking the plants in tap water. Shoots were excised just above the first node and fresh root and shoot mass recorded. Afterwards the root and shoots were placed in brown paper bags and labelled according to treatments.

Root length measurements were recorded by scanning the roots with a Brother™ scanner (MFC7420) and saving the images as black and white AVI files. The images were processed using the program DeltaTscan® and the total root length recorded. The roots were again placed into their original brown paper bags.

The samples were then dried in an oven (Protea, I80E) at a temperature of 70°C for three days. The dry mass data was recorded when the samples reached a constant mass.

3.2.2 Follow-up greenhouse trial two with most promising selected PGPR strains

Isolates from the PGPR screening trial were selected on the basis of their growth stimulating capabilities in the PGPR screening trial and previous trials conducted on other grain crops (Pretorius, 2012; Hassen, 2010).

Four tons of field soil from the selected Towoomba Agricultural Development Centre (ADC) trial site (Huttons ecotope) was collected and used in this greenhouse trial. The trial was conducted on the same basis as the PGPR screening trial described in section 3.2 with the following changes: the selected inoculants were A08, A26, A32, A40, S2-08, S1-08, S4-08, S6-08 and the commercial product, Bacup®. The treatments were only replicated four times and not five times as in the screening trial in section 3.2 and five plants per pot were left to grow until harvesting in the pasteurized Huttons ecotope.

Fusarium isolations were made by using Rose-Bengal-Glycerol-Urea (RBGU) medium as described by Hassen *et al*. (2007) from the Towoomba ADC soil. The *Fusarium* isolates were sent to the Agricultural Research Council (Mycology unit, Plant Protection Research Institute, Biosystematics Division, Agricultural Research Council, Queenswood, Pretoria, South Africa) for identification.

Soil analysis was done by the soil analysis laboratory (Department of Plant Production and Soil Sciences, University of Pretoria) to determine phosphate, calcium, magnesium and potassium concentrations as well as pH, clay %, soil texture and colour. The nutrient analyses of the Towoomba ADC trial soils showed no nutrient deficiencies (Appendice A, table A, sample 3530) except for phosphorus. The soil was taken from the same area for both the follow-up evaluation PGPR and biocontrol trials (chapter 5). The ratio of Ca to Mg in the soil was close to the recommended value according to the fertilisation guidelines in the Maize Production Manual (ARC 2008) of 3:1 with 2,7:1 for the follow-up-evaluation PGPR trial and 2,8:1 for the biocontrol trial (chapter 5).

According to the fertilizer guidelines in the maize production manual (ARC, 2008) the recommended soil nutrient ratio for calcium, magnesium, potassium and sodium is 65:23:10:2. If the soil analysis (Appendix A, table A, sample 3530) of the trial soil is considered the ratio for Ca, Mg, K and Na was close to the recommended values of the ARC (2008) at a ratio of 59:22.4:11.4:6.3 respectively. However, the phosphorus level in the trial soil was 23% of the recommended 75kgP/ha (ARC, 2008) and was therefore supplemented by adding 0.18g of superphosphate (10.5%) per kilogram of soil. Nitrogen was amended to a level of 140kg/ha by adding 0.47ml nitrogen (147.6g/l) per kg of soil. Data collected included leaf length, root and shoot wet mass and total wet mass data of the maize seedlings.

3.3 Statistical analysis

For this experiment each treatment consisted of 5 plants per pot which was replicated 4 times and arranged in a (CRD). All data was captured and analysed with the statistical package SAS 9.2 using Proc GLM procedures at p=0.05. Means were separated using the Dunnetts test by comparing the treatments to the control if significant differences were observed at $p<0.05$.

3.4 Results and discussion

3.4.1 Preliminary screening trial one

Results of this trial are presented in Table 3.1.

The bacterial cell count before planting, verified via viable counting, was a minimum of $3.1X10^{6}$ CFU/ml for A-39 and a maximum of $1.7X10^{10}$ CFU/ml for A-24 with a mean value for all isolates of $8.0X10^{7}$CFU/ml. Although specific trends in terms of plant growth enhancement were observed, there were no significant differences between the treatments which support the findings of Gravel *et al.* (2007) who reported a similar (non-significant) trend in plant growth enhancement when four week old tomato plants were drenched with 200ml inoculum comprising *Penicillum brevicompactum, Trichoderma atroviride, Pseudomonas marginalis*, or *Penicillium putida*.

There were three possible reasons for this non-significant result. Firstly, the duration of the trial may have been too short thereby limiting any beneficial effect. Secondly, the inoculants selected might not have had intrinsic plant growth enhancing capabilities. However, a trend i.t.o. plant growth enhancement was observed in the first screening trial, similar to those of Gravel *et al.* (2007) as mentioned above. The third possibility might have been that there were sub-optimal nutrient levels in the soil. According to Adesemoye *et al*. (2009), PGPR in combination with fertilizers can improve the absorption efficiency of the nutrients by the plant. It was shown that a 25% increase in fertilizer efficiency could be obtained by using PGPR in conjunction with fertilizers but the data showed minimal to no positive effect when compared to the control at levels below 75% recommended fertilizer rates. Egener *et al*. (1999) also observed that the presence of nitrogen supressed the expression of genes associated with nitrogen fixation in most diazotrophs which supports the optimal fertilization findings as observed by Adesemoye *et al.* (2009).

Analysis of the soil used in the Preliminary screening trial (greenhouse trial one) showed deficiencies for the normal growth requirements for maize production (Appendix A; Table A, lab no 1753). It is probable that the various nutrient deficiencies were most likely the limiting factor that could have curtailed any beneficial effect of the PGPR, resulting in statistically non-significant results. Adesemoye *et al*. (2009) provided evidence that a threshold level of 75% of the recommended fertilizer rate could be used in conjunction with PGPR to have the same effect as a 100% fertilizer application rate without PGPR. The soil analyses showed a phosphorus (P), potassium (K) and magnesium (Mg) deficiency of 47.78%, 65% and 5% below the recommended rates of 27mg/kg, 100mg/kg and 40mg/kg, respectively, for maize production on sandy ambic soils (ARC, 2008). It is also well below the threshold levels described by Adesemoye *et al*. (2009). Although the calcium (Ca), magnesium (Mg) ratio is within the 8:1 ratio the ideal ratio between Ca: Mg: K: Na should be 65:23:10:2 respectively (ARC, 2008). Furthermore the soil analyses showed the Ca: Mg: K: Na ratio to be 61:13.52:12.46:12.81 of the recommended levels according to ARC (2008) guidelines. The ratio of nutrients is not ideal for maize production therefore this may support the observations made in this study.

The results obtained for the inoculants A-7, A-42 and A-19 showed a trend towards decreased root length and a decrease in dry root weight respectively, compared to that of the control (Table 3.1), although this was not statistically significant. The most profound suppression of root growth was recorded for A-19.

Although the results of the data collected during the trial were non-significant, there were a few inoculants that showed a positive effect on plant mass. For example, A-08, A-26, A-32, A-40 and S2-08 increased total dry weight by 28.32%, 10.79%, 24.88%, 19.35% and 7.16%, respectively. These inoculants were thus selected for further study to go into the Follow-up greenhouse trial two (3.2.2) and the Greenhouse biocontrol trial (5.2.8).

3.4.2 Follow-up greenhouse trial two with most promising PGPR strains

Results of the follow-up greenhouse trial two are shown in Table 3.2.
The inoculants that showed the most significant effect on root mass was S2-08 increasing the root mass by 22% and S1 that decreased the root mass by 29% in comparison to the control. The treatments that had a negative effect on plant mass compared to the control were S1-08, S6-08, A-08, A-32 with final root weights of 35.60g, 39.30g, 40.03g and 46.10g, respectively, compared to that of the control being 50.15g. The only treatments that resulted in a root mass increase were A-26, A40, Bacup®, S4-08 and S2-08 with final fresh root weights of 55.45g, 56.35g, 56.63g, 58.90g and 61.28g, respectively, compared to the control at 50.15g.

Only the treatments with S2-08, S4-08 and A40 increased shoot mass with a final mass of 73.03g, 73.80 and 75.88g respectively, with A40 being the best performing inoculant resulting in a percentage increase of 4.98% over that of the control. On the other hand, the lowest shoot weight of 51.25g was recorded for S6-08 followed in ascending order by S1-08, A-08, A-32, Bacup® and A-26 with 58.78g, 61.78g, 67.70g, 69.20g, and 70.65g, respectively, compared to the control weight of 72.28g.

In terms of total plant mass the best performing isolate overall was S2-08 with an increase of 9.70% over the untreated control. Isolate S6-08 resulted in a decrease of 26.04% compared to the control. Isolates S1-08, A-08 and A-32 also showed an

overall decrease of 22.91%, 16.85% and 26.03% respectively when compared to the mass of the control at 122.43g. Bacup®, A-26, A40, and S4-08 resulted in a minimal overall weight increase over that of the control (125.83g, 126.10g, 132.23g, and 132.70g, respectively). There were however no statistically significant differences between these treatments.

With regards to the root length data all treatments resulted in a reduced root length in comparison to the control. S6-08 decreased the root length most significantly by 17%, strain A40 had the least effect, decreasing root length by 1.8%. Isolates S1-08, A-08, A-26, S4-08, S2-08, A-32 and Bacup® showed signs of root growth inhibition when compared to that of the untreated control. For each treatment, effects on plant mass and root length were similar, i.e. increasing or decreasing the values.

Although the untreated control had the greatest average root length, S2-08 showed a 9.7% higher total shoot mass, albeit not statistically significant. These results emphasize the importance not only of strain selection, but also of soil type, soil nutrient levels and inoculum dose when screening trials with rhizobacteria are conducted.

3.5 Conclusion

In this study, multiple comparisons of various growth parameters indicated that even though the control outperformed all the other treatments i.t.o. shoot length, the plant mass data indicated otherwise with bacterial strain S2-8 for example resulting in an increase of 9.70%. Although no statistical differences were found during data analyses this does not mean that these results are of no value because certain trends were evident. Similarly Jarak *et al.* (2012) found no significant differences between the uninoculated (control) maize and maize treated with a mixture of *Azobacter chroococcum*, *Bacillus* spp. and *Pseudomonas* spp. when shoot length was evaluated, but when yield was assessed a significant increase of 12.2% over the control was obtained. In another study Jarak *et al.* (2012) reported no significant differences in growth of maize inoculated with a product called Combat® compared to the uninoculated control and only found significant differences between treatment

and control plants i.t.o. yield. Another study by Pholo (2009) corroborates these findings.

The reduction in plant growth observed with some treatments in the current study corresponds to those of other researchers such as Miller and Bassler (2001) who reported that rhizobacteria applied at high cell concentrations may suppress plant growth as opposed to enhancing growth. The effect of different PGPR inoculum concentrations on plant growth was further investigated in the current study (Chapter 4).

Table 3.1. Effect of rhizobacteria on growth of maize seedlings in the greenhouse (Preliminary screening trial, greenhouse trial one)

	Seedling mass in grams							% Change in seedling mass compared to the control						
	Fresh mass			Dry mass				Fresh mass**			Dry mass**			
Rhizobacterial isolate*	Root	Shoot	Total	Root	Shoot	Total	Total Root Length	Root	Shoot	Total	Root	Shoot	Total	Total Root length
A-04	4.44[a]	1.73[a]	6.07[a]	0.47[a]	0.29[a]	0.75[a]	7857.43[a]	-23.75	6.33	-15.78	-1.66	17.62	4.99	-5.56
A-06	4.93[a]	1.89[a]	6.67[a]	0.51[a]	0.28[a]	0.77[a]	8917.40[a]	-15.35	15.99	-7.40	6.49	13.89	9.04	7.18
A-07	5.64[a]	2.25[a]	7.82[a]	0.43[a]	0.32[a]	0.73[a]	6484.62[a]	-3.08	38.31	8.51	-9.67	26.33	2.75	-22.06
A-08	7.02[a]	2.35[a]	9.15[a]	0.61[a]	0.33[a]	0.91[a]	8329.17[a]	20.60	44.09	26.95	26.42	31.92	28.32	0.11
A-09	4.46[a]	1.89[a]	6.19[a]	0.48[a]	0.32[a]	0.77[a]	7265.04[a]	-23.40	15.71	-14.09	-0.51	26.77	8.90	-12.68
A-10	5.15[a]	1.74[a]	6.76[a]	0.43[a]	0.27[a]	0.68[a]	7562.07[a]	-11.43	6.66	-6.21	-10.82	8.10	-4.29	-9.11
A-19	2.99[a]	1.44[a]	4.38[a]	0.36[a]	0.29[a]	0.63[a]	5688.40[a]	-48.63	-11.66	-39.24	-25.19	14.90	-11.35	-31.63
A-22	4.56[a]	1.85[a]	6.32[a]	0.43[a]	0.28[a]	0.70[a]	7483.86[a]	-21.67	13.66	-12.30	-9.82	13.77	-1.68	-10.05
A-24	5.10[a]	2.05[a]	6.98[a]	0.51[a]	0.33[a]	0.82[a]	7988.88[a]	-12.39	25.49	-3.11	6.21	31.27	14.86	-3.98
A-25	4.86[a]	2.06[a]	6.89[a]	0.48[a]	0.30[a]	0.76[a]	7501.33[a]	-16.54	26.15	-4.41	0.58	19.93	7.25	-9.84
A-26	7.64[a]	2.38[a]	9.78[a]	0.50[a]	0.31[a]	0.79[a]	8664.47[a]	31.25	45.81	35.80	4.46	22.80	10.79	4.14
A-28	5.44[a]	1.93[a]	7.19[a]	0.47[a]	0.31[a]	0.76[a]	8293.40[a]	-6.48	18.21	-0.15	-1.49	22.32	6.72	-0.32
A-29	4.91[a]	1.99[a]	6.96[a]	0.50[a]	0.31[a]	0.75[a]	6557.84[a]	-15.69	22.04	-3.39	4.12	22.60	5.10	-21.18
A-32	6.75[a]	2.18[a]	8.71[a]	0.60[a]	0.31[a]	0.89[a]	9069.66[a]	16.01	33.93	20.86	24.31	25.96	24.88	9.01
A-33	4.91[a]	2.09[a]	6.92[a]	0.48[a]	0.34[a]	0.79[a]	8135.32[a]	-15.67	28.15	-3.93	-0.31	34.10	11.69	-2.22
A-34	6.29[a]	2.18[a]	8.35[a]	0.52[a]	0.29[a]	0.79[a]	7805.01[a]	8.12	33.76	15.84	7.39	17.01	10.71	-6.19
A-36	6.54[a]	2.09[a]	8.44[a]	0.46[a]	0.28[a]	0.71[a]	7954.77[a]	12.45	28.15	17.11	-5.08	10.90	0.15	-4.39
A-37	5.15[a]	1.86[a]	6.79[a]	0.39[a]	0.25[a]	0.62[a]	7052.05[a]	-11.43	13.88	-5.69	-17.82	-1.13	-12.06	-15.24
A-38	4.84[a]	2.08[a]	6.86[a]	0.48[a]	0.32[a]	0.78[a]	7420.63[a]	-16.88	27.48	-4.74	0.00	26.85	9.27	-10.81
A-39	6.22[a]	2.23[a]	8.30[a]	0.48[a]	0.32[a]	0.78[a]	7445.59[a]	6.80	37.03	15.22	-0.45	28.72	9.62	-10.51
A-40	7.52[a]	2.38[a]	9.74[a]	0.54[a]	0.33[a]	0.85[a]	8986.46[a]	29.20	46.14	35.18	12.04	33.21	19.35	8.01
A-41	4.84[a]	2.13[a]	6.91[a]	0.45[a]	0.32[a]	0.75[a]	7531.28[a]	-16.88	30.76	-4.03	-6.85	29.40	5.66	-9.48
A-42	4.42[a]	1.94[a]	6.30[a]	0.42[a]	0.29[a]	0.69[a]	6270.80[a]	-24.02	19.10	-12.55	-13.08	17.90	-2.39	-24.63
A-43	4.93[a]	1.84[a]	6.63[a]	0.49[a]	0.29[a]	0.76[a]	8261.78[a]	-15.35	12.83	-8.04	2.80	14.09	6.70	-0.70

*Rhizobacterial isolates from the UP-PGPR culture collection.

** % change in mass [(treatment - control)/ control x 100], therefore negative values indicate treatments with a lower mass than the untreated control and positive values are treatments with a higher mass than the untreated control.

***Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference

Table 3.1. (continued). Effect of rhizobacteria on growth of maize seedlings in the greenhouse (Preliminary screening trial, greenhouse trial one)

(LSD) tests using the GLM procedure. The treatment means were compared with the control using the Dunnetts test if significance was observed

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	Seedling mass in grams							% Change in seedling mass compared to the control**						
	Fresh mass (g)			Dry mass (g)				Fresh mass			Dry mass			
Rhizobacterial isolate*	Root	Shoot	Total	Root	Shoot	Total	Total root length	Root	Shoot	Total	Root	Shoot	Total	Total Root Length
A-44	6.53[a]	2.05[a]	8.45[a]	0.52[a]	0.27[a]	0.77[a]	8447.32[a]	12.14	25.49	17.34	8.37	8.75	8.50	1.53
A-45	5.67[a]	2.14[a]	7.65[a]	0.43[a]	0.28[a]	0.70[a]	6854.87[a]	-2.56	31.09	6.24	-9.65	12.31	-2.07	-17.61
A-46	4.75[a]	2.12[a]	6.76[a]	0.44[a]	0.33[a]	0.75[a]	7128.59[a]	-18.32	30.21	-6.18	-8.37	30.42	5.02	-14.32
AFP1-1	5.16[a]	2.29[a]	7.36[a]	0.49[a]	0.33[a]	0.80[a]	7399.83[a]	-11.34	40.70	2.10	2.65	33.09	13.15	-11.06
AZO MARKET***	5.79[a]	2.02[a]	7.79[a]	0.57[a]	0.33[a]	0.87[a]	8506.39[a]	-0.57	24.21	8.15	17.76	31.19	22.39	2.24
S2-08	5.18[a]	1.92[a]	6.89[a]	0.49[a]	0.29[a]	0.76[a]	7384.85[a]	-11.02	17.93	-4.40	2.11	16.73	7.16	-11.24
CONTROL	5.82	1.63	7.20	0.48	0.25	0.71	8320.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Rhizobacterial isolates from the UP-PGPR culture collection.

** % change in mass [(treatment - control)/ control x 100] therefore negative values are treatments that are less than the untreated control and positive value are treatments with a higher mass than the untreated control.

***Commercial product AZOMARKET® from BASF©.

****Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure. The treatment means were compared with the control using the Dunnetts test if significance was observed

Table 3.2. Effect of selected rhizobacterial isolates on growth of maize seedlings in the greenhouse (follow-up greenhouse trial two (3.2.2)).

Inoculant*	Seedling mass in grams				% Change in seedling mass in comparison with the control**			
	Fresh mass (g)				Fresh mass			
	Root	Shoot	Total	Plant length*****	Root	Shoot	Total	Plant length****
A-08	40.03[a]	61.78[a]	101.80[a]	48.95[a]	-20.18	-14.53	-16.85	-9.40
A-26	55.45[a]	70.65[a]	126.10[a]	50.10[a]	10.57	-2.26	3.00	-7.27
A-32	46.10[a]	67.70[a]	113.80[a]	52.30[a]	-8.08	-6.34	-7.05	-3.20
A-40	56.35[a]	75.88[a]	132.23[a]	53.05[a]	12.36	4.98	8.00	-1.81
S1-08	35.60[a]	58.78[a]	94.38[a]	48.64[a]	-29.01	-18.68	-22.91	-9.98
S2-08	61.28[a]	73.03[a]	134.30[a]	51.61[a]	22.19	1.04	9.70	-4.48
S4-08	58.90[a]	73.80[a]	132.70[a]	50.85[a]	17.45	2.10	8.39	-5.89
S6-08	39.30[a]	51.25[a]	90.55[a]	44.75[a]	-21.64	-29.10	-26.04	-17.18
BACUP®***	56.63[a]	69.20[a]	125.83[a]	52.80[a]	12.92	-4.26	2.78	-2.28
CONTROL	50.15[a]	72.28[a]	122.43[a]	54.03[a]				

*Rhizobacterial isolates from the UP-PGPR culture collection.
** % change in mass [(treatment - control)/ control x 100].
***Commercial product.
**** Plant length determined by measuring in centimetres from the first node from the soil layer to the apical leaf tip.
*****Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test if significance was observed.

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Chapter 4

Assessment of growth promotion of maize at various soil nutrient levels, PGPR inoculum dosages and with different application methods.

Abstract

There is an optimal fertilizer rate at which PGPR are most effective indicating that fertilizer and PGPR dosages are dependent on each other. The relationship between PGPR efficacy and fertilizer rate and PGPR efficacy and inoculum dosage was tested in this study by evaluating fertilizer rate and inoculum dosages in two separate trials i.e. nutrient evaluation trial and a dosage response trial. The nutrient evaluation trial assessed PGPR effectiveness at a constant dose of $8X10^8$cfu/ml at various nitrogen and phosphate levels in a split plot arrangement of a CRD. The effect of PGPR dose on plant growth was evaluated in a dose response trial in which three inoculum concentrations were used to determine the optimum dosage. In this trial an algenate inoculum carrier was compared to the standard soil drench technique to evaluate the effect of different inoculation techniques. To evaluate the effect of nutrient levels on PGPR efficacy a trial was conducted in which each PGPR treatment was evaluated at three levels of phosphate (0kg/ha, 75kg/ha and 150kg/ha) and three nitrogen levels (0kg/ha, 140kg/ha and 280kg/ha), over a period of 82 days. The optimum performances of the isolates were in the 75kg/ha range for phosphate. However, plant biomass still increased in the 200kgN/ha range. Within the nitrogen range a slight decrease in the biomass increase rate was observed with an increase in nitrogen dose that could be summarized by the logarithmic function as $y = 7.7994\ln(x) + 5.6732$. At nitrogen level N0 the mean dry weight was 5.85g, at nitrogen N1 the mean dry weight was 10.60g and at N2 the mean dry weight was 14.54g. This decrease was only slight with a dry weight mean value for all the treatments at N0 level of 5.85g, N1 at 10.60g and N2 at 14.54g.

The dose response trial was done in the same manner as the nutrient reaction trial except that the nutrient level was kept constant at 75kg/ha phosphate and 140kg/ha nitrogen. The PGPR were applied at 40ml dose increments up to 120ml per pot. The optimum inoculum dosage was found to be 120ml/pot as a soil drench treatment.

Results from the trial indicate that both fertiliser levels and PGPR dose have an effect on the ability of the PGPR to enhance plant growth. For the rates tested it was found that the optimal phosphate level was 75 kg/ha. However plant growth increased as nitrogen dose increased up to the maximum level of evaluation at 200kg/ha. The data suggest that there is a direct correlation between PGPR dose and plant growth as can be seen from the data that the optimum PGPR dose was at 120ml (soil drench). This application method however was found not to be a viable due to the large volume of soil drench that would be needed under standard agricultural practices.

4.1 Introduction

Inoculation of sorghum with PGPR (*Bradyrhizobium japonicum*, *R. leguminosarum* var. *viceae, Azorhizobium, Rhizobium*, *Rhizobium leguminosarum* var. *viceae*, *caulinodan*, *Sinorhizobium meliloti*) can affect phosphate uptake by influencing the transporter activity of the root plasma membrane (Matiru & Dakora, 2004). Zahir *et al*. (1998) inoculated maize seed with four isolates (two *Azotobacter* spp. and *Pseudomonas* spp.) under field conditions receiving NPK at 150-100-100 kg/ha and found that collective inoculation of the isolates considerably increased grain yield compared to the uninoculated plants. Vedderweiss *et al*. (1999) found that maize inoculated with *Azospirillum* spp. at a concentration of 10^6 cfu/ml enhanced fresh root and shoot weight of seedlings while Stancheva *et al*. (1992) reported that inoculation of maize with *Azospirillum brasilense* strain 1774 in a blend with 100kg N/ha fertilizer gave the same result as 200kg N/ha of non-inoculated plants. These results were further supported by Dobbelaere *et al*. (2001) who showed that inoculated maize had a higher nitrogen content compared to that of the untreated control. Gholami (2009) inoculated maize with six different strains of *Azospirillum* spp. and *Pseudomonas* spp. which significantly increased all the growth parameters that were evaluated which included; seed germination, seed vigor, plant height, dry weight and most importantly yield. The findings of Gholami (2009) were complemented by a study conducted by Mirshekari *et al.* (2012) that showed the complimentary stimulatory effect of a mixture of *Azospirillum lipoferum* and *Azobacter chroococcum* that increased the overall plant health and yield of maize.

Richardson *et al.* (2001) reported that various microorganisms could solubilize phosphates *in vitro* and therefore have the potential to increase soil fertility. This observation may be true not only for phosphates but to all the growth promoting chemicals secreted by these PGPR. In addition, Ekin *et al.* (2009) showed that there is a link between nitrogen fertilization and the *Bacillus* inoculants they used in their experiments on potato growth and yield. They found that when the *Bacillus* spp. were combined with nitrogen, the growth parameters were significantly higher than with only the nitrogen treatments.

A study conducted by Adesemoye (2009) not only supported the nitrogen efficiency findings of Ekin *et al.* (2009) but also found that at a nutrient dose of 75% nitrogen, phosphorus and potassium in combination with a mixture of *Bacillus amyloliquefaciens* and *B. pumilus* had the same effect on tomato yield as that of 100% fertiliser dose without PGPR inoculation. However, they also showed minimal to no positive effect compared to the control at levels below 75% of the recommended fertilizer rates, suggesting that there is an optimal level of fertilizer at which the PGPR are most effective at enhancing plant growth. Egener *et al*. (1999) also observed that the presence of nitrogen mediates the expression of genes associated with nitrogen fixation in most diazotrophs which supports the findings that fertilizer level influences PGPR activity.

In addition to fertilizer affecting the efficacy of PGPR, it is also known that the number of PGPR present in the rhizosphere have an impact on the degree of plant growth promotion observed. The number of colony forming units (CFU) that are applied is of the utmost importance according to the findings of Racke & Sikora (1992). The experiment was conducted as a biocontrol experiment without any mode of action evaluation for growth stimulating capabilities. They found that the ideal cfu density of *A. radiobacter* and *B. sphaericus* on potato tubers were at a concentration of $9.7X10^{8}$cfu/ml and $3.16X10^{9}$cfu/ml respectively.

The findings of Kifle & Laing (2011) supported those of Racke & Sikora (1992) in regards to optimum PGPR dosage by linking the dose of fertilizer and the PGPR dose. They found that high fertilizer doses and high PGPR inoculum doses were less

effective than the inverse and could be contributed to increased substrate requirements of the inoculants. The most interesting finding from a commercial perspective was that the $1X10^8$ cfu/ml dose applied as a seed treatment was the most economical as it was relatively cheap to produce and apply especially when the crop size did not allow for effective drenching. In this study the fertilizer rate and inoculum dosage were independently tested to enable to ascertain whether there is an optimum fertilizer and dosage level to increase PGPR effectiveness as described by Racke & Sikora (1992).

4.2 Materials and methods

4.2.1 Assessment of the effect of PGPR isolates at different soil nutrient levels.

Huttons type soil (for analysis see appendix A) was taken from Towoomba Agricultural Development Centre at location 24^o55'22,44"5, 20^o20'14.91"E and transferred to the L.C de Villiers experimental farm at the University of Pretoria. The soil was pasteurized at 80^oC and left to cool before ten 100ml soil samples were taken and thoroughly mixed. From this mixture a 100ml global soil sample (sample number 1234) was taken and sent for nitrogen and phosphorus analysis at the soil laboratories of the University of Pretoria.

The existing soil nutrient levels indicated by the chemical analysis were used as a benchmark from which additional phosphorous and nitrogen were supplemented as follows: Superphosphate (Omnia, Bryanston, South Africa) was ground to a fine powder in a mortar and pestle. The powdered superphosphate was added to the soil at the required dosage and the soil mixed thoroughly. The treatments were as follows: P0= no phosphate added, P1=25mg/kg phosphate added to 7.38kg of soil per pot (equivalent to 75kg/ha) and for P2=50mg/kg phosphate was added to 7.38kg of soil per pot (equivalent to 150kg/ha). Nitrogen levels were adjusted after seedling emergence by application of NH_4OH solution as follows: N=0 received no nitrogen application, N1 received 3.5ml NH_4OH solution per pot at a concentration of 6g/L NH_4OH per litre dH_2O (equivalent to 140kg/ha) and each pot of the N2 treatment received 3.5ml of a 295.2g/L NH_4OH per litre dH_2O solution (equivalent to 280kg/ha).

A control was included with a phosphate and nitrogen level of P1 (75kgP/ha) and N1 (140kgN/ha) according to recommended agricultural practices for maize.

The trial was separated into nine separate trials according to phosphate levels (0; 75kgP/ha; 150kgP/ha) and 3 nitrogen levels (0kgN/ha; 140kgN/ha; 280kgN/ha) using a completely randomised design (CRD). Bacterial treatments consisted of application of the individual bacterial isolates S1-08, S2-08, S3-08, S4-08 and two commercial products namely Bacup® (BASF, Kwazulu Natal, South Africa) and Brus® (Stimuplant, Gauteng, South Africa) respectively. Each treatment was replicated five times with a replicate comprising of five pots containing two plants each.

The following is a summary of the individual trials with the 6 bacterial treatments applied to each fertiliser level respectively:

Trial one: Phosphate 0 + Nitrogen 0
Trial two: Phosphate 0 + Nitrogen 1
Trial three: Phosphate 0 + nitrogen 2
Trial four: Phosphate 1 + Nitrogen 0
Trial five: Phosphate 1 + Nitrogen 1
Trial six: Phosphate 1 + nitrogen 2
Trial seven: Phosphate 2 + Nitrogen 0
Trial eight: Phosphate 2 + Nitrogen 1
Trial nine: Phosphate 2 + nitrogen 2

The bacterial strains were grown in nutrient broth (Stimuplant©). The final cell concentrations of each strain were:

- S1 at $8.4X10^8$ cfu/ml
- S2 at $8.0X10^8$ cfu/ml
- S3 at $8.4X10^8$ cfu/ml
- S4 at $2.36X10^9$ cfu/ml

Maize seed from the cultivar ZM523 was obtained from the Limpopo Department of

Agriculture, Research Section, Towoomba ADC in 2009. The seed were surface sterilized by immersing in 70% ethanol for 3 minutes and subsequently for 1 minute in 3% hypochloride solution. The seeds were then rinsed five times with sterile dH_2O. After surface sterilization the seed were primed by submerging for 3 hours in dH_2O.

Two hundred and seventy 5L plastic pots were surface sterilized by submerging them in a 0.01% solution of hypochloride for 12 hours and rinsing them with distilled water. A polyurethane mesh of 200 mm X 200 mm was placed in the bottom of each pot before filling with pasteurized sandy loam topsoil. Of the pasteurized soil, 7.38kg was placed in each pot and five sterile primed maize seeds were planted in a heptagon formation at a depth of 2.5cm below the soil surface in each pot. After germination plants were thinned to two plants per pot. The inoculants were added at 10ml per treatment as a soil drench directly after planting by pipetting twice with a 5ml pipette. Sterile pipettes were used after each individual treatment was applied.

The plants were allowed to grow for 82 days at prevailing greenhouse temperatures ranging from 15^{o}C to 35^{o}C at the University of Pretoria's Hatfield experimental farm before harvesting. The soil was removed by soaking the plants in tap water. Shoots were excised just above the first node and fresh root and shoot mass recorded. After recording the fresh mass the root and shoots were placed in brown paper bags labelled according to treatments.The samples were then dried in an oven (Protea, I80E) at a temperature of 70°C for several days until the daily weight remained constant, before recording the dry mass.

4.2.2 Assessment of PGPR dosages and selected methods of application

The trial was conducted in the same manner as described in 4.2.1 except that the nutrient levels were kept constant at P1 and N1. The seeds were surface sterilized as described in 4.2.1.

Algenate seed coating (treatment T4 and T5) was prepared as described in appendix A.5 to encapsulate the rhizobacterial inoculants on the seeds. The algenate solution was prepared and autoclaved at 120^{o}C for 20min and left to cool in the laminar flow. The rhizobacterial inoculants were added to the prepared algenate at label

recommendations (200g isolate to 50kg of maize seed). The maize seeds were submerged in this algenate-isolate-solution. Individual seeds were then submerged for 30 s in a 0.1M $CaCl_2$ solution and rinsed 3 times with dH_2O and left to dry in the laminar flow cabinet.

The treatments were as follows:
- T1: 40ml of S2-08 applied per pot as a soil drench
- T2: 80ml of S2-08 applied per pot as a soil drench
- T3: 120ml of S2-08 applied per pot as a soil drench
- T4: S2-08 applied to seed in algenate encapsulation
- T5: Brus® applied to seed in algenate encapsulation per pot

Two controls were included - control 1 comprised of 120ml sterile dH_2O applied per pot and control 2 comprised of an uninoculated algenate seed treatment

The plants were allowed to grow for 82 days before harvesting. The soil was removed from the root systems by soaking in tap water. Roots were excised from the shoots just above the first node, and wet root and shoot mass determined. Subsequently the fresh roots and shoots were placed in brown paper bags and labelled accordingly. The samples were dried in an oven (Protea, I80E) at a temperature of 70°C for several days until the samples reached a daily constant mass.

4.3 Statistical analysis

All data were captured and analysed using proc GLM procedures of SAS 9.2 at P=0.05. Means were separated using Fishers protected LSD test if significance was observed.

4.4 Results and discussion

4.4.1 Effect of PGPR isolates at different soil nutrient levels

From the results (Table 4.1) it can be seen that there were no significant differences between different PGPR treatments when the bacterial isolates were evaluated at the P=0 and N=1 levels as well as at the P=1 and N=1 levels. Brus® had the most

significant growth enhancing effect under phosphate limited conditions (P=0) at N=0 (0kgN/ha) with a seedling wet mass of 15.26g but then decreases to 11.98g at the N=2 level (280kgN/ha). Overall it is noteable that there was a decline in wet and dry mass when the rhizobacteria were applied at higher levels of soil nitrogen content except for the treatments S2-08 and Brus® at N=1 level.

At P=1 level (75kgP/ha) there was a constant increase in wet and dry mass of the maize plants in all the PGPR treatments as the nitrogen level increased. S4-08 was the best performer at the N=0 level with a wet mass of 50.07g and 6.86g dry weight which were significantly different to both the commercial products. The best performing isolate at the P=1 X N=1 level was S3-08, which resulted in a fresh mass of 74.52g closely followed by S4-08 at 74.49g but S4-08 had a final dry weight of 11.73g which exceeded S3-08 dry weight of 11.68g. The biomass of the treatments peaked at a wet and dry mass of 70.70g and 10.82g for S2-08, 62.12g and 10.74g for S1-08 followed by Brus® at 60.46g & 9.79g and Bacup® at 51.27g and 8.87g, respectively.

At P=1 X N=2 level isolate S4-08 did not perform as well as S1-08, S2-08 and S3-08. All the treatments outperformed the commercial products (Brus® & Bacup®) across all the nitrogen dosages for the P=1 level. These results confirmed those of Racke & Sikora (1992) who found a link between PGPR concentration and fertilizer when determining the efficiency of PGPR. This effect can be seen when looking at the P=1 X N=2 levels.

When phosphate levels increased to the P=2 level (150kg/ha) the only significant differences observed in the N=0 level were for the treatments S4-08 and Bacup® when fresh mass was evaluated. When nitrogen was increased from N=0 to N=1 levels the fresh mass for S1-08, S2-08 and S3-08 increased but the dry mass decreased. This wet weight increase and dry weight decrease continued as the nitrogen dose increased to the N=2 (280kgN/ha) level. In most of the treatments the dry mass was lower than in the N=0 fertilizer level except for the S3-08 and the Bacup® treatment that showed a small, statistically non-significant increase of 0.35g and 0.33g, respectively.

The optimum nutrient level for all the treatments were in the P=1 and N=2 range. The S-inoculants still outperformed both the commercial treatments. For the four S-inoculants, treatment S1-08 had the highest wet and dry mass of 103.01g and 15.97g followed by S3-08, S2-08 and S4-08.

According to the data in Table 4.1 the optimum performance of the isolates is in the Phosphate 75kg/ha range but the plant biomass still increased even to the 200kgN/ha range. There is however a slight decrease in the rate of biomass increase at the 200kgN/ha level which could be summarized by the logarithmic function as y = 7.7994ln(x) + 5.6732. The mean dry weight values for all the treatments at a N0, N1 and N2 level was 5.85g, 10.60g, 14.54g respectively. In a study on PGPR inoculation of barley Mirshekari (2012) found a direct relation between yield and nitrogen and/or phosphate levels when barley was inoculated with *Azotobacter chroococcum* and *Azospirillum lipoferum.* Their data indicate that the higher the nitrogen and/or phosphate levels the lower the yield increase due to PGPR treatment. The data from our study supports these findings of Mirshekari (2012).

4.4.2 Assessment of PGPR dosages and selected methods of application

The data is presented in Table 4.2. All the soil drench applications showed a tendency of outperforming both the controls although this was not statistically significant. The optimum dosage for S2-08 was 80ml/pot (T2) resulting in 16.78% and a 23.28% increases of wet and dry mass respectively in comparison with control 1 and 20.78% and 25.85% increases in wet and dry mass respectively compared to control 2. The 40ml/pot (T1) and 120ml/pot (T3) treatments showed an increase in biomass over the controls with the 40ml/pot treatment (T1) at a 8.36% and 16.36% and 12.08% and 18.78% increase over the controls wet and dry mass respectively. The 120ml/pot treatment (T3) showed increases of 9.05% and 15.96% and 12.79% and 18.37% over that of control 1 and control 2 wet and dry mass. The inoculant *Bacillus cereus* (Table 5.2) used in this study was applied at the same concentration as the concentration recommended by Kifle & Laing (2011) but according to our data the growth promoting effect tapers off at high doses as can be seen in the data from treatment T3. This observation of optimum dose corresponds with the findings of

Pholo (2009) who inoculated maize seed with the commercial PGPR product called Combat®. Pholo (2009) results on 21 day old maize seedlings indicated that root and shoot development was negatively affected compared to the uninoculated control at both low and high dosages. On the other hand growth was promoted at an optimum dosage of 25mg/kg to 50mg/kg.

In this study, although not statistically significant, the algenate seed treatments (T4 and T5) showed poorer growth than those of the control treatments. Treatment T5 inoculated with the commercial product Brus® had the least negative growth impact with a decline of 4.78% and 1.64% when compared to control 1 and 1.51% and 0.41% compared to control 2 i.t.o wet and dry mass. The S2-08 algenate seed coating (T4) had the most significant negative impact on seedling growth resulting in a decrease of 17.61% and 15.21% compared to control 1 and 14.78% and 13.35% to control 2 i.t.o. wet and dry mass.

The results with the algenate treatments (T4 and T5) contrasts with the results obtained by Trivedi *et al.* (2005) who tested various carriers for PGPR application of *Bacillus subtilis and Pseudomonas corrugata* on maize, one of which was an algenate seed coating. In their experiment they determined the ability of PGPR to colonize the rhizosphere at seven day intervals over a growth period of 42 days. They found that the algenate based formulations were the most effective when compared to charcoal and broth formulations. They also observed that the algenate based inoculants had an initial low colony count but this increased over time while the charcoal and broth formulations peaked at seven days followed by a decline. Fravel *et al.* (1985) contributed the slow increase of the algenate based formulations to the algenate providing protection and causing slow release of the inoculants into the rhizosphere. A possible reason why no significant differences were obtained in the current trial in contrast with the results of Trivedi *et al.* (2005) might be the fact that the current trial was conducted over an 82 day period whereas the trial of Trivedi *et al.* (2005) was conducted over a period of 42 days.

4.5 Conclusion.

The results of the trial assessing the effect of different soil nitrogen and phosphate levels on PGPR performance (Table 4.1) indicated that there is a direct relation between nutrient dose (N and P) and PGPR efficacy, corresponding with the findings of Mirshekari (2012). This relation between nutrient dose and PGPR efficacy should be extensively evaluated in future research in order to optimize PGPR efficacy and limit the detrimental effects as mentioned by Adesemoye *et al*. (2009).

When data from the PGPR application trial is considered (Table 4.2), it is seen that the broth treatments performed better than the control treatments, but the control treatments performed better than the algenate treatments. This contradicts the results obtained by Trivedi *et al.* (2005). The reason for the contradicting results is unknown because the maize germination percentage in the current study was 100%, but it could be that there was a build-up of growth stimulating or biocontrol compounds that may have retarded the growth of the maize seedlings to the extent that there was an inhibitory effect within the algenate capsule.

It is important to consider the economic viability when evaluating PGPR's and to adapt screening techniques/trials to realistically represent how the final product would be used commercially. As seen in this study, treatment T3 was the best performing dose but this type of liquid application is uneconomical under normal agricultural practices in South Africa. If the total amount of broth applied per ha is calculated that would be needed to effectively implement this treatment method i.e. 80ml per 5 litre of soil at a depth of 0.2m, it would amount to 32000L/ha PGPR broth needed, which is not economically viable. For this reason it was decided to use powder formulations as a carrier for the PGPR inoculants in the field trials according to commercial practices (chapter 6). This would allow the results to be compared with commercial products on the market and used as a standard for evaluation in the field trials.

Table 4.1 Effect of PGPR isolates on maize growth at different levels of phosphate and nitrogen in the soil (greenhouse experiment)

		Seedling mass (g)					
Phosphate level	**Inoculant ***	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**	**Dry**
P0**	S1-08	15.24^{c}	2.49^{bc}	8.06^{a}	1.33^{a}	4.88^{a}	0.82^{a}
	S2-08	8.36^{a}	1.42^{a}	10.36^{a}	1.38^{a}	9.54^{bc}	1.44^{bc}
	S3-08	10.08^{ab}	1.39^{a}	8.80^{a}	1.29^{a}	7.37^{ab}	1.19^{ab}
	S4-08	14.79^{c}	2.56^{bc}	9.79^{a}	1.77^{a}	8.33^{abc}	1.58^{bc}
	BRUS®	15.26^{c}	2.68^{c}	12.06^{a}	1.78^{a}	11.98^{c}	1.85^{c}
	Bacup®	13.34^{bc}	2.02^{ab}	11.02^{a}	1.24^{a}	6.31^{ab}	0.86^{a}
P1**	S1-08	42.43^{bc}	6.69^{b}	62.12^{a}	10.74^{a}	103.0^{c}	15.97^{b}
	S2-08	42.13^{bc}	5.90^{ab}	70.70^{a}	10.82^{a}	91.09^{b}	15.49^{b}
	S3-08	39.17^{ab}	5.60^{ab}	74.52^{a}	11.68^{a}	91.81^{bc}	14.56^{ab}
	S4-08	50.07^{c}	6.86^{b}	74.49^{a}	11.73^{a}	78.62^{a}	15.33^{b}
	BRUS®	34.10^{ab}	4.97^{a}	60.46^{a}	9.79^{a}	72.59^{a}	12.84^{a}
	Bacup®	33.30^{a}	5.09^{a}	51.27^{a}	8.87^{a}	73.72^{a}	13.08^{a}
P2**	S1-08	35.61^{a}	11.21^{a}	40.68^{a}	5.32^{a}	59.11^{a}	8.09^{a}
	S2-08	27.30^{a}	10.85^{a}	60.30^{c}	4.30^{a}	45.05^{a}	10.40^{a}
	S3-08	32.96^{a}	9.13^{a}	45.07^{ab}	5.03^{a}	57.67^{b}	9.48^{a}
	S4-08	60.78^{b}	11.58^{a}	55.82^{c}	11.35^{b}	54.77^{ab}	9.87^{a}
	BRUS®	43.41^{a}	10.01^{a}	35.33^{ab}	8.58^{a}	55.82^{ab}	6.36^{a}
	Bacup®	51.27^{b}	9.30^{a}	50.57^{bc}	9.55^{b}	70.93^{c}	9.63^{a}
Nitrogen level		**N0****		**N1****		**N2****	

*Rhizobacterial isolates from the UP-PGPR culture collection (S1-08, S2-08, S3-08, and S4-08. Brus® and Bacup® are commercial products).

**"N" designate nitrogen, "P" designate Phosphate N0= No nitrogen, N1=140kgN/ha, N2=280kgN/ha, P0= No phosphate, P1= 75kgP/ha, P2= 150kgP/ha

***Treatment means within the same corresponding columns of phosphate and nitrogen levels followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Fishers protected test if significance was observed.

Table 4.2 Effect of rhizobacterial isolates on growth of maize at various inoculum dosages and with different application methods.

Treatments*	Seedling mass(g)		% Change in seedling mass compared to the control**			
	Total		Control 1		Control 2	
	Wet	Dry	Wet	Dry	Wet	Dry
T1	68.64[a]	14.55[a]	8.35	16.40	12.07	18.78
T2	73.97[a]	15.41[a]	16.76	23.28	20.77	25.80
T3	69.08[a]	14.50[a]	9.04	16.00	12.78	18.37
T4	52.19[a]	10.61[a]	-17.62	-15.12	-14.79	-13.39
T5	60.32[a]	12.30[a]	-4.78	-1.60	-1.52	0.41
Control 1 (dH_2O)	63.35[a]	12.50[a]	0.00	0.00	3.43	2.04
Control 2 (Algenate)	61.25[a]	12.25[a]	-3.31	-2.00	0.00	0.00

* T1: 40ml of S2-08 (soil drench), T2: 80ml of S2-08 (soil drench), T3: 120ml of S2-08 (soil drench), T4: algenate seed coating with S2-08, T5: algenate seed coating with Brus®, Control 1: 120ml of dH_2O (soil drench), Control 2: uninoculated algenate seed coating.
** Change in seedling mass calculated as (100/control X treatment) -100 = %
***Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test if significance was observed.

4.6 References

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Chapter 5

Assessment of selected Rhizobacterial isolates for their ability to suppress the causal agent of Fusarium root/crown rot of maize in the greenhouse.

Abstract

Maize is grown throughout the world for its high carbohydrate content. This crop is targeted by various pathogens, of which fungi are rated as the second most important pathogens after insects affecting yield of maize. Various *Fusarium* spp. are linked with maize yield reductions, but trying to quantify the yield losses caused by these pathogens are difficult because of the inconsistency in the damage caused, by what is referred to as the root rot complex. Factors to be considered during development of a successful biological control agent include reliability and effectiveness under field conditions. In this study*, Fusarium* species were isolated from three different diseased maize stalk samples. These isolates were identified morphologically as *Fusarium subglutinans* and *F. graminearum,* and were used in the pathogenicity and biocontrol trials. Millet seed inocula of these isolates were prepared and used in a pathogenicity experiment in the greenhouse. Parameters measured included disease severity rating, biomass of host plant, root rot severity and also isolation frequency. Although close to a 100% infection rate was recorded for all the treatments, pathogenicity results based on disease ratings were variable. However, a consistent effect of the *Fusarium* isolates on biomass was observed when root rating was linked to plant wet and dry mass. This can be described as an algorythm for dry weight as $y = -0.1982x^2 + 0.4122x + 7.1913$ and $y = -0.7379x^2 + 0.0864x + 47.369$ for wet weight. Due to the irregular results obtained, it was decided to use soil naturally infested with *Fusarium*. The most effective biocontrol agents (identified in earlier experiments) were treatment with strain S2-08 and the commercial product Bacup® respectively, resulting in 19.38%, 22.22% and 25.24%, 30.11% increase in fresh and dry mass over the untreated, *Fusarium* infected control respectively. The data suggested that these inoculants can potentially be effective biological control agents against *Fusarium* spp. but they should be extensively evaluated under in-field conditions.

5.1 Introduction

Maize is grown throughout the world as a staple food crop in many countries, especially in Africa. It is relatively easy to cultivate and high yields per hectare are possible (Asiedu, 1989). In field and in storage maize is targeted by various pests and pathogens of which insects and fungi are rated number one and two in importance, respectively (Ominski *et al*., 1994; Gwinner *et al*., 1996). The fungal pathogens can be divided into two groups based on their isolation frequencies (Hugo, 1995). The first group are the root pathogens e.g. *Fusarium* spp. and the second group are the root colonizers which include genera such as *Phoma* and *Curvularia*

Various *Fusarium* spp. are associated with maize diseases e.g. *F. proliferatum, G. moniliforme, F. graminearum and F. anthophilum* (Scott, 1993; Munkvold & Desjardins, 1997). Trying to quantify yield losses caused by root rot pathogens is a major hurdle because of the inconsistency of disease severity under similar environmental conditions. Severe losses have been reported but taking into consideration the complexity of this disease and the symptoms involved makes in-field quantification attributed exclusively to a primary pathogen very difficult (Hugo, 1995).

According to Eilenberg (2001) the term biological control implies a living organism being used to limit the impact or population of a pest/pathogen on a crop or as stated by Bulgarelli *et al.* (2013) as “the process of suppressing a deleterious/ pathogenic living organism with a living organism”. The term “Plant Growth Promoting Rhizobacteria” (PGPR) is used to describe bacteria that are associated with the rhizosphere of the plant that enhance plant health either directly by contributing substances which include hormones, nutrients etc. or indirectly by limiting pathogen growth or damage (Alabouvette *et al.,* 2006).

Pal & Mc Spadden (2006) grouped the various mechanisms for biocontrol activity, e.g. parasitism, competition, induced resistance, into three categories, namely indirect antagonism where the pathogen is 1) indirectly affected e.g. induced systemic resistance, induced systemic tolerance and competition 2) direct

antagonism where the pathogen is directly affected by targeting the pathogen e.g. parasitism and lastly 3) mixed pathogen antagonism where the pathogen is directly affected but not specifically targeted e.g. antibiosis.

According to Berg (2009) the most numerous biocontrol agents on the market are *Bacillus* spp., *Trichoderma* spp. and *Pseudomonas* species. The reason for this small selection of species in the market is due to the extensive selection and evaluation under greenhouse and field conditions by researchers in order to produce an effective biocontrol agent. Factors to be considered, according to Shoda (2000) and Campbell (1989), to develop a successful biological control agent is that it should be economical, reliable and effective under field conditions. The objective of the study in order to develop an effective biological control agent is to screen for biocontrol activity under greenhouse conditions. Subsequently isolates showing biocontrol activity would be extensively evaluated under various field conditions (chapter 6).

5.2 Materials and methods

5.2.1 Pathogen isolation

Fusarium species were isolated from three different maize stalks (collected from Towoomba Agricultural Development Centre) showing symptoms of root and stalk rot. The pathogens were isolated after surface sterilizing the maize stalks by submerging them in a 3% sodium hypochloride solution for 5 seconds followed by spraying with 70% ethanol and drying in the laminar flow. The maize stalks where then split in two using a sterile scalpel. A 5 cm piece of the vascular system that had a pink discoloration was aseptically removed using sterile forceps and transferred to a sterile Petri dish were it was cut into 5mm segments using a sterile scalpel. The vascular system segments were then transferred to Petri dishes containing Rose-Bengal-Glycerol-Urea (RBGU) medium selective for *Fusarium* spp. according to van Wyk *et al.* (1989). Two solutions were prepared. The first solution comprised 10 ml glycerol, 0,5g Rose Bengal and 12g bacteriological agar (BA) (Merck, Johannesburg, South Africa) dissolved in 800ml of dH_2O and autoclaved at 120°C for 20min. A second solution was prepared by dissolving 1.0g of urea, 0.5g L-alanine, 1g PCNB

and 0.25g of chloramphenicol (Merck, Johannesburg, South Africa) in absolute ethanol. This solution was added to 200 ml sterile dH_2O and mixed by gentle swirling. Once the first solution containing the agar had cooled to approximately 50°C, the second solution was added to it and the agar poured into Petri dishes immediately.

Three 5 mm segments of vascular tissue were transferred to a plate and incubated for 7 days at 25^{o}C. For pure-culturing 5mm diameter discs were cut from the margins of fungal colonies by means of a sterile cork borer and transferred to half strength PDA (½ PDA). The ½ PDA was prepared by dissolving 19.5g PDA powder (Merck, Johannesburg, South Africa) in 1 litre of dH_2O. The ½ PDA solution was autoclaved for 20min at 120°C and left to cool on the laboratory bench. After cooling the ½ PDA was aseptically poured into sterile Petri dishes and left in a laminar flow cabinet to cool down. The inoculated plates were incubated for 7 days at room temperature and the morphology of the growing cultures noted.

5.2.2 Single spore culture preparation

For preparation of single spore cultures, the *Fusarium* isolates were aseptically plated onto Oatmeal agar (OMA) (appendix A.10) prepared according to Atlas (2004) to induce sporulation. The OMA plates were incubated for 7 days at room temperature. Spore formation on the OMA plates was verified by observing stained (0.5% lactophenol analine blue) fungal material using a light microscope at 40X magnification.

Single spore cultures were prepared from the sporulating *Fusarium* spp. colonies on OMA according to the procedure described by Choi *et al*. (1999). A 2cm X 2cm block of fungal growth from the OMA culture was transferred to a sterile Petri dish containing 5ml sterile dH_2O. After swirling of the petri dish containing the block of culture and water, 1ml of the resulting suspension was transferred to a sterile Petri dish and another 1ml of sterile dH_2O was added to dilute the spore suspension. One hundred microlitres of this suspension was aseptically transferred to water agar (0.6% bacteriological agar) plates and spread across the surface with a sterile inoculation loop. These plates were then incubated at room temperature for 10

hours. Germinated spores were viewed under the stereomicroscope. Single spores were picked up with a sterile hypodermic needle and transferred to the centre of a ½ PDA plate. These plates were sealed with Parafilm, labelled as MB-2 and Unknown isolates and incubated at 25^{o}C for 2 weeks.

Two of the cultures that showed morphological differences were sent for identification to the Mycology unit at the Agicultural Research Council (Plant Protection Research Institute, Biosystematic division, Private Bag X134, Queenswood, Pretoria, South Africa).

5.2.3 Pathogenicity trial

The *Fusarium* isolates isolated (Table 5.1) from the infected Towoomba ADC maize stalks and various other unidentified *Fusarium* spp. from the University of Pretoria's culture collection were grown on sterile millet seed for 2 weeks at room temperature. The millet seed bags were prepared by using autoclaveable polyurethane bags that contained 100ml millet seed and 200ml dH_2O and sealed using a sonic sealer. These bags were subsequently placed into another autoclave bag. The dual bag system was then sealed with an impulse sealer, pierced with a needle and autoclaved three successive times over a period of three days at 120°C for 20min.

The sterile millet seed containing bags were inoculated with pure single spore cultures of all the *Fusarium* isolates (Table 5.1) as follows. One corner of the bag was aseptically cut open with a sterile scalpel and 5 X 1cm blocks of the fungal cultures growing on ½ PDA blocks were deposited into the bags with millet seed. After inoculation, the millet bags were sealed using the sonic sealer. For each *Fusarium* isolate three millet seed bags were prepared. As a control only sterile ½ PDA blocks were transferred to the millet seed. To ensure even growth distribution the bags were mixed by manually shaking the bags twice per week.

5.2.3.1 Pathogenicity test, greenhouse trial

Fifty seven, five litre plastic pots were surface sterilized by submerging them in a 0.01% solution of hypochloride for 12 hours followed by rinsing with distilled water. A polyurethane mesh of 200mm X 200mm was placed in the bottom of each pot before

filling ¾ of the pot with steam pasteurized loamy topsoil. Thirty grams of millet seed inoculum of the pathogen was mixed by hand with 500ml of steam pasteurized loamy topsoil. The control pots contained soil with sterile millet seed in the top layer. Maize seed (cultivar ZM 523) was surface sterilized by submerging the seed in 70% ethanol for 3 minutes and then again for 1 minute in 3% sodium hypochloride. The seed was subsequently rinsed five times with sterile distilled water. After surface sterilization, the seed was primed by submerging in sterile dH_2O for three hours before planting. Five seeds were planted per pot in a heptagonal formation at a depth of 2.5cm and each treatment was done in triplicate.

The pots were watered daily and above ground symptoms noted at seven day intervals. Greenhouse temperatures ranged from 15°C to 35°C. Sixty days after planting four of the five plants per pot were removed by hand, washed in tap water; root rot symptoms assessed and the level of *Fusarium* infection determined by means of isolations. Root rot symptoms were assessed according to a root rot disease index (RDI) score from 0 - 5 as amended from Soonthornpoct *et al*. (2000) as follows: an index value of zero implied no root rot, 1= <20% of the roots are affected, 2= 21-40%, 3= 41-60%, 4=61-80% and 5=81-100% of all the roots are affected by root rot. The fifth plant per pot was left to grow for another 13 days before harvesting to evaluate root rot damage.

5.2.3.2 Determining Fusarium incidence in the roots of maize plants (isolation frequency)

The four plants used to determine disease incidence were harvested and classified (see section Pathogenicity test, greenhouse trial). The roots were used to determine the incidence of the inoculated *Fusarium* (isolation frequency). For this purpose four roots were removed per plant and combined into a pooled sample. From this sample, 12 roots per treatment were randomly selected and surface sterilized by placing the roots in a 3% sodium hypochloride solution for 15 seconds, followed by immersion in 70% ethanol for another 5 seconds. After surface sterilization the roots were washed three times with sterile dH_2O. Each root was aseptically cut into ten 2mm long segments with a sterile scalpel in a sterile petridish. These were plated out on RBGU-medium selective for *Fusarium*. The plates were incubated for 7 days at

25^{o}C before examining for fungal growth and morphology. *Fusarium* incidence (isolation frequency) was calculated as: number of segments showing *Fusarium* growth divided by the total number of segments evaluated times 100.

5.2.3.3 Comparing root rot damage and plant mass

To determine the effect of *Fusarium* on plant mass, the remaining plants in the pots were harvested 73 days after planting. The plants were removed from the pots by gently removing the soil by hand and washing the roots in tap water until no soil remained. The roots and shoots were separated by cutting the shoots at the first node just above the area where there was root formation. Shoot lengths and wet and dry root and shoot masses were determined by weighing and root rot assessed as described in section "Determining *Fusarium* incidence in the roots of maize plants".

5.2.3.4 Incidence of Fusarium in naturally infested soil

Due to the variable results in the pathogenicity trial with the individual *Fusarium* isolates, Hutton ecotope soil that was naturally infested with *Fusarium* (previously planted with maize) was obtained from the Limpopo Department of Agriculture Research Station, Towoomba ADC (Latitude:24°55'22.44"S, Longitude:20°20'14.91"E at an altitude of 1164m above sea level). This soil was transferred to the greenhouse at the University of Pretoria's experimental farm, the *Fusarium* density in the soil determined as described below and the soil subsequently used for the biocontrol trial.

Fusarium spp. isolations were made from a maize stalk that was planted in this Towoomba ADC soil according to the same procedure described in section 5.2 1. The isolates were sent to the Mycology unit at the Agicultural Research Council (Plant Protection Research Institute, Biosystematic division, Private Bag X134, Queenswood, Pretoria, South Africa) for identification.

Some of the soil was pasteurized by steaming at 80^{0}C for 3 hours and subsequently left to cool down to ambient temperature. From the pasteurized and unpasteurized soil a 100ml representative sample was taken and the effectiveness of soil pasteurization and incidence of *Fusarium* determined by means of spread plating of

a dilution series. The soil was sent for analysis to the soil laboratory at the University of Pretoria (Appendix A, Table A, 3531)

Distilled water and test tubes were autoclaved at 121^0C for 20min. One gram of pasteurized and unpasteurized soil was weighed out and added to a sterile test tube together with 10ml of sterile dH_2O and the soil suspension vortexed thoroughly. Subsequently 100_{uL} volumes of the soil suspension were serially diluted by successive transfers to a series of test tubes until a dilution of 10^{-9} was reached. Three aliquots of 100_{uL} from each test tube were plated out on agar plates containing RBGU medium. The plates were sealed with Parafilm and incubated for 7 days at 25^oC before examining for fungal growth and recording the incidence of *Fusarium* in the samples. Controls were prepared by plating from the un-inoculated sterile test tubes.

5.2.4 Biocontrol trial in the greenhouse

The naturally infested field soil (5.2.3) was used in this trial. The serial dilutions indicated that an average of 33cfu/ml of *Fusarium* occurred per gram soil. The rhizobacterial isolates were grown according to the same procedures as described under 5.2.3 with the exception that the broth volume was 200ml instead of 150ml. After the PGPR trial (section 3.2.3) the bacterial isolates that showed the most promise were selected to include in the biocontrol trial. The isolates selected were A-08A-26A-32, A-40 and S2-08. In addition new isolates which showed promise in other studies on cereals (Pretorius, 2012) were included namely S1-08, S4-08 and S6-08. Bacup® was included as a commercial standard.

Eighty 5L plastic pots were surface sterilized by submerging in a 0.01% hypochloride solution for 12 hours before rinsing three times with dH_2O. A 200mm X 200mm plastic mesh was placed in the bottom of the pot and the pots were filled with 3.85 kg of the naturally infested soil from Towoomba ADC. Maize seed (cultivar ZM523) obtained from the Limpopo Department of Agriculture, Research section, Towoomba ADC in 2009 was surface sterilized in 70% ethanol for 3 min. and then for in 3% sodium hypochloride solution for 1 min. The seeds were then rinsed five successive times with sterile dH_2O. After surface sterilization the seed were primed by

submerging in sterile distilled water for 3 hours. Five primed seeds were planted in a heptagon formation at a depth of 2.5cm in each pot. Four pots per treatment were arranged in a completely randomized design (CRD) on a greenhouse bench.

The 200ml bacterial inoculum suspensions prepared as described under 3.2.2 were used to inoculate the seed. Fifty millilitres of inoculum was evenly added over the soil per pots using a 5ml pipette. The treatment comprised of the selected single bacterial strain and the control was only inoculated with ½ strength sterile Ringers solution. Each of the pots was labelled and watered daily with tap water. Germination date and shoot lengths were recorded weekly by measuring the plants from the base of the stem to the apical leaf. The plants were grown for 74 days. At harvesting the roots were excised from the stems and fresh root and shoot mass determined by weighing. Dry root and shoot weight was also determined after drying of the samples in a drying oven at 70°C until the sample weight remained constant.

5.4 Statistical analysis

All data was captured and analysed by importing the data from an excel spread sheet to the statistical package SAS 9.2. The data was analysed with proc GLM at a p=0.05 level and the means compared to the control using the Dunnetts test at p=0.05. Root rot severity and maize growth parameters were compared by means of regression analysis using SAS 9.2 at p=0.05.

5.5 Results and discussion

5.5.1 Pathogenicity test

The incidence and effect of various *Fusarium* spp. on maize under greenhouse conditions is shown in table 5.1. Only the *Fusarium* isolates GFus1B, Unknown 10, Unknown 5 and Unknown 9 caused a reduction in growth of maize plants in comparison with the uninoculated control plants. The plants growing in *Fusarium* infested soil showed 95% or more infection by *Fusarium*. A similarly high infection rate was observed by Harvey *et al.* (2008) who recorded a 100% infection rate of *Fusarium graminearum* in maize under field conditions after a 12 week growth period. Harvey *et al.* (2008) not only observed the high infection rate by *Fusarium*

graminearum but also reported that maize grain yield was not significantly affected. In line with the findings by Harvey *et al.* (2008) the results of the current study indicate that although some of the isolates were non-pathogenic, it did not limit *Fusarium* spp.'s ability to infect maize. Symptomless infections by *Fusaria* is a common phenomenon on many crops (Munkvold & Desjardins., 1997). It is also a well-known fact that disease expression in *Fusarium* infected plants is strongly linked to stress factors on the plant (El Meleigi *et al*., 1983; Schneider & Pendery, 1983; Velluti *et al*., 2000). Therefore it can be concluded that in the current trial, disease development was not favoured due to the ideal growth conditions in which the plants were growing.

Hugo (1995) found contradicting results when trying to correlate root rot symptoms caused by fungal pathogens with maize yield decreases. The variability in pathogenicity of *Fusarium* spp. are evident in a study conducted by Asran & Buchenauer (2002) who reported varying degrees of root rot symptoms when the root systems of 10 day old maize seedlings were inoculated with *Fusarium graminearum*. From the results of the current study (Table 5.1) it is evident that after a 60 day growth period, all the plants were infected to a high degree with the various *Fusarium* species even though most of the treatments showed no symptoms (above or below ground).

In this study, disease severity as measured according to the relative disease index (RDI scale) ranged from zero to severe when plants were grown in soil infested with the *Fusarium* isolates "unknown 63" and MB2 (table 5.1). However, no disease was observed on plants inoculated with the G-Fus isolates from the University of Pretoria's culture collection. This lack of pathogenicity in the G-Fus isolates may be due to loss of pathogenicity after long term storage and successive plating on artificial media. This observation emphasizes the necessity of using freshly isolated pathogen isolates for biocontrol trials. The quantification of root rot is usually associated with root discoloration and the inhibition of root development.

5.5.2 Comparing root rot severity and growth parameters of maize growing in Fusarium infested soil in the greenhouse.

The *Fusarium* isolates "Unknown 9" and "Unknown 10" had the greatest effect on all of the growth parameters of maize plants resulting in a total wet biomass reduction of

26.30% and 23.25% and a dry biomass reduction in dry mass of 30.82% and 17.17%, respectively, in comparison with the uninfected control (table 5.1). Most of the isolates were identified as *F. graminearum* (Table 5.1). Even though single spore cultures of the *Fusarium* isolates were used in the study, a range of root rot severities from zero (asymptomatic) to severe were recorded between replicates of the same *Fusarium* strain. The high variability recorded between replicates inoculated with the same *Fusarium* isolate in the current study concur with the findings of Hugo (1995) and Cumagun *et al.* (2004).

Fusarium subglutinans (Table 5.1) gave similar, variable results with regards to pathogenicity as *F. graminearum.* In contrast *F. subglutinans* resulted in growth enhancement with an average increase in wet and dry biomass of 23.28% and 40.48% compared to the control. A study conducted by Pastircak (2004) also found a stimulatory effect when maize seed germination activity increased after a 16 hour soaking period in a *F. moniliforme* suspension. Yates *et al.* (2005) conducted maize field trials over a three year period and their results also showed a stimulatory effect of *Fusarium* i.t.o. significant yield and vegetative growth increases when the maize seed was inoculated with *Fusarium verticillioides* before planting. It was found by Nicholson *et al.* (1998) that *Fusarium graminearum* produced the growth hormone gibberrelic acid that is associated with the Bakanae disease in rice.

The non-significance of the shoot length results in Table 5.1 and Fig. 3 clearly indicated the small effect all the *Fusarium* isolates had on shoot development. The data indicated that even though there was root damage ranging from moderate to severe, no significant above ground symptoms were observed. This observation corresponds with the findings of Richardson (1942) and Gaur (1990). Richardson (1942) described the above ground symptoms of root rot on maize as very deceptive since they may only appear when the root systems are rotten to the extent that only a few secondary roots are still functional.

The relationship between the RDI for all isolates (Figs. 4 and 5) and maize biomass can be described as a function rate of $y= -0.7379x^2 + 0.0864x + 47.369$ and $-0.1982x^2 + 0.4122x + 7.1913$ for total wet and dry mass, respectively. Although the regression analysis data for the total wet and dry mass was significant but with non-

significant plant biomass observations, there still is a trend observed between the RDI and plant biomass. This indicates that an RDI could be developed to estimate maize yield losses and future research in this area might be worthwhile.

In section 5.2.3 (*Determining Fusarium incidence*)", a low level of *Fusarium* (5cfu/g of soil) survived the steam treatment. In the unpasteurized soil, a count of 33cfu/ml was recorded at the lowest dilution rate. Pasteurization of the soil therefore reduced the inoculum density of *Fusarium* by 86.8%. If sterile soil is needed then a second cycle of steaming could be implemented and the duration of steaming extended but this may have an unknown negative effect such as the release of toxic substances in the soil.

5.5.3 Biocontrol trial in the greenhouse

The most effective rhizobacterial strains identified in the biocontrol trial were S2-08 and the commercial product Bacup® resulting in 19.38% and 22.22% increase in wet mass and 25.24% and 30.11% increase in dry mass respectively in comparison with the infected control. The rest of the inoculants either inhibited growth e.g. S6-08 resulting in a decrease of wet and dry mass of 7.29% and 13.36% compared to the control or had very little positive effect across all parameters evaluated. Although the data is non-significant when compared to the control a trend towards biocontrol activity can be seen.

5.6 Conclusion

The results of the current study support the statement by Kloepper *et al*. (1993) that plant growth stimulation and biocontrol should be viewed as "two sides of the same coin". In the current study the bacterial inoculants showed growth stimulating capabilities (chapter 4) and biocontrol activity. Even though the greenhouse trial data was not statistically significant, there was an effect where the pathogen was inhibited to such an extent that a 30.11% increase in dry biomass of plants treated with the commercial product Bacup® was recorded over that of the control. The obvious difficulty of obtaining clear pathogenic effects with *Fusarium* inoculation makes it equally difficult to obtain statistically significant biocontrol results.

Table 5.1 Effect of *Fusarium* on maize growth and incidence of *Fusarium* in the roots of maize seedlings grown in soil inoculated with individual *Fusarium* isolates (Pathogenicity trial)

			Seedling mass (g)									% Change in seedling mass compared to the control						
			Fresh mass			Dry mass						Fresh mass				Dry mass		
Isolate code	Identification ****	Shoot length (cm)***	Shoot	Root	Total	Shoot	Root	Total	Root rot Rating	Isolation frequency *****	Shoot length	Shoot	Root	Total	Shoot	Root	Total
unkn 3*	F. graminearum	456.67[a]	28.03[a]	12.10[a]	40.13[a]	4.07[a]	1.72[a]	5.79[a]	0.33	100%	-14.91	-5.29	17.03	0.48	-7.37	22.51	-0.11
unkn 5*	F. graminearum	461.67[a]	24.02[a]	10.92[a]	34.94[a]	3.81[a]	1.48[a]	5.29[a]	2.33	100%	-13.98	-18.84	5.68	-12.50	-13.21	5.21	-8.74
unkn 8*	F. graminearum	443.33[a]	36.68[a]	12.52[a]	49.19[a]	5.77[a]	1.95[a]	7.72[a]	0.00	95.83%	-17.39	23.92	21.09	23.19	31.51	38.63	33.24
unkn 9*	F. graminearum	443.33[a]	24.92[a]	4.51[a]	29.43[a]	3.31[a]	0.70[a]	4.01[a]	5.00	96.00%	-17.39	-15.79	-56.37	-26.29	-24.53	-50.47	-30.82
unkn 10*	F. graminearum	441.67[a]	21.32[a]	9.33[a]	30.65[a]	3.39[a]	1.38[a]	4.77[a]	4.67	100%	-17.70	-27.95	-9.77	-23.25	-22.70	-2.13	-17.71
MB2-1A*	F. graminearum	550.00[a]	41.39[a]	14.74[a]	56.13[a]	6.38[a]	2.11[a]	8.49[a]	1.00	100%	2.48	39.85	42.57	40.55	45.33	50.00	46.46
MB2-1*	F. subglutinans	488.33[a]	30.81[a]	11.78[a]	42.59[a]	5.07[a]	1.89[a]	6.95[a]	3.67	100%	-9.01	4.09	14.00	6.65	15.41	34.12	19.95
MB2-4*	F. graminearum	460.00[a]	27.99[a]	9.92[a]	37.91[a]	4.43[a]	1.51[a]	5.93[a]	3.67	100%	-14.29	-5.43	-4.00	-5.06	0.84	7.11	2.36
63-4	F. graminearum	470.00[a]	31.18[a]	9.66[a]	40.84[a]	5.25[a]	1.78[a]	7.03[a]	2.67	100%	-12.42	5.35	-6.51	2.28	19.59	26.78	21.33
63-6	F. graminearum	443.33[a]	24.58[a]	11.46[a]	36.04[a]	4.28[a]	1.66[a]	5.95[a]	4.00	100%	-17.39	-16.94	10.84	-9.75	-2.43	18.25	2.59
63-9	F. subglutinans	486.67[a]	39.50[a]	16.37[a]	55.87[a]	6.48[a]	2.85[b]	9.33[b]	0.33	100%	-9.32	33.46	58.40	39.92	47.68	102.61	61.01
G-FUS	Unknown	510.00[a]	38.85[a]	14.79[a]	53.64[a]	6.34[a]	1.77[a]	8.11[a]	0.00	100%	-4.97	31.28	43.08	34.33	44.50	25.59	39.91
G-FUS?	Unknown	466.67[a]	30.62[a]	9.63[a]	40.25[a]	4.99[a]	1.86[a]	6.85[a]	0.00	100%	-13.04	3.46	-6.87	0.78	13.59	32.23	18.11
G-FUS1B	Unknown	396.67[a]	22.10[a]	11.54[a]	33.64[a]	2.94[a]	1.33[a]	4.27[a]	0.00	100%	-26.09	-25.33	11.64	-15.76	-32.95	-5.69	-26.34
G-FUS-2	Unknown	420.00[a]	39.03[a]	16.15[a]	55.18[a]	5.98[a]	2.33[a]	8.31[a]	0.00	98.18%	-21.74	31.87	56.24	38.18	36.22	65.88	43.42
G-FUS-4	Unknown	528.33[a]	41.72[a]	13.24[a]	54.96[a]	6.59[a]	1.96[a]	8.55[a]	0.00	95%	-1.55	40.97	28.06	37.63	50.11	39.10	47.44
Control		536.67[a]	29.60[a]	10.34[a]	39.93[a]	4.39[a]	1.41[a]	5.80[a]	0.00	0%	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* all unknown & MB2 isolates originated from Towoomba ADC maize stalks, isolates G-Fus & 63 from the University of Pretorias' culture collection.
** Unkn abbreviation for unknown isolates
*** Shoot length (cm) determined by measuring from the first node from the soil layer to the apical leaf tip.
**** Pathogen isolates MB2-1 & 63-4 identified as *Fusarium subglutinans* (Wollen & Reinking) P.E Nelson, Toussoun & Marassas (PRI10207) and (*Fusarium graminearum* Schwabe (PRI 10208) by Mycology unit, Plant Protection Research Institute, Biosystematics Division, Agricultural Research Council, Queenswood, Pretoria, South Africa.
***** Isolation frequency [(100)/ 120) *(number of segments positive for *Fusarium* growth)].
*****Treatment means followed by the same letter within the same column do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test.

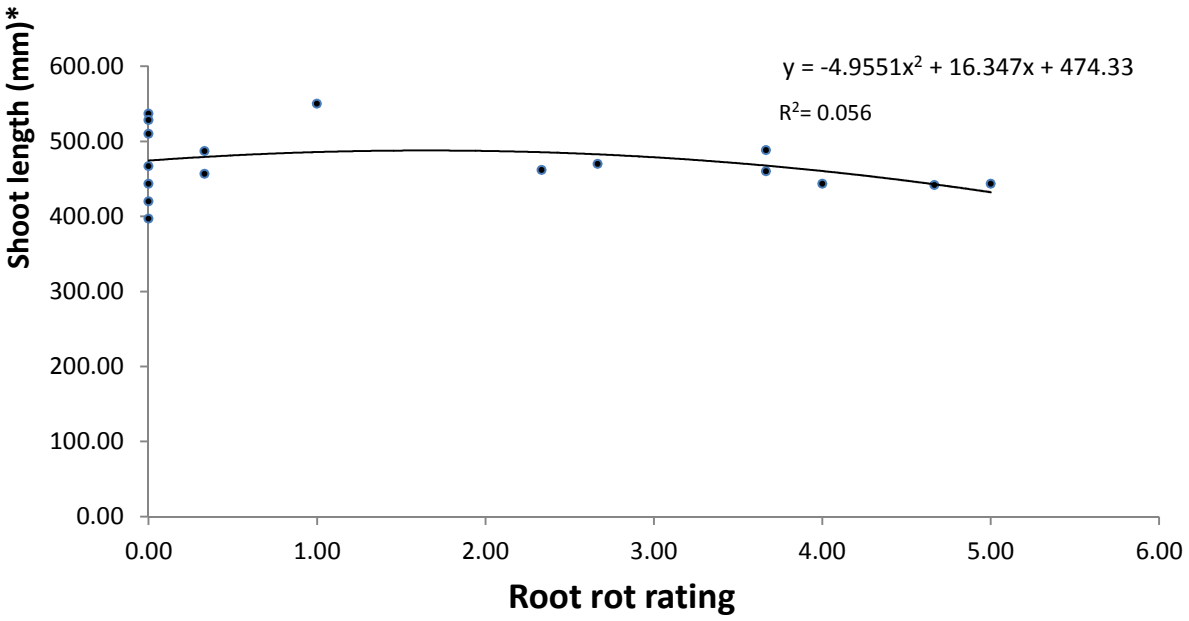

Figure 3 Relationship between root rot severity and shoot length of Z*ea maize* plants grown for 74 days in soil artificially infested with *Fusarium* (all *Fusarium* isolates listed in table 5.1) in the greenhouse. Graph shows the relationship between maize shoot length and root rot severity. Regression analysis performed using SAS 9.2 at p=0.05. *Shoot length measured from first node to apical leaf.

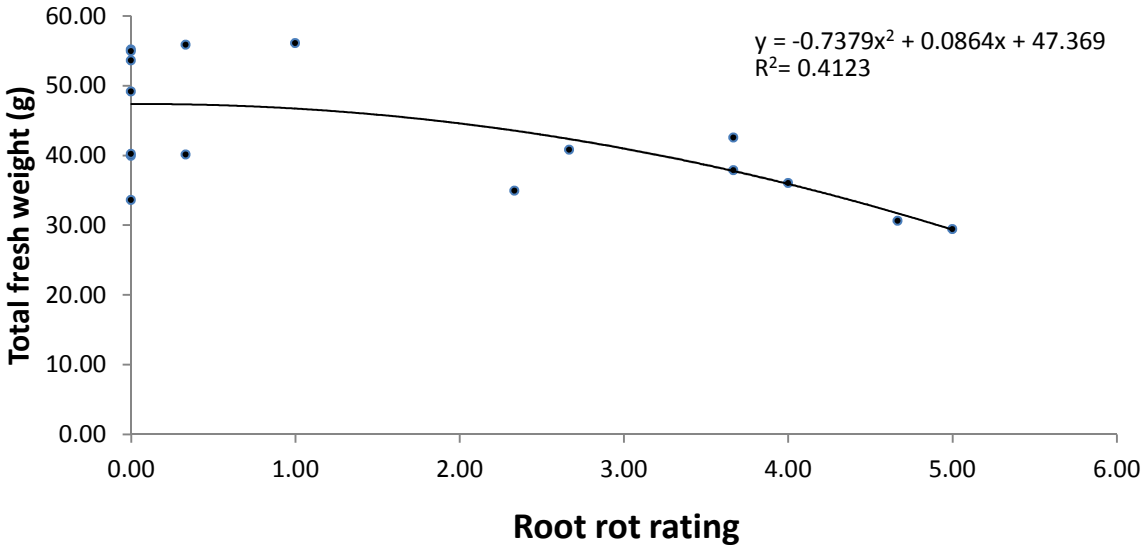

Figure 4: Relationship between root rot severity and fresh mass of Z*ea maize* after 74 days growth in soil artificially infested with *Fusarium* (all *Fusarium* isolates listed in table 5.1) in the greenhouse. The graph indicates the relationship between fresh weight and root rot severity. Regression analysis performed using SAS 9.2 at p=0.05.

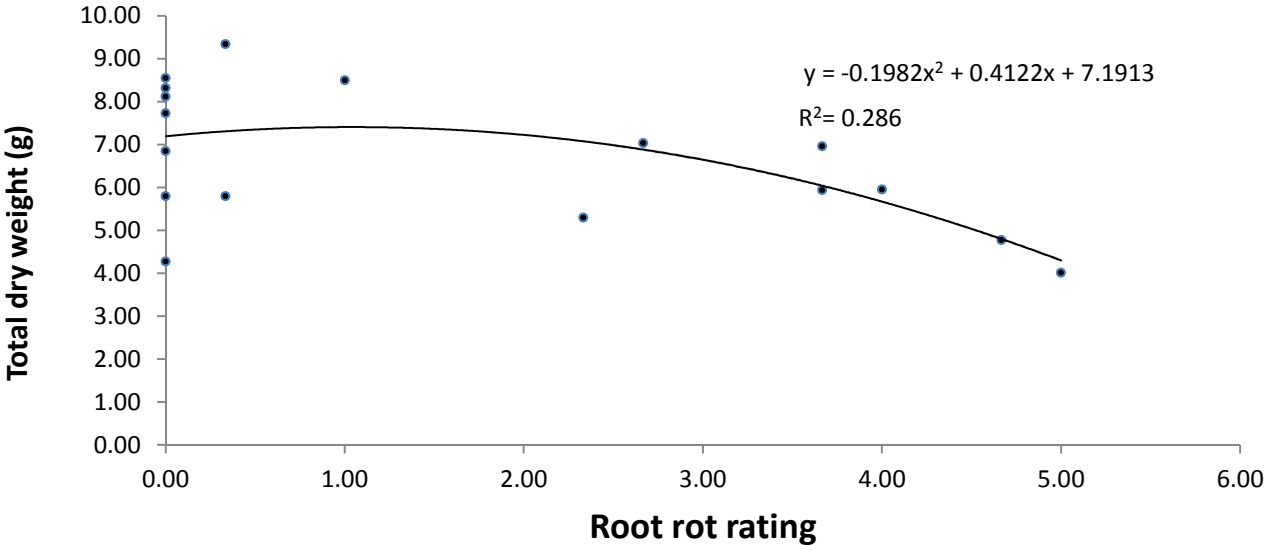

Figure 5: **Re**lationship between root rot severity and dry weight of Z*ea maize* plants grown for 74 days in soil artificially infested with *Fusarium* (all *Fusarium* isolates listed in table 5.1) in the greenhouse. Graph shows the relationship between and root rot severity. Regression analysis performed using SAS 9.2 at p=0.05.

Table 5.2. Effect of rhizobacteria on growth of maize in soil naturally infested with *Fusarium* in the greenhouse (biological control trial)

	Seedling Mass (g)			% change in seedling mass and length compared to the control		
Inoculant*	**Total fresh weight**	**Total dry weight**	**Shoot length****	**Total fresh weight**	**Total dry weight**	**Shoot length****
A-32	29.36 [a]	6.56 [a]	18.46 [a]	4.97	5.64	6.64
A-40	29.17 [a]	6.44 [a]	17.81 [a]	4.29	3.70	2.89
S1-08	29.21 [a]	6.82 [a]	18.04 [a]	4.43	9.82	4.22
S2-08	33.39 [a]	7.59 [a]	20.15 [a]	19.38	22.22	16.41
S4-08	26.89 [a]	6.16 [a]	17.21 [a]	-3.86	-0.81	-0.58
S6-08	25.93 [a]	5.38 [a]	16.36 [a]	-7.29	-13.37	-5.49
Bacup®	35.03 [a]	8.08 [a]	17.68 [a]	25.24	30.11	2.14
Control	27.97 [a]	6.21 [a]	17.31 [a]	0.00	0.00	0.00

* Isolates from the University of Pretoria culture collection.
** Plant length determined by measuring (cm) from the first node from the soil layer to the apical leaf tip.
***Treatment means followed by the same letter within the same column do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test.

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Chapter 6

Efficacy of selected Rhizobacterial isolates as biofertilizers on maize in the field.

Abstract

Maize is the most important grain crop in South Africa and is a staple food for the majority of people in Africa. The beneficial effects of PGPR inoculation have been well documented, but obtaining reproducible results is often a problem under field conditions where many factors can affect their performance. In this study, field trials were conducted over a three year period at the Limpopo Department of Agriculture Research Station Towoomba in Huttons, Arcadia and Shortlands soil ecotopes. The objectives of the field trials were to evaluate the effect of PGPR inoculation on maize yield as set by commercial practices in the Springbok flats of the Limpopo Province. All the field trials were planted at a planting population of 22 000 plants per hectare at recommended fertilizer levels before harvesting at a grain moisture level of 12%. During the 2010/2011 growing season seed treatments with the treatment mixture S1 and S2-08 resulted in an increase in maize yield of 88.48% and 8.4%, respectively, compared to the untreated control in both the Huttons and Shortlands soil ecotope. After the final results were obtained from the field trials, isolates the S1-08, S2-08 and S3-08 were identified as *Bacillus cereus* strains. All three strains tested positive for the production of human diahoreal toxin. Only one of the S-inoculants, S7-08 did not produce this toxin and was thus also included with the isolates T-19, A-40, T-26, A-29 and the commercial product Brus® to the field study in order to compensate for the removal of the *Bacillus cereus* isolates. Consistent rainfall during the 2011/2012 season is probably the reason for the improved yields obtained for the second field trial i.e. average between all treatments at 1.60 tha^{-1} and 6.59 tha^{-1} for the Huttons and the Shortlands trial respectively. Under dry land conditions the results indicated that the less fertile/ lower the clay content, the greater the plant growth enhancement by the rhizobacteria when all three soil types are compared. Interestingly all the treatments in the Arcadian soils resulted in a reduction in yield compared to the control. It is possible that the high clay content of the Arcadian soil may have a negative effect on the PGPR activity. During the 2012/2013 growing season, field trials were not as successful as the previous year

as poor rainfall led to the failure of the crop planted in Hutton and Arcadian soils where the trials did not receive supplementary irrigation while those in the Shortlands did. In these 2012/2013 trials rhizobacterial strain S-7 was the best performing isolate resulting in a total grain yield of 5.11 tha^{-1} closely followed by T-19 with 4.76 tha^{-1} compared to the control yield of 3.82 tha^{-1} for the Shortlands soil ecotope trial. Data over the three seasons gave a clear indication of the potential of PGPR in improving maize yield in the agricultural sector, but as previously stated variability in field performance is a major problem.

6.1 Introduction

There is ample data to support the use of biological applications in the agricultural sector to increase yield and plant health. Chen *et al*. (1994) gives an extensive overview of the YIB project conducted in China that serves to indicate the importance of PGPR application in agriculture. This project covers 28 provinces in China with research ranging from basic studies to product formulation with three bacterial species namely *B. cereus, B. firmus* and *B. lichniformis.* Good results have been obtained from this large, 3.3 million hectare project with grain yield increases as high as 16% and 11% for wheat and maize respectively. This inventiveness clearly shows how much effort and money is being spent in this PGPR field. Similarly various other researchers have reported beneficial effects of PGPR inoculation on various other crops (Zahir *et al*., 2004). In a study conducted by Zahir *et al*. (1998) maize seed was inoculated with four isolates each of *Azotobacter* spp. and *Pseudomonas* spp. under field conditions at fertiliser levels of 150-100-100 kg/ha NPK. Collective inoculation of the isolates resulted in a considerable increase in maize grain yield of 19.8%, and cob weight, cob length, 1000 grain weight, plant height, nitrogen content in the straw and grain by 21.3%, 20.6%, 9.6%, 8.5% 18% and 19.8%, respectively, compared to the non-inoculated control plants. Vedderweiss *et al*. (1999) found that maize inoculated with *Azospirillum* spp. at a concentration of 10^6 cfu/ml enhanced fresh root and shoot weight of seedlings and Stancheva *et al*. (1992) reported that inoculation of maize with *Azospirillum brasilense* strain 1774 in a blend with 100kg N/ha fertilizer gave the same result as an application of only 200kg N/ha. This model of increased nitrogen utilization by the

plant is supported by Dobbelaere *et al*. (2001). A list of responses of different crops to PGPR is illustrated in table III in Zahir *et al*. (2004).

The positive effects of PGPR inoculation have been well documented, but inconsistent results with respect to plant growth promotion are often experienced in field trials (Zahir *et al*., 2004). Furthermore, the establishment and proliferation of PGPR while outcompeting native flora complicates their use (Smith *et al.,* 1992). The potential of PGPR application in agriculture to promote plant health and the concomitant inconsistency under field conditions has been extensively documented. For these reasons the objective of the field trials were to evaluate the selected PGPRs' under normal field conditions for the promotion of plant health and ultimately yield increase of maize over three seasons.

6.2 Biofertilizer field trial - 2010/2011 growing season.

6.2.1 Material and methods

The field trials were conducted at the Towoomba Research Station Limpopo Department of Agriculture, on the southern part of the Springbok flats, approximately 4 km south east of Bela Bela in the Limpopo Province (28°21'E, 24°25'S; 1 184 m above sea level). Towoomba Research Station is situated in the summer rainfall area with a long-term average annual rainfall (60 year average) of 627 mm per annum (Towoomba weather station data). The rainfall distribution during the season is highest during the period November to February and lowest during May to August. The annual rainfall distribution is erratic, and rain often occurs in short bursts of high intensity, associated with thunderstorms and lightning. Hail occurs sporadically while seasonal droughts often occur during mid-January to mid-February. According to the 50-year average, the long-term daily average maximum and minimum temperatures at Towoomba vary between 29.7°C and 16.5°C for December and 20.8°C and 3.0°C for July, respectively (Towoomba weather station data). Light frost occurs sporadically during June and July. Air temperatures above 30°C and below freezing point can be expected for 87 and 8 days of the year, respectively.

The trial was replicated in two different soil ecotopes in the 2010/2011 growing season. The first trial was planted exclusively under dry land conditions in a Huttons ecotope on the Towoomba ADC Research Station. The other trial was planted under supplemented irrigation in the form of a fixed overhead floppy irrigation system in Shortlands soil ecotope approximately 3km from the Huttons trial sites. Soil samples were taken according to the procedures set out in annexure A.2 and sent for nutrient analysis.

The fields were prepared by firstly spraying the trial areas with herbicide at a rate of four liters of Glycine Glyphosate/ha at a concentration of 450g/ai/L. The area was left to rest for two to three weeks for the chemicals to take effect. The lands were then ripped, ploughed and disked to loosen up and even out the soil. Planting commenced as soon as the soil moisture reached 60% at a depth of one meter and when rain was predicted in regular weekly cycles.

Both trials consisted of five treatments and a control with each treatment replicated 6 times. Untreated maize seed (Pioneer cultivar P1615R) was first treated with Thiram® to prevent fungal diseases before seed treatment with the rhizobacterial strains commenced. The treatments were with inoculants S1-08, S2-08, and a S1-08 and S2-08 mixture, the commercial products PTo4 (Hoxies, Gauteng, South Africa) and Brus® (Stimuplant, Gauteng, South Africa). The product were used according to factory specifications at one liter of inoculant per four kilograms of seed for the treatment PTo4 and 200g perlite powder per 50kg of maize seed for Brus®. For each treatment, 5kg of seed was prepared by adding 20g of perlite powder inoculated with the isolates (prepared by Stimuplant®) and thoroughly mixed with Stimulum® (sticker prepared by adding 100ml of dH_2O). For the PTo4 treatment 5kg of seed was prepared by adding 1.25L of the product to the seed before planting. For the control only Thiram® was added to the seed. Planting was done with a Monosem planter at a density of 22 222 plants per ha. Each treatment replicate consisted of a 200m long row with an intra row spacing of 50cm and an inter row spacing of 90 cm.

The treatments were replicated six times in a completely randomized design with one 200m row constituting one replicate. Two extra hectares for each trial area were also planted and this served as a buffer zone. Six replications per treatment were harvested.

For the trial in the Shortlands soil ecotope fertilizer was added to achieve at recommended levels (ARC, 2008) according to the soil analysis in Table A (calculations done according to appendix A.3) viz. 285.71kg superphosphate at a concentration of 10.5% per hectare which is equivalent to 45.00kg phosphate per hectare. Nitrogen was applied by adding 325.64kg of limestone ammonium nitrate (LAN 28) at a concentration of 280g/kg which is equivalent to 91.18kg N per hectare.

For the trial in the Huttons ecotope fertilizer was added to achieve the recommended levels (ARC, 2008) according to the soil analysis in Table A viz. 480.00kg/ha superphosphate (10.5%) per hectare which is equivalent to 50.4kg phosphate per hectare and nitrogen at 316.96kg/ha (280g/kg LAN) which is equivalent to 88.75kg/ha.

Weeds were controlled by manual and mechanical weeding up to a 4 leaf stage. After this glyphosate (280g/ai/L) was applied at a rate of four liters per hectare when significant weed establishment had occurred in the field. Bollworm infestations were treated with Deltamethrin at manufacturers recommended rate using a boom sprayer at a four and ten leaf stage of plant development.

6.3 Data collection

All trials were harvested by hand. For the Huttons ecotope the following was recorded: germination date, growth rate, wet and dry mass and grain yield at 12% moisture.

Shortlands soil ecotope: Only grain yield was taken at 12% moisture. The reason for only recording yield was the farmer did not allow plants to be harvested during the growing season because this would reduce his yield per hectare.

6.4 Statistical analysis

All data was captured and analysed by importing the data from an excel spread sheet to the statistical package SAS 9.2. The data was analysed with proc GLM at a p=0.05 level and the means separated using the Dunnetts test and compared to the control if significance was observed at $p<0.05$.

6.5 Results and discussion

In the field trial conducted in the Huttons ecotope, significant differences were observed with respect to initial dry mass at 29 days (Table 6.1). Compared to the untreated control, the treatments with S1 and S2-08 mixture resulted in a 168.19% increase in dry mass followed closely by the PTo4 treatment with a 168.07% increase. The significant observation extended to the 42 day interval but convergence of the control dry matter and the other treatments at the 62 day interval, resulted in a non-significant observation with only a 28.11% increase over the control by the best performing isolate mixture S1 and S-08. The same non-significant observation is supported by Myresiotis *et al*. (2014) which showed that maize root biomass increased but stalk biomass on the other hand did not increase when inoculated with *Bacillus subtilis*.

In terms of plant length no significant differences were observed between the control and the treatments. A similar observation was made by Schoebitz *et al.* (2013) who reported that an increased phosphate uptake of up to 50% over a 60 day growth period did not significantly affect the dry mass of wheat plants. This could be contributed to the genetic potential of the maize that limits plant length under ideal conditions. However, another observation was made by Shoebitz *et al.* (2013) that plant mass correlated with yield in some respects. In the Huttons ecotope trial, dry mass and grain yield of the best performing isolates (S1 & S2-08, PTo4 and Brus®) were observed. The isolates showed a final dry mass increase of 28.11%, 13.65% and 13.63% which when compared to the grain yield increase of 88.48%, 57.27% and 49.11%, respectively, supported the positive correlation observation made by Shoebitz *et al.* (2013).

A similar trend was observed in the trial conducted in the Shortlands soil. Although the data was non-significant there was still a clear trend of yield increase of 8.14% and 5.87% for the S1 and S2-08 mixture and Brus® respectively. The other treatments had no or a slightly negative effect on yield (6.12% reduction for treatment with S1) compared to the control.

Differences in the grain yield between experiments could be attributed to the different soil types as the Huttons ecotope soil is classified as highly fertile and the Shortlands ecotope as shallow and marginal (Soil Classification Working Group, 1991). Another factor to take into consideration is that the Huttons ecotope was planted exclusively under dry land conditions whereas the Shortlands trial received supplementary irrigation. This supplementary irrigation could also explain the limited grain yield variability between the control and the bacterial treatments as the maize plants had limited moisture stress thereby alleviating any limiting factor which could affect grain yield. Unfortunately, after the final results were obtained from the field trials rhizobacterial isolates S1-08, S2-08 and S3-08 were identified as *Bacillus cereus* species and S4-08 as *Stenotrophomonas maltophili* (Table B) that are reported as possible human pathogens.

The *B. cereus* isolates were sent to the National Health Laboratory Services (NHLS) at the University of the Witwatersrand (Infection Control Services, Department of Clinical Microbiology and Infectious Diseases, Hougton, Johannesburg, South Africa) for tests to determine whether they produce toxins harmful to humans and animals. Isolates S1-08, S2-08, S3-08, S6-08 tested positive for a human diahoreal toxin with the latex agglutination test and were therefore not included in the follow-up field trials. However, isolate S7-08 did not produce the toxin and was thus included in the 2011/2012 field evaluation trials. The isolates which tested positive for the human diahoreal toxin could not be considered for commercialization. The increases in plant mass and yield that resulted from the rhizobacterial treatments can be ascribed to direct plant growth promoting activity but possibly also to indirect growth enhancement due to biocontrol activity. The latter mechanism is a possibility in light of the high levels of *Fusarium* which were detected in the soil (Chapter 5, section 5.2.7).

Table 6.1 Effect of rhizobacteria on growth and yield of maize under field conditions during the 2010/2011 season

	Huttons ecotope									% change in maize growth parameters compared to the Control								
	Dry weight (g)			Plant length (cm)****				Grain yield (kg/ha)**		Dry weight (g)			Plant length (cm)****				Grain yield (kg/ha)**	
	Days ***			Days***						Days **			Days**					
Treatments*	29	42	61	29	42	61	91	Huttons	Short lands**	29	42	61	29	42	61	91	Huttons	Short lands **
S1-08	8.31[a]	76.33 [b]	181.33 [a]	71.50 [a]	135.67 [a]	167.33 [a]	218.00 [a]	1446.90[a]	2750.20[a]	-0.24	113.99	9.23	1.17	19.71	-5.10	-1.06	14.25	-6.11
S2-08	8.34[a]	85.33[b]	181.33 [a]	74.33 [a]	143.00 [a]	174.00 [a]	220.33 [a]	1378.48[a]	2852.28[a]	0.12	139.22	9.23	5.18	26.18	-1.32	0.00	8.84	-2.63
S1 & S2-08	22.34[b]	61.67 [b]	212.67 [a]	80.83 [a]	134.33 [a]	188.33 [a]	216.33 [a]	2387.13[b]	3173.01[a]	168.19	72.89	28.11	14.38	18.53	6.81	-1.82	88.49	8.32
Brus®	19.00[b]	54.33 [b]	188.63 [a]	80.67 [a]	125.33 [a]	178.33 [a]	221.33 [a]	1888.45[a]	3101.51[a]	128.09	52.31	13.63	14.15	10.59	1.13	0.45	49.11	5.88
PTo4	22.33[b]	68.00 [b]	188.67 [a]	86.00 [a]	135.33 [a]	174.00 [a]	220.00 [a]	1991.80[a]	2755.71[a]	168.07	90.64	13.66	21.69	19.41	-1.32	-0.15	57.27	-5.93
Control	8.33[a]	35.67 [a]	166.00 [a]	70.67 [a]	113.33 [a]	176.33 [a]	220.33 [a]	1266.47[a]	2929.32[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* S1-08 and S2-08 are isolates from the University of Pretoria's PGPR culture collection. Brus® is a commercial product of Stimuplant (Gauteng, South Africa), PTo4 (experimental commercial product from Brazil with unknown manufacturer and content).

** Yield calculated by converting the grain moisture content of each treatment and recalculating the yield to 12 % moisture content. Yield for Shortlands soil ecotope included and all other growth parameters excluded due to farmers restriction on pre-mature plant harvesting.

*** Number of days since planting.

**** Plant length determined by measuring in centimetres from the first node from the soil layer to the apical leaf tip.

***** Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedures and separated with the Dunnetts test if significances was observed.

6.6 Biofertilizer field trial- 2011/2012 growing season.

6.6.1 Materials and methods

The 2011/2012 field trials were planted on the 19th of December 2011 on Towoomba ADC on Huttons and Arcadian soil ecotopes. The Shortlands ecotope trial was planted on the 28th of December 2011.

The trial layout was exactly as in the field trials conducted during the 2010/2011 season (6.2.1). Fertilisation was applied based on the soil analysis received from the University of Pretoria's Soil Science laboratory to the same levels as in the 2010/2011 field trial i.e. Nitrogen applied at 100kg/ha and phosphate at 75kg/ha. These calculations are according to Table A and Appendix A.3 and amounted to 54.9kg/N/ha and 47.97kg/P/ha as needed for the Huttons soil, 45.9kg/P/ha and 64.48kg/N/ha as needed for the Shortlands soil whereas the Arcadian soils had to be amended with 57kg/P/ha and 45.45kg/N/ha.

The only differences between the 2010/2011 trial materials and methods were new PGPR isolates were used in the 2011/2012 field trials. Five new strains were included in the 2011/2012 trials based on their performance in other trials (Pretorius, 2012). The only strain included from the previous field trials was S 7-08 (non-toxin producing *B. cereus*). The other strains included were T-19, A-40, T-26, A-29, S7-08 and the commercial product Brus® (Stimuplant©, Gauteng, South Africa). The treatments were prepared by Stimuplant© by inoculating sterile perlite powder with a broth culture of the bacterial isolates and planted according to Stimuplant© commercial recommendations (200g powder per 50kg of maize seed). For the control treatment the seed was coated with sterile Perlite© powder.

Maize seed (cultivar was P1615R, Roundup ready®) were obtained from Pioneer© (Rosslyn, Gauteng, South Africa). This is a yellow maize cultivar that was developed to be adapted to hot regions and has a short growing period, with a yield potential of 13.52t/ha under irrigation with 0% lodging and a mean head count of 1.23 heads per plant.

These trials were planted in three different soil types namely the Huttons, Arcadian and Shortlands soil ecotope. The trial areas were exact replicas of that of the previous 2010/2011 growing season with the exception that a larger area was planted to minimize the effect of vermin damage in the trial sites.

The only constraint encountered during these trials was that the farmers did not allow collection of samples for determining plant height and fresh and dry mass during the growing season as this would have caused loss of yield and revenue.

The trials were successfully protected from vermin with most of the damage located in the buffer zones around the trials. In total 280 plants were harvested per replicate at approximately 12% grain moisture for each soil type to determine yield. From each replicate, after harvesting, three individual grain samples were taken and the average moisture content calculated to re-calculate the grain yield mass of all replicates to a standard 12% moisture content.

6.7 Statistical analysis

All data was captured and analysed by importing the data from an excel spread sheet to the statistical package SAS 9.2. The data was analysed with proc GLM at a $p=0.05$ level and the means separated using the Dunnetts test and compared to the control if significance was observed at $p<0.05$.

6.8 Results and discussion

The major difference between the 2010/2011 and the 2011/2012 growing seasons was the rainfall pattern. During the 2011/2012 season rainfall only occurred later in the season but was more constant and occurred at more regular intervals. This is reflected in the yield when the two seasons are compared with a mean difference in total yield of all the treatments of $1.60 tha^{-1}$ and $6.59 tha^{-1}$ for the Huttons and the Shortlands ecotope trials, respectively.

The 2011/2012 season produced very good yields (Table 6.2) ranging from 2.97 t/ha for the S7 treatment and 3.62 t/ha for the Brus® treatment in the Huttons ecotope soil whereas in the Arcadian ecotope soil treatment with A-26 resulted in 4.08 t/ha compared to 5.05 t/ha for the untreated control..

Under dry land conditions the rhizobacterial treatments resulted in greater yield increases in the less fertile soil with lower clay content compared to the control treatments. The results support the findings of Egamberdiyeva (2007) which indicated that the PGPR inoculants had a greater effect on plant growth stimulation in lower potential soil as in the more fertile soils when they evaluated PGPR in two different soil types. Although the Huttons ecotope is considered as fertile and Shortlands as one of the poorest, the Arcadian soils have amongst the highest soil fertility on the Springbok flats in the Limpopo Province. It was found by Paglia & De Nobili (1993) that soil porosity has a dramatic effect on plant enzyme activity and root development. They showed that urease activity dramatically decreased when soil pore size decreased while phosphatase activity was not significantly influenced by pore size and this could explain the negative yield observations in the Arcadian soils with their high clay content.

Yields in the Shortlands soil ecotope ranged from 7.55 t/ha in the treatment with strain A-26 to 11.41 t/ha in the treatment with strain T-19. The probable reason for this drastic increase compared to the Huttons and Arcadia ecotope trials was due to supplementary irrigation given at critical times. The difference between the Shortlands ecotope untreated control and the T-19 treatment was 2.87t/ha. With the large sample sizes collected per replicate the co-efficient of variation (CV) for the Shortlands ecotope trial remained at an acceptable level of 18% even though non-significant. The fact that differences between treatments were not statistically significant does not negate the fact that there was a clear trend of improved yield in the bacterial treated plants compared to the untreated controls, demonstrating the potential of biological fertilizers to increase yield in the Agricultural sector.

Table 6.2 Effect of rhizobacteria on growth and yield of maize under field conditions during the 2011/2012 season

Treatments*	Yield (kg/ha)*****			% Change in yield compared to the control**		
	Huttons	Arcadia	Shortlands	Huttons	Arcadia	Shortlands
T-19	3421.94[a]	4743.75[a]	11413.92[a]	+13.26	-6.00	33.69
Brus®	3621.95[a]	4293.58[a]	9958.83[a]	19.88	-14.92	16.65
S7	2971.84[a]	4901.32[a]	9254.66[a]	-1.64	-2.88	8.40
A40	3438.74[a]	4239.55[a]	8742.95[a]	13.82	-15.99	2.41
A26	3600.44[a]	4075.96[a]	7550.38[a]	19.17	-19.24	-11.56
T29	3242.69[a]	4187.07[a]	11132.34[a]	7.33	-17.03	30.39
Control	3021.29[a]	5046.71[a]	8537.51[a]	0.00	0.00	0.00

*T19, S7 A40 and T29 are rhizobacterial isolates from the University of Pretoria's PGPR culture collection. Brus® is a commercial product of Stimuplant (Gauteng, South Africa)

** % change in mass [(100/Control)* (treatment-100] therefore negative values indicate a reduction in yield compared to the untreated control.

***Treatment means followed by the same letter within the same column do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test.

***** Yield kg/ha at a moisture percentage of 12%.

6.9 Biofertilizer field trial - 2012/2013 growing season

6.9.1 Materials and methods

The 2011/2012 data was used as a baseline for comparison of inoculant performance consistency during the 2012/2013 season. Soil samples were taken at all trial locations and subjected to chemical analysis. Results of the soil analyses indicated that the soil nitrogen was up to commercial recommendations according to the 2011/2012 trial requirements. The phosphate shortage was corrected with super phosphate. Fertiliser requirements were as follows: 84.66kg/N/ha and 84.66kg/P/ha needed for the Huttons soil, 56.4kg/P/ha needed for the Shortlands soil while the Arcadian soils required 68.4kg/P/ha and 22.72kg/N/ha. All trials were planted to the same criteria as in the 2011/2012 field trial. The sizes of the trials were increased to compensate for vermin damage. The Shortlands trial was planted on the 13th of December 2013 and the Huttons and Arcadia ecotope trials were planted on the 14th of January 2013.

6.10 Statistical analysis

All data was captured and analysed by importing the data from an excel spread sheet to the statistical package SAS 9.2. The data was analysed with proc GLM at a p=0.05 level and the means separated using the Dunnetts test and compared to the control if significance was observed at $p<0.05$.

6.11 Results and discussion

The results are shown in Table 6.3. The trials in the Huttons and Arcadia soils failed due to drought. They were conducted under dry land conditions whilst the trial in the Shortlands soil was irrigated. Overall rainfall was sufficient but the frequency of showers were dispersed (appendix A11: 2012/2013 rainfall) and characterised by high temperatures between showers. These conditions induced premature reproductive growth and ultimate failure of the Arcadian and Huttons trials.

The Shortlands trial was harvested by hand when grain had matured and yields were calculated at 12% moisture content. The data show that S-7 was the best performing

inoculant resulting in 5.11t/ha followed in descending order by T-19, A-26, T-29, Brus®, Control and A-40 yielding 4.7 t/ha 4.5 t/ha, 4.3t/ha, 3.8 t/ha, 3.8 t/ha and 3.5 t/ha respectively. Treatment with A-40 yielding 3.5t/ha, was the only treatment that resulted in a reduction of yield compared to the control at 3.8t/ha

The strain which gave the most consistent results was T-19 resulting in yield increases of 0.9t/ha (i.e. 24.6% increase) during the 2012/2013 season and 2.8t/ha (i.e.33.6% increase) during the 2011/2012 season, compared to the controls, in Shortlands soil.

Table 6.3 Effect of selected PGPR strains on yield of maize under field conditions during the 2012/2013 season

	Yield (kg/ha)****	% Change in yield against control **
Treatment*	Shortlands ecotope	
T19	4755.75[a]	24.63
Brus®	3870.44[a]	1.43
S7	5118.78[a]	34.14
A40	3595.18[a]	-5.79
A26	4500.34[a]	17.93
T29	4342.61[a]	13.80
Control	3816.00[a]	0.00

*T19, S7, A40, A26 and T29 are isolates from the University of Pretoria's PGPR culture collection whereas Brus® is a Commercial product from Stimuplant© (Gauteng, South Africa).
** % change in mass [(100/Control)* treatment-100] therefore values less than zero are treatments that caused a reduction in yield compared to the untreated control.
***Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure and separated with the Dunnetts test.
**** Grain yield calculated at a moisture percentage of 12% per hectare.

Babalola (2010) states that variation in PGPR results under field conditions can be mainly attributed to climatic conditions e.g. soil type, temperature, water, soil texture etc. On the other hand Gosling *et al*. (2006) found that PGPR inoculants must be more efficient at promoting growth than the native PGPR or the result would be a decrease in yield. The observations made by Babalola (2010) and Gosling *et al*. (2006) are supported by the observations made in the current study where the field

results were not only influenced by the climatic conditions but growth inhibition was also observed with some bacterial treatments when compared to the controls.

6.12 Conclusion

The data in the current study supports the findings of Nelson (2004), who conclude that it is difficult to achieve consistent PGPR field performance with the heterogeneity of abiotic, biotic factors and competition with the indigenous organisms. According to Bashan (1998) the goal is to increase field consistency by combining an effective PGPR isolate with a formulation that enables the inoculant to be effectively transferred into the rhizosphere and counter environmental conditions as described by Wu *et al.* (2012). When the study is considered as a whole, the treatment T-19 could possibly meet the requirements set by Bashan (1998). Although this treatment did not perform consistently over each season, it gave the most consistent results of all the treatments. Over the three seasons of field evaluation, it can safely be said that the results obtained over the period show the potential of PGPR as a beneficial factor in the agricultural sector.

6.13 References

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Chapter 7

Identification and modes of action of the best performing rhizobacterial isolates

Abstract

Various studies have confirmed that growth stimulation by PGPR cannot be contributed to one primary factor only but rather various coherent PGPR mechanisms that influence various plant growth systems. The objective of the current study was to elucidate the modes of action of the effective biocontrol and plant growth promoting strains tested thus far (previous chapters). The bacterial isolates assessed in the field trials were identified and evaluated for their capability to fix nitrogen, solubilize phosphate, produce phytohormones and act as biocontrol agents via antibiosis against *R. solani* and *F. oxysporum.* Identification via 16srRNA sequencing confirmed *Lysinibacillus sphearicus* strain T-19, *Bacillus cereus* strain S7, *Brevundiomonas vesicularis* strain A40, *Chryseobacterium* strain A26 and *Paenibacillus alvei* strain T29. The data indicated that the only strains that did not solubilize phosphate were T-19 and the commercial product Brus® whereas A-40 showed the best *in vitro* phosphate solubilisation activity. On the other hand, T29 showed the best *in vitro* activity as an atmospheric nitrogen fixer, proliferating on nitrogen free substrate and also producing ammonia. The commercial product, Brus® showed the most prominent *in vitro* antibiosis activity against *R. solani*. Almost all the strains had more than one mechanism that could promote plant growth which confirms the multiple PGPR mechanisms reported in literature.

7.1 Introduction

Plant growth promoting rhizobacteria influence plant health via a variety of direct and indirect mechanisms. According to Zahir *et al*. (2004) and Glick *et al*. (2007) direct mechanisms influence the plant directly by affecting plant growth regulator balance, enhancing the nutritional status of the plant or inducing systemic resistance within the plant. Indirect mechanisms (biocontrol) on the other hand involve mechanisms that alleviate the negative effects that pathogens may have on plant health.

Viveros *et al*. (2010) further classified PGPR according to their primary mechanism into three categories as either a biofertilizer, phytostimulant or a biopesticide. Plant growth promoting rhizobacteria with the ability to increase the availability of nutrients to the plant e.g. through nitrogen fixation (Boddey & Dobereiner., 1995) and phosphate solubilization (De Freitas *et al*., 1997; Yang *et al.,* 2009) are classified as biofertilizers. Phytostimulants are grouped according to their ability to supply or change growth regulator levels within the plant e.g. ethylene, IAA, gibberrelic acid, auxin and cytokinins (Glick, 1995; Jalili *et al*., 2009). Lastly, biopesticides are classified as PGPR that suppress or control phytopathogens via the excretion of antibiotics, enzymes, siderophores, HCN, antifungal metabolistes and/or competing for nutrients (nich exclusion) within the rhizosphere (Flaishman *et al*., 1996; Lugtenberg & Kamilova, 2009; Qin *et al*., 2011)

Kloepper (1993) stated that it is difficult to separate growth promotion and biocontrol, but that these effects should rather be viewed as two sides of the same coin. The results of Banchio *et al.* (2008) supported this by indicating the difficulty to contribute the plant growth stimulating effect of PGPR on a primary factor as various isolates contained more than one mechanism for growth promotion. In addition to the complications mentioned by Banchio *et al.* (2008), other factors must also be considered for a successful PGPR organism. These factors include that the PGPR must be rhizospheric proficient and able to survive and propagate in the rhizospheric soil (Cattelan *et al*., 1999).

7.2 Materials and methods

7.2.1 Cultures

All bacterial cultures were obtained from the UP-PGPR culture collection. Isolates were maintained using Microbank™ beads (Pro-Lab Diagnostics) stored at -70°C and streaked onto nutrient agar (Biolab, Wadeville) as needed. Fungal cultures were obtained from the Department of Microbiology and Plant Pathology's fungal culture collection. *Rhizoctonia solani* UPGH122 was grown on potato dextrose agar (Biolab, Wadeville) and *Fusarium oxysporum* UPGH 132 and *Fusarium graminearum* WP4F on half strength potato dextrose agar.

7.2.2 Identification

For identification, a pure culture of each PGPR isolate was sent to Inqaba Biotechnical Industries (Hatfield, Gauteng, South Africa) for sequencing of the 16SrRNA gene region. The isolates were identified based on species relatedness to other strains based on BLASTN searches in the NCBI data libraries. At Inqaba the DNA was extracted with Zymo Fungal/Bacterial DNA extraction kit (Zymo Research Corp.), the PCR performed using DreamTaq (Fermentas Life Sciences, DreamTacTM Green PCR Master Mix) and the primers 27-F and 1492-R. The sequencing reaction was performed with ABI Big Dye v3.1 and the clean-up performed with the Zymo Sequencing Clean-up kit (ZR-96, DNA Sequencing Clean-up KitTM).

7.2.3 Modes of action

7.2.3.1 Mineral phosphate solubilisation

Phosphate solubilisation was evaluated according to the procedures described by Nautiyal (1999) in Pikovskaya amended medium (Appendix A.6). The agar medium was prepared by amending bacteriological agar (Biolab, Wadeville) with 10g/l glucose, 5g/l NH_4Cl, 1g/l $MgSO_4.7H_2O$ and 5mg/ml $Ca_3(PO_4)_2$ and adjusting the pH to 7.2. The media was then autoclaved at 121^0C for 20 minutes and left to cool to handling temperature before pouring into sterile 90mm petri-dishes in a laminar flow cabinet.

The rhizobacterial strains were stab-inoculated into the Pikovskaya amended media with a flame sterilised inoculation needle. Four strains were inoculated at a 90^0 angle per plate using five replicates per strain. The plates were labelled, sealed with Parafilm and incubated for 5 days at room temperature. A positive reaction for phosphate solubilisation was recorded when a clear halo developed around the bacterial colony in the Pikovskaya medium.

7.3.2.2 Assessment of atmospheric nitrogen fixing ability

The rhizobacterial strains were evaluated for nitrogen fixation and ammonia production. The Nessler's reagent test (Dye, 1962) was also used to test for nitrogen production in nitrogen free media in order to test for nitrogen leakage.

7.3.2.2.1 Nitrogen fixation – growth in N free medium

Winogradsky nitrogen free medium (Appendix A.10) was prepared according to Tchan & New (1984) by adding the following to 1L dH_2O and the pH adjusted to 7.2 with NaOH:

- 50.0g/L KH_2PO_4
- 25g/L$MgSO_4$• $7H_2O$
- 25g/L NaCl;
- 1g/L $FeSO_4$•$7H_2O$
- 1g/L Na_2MoO_4•2 H_2O
- 1g/L $MnSO_4$•$4H_2O$

Five millilitre of this solution was then added to 0.1g $CaCO_3$ in 1L of dH_2O before sterilizing at 120°C for 20 min and labelled as the stock solution.

Ten grams of sucrose mixed with 200ml distilled water was autoclaved separately at 120°C for 20 min. This was then subsequently added to 300ml distilled water and 2.5ml of the prepared stock solution.

The nitrogen fixation test was performed in semi-solid and on solid agar plates. For the agar plates, 7g of bacteriological agar was added to 500ml of distilled water and for the semi-solid media 1.5g was added to test the test tubes.

The bacterial strains were transferred to the agar plates by means of a flame sterilised inoculation loop and stab inoculated into the semi-solid media by means of a flamed inoculation needle. All treatments were replicated twice. The agar plates and semi-solid media containing test tubes were incubated for 10 days at 25^{o}C before evaluating colony growth and pellicle formation for the two test methods respectively according to Baldani & Dobereiner (1980) and Caceres (1982).

7.3.2.2.2 Detection of ammonia production with Nessler's reagent

Ammonia production was tested for according to the procedures described by Rana *et al.* (2012). Test tubes were sterilized by autoclaving at 120^{o}C for 20min before adding one millilitre sterile peptone water medium (Biolab, Wadeville). The tubes were aseptically inoculated with a flamed inoculation loop before sealing with Parafilm and labelled accordingly. This was done in triplicate for all treatments before incubating for 3 days at 25^{o}C on a rotary shaker. A positive test for ammonia was indicated by a brown/yellow colour change after adding 0.1 ml of Nessler's reagent (Dye, 1962).

7.3.2.3 IAA production

IAA production was tested using the S2/1 method as described by Glickman *et al.* (1995). One hundred millilitres of sterile nutrient broth was inoculated with each bacterial strain respectively placed on a rotary shaker for 48 hours at 25°C and 150rpm. After his period the cultures were transferred to sterile 50ml conical tubes and centrifuged at 3000 x g for 10 minutes. From this one millilitre of the supernatant was transferred to a test tube and two millilitre of Salkowski's reagent. The Salkowski reagent was prepared by slowly adding 4.5g $FeCl_3$ to one litre of 10.8 M (67%) H_2SO_4. As a control only Salkowski reagent was added to the sterile nutrient broth. A colour change to brown in the solution was recorded as a positive for IAA production.

7.3.2.4 Dual culture assay to determine antibiosis

Inhibition of fungal pathogens (both *R. solani and F. oxysporum*) by the bacterial isolates was determined by means of the dual culture method on Water-yeast agar (WYA) and Potato dextrose agar (PDA), respectively. Water yeast agar was used because it is a minimal medium that mimics the carbon-limiting environment of soil. The WYA comprised of 20g agar, 5g NaCl, 1g KH_2PO4, 0.1g yeast extract in 1L distilled water. A single isolate of the bacterium to be tested was stab inoculated in three places equidistant from the centre and each other onto a WYA plate. A 5mm diameter fungal plug from a fresh fungal culture was then placed in the centre of the plate between the bacterial inoculation spots (De Boer *et al.,* 2007).

Three replicate plates were used for each bacterial strain. When the fungal colonies on the control plate without bacteria reached the edge of the plate (after approximately four days), the growth towards and away from the bacterial colonies was recorded and the percentage inhibition of mycelial growth calculated. The percentage inhibition of mycelial growth was calculated by means of the formula [(R2-R1)/R2] x100, with R1 being the distance of mycelial growth towards the bacterial colonies and R2 the maximum mycelial growth on the control plate.

7.4 Results and discussion

7.4.1 Identification

The bacteria isolated were diverse and include *Lysinibacillus sphearicus* (T19), *Bacillus cereus* (S7), *Brevundiomonas vesicularis* (A-40), *Chryseobacterium* (A26) and *Paenibacillus alvei* (T29).

7.4.2 Mineral phosphate solubilisation

The only bacterial strain that did not solubilize phosphate was isolate T-19 (*Lysinibacillus sphearicus*) and the commercial product Brus®. Strain A-40 (*Brevundiomonas vesicularis*) showed the greatest phosphate solubilisation activity with a halo formation of more than 3mm (Figure 6).

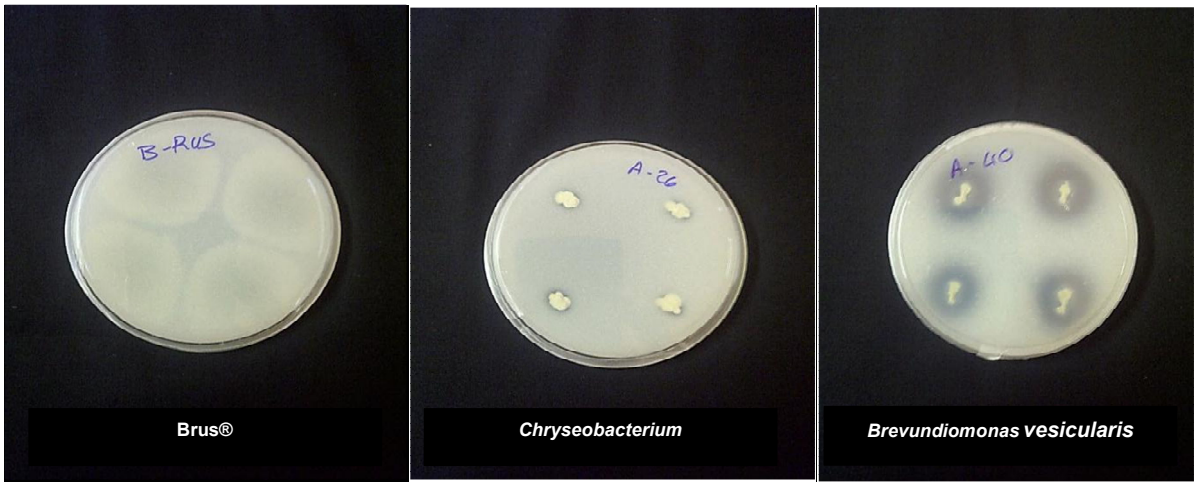

Figure 6: Example of Phosphate solubilisation activity of some of the rhizobacterial strains on Pikovskaya amended medium.

7.4.3 Assessment of atmospheric nitrogen fixing ability

The results of the study indicated that the rhizobacterial strain T29 (*Paenibacillus alvei)* has the best atmospheric nitrogen fixing ability, testing positive in the semi-solid medium and also proliferating on the solid N-free medium. On the other hand, all the bacterial strains tested positive for ammonia production except for S7 (*Bacillus cereus)* although this strain did grow in the nitrogen free semi-solid and solid media.

7.4.4 IAA production

All the strains tested positive for production of the plant growth hormone IAA except for the commercial inoculant Brus®.

7.4.5 Dual culture assay to determine antibiosis

In the dual culture assay (Figure 7) only two of the bacterial strains showed antibiosis activity against *R. solani*. viz. T-29 (2% inhibition) and the commercial product Brus® (3.56 % inhibition).

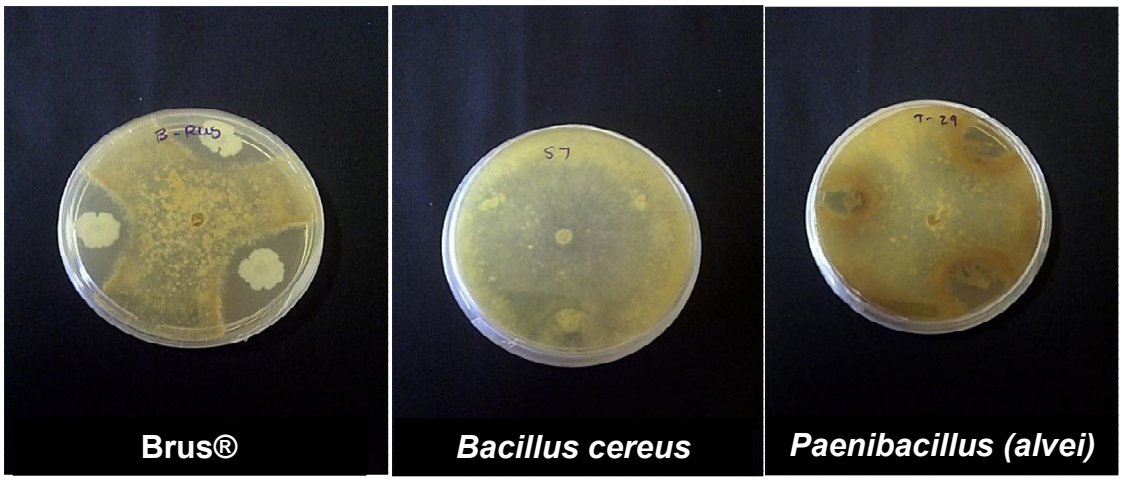

Figure 7: *In vitro* inhibition of *Rhizoctonia solani* by some of the PGPR strains during the dual culture tests.

7.5 Conclusion

As is evident from the data in Table 7.1, the selected rhizobacterial strains each tested positive for a number of different direct or indirect plant growth promoting modes of action. Based on these findings it can therefore be expected that a combination (mixture) of these selected strains will produce a robust and effective plant growth enhancing/ biofertilizer product needed to help address food security issues in Africa as stated by Mwaniki (2006).

Table 7.1 Specific modes of action exhibited by the rhizobacterial strains

Bacterial strain*	Identification(16srRNA)	Phosphate solubilisation[a]	Nitrogen fixation[b]			IAA production[d]	Antibiosis (Dual culture)[e]
			Semi-solid medium	Solid medium	Nessler's reagent[c]		
T19	*Lysinibacillus spp (sphearicus / fusiformis)*	-	+	0	+	+	0
Brus®	Commercial (unknown)	-	-	-	-	-	3.56
S7	*Bacillus cereus*	+	+	1	-	+	0
A40	*Brevundiomonas vesicularis*	+++	-	1	+	+	0
A26	*Chryseobacterium*	+	+	1	+	+	0
T29	*Paenibacillus (alvei)*	+	+	2	+	+	2

[a] Phosphate solubilisation was assessed on Pikovskaya medium where a clearing zone constituted a positive reaction : – = no clearing zone, + = 0-1 mm, zone, ++ = 1-2 mm zone, +++ = 2-3mm zone.

[b] Nitrogen fixation was determined by colony formation in the solid media and pellicle formation in the semi-solid media by bacterial strains inoculated in N-free media;0= no colony formation, 1= small colony formation, 2= profuse colony formation, - = no pellicle; + = presence of pellicle.

[c] Production of ammonia was indicated by a colour change to yellow brown +=positive for ammonia production -= no ammonia production

[d] IAA production was indicated by a colour change to yellow-brown +=IAA produced, -= no IAA production

[e] Percentage mycelial growth inhibition of *R.solani* calculated as [(R – r)/ R] x 100 where R is mycelial growth away from the bacterial colony (the maximum growth of fungal mycelia) and r is the mycelial growth towards the bacterial colony.

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Appendix A

Table A: Results of soil analyses for different soils used in the various biofertilizer trials (soil analysis conducted by the Soil Science Laboratory, Department of Plant Production and Soil Sciences, University of Pretoria)

				Ammonium Acetate Extractable									
				P bray I	Ca	K	Mg	Na	NH_4	NO_3	Coarse sand	Silt	Clay
Year	Lab no.	Field no.	pH water	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	%	%	%
2009	1753	Greenhhouse trial 1	5.8	12.9	172	35	38	36					
2009	3530	Greenhouse trial 2	6.6	5.9	848	161	316	89					
2009	3531	Biocontrol	6.3	63.7	711	228	252	91					
2009	1234	Nutrient trial	0	0	0	0	0	0	0	0			
2010	2375	Shortlands	7.2	15	2703	269	539	70	1.23	1.71			
2010	2287	Huttons soil	5.7	8.2	1249	473	505	76	1.79	1.96	56	15	25
2010	2288	Arcadian	7.8	5.1	8589	570	1348	193	0.5	7.78	28.6	16.9	50.6
2011	1418	Arcadian	7.8	6.0	8556	406	1231	300	5.34	9.81			
2011	1419	Huttons	6.1	6.7	1930	352	465	56	6.87	9.12			
2011	1420	Shortlands	7.1	9.7	1989	202	435	92	9.53	12.03			
2012	1046	Huttons	6	11.7	1493	339	488	28	10.86	17.36	60.4	26.9	26.9
2012	1051	Shortlands	7.1	18.8	2656	71.3	713	65	9.07	32.48	66.1	12.5	17.5
2012	1052	Arcadia	7.8	2.2	3235	391	1466	374	8.06	17.7	31.3	15.6	49.4

A.2 Soil sampling procedure

The sampling method was conducted according to ARC (2008). Twenty top soil samples were taken at random within the trial site with a 75mm diam. auger and five subsoil samples were taken at a depth of 300mm and 600mm soil respectively. These samples were then thoroughly mixed and a 500g sub-sample taken and sealed in plastic bags and transported to the soil analysis laboratory, Department of Plant Production and Soil Sciences at the University of Pretoria for chemical analysis.

A.3 Calculation of fertiliser requirements for field soils

Amount of soil per hectare

One hectare = 100 meters X 100 meters

= 10 000m^2

Soil volume = surface X depth X Litre/m^2

=10 000m^2 X 0.2m X 1000

=2000 000L

Thus soil type (kg/l) x soil volume per hectare x nutrient (soil analysis).

$$= \frac{\text{amount of nutrient per hectare in milligram}}{1000\ 000}$$

= kg nutrient per hectare

Nutrient needed per hectare

= target amount at 75kg/ha for Phosphate and 100kg/ha for Nitrogen

= target amount – kg nutrient/ha

$$= \frac{\text{needed amount per hectare}}{\text{Nutrient concentration per bag}}$$

= amount of bags needed per hectare to correct nutrient levels.

A.4 Nutrient agar (NA) preparation

NA media were prepared by dissolving 37g Nutrient agar (Merck, Johannesburg, South Africa) in one litre of dH_2O. The glass bottle containing the nutrient agar solution was then sealed with a double layer of foil and subsequently autoclaved (Gentinge, Geza 400) at 120^0C for 20 minutes and left to cool to easy handling temperature before casting in 90mm sterile petri-dishes (Merck, Johannesburg, South Africa)

A.5 Algenate seed coating preparation

Procedures adapted from Trivedi *et al*. (2005)

The maize seeds were prepared by surface sterilizing in 70% ethanol for 3 minutes and subsequently for 1 minute in 3% sodium hypochloride solution. The seeds were then rinsed five successive times with sterile dH_2O, and dipped for 5 seconds in 2% wt/vol algenate which contained the respective rhizobacterial strains at 10^9cfu/ml. The seeds were removed from the algenate solution, immediately immersed in 0.1M $CaCl_2$ (calcium chloride) for 30 seconds and rinsed three successive times with sterile distilled water (dH_2O). The seeds were then incubated in tryptophan yeast extract broth for 24 hours and washed again with sterile dH_2O before leaving to dry in the laminar flow cabinet for an hour.

A.6 Pikovskaya's agar medium

Procedures were conducted as described by Nautiyal (1999). Pikovskaya media was amended with 10g/l glucose, 5g/l NH_4Cl, 1g/l $MgSO_4.7H_2O$ and 5mg/ml $Ca_3(PO_4)_2$ and the pH adjusted to 7.2 with 1M NaOH. The medium was autoclaved at 121^0C for 20 minutes and left to cool to handling temperature before pouring into sterile 90mm petri-dishes in the laminar flow.

A.7 Salkowski reagent

The reagent was prepared as described by Glickmann and Dessaux (1995), by slowly adding 12g of $FeCl_3$ per litre of 7.9M H_2SO_4.

A.8 Sterile nutrient broth

This was prepared by adding 16g nutrient broth powder to one litre of distilled water and autoclaved at 121^0C for 20 minutes.

A.9 Nfb media

Nfb medium was prepared as described by Bhavanath *et al*. (2009). The Nfb medium comprised of 5g/l malate as a carbon source, 5g K_2HPO_4, 0.6g KH_2PO_4, 1.8g $MgSO_4$ in 7 X H_2O, 0.2g NaCl, 0.1g $CaCl_2$ in 2X H_2O, 0.2g Bromomthymol blue at a concentration of 0.5% w/V in 0.2M KOH, 2ml Fe-EDTA (1.6%w/v), and 4ml KOH. A 2ml volume of trace element was also added which consisted of 100mg/l $ZnSO_4$, 30mg/l $MnCl_2$ dissolved in 4X H_2O, 300mg/l H_3BO_3, 200mg/l $CoCl_2$ dissolved in 6X H_2O, 10mg/l $CuCl_2$ dissolved in 2X H_2O, 20mg/l $NiCl_2$ dissolved in 1X H_2O, 30mg/l Na_2MoO_4 dissolved in 2X H_2O.

To this Nfb medium, 1ml of the vitamin solution was also added which comprised of 10mg/l Ribloflavine, 50mg/l Thiamin-HCL dissolved in 2XH_2O, 50mg/l nicotinic acid, 50mg/l Pyrodixin-HCL, 50mg/l Ca-panthotenate, 100mg/l biotin, 200mg/l folic acid and 200mg/l vitamin B_{12}.

To the Nfb medium that was amended with the vitamin solution, 1L dH_2O and 1.8g of agar was added (Merck chemicals, Johannesburg, South Africa). The pH was adjusted to 6.5 with a 10N KOH solution before sealing in a two litre Erlen Meyer flask with a double foil layer and autoclaving at 121^0C for 20 minutes.

A.10 Winogradsky nitrogen free media

Winogradsky nitrogen free media solution was prepared according to (Tchan & New, 1984) by adding the following to 1L dH_2O:

- 50.0g/L KH_2PO_4
- 25g/L$MgSO_4$• $7H_2O$
- 25g/L NaCl;
- 1g/L $FeSO_4$•$7H_2O$
- 1g/L Na_2MoO_4•2 H_2O

- 1g/L $MnSO_4 \cdot 4H_2O$

Five millilitre of this prepared 1L dH2O solution was then added to 0.1g $CaCO_3$ in 1L of dH_2O before sterilizing at 120°C for 20 min and labelled as the stock solution.

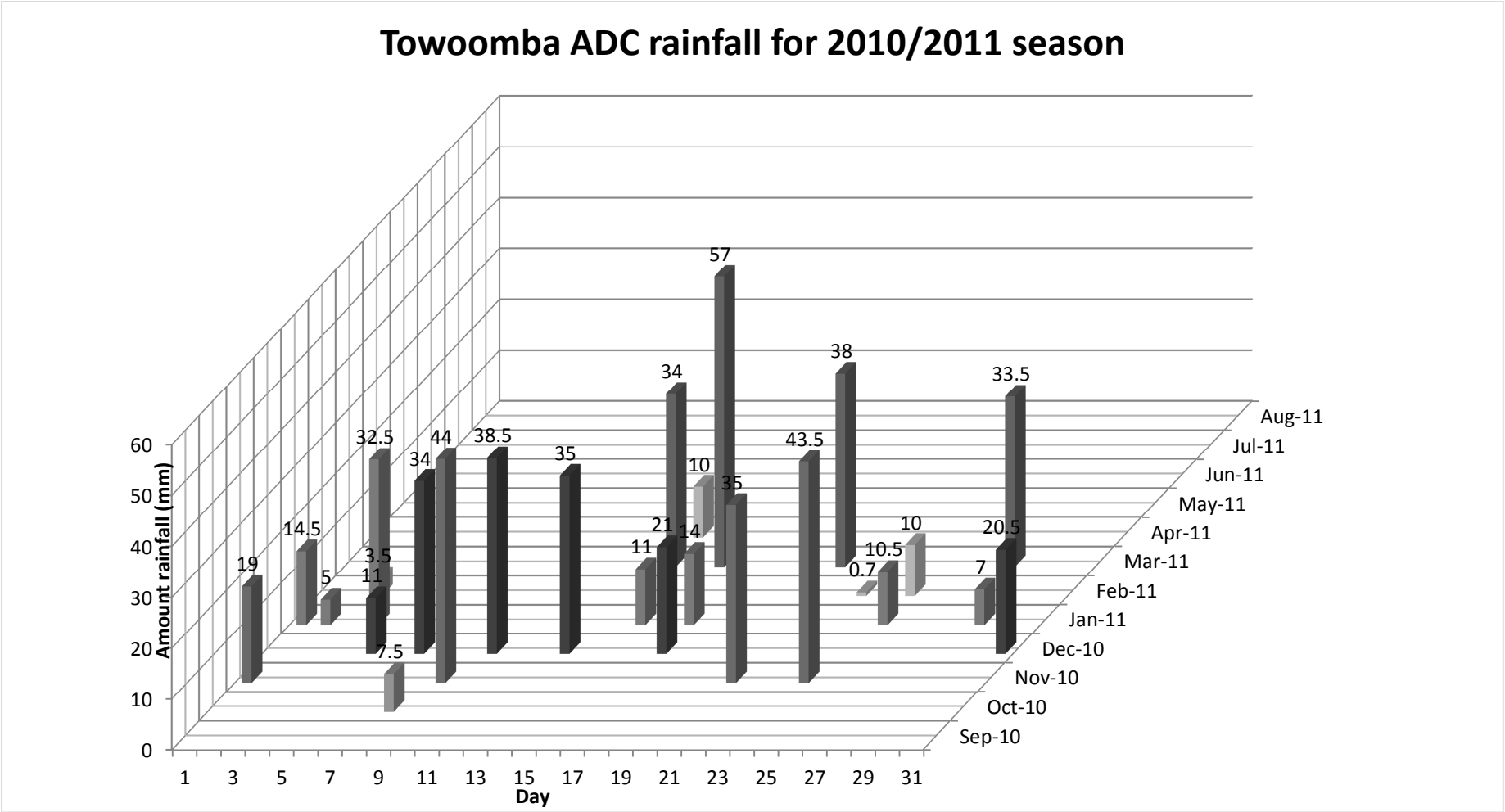
Ten grams of sucrose mixed with 200ml distilled water was autoclaved separately at 120°C for 20 min. This was then subsequently added to 300ml distilled water and 2.5ml of the prepared stock solution.

The nitrogen fixation test was performed on semisolid medium and agar plates. To prepare the agar plates, 7g of bacteriological agar was added to 500ml of distilled water in an Erlen Meyer flask and sealed with a double foil layer before autoclaving at 120°C for 20 min. The semi-solid media was prepared in the same manner as the agar plates but only 1.5g agar was used. The petri-dishes were poured in the laminar flow, under aspetical conditions when the agar cooled to handling temperature.

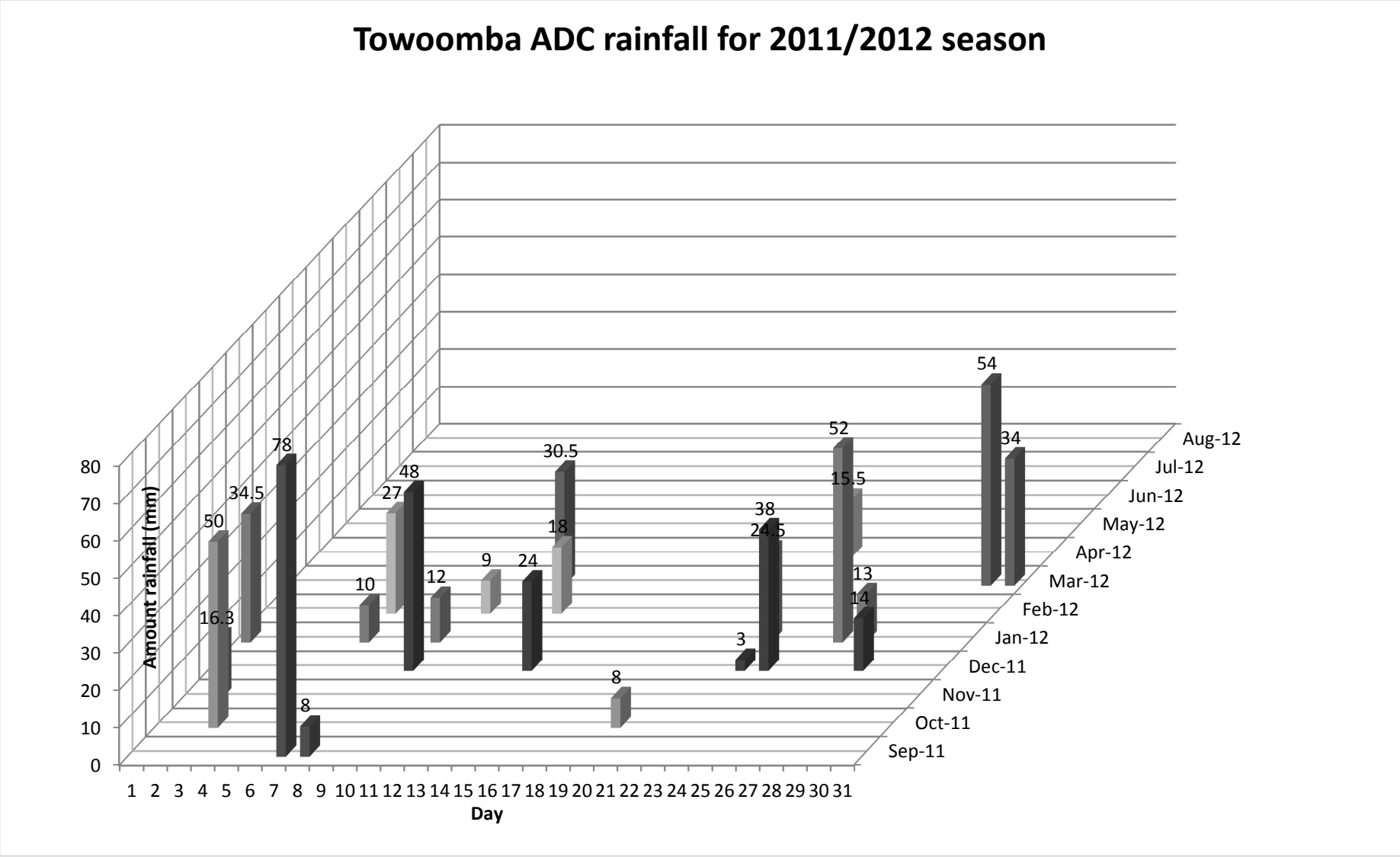
A.11 Oatmeal media

Oatmeal agar was prepared according to Atlas (2004) by adding 60g of oatmeal to one litre of dH_2O and 12.5g of nutrient agar to a 2L Erlen Meyer flask. This was mixed thoroughly before sealing the Erlen Meyer flask with a double layer of foil and autoclaved at 121^0C for 20min. This mixture was left to cool before aseptically pouring the agar into sterile petriplates in the laminarflow cabinet.

A.12 Rainfall data recorded at Towoomba research station during 2010-2011 season.



A.13 Rainfall data recorded at Towoomba research station during 2011-2012 season.



A.14 Rainfall data recorded at Towoomba research station during 2012-2013 season.

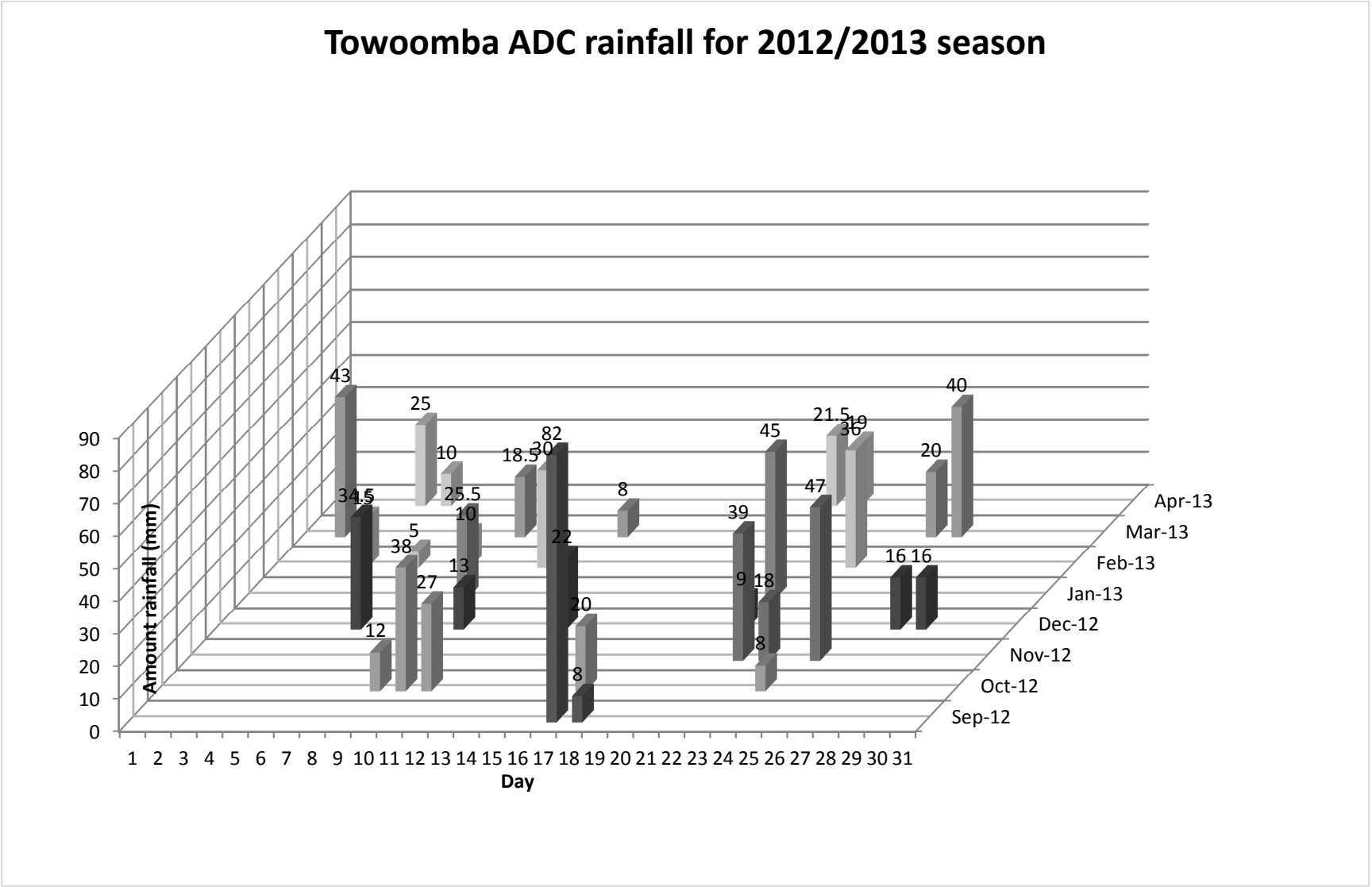


Table B: Summary of the different trials and experiments conducted for PGPR efficacy and strain selection.

Inoculant code	16s Identification	GP1 (GREENHOUSE SCREENING FOR PGPR INOCULANTS)	GP2 (SELECTION OF BEST PERFORMING INOCULANTS AND RE-TRIAL IN HUTTONS ECOTOPE)	GP3 (BIOCONTROL TRIAL from separate F.graminearum pathogenicity selection greenhouse trial.)	GP4 (NUTRIENT LEVEL OPTIMISATION TRIAL)	GP5 (Dose response optimisation trial	2010/2011 field trials			2011/2012 field trials			2012/2013 field trials			Lab work		
							Soil ecotope			Soil ecotope			Soil ecotope			Dual culturing	IAA production	Nitrogenase activity
							Huttons	Shortlands	Arcadia	Huttons	Shortlands	Arcadia	Huttons	Shortlands	Arcadia			
A-04		✓																
A-06		✓																
A-07	*Bacillus aryabhattai*	✓																
A-08	*B. arabyhattai*	✓	✓	✓												✓		
A-09		✓																
A-10		✓																
A-19		✓																
A-22		✓																
A-24		✓																
A-25		✓																
A-26	*Chryseobacterium*	✓	✓	✓						✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓	✓	✓
A-28		✓																
A-29	*Stenotrophomonas maltophilia*	✓																
A-32	*Stenotrophomonas*	✓	✓	✓						✓						✓		

A-33		✓																
A-34		✓																
A-36		✓																
A-37		✓																
A-38		✓																
A-39		✓																
A-40	*Brevundiomonas vesicularis*	✓	✓	✓						✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓	✓	✓
A-41		✓																
A-42		✓																
A-43		✓																
A-44		✓																
A-45		✓																
A-46		✓																
AFP1-1		✓																
AZOMARKET		✓																
S1-08	*B. cereus(+toks)*	✓	✓	✓	✓	✓	✓	✓	Failed (drought)	discontinued (toxic)						✓		discontinued (toxic)
S2-08	*B. cereus(+toks)*	✓	✓	✓	✓	✓	✓	✓	Failed (drought)	discontinued (toxic)						✓		discontinued (toxic)
S3-08	*B. cereus(+toks)*		✓	✓	✓	✓												
S4-08	*Stenotrophomonas maltophila*		✓	✓	✓	✓				discontinued (toxic)						✓		discontinued (toxic)
S6-08	*Chryseomonas luteola*		✓	✓						discontinued (toxic)						✓		discontinued (toxic)

S7-08	*Bacillus cereus*									✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓	✓	✓
T-19	*Lysinibacillus spp (sphearicus / fusiformis)*									✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓	✓	✓
T-29	*Paenibacillus (alvei)*									✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓	✓	✓
Brus®					✓	✓	✓	✓	Failed (drought)	✓	✓	✓	Failed (drought)	✓	Failed (drought)	✓		
BACUP®			✓	✓	✓	✓												
S1&S2 mixture							✓	✓										