Efficacy of selected rhizobacterial isolates for biocontrol of *Rhizoctonia solani* and growth promotion of maize in South Africa

By

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DECLARATION

I, Nicole Rudolph, declare that the thesis/dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ……..

DATE: ……...

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"*Even though I walk through the darkest valley I will fear no evil, for you are with me; your rod and your staff, they comfort me*". (Psalms 23:4)

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SUMMARY

Plant growth promoting rhizobacteria (PGPR) are bacteria that colonize the volume of soil surrounding plant roots (known as the rhizosphere) which, when in association with a suitable host plant, promote plant growth. The growing demand for sustainable crop production strategies has placed increasing emphasis on PGPR and their potential use in agriculture. The current study therefore focused on the beneficial effects of PGPR isolates (selected from the PGPR culture collection at the University of Pretoria) on maize (*Zea mays* L.) and the ability of these isolates to control *Rhizoctonia solani* Kühn.

The seed quality and vigour of two maize cultivars (PAN 6236B and PAN 6Q-308B) was determined using the standard germination, moisture and various vigour tests. PAN 6Q-308B was found to have lower vigour and was selected for use in the remainder of the tests performed in the study. In a modified seed germination bioassay the percentage germination, seedling mass, shoot and root length of PAN 6Q-308B was increased by selected PGPR isolates. The vigour index (VI) of PAN 6Q-308B was also increased by five isolates (S6, S7, T19, T29 and A32) which were then selected for evaluation in the greenhouse.

In the greenhouse pot trial isolates T19, S7 and T29 gave the most promising results in terms of increasing the dry mass of maize seedlings. These isolates were evaluated in a formulation efficacy trial where they were applied individually and in combination as a soil drench, seed treatment and liquid suspension or powder formulation applied to a pelletised compost carrier. The four most effective treatments at increasing the dry root mass of maize included a soil drench application of either isolate T29 or T19, a soil drench application of the consortium of isolates T19, T29 and S7, and a powder formulation of isolate T29 applied onto the compost pellet carrier.

Isolates A08, A07 and T19 were the most effective at reducing the disease symptoms of *R. solani* Kühn and increasing dry root matter above that of the *R. solani* inoculated control in a seedling tray trial. Pot trials were conducted to assess the efficacy of isolates A08, A07 and T19 for control of *R. solani*, when applied as a seed treatment or soil drench. All three isolates failed to control the pathogen when applied as a seed treatment but a soil drench of isolate T19 was found to be very effective.

A six month shelf-life study was conducted to determine the survival of the PGPR isolates T19, S7 and T29 applied to a novel carrier comprising pelletised composted chicken

manure. The isolates were individually applied to the pellets in a liquid or powder form. The latter was found to be the most suitable for supporting the survival of the PGPR isolates up to six months at 25˚C. The study revealed that the viability of the isolates decreased more rapidly at a temperature of 35˚C as opposed to 25˚C, and that the cell numbers of isolate T19 remained the most stable throughout the six months incubation period in comparison with the other isolates.

CHAPTER 1

General introduction

1.1 Background

The most important agricultural commodity in South Africa (RSA) is maize (*Zea mays* L.). It is a major feed grain used for local human and animal consumption and is an important trade crop being exported primarily to African countries, Asia and Europe. White maize accounts for 60% of the total maize produced in the country and yellow maize for the remaining 40% (NAMC, 2003). Approximately 94% of white maize produced is consumed as a staple food in the form of maize meal, particularly by the poor throughout Africa, whereas yellow maize is primarily used to feed livestock. About 10% of the maize produced is used for seed and industrial uses with Monsanto being the largest maize seed company in RSA (DAFF, 2012).

The maize industry is comprised of an estimated 9000 commercial farmers which produce the bulk of the South African crop and employ approximately 150 000 workers. A smaller portion of maize is produced by thousands of small-scale emerging farmers of which the number is unknown (DAFF, 2012). Maize is primarily grown on dry land, in areas with an even distribution of rain exceeding 350mm per year (Du Plessis, 2003). Planting usually takes place during October to December and the total area planted is between 3.8 and 4.8 million ha of land per year. The major maize growing areas in RSA produce around 83% of the total maize in the country and are found in the Mpumalanga, North West and Free State provinces (Figure 1.1) (DAFF, 2012).

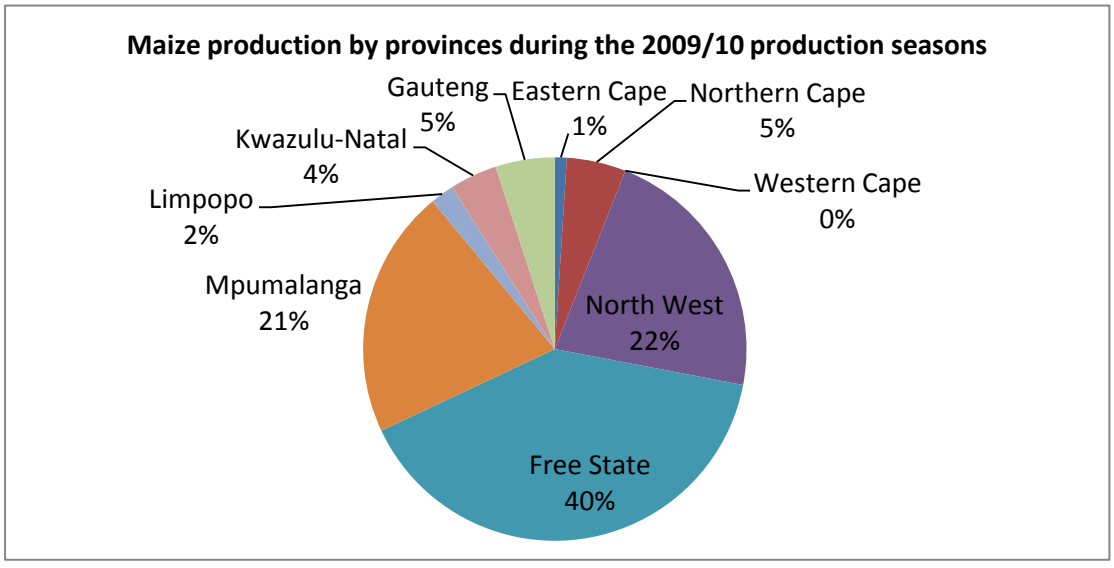

Figure 1.1: Maize production areas in South Africa (DAFF, 2012)

Despite maize being one of the most important grain crops in the country, maize production in RSA has been below the global average since 1961. In 2012, RSA produced 1.8 tons/ha less than the global average of 4.8 tons/ha and accounted for a mere 1.4% of global production and 18.3% of maize production in Africa (FAO, 2013). Africa has the lowest crop production per unit of land cultivated in the global agricultural sector (Thomson, 2008). Numerous factors are responsible for the low maize yield in African countries. In general, African soil is deficient in phosphorus (Materechera and Morutse, 2009; Yazdani *et al*. 2011), is high in toxic aluminium, is acidic and therefore requires addition of costly fertilizers before optimum crop production can be achieved. Also, limited access to water, unpredictable drought conditions (Thomson, 2008) and climate change are major factors which limit maize production (Jones and Thornton, 2003). The effect of climate change on rainfed smallholder maize production in RSA, is predicted to reduce maize yield with 968 kg/ha by 2055 according to a simulation model by Jones and Thornton (2003).

Biotic factors such as insects, weeds, viruses, bacteria and fungi also affect maize production adversely. Insect damage, for example by stem borers, causes severe yield losses which may range from 15-40% depending on the environmental conditions (AATF, 2004). *Striga* spp. (witchweed) is an aggressive parasitic weed that causes maize losses of between 20-80% and is a major problem especially in sub-Saharan African countries (Thomson, 2008). On average, 10.9% of global maize production is lost due to disease caused by various plant pathogens (Pinstrup-Andersen, 2001); however losses of up to 90% have been reported in localized areas (Ngoko *et al*., 2002). Important maize pathogens which can be very destructive under favourable conditions in RSA include *Xanthomonas campestris* pv *zeae* (bacterial leaf streak), Maize streak virus (maize streak disease), *Cercospora zeae*-maydis Tehon & E.Y. Daniels (grey leaf spot), *Stenocarpella maydis* Berk & B. Sutton (Diplodia stem and cob rot) and *Puccinia sorghi* Schwein (common rust) (Kloppers *et al.* 2005). Another pathogen which is also of considerable economic importance is the basidiomycete *Rhizoctonia solani* Kühn. The pathogen is highly biodiverse, is widespread and can be responsible for serious crop losses of maize and many other important crops (Ithurrart *et al*. 2004; Pfahler and Petersen, 2004; Bolton *et al*. 2010). Grain yield losses of up to 30% have been reported as a result of *R. solani* infections (Pfahler and Petersen, 2004).

The agricultural sector relies heavily on chemical pesticides and fertilizers for plant disease control (Cummings, 2009) and increased soil fertility (Bashan and de-Bashan, 2005). Strict regulatory processes regarding environmental and health problems associated with the use of synthetic chemicals (Holm *et al*. 2005; Stark, 2008), has however lead to the withdrawal of

numerous products from the agricultural market (Chincholkar and Mukerji, 2007; EPA, 2010). This represents a serious challenge for farmers who rely on these products for crop protection and has compelled industries, researchers and the farmers themselves to consider alternative approaches which are more ecologically compatible (Berg, 2009; Al-Taweil *et al.* 2010). Consequently, the utilization of plant growth promoting bacteria in sustainable crop production has become increasingly popular (Hayat *et al*. 2010). It was in the 1970's that Kloepper and Schroth first described soil bacteria that colonized plant roots and enhanced plant growth and in 1981 introduced the term plant growth promoting rhizobacteria (PGPR) (Bhattacharyya and Jha, 2012). Today, PGPR play a pivotal role in agriculture (Hayat *et al*. 2010) and numerous commercial products are available for production of important crops such as rice (*Oryza sativa* L.), maize, tomatoes (*Solanum lycopersicum* L.) and wheat (*Triticum aestivum* L.) (Berg, 2009).

PGPR are a group of free-living rhizosphere inhabiting bacteria that stimulate plant growth either directly by enhancing the plant's nutrient status, or indirectly by inhibiting plant pathogens (Vessey, 2003; Kumar *et al*. 2011). Generally, approximately 2 - 5% of cultivable microorganisms isolated from plant associated microenvironments are PGPR (Antoun and Prévost, 2005). These beneficial bacteria have various traits such as nitrogen fixation, phosphate solubilisation, iron sequestration, antibiotic production and so forth which benefit the plant and ultimately increase crop yields (Bhattacharyya and Jha, 2012).

The use of PGPR on graminaceous crops has been researched for more than 70 years (Cummings, 2009) and their positive effect on maize yield has been demonstrated both in laboratory and field trials (Kumar *et al*. 2007; Adesemoye *et al.* 2008; Naveed *et al*. 2008). *Azospirillum irakense* Khammas *et al*. 1991 was able to enhance germination, stimulate plant growth, increase dry matter content, improve the uptake of nitrogen and increase the nitrogen content in maize grains (Dobbelaere *et al*. 2002). In some cases, maize growth may be stimulated by more than 100% after inoculation with PGPR isolates such as *Bacillus* and *Pseudomonas* spp. (Kumar *et al*. 2007). Furthermore, numerous PGPR have demonstrated their potential in sustainable crop production systems being able to improve yields of maize which received suboptimal fertilizer applications (Adesemoye *et al*. 2008). PGPR have also demonstrated their ability to control important maize pathogens. For example, *Burkholderia cepacia* Palleroni & Holmes significantly reduced Pythium root rot on maize (Hebbar *et al*. 1998), *Pseudomonas* and *Bacilli* spp. strongly inhibited root rot caused by *Fusarium* spp. and charcoal rot caused by *Macrophomina phaseolina* (Tassi.) Goid (Pal *et al*. 2001), and *Bacillus subtilis* controlled banded leaf and sheath blight of maize caused by *R. solani* (Muis and Quimio, 2006).

PGPR thus have significant potential to improve maize yield in RSA through their growth promoting and disease supressing abilities. Furthermore, the effective utilization of PGPR could help reduce the excessive use of synthetic pesticides and fertilizers thereby increasing the profitability of the crop and promoting sustainable cultivation of maize in RSA. Since RSA is a participant of the United Nations Global Compact that promotes the development of sustainable practices (UNGC, 2013) research regarding PGPR on maize would be a valuable contribution.

1.2 Aim

Considering the aforementioned, the primary aim of this study was to investigate the efficacy of selected rhizobacteria from the University of Pretoria's-PGPR culture collection for the growth promotion of maize and biocontrol of *R. solani* on maize.

1.3 General objectives

The objectives addressed in this study were to:

- Assess the effect of PGPR on the seed germination, vigour and growth of maize seedlings of a reduced vigour cultivar, *in vitro* (chapter 3).

- Determine the ability of selected PGPR isolates, applied as two conventional and two novel formulations, to promote the growth of maize seedlings in the greenhouse (chapter 4).

- Evaluate the potential of rhizobacterial isolates to control *R. solani* on maize seedlings in laboratory and greenhouse trials (chapter 5).

- Determine the survivability of three selected PGPR isolates as a novel powder or liquid formulation on a carrier comprising composted chicken manure pellets, applied either singly or in combination (chapter 6).

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CHAPTER 2

Literature review of *Rhizoctonia solani* as a pathogen on maize seedlings and plant growth promoting rhizobacteria in agriculture

2.1 Introduction

Maize (*Zea mays* L.) is the staple food source and primary field crop grown in South Africa (RSA). More than 50% of farmland area in the country is planted with maize (Durand, 2006) and about 9 million tons on average has been produced every year since 1999 (FAO, 2010). To satisfy future demands for maize, an annual increase of 3% is required (Durand, 2006). According to Pratt *et al*. (2003), however; the demand is expected to double by the year 2020 in line with the projected global population increase of 35% from 1995 (Pinstrup-Andersen, 2001). One of the many challenges that need to be overcome before this demand can be met is the managing of pests and diseases. In Africa, the percentage crop losses due to plant disease during 1988-1990 were estimated at 15.7%. Rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) production suffer the largest crop losses due to plant diseases and approximately 10.9% of maize produced worldwide is lost to pathogen infections (Pinstrup-Andersen, 2001).

Diseases caused by the fungus *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) can have devastating effects on maize crops (Campbell, 1985). The fungus is frequently associated with maize seedling diseases (Hebbar *et al*. 1998) such as seed rots and damping-off of germinating seeds or weak seedlings (Sneh *et al*. 1996). Seedling diseases generally occurs when environmental conditions retard or decrease seed germination or seedling growth (Mc Gee, 1988). Mature maize plants are also affected by the pathogen which may result in crown and root rot, leaf and sheath blight and yellowing or wilting (Buddemeyer *et al*. 2004). Controlling *R. solani* on maize is challenging since the pathogen can survive in the soil or in plant debris and on roots of weeds and a variety of hosts used in maize crop rotations (Sumner and Bell, 1986).

For many years the extensive use of agrochemicals to control plant pathogens and ensure optimum crop yields was widely accepted (Niranjan *et al.* 2003b; Cummings, 2009). However, due to the increased cost and hazardous effect of chemicals on the environment and human health, consumers have been demanding the use of safer products which support sustainable crop production (Paulitz, 2006; Ongena and Jacques, 2008; Cummings, 2009; Al-Taweil *et al.* 2010). Nevertheless, many pesticides which are amongst the most

hazardous chemical products are still being used for crop protection in RSA (Pesticide Act 36 of 1947, 2010). The development of novel agriculture products which involves the use of beneficial microorganisms could offer an environmentally friendly alternative to the use of synthetic chemicals (Ongena and Jacques, 2008; Berg, 2009).

The application of soil microorganisms or more specifically plant growth promoting rhizobacteria (PGPR) in sustainable crop production programs has received much interest worldwide from farmers, industries and researchers (Zahir *et al.* 2004; Nezaret and Gohlami 2009; Kumar *et al.* 2011). Inoculation of plants with these bacteria are known to increase germination rate, seedling vigour, root and shoot weight (Niranjan *et al.* 2003a), leaf area (Gholami *et al.* 2009), nitrogen as well as magnesium, protein and chlorophyll content and consequently yield in a number of crops (Lucy *et al.* 2004). The role of PGPR as biological control agents of plant pathogens has also gained interest over the past few years, specifically in the production of organic foods (Fravel, 2005; Janczura *et al*. 2006; Babalola, 2010). PGPR have demonstrated their effectiveness in controlling numerous diseases (Compant *et al*. 2005; Pérez-García *et al*. 2011) including *R. solani* on a number of crops (Howell and Stipanovic, 1978; Muis and Quimio, 2006) including maize (Demirci *et al*. 2009).

2.2 *Rhizoctonia solani* as a pathogen on maize seedlings

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) is a common soil-borne fungus that infects a broad array of important agricultural crops worldwide (García *et al*. 2006). The pathogen has been reported on more than 150 plant species (Hsiang and Dean, 2001) which include important agricultural crops such as potatoes (*Solanum tuberosum* L.) (Demirci *et al*. 2009), cotton (*Gossypium* spp.) (Howell and Stipanovic, 1978), tomatoes (Cardinale *et al*. 2006), beans (*Phaseolus vulgaris* L.) (Nik and Yap 1979), sugar beet (*Beta vulgaris* L.) (Scholten *et al*. 2001) and cereals (Li *et al*. 1998). *Rhizoctonia solani* causes a variety of diseases depending on the host plant and fungal strain involved (García *et al*. 2006). Disease examples include, sharp eyespot of barley (*Hordeum vulgare* L.) (Mazzola, 1996), foliar blight of cabbage (*Brassica olearacea* L.) (Tu *et al*. 1996), soybean (*Glycine max* L.) stem rot and black scurf of potato (Pascual *et al.* 2000).

Identification and classification of *Rhizoctonia* species is based on classical, biochemical and molecular methods. Classical methods involve comparative studies of the teleomorphic reproductive stage of *Rhizoctonia* and differentiation based on the dolipore septum. The most important classical method however, is the system of anastomosis grouping that is based on hyphal fusion of different strains. If fungal hyphae of different strains are compatible, the strains are placed within the same anastomosis group (AG) and it is within these groups that biochemical and molecular approaches are further used to solve taxonomical questions (Liu *et al*. 1990; Sneh *et al*. 1996). At least 12 AGs have been described and these groups have been further divided into subgroups based on morphology, pathogenicity, DNA complementarity etc. (García *et al*. 2006). *Rhizoctonia* maize diseases are caused by strains from several AGs (Buddemeyer *et al*. 2004).

Currently, seed treatment with fungicides is the foremost method of disease control; however, fungi and bacteria are increasingly being investigated for their biocontrol ability against root pathogens such as *R. solani* (Hebbar *et al*. 1998; Ugoji and Laing 2007; Demirci *et al*. 2009). Development of current and future control strategies of *R. solani* relies on knowledge concerning the pathogens epidemiology, and disease cycle (Sneh *et al*. 1996). These topics will be discussed in this section.

2.2.1 Epidemiology and disease cycle of *Rhizoctonia solani*

Epidemiology refers to factors that influence disease occurrence, spread and severity. For disease to occur, a source of pathogen inoculum, a susceptible host and favourable

environmental conditions are required (Agrios, 2005). *Rhizoctonia solani* produces three types of inoculum namely hyphae, sclerotia and basidiospores. Generally, the pathogen occurs in nature as vegetative mycelium and sometimes sclerotia. The fungus is visible within soil or on infected plant debris as dark brown hyphae and/or small brown to black survival structures (sclerotia) (Sneh *et al*. 1996).

Infection is initiated when fungal hyphae, which are attracted by plant exudates, touch the plant and attach to its surface (Figure 2.1). Infection structures (appressorium or infection cushion) are then formed and penetration of plant cells follow. The mycelium invades the host and uses the plant nutrients for continued fungal growth and development of new inoculum (Keijer, 1996). Under favourable conditions the fungus grows rapidly and aggressively within soil, increasing significantly to such an extent that once established within a field, it survives there indefinitely (Baker and Martinson, 1970).

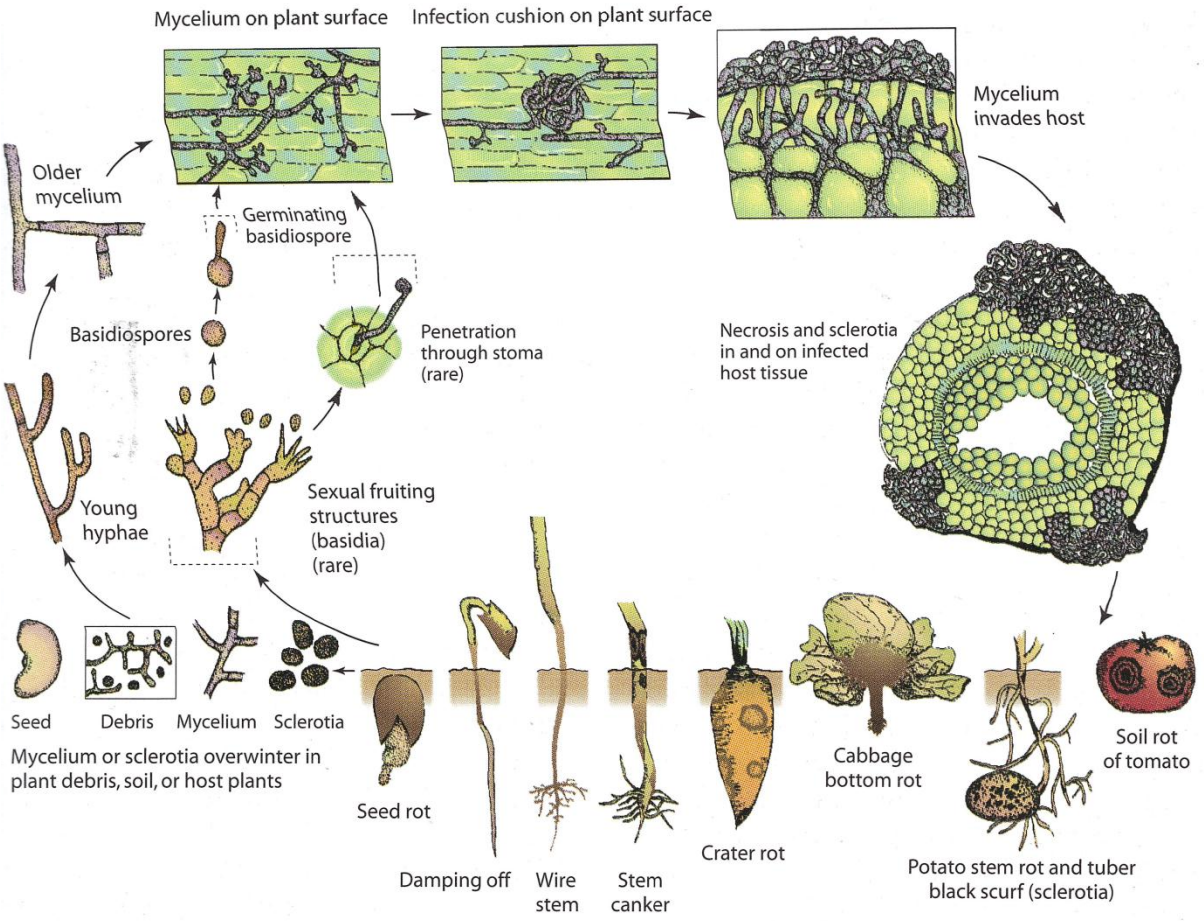

Figure 2.1: Disease cycle of *Rhizoctonia solani* (*Thanatephorus cucmeris*) (Agrios, 2005)

Rhizoctonia basidiospores (sexual spores) are fragile and sporadic and their development, survival and dissemination are highly dependent on environmental factors (Pascual *et al*. 2000). A high relative humidity or free water is required for basidiospores to germinate and when exposed to direct sunlight for longer than 60min germination fails to occur. Also, basidiospores are unable to survive in soil for long periods unless deposited directly on host plant parts where nutrients are readily available. Basidiospores are less important as a source of inoculum compared to hyphae and sclerotia. However, during extended cool and wet conditions basidiospores play an important role in long distance dispersal of the pathogen (Sneh *et al*. 1996).

Weeds and other rotation crops may serve as a reservoir for *R. solani* inoculum (Schillinger and Paulitz 2006). The fungus may also be transmitted by infected seeds that harbour the pathogen externally or internally within layers of the seed coat. Seed transmission may result in infection of soil previously free of *R. solani.* This emphasizes the importance of using pathogen-free seed or treated seed in which the fungus has been eradicated (Baker and Martinson, 1970; Laemmlen, 2001).

Rhizoctonia solani is disseminated by rainfall, flood or irrigation water and accidental transfer of contaminated soil on tools, machinery, seeds or propagative plant parts (Agrios, 2005). Whether infection will take place depends strongly on the growth rate of the fungus and the time it takes for seedlings to emerge. Seedlings that emerge and grow at a fast rate have an increased chance of escaping infection. Damaged seeds with poor seedling vigour are thus more susceptible than healthy seeds since the former mentioned develops at a slower rate (Baker and Martinson, 1970). Also, adverse conditions that delay seedling development increases disease incidence and disease severity, specifically of damping-off. Such conditions include prolonged periods of cold and wet soil with temperatures ranging at 10-13˚C and lower (Rane and Ruhl, 2002).

Increases in disease severity have been correlated with high inoculum densities (Paula *et al*. 2008). Inoculum potential is influenced by most environmental factors which include temperature, moisture and nutrient levels, microbes, soil compaction and soil texture. Inoculum levels are lower in clayey soils where compaction is more intense as compared to sandy soils where disease development is favoured (Sumner, 1996). Percentage moisture in the soil and air also contributes significantly to inoculum survival. Symptoms of damping-off are most severe between 20-80% soil moisture. Above 80% moisture, aeration is restricted and oxygen is limited thereby decreasing inoculum survival (Baker and Martinson, 1970).

2.2.2 Disease symptoms associated with damping-off

In general, damping-off is the most common symptom associated with *R. solani* infections of most hosts (Agrios, 2005). Maize seedlings may be affected prior to emergence (pre-emergence damping-off) or after emergence (post-emergence damping-off). The pre-emergence phase of damping-off may result from planting seed in infested soil or may be an extension of seed decay resulting from planting of infected seeds. Seed decay is the rotting of seeds prior to germination whereas damping-off causes seed rot after germination (Baker and Martinson, 1970; Campbell, 1985).

Post-emergence damping-off of maize occurs after the seedling has emerged through the soil surface (Baker and Martinson, 1970). Symptoms develop during the juvenile stage when the seedling is most susceptible. If conditions are unfavourable for disease development, symptoms decline with maturation as the plant tissues strengthen (Baker and Martinson, 1970). More than often however, damping-off leads to rapid death of the maize seedling (Campbell, 1985). Seedling death results from rotting of the hypocotyl at soil level which eventually causes the seedling to fall over. Infection often spreads downwards to the roots resulting in the characteristic 'spear-tip' symptom and root rot. Red-brown, sunken lesions develop on the roots and occasionally the hypocotyl and small, black sclerotia may be present on the infected plant parts (Wiese, 1987; Burgess *et al*. 2008).

2.2.3 Disease control

2.2.3.1 Chemical control

Disease control of *R. solani* is very difficult once the pathogen has established within a field (Laemmlen, 2001). The majority of control methods are therefore aimed at reducing inoculum levels and preventing distribution of *R. solani* into uninfected soil (Azzopardi *et al*. 2002). Application of fungicides containing pentachloronitrobenzene, thiophanate methyl and iprodione (Laemmlen, 2001) is the most widely recognized control method for *Rhizoctonia* diseases (Leach and Garber, 1970; Kataria and Gisi, 1996). The fungicide validamycin is especially important for control of *R. solani* strains on maize (Azzopardi *et al*. 2002). However, in South Africa, Celest® XL (Syngenta, 2013) and Acronis® (BASF, 2013) are generally used as fungicides for *Rhizoctonia* control on maize.

Since *R. solani* is such a versatile species, fungicides may be applied either as a foliar, soil or seed treatment depending on the host-pathogen interaction involved (Leach and Garber, 1970). Fungicidal seed treatment is applied to the majority of commercially produced maize to provide protection after planting, for up to 2-3 weeks against fungal infections of specifically damping-off (Kataria and Gisi, 1996; Munkvold and O'Mara, 2002). Soil applications in the form of drenches or granules have a longer lasting effect than seed treatments protecting crops such as potatoes, sugarbeet and cereals throughout the growing season. Foliar sprays are applied to aerial plant parts to control mostly stem and leaf blights caused by *R. solani* (Kataria and Gisi, 1996).

2.2.3.2 Biological control

Biological control of soil-borne pathogens, specifically *Rhizoctonia* species, has been well documented. Several successful biocontrol products have been commercially available for many years (Table 2.1) forming an integral part of crop production (Howell and Stipanovic, 1978; Hebbar *et al*. 1998; Jung *et al*. 2003). Today considerable research is still being done on development of new biocontrol formulations (Ugoji and Laing, 2007; Demirci *et al*. 2009; Montealegre *et al*. 2010).

A fungal species commonly associated with possessing biocontrol activity against *Rhizoctonia* diseases is *Trichoderma* sp. (Montealegre *et al*. 2010). Rini and Sulochana (2007) screened 26 *Trichoderma* isolates obtained from the rhizosphere of tomatoes for biocontrol efficiency against *R. solani* causing tomato root rot. Eleven of the *Trichoderma* isolates supressed mycelial growth of the pathogen *in vitro* with *Trichoderma pseudokoningii* Rifai TR17, *Trichoderma longibrachiatum* Rifai TR2 and *Trichoderma viride* Pers. TR22 resulting in the most inhibition namely 54%, 42% and 41% respectively (Rini and Sulochana, 2007). In a study by Rehman *et al*. (2012) *Trichoderma harzianum* Rifai and *Trichoderma viride* significantly reduced growth of *R. solani in vitro* by 85.5% and 83%, respectively, compared to the control. In field trials disease incidence of damping-off was reduced and percentage germination and seedling vigour of cauliflower was increased when treated with these antagonists compared to the untreated control (Rehman *et al*. 2012).

Table 2.1: Developed biocontrol formulations to supress *Rhizoctonia* diseases (Adapted from Lewis and Kuilk, 1996; Nakkeeran 2005)

Product	Biocontrol agent	Host	Manufacturer
Kodiac	*Bacillus subtilis*	beans, cotton, soybeans, peanuts	Gustafson, Inc. Plains, Texas, USA
Deny	*Burkholderia cepacia*	alfalfa, barley, beans, cotton, peas, sorghum, vegetable crops, wheat	Stine Microbial Products, Shawnee, KS
GlioGard	*Gliocladium virens*	bedding plants	W. R. Grace-Conn. Columbia, Maryland, USA
SoilGard	*Gliocladium virens*	bedding plants	W. R. Grace-Conn. Columbia, Maryland, USA
Rhizo-Plus	*B. subtillis*	greenhouse grown crops, forest tree seedlings, ornamentals	KFZB Biotechnik GMBH, Berlin, Germany.
Trichodex	*Trichoderma harzianum*	tomatoes, strawberries	Machteshim, Ltd. Beer Sheva, Israel
Campanion	*B. subtilis*	horticultural crops, turf	Growth products, USA
Intercept	*Pseudomonas cepacia*	maize, vegetables, cotton	Soil Technologies Corp, USA

Bacterial species commonly known to have antagonistic effects against *R. solani* include *Pseudomonas* (García *et al*. 2006; Rini and Sulochana, 2007) and *Bacillus* species (Jung *et al*. 2003). According to a study by Howell and Stipanovic (1978), the survival of cotton seedlings increased by 49% when seeds were treated with *Pseudomonas fluorescens* before planting in *R. solani* infested soil. Ugoji and Laing (2007) studied the biocontrol effect of *Bacillus* against *R. solani* on maize seedlings in rhizotrons. After a four week period results showed that *Bacillus* B77 and B81 effectively controlled *R. solani* (Figure 2.2) as indicated by the significant increase in root area and root and shoot dry mass in comparison with the untreated control. Isolate B81 had the strongest antagonistic effect towards *R. solani* controlling the pathogen by 48% and 35% as reflected by shoot and root dry mass (Ugoji and Laing, 2007). The mechanisms associated with biological control of plant pathogens using rhizosphere bacteria are further discussed in section 2.

Figure 2.2: Control of *R. solani* (RH) on maize by means of *Bacillus subtilis* Ehrenberg B77 and B81. Maize root (R), seed treatment (ST) (Ugoji and Laing, 2007).

2.2.3.3 Cultural practices

In addition to chemical and biological control, numerous crop management practises can be used to limit *R. solani* infections (Leach and Garber, 1970; Azzopardi *et al*. 2002; Agrios, 2005). Rotation with non-host crops for 1-4 years is one of the oldest and most used methods of disease control (Leach and Garber, 1970; Azzopardi *et al*. 2002). However, finding a non-host crop may sometimes be complicated since *R. solani* has such a broad host range. It is therefore important to ensure that crops used in the rotation sequence are not attacked by the same AG group already present in the soil (Schillinger and Paulitz, 2006).

In fields with no-till or minimum tillage practices, overlying plant residue create wet and cool soil conditions which enhance *Rhizoctonia* diseases (Baker and Martinson, 1970). Tilling the field allows moisture to evaporate rapidly so that soil can dry before fungal growth proliferates. Also, tillage reduces inoculum build-up by breaking up hyphal networks and altering microbial activity within soil to inhibit *R. solani* (Paulitz, 2006).

Other cultural methods that can limit *R. solani* infections include shallow planting of seeds to limit exposure of the seedling to the pathogen and to prevent damaging the seeds (Laemmlen, 2001). The time of planting also significantly influences disease incidence. Planting under optimal conditions for seed germination and seedling growth allows the plant

to escape infection by pathogens. Lastly, as mentioned previously the farmer should ensure that seeds, tubers and other propagating material are free of *R. solani* before planting (Leach and Garber, 1970).

2.3 Mechanisms and applications of plant growth promoting bacteria

Plant growth promoting rhizobacteria deploy a variety of mechanisms to promote plant growth either directly or indirectly. In general, bacteria that stimulate plant growth directly produce beneficial compounds that are utilized by the plant or assist plants in nutrient uptake from the surrounding soil environment (Verma *et al.* 2010). Direct modes of action may include nitrogen fixation, production of plant hormones and increasing availability of nutrients (which are mostly limited) for plant uptake (Van Elsas *et al.* 2007).

Direct growth promotion excludes the control of phytopathogens which relates to indirect growth promotion where the primary aim is to reduce or prevent plant disease occurrence (Zahir *et al.* 2004; Verma *et al.* 2010). Indirect modes of action may include production of antibiotics or lytic enzymes by PGPR for pathogen suppression, competition with plant pathogens for nutrients, induction of systemic resistance within the plant and synthesis of siderophores which chelate iron making it unavailable to soil inhabiting pathogens (Hayat *et al.* 2010; Labuschagne, 2010).

Certain generic terms have been adopted to class PGPR according to the primary mechanisms/activities by which they enhance plant growth (Labuschagne, 2010; Martínez-Viveros *et al.* 2010). According to Labuschagne (2010), PGPR can be classified as biological control agents that reduce or prevent plant disease, biopesticides that control plant pests, or biofertilizers that increase plant growth through improved nutrient acquisition (Figure 2.3). However, there is some inconsistency in the literature concerning the different classifications being used since numerous bacteria exert more than a single mode of action (Martínez-Viveros *et al.* 2010). Martínez-Viveros *et al*. (2010) also described three classes of PGPR, however, they grouped biopesticides and biocontrol agents into one class. PGPR that synthesize growth regulators were classed as phytostimulators and the third class i.e. biofertilizers was as described by Labuschagne (2010). For the purpose of this review the classification of PGPR according to Labuschagne (2010) will be adopted and the two most applicable classes to this study i.e. biocontrol and biofertilization will be discussed.

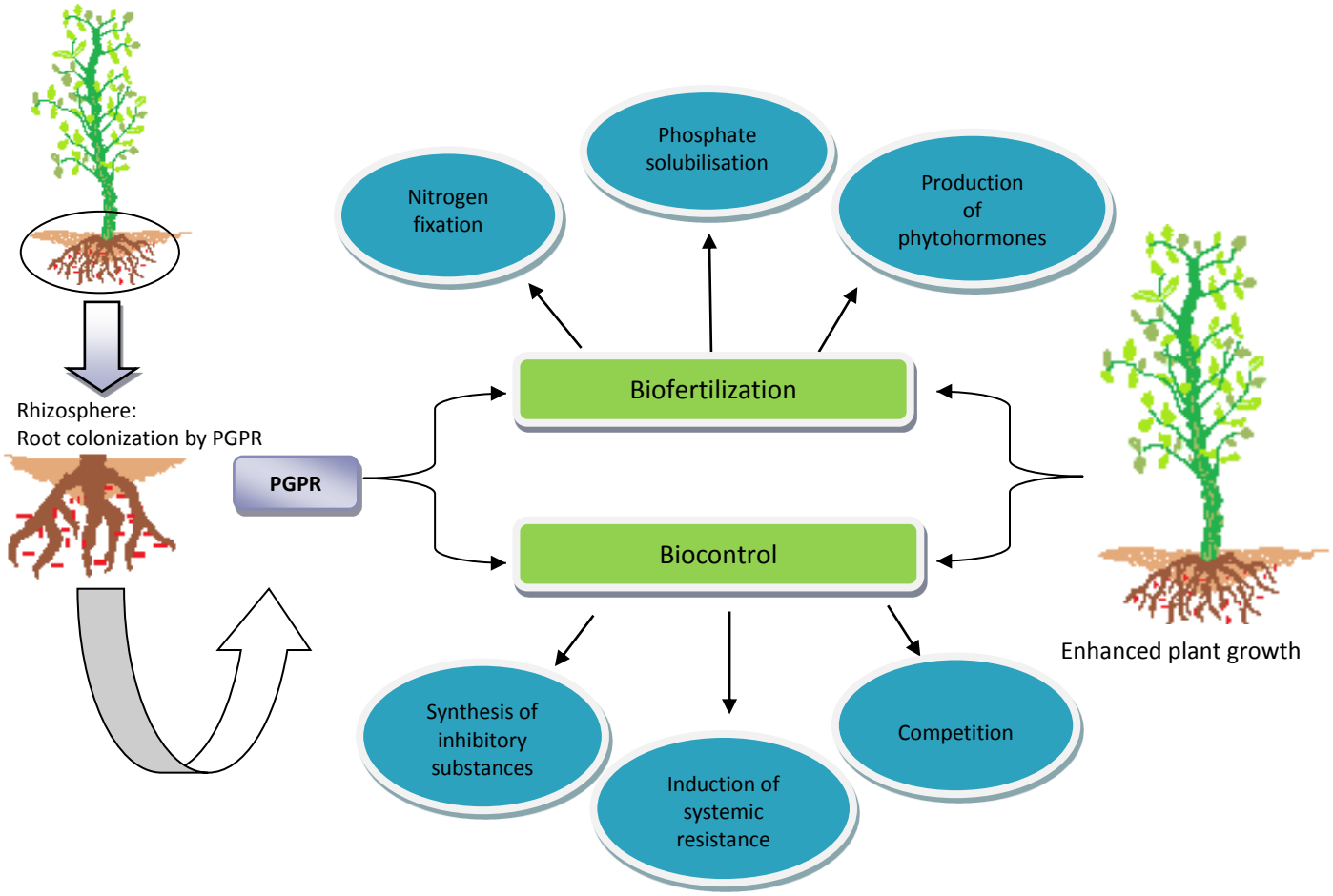

Figure 2.3: Important modes of action of PGPR for promoting plant growth (Adapted from Kumar *et al.* 2011)

2.3.1 Biological control

Phytopathogens are a major threat to crop production causing a significant decrease in plant performance, quality and yield (Martínez-Viveros *et al.* 2010; Kumar *et al.* 2011). At present, no less than 15-20% of all crops are lost due to pathogen infections occurring either in the field, or at some stage during preparation, processing or presentation to the customer (Dixon and Tilston, 2010). In extreme case for example canola infected with *Sclerotinia sclerotiorum* (Lib.) de Bary (the cause of stem rot), severe pathogen infection may wipe out the entire crop. Even losses as small as 1-5% annually, of an essential crop such as rice, can have a devastating economic impact resulting in huge financial losses (Chithrashree *et al.* 2011).

Currently, the primary means of controlling plant diseases is by use of fungicides and pesticides (Martínez-Viveros *et al.* 2010). Due to increased pathogen resistance to chemical pesticides, high production costs and detrimental environmental effects of these chemicals, alternative methods to replace/reduce pesticide usage are becoming more and more popular (Janczura *et al.* 2006; Kumar *et al.* 2011). In this context, biological control agents have become an integral part of agriculture over the past few years (Saharan and Nehra, 2011).

Certain PGPR are capable of suppressing a broad spectrum of plant diseases caused by bacteria, fungi, nematodes and in some cases viruses (Ramamoorthy *et al.* 2001; Saharan and Nehra, 2011). To date, most research concerning bacterial biological control agents have focused on species of *Bacillus, Pseudomonas, Streptomyces* and *Burkholderia*. Nonetheless, a large diversity of other biocontrol agents also contributes toward agricultural production (Kumar *et al.* 2011). The mechanisms by which PGPR control plant pathogens include predominantly synthesis of inhibitory substances, competition for space and nutrients and induced systemic resistance in host plants (Compant *et al.* 2005; Saharan and Nehra, 2011).

2.3.1.1 Synthesis of inhibitory substances

a) Antibiotic production

Antibiotics are organic chemical compounds of low molecular weight produced by a variety of microbes that primarily occur in the soil (Martínez-Viveros *et al.* 2010; Stewart *et al.* 2010). Antibiotic-mediated suppression of plant pathogens is one of the most studied and effective mechanisms of PGPR (Zahir *et al.* 2004; Compant *et al.* 2005). Most biocontrol PGPR are capable of synthesizing one or more antibiotics that kill or inhibit the growth of other microorganisms (Labuschagne, 2010). The means by which antibiotics affect pathogens may involve several mechanisms including interfering with protein or cell wall synthesis, affecting cell membrane integrity or disturbing nucleic acid metabolism (Van Elsas *et al.* 2007). Antibiotics produced by PGPR possess numerous properties that may be antioxidant, antitumor, phytotoxic, cytotoxic, antihelminthic, antiviral, antibacterial and antifungal (Fernando *et al.* 2005).

Although a wide range of antibiotics are produced by different genera of biocontrol strains (Raaijmakers *et al.* 2002), researchers have concentrated mostly on *Pseudomonas* and *Bacillus* species (Kumar *et al.* 2011). Antibiotics produced by *Pseudomonas* species include viscosinamide, butyrolactones, oomycin A, phenazine-1-carboxcylic acid, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, ecomycins, cepaciamide A and pseudomonic acid (Fernando *et al.* 2005). DAPG is considered one of the most important antibiotics produced by PGPR in view of its antifungal (Loper and Gross, 2007), antibacterial (Velusamy *et al.* 2006) and antihelminthic properties (Cronin *et al.* 1997). Field trials by Velusamy *et al.* (2006) showed that rice bacterial blight was inhibited by DAPG-producing *Psudomonas fluorescence* Migula by up to 64%. The compound is also suppressive to the

destructive take-all disease of wheat caused by the fungus *Gaeumannomyces graminis* var. tritici J. Walker. A variety of seedling and root diseases are suppressed by strains of *P. fluorescens* that synthesise DAPG (Babalola, 2010). For example, Pythium root rot of cucumber (*Cucumis sativus* L.) was significantly inhibited by enhancing DAPG and pyolutoerin production in *P. fluorescens* strain CHA0 (Raaijmakers *et al.* 2002).

Various strains and species of *Bacillus* are also known to produce a range of disease suppressive antibiotics (Stewart *et al.* 2010). For instance, the antifungal antibiotic zwittermycin A produced by *Bacillus cereus* Frankland & Frankland strain UW85, inhibited damping-off of alfalfa caused by *Phytophthora medicaginis* E.M. Hansen & D.P. Maxwell (Silo-Suh *et al.* 1994). Zwittermycin A is inhibitory against numerous other fungal pathogens as well as oomycetes. The antibiotic is also produced by *Bacillus thuringiensis* Berliner where it is known to be insecticidal (Fernando *et al.* 2005).

Certain strains of *B. subtilis* produce a cyclic lipopeptide antibiotic, surfactin (Fernando *et al.* 2005). The compound demonstrated antibacterial activity against *Pseudomonas syringae* infection in *Arabidopsis thaliana* (L.) (Kumar *et al.* 2011). *Bacillus subtilis* also produces the antibiotic bacillomycin which is in the same family as the iturin class. Compounds such as iturin A and bacillomycin D, bacillomycin F and bacillomycin L are very effective against fungal pathogens (Fernando *et al.* 2005). According to a study by Moyne *et al.* (2001), two peptide analogs of bacillomycin D produced by *B. subtilis* strain AU195 demonstrated powerful antifungal activity against *Aspergillus flavus* Link. In another study, *B. subtilis* stain RB 14-C significantly reduced *R. solani* infection on tomato upon introduction into the soil. Disease suppression was correlated with production of iturin A by RB 14-C (Stewart *et al.* 2010). Iturin A also controls various other pathogens such as *Sclerotinia sclerotiorum*, *Fusarium oxysporum* f. sp. *lini* and *Pythium ultimum* Trow (Fernando *et al.* 2005).

b) Siderophore production

Iron is frequently present in the soil as ferric iron, a form which is mostly insoluble and unavailable for direct use by microorganisms (Van Elsas *et al.* 2007). Therefore, certain microbes have developed the ability to produce small iron-binding molecules, termed siderophores (Stewart *et al.* 2010). Siderophores chelate ferric iron and transport it across the bacterial cell membrane into the cell, thereby depleting the surrounding soil environment of iron. Consequently siderophore producing bacteria help to protect plants against pathogen attack since low concentrations of iron in the soil limit pathogen growth (Hayat *et al.* 2010).

Siderophores are synthesized by various bacterial genera including *Bradyrhizobium,* (Jadhav *et al.* 1994; Arora *et al.* 2001), *Bacillus*, *Agrobacterium* (Zahir *et al.* 2004), *Serratia* and *Streptomyces* (Martínez-Viveros *et al.* 2010). Most siderophore related studies have, however, primarily focused on *Pseudomonas* spp. due to their proposed role in the control of soil-borne pathogens and disease suppressive soils (Scher and Baker 1982; Loper and Buyer, 1991; Stewart *et al.* 2010). *Pseudomonas* spp. produce primarily two types of siderophores termed pseudobactins and pyochelins (Labuschagne, 2010; Stewart *et al.* 2010). To assess the importance of siderophores in biological control, mutant *Pseudomonas* strains that are deficient in siderophore production are primarily used (Stewart *et al.* 2010).

Control of soil inhabiting pathogens through siderophore producing *Pseudomonas* spp. has previously been reported. According to a study by Scher and Baker (1982), a siderophore producing strain of *Pseudomonas putida* caused a significant reduction in Fusarium wilt of radish (*Raphanus sativus* L.), flax (*Linum usitatissimum* L.) and cucumber. The soil became suppressive to *Fusarium oxysporum* due to reduced iron conditions after *P. putida* was added to the soil. In another study, *P. fluorescens* Migula strain 3551 reduced seed colonisation and pre-emergence damping-off of cotton caused by *Pythium ultimum*. The increased disease control was directly related to siderophore production of *P. fluorescens* strain 3351 (Loper, 1988). In a study conducted by Kloepper *et al.* (1980), soil inoculated with either fluorescent *Pseudomonas* strain BI0 or the siderophore (pseudobactins) it produces, became suppressive to both *F. oxysporum* and *G. graminis* var. *tritici.*

c) Secretion of lytic enzymes

Another mechanism by which bacteria suppress soil-borne pathogens directly is by producing and releasing lytic enzymes (Ordentlich *et al.* 1988; Whipps 2001). These enzymes hydrolyze compounds such as proteins, chitin and cellulose consequently inhibiting hyphal growth of pathogenic fungi (Pal and Gardener, 2006). Particularly PGPR that produce the enzymes chitinases has been reported to deliver satisfactory levels of disease control (Jung *et al.* 2003; Compant *et al.* 2005; Fernando *et al.* 2007; Hariprasad *et al.* 2011). This enzyme degrades chitin which is one of the main constituents of fungal cell walls (Labuschagne, 2010; Hariprasad *et al.* 2011).

In a study by Ordentlich *et al.* (1988), the soil inhabiting bacterium, *Serratia marcescens* Bizio, significantly inhibited the fungus *Sclerotium rolfsii* Sacc. by up to 75% under greenhouse conditions. Results indicated that fungal inhibition was due to the chitinolytic activities of *S. marcescens* causing deformation and lyses of the pathogens hyphal tips.

Hariprasad *et al.* (2011) demonstrated that the synthesis of extracellular chitinases by *B. subtilis* isolate CRB20 caused a significant reduction in Fusarium wilt of tomatoes in the greenhouse. Furthermore, Jung *et al.* (2003) found that the chitinolytic bacterium, *Paenibacillus illinoisensis* KJA-424, inhibited hyphal growth of *R. solani.* Swelling and lyses of the fungal hyphal tips were observed under the light and scanning electron microscope (Jung *et al.* 2003).

Besides chitin, β-1.3-glucan is another major component of fungal cell walls. Bacteria that produce the enzyme β-1.3-glucanase catalyse the breakdown of β-1.3-glucan linkages and therefore weaken the fungal cell wall rendering these bacteria potential biocontrol agents. Hong and Meng (2003) purified the hydrolytic enzyme β-1.3-glucanase from a *Paenibacillus* strain to determine the enzymes antifungal activity against *Pythium aphanidermatum* (Edson) Fitzp. and *R. solani* AG-4. By observing the fungal hyphae under a phase-contrast microscope, it was clear that β-1.3-glucanase damaged the cell walls of the growing hyphae (Hong and Meng, 2003). In a similar study, Park *et al.* (2012) purified β-1.3-glucanase from a *Streptomyces torulosus* Lyons & Pridham PCPOK-0324 culture. *In vitro* trials revealed a strong antifungal effect towards *Phytophthora capsici* Leonian and *R. solani* inhibiting mycelial growth on PDA amended with purified β-1.3-glucanase (Park *et al.* 2012).

2.3.1.2 Induction of systemic resistance

Various natural defence mechanisms present within plants allow for protection against biotic stresses such as insects, parasites, viruses and pathogens. However, pathogens are able to overcome these mechanisms by suppressing the plants resistance reactions, evading the defence responses, or not triggering resistance mechanisms at all. In order to reduce disease intensity, resistance mechanisms within plants need to be activated by a stimulus before infection by a virulent pathogen occurs (Van Loon *et al.* 1998; Dutta *et al.* 2008). This phenomenon is referred to as induced resistance, and is defined as the physiological state of enhanced resistance that develops within a plant, in response to appropriate biotic stimuli (Van Loon *et al.* 1998; Choudhary *et al.* 2007).

Generally, the increased defence is not limited to the area of primary infection, but is also detected in non-infected plant tissues (Loon *et al.* 1998) and is therefore referred to as systemic resistance. The two primary types of systemic resistance is systemic acquired resistance (SAR) and induced systemic resistance (ISR). The latter is triggered by PGPR whereas SAR is activated by virulent, avirulent and non-pathogenic microorganisms

(Ramamoorthy *et al*. 2001). Also, ISR requires jasmonate and ethylene to regulate various pathways and does not involve accumulation of pathogenesis-related proteins and salicylic acid as in the case of SAR (Choudhary *et al.* 2007).

PGPR elicit ISR in plants by strengthening the cell wall structure and triggering certain reactions that activate production of defence chemicals (Ramamoorthy *et al.* 2001; Saharan and Nehra, 2011). For example, enhanced cell wall lignification was detected in roots of bean seeds treated with *Pseudomonas putida* (Anderson and Guerra, 1985). In another study, *Pseudomonas fluorescens* strain 63-28 induced defence reactions in peas leading to the formation of physical barriers enhancing resistance. This involved callose-enriched wall appositions and increased phenolic compounds within cell walls, which enhanced resistance against *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou *et al.* 1996). Similar results were obtained by M'Piga *et al.* (1997) when tomato plants were treated with *P. fluorescens* strain 63-28 i.e. callose depositions and increased phenolic compounds in the cell wall. The induced resistance proved to be effective in that fungal colonization by *F. oxysporum* f. sp. *radicis-lycopersici* was significantly reduced and restricted to intercellular spaces of the roots outer cortex (M'Piga *et al.* 1997).

Besides *Pseudomonas* strains, *Bacillus* PGPR are also known to produce ISR within plants of agricultural importance. In rice for example, seed bacterization with *Bacillus pumilis* strain SE34 and *B. subtilis* GBO3 induced systemic resistance against *Xanthomonas oryzae* pv. *oryzae* which causes bacterial leaf blight. An increased accumulation of defence enzymes such as phenylalanine ammonia lyase and polyphenol oxidase was detected. These enzymes are required for the production of lignin, phytoalexins and phenolics which play an important role in disease resistance (Chithrashree *et al.* 2011). In a study by Enebak and Carey (2000), systemic resistance was induced in loblolly pine seedlings following seed treatment with three strains of *B. pumilus.* Significant resistance was achieved against loblolly rust caused by *Cronartium quercuum* f. sp*. fusiforme* Burds. & G.A. Snow (Enebak and Carey, 2000). Several other strains of *Bacillus* such as *B. amyloliquifaciens*, *B. cereus*, *B. pasteurii* and *B. mycoides* have also been reported to induced systemic resistance in a number of crops including bell pepper (*Capsicum annuum* L.), cucumber, tobacco (*Nicotiana tabacum* L.), sugarbeet, tropical crops and watermelon (Citrullus lanatus (Thunb.) Matsum & Nakai) (Kloepper *et al*. 2004).

2.3.1.3 Competition

Another mechanism by which PGPR control plant pathogens, is through competition with other microorganisms for nutrients and appropriate niches (Chet *et al.* 1990; Bashan and de-Bashan, 2005; Compant *et al.* 2005). Nutrients are released in the soil as root or seed exudates and are mostly limited from a microbial point of view. Consequently, the most suitable microbial niche is in the rhizosphere surrounding the roots or the root surface itself (Pal and Gardener, 2006; Raaijmakers *et al.* 2009). PGPR thus need to successfully colonize the rhizosphere and host plant roots to displace competing microorganisms such as plant pathogens (Chet *et al.* 1990; Pal and Gardener, 2006). Inability to colonize the rhizosphere or plant roots renders that strain of PGPR infective as a biological control agent, regardless of the modes of action involved (Montealegre *et al.* 2003; Raaijmakers *et al.* 2009).

Yan *et al.* (2003) compared the application of PGPR as seed treatments to mixing PGPR into Promix™ soilless medium prepared for tomato transplants. Results showed a significant increase in growth of tomatoes planted after *P. fluorescens* strain SE34r was mixed into soilless medium compared to seed treatment application. Application of PGPR into soilless medium resulted in uniform colonization throughout the roots whereas seed inoculation had considerably higher numbers in the taproots and upper roots than the rest of the root system. The difference in growth promotion resulting from different application methods may therefore be due to variation in colonization patterns, and demonstrates the importance of effective colonization (Yan *et al.* 2003).

Buddrus-Schiemann *et al.* (2010) conducted a study to establish the colonization of the growth promoting *Pseudomonas* sp. strain DSMZ 13134 which is present in the commercial product Proradix®. Confocal laser scanning microscopy results showed that *Pseudomonas* sp. strain DSMZ 13134 effectively colonized the rhizoplane as well as the entire root system of barley. The bacterial strain proved to be an excellent competitor and successfully displaced seed- and soil-borne bacteria in both greenhouse and field trials (Buddrus-Schiemann *et al.* 2010). The importance of effective root colonization cannot be over emphasized and research in this regard is of great importance when dealing with potential biocontrol agents.

2.3.2 Biofertilization

The term ‘biofertilizer’, has several different definitions and interpretations (Bashan, 1998; Mahdi *et al.* 2010; Martínez-Viveros *et al.* 2010). The word has been used in the literature in a broad sense describing anything from manure to plant extract products. According to Vessey (2003), a biofertilizer is a preparation containing living microorganisms that directly stimulate plant growth by facilitating nutrient uptake after effectively colonizing plants or the surrounding soil area, when applied as a soil, seed or plant surface treatment. This definition therefore excludes organic fertilizers such as manure since aforementioned does not rely on living microorganisms to promote plant growth but rather depends on organic compounds which as a result of product decay stimulate plant growth (Vessey, 2003). For the purpose of this review, the definition for biofertilizers according to Vessey (2003) will be adopted.

The use of biofertilizers as an alternative or supplement to chemical fertilizers has increased significantly over the past few years (Martínez-Viveros *et al.* 2010). One of the primary reasons is that compared to synthetic chemicals, biofertilizers offer a more economical approach (Bloemberg and Lugtenberg, 2001; Mahdi *et al.* 2010). Many developing countries rely on biofertilizers to enhance food security, specifically in low input crop production systems where smallholder farmers cannot afford costly chemicals (Harman *et al.* 2010). In addition, biofertilizers promote sustainable agriculture by maintaining soil fertility through natural biological systems involving microorganisms (Adesemoye *et al.* 2009). The use of biofertilizers in crop production has therefore become an important research topic with PGPR being the focus of these studies (Vessey, 2003; Adesemoye *et al.* 2009; Al-Taweil *et al.* 2010; Martínez-Viveros *et al.* 2010).

PGPR play an important role as biofertilizers by increasing the amount of available nutrients for plant uptake (Yazdani *et al.* 2011). Nitrogen (N) and phosphorous (P) are essential growth elements that are primarily inaccessible to plants (Kumar *et al.* 2011). Certain PGPR can fix atmospheric nitrogen and solubilise soil phosphates converting these nutrients into forms available for plant uptake (Mahdi *et al.* 2010; Martínez-Viveros *et al.* 2010). Besides N fixation and phosphate solubilisation, PGPR also stimulate plant growth directly by producing phytohormones or plant growth regulators (Boiero *et al.* 2007). PGPR that utilize above mentioned modes of action will be discussed in more detail below.

2.3.2.1 Nitrogen fixation

Nitrogen is essential for plant growth and development being a requirement for enzyme, protein, chlorophyll, DNA and RNA synthesis (Hayat *et al.* 2010). For plants to be able to utilize N however, it must be converted from its atmospheric form, dinitrogen to ammonia. This process of conversion involves N-fixing bacteria such as rhizobia or PGPR, and is referred to as biological N fixation (Keyeo *et al*. 2011). Rhizobia form a symbiotic relationship involving nodule formation with primarily legumes, where the bacterium fixes N for the plant in exchange for fixed carbon released from plant roots. Similarly, PGPR fix N in return for carbon. However, these bacteria are free-living and establish an associative rather than a symbiotic interaction with plants (Bloemberg and Lugtenberg, 2001).

PGPR that have been identified as N-fixing bacteria include *Azotobacter, Azospirillum, Acetobacter*, *Herbaspirillum* (Bloemberg and Lugtenberg, 2001), *Enterobacter, Pseudomonas* (Hayat *et al.* 2010) and *Paenibacillus* (Kumar *et al.* 2011). These bacteria fix N under limiting oxygen conditions by using a nitrogenase complex (Bloemberg and Lugtenberg, 2001). Increased N uptake and yield in C3 and C4 crops such as maize, wheat, rice, sugarcane (*Saccharum* L.) and cotton treated with N-fixing PGPR has previously been reported (Hayat *et al.* 2010). Many of these reports include crop inoculation with specifically *Azospirillum* strains (Kennedy *et al.* 2004; Zahir *et al.* 2004). For example Rodrigues *et al*. (2008) reported that strains of *Azospirillum amazonense* enhanced N uptake, grain dry matter and amount of panicles of rice. Similarly, Saubidet *et al.* (2002) detected increased N content, grain yield and plant mass of wheat inoculated with strains of *Azospirillum brasilense*.

PGPR with N-fixing ability contribute significantly to sustainable agriculture by reducing the level of synthetic fertilizers required (Adesemoye *et al.* 2009). Keyeo *et al*. (2011) reported that N-fixing *Enterobacter* strain L2 in combination with minimal nitrogen fertilizer application (25%), promoted rice growth to such an extent that yields were comparable to those obtained for 100% N application. Also, chlorophyll and protein content of rice leaves treated with strain L2 in the absence of fertilizers were comparable to those observed where 100% nitrogen fertilizer was applied (Keyeo *et al*. 2011). Ekin *et al.* (2009) reported that inoculation of potato tubers with *Bacillus* sp. OSU-142 (N-fixing bacterium) in the field resulted in similar potato tuber yields as obtained when nitrogen was applied at 120 kg/ha. In combination however, all growth parameters were increased significantly and resulted in considerably higher yields than with individual application of either *Bacillus* or N (Ekin *et al.* 2009).

2.3.2.2 Phosphate solubilisation

Phosphorous (P) plays an essential role in plant growth by affecting seed, root and flower development, stem and stalk strength and resistance to phytopathogens (Khan *et al.* 2009). Although a large reserve of P is present in the soil, this nutrient is mostly limited for plant uptake because it primarily occurs in the insoluble form (Chabot *et al.* 1996; Vessey, 2003). Soluble forms of P are present in minute micromolar amounts or in concentrations of 1.0 mg/kg (Khan *et al.* 2009), which is very low compared to the majority of other soil minerals present in minimolar quantities (Kumar *et al.* 2011). Plants can only absorb P from the soil solution when it is converted to the soluble form known as phosphate (Pi, $H2PO4^-$) (Kumar *et al.* 2011). Application of P fertilizers to increase soluble P in soil is not very efficient, since P fixation and precipitation with calcium (Ca), iron (Fe) and aluminium (Al) occurs (Chabot *et al.* 1996; Khan *et al.* 2009). Addition of phosphate solubilizing bacteria however, may help increase the amount of available P in the soil (Chabot *et al.* 1996).

A variety of rhizospheric bacteria are capable of solubilising P and converting it to plant-available forms (Vessey, 2003). These bacteria are referred to as phosphate solubilizing bacteria (PSB). The mode of action of PSB involves synthesis of organic acids, which solubilise insoluble phosphates by chelating Al, Fe and Ca cations, consequently decreasing the soil pH and preventing phosphate soil adsorption (Khan *et al.* 2009). Certain PGPR are known to possess P solubilising characteristics in addition to plant growth promoting activities (Chabot *et al.* 1996). For example, *Serratia* sp. strain 22b and *Pseudomonas* sp. strain 24, increased dry matter content of lettuce (*Lactuca sativa* L.) and maize shoots by converting insoluble P to plant-available forms (Chabot *et al.* 1996). In a study by Hameeda *et al.* (2008), maize inoculation with *Serratia marcescens* EB 67 enhanced P uptake resulting in a significant increase in dry weight under both greenhouse and field conditions. Inoculation also promoted root and shoot length as well as vigour index of maize in papertowel trials (Hameeda *et al.* 2008).

Several studies have shown that application of PGPR with phosphate solubilising activity in conjunction with P fertilizers results in better yields compared to application of either one alone (Stamford *et al.* 2003; Ekin, 2010; Yazdani *et al.* 2009). When sunflower seeds were treated with PGPR *Bacillus* (strain M-13), kernel ratio increased by 3.4%, oil yield by 24.7% and P content by 13.2%. However, in combination with P fertilizers results were even more promising. Significant increases of 7.7%, 84.8% and 28% were obtained for kernel ratio, oil yield and P content, respectively (Ekin, 2010). Sundara *et al.* (2002) also reported that combined application of P fertilizer and PSB delivered greater effects than individual

application. Addition of PSB *Bacillus megaterium* var. *phospaticum* to sugarcane reduced the P fertilizer rate generally required for sugarcane production by 25%. In other words, the same results were obtained when 100% P fertilizer was applied as when 75% P fertilizer was applied in addition with PSB (Sundara *et al.* 2002).

Worth mentioning however, is that even though bacteria may act as PSB, these microorganisms do not necessarily have plant growth promoting activity. For example, no growth promoting effects were observed when soybean seedlings were treated with PSB in a study by Cattelan *et al.* (1999). PSB that do however promote growth, are of great importance as potential biofertilizers in agriculture, and future crop production is highly dependent on these bioinoculants as a source of available phosphorous (Kumar *et al.* 2011).

2.3.2.3 Production of phytohormones

The production of phytohormones is considered one of the most important modes of direct plant growth promotion by PGPR, following nitrogen fixation (Boiero *et al.* 2007). Phytohormones are known to affect plant growth by interfering with processes such as cell division, elongation and differentiation of specifically the roots (Kumar *et al.* 2011). Since roots are responsible for the uptake of plant nutrients and water from the soil, a well-developed root system is a prerequisite for increased plant growth (Adesemoye *et al.* 2009).

Increased seed germination and vigour index (VI) of crops is often associated with phytohormone producing PGPR. Vigour index gives an indication of a seedlings health and therefore ability to survive in the field under variable, mostly unfavourable environmental conditions (Milosevic *et al*. 2010). VI is determined by taking into account percentage seed germination and seedling length (Miguel and Fihlo *et al*. 2002). Seed treatment of a variety of crops including maize, soybean (Cassan *et al*. 2009), tomato (Manikandan *et al*. 2010), safflower (*Carthamus tinctorius* L.) (Govindappa *et al*. 2010), pearl millet (*Pennisetum glaucum* L.) (Niranjan *et al*. 2004), wheat (Shaukat *et al*. 2006) and sunflower (*Helianthus annuus* L.) with phytohormone producing PGPR strains, have resulted in significant increases in VI above the control. The primary hormone associated with increased VI is indole-3-acetic acid (IAA). Approximately 80% of rhizosphere inhabiting bacteria are known to produce IAA (Hayat *et al.* 2010). Low concentrations of this phytohormone is generally known to stimulate root growth by promoting primary root length and development of adventitious and lateral roots (Khakipour *et al.* 2008; Shahab *et al.* 2009). The growth

promoting effects of IAA producing PGPR have been illustrated on numerous agricultural crops (Table 2.2).

Another plant hormone produced by PGPR is cytokinin. Treatment of plants with cytokinins primarily results in enhanced cell division and tissue expansion which may stimulate seed germination, leaf growth, bud formation and reproductive development (Timmusk *et al.* 1999; Vessey, 2003). Although identification and quantification of cytokinins is usually complicated due to the small amount available in biological samples, researchers have shown that plant growth promotion by PGPR is somehow associated with cytokinin biosynthesis (Hayat *et al.* 2010). PGPR that are known to produce cytokinins generally include *Bacillus* spp. (Arkhipova and Anokhina, 2009) *Paenibacillus polymyxa* Prazmowski (Timmusk *et al.* 1999) and *P. fluorescens* (Salamone *et al.* 2001). In a study by Arkhipova and Anokhina (2009), wheat inoculation with cytokinin-producing *Bacillus* sp. significantly increased dry and wet mass of plants compared to the control. In another study, Arkhipova *et al*. (2007) showed that addition of the cytokinin-producing *Bacillus* strain IB-22 to drought stressed lettuce plants increased shoot mass.

Table 2.2: Indole-3-acetic acid producing PGPR known to promote plant growth of numerous crops (adopted from Vessey, 2003)

PGPR	Host	Reference
Aeromonas veronii	Rice	Mehnaz *et al.* 2001
Agrobacterium sp.	Lettuce	Barazani and Friedman, 1999
Alcaligenes piechaudii	Lettuce	Barazani and Friedman, 1999
Azospirillum brasilense	Wheat	Kaushik *et al.* 2002
Azotobacter sp.	Maize	Zahir *et al.* 2000
Bacilllus thuringiensis	Mung beans	Shahab *et al.* 2009
Bacillus spp.	Spinach and wheat	Çakmakçı *et al.* 2007
Bradyrhizobium sp.	Radish	Antoun *et al.* 1998
Comamonas acidovorans	Lettuce	Barazani and Friedman, 1999
Enterobacter cloacae	Rice	Mehnaz *et al.* 2001
Enterobacter sp.	Sugarcane	Mirza *et al.* 2001
Enterobacter sp.	Rice	Keyeo *et al.* 2011
Rhizobium leguminosarum	Radish	Antoun *et al.* 1998

Gibberellins or gibberellic acid (GA) is another class of phytohormones that regulate plant growth by affecting various physiological processes. Enhanced shoot length is the most prominent effect GA has on plants (Vessey, 2003); however, increased seed germination and nodule formation have also been reported (Cassan *et al.* 2009). Approximately 130 GAs produced by bacteria, fungi and plants have been identified, with GA_1, GA_3 and GA_4 being the most important regarding shoot elongation (Cassan *et al.* 2001). In the study by Cassan *et al.* (2001), two dwarf rice mutants incapable of GA_1 synthesis were inoculated with two

Azospirillum spp. strains and supplemented with 3-deoxy GA_{20} glucosyle ester, the precursor required for GA_1 biosynthesis. Hydrolysis of 3-deoxy GA_{20} glucosyle ester to GA_1 by the two *Azospirillum* spp. strains resulted in increased shoot length of the dwarf rice mutants. *Azospirillum* spp. are well known in agriculture for their plant growth promoting effect. The study by Cassan *et al.* (2001) demonstrates their importance in the synthesis of GA_1 and the role of GA_1 in plant growth.

2.3.2.4 Production of siderophores

As mentioned before, iron exists in the soil in the ferric form, which as with microorganisms is also unavailable uptake by plants (Bar-ness *et al*. 1992). Research has demonstrated that plant roots can acquire and utilize iron from microbial siderophores in the soil. In a study by Mahmoud and Abd-Alla (2001) *in vivo* and *in vitro* results showed that nodulation, nitrogenase activity, dry mass and nitrogen content of mungbean (*Vigna radiata* L.) was significantly increased above the control after inoculation with *Bradyrhizobium*. Chemical assays indicated that these bacteria produced hydroxamate siderophores which served as a source of iron to the plants (Mahmoud and Abd-Alla, 2001). Other microbial siderophores which have also been shown to enhance plant growth include the catechol siderophore and the catechol-hydroxamate-hydroxy-acid siderophores (Crowley *et al*. 1998).

The mechanism for obtaining iron from siderophores varies between plant species and therefore only certain siderophores can be utilized by individual plant species (Crowley *et al*. 1998). Oats, maize, cotton and sunflower are known to obtain iron from ferrioxamine B, a hydroxamate siderophore (Bar-ness *et al*. 1992). The mechanism by which plants utilize siderophores is unclear; however, it has been suggested that the plant enzyme, NADH:nitrate reductase plays a role in catalysing the reduction of hydroxamate siderophores (Smarrelli and Castignetti, 1986). It has also been proposed that plants obtain inorganic iron by a mechanism involving a plasma membrane bound reductase-carrier protein which transports iron across the root cell membranes (Romheld and Marschner, 1983). Although, not well understood, microbial siderophores play an important role in direct plant growth promotion and further studies about their use in iron deficient soils are important.

2.4 Factors affecting PGPR survival and activity in the soil

Developing PGPR products for large-scale commercial use is generally a slow and challenging process requiring major research efforts (Nakkeeran, 2005; Spadaro and Gullino, 2005). Years of extensive research in simplified environments have delivered many promising PGPR strains; however, numerous failures to replicate the same successful results in the field have been reported (Table 2.3) (Van Veen *et al.* 1997, Bashan and de-Bashan, 2005; Martínez-Viveros *et al.* 2010; Saharan and Nehra, 2011). This constitutes the foremost limitation in commercialization of PGPR products namely the fact that success achieved with regards to plant growth promotion or biocontrol in laboratory or greenhouse trials, does not necessarily mean success in the field (Fuentes-Ramirez and Caballero-Mellado, 2005; Maiyappan *et al.* 2010; Roy, 2010).

Failure of PGPR to perform in field trials is primarily attributed to the rapid decrease in bacterial numbers after inoculation into the unpredictable soil environment (Van Veen *et al.* 1997; Roy, 2010). This phenomenon whereby bacterial growth or survival is inhibited when applied to natural soil is known as soil microbiostasis (Ho and Ko, 1985). Various abiotic factors for example soil pH, texture and temperature as well as biotic factors such as the presence of indigenous microorganisms determines the degree of soil microbiostasis (Van Veen *et al.* 1997; Mahdi *et al.* 2010). Many other factors, specifically adverse climatic conditions that cause stress to bacterial inoculants also contribute to the inconsistent performance of PGPR in the field (Maiyappan *et al.* 2010).

Although many successful PGPR products are already available on the market, concerns have been raised regarding the practical application of PGPR in agricultural soils due to inconsistent performance (Saharan and Nehra, 2011). If however, the influence of ecological factors on PGPR populations is known, one will be able to predict the conditions under which growth promotion can be achieved consistently (Green *et al.* 2006; Schweitzer *et al.* 2008; Saharan and Nehra, 2011). PGPR inoculant formulations can then be developed in favour of the beneficial bacterial to increase their chances of survival under adverse conditions (Bashan, 1998; Roy, 2010). Consequently, it is essential to obtain a better understanding of how the biotic and abiotic factors influence PGPR survival following their release into the soil (Table 2.4) (Van Veen *et al.* 1997)**.**

Table 2.3: Plant crop response to PGPR inoculation under different experimental conditions
(Adapted from Martínez-Viveros *et al.* 2010)

Plant	PGPR inoculum	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Maize (*Zea mays* L.)	*Azotobacter*	-IAA production	-Straw yield (t ha^{-1}) -Fresh biomass (t ha^{-1}) -Plant height (cm) -Fresh cob weight (g) -Cob length (cm) -1000-grain weight (g)	17 12 7 13 6 7	-Field experiment -Non-commercial strains	Zahir *et al*. 2005
Maize (*Zea mays* L.)	*Pseudomonas fluorescens* (MPp4), *Burkholderia sp*. (MBp1,MBf21 and MBf15)	-IAA production and antagonsim against *Fusarium verticillioides*	-Shoot length (cm) -Longest root length (cm) -Shoot fresh weight (g) -Root fresh weight (g) -Plants showing disease symptoms (%) -Disease reduction (%)	30-32 47-63 24-32 76-88 10-30 60-87	-Microcosm and greenhouse experiments -Non-commercial strains -Not proven at field level	Hernández-Rodriguez *et al*. 2008
Oat (*Avena sativa* L)	*Azospirillum* sp.(Ch06 and Ch08) *Azotobacter* sp. (Ch05) *Pseudomonas* sp. (Ch09)	IAA production and acetylene reducing activity	-Root length (mm) -Root area (cm^2) -Shoot dry weight (mg $plant^{-1}$) -Total N (mg $plant^{-1}$) -Proportion of plant N fixed from atmosphere (% Ndfa)	-12-23 8-500 6-93 -50-50 50-64	-*In vitro* -Non-commercial strains -Not proven at field level	Yao *et al*. 2008
Sorghum (*Sorghum bicolour* (L.) Moench)	*B. cereus* (KBE7-8) *B. cereus* (NAS4-3) and *Stenotrophomonas maltophilia* (KBS9-B)	Siderophore production, IAA production and phosphate solubilization	-Shoot height (mm) -Shoot fresh weight (g) -Shoot dry weight (g) -Chlorophyll (spad units) -Leaf width (mm) -Root length (mm) -Root dry weight (g)	104-182 1133-2255 180-260 68-78 103-326 214-279 1300-1525	-Greenhouse pot trial -Non-commercial strains -Not proven at field level	Idris *et al*. 2009

[a] Percentage increase over non-inoculated control

Table 2.3: Plant crop response to PGPR inoculation under different experimental conditions
(Adapted from Martínez-Viveros *et al.* 2010)

Plant	PGPR inoculum	PGPR mechanisms involved	Plant growth parameter (measure unit)	Increased plant parameters[a] (%)	Assay condition and limitation	Reference
Sweet cherry cv. 0900 Ziraat (*Prunus avium* L.*)*	*Pseudomonas* BA-8 and *Bacillus* OSU-142		-Yield per trunk cross-sectional area (kg cm-2) -Fruit weight (g) Fruit diameter (mm) Total soluble solid (%) -Titretable acidity (%) -Shoot length (cm) -Shoot diameter (mm)	11-22 1-5 0.2-1 1-4 -0.4-3 11-29 -0.5-0.7	-Field experiment -Non-commercial strains	Esitken *et al*. 2006
Tomato cv Rio Fuego (*Lycopersicon esculentum*)	*Bacillus subtilis* BEB-ISbs (BS13)		-Yield plant $^{-1}$ (g) -Marketable grade yield (%) -Weight/fruit (g) -Length (cm) -Diameter (cm)	21-25 6-20 18-29 9-18 4-5	-Greenhouse experiments -Non-commercial strains -Not proven at field level	Mena-Violante and Olalde-Portugal, 2007
Tomato cv Mairoku (*L.esculentum* Mill.)	*Azospirillum brasilense* CW903, *Burkholderia pyrrocinia* CBPB-HOD, *Methylobaterium oryzae* CBMB20	IAA production, P solubilizing and N fixing	- Shoot length (cm) -Root length (cm) -Stem girth(mm)	8-13 1-13 5-11	-Greenhouse experiments -Non-commercial strains -Not proven at field level	Madhaiyan *et al*. 2010

[a] Percentage increase over non-inoculated control

Table 2.4: Factors influencing bacterial survival in soils
(Adapted from Van Veen *et al.* 1997)

Origin	Factor	Effect
Biotic	Competition	Population size decrease
	Predation	Population size decrease
	Plant roots	Release of organic compounds enhances survival
Abiotic	Clay minerals	Protection against predation and toxic chemicals
	Water tension	High tension: water shortage, high osmolarity; low tension: anaerobism, increased nutrient availability by diffusion
	Organic carbon	Selection for copiotrophic or oligotrophic species; limited organic carbon results in starvation and reduction in activity
	Inorganic nutrients (N, P)	Limitation results in starvation
	pH	Selection for species; release of nutrients (e.g., P) or toxic compounds (e.g., Al^{3+}).
	Temperature	Metabolic activity as well as biotic (predatory) pressure affected.
	Chemicals (toxic waste)	Inhibition of sensitive organisms; selection of biodegradative, resistant, or tolerant forms

2.4.1 Biotic factors

2.4.1.1 Competition

In general, PGPR are introduced into the soil at concentrations of 10^8 to 10^9 CFU/ml (Martínez-Viveros *et al.* 2010). The concentration at which inoculated bacteria persist in the soil is however influenced to a large extent by the presence of native microflora (Van Veen *et al.* 1997; Antoun and Prévost, 2005). In sterilized soil where indigenous microorganisms are absent, inoculants remain viable at concentrations of 10^7 to 10^8 CFU/g soil for several weeks. On the contrary, in non-sterilized soil with thriving native microflora a rapid reduction in bacterial numbers is observed on a weekly basis (Martínez-Viveros *et al.* 2010). For example, Viebahn (2003) found that within 132 days (from the period of sowing to wheat harvesting), bacterial numbers of genetically modified strains of *Pseudomonas putida* inoculants decreased by five orders of magnitude.

This decrease in bacterial inoculant densities is primarily due to competition between indigenous microorganisms and PGPR inoculants for space and nutrients (Cummings, 2009). Indigenous microflora have an added advantage in that they are present in their natural habitat and thus often better adapted than incoming microbes (Roy, 2010). It is therefore imperative that PGPR formulations are developed in such a manner as to provide the beneficial bacteria with favourable conditions enabling them to compete successfully.

Roy (2010) determined the survivability of PGPR strain *Azotobacter chroococcum* (SDSA-I12/2) in four different inoculant carriers namely charcoal, lignite, cured compost and vermicompost. Field trial results revealed that *A*. *chroococcum* survived best in vermicompost and delivered the highest autumn rice yields compared to the other carrier based inoculants. This indicates that vermicompost provided a suitable micro-environment for *A*. *chroococcum* enabling the bacteria to compete successfully and survive sufficiently in the soil (Roy, 2010).

2.4.1.2 Predation

PGPR are also subjected to predatory microorganisms such as protozoa. Protozoa play an important part in the soil ecosystem by maintaining the nutrient cycle; however, grazing protozoa consume large amounts of the bacterial community which may include PGPR inoculants (Antoun and Prévost, 2005). Heijnin *et al.* (1995) showed that increases in protozoan numbers and activity in the soil, correlated with a reduction in bacterial inoculant concentrations in the rhizosphere.

Other predatory microorganisms may also include nematodes, invertebrate eukaryotic worm-like animals that are primarily free-living in the soil. Although some nematodes parasitize plants, the vast majority consume microorganisms such as bacteria and therefore contribute to reducing incoming microbe populations (Decraemer and Hunt, 2006). The presence of certain nematodes in the soil however, has also been associated with having beneficial effects on PGPR inoculants. Knox *et al*. (2003) found the presence of three species of nematodes namley *Acrobeloides thornei* Brzeski*, Cruznema* sp, and *Caenorhabditis elegans* (Maupas) in a microcosm system significantly enhanced wheat rhizosphere colonization by *Pseudomonas corrugata* Roberts & Scarlett*, P. fluorescens* and *B. subtilis.*

2.4.1.3 Plant effect

Among factors affecting the activities of bacteria in the soil, plants play an important role (Smith *et al.* 1999; Schweitzer *et al.* 2008). The manner in which plants affect microbial populations is by excreting an array of organic compounds from their roots (Bais *et al.* 2006). These root exudates affect numerous biological, chemical and physical interactions in the rhizosphere, and is therefore referred to as the “rhizosphere effect” (Antoun and Prévost, 2005). Root exudates include ions, enzymes, mucilage, gasses such as ethylene and many other carbon containing-compounds (Kang and Mills, 2004; Bais *et al.* 2006). These

compounds affect chemotaxis of PGPR in the vicinity of plant roots and flagellar motility of the bacteria. PGPR are also dependant on root exudates as a source of essential nutrients in the rhizosphere and are thereby attracted to the plant roots (Bais *et al.* 2006).

Several studies have demonstrated the effect of plant species on bacterial community composition in the rhizosphere (Lemanceau *et al.* 1995; Kang and Mills, 2004; Schweitzer *et al.* 2008). Different plant species excrete different root exudate compounds which attract different bacteria and contribute to changes in bacterial communities (Bais *et al.* 2006). Fons *et al*. (2003) determined whether saponins excreted from gypsophila (*Gypsophila paniculata* L.) could alter and select for certain rhizobacteria in the rhizosphere of subterranean clover (*Trifolium subterraneum* L. 'Mount Barker'). Results showed that *Aquaspirrillum* spp, which dominates the rhizosphere of gypsophila, became the main bacterial genus in the rhizosphere of clover. In addition, a significant reduction was observed in the presence of *Acinetobacter* spp and *Chryseomonas* spp, the primary bacteria previously detected in clover rhizosphere (Fons *et al.* 2003).

Other factors that may also influence soil microbial activity includes plant genotype, (Smith and Goodman, 1999) and plant growth characteristics. Schweitzer *et al.* (2008) demonstrated that 77% of variation in population size (based on microbial biomass nitrogen) and composition of microbial communities (measured with phospholipid fatty acid biomarkers) were due to genotypes of *Populus angustifolia* E. James. Concerning plant growth habits, Kuske *et al.* (2002) observed significant differences in the composition of bacterial communities in the rhizosphere of three species of grass with different growth habits. The roots of *Bromus tectorum* L. are cold resistant and can continue growing in the winter as opposed to *Stipa hymenoides* Roem. & Schult. and *Hilaria jamesii* (Torr). Benth. which go into dormancy. Where *S. hymenoides* has a deep fibrous root the other two grass species have shallow roots with *H. jamesii* forming rhizomes that covers the soil surface. The variation in bacterial community composition was ascribed to the different root growth characteristics of three grass species that change rhizospheric conditions (Kuske *et al.* 2002).

2.4.2 Abiotic factors

2.4.2.1 Soil type

Apart from biotic factors, abiotic soil factors also influence the performance of bacterial inoculants in the soil. Soil type in particular has a major effect on microbial activity. This has been confirmed in a number of reports where survival, composition and genetic diversity of bacterial communities in different soil types have been studied (England *et al*. 1993; Horwarth *et al*. 1998; Øvreås and Torsvik, 1998; Dalmastri *et al*. 1999; Garbeva *et al*. 2004). In a study by Van Elsas *et al*. (1986), changes in survival patterns of fluorescent pseudomonas inoculants were correlated to differences in soil type. Higher numbers of fluorescent pseudomonas were detected in clayey soil with a fine texture than in coarser sandy soil (Van Elsas *et al*. 1986). Postma and Van Veen (1989) obtained similar results in that *Rhizobium leguminosarum* (Frank) biovar. trifolii survived better in silt loam soil type which contains clay aggregates to which bacteria can adhere, than in loamy sand.

The fine texture of clayey soils is known to create a larger total pore space than coarser textured soil but pore sizes are smaller and form more stable aggregates (England *et al*. 1993). Smaller pore sizes have been shown to be impenetrable to grazing protozoa (Wright *et al*. 1995) and toxic chemicals such as chloroform thereby protecting bacteria colonizing these sites from predation and adverse conditions. The survival of bacterial inoculants is therefore also dependant on their ability to reach and colonize these protective sites subsequent to inoculation (Van Veen *et al.* 1997).

2.4.2.2 Moisture and temperature

Postma and Van Veen (1989) investigated the effect that different moisture levels have on inoculant movement, distribution and localization in the soil. Their study showed that bacterial survival was significantly increased when inoculated into initially dry soil as compared to inoculation into wet soil. In soil with initially high moisture content, it is suspected that the water fills the smaller pore spaces first creating a barrier to incoming microbes. The bacterial cells then localize within the larger pore spaces where they are more exposed to predators as compared to in the smaller protective sites (Vargas and Hattori, 1986). Within dryer soil with higher water potentials, fewer pores are filled with water and inoculant cells can migrate to the smaller soil spaces. Inoculating at the correct soil moisture

content therefore aids bacterial movement and distribution to protective microhabitats within the soil thereby enhancing their survival (Postma and Van Veen, 1989; Wright *et al*. 1995).

The moisture content and temperature maintained within soil following bacterial inoculation, also affects inoculant survival. O'Callaghan *et al*. (2001) inoculated soil microcosms at different moisture contents (13%, 23% and 30% (w/w)) with *P. fluorescens* and incubated the microcosms at different temperatures (10°C, 15°C or 20°C). Results showed that *P. fluorescens* preferred a soil moisture content of 23% as was indicated by the highest bacterial numbers present in soil after 110 days at 10°C. At 30% moisture content the inoculant was present in soil at a slightly lower concentration than at 23% moisture content at 10°C. In the dry soil at 13% moisture level the concentration of *P. fluorescens* decreased the fastest compared to the other treatments resulting in the lowest concentration of viable bacteria. Furthermore, as temperature increased, inoculant levels decreased at all soil moisture contents with no bacteria being viable at 20°C after 54 days (O'Callaghan *et al*. 2001). The influence of soil moisture and temperature on inoculant survival should thus be taken into account when considering the use of PGPR products.

2.4.2.3 Nutrient status

The quantity of microorganisms that can be maintained within soil is largely dependent on substrate availability of specifically organic matter within the soil (Kobabe *et al*. 2004). Since organic matter is difficult to degrade and is often located in sites inaccessible to microbes, the availability of nutrients in natural soils for use by indigenous microorganisms is generally low. Introduction of additional microbes to soil sites deficient in essential nutrients may therefore result in starvation of microorganisms which may include inoculant bacteria (Van Veen *et al.* 1997). This once again emphasizes the importance of applying PGPR that are able to compete vigorously with indigenous microorganisms for the necessary nutrients.

Other abiotic factors which should not be overlooked when considering the use of PGPRs include the use of soil amendments such as pesticides and herbicides which can be harmful to microorganisms. Soil pH also influences the efficiency of PGPR by selecting for certain microbial species, affecting nutrient availability and bacterial activity (Wardle, 1992). Ho and Ko (1985) studied the effect of pH on soil bacteriostasis (microbiostasis specifically against bacteria). When soil pH was increased from 6.3 to 8.4 with addition of NaOH, bacteriostasis was absent, indicating that acidic soils inhibit bacterial activity of most bacterial species (Ho and Ko, 1985; Wardle, 1992).

In conclusion, PGPR are influenced by many biotic and abiotic factors which have a major effect on the activity and survival of these bacteria in the soil. Inconsistent results can therefore be expected in fields with different ecological conditions; however, the degree of variability depends on how favourable the target environment is or can be made to suit the needs of the introduced bacteria. With careful consideration of environmental factors together with development of appropriate PGPR formulations (to be discussed in the next section) successful application in terms of survival and consistent performance of PGPR in the soil can be achieved.

2.5 Inoculant formulations and commercialization

Before commercialization of potential PGPR inoculants is possible, large scale production of the particular bacterial strain and its conversion into an appropriate formulation is required. “Formulation is the industrial "art" of converting a promising laboratory-proven bacterium into a commercial field product” (Bashan, 1998). The primary function of an inoculant formulation is to ensure the survival of the beneficial bacteria in the soil by providing a suitable micro-environment. The success or failure of PGPR inoculants is therefore determined by the formula in which the bacteria are applied to the soil (Nakkeeran *et al.* 2005).

Development of poor formulations is often the reason why many promising bacteria described in the scientific literature are never commercialized. Except for obtaining a potential PGPR isolate with all the necessary characteristics, various aspects need to be considered when formulating potentially useful PGPR isolates. For example: the characteristics and type of formulation to be used, the method and time of delivery of the product and the time and application technology to be used (Bashan, 1998; Banerjee and Yesmin, 2004; Nakkeeran *et al.* 2005).

2.5.1 Characteristics required of a PGPR isolate for formulation development

Before a PGPR isolate should be considered for formulating, thorough research should be done to determine whether the bacteria in question possess the required characteristics. This is the first step ensuring the success of an inoculant formulation (Nakkeeran *et al.* 2005).

An ideal PGPR isolate for formulation should:

1. promote plant growth
2. inhibit plant pathogens
3. have multiple modes of action
4. be able to compete effectively
5. be compatible with other PGPR
6. not be harmful to the environment
7. tolerate high temperatures and toxic compounds such as oxidizing agents
8. be mass produced easily and
9. have high rhizosphere competence (Nakkeeran *et al.* 2005).

Discovering a PGPR isolate with all of these traits is virtually impossible. However, researchers should aim to obtain isolates with as many of these characteristics as possible. Alternatively, researchers are increasingly developing PGPR formulations containing multiple bacterial strains with different traits. PGPR formulations containing compatible strain

mixtures have been shown to render better or more consistent field trial results than formulations with single strains (Domenech *et al*. 2006; Maiyappan *et al*. 2011). This can be expected since mixed strain formulations are more likely to perform better under a wide range of environmental conditions due to the presence of stains with different genetic traits. Also, the presence of several different strains provides a product with multiple modes of action (Nakkeeran *et al.* 2005).

2.5.2 Mass production and development of formulations

The next step after obtaining the best possible bacterial isolate is the mass production and development of a suitable formulation by either liquid or solid fermentation technologies (Nakkeeran *et al*. 2005; Spadaro and Gullino 2005). Mass production of bacterial inoculum involves a shift from the small-scale laboratory produced inoculum in 250 ml Erlenmeyer flasks to production of 10,00 litres of cells in large fermenters. With contamination already being a problem during small-scale production, it is one of the biggest challenges encountered during scaleup. The presence of contaminant microorganisms may influence the performance of the final product since it will not be representative of the initial product produced on small-scale in the laboratory during research trials. Quality control and check points throughout the production process are thus crucial during formulation development (Mathre *et al*. 1999).

As mentioned before, the type of formulation in which the final product will be available determines whether the PGPR isolate in question will be successful or not (Nakkeeran *et al.* 2005). The major portion of a formulation consists of carrier materials (organic or inorganic) which are required to deliver the correct amount of viable bacterial cells at the right time. Other ingredients that make up the formulation may include diluents, dispersants, activators, contaminant suppressants and membrane stabilizers (Spadaro and Gullino, 2005). The formulation itself is the final form in which the bacterial isolate is transferred from the factory to the field.

There are a number of important factors that need to be taken into account when designing formulations with the aim of commercialization. For example, formulations must be compatible with agrochemicals and agricultural equipment already available on the market and must be environmentally friendly i.e. biodegradable and nontoxic (Spadaro and Gullino 2005). Also, formulations must be easy to handle and safe to work with during transport, storage and application (Bashan, 1998). Very importantly, raw materials and ingredients

needed for the formulation must be inexpensive and readily available so that production costs are kept to a minimum (Nakkeeran *et al*. 2005).

2.5.3 Types of carriers for formulation

The type of carrier material varies from formulation to formulation and no one universal carrier or formulation is yet available. The absence of a universal carrier can be expected considering that each PGPR strain requires a uniquely designed formulation suited to the complex soil-plant-microorganism interaction (Antoun and Prévost, 2005). For this reason numerous carriers such as vermiculite, talc (Bora *et al.* 2004), lignite (Roy *et al.* 2010), alginate (Bashan and Gonzalez, 1999), kaolinite, montmorillonite, turf, pressmud, peat, zeolite, etc have been used and tested in laboratory and field trials (Nakkeeran *et al*. 2005). Some of the most common carriers will be discussed.

Talc, which is more commonly known as soapstone, is a natural mineral that is readily available. The low moisture absorption of talc increases the storage life of talc based products, making it a good carrier for PGPR formulations (Nakkeeran *et al*. 2005). Bora *et al*. (2004) showed that talc-based formulations were able to maintain a high number of viable cells of between 10^9 and 10^{10} cfu/g of *P. putida* strain 30, after 6 months of incubation at 4˚C. Furthermore, these cells were in good physiological condition maintaining their ability to control *F. oxysporum* f. sp. *melonis* in field trials by reducing wilt of muskmelon by 63% compared to the control.

Peat has often been used as carriers for PGPR formulations primarily because of its low-cost (Cummings, 2009). Peat is formed in wetland conditions and consists of a combination of partially decayed vegetation which includes various plants such as reeds, sedges and primarily mosses (Nakkeeran *et al*. 2005). Rabindran and Vidhyasekaran (1996) developed a peat based formulation of *P. fluorescens* which significantly increased rice yield and effectively controlled *R. solani* causing rice sheath blight. There are however numerous drawbacks associated with peat. Since the formation of peat is dependent on the decay of varying plant sources the quality of peat varies and it contains many contaminants. A variation in quality between different peat batches may influence the overall effectiveness of the PGPR formulation leading to inconsistent results. Furthermore sterilization of certain peats to eliminate contaminants may release substances that are toxic to the bacterial inoculants reducing the concentration of PGPR in the formulation (Bashan, 1998; Cummings, 2009).

Vermiculite is commonly used as a potting mixture or carrier within bacterial formulations due to its high porosity that ensures a large water-holding capacity and adequate pore space for bacterial cells to occupy (Nakkeeran *et al*. 2005). Vermiculite based formulations are known to have a long shelf-life delivering a high concentration of beneficial bacteria as was shown in the study by Sangeetha and Stella (2012). In their study PGPR isolates i.e *Azospirillum lipoferum* VAZS-18, *Azotobacter chroococcum* VAZB-6, *Bacillus megaterium* VBA-2 and *P. fluorescens* VPS-19 remained viable for six months at concentrations of 10^8CFU/g at 30˚C, 35˚C and 40˚C. A reduction in bacterial numbers was however observed at 35˚C and 40˚C compared to 30˚C (Sangeetha and Stella, 2012). Vermiculite does however, have one disadvantage in that it cannot be used as a seed treatment because of its tendency to fall off seeds (Bashan, 1998).

2.5.4 Delivery and application of PGPR formulations

Much attention should be devoted to the correct delivery and application of PGPR formulations as this is the final step in determining the success of the product. Inoculants must be delivered at the right time to the site where the PGPR will be most effective using standard farming machinery that is currently available on the market (Nakkeeran *et al*. 2005; Spadaro and Gullino, 2005). Although the application of microbial formulations is more complex compared to that of agrochemicals, less research has been done on delivery of biological products than chemicals. Research efforts should rather be aimed at improving the delivery and application technology of microbial formulations considering the benefits associated with the use of biological products (Nakkeeran *et al*. 2005).

PGPR can be delivered to the site of action either as a seed treatment, seedling dip, soil application, foliar spray, fruit spray or as a combination of delivery methods (Nakkeeran *et al*. 2005). The method of inoculant delivery determines in which form formulations should be developed i.e. as slurries (Bashan, 1998), powders (Bora *et al*. 2004), liquids (Manikandan *et al*. 2010) or granules (Al-Taweil *et al*. 2010). If a seed treatment is required slurries are mostly preferred. Granules on the other hand can be applied directly to the soil and is often combined with potting mix. Liquid formulations are particularly useful when applied through drip irrigation systems (Spadaro and Gullino, 2005) or as foliar or fruit sprays against leaf and fruit diseases (Bashan, 1998). Lastly, powder formulations are best suited for root dips (Spadaro and Gullino, 2005) and are also often used as seed treatments (Bora *et al*. 2004).

PGPR inoculants can also be delivered in the form of capsules. This involves encapsulation of bacterial cells within polymeric material such as alginate (John *et al*. 2005). There are numerous advantages associated with encapsulation of inoculants for example the microorganisms are protected against environmental stresses and are released gradually into the soil as the polymers degrade, giving the cells time to adapt (Bashan, 1998; Rekha *et al*. 2007). Compared to other forms of inoculants however, polymers are more expensive and encapsulation is more labour intensive. Further research efforts on the use of encapsulated inoculants should be focused on making the formulation more cost effective (John *et al*. 2005).

2.5.5 Commercial aspects

For PGPR to become a significant component of crop production and protection, PGPR products need to be commercialized. Although the costs associated with commercialization of microbial products are lower compared to that of chemicals, expenses are still high (Harman *et al*. 2010). Particularly the high cost of registration and toxicology studies limits the commercialization of PGPR products worldwide. Toxicology testing on microbial products is critical to ensure the microorganisms within the product are not toxic or harmful to humans, animals or plants (Cook, 1996). High costs specifically restrict the development of PGPR formulations in developing countries and for use on minor crops with a small market (Mathre *et al*. 1999). The environmental protection agency should aim to minimize these expenses and relax the stringent formalities associated with registration to motivate industries to commercialize the products (Nakkeeran *et al*. 2005).

An alternative or perhaps more promising solution to above mentioned constraint would be for scientific organizations and industries to establish long term partnerships where corporate resources could be used to cover the costs of commercialization (Pal and Gardener, 2006). Industry inputs are required to accomplish all stages of commercialization from extensive research trials to successful development of microbial formulations as well as toxicology tests, patent protection of bacterial strains, registration and marketing of the final product. Industries should support research efforts of academic institutions to standardize application rates, methods of delivery, shelf-life and quality control so that the product is acceptable on the market resulting in financial returns on investment. By working together to achieve a common goal research institutions and corporate bodies could contribute significantly to the success of PGPR products in the agricultural sector (Nakkeeran *et al*. 2005).

Another factor which plays an important role in promoting commercialization of PGPR products is education. Individuals involved with the commercialization of PGPR products should be properly educated concerning the benefits, mode of action, methods of application and safety of PGPR products. This includes government extension workers, policy makers as well as commercial companies that manufacture and sell microbial products (Nakkeeran *et al*. 2005). Most importantly, the end user or farmer which is mostly familiar with the use of agrochemicals should be informed on aforementioned aspects. The necessary education can be provided by hosting farmers days, giving field demonstrations and providing training sessions. In this manner the demand for PGPR products will be increased substantially, industries will invest with more confidence and ultimately sustainable agriculture will be promoted (Harman *et al*. 2010).

2.6 References

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CHAPTER 3

Effect of PGPR on seed germination, vigour and seedling growth of a maize cultivar with reduced seed vigour

Abstract

Seed quality is one of the most important factors influencing crop quality and yield. Seed germination and vigour which determine seed quality are therefore important characteristics that need to be taken into account by farmers before planting. As found in this study, plant growth promoting rhizobacteria (PGPR) have the ability to enhance seed germination and vigour as well as seedling growth of maize. Moisture, germination and vigour tests were performed on two maize (*Zea mays* L.) cultivars (PAN 6236B and PAN 6Q-308B) to determine seed quality. PAN 6Q-308B had the lower percentage germination and vigour tests (cold test, radicle emergence test, accelerated ageing test) indicated that the cultivar had a lower vigour compared with PAN 6236B. Eight PGPR isolates (T19, T29, A32, S6, S7, A08, A07 and T22) were applied as seed treatments in a modified seed germination bioassay to determine their effect on the lower vigour maize cultivar (PAN 6Q-308B). Results showed that three of the isolates (S6, A32, and T29) significantly increased percentage germination by up to 7.8%. Also, plant dry mass of maize seedlings treated with S6, S7, A32, T29 and T22 was significantly higher (20.1% to 29.7%) when compared to the untreated control. The vigour index of the maize cultivar was significantly enhanced by 16.5-22% by five of the isolates (S6, S7, T19, T29 and A32) of which S6 proved to be most promising. Thus, the present study demonstrated that seed inoculation with PGPR can increase early growth parameters of maize seedlings and enhance germination and vigour of maize seed of low vigour cultivars.

3.1 Introduction

Within seed lies the potential to produce new, healthy crop stands which are necessary for food security and the survival of the human population. Seed potential is determined by the quality of seed, which forms the foundation for successful grain crop production (Ellis *et al*. 1993). The seed stock chosen by the grain producer can have a significant detrimental effect on crop yield if seed quality is low (Msuya and Stefano, 2010). Determining the quality of a seed lot before planting provides farmers with additional information that is useful when making important decisions such as fertilizer, pesticide and seed treatment applications or seed storage conditions after harvest (Erker, 2008). Quality seed is also essential for the seed trade industry which plays an important role in a country's economy. In 2008, the value

of global seed production was around R292 billion of which the South African market contributed approximately R2.4 billion (Milosevic *et al*. 2010).

Various tests are available to determine the quality of seed. Two of these tests involve determining seed germination and vigour since these are key indicators of seed quality (Milosevic *et al*. 2010). Germination tests give an indication of the maximum germination potential of a seed lot since seed is subjected to ideal environmental conditions. Vigour tests on the other hand represent the ability of seeds to germinate under unfavourable conditions which are often encountered in the field. Therefore, although a seed lot may have a high percentage germination in the standard germination test, field emergence may be poor and the lot is then referred to as a low-vigour seed lot (Powell, 2009). Vigour tests help to establish the true potential of seed by providing additional information to the standard germination test. Although vigour is difficult to define, the term refers to all seed properties that enable rapid and good germination in unfavourable environmental conditions (ISTA, 2012). Vigour indicates, for example, the storage potential of seed, the uniformity of seed emergence and rate of seedling development (Miguel and Fihlo, 2002; ISTA, 2012).

Studies involving bacterial strains such as *Pseudomonas, Azotobacter, Azospirillum* (Shaukat *et al*. 2006) and *Bacillus* (Niranjan *et al*. 2003; Kloepper *et al*. 2004) have indicated that certain bacteria can increase seed germination, vigour, seedling growth and yield of important crops. Such bacteria play an important role in crop production and are termed plant growth promoting rhizobacteria (PGPR) (Martínez-Viveros *et al*. 2010). Application of PGPR to cereals such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) could help satisfy the world food demand especially in developing countries such as South Africa where these crops are the main source of carbohydrates (Pinstrup-Andersen, 2001). Indeed, increases in seed germination, rate of germination, vigour and yield of wheat (Shaukat *et al*. 2006), pearl millet (*Pennisetum glaucum* L.) (Niranjan *et al*. 2004) and maize (Nezaret and Gholami, 2009) have been reported when PGPR were applied as seed treatments to these crops. Application of PGPR in agriculture could also limit the use of harmful agrochemicals (Martínez-Viveros *et al*. 2010) and provide a cheaper approach for crop production compared to use of synthetic chemicals, thereby motivating continued farming of crops (Pinstrup-Andersen, 2001).

The first objective of this study was to determine and compare the seed quality of two maize cultivars (PAN 6236B and PAN 6Q-308B) in order to select the cultivar with the lower vigour for further testing. The second and primary objective was to determine if selected PGPR strains could enhance seed germination, vigour and growth parameters of a maize cultivar

with reduced vigour *in vitro.* Based on these results the five best performing PGPR isolates, with regards to enhancing seed vigour, would then be selected for further research.

3.2 Materials and Methods

3.2.1. Seed quality tests

Untreated seed of two maize cultivars (cv) PAN 6Q-308B and PAN 6236B were used in all the seed tests unless otherwise stated. The seed was obtained from PANNAR (Pty) Ltd., Bapsfontein, South Africa.

3.2.1.1 Moisture test

Two maize seed samples (5g) of each cultivar was selected at random and ground to a fine powder, respectively, using a coffee grinder (Mellerware, South Africa). The ground seed was placed in a glass Petri dish and the mass was determined. The Petri dish with lid removed was then placed in a drying oven (Labex (Pty) Ltd, South Africa) for four hours at 130˚C to remove all moisture from the seed. After the drying period, the seeds were covered with the lid and left to cool in a desiccator for 40 minutes. The Petri dish containing the ground seeds was weighed again and percentage seed moisture content was calculated (ISTA, 2012).

3.2.1.2 Germination test

Both maize cultivars were subjected to a standard germination test according to the International Seed Testing Association (ISTA) rules as follows: Fifty seeds were placed with the radicle pointing downwards, on three layers of moistened (sterile distilled water) germination paper (12 x 22cm) obtained from Agricol (Edms) Bpk, Brackenfell, South Africa. Seeds were evenly spaced in rows of 17, 16 and 17 seeds per row. A single moistened germination paper was placed over the seeds and all the sheets were rolled up and placed upright in a plastic bag for seven days at 25˚C (ISTA, 2012) for germination to take place. After seven days, seedlings were evaluated as normal/abnormal according to the ISTA rules and percentage germination was calculated. Four replicates of 100 maize seeds were tested.

3.2.1.3 Cold test

To determine seed vigour, PAN 6Q-308B and PAN 6236B seeds were subjected to the cold test. Fifty seeds were placed on moistened germination paper as for the standard germination test and each row of seeds was covered with a thin layer of soil collected from a

maize field at PANNAR, Bapsfontein, South Africa. The soil was not sterilized since the purpose of the cold test is to subject seeds to pathogens naturally present within the soil from a field planted with maize. The seeds were then incubated for seven days at 5˚C followed by incubation for seven days at 25˚C. Four replicates of 50 seeds were done. Evaluation of seedlings as normal/abnormal was done and percentage germination was determined (ISTA, 2012).

3.2.1.4 Accelerated ageing test

The storage potential of the two maize seed cultivars was established using the accelerated ageing test. This test is also a measure of seed vigour. Twenty five maize seeds were selected at random. Seeds were placed in a porous cloth bag on a grid over a saturated salt solution in a plastic container. The container was then incubated at 45˚C for four days. After incubation the germination test as described above was conducted and percentage germination was determined (ISTA, 2012). Four replications were completed for each cultivar.

3.2.1.5 Radicle emergence test

The same procedure as for the standard germination test was followed except that seeds were placed in two rows, one of 12 and one of 13 seeds, on the germination paper. The paper rolls containing the seeds were placed in plastic bags and incubated at 20˚C for 66h to allow the radicles to emerge. The maize cultivar (PAN 6236B) that performed best in the previous seed tests served as the control and PAN 6Q-308B as the treatment. Eight replicates of 25 seeds per cultivar were included.

After incubation all seeds of which the radicle had emerged and was equal to or longer than 2mm were recorded and the number of radicles emerged was converted to percentage emergence for each replicate. Average radicle emergence was determined and compared to the control to establish seed vigour of PAN 6Q-308B (ISTA, 2012).

3.2.2 Effect of PGPR on seed germination and vigour

3.2.2.1 PGPR isolates and inoculum preparation

Eight PGPR isolates viz. T19 (*Lysinibacillus sphaericus*), T29 (*Paenibacillus alvei*), A32 (*Stenotrophomonas* sp.), S6 (*Bacillus cereus*), S7 (*Bacillus cereus*), A08 (*Bacillus aryabhattai*), A07 (*Bacillus aryabhattai*) and T22 (*Paenibacillus alvei*) were obtained from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa for use in this study. The rhizobacterial isolates were

previously identified as part of two studies by Hassen (2007) and Pretorius (2012), respectively. Pure cultures of the isolates were sent to Inqaba Biotechnical Industries (Hatfield, South Africa) for sequencing of the 16sRNA gene region. 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 Bid Dye v3.1 and the clean-up performed with the Zymo Sequencing Clean-up kit (ZR-96, DNA Sequencing Clean-up KitTM).

Bacterial cultures were revived from storage in the frozen form at -70˚C by selecting and streaking a single Microbank™ bead (Pro-Lab Diagnostics) containing the bacterial isolate onto fresh nutrient agar (NA) (Biolab, Wadeville, South Africa). The bacterial cultures were then incubated at 25˚C for 24h. A single bacterial colony was selected and sterile nutrient broth (100ml) (Biolab, Wadeville, South Africa) was inoculated with each isolate consecutively and incubated at 37˚C on a rotary shaker at 200rpm for 18h. The serial dilution technique was used to determine the concentration of bacterial suspensions. Dilutions were prepared by pipetting 1ml of bacterial suspension into 9ml sterile ringers in a test tube from which a 8-fold dilution range was prepared. From each dilution 100µl volumes were spread plated in duplicate onto NA and plates were incubated for two days at 25˚C. Colonies were counted and a concentration of between 10^8 and 10^9 CFU/ml was obtained.

3.2.2.2 Seed inoculation

Maize seeds (30g) of the lower vigour cultivar, PAN 6Q-308B, were surface sterilized for 2 minutes in a 1% sodium hypochlorite solution and rinsed five times in sterile distilled water (Nezaret and Gholami, 2009). For inoculation, 1% carboxymethyl cellulose (CMC) (Sigma-Aldrich, South Africa) was added to the bacterial suspension to serve as an adhesive to improve adhesion of bacterial cells that to the seeds. Seeds were then soaked in the CMC-bacterial suspension (10^8 CFU/ml) for 30 minutes in a sterile glass beaker. For the control treatment seeds were soaked in uninoculated nutrient broth amended with 1% CMC. Seeds were then air-dried in a laminar flow cabinet (Kumar *et al*. 2011).

To determine the number of bacterial cells retained per seed, a single seed was vortexed for 2 minutes in 9ml sterile ringers solution and a dilution series was prepared. Aliquots (0.1ml) were spread plated onto NA plates and were incubated at 25˚C for 24h. Between 10^4 and 10^5 CFU/seed was obtained. Three replications of the serial dilution were performed.

3.2.2.3 Modified seed germination bioassay

After seeds were treated with the various PGPR strains the standard germination test was conducted. Untreated maize seeds served as the control. Following the incubation period of seven days, seedlings were evaluated as normal/abnormal according to ISTA guidelines and percentage germination was determined. Root and shoot length of individual seedlings were measured and fresh mass was determined. Dry mass was recorded after drying seedlings in brown paper bags in an oven (Labex (Pty) Ltd, South Africa) at 75˚C until the weight remained constant. Vigour index was determined using the following formula: Vigour index (VI) = (mean root length + mean shoot length) × % germination (Nezaret and Gholami, 2009). The experiment was repeated and consisted of four replications of 100 seeds each.

3.2.3 Experimental design and statistical analysis

Percentage germination was arcsine transformed to normalize data before statistical analyses were done; however, values are presented as untransformed data (Govindappa *et al*. 2010). All data was statistically analysed by one way ANOVA (complete randomized design) and Fisher's Least Significant Difference test ($p = 0.05$) was used to detect significant differences between means of treatments. Statistical analysis was performed using GenStat Discovery Edition four.

3.3 Results

3.3.1 Seed quality tests

The moisture content of PAN 6Q-308B and PAN 6236B seed was 12.1% and 12.5% respectively. The results of the seed tests are presented in Table 3.1. According to the standard germination test, seeds of cv. PAN 6236B had a high percentage germination of 95% whereas only 85% of PAN 6Q-308B seeds germinated. Cold test results showed that 85% of PAN 6236B seed germinated which was significantly higher (17%) than that of PAN 6Q-308B seed. In the four day accelerated ageing test, seed germination of PAN 6Q-308B was reduced by 17% whereas a 5% reduction was observed in PAN 6236B seeds when compared to the standard germination test. Only 68% of PAN 6Q-308B seeds germinated during the cold test. During the radicle emergence test 86% of PAN 6Q-308B and 92% of PAN 6236B seeds developed an emerging radicle after 66h of incubation at 20˚C. All seed test results indicated that PAN 6236B seeds had a significantly higher germination percentage than PAN 6Q-308B.

Table 3.1: Percentage seed germination of maize cultivar PAN 6Q-308B and PAN 6236B during various seed tests performed according to ISTA standards

	Germination (%)			
Seed test	**PAN 6Q-308B**	**PAN 6236B**	CV (%)	LSD
Standard germination*	85 b	95 a	5.9	4.58
Cold test*	68 b	85 a	6.4	4.22
Accelerated ageing (4 day)*	68 b	90 a	5.5	3.79
Radicle emergence*	86 b	92 a	5.9	4.43

Values are means of two experiments consisting of four replications of 100 seeds each for the standard germination test, four replications of 50 seeds each for the cold test, four replications of 25 seeds each for the accelerated ageing test and eight replications of 25 seeds each for the radicle emergence test. *In rows, mean results of percentage germination of the two cultivars was compared statistically and values followed by the same letters for each test respectively do not differ significantly (p = 0.05) according to Fisher's least significant difference (LSD) test.

3.3.2 Effect of PGPR on seed germination, vigour index and growth of maize seedlings

Fresh shoot mass of PAN 6Q-308B was significantly enhanced by the application of most PGPR isolates to the seeds except isolates A07, A08 and T22 (Table 3.2). In contrast to the other isolates, A07 actually caused a 7.4% reduction in fresh shoot mass in comparison to the untreated control, although, the difference was not significant. The highest increase in shoot fresh mass was obtained for maize seeds treated with isolates T19 (35.3%) and S6 (35.4%).

PGPR application to maize seed had no effect on seedling root fresh mass compared to the control except for isolate S7 which increased fresh mass by 41.8%. A significant increase in total seedling fresh mass was observed when seeds were treated with isolates S6, A32 and T19.

Among the eight isolates tested on maize seed, four (S6, A32, T29, T19) had a significant effect on shoot dry mass (Table 3.2) compared to the untreated control. Isolate S6 performed the best concerning increasing dry shoot mass. The only maize seed treatment which significantly improved root dry mass was PGPR isolate S7. In terms of total dry mass, significant increases above the control of between 20.1% to 29.5% were obtained when seeds were treated with PGPR isolates S6, S7, A32, T29 and T19. The highest increase in total dry mass was obtained when seeds were treated with isolate S6.

Table 3.2: Effect of seed treatment with PGPR isolates on shoot and root mass of maize seedlings *in vitro*

	Seedling mass in grams						Percentage change in mass**					
	Fresh mass*			Dry mass*			Fresh mass			Dry mass		
Isolate	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**
Control	35.98 ab	8.23 a	44.21 ab	2.81 a	1.13 a	3.94 a	0	0	0	0	0	0
S6	48.7 f	10.3 ab	59.00 cd	3.79 f	1.32 ab	5.11 c	35.36	25.23	33.48	34.85	16.12	29.46
S7	40.71 cd	11.66 b	52.37 bc	3.19 abc	1.6 c	4.79 bc	13.16	41.75	18.48	13.4	41.34	21.5
A07	33.31 a	9.04 ab	42.35 a	3.04 abc	1.28 a	4.32 ab	-7.4	9.82	-4.2	8.02	13.01	9.46
A32	46.97 ef	9.56 ab	56.53 cd	3.58 def	1.15 a	4.73 bc	30.55	16.19	27.88	27.52	1.71	20.1
A08	36.07 ab	11.63 ab	47.7 abc	2.73 a	1.31 ab	4.04 a	0.25	41.34	7.9	-2.83	15.88	2.55
T22	38.52 bc	10.43 ab	48.95 bc	2.93 ab	1.23 a	4.16 ab	7.06	26.8	10.74	4.2	8.6	5.46
T29	43.52 de	9.39 ab	52.91 bcd	3.47 cde	1.28 a	4.75 bc	20.97	14.17	19.7	23.42	12.73	20.35
T19	48.67 f	10.6 ab	59.27 d	3.57 ef	1.43 ab	5.00 c	35.28	28.85	34.08	27.07	26.13	26.8
LSD	4.328	3.431	6.165	0.573	0.309	0.651						
CV (%)	10.5	34	12	17.7	23.7	14.3						

*In each column, mean values followed by the same letters do not differ significantly according to Fisher's LSD test at p = 0.05
**Percentage change in mass= [(treatment-control)/control x 100], therefore negative values indicate a decrease in mass compared to the control and positive values an increase in mass compared to the control. Values are means from two repeated experiments with four replications of 100 seeds each.

Root and shoot length of maize seedlings after seed treatment with PGPR isolates are shown in Figure 3.1. Isolate S6, A32, T29 and T19 promoted shoot length significantly whereas all other isolates (S7, A07, A08 and T22) had no significant effect on shoot length. Three isolates (A07, A08 and T22) reduced shoot length compared to the control although the reduction was not statistically significant. Root length was significantly increased above the untreated control when seeds were treated with T19 and S7. Isolate T22 slightly inhibited root length as compared to the control however statistical differences were not significant.

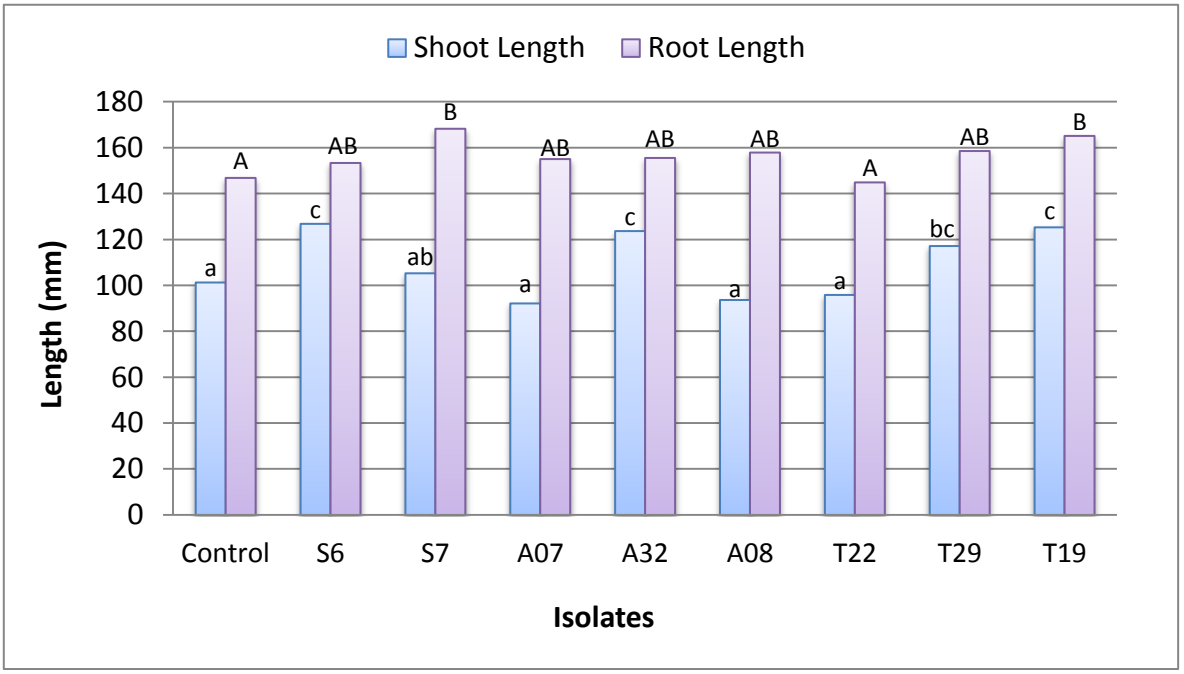

Figure 3.1: Effect of seed treatment with PGPR isolates on shoot and root length of maize seedlings *in vitro.* Values are means from two repeated experiments with four replications of 100 seeds each. Means with the same letter are not significantly different according to Fisher's LSD test at p = 0.05. (Root length: LSD= 17.11; CV= 11%. Shoot length LSD= 15.39; CV= 14.1%)

In comparison to the untreated control, five of the eight PGPR isolates (S6, S7, A32, T22, T29) enhanced seed germination of PAN 6Q-308B significantly under laboratory conditions (Figure 3.2). The highest percentage germination (92%) was obtained when seed was inoculated with isolate T22 representing an increase of 7.8% in comparison with the control. The lowest percentage germination (86.3%) resulted from treatment with isolate A07. Inoculations of seed with isolates A07, A08 and T19 had no significant effect on germination when compared to the untreated control.

Seed treatment with PGPR isolates S6, S7, A32, T29 and T19 resulted in a significant increase in VI of maize compared to the untreated control (Figure 3.2). The highest values recorded for VI ranged between 2501 and 2560 for isolates T29, T19, A32 and S6 representing increases of 19.2 and 22%, respectively, in comparison with the untreated

control. No statistical differences were obtained between VI values of maize seed treated with PGPR isolates A07, A08, T22 and the untreated control.

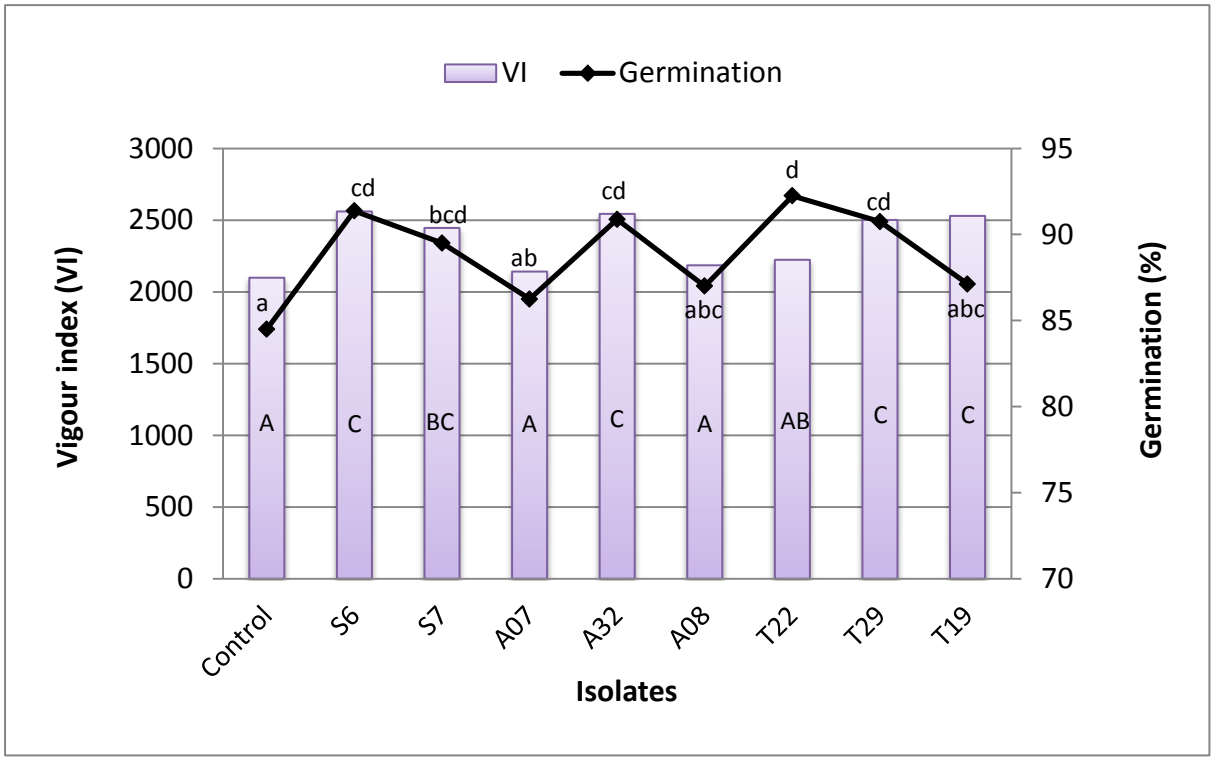

Figure 3.2: Effect of seed treatment with PGPR isolates on vigour index and percentage germination of maize seed *in vitro.* Vigour index and seed germination values are means from two repeated experiments with four replications of 100 seed each. Means with the same letter are not significantly different according to Fisher's LSD test at p = 0.05. (LSD= 245.7; CV= 10.4%)

3.4 Discussion

Seed moisture content is considered an important parameter when determining the market value of seed. Moisture content of seed is a measure which reflects the rate of seed quality decline during storage. The higher the moisture content, the faster seed ages, becomes more susceptible to fungal infections and ultimately loses quality (Powell, 2009). In the current study, the moisture content of cv. PAN 6Q-308B and PAN 6236B seeds was 12.1 and 12.5% which is acceptable since the maximum acceptable moisture content value for maize seed is 12.5% (Semple and Kirenga, 1994). These results indicate that seed moisture content of the two maize cultivars did not significantly influence seed performance during the standard germination and vigour tests that followed.

The minimum percentage germination required for maize seed in the standard germination test for seed to be certified is 90% according to national laboratory standards applicable to COMESA member states (Mujaju, 2010). Germination of PAN 6236B seeds was 5% above

this value whereas PAN 6Q-308B was 5% below this value. The performance of the cultivars in vigour tests in which a significantly higher percentage germination was obtained for PAN 6236B seeds compared to PAN 6Q-308B in all tests coincide with the standard germination results. Furthermore, vigour tests are more complex and sensitive than the standard germination test and therefore provide a more accurate indication of seed quality and field performance (Miguel and Fihlo *et al*. 2002). The four day accelerated ageing test indicated that seed of PAN 6236B will age at a slower rate than seed of PAN6Q-308B during storage. Also, the rate of germination of PAN 6236B, as determined by the radicle emergence test was faster than that of PAN6Q-308B. A high rate of radicle emergence at the beginning of the germination period is an early indication of high vigour seed (ISTA, 2012). No fixed standard has been proposed for percentage germination during the cold test (Fessel *et al*. 2006). Nevertheless, current results showed that PAN 6236B performed significantly better under stressful conditions than PAN 6Q-308B. Based on these findings, PAN 6Q-308B seeds was classified as the cultivar with the lower seed vigour and was therefore selected for use in further experiments to investigate whether germination, growth and vigour could be enhanced by seed treatment with PGPR.

The study showed that seed treatment of cv. PAN 6Q-308B with selected PGPR isolates benefitted maize plants during the early growth stages by increasing seed germination, seedling growth and hence VI of seedlings. Increased vigour improves a seedlings ability to withstand infections by pathogens and survive under harsh environmental conditions. Seed inoculation with isolates S6, A32, and T29 significantly enhanced VI, percentage germination and shoot length in comparison with the control and isolate S7 and T19 increased VI and root length significantly. The greatest enhancement in VI was obtained with isolate S6 whereas T22 promoted percentage germination the most. Seed treatment of lower quality maize seeds such as that of PAN6Q-308B with PGPR such as isolate T22, can improve germination percentage by up to 7.8%, which is comparable to the percentage germination of good quality seed such as that of PAN 6236B. Similar results have been reported in earlier studies on maize in which seed germination was enhanced by up to 18.5% and VI by more than 100% compared to the control (Nezaret and Gholami, 2009). Increases in early seedling growth due to seed treatment with various PGPR strains were also reported in other grains such as pearl millet (Niranjan *et al*. 2003), wheat (Shaukat *et al*. 2006) and rice (Chithrashree *et al*. 2011).

The mode of action by which PGPR affects germination, growth and vigour parameters was not determined in the present investigation. However, findings from other studies have

suggested that hormone production by PGPR play a role (Lucangelli and Bottini, 1997; Zahir *et al*. 2000; Boiero *et al*. 2007; Shahab *et al*. 2009). For example, gibberellic acid (GA_3) has been associated with increased shoot growth and germination of seedlings (Lucangelli and Bottini, 1997). It has often been reported that indole 3-acetic acid (IAA) affects root growth either positively or negatively depending on the concentration (Barazani and Friedman, 1999; Ashrafuzzaman *et al*. 2009; Shahab *et al*. 2009). Higher concentrations of IAA are mostly known to inhibit root growth (Saharan and Nehra, 2011). For example, phytohormone producing strains of *Azospirillum brasilense* and *Bradyrhizobium japonicum* promoted early development of maize and soybean in terms of seed germination, seedling growth and root volume, although root length was reduced (Cassan *et al*. 2009). Phytohormone identification by gas-chromatography-mass spectrometry revealed that both bacterial strains produced GA_3, which was suspected to be the cause for increased shoot growth and germination. Also, high concentrations of IAA was detected and associated with decreased root length (Cassan *et al*. 2009). Similar results were obtained in the current study with PGPR isolate T22 which inhibited root length compared to the untreated control while increasing germination and shoot and root dry mass.

Isolate A07 and A08 had no significant effect on any of the growth parameters of maize seedlings. This does not however indicate that these isolates are not potential growth promoting bacteria on maize since A07 and A08 may function by alternative modes of action which are not taken into account in the *in vitro* germination and vigour index tests. For example, siderophore production or phosphate solubilisation which can only be utilized by PGPR when a relevant source of nutrients such as soil or growth media as opposed to germination paper, is present. In the current study seed was germinated in germination paper in the absence of soil or other growth media, therefore, even though the modified germination test is a quick method for screening PGPR for their effect on early growth of seedlings, the true potential of some isolates may not be adequately assessed using only these tests. It should be mentioned however that isolates A07, A08 and T22 reduced shoot length compared to the untreated control and although these isolates may possibly enhance maize growth by other mechanisms of action than phytohormone production these isolates were not selected for further experiments in the current study.

In conclusion, PGPR have the ability to promote seedling growth and improve vigour and germination of maize seed with lower vigour. In general, five of the isolates (S6, S7, T19, T29 and A32) positively influenced early growth of maize seedlings and were selected for further investigations in the current study.

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CHAPTER 4

Efficacy of plant growth promoting rhizobacteria formulations for growth promotion of maize

Abstract

The development of plant growth promoting rhizobacteria (PGPR) into formulations which ensure delivery of an effective product is an important and challenging aspect in microbiological research. In this study, the growth promoting effect of five PGPR isolates (*Stenotrophomonas* sp. A32, *Bacillus cereus* S6, *B. cereus* S7, *Lysinibacillus sphaericus* T19 and *Paenibacillus alvei* T29) on maize was determined to select the three most effective strains for formulation development. Significant increases in dry and fresh root and shoot mass of maize was obtained after treatment with isolates S7, A32, T19 and T29. Isolate T19, S7 and T29 significantly enhanced total dry mass by 31.8, 32.5 and 34.3%, respectively, and was selected for the formulation efficacy trial. The isolates were evaluated singly and in combination as a conventional seed treatment and soil drench. In addition, a liquid cell suspension and powder formulation of the individual rhizobacterial isolates and consortium of isolates, respectively, was inoculated onto a novel compost carrier and was also evaluated. The most effective treatment in terms of growth promotion was the soil drench formulation in which isolates T29, T19 and the consortium of T19, S7 and T29 isolates enhanced dry root mass by 70, 65.6 and 82.7% in comparison with the positive control. None of the isolates were effective as seed treatments or inoculants applied as a liquid cell suspension onto the pelletised compost carrier. Only isolate T29 performed significantly well when applied in a perlite powder form to the compost pellets, increasing dry root mass by 65.6%. These results suggest that the success of PGPR in agriculture is strongly dependant on the formulation in which inoculants are applied to the soil. From this study it can be concluded that the soil drench formulation of PGPR is a promising formulation for continued research in field trials.

4.1 Introduction

Graminaceous crops play an important role in agriculture all over the world in ensuring food security. Cereals contribute approximately 1292kcal/day to the human diet which is substantially higher than animal products (501Kcal), fruit (92Kcal) and vegetables (87Kcal) (FAO, 2009). Due to the increasing demand for food worldwide the application of plant growth promoting bacteria (PGPR) to cereals has been an on-going research topic for over seventy years (Cummings, 2009). Studies specifically on maize have included mostly

bacterial species of *Bacillus* (Kumar *et al.* 2007), *Pseudomonas* and *Azospirillum* (Naveed *et al*. 2008; Cassan *et al*. 2009; Gholami *et al*. 2009; Walker *et al*. 2012) as inoculants. These bacterial species have been found to enhance root and shoot growth, seed germination and vigour, nutrient uptake and grain yield of maize (Gholami *et al*. 2009). Some of the PGPR products that are commercially available in South Africa include Landbac®, Biostart® (Microbial Solutions, 2013), BacUp® (Becker Underwood, 2013) and B-Rus which is in the process of being registered (Stimuplant, 2013).

Other than synthetic chemicals, production and commercialization of microbial inoculants pose a special difficulty in that the active ingredient within the solution is a live organism (Mcintyre, 1991). Consequently, it has often been found that microbial inoculants yield satisfactory results within controlled environments but fail to perform effectively under natural field conditions (Bashan and de-Bashan, 2005; Hernández-Rodriguez *et al*. 2008; Saharan and Nehra, 2011). However, through the development of microbial inoculants into adequate formulations consistent satisfactory results can be achieved (Mcintyre, 1991; Nakkeeran *et al.* 2005). The primary purpose of a formulation is to ensure the survival of the bacterial cells after delivery into the soil (Ramamoorthy *et al.* 2001). Microbial formulations may consist of powders, fine dusts, pellets, gels (Lewis, 1991) or liquids (Manikandan *et al*. 2010) depending on the specific requirements of the microorganism. Application of PGPR as seed-treatments (Raupach and Kloepper, 1998; Bora *et al*. 2004; Pereira *et al*. 2009; Manikandan *et al*. 2010) and soil drenches (Rabindran and Vidhyasekaran, 1996; Ramamoorthy *et al.* 2001; Anandaraj and Delapierre, 2010) has been well documented. For example application of *Bacillus amyloliquefaciens* (ex Fukumoto) to maize as a seed treatment resulted in significant protection against *Fusarium verticillioides* root rot (Pereira *et al*. 2009) and a soil drench of *Bacillus* spp. effectively reduced disease severity of *Peronospora tabacina* Adam (blue mold) on tobacco (Zhang *et al*. 2002).

By promoting the use of PGPR products in South Africa, a safer, environmentally friendly and sustainable agricultural system could be fostered. In order to achieve this however, reliable PGPR products with consistent efficacy needs to be produced (Nakkeeran *et al*. 2005). In this context, the growth promoting effect of PGPR isolates on maize in the greenhouse was determined, and the most promising plant growth enhancing isolates were obtained. These selected isolates were applied to maize as a seed treatment and soil drench. A novel formulation consisting of PGPR strains applied to a pelletised compost carrier either as a liquid cell suspension or a dry powder formulation was also evaluated in the formulation efficacy trial. The primary purpose of this study was therefore to determine

the most effective PGPR formulation in terms of growth promotion of maize in the greenhouse.

4.2 Materials and Methods

4.2.1 *In vivo* screening of rhizobacterial isolates for growth promotion of maize

4.2.1.1 Inoculum preparation

Rhizobacterial isolates T19 (*Lysinibacillus sphaericus*), T29 (*Paenibacillus alvei*), S6 (*Bacillus cereus*), S7 (*B.cereus*) and A32 (*Stenotrophomonas* sp.) were selected for this trial based on their positive influence on vigour index of maize as reported in chapter 3. Bacterial suspensions of these isolates were prepared as described in chapter 3 section 3.2.2.1. The suspensions were transferred to 50ml conical plastic tubes and bacterial cells were harvested by centrifuging (5804R Eppendorf centrifuge) at 1500rpm for 10 minutes after which the supernatant was discarded and the pellet was re-suspended in 100ml sterile ringers. To ensure that the pellet had dissolved completely the suspension was vortexed for 2 minutes. The control treatment consisted of sterile ringers only.

4.2.1.2 Greenhouse trial

A sample of the soil used for the trial was analysed at the Department of Plant Production and Soil Science laboratory, University of Pretoria, Pretoria, South Africa. The soil was a loamy sand and consisted of 85% sand, 4% silt and 11% clay. The physio-chemical properties of the soil was as follows: pH (H_2O) 6.6, pH (KCl) 6, Bray-1 P (phosphorous) 10mg/kg and P sorbed 3mg/l.

Plastic pots (20cm diameter) were filled with 4kg steam sterilized soil each and placed in the greenhouse at the Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa. Prior to planting, superphosphate was mixed into the soil at a rate of 0.5g/kg soil. Four surface sterilized (2 minutes in a 1% sodium hypochlorite solution) maize seeds were planted per pot and 100ml of the bacterial inoculum suspensions of each PGPR isolate, respectively, was poured directly over the area where the seeds were planted. Seedlings were thinned to two plants per pot one week after planting. Limestone ammonium nitrate was applied at a rate of 100kg/ha at one and three weeks after planting to supply the necessary nitrogen. Plants were watered with tap water every second day for a period of four weeks and the temperature was maintained between 15-25˚C.

After four weeks plants were harvested by gently washing the soil from the roots in a bucket of water. Roots were excised from the shoots using a pair of scissors and fresh shoot and root mass was recorded. The roots and shoots were then placed in brown paper bags, dried in an oven (Labex (Pty) Ltd, South Africa) at 75˚C until a constant weight was reached, and dry mass was determined by weighing.

4.2.1.3 Experimental design and statistical analysis

Pots were placed in a completely randomized block design on benches within the greenhouse. Five replicates of each treatment were included with a replication consisting of one pot containing two plants. The experiment was performed twice. Data was evaluated by one way ANOVA using GenStat Discovery Edition four. Means were separated using Fisher's Least Significant Difference test (p = 0.05).

4.2.2 Efficacy of different formulations of selected rhizobacterial isolates for growth promotion of maize

4.2.2.1 Inoculum preparation

Isolates S7, T19 and T29 were selected based on the results from aforementioned screening trial, for use in the formulation efficacy trial. Treatments consisted of isolates applied singly and in combination as a liquid cell suspension soil drench, a liquid cell suspension applied to a pelletized compost carrier, a dry powder applied to a pelletized compost carrier and a dry powder seed treatment.

a) Soil drench inoculum

Sterile nutrient broth (NB) (100ml) was inoculated with a single pure colony of isolate S7, T19 and T29, respectively, using a sterile inoculation loop. For the inoculum mixture the NB was inoculated with one colony of every isolate. The bacterial broth (Figure 4.1A) was placed on a shake incubator (200rpm) for 18h at 37˚C. The control consisted of uninoculated nutrient broth. Serial dilutions were done as described in section 3.2.2.1 of chapter 3 and the bacterial concentration obtained was 10^8 CFU/ml.

b) Liquid and powder inoculum for application onto pelletized compost carrier

The untreated compost pellets (Rescue®) consisted of organic composted chicken manure and was manufactured by and obtained from Ag-chem Africa, 288 Mundt St, Silverton, Pretoria, South Africa. The nutrient content of the compost pellets was determined by the Department of Plant Production and Soil Science laboratory, University of Pretoria, Pretoria,

South Africa. Pellet analysis were as follows: pH (H_2O) 7.6, Bray-1 P 1388.3mg/kg, Ca 3908mg/kg, K 15935mg/kg, Mg 2092mg/kg and Na 4823mg/kg.

Bacterial suspensions of the isolates were prepared as for the soil drench inoculum (4.2.2.1a). Bacterial cells were collected after centrifugation (1500rpm for 10 minutes) and discarding of the supernatant. To prepare the powder treated compost pellets, cells were re-suspended in 21ml sterile ringers and the suspension was injected by means of a sterile syringe into a sealed heat resistant plastic bag containing 200g autoclaved Filtraflo® perlite (INFIGRO Natural Technologies (Pty) Ltd, 4 Turk street, Clayville, Olifantsfontein 1666, Johannesburg, South Africa) with a particle size of 15µm. The opening created by the needle during inoculation was resealed using masking tape and the bag was manually kneaded until the contents inside was thoroughly mixed. The inoculated perlite bag was then incubated at 25˚C for six days. This was also the method of inoculum preparation for the seed treatment as described below. After incubation the perlite inoculum was applied to the compost pellets by adding perlite to the pellets at a rate of 3g/500g in a round, plastic, 5L mixing bowl and the contents were mixed. The consortium of inoculants was applied to the compost pellets by blending 1g of perlite inoculum of each isolate and applying the mixture to 500g pellets. The treated pellets were then sealed in polypropylene bags (500g pellets/bag) until used. Untreated pellets were used for the control treatment.

To apply the bacterial cell suspensions onto the compost pellets, bacterial cells collected during centrifugation were re-suspended into 15ml sterile ringers solution and sprayed evenly onto the pellets (500g) using a pressure gun. The consortium of isolates was applied by blending 5ml bacterial suspension of each isolate and spraying the mixture onto 500g pellets. After thoroughly mixing the treated pellets in a mixing bowl the pellets were placed in woven polypropylene bags until used. Untreated pellets served as the control.

The bacterial cell concentrations on the two pellet formulations (i.e. liquid cell suspension treated pellets and perlite treated pellets) was determined by means of a serial dilution. A sample of 10g of pellets per polypropylene bag was taken and vortexed (30 seconds) in 90ml sterile ringers (Merck, 64271 Darmstadt, Germany). The pellet suspension (1ml) was pippeted into 9ml sterile ringers and a dilution range (10^2-10^6) was prepared. From the dilutions 0.1ml was pipetted onto NA (Biolab, Wadeville) amended with polymyxin B antibiotic (10mg/L) and plates were incubated at 25˚C for 24h. Three replications were done. The bacterial concentration on compost pellets with perlite inoculum containing individual isolates S7, T19 and T29 was determined to be 10^5 CFU/g pellets. The bacterial count for pellets treated with liquid suspensions of isolate S7, T19 and T29 was 10^6 CFU/g pellets.

The concentration of bacteria on pellets treated with the consortium of isolates as perlite powder and liquid cell suspension was 10^5 CFU/g pellets and 10^6 CFU/g pellets, respectively.

c) Seed treatment

Maize seeds (PAN 6Q-308B) were surface sterilized by washing seeds in a 1% sodium hypochlorite solution for 2 minutes followed by rinsing in sterile distilled water. Inoculum of the respective rhizobacterial isolates was prepared as described above (4.2.2.1b) by injecting 21ml of the centrifuged bacterial cell suspensions into plastic bags containing 200g sterile perlite and incubating the bags for six days at 25˚C. Uninoculated perlite was used for the control treatment.

The inoculum was applied by coating the seeds in a plastic bag as follows: A seed sample of 25g was placed in a plastic bag and 2ml of 1% carboxymethyl cellulose (CMC) (Sigma-Aldrich, South Africa) solution was added to the seeds to act as a sticker. The bag was inflated (by blowing into it), sealed and shaken until seeds were evenly coated with the CMC. The perlite inoculum was added to the seeds at a rate of 4g/kg seed and the bag was shaken again. This was done individually for every rhizobacterial isolate. The seed treatment with the consortium of three bacterial isolates was done by combining 5g inoculated perlite per isolate, mixing thoroughly and applying to the seeds at 4g/kg seed, as with the individual isolate application. After seeds were coated uniformly the bag was opened and seeds were left to dry overnight in a laminar flow (Figure 4.1B) (Kifle and Laing, 2011). The bacterial concentration on the seeds was determined by performing serial dilutions as described in section 3.2.2.2 of chapter 3 and was found to be approximately 10^5 CFU/seed.

Figure 4.1: (A) Bacterial cell suspension in nutrient broth which was used for the soil drench treatment; (B) Maize seeds in polyethylene bags after treatment with the perlite powder formulation of bacterial isolates; (C) Illustration of method of application of compost pellets during planting of maize in the formulation efficacy greenhouse trial.

4.2.2.2 Greenhouse trial

The properties of the soil used in this pot trial were determined by the Department of Plant Production and Soil Science laboratory. The results obtained were as follows: pH (H_2O) 5.3, Bray-1 P 10.7mg/kg,145mg Ca/kg, 25mg K/kg, 31mg Mg/kg and 28mg Na/kg. The soil was steam pasteurised and superphosphate was added at a rate of 0.5g/kg soil to all pots except those in which compost pellet treatments were to be applied. Analysis of pellet samples revealed that adequate amounts of nutrients could be supplied by the pellets and therefore no additional fertiliser was added to the soil.

Four maize seeds were planted in a row down the centre of each pot to mimic field planting actions. Only the seeds used for the soil drench and compost pellet treatments were surface disinfected prior to use in 1% sodium hypochlorite for 2 minutes and rinsed in sterile distilled water. Seeds treated with the PGPR powder inoculum were not surface disinfected before planting. For the soil drench treatment, a volume of 20ml inoculum of each of the three rhizobacterial isolates (T19, T29 and S7) was poured directly over the area where the seeds were planted. The compost pellets treated with the powder and liquid suspension formulation of isolates were applied by placing inoculum adjacent to and at the same depth as the planted maize seeds in a 3cm wide band at a rate of 6.2g/pot which is equivalent to 250kg/ha (Figure 4.1C). The rate of compost pellets used and the manner of pellet placement was done to mimic field conditions. All treatments consisting of the consortium of three bacterial isolates (T19, S7 and T29) were applied in the same manner as the individual isolate treatments.

One negative and three positive controls corresponding to the seed treatment, soil drench and compost pellet treatment were used in the formulation efficacy trial. The negative control (C) also referred to as the untreated control consisted of untreated maize seed planted in pots receiving no treatment. The positive control corresponding to the soil drench treatment consisted of uninoculated nutrient broth applied as a soil drench to planted maize seeds (CSD). Maize seed coated with uninoculated perlite powder was the positive control (CST) for maize seed treated with perlite inoculum containing the individual isolates or consortium of three isolates. These positive controls were included to ensure that treatment effects were not influenced by components of the formulations other than the bacteria. The third positive control (CP) was untreated seed in combination with untreated compost pellets which was included for the purpose of determining the influence of the compost pellet on maize growth.

Plants were watered every second day for six weeks. Greenhouse temperature ranged between 18 and 28˚C. One week after sowing seedlings were thinned to two seedlings per

pot and 3.5ml of an 80g ammonium nitrate/L water stock solution was applied per 4kg pot (the equivalent of 100kg/ha) to treatments which did not receive compost pellets. Plants were fertilized again after three weeks of growing. Plants were harvested six weeks after planting. Roots were washed free from soil under running tap water and excised using a pair of scissors. Roots and shoots were dried in an oven at 70˚C until dry (i.e. constant weight was reached). Dry mass of roots and shoots was determined by weighing.

4.2.2.3 Experimental design and statistical analysis

Pots were placed on greenhouse benches in a completely randomized block design. Four replicates of each treatment were included with each pot containing two plants representing a replication. The experiment was repeated once. The data was subjected to ANOVA using GenStat Discovery Edition four and the mean separation was done by Fisher's Least Significant Difference test (LSD) ($p = 0.05$)

4.3 Results

4.3.1 *In vivo* screening of PGPR isolates for growth promotion of maize

All five rhizobacterial isolates had a significant root growth promoting effect in terms of fresh and dry root weight of inoculated maize compared to the uninoculated control (Table 4.1). The percentage increase recorded for dry root mass after the bacterial treatments were applied ranged from 34 to 41.8%. The bacterial isolates S7, A32, T19 and T29 promoted shoot growth of maize significantly since percentage dry and fresh shoot mass was increased by 27-34.1% and 29-48.8% respectively. Total fresh and dry weight was also enhanced significantly by all bacterial isolates tested compared to the untreated control. Although growth enhancement between the various bacterial isolates did not differ significantly, isolate T19, T29 and S7 were selected for the formulation efficacy trials. Excluding isolate A32, these isolate treatments were the most effective in increasing total dry mass of maize. Identification of isolate A32 indicated that the bacterium belonged to the genus *Stenotrophomonas* which is considered a human pathogen. Therefore, due to the risk associated with isolate A32 it was not considered for the formulation efficacy trial.

4.3.2 Efficacy of different formulations of selected rhizobacterial isolates for growth promotion of maize

The results obtained for rhizobacterial isolates applied as different types of formulations to maize are presented in Figure 4.2-4.5. No statistical differences were recorded between the untreated control (C) and the controls of the various formulations (CST= seed treated with

uninoculated perlite, CP= untreated pellet applied to maize and CSD= uninoculated nutrient broth applied as a soil drench) for any of the growth parameters measured. This indicates that the components within the formulations i.e. nutrient broth, perlite powder and compost pellets on their own did not have any significant growth promoting effects on maize and that enhancements in growth parameters were due to the bacterial isolates only (Figure 4.2- 4.5).

The soil drench treatment of isolate T19 (T19SD) significantly enhanced dry root mass by 69% compared with the untreated control (C) and by 70% compared with the positive control (CSD) which consisted of uninoculated nutrient broth (Figure 4.2). Maize seed treatment with isolate T19 failed to enhance plant growth considerably in comparison with the untreated control (C) and positive control (CST) consisting of seed treated with uninoculated perlite. Inoculation of isolate T19 onto the pelletized compost carrier as a liquid suspension (T19LP) or dry powder formulation (T19PP) did not result in any significant changes in plant dry mass compared to the negative control and pellet control (CP) where untreated pellets were applied to maize. A general trend showing a 9% increase in root dry mass and 8.1% increase in shoot dry matter in comparison with the negative control was however observed after application of both these treatments.

The soil drench treatment of isolate S7 (S7SD) enhanced dry root mass by 48.1% and dry shoot mass by 29.1% compared to the control treatment (CSD) where uninoculated nutrient broth was applied, however, statistical differences were not obtained (Figure 4.3). Similarly, the seed treatment application of isolate S7 (S7ST) to maize seedlings enhanced dry root mass by 28.8% and shoot mass by 29.1%, respectively, compared to the corresponding control (CST) where seeds were treated with uninoculated perlite powder, but no statistical differences were obtained (Figure 4.3). The compost pellets treated with a dry powder formulation of isolate S7 (S7PP) increased dry root mass by 39.5%, however, results were not significantly different when compared to the control (CP) where untreated pellets were applied to maize. Also, application of isolate S7 as a liquid suspension (S7LP) to compost pellets did not result in a significant increase in plant mass compared with the untreated pellet control (CP), however, a general increase of 16% in dry shoot mass was observed.

When isolate T29 was applied as a soil drench treatment (T29SD) to maize plants, results showed that root as well as shoot dry mass was considerably higher than the untreated (C) and positive control (CSD) (Figure 4.4). The percentage increase in root and shoot dry mass for maize seedlings treated with T29SD was 53.6 and 46.8%, respectively, compared with the positive control (CSD) which consisted of uninoculated nutrient broth. Dry root mass was increased significantly by up to 65.7% when the compost pellets treated with the dry powder

inoculum of isolate T29 (T29PP) was applied to maize plants as compared to when untreated pellets were applied to maize. However, the effect of the compost pellets inoculated with isolate T29 as a liquid suspension (T29LP) on dry root mass was not meaningful according to statistical analysis, although dry root mass was generally increased by 40%. Inoculation of maize seeds with a perlite powder containing isolate T29 (T29ST) did not result in any significant plant growth promotion.

When all isolates (T19, S7 and T29) were applied simultaneously in a mixture as a soil drench (SDM), both root and shoot dry mass were significantly higher than the untreated control (C) (Figure 4.5). Also, compared with the control (CSD) consisting of uninoculated nutrient broth, the SDM treatment increased root mass considerably by 82.7% and shoot mass by 38.2%. The treated compost pellets inoculated with a liquid cell suspension (LPM) or perlite powder formulation (PPM) of the consortium of three isolates did not have a meaningful effect on maize growth. However, the LPM and PPM treatments increased dry root mass by 19.1 and 21.7%, respectively, in comparison with the uninoculated compost pellet control (CP). Although the general trend observed was that seed treatment with the consortium of isolates increased dry root and shoot mass with up to 20%, the increase was not significantly different from the seed treatment with uninoculated perlite (CST). No statistical differences were found between the mixture of isolates vs. single isolate treatments applied in any formulation (data not shown).

Table 4.1: The growth promoting effect of rhizobacterial isolates on maize after four weeks in the greenhouse

	Seedlings mass in grams						Percentage increase in mass**					
	Fresh mass *			Dry mass*			Fresh mass			Dry mass		
Isolates	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**
Control	7.32 a	3.01 a	10.33 a	0.62 a	0.24 a	0.85 a	0	0	0	0	0	0
S6	8.52 ab	4.18 b	12.7 b	0.72 ab	0.33 b	1.06 b	16.36	38.87	22.92	17.78	41.03	24.24
S7	9.48 bc	4.45 b	13.93 b	0.78 b	0.34 b	1.12 b	29.47	47.84	34.82	27.93	41.87	31.81
A32	10.9 c	4.17 b	15.07 b	0.80 b	0.32 b	1.13 b	48.87	38.54	45.86	30.18	36.81	32.02
T19	10.01 bc	4.35 b	14.03 b	0.79 b	0.32 b	1.13 b	33.02	42.52	35.79	29.74	39.81	32.54
T29	9.74 bc	4.29 b	14.36 b	0.83 b	0.33 b	1.14 b	36.71	44.52	38.99	34.12	34.66	34.27
LSD	1.929	0.868	2.318	0.112	0.057	0.148						
CV (%)	23	23.6	19.2	16.4	20.1	15.3						

*In each column, mean values followed by the same letters do not differ significantly according to Fisher's LSD test at p = 0.05
**Percentage change in mass= [(treatment-control)/control x 100], positive values indicate an increase in mass compared to the control. Values are means from two repeated experiments with five replications.

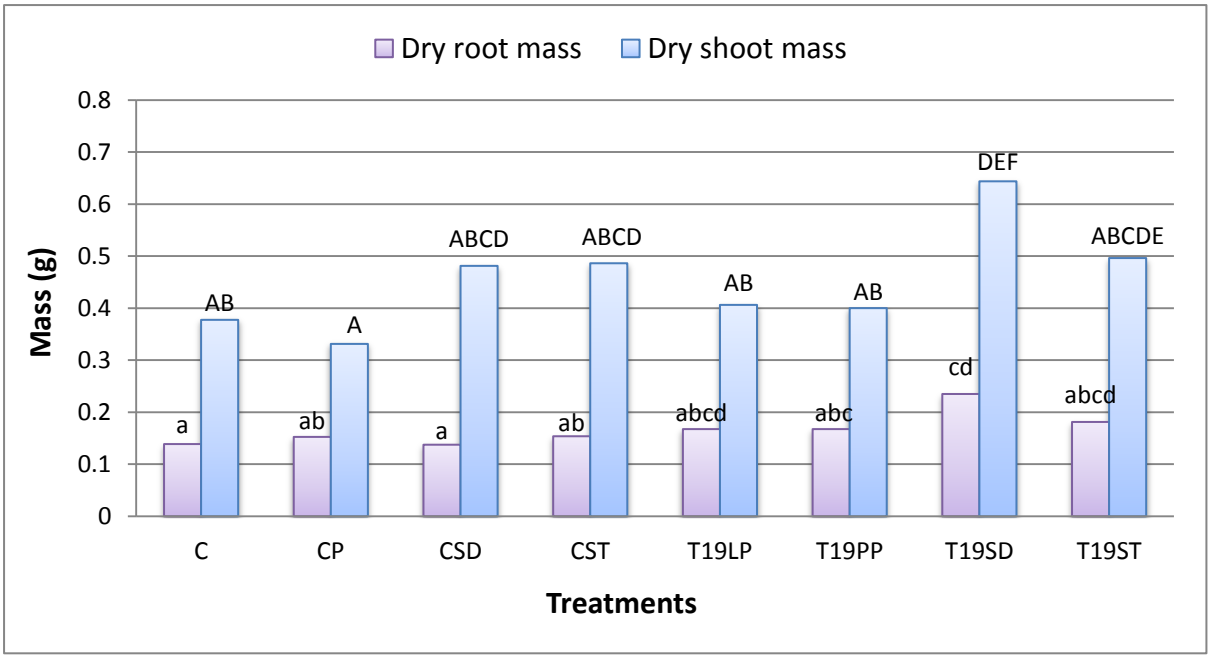

Figure 4.2: Effect of isolate T19 applied in four different formulations on growth of maize in the greenhouse. Mean values of two experimental trials with four replications of two plants each are presented for dry root and shoot mass respectively. Means with different letters indicate significant differences according to Fisher's LSD test (p = 0.05). C=negative control; CP=control pellet, CSD=control soil drench, CST=control seed treatment, LP=pellet treated with inoculated nutrient broth, PP=pellet treated with inoculated perlite powder, SD=soil drench, ST=seed treatment. (Dry root mass: LSD= 0.071; CV= 38%. Dry shoot mass: LSD= 0.171; CV= 35.6%)

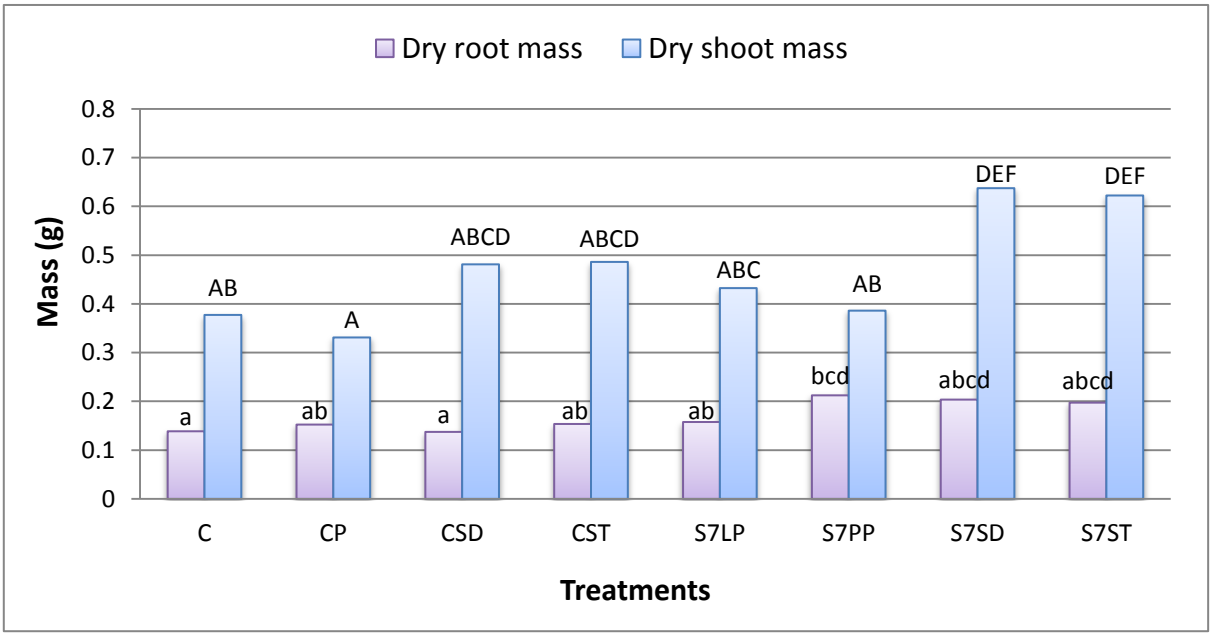

Figure 4.3: Effect of isolate S7 applied in four different formulations on growth of maize in the greenhouse. Mean values of two experimental trials with four replications of two plants each are presented for dry root and shoot mass respectively. Means with different letters indicate significant differences according to Fisher's LSD test (p = 0.05). C=negative control; CP=control pellet, CSD=control soil drench, CST=control seed treatment, LP=pellet treated with inoculated nutrient broth, PP=pellet treated with inoculated perlite powder, SD=soil drench, ST=seed treatment. (Dry root mass: LSD= 0.071; CV= 38%. Dry shoot mass: LSD= 0.171; CV= 35.6

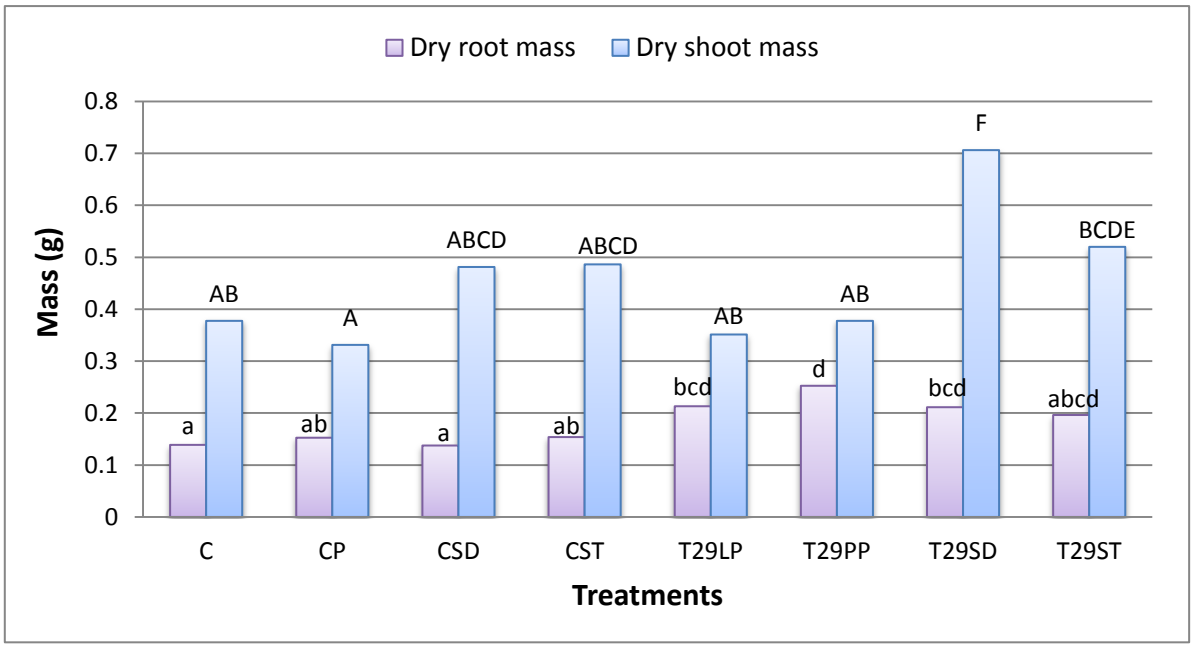

Figure 4.4: Effect of isolate T29 applied in four different formulations on growth of maize in the greenhouse. Mean values of two experimental trials with four replications of two plants each are presented for dry root and shoot mass respectively. Means with different letters indicate significant differences according to Fisher's LSD test (p = 0.05). C=negative control; CP=control pellet, CSD=control soil drench, CST=control seed treatment, LP=pellet treated with inoculated nutrient broth, PP=pellet treated with inoculated perlite powder, SD=soil drench, ST=seed treatment. (Dry root mass: LSD= 0.071; CV= 38%. Dry shoot mass: LSD= 0.171; CV= 35.6%).

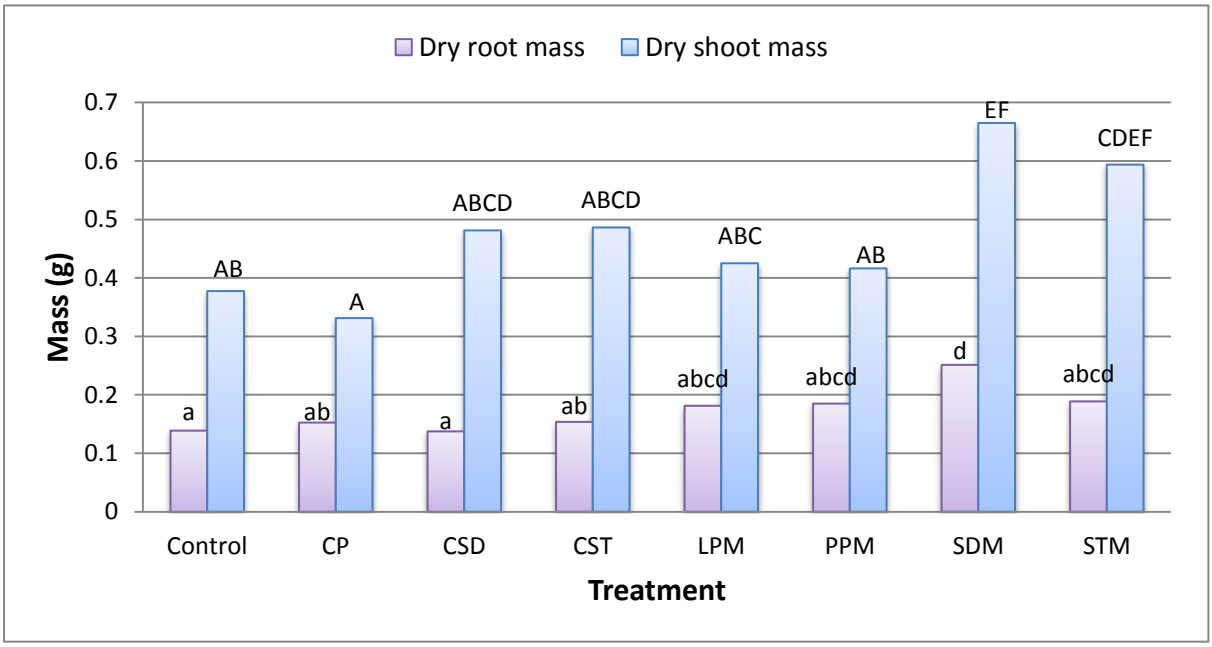

Figure 4.5: Effect of a consortium of all three isolates (T19, S7 and T29) applied in four different formulations on growth of maize in the greenhouse. Mean values of two experimental trials with four replications of two plants each are presented for dry root and shoot mass respectively. Means with different letters indicate significant differences according to Fisher's LSD test (p = 0.05). C=negative control; CP=control pellet, CSD=control soil drench, CST=control seed treatment, MLP=pellet treated with inoculated nutrient broth, PPM=pellet treated with inoculated perlite powder, SDM=seed drench mix, STM=seed treatment mix. (Dry root mass: LSD= 0.071; CV= 38%. Dry shoot mass: LSD= 0.171; CV= 35.6%)

4.4 Discussion

Maize is known to produce various root exudates such as sugar-, amino acid- and carbohydrate- containing compounds into the soil. Studies have shown that these nutrients are utilized by rhizosphere mycoflora and may therefore be useful in supporting the growth of newly introduced inoculants (Kozdrój *et al*. 2004). In the current study five rhizobacterial isolates (*Stenotrophomonas* sp. A32, *Bacillus cereus* S6, *B. cereus* S7, *Lysinibacillus sphaericus* T19 and *Paenibacillus alvei* T29) were tested in a greenhouse trial to determine their beneficial effects on the growth of maize. The results showed that the bacterial isolates enhanced root and shoot growth of maize by up to 41.8 and 34.1%, respectively. The Bacilli bacteria are well known in the agricultural sector for their plant growth promoting and biological control ability (Lee *et al*. 2006; Ahmad *et al*. 2008; Kumar *et al*. 2012). Zhang *et al*. (2004) demonstrated that *B. pumilus* SE34 and *B. pasteurii* C-9 promoted tobacco growth after seed treatment with these strains (Zhang *et al*. 2004). Kumar *et al*. (2007) also reported considerable increases of over 100% in root dry mass after maize was inoculated with *Bacillus megaterium* and *B. subtilis,* respectively (Kumar *et al*. 2007).

The success of the Bacilli class bacteria as growth promoters and biocontrol agents can be attributed to the wide range of mechanisms utilized by these bacteria (Avis *et al*. 2008). Previous work has shown that the Bacilli isolates T19, T29, S6 and S7 used in the current study exhibits a broad spectrum of mechanisms of plant growth enhancement and biocontrol. The mechanisms, determined by *in vitro* assays, included phosphate solubilisation and the production of antibiotics, siderophores, chitinase and indole acetic acid and growth in nitrogen free media indicating nitrogen fixation (Hassen, 2007; Pretorius, 2012). Although bacterial metabolite production *in vitro* may vary from *in vivo* production, the beneficial growth promoting effects of the rhizobacterial isolates in this study is most probably as a result of the bacteria utilizing some of the modes of action detected in the laboratory assays (Smyth *et al*. 2011).

Based on the growth promoting efficacy of isolates T19, T29 and S7 in the aforementioned screening trial, these isolates were used in the formulation efficacy trial. The formulation in which a rhizobacterial isolate is applied is a vital aspect determining the success or failure of the microbial inoculant in the soil (Xavier *et al*. 2004). The Bacilli are an attractive option for developing of commercially feasible microbial formulations owing to their ability to form endospores (Lee *et al*. 2006). Endospores are able to withstand desiccation due to radiation, high temperatures and toxic compounds allowing for development of a potentially stable formulation (Laloo *et al*. 2008) with a long shelf-life (Wiwattanapatapee *et al*. 2004). Hence,

the efficacy of different formulations of rhizobacterial isolates T19, T29 and S7 was evaluated in the current study.

In general, most isolates (T19, T29 and the consortium of isolates) performed best in terms of growth promotion when applied as a soil drench formulation. The soil drench treatment containing isolate T19 viz. T19SD was the most effective of all treatments concerning individual application of isolates. The treatment increased dry root mass by 70% compared to the control where uninoculated nutrient broth was applied (CSD). Although no significant differences were detected between individual isolate treatments vs. the consortium of isolates, the SDM increased dry root mass by 12% more than the best performing individually applied isolate (T19SD). Similar results were obtained by Domenech *et al*. (2006) when combined application of three PGPR strains i.e. *Bacillus subtilis*, *B. amyloliquefaciens* and *Pseudomonas fluorescens* promoted better growth of tomato and pepper in comparison with individual strains. Jetiyanon *et al*. (2003) also found that strain mixtures of *Bacillus amyloliquefaciens* strain IN937a and *B. pumilus* strains IN937b gave better control of a broad-spectrum of diseases on various crops than application of the individual strains. The greater success of the combined application of PGPR strains is suspected to be due to the synergistic effect of the different modes of action of each strain (Nakkeeran *et al*. 2005).

One of the primary reasons for the efficiency of the soil drench formulation as compared to the seed- and compost pellet treatments may be due to a difference in inoculum concentration applied. A substantially higher concentration of bacterial cells could be delivered to the soil in the soil drench formulation (± 2 x 10^9 CFU/pot) compared to the seed treatment (± 4 x 10^5 CFU/pot) and compost pellets treated with a liquid cell suspension (± 6.2 x 10^6 CFU/pot) or dry powder (± 6,2 x 10^5 CFU/pot) formulation respectively. Kifle and Laing (2011) reported that no significant differences in growth parameters of lettuce compared to the untreated control were found when *B. subtilis* was applied at 10^5 CFU/seed. However, at a concentration of 10^8 CFU/seed the treatment effectively promoted lettuce growth (Kifle and Laing, 2011). In the current study the concentration of inoculum delivered via the seed was 10^5 CFU/seed and may therefore have been too low.

Okon and Itzigsohn (1995) suggested that the optimal bacterial concentration to be delivered to the soil should be between 10^9 to 10^{10} cells g-1 or ml-1. Obtaining such high concentrations on the seed or pellet surface is problematic especially when a carrier (such as perlite powder) is used (Okon and Itzigsohn, 1995). Only a small volume of bacterial suspension can be applied to the perlite before it is saturated thereby restricting the

concentration applied to the seed and compost pellet. Furthermore, the concentration of bacteria introduced into the soil as a seed treatment or as inoculum on a compost pellet carrier is dependent on the size and number of seeds planted or pellets used. Nevertheless, isolate T29 caused a significant increase (65.5%) in dry root mass when applied to soil on the pellet carrier as a perlite powder inoculum. As demonstrated by Pretorius (2012) this isolate has multiple modes of action which include phosphate solubilisation, siderophore production, ability to fix atmospheric nitrogen and biocontrol activity. These characteristics may enable isolate T29 to adapt more easily within the soil compared to the other isolates which are not so versatile. When applied to the compost pellet carrier as a liquid suspension, isolate T29 generally increased dry root mass with 40%, however, statistical differences were not recorded. It is also worth mentioning that isolate S7 enhanced dry root mass with 39.5% when inoculated onto the compost pellet as a dry powder formulation however statistical differences were not obtained.

A possible solution for increasing the bacterial concentration on the seed and compost pellet is to increase the initial cell count in the bacterial suspension. This may be accomplished by addition of substances such as cassava starch, sodium alginate (Tittabutr *et al*. 2007), trehalose, glycerol (Manikandan *et al*. 2010) or molasses (Nita *et al*. 2012) to the growth medium as an added source of nutrients. As illustrated by Tittabutr *et al*. (2007) additives can improve the quality of inoculants by providing increased protection of bacterial cells against environmental stresses, enhancing adhesion to seed and stabilizing the formulation. In their study addition of gum arabic, cassava starch or polyvinyl alcohol to yeast extract mannitol medium increased cell numbers of several Rhizobia strains above cell counts obtained when strains were inoculated into the growth medium alone (Tittabutr *et al*. 2007).

Adhesives can also be added to improve the quality of the carrier material. In the current study 1% carboxymethyl cellulose was used as an adhesive for the seed treatment. However, the application of a higher concentration of CMC or perhaps an alternative adhesive could possibly have increased the survival of the PGPR isolates on maize seed. Daza *et al*. (2000) found that perlite supplemented with 10% sucrose supported the survival of *Bacillus megaterium, Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli* during storage in polyethylene bags for longer and at higher cell concentrations compared to the use of other additives (2% carboxymethyl cellulose, 25% glycerol and 50% polyethyleneglycol).

Other aspects such as root colonisation studies of isolates T19, T29 and S7 specifically on maize should also be conducted even though such studies on wheat has already indicated

good colonisation (Pretorius, 2012). The viability of inoculants on the seed and pellet surface also needs to be investigated. In general, the bacterial concentration on seeds is known to decrease substantially shortly after seed application and research efforts should be focused on minimizing this loss (Date, 2001). Movement of the bacteria from the seeds and pellets to the rhizosphere may also influence the effectiveness of these treatments (Pal *et al*. 2001; Wiwattanapatapee *et al.* 2004). In the current study it was observed that the compost pellets dissolved at a slow rate in the soil. It is possible that the bacterial cells were trapped within the surface cracks of the pellets and inoculum release occurred at a slow rate thereby delaying the beneficial effects of the bacterial isolates. The slow disintegration of the compost pellets may also explain why the pellet control containing the untreated pellets did not enhance maize growth compared to the negative control. Materechera and Morutse (2009) studied the potential of chicken manure to be used as a source of phosphorous for dry land maize production in South Africa. In their study the benefit of the chicken manure application only became apparent at the end of the growing season. They concluded that since nutrients within the chicken manure are mostly present in organic forms, the slow process of mineralization first needs to take place for nutrients to be available for plant uptake (Materechera and Morutse, 2009). The benefit of the compost pellet would therefore only become apparent over a longer period of time such as in field applications during a full growing season. Alternatively, additives such as disintegrants could aid in faster disintegration of the compost pellet thereby triggering quick release of nutrients and rhizobacterial isolates into the soil (Wiwattanapatapee, 2004).

In conclusion, the development of effective PGPR formulations is a challenging task that requires much research efforts. From the current study it was clear that the efficacy of rhizobacteria in the soil depended significantly on the formulation type. Individual rhizobacterial isolates were demonstrated to dramatically enhance growth of maize when applied as a soil drench. The soil drench application of isolate T19 performed better than all other treatments in terms of plant growth promotion. Further refinement of formulations is however necessary for successful application of inoculants as seed treatments or on compost pellet carriers. A general trend towards increased root mass of maize was observed after treatment with rhizobacterial isolates inoculated onto compost pellets. However, the only rhizobacterial isolate used in the current study which significantly enhanced dry root mass of maize when applied as a dry powder to the compost pellets was isolate T29. Although the consortium of isolates did not perform significantly better than individual application of isolates, the soil drench consortium of isolates enhanced plant mass considerably compared to the untreated control. Research pertaining to the synergistic effects of the rhizobacterial isolates would be helpful to improve the effectiveness of the

consortium formulations. Future research should also involve survival studies of these isolates within formulations and performance of these isolates in field trials.

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CHAPTER 5

The biocontrol activity of rhizobacteria against *Rhizoctonia solani* on maize

Abstract

Rhizoctonia solani Kühn is the causal agent of pre- and post-emergence damping-off of maize seedlings and many other economically important crops. The present study evaluated the potential of rhizobacterial isolates for biological control of a pathogenic isolate of *R. solani*. Seven rhizobacterial isolates viz. T19, T29, T22, S6, S7, A08 and A07 belonging to the Bacilli class were applied to *R. solani* infected maize seedlings in seedling trays. Dry root mass was significantly increased by 53.2, 54.1 and 68.2% after treatment with isolates A08, A07 and T19, respectively. In addition, disease severity was reduced by these isolates and control treatments demonstrated that these isolates had root growth promoting effects. *In vitro* dual culture trials showed that isolates T22, S7, A08 and A07 exerted antibiosis towards the pathogen, however, no biocontrol with isolate T22 and S7 was observed *in vivo*. Based on the efficacy of the rhizobacterial isolates in seedling trays, isolates A08, A07 and T19 were selected for evaluation as seed treatments and soil drenches against *R. solani* in pots. The only rhizobacterial treatment to inhibit *R. solani* was a soil drench of isolate T19 which enhanced dry shoot mass and total dry mass of infected seedlings by more than 100%.

5.1 Introduction

One of the major problems associated with the use of pesticides for disease control is that pathogens tend to develop resistance against synthetic chemicals. Once pathogens have developed resistance, effective disease control using chemicals can only be achieved by increasing pesticide doses which eventually results in over use of chemicals (Pinstrup-Anderson, 2001). Excessive use of chemicals has multiple detrimental effects on the environment and increases input costs substantially (Orhan *et al*. 2006). Nevertheless, due to the high crop quality and yield obtained as a result of the use of synthetic pesticides, hazardous chemical products have dominated the market place for many years (Bashan, 1998; Bashan and de-Bashan, 2005; Cummings, 2009b). However, consumers' disapproving attitude towards the use of pesticides (Pal and Gardener, 2006) has generated an increasing trend towards the use of more environmentally friendly products such as biological control agents (Babalola, 2010).

Rhizobacteria that reduce or prevent plant disease by inhibiting plant pathogens are referred to as biocontrol agents (Maheshwari, 2010). Biological control agents offer a range of alternative modes of action to synthetic chemicals and when applied in rotation with chemicals may help decrease the chances of pathogens developing resistance (Fravel, 2005). Effective control of monocyclic, soil-borne and post-harvest diseases has been achieved through application of biocontrol agents (Pal and Gardener, 2006). A common soil-borne pathogen that has been the target of several biocontrol research studies is *Rhizoctonia solani* (Howell and Stipanovic, 1978; Jung *et al*. 2003; Montealegre *et al*. 2003; Demirci *et al*. 2009; Montealegre *et al*. 2010). This fungal pathogen is able to survive in the soil for many years and causes extensive damage to a wide range of hosts worldwide (Pfahler and Petersen, 2004). Grain crop losses of between 11-40% have been reported due to *R. solani* infections (Akhtar *et al*. 2009). On maize, the pathogen causes sheath and leaf blight (Li *et al*. 1998), crown and brace rot (Sumner and Minton, 1989), and wilting, yellowing and damping-off (Buddemeyer *et al*. 2004). Various biocontrol products are available commercially for the control of *R. solani*. Some of these products include AtEze™, Kodiac®, Trichodex®, Gliogard™ and Intercept™ (Nakkeeran, 2005). Most of the available commercial products have been registered and manufactured outside the borders of South Africa with only a few including TrichoPlus™, Subtilex® NG (Becker Underwood, 2013) and Eco-T® (Plant Health Products, 2013), produced locally.

Considering the above mentioned aspects, the aim of this study was to screen for effective rhizobacterial isolates with biocontrol activity against *R. solani* on maize in dual culture and seedling tray trials. The most promising rhizobacterial isolates showing biocontrol of *R. solani* would then be further evaluated in pots in greenhouse trials. The results obtained, if promising, would serve as the starting point for the future development of a locally produced biocontrol product that could improve the country's economy and contribute to South Africa's agricultural sector by controlling *R. solani* diseases.

5.2 Materials and Methods

5.2.1 Pathogenicity evaluation of *Rhizoctonia solani* isolates 10375 and 10376

5.2.1.1 Inoculum preparation

A maize pot trial was conducted to evaluate the pathogenicity of two *Rhizoctonia solani* isolates (*R. solani* 10375 and 10376) obtained from the Agricultural Research Council (ARC), Plant Protection Research Institute, Roodeplaas, Pretoria, South Africa. The original

fungal isolates were obtained from the ARC in sterile McCartney bottles on potato dextrose agar (PDA) (Biolab, Wadeville, South Africa) slants. Inoculum was prepared by cutting a 5mm x 5mm block from the actively growing fungal culture on the agar slant and placing the block in the centre of a Petri dish (9cm) containing PDA. The Petri dish was incubated for seven days under normal light at 25˚C after which sub-cultures were prepared by cutting a 5mm x 5mm block from the edge of the fungal culture and placing it on PDA in a Petri dish. After 5 days of incubation at 25˚C the fungal cultures were covered with 23g of steam sterilized and thrice autoclaved soil. Seven days after incubation under normal light at 25˚C the soil was overgrown with *R. solani* and was scraped off the surface of the PDA culture and was used as the inoculum source in the pathogenicity trial (Yang *et al*. 2008).

5.2.1.2 Greenhouse trial

Maize seeds (PAN 6Q-308B) were surface sterilized in 1% sodium hypochlorite (2 minutes) and rinsed thoroughly in distilled water. Four seeds were planted in each pot (12cm diameter) which was filled with steam sterilized soil. The same soil type as in chapter 4 section 4.2.1.2 was used. The *R. solani* inoculated soil inoculum prepared in the Petri dishes was added to each pot adjacent to the planted seeds at a rate of 20g per pot. For the control treatment the pots were inoculated with autoclaved soil. The plants were watered every second day with tap water and a temperature of between 10-28˚C was maintained in the greenhouse.

The plants were harvested after four weeks by carefully rinsing the soil from the roots under running tap water. The roots were rated for disease severity according to a disease severity scale as described by Harvenson *et al*. (2005) where 0= no disease symptoms; 1= up to 25% of root surface necrotic; 2= 26-50% of root surface necrotic but no rotting of internal tissue; 3= 51-75% of root system rotten including internal tissue; 4= more than 75% of roots rotten; 5= 100% of roots rotten/no roots. After scoring the seedlings, one plant from every replicate for every treatment was selected and two small root pieces was cut from every seedling using a sterile scalpel. The root pieces were surface sterilized in 1% sodium hypochlorite for 3 minutes and rinsed five times in sterile distilled water. Roots were blotted dry on sterile blotter paper and placed on water agar (Agar Bacteriological, Biolab, Wadeville, South Africa) in a Petri dish. The Petri dishes were incubated under normal light at 25˚C for seven days and observed under the microscope to confirm the presence of *R. solani*. Root and shoot wet mass of the remaining plants was recorded and plants were placed in brown paper bags in an oven (Labex (Pty) Ltd, South Africa) at 70˚C until the weight remained constant, after which dry mass was determined.

5.2.1.3 Experimental design and statistical analysis

Five replications were included with four plants per replicate and the trial was conducted twice. Pots were arranged on greenhouse benches in a completely randomized block design. Data was analysed through one way ANOVA where means were separated according to Fishers protected LSD test using GenStat Discovery Edition four.

5.2.2 *In vitro* dual culture trial to determine antibiosis of rhizobacterial isolates

Seven rhizobacterial isolates indicated in Table 5.1 were tested for antibiotic production in a dual culture trial with *R. solani* isolate 10375 which was found to be the more pathogenic of the two *R. solani* isolates.

Table 5.1: Isolate codes and identity of rhizobacteria used in the current study

Number	Isolate code	Isolate identity
1	T19	*Lysinibacillus sphaericus*
2	T29	*Paenibacillus alvei*
3	T22	*Paenibacillus alvei*
4	S6	*Bacillus cereus*
5	S7	*Bacillus cereus*
6	A08	*Bacillus aryabhattai*
7	A07	*Bacillus aryabhattai*

All isolates were obtained from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa. Bacterial cultures were revived from storage by streaking a single Microbank™ bead (Pro-Lab Diagnostics) containing the bacterial isolate onto fresh nutrient agar (NA) (Biolab, Wadeville, South Africa) and incubating it for 24h at 25˚C in the dark.

For the dual culture trial a single fungal block (5mm) from an actively growing culture of *R. solani* 10375 was placed in the centre of a 9mm Petri dish containing sterile solidified potato dextrose agar (PDA) (Biolab, Wadeville, South Africa). The rhizobacterial isolates were spot inoculated onto the PDA by pricking a single bacterial colony from the freshly prepared rhizobacterial cultures prepared above, with a sterile toothpick and inoculating three spots equidistantly around the fungal block. Petri dishes were incubated at 25˚C for seven days in the dark. Percentage inhibition of *R. solani* was determined using the following formula:

% inhibition of mycelial growth = [(R2 – R1)/R2] x 100

Where R1 = Distance of mycelial growth towards bacterial spots

R2 = Maximum mycelial growth of control between spots

5.2.3 Screening of rhizobacterial isolates for biocontrol activity against *R. solani* on maize in seedling trays

5.2.3.1 Inoculum preparation

The same rhizobacterial isolates which were used in the dual culture trial (S6, S7, A08, A07, T19, T29 and T22) were used in this seedling tray trial. Bacterial suspensions of these isolates were prepared as described in chapter 3 section 3.2.2.1. Since *R. solani* 10375 was the more pathogenic of the two *Rhizoctonia* isolates it was used in this trial. The fungus was grown by cutting a 5mm x 5mm fungal block from the edge of an actively growing culture and placing it on PDA in the centre of a Petri dish. The Petri dish was incubated under normal light at 25˚C for seven days or until the entire surface of the PDA in the Petri dish was covered with fungal growth.

5.2.3.2 Greenhouse trial

Plastic seedling trays (10cm x 15cm) were surface sterilized in 2% sodium hypochlorite for 10 minutes and were rinsed thoroughly with tap water before use. The seedling trays were filled with steam sterilized soil of which the properties were described in chapter 4 section 4.2.1.2. Additional phosphorous was added to the soil at a rate of 0.5g superphosphate/kg soil. Each cell of the seedling tray was inoculated with two *R. solani* agar plugs (5mm diam.). After three days, two surface sterilized (2 minutes in 1% sodium hypochlorite) maize seeds were planted in each seedling tray cell. Seven days after planting, seedlings were thinned to one plant per cell and 10ml of the bacterial inoculum of each isolate, respectively, was poured into each cell. Seedling trays were placed on greenhouse benches in a completely randomized block design and the greenhouse temperature was maintained between 15 - 30˚C. Seedlings were watered on a daily basis with tap water.

There were four control treatments: 1) Plants drenched with uninoculated nutrient broth; 2) Plants inoculated with *R. solani* 10375 only; 3) Plants inoculated with rhizobacterial isolates only and 4) a commercial control where Celest® XL (Syngenta, 94 Bekker Street, Midrand, South Africa) was applied as a seed treatment. For the commercial control, seeds were surface sterilized as in section 5.2.1.2 above and placed in a plastic bag. Celest XL® was added at the recommended rate of 1ml/kg seed using a pipette, the bag was closed and

shaken until seeds were uniformly covered. The bag was reopened and placed in a laminar flow overnight for the seeds to dry.

Plants were harvested, evaluated and scored after four weeks in the same manner and according to the same disease severity scale as for the pathogenicity trial described in section 5.2.1.2. After scoring the seedlings, the wet mass was determined after excising the roots from the shoots of seedlings. Dry mass was also determined after placing the roots and shoots in brown paper bags to dry in an oven at 70˚C.

5.2.3.3 Experimental design and statistical analysis

Two independent experiments were conducted and each consisted of five replicates where one seedling tray with six cells represented one replicate. All data were analysed by ANOVA and mean contrasts were performed using Fisher's protected least significant difference test with p = 0.05 as the level of significance. Statistical analysis was performed using GenStat Discovery Edition four.

5.2.4 Biocontrol activity of selected rhizobacterial isolates applied as a soil drench and seed treatment against *R. solani* in a maize pot trial

5.2.4.1 Inoculum preparation

The three rhizobacterial isolates that performed the best in the biocontrol of *R. solani* on maize in the seedling trays were isolates A07, A08 and T19. These isolates were selected for the biocontrol trial performed in pots. The isolates were applied to maize as a seed treatment and soil drench, respectively. The soil drench inoculum was prepared as described in chapter 3 section 3.2.2.1. The bacterial seed treatment inoculum (perlite based) was prepared and applied to seed in the same manner as for the formulation efficacy trial described in chapter 4 section 4.2.2.1.C and the final concentration per seed was 10^5 CFU/seed. The pathogen inoculum was prepared by growing fungal cultures of *R. solani* 10375 on PDA at 25˚C for 7 days as described in section 5.2.3.1.

5.2.4.2 Greenhouse trial

Pots containing steam sterilized soil (the same as described in chapter 4 section 4.2.1.2) amended with 0.5g superphosphate/kg soil was inoculated with five *R. solani* 10375 agar plugs (5mm diam.). Agar plugs were arranged by placing one in the centre of the pot and the remaining four equidistantly around the centre plug. Three days after inoculating the soil with *R. solani* four surface sterilized (2 minutes in 1% sodium hypochlorite) maize seeds were

planted in a row down the centre of each pot. The bacterial suspension was then poured over the area where the untreated seeds were planted (20ml/pot). The treated seed was planted in the same manner by using sterile tweezers so as to not remove the bacterial perlite inoculum. After germination, the seedlings were thinned to two plants per pot.

There were five control treatments: 1) An untreated control i.e. uninoculated maize plants; 2) Plants grown in soil inoculated with *R. solani* 10375 only 3) Plants inoculated with rhizobacterial isolates applied as a soil drench 4) Plants inoculated with rhizobacterial isolates as a seed treatment and 5.) A commercial control where Celest® XL was applied as a seed treatment (1ml/kg seed) using the same method as for the seedling tray trial in section 5.2.3.2.

The temperature in the greenhouse ranged between 15 and 31˚C and plants were watered with tap water every second day. Plants were harvested after four weeks and evaluated for disease using the same disease rating scale as for the pathogenicity and seedling tray trial (section 5.2.1.2). Wet root and shoot mass was recorded after excising roots from shoots using a pair of scissors. Dry plant mass was obtained by weighing the root and shoots after drying in brown paper bags in an oven until a constant weight was reached.

5.2.4.3 Experimental design and statistical analysis

The experiment consisted of five replicates per treatment where two plants per pot represented one replicate. The trial was repeated once. Statistical analysis of all data was done by one way ANOVA using GenStat Discovery Edition four. Significant differences between treatments were detected using Fisher's protected least significant difference test ($p = 0.05$).

5.3 Results

5.3.1 Pathogenicity evaluation of *Rhizoctonia solani* 10375 and 10376

The pathogenicity of two *R. solani* isolates (10375 and 10376) from the ARC was determined in a greenhouse pot trial. The uninoculated control treatment showed no symptoms of *R. solani* infection and thus 100% of the control seedlings were scored a rating of 0 on the disease severity scale of Harvenson *et al*. (2005) (Table 5.2). Maize seedlings planted in *R. solani* 10375 infested soil showed symptoms of pre- and post-emergence damping-off whereas *R. solani* 10376 only caused post-emergence damping-off of seedlings (Figure 5.1). Disease symptoms were primarily observed on the roots of infected seedlings

and microscopic examination of fungal isolations made from the roots confirmed the presence of *Rhizoctonia solani* on infected seedlings. The roots of seedlings treated with *R. solani* 10375 were few, weak, soft and brown and many sclerotia were visible on the roots. Evaluation of *R. solani* 10375 infected roots according to the disease scale revealed that 51.9% of seedlings had a rating of 4 and 48.1% of seedlings had a rating of 5 (Table 5.2). On the other hand, most seedlings (34.3%) infected with *R. solani* 10376 had a disease severity rating of 1 and only 8.6% and 2.8% of seedlings were severely infected resulting in a disease rating of 4 and 5, respectively.

Table 5.2: Percentage of maize seedlings with *R. solani* disease ratings according to the scale of Harvenson *et al.* (2005)

	Disease severity scale*					
Isolate	**0**	**1**	**2**	**3**	**4**	**5**
Control	100%	0%	0%	0%	0%	0%
R. solani 10375	0%	0%	0%	0%	51.9%	48.1%
R. solani 10376	11.4%	34.3%	31.5%	11.4%	8.6%	2.8%

*Disease severity scale: 0= no disease symptoms; 1= up to 25% of root surface necrotic; 2= 26-50% of root surface necrotic; 3= 51-75% of root system rotten including internal tissue; 4= more than 75% of root system rotten; 5= 100% of roots rotten/no roots

Figure 5.1: Uninoculated control or healthy maize seedlings (left) versus seedlings grown in soil inoculated with *R. solani* 10376 (centre) and 10375 (right).

The pathogenic effect that the *R. solani* isolates had on maize root and shoot growth is indicated in Table 5.3. The shoot-, root- and total fresh mass of seedlings were significantly reduced by both pathogens compared to the uninoculated control. The *R. solani* isolates also reduced dry root mass considerably when matched with the healthy control. *Rhizoctonia solani* 10376 had no significant effect on dry shoot mass and total dry mass of seedlings whereas *R. solani* 10375 reduced these parameters significantly.

Table 5.3: The effect of *R. solani* isolate 10375 and 10376, respectively, on maize seedlings

	Seedling mass in grams					
	Fresh mass*			Dry mass*		
Isolate	**Shoot**	**Root**	**Total**	**Shoot**	**Root**	**Total**
Control	2.41 c	1.81 c	4.22 c	0.23 b	0.23 c	0.46 c
R. solani 10375	0.80 a	0.14 a	0.94 a	0.15 a	0.12 a	0.27 a
R. solani 10376	1.73 b	0.89 b	2.62 b	0.21 b	0.18 b	0.39 bc

*In each column, mean values followed by the same letters do not differ significantly according to Fisher's LSD test at P=0.05.

5.3.2 *In vitro* dual culture trial to determine antibiosis of rhizobacterial isolates

Four of the rhizobacterial isolates viz. T22, S7, A08 and A07 showed inhibition of fungal growth around the point of bacterial inoculation on the NA media. Isolate A08 gave the highest percentage of inhibition which was 75.2% followed by isolate S7 resulting in 77.3% inhibition of *R. solani* mycelial growth. The percentage fungal growth inhibition obtained when isolate T22 and A07 was spot inoculated on the NA was 58.4% and 8.9%, respectively.

5.3.3 Screening of rhizobacterial isolates for biocontrol activity against *R. solani* 10375 on maize in seedling trays

The untreated control was healthy and showed no symptoms of *R. solani* infection. All seedlings of the untreated control were rated 0 based on the diseases severity scale of Harvenson *et al*. (2005). No significant differences were obtained for seedling mass measured for the untreated and nutrient broth control indicating that nutrient broth which was used for the seed drench treatments had no significant effect on plant growth. The *R. solani* inoculated control showed that *R. solani* 10375 had a significant effect on the roots of maize seedlings by significantly reducing fresh- and dry root matter content by 24 and 33.4%, respectively, compared to the untreated control (Table 5.4). As in the pathogenicity trial, disease symptoms were not observed on the shoots of seedlings, however, the roots were severely infected and the *R. solani* inoculated control had a disease severity rating of 4. No significant differences were obtained between the growth parameters measured for the commercial control fungicide treatment and the untreated control.

Table 5.4: Effect of rhizobacterial isolates on maize seedlings in seedling trays inoculated with *Rhizoctonia solani* 10375

	Seedling mass in grams					
	Fresh mass*			Dry mass*		
Treatment	**Root**	**Shoot**	**Total**	**Root**	**Shoot**	**Total**
Untreated control	6.36 cd	9.88 abcd	16.24 abc	0.76 cde	0.86 abc	1.61 abc
Nutrient broth control	6.66 cd	11.85 cdefg	18.51 cd	0.75 cde	0.88 abc	1.63 abc
Commercial control	5.76 bc	11.05 abcdef	16.81 bc	0.65 abcd	0.91 abcd	1.57abc
R. solani inoculated control	4.86 ab	9.11 a	13.97 ab	0.50 ab	0.86 abc	1.36 ab
S7 only	9.25 g	14.33 h	23.58 e	0.95 ef	1.19 e	2.14 d
S7 & *R. solani*	4.33 a	10.23 abcd	14.56 ab	0.49 a	0.98 abcde	1.47 ab
T19 only	6.63 cd	11.56 bcdefg	18.19 cd	0.98 f	1.11 de	2.09 d
T19 & *R. solani*	7.63 def	12.96 fgh	20.59 ef	0.85 def	1.00 abcde	1.84 cd
T29 only	8.19 efg	12.5 efgh	20.69 cd	0.93 ef	1.20 e	2.12 d
T29 & *R. solani*	4.64 ab	10.19 abcd	14.83 ab	0.51 ab	1.04 bcde	1.55 ab
S6 only	7.77 def	11.05 abcdef	18.82 cd	0.63 abc	0.99 abcde	1.62 abc
S6 & *R. solani*	4.60 ab	9.60 ab	14.20 ab	0.61 abc	0.85 ab	1.46 ab
A08 only	9.02 fg	13.72 gh	22.74 e	1.00 f	1.07 cde	2.07 d
A08 & *R. solani*	5.51 abc	9.61 abc	15.12 ab	0.77 cde	0.85 ab	1.62 abc
A07 only	7.62 def	11.64 bcdefg	19.26 cd	1.02 f	1.07 bcde	2.09 d
A07 & *R. solani*	4.38 ab	9.16 a	13.54 a	0.78 cde	0.81 a	1.59 abc
T22 only	7.38 de	11.87 defg	19.25 cd	0.70 bcd	0.99 abcde	1.69 bc
T22 & *R. solani*	4.52 ab	10.44 abcde	14.96 ab	0.47 a	0.85 ab	1.31 a
LSD	1.412	2.249	3.076	0.199	0.223	0.326
CV (%)	25.1	22.8	19.8	30.4	25.8	21.6

*In each column, mean values followed by the same letters do not differ significantly according to Fisher's LSD test at P=0.05. Values are means of two experiments consisting of five replicates each where one seedling tray with six cells represented one replicate.

Root mass of seedlings treated with rhizobacterial isolates T22 or T29, in addition to inoculation with *R. solani,* was significantly reduced below that of the untreated control. Disease symptoms of seedlings treated with isolate T22 and T29 were evaluated and scored a value of 3 and 4, respectively, based on the disease severity scale. Treatment with isolate T29 in the absence of the pathogen increased dry root mass by 22.5% although this was not statistically significant. Nevertheless, dry shoot mass was significantly promoted by up to 40% compared to the untreated control. Isolate S7 applied as a soil drench treatment also promoted shoot dry mass and total dry mass by 38.5% and 32.5%, respectively, compared to the untreated control, in *R. solani* free soil. However, no significant differences were obtained between growth parameters of *R. solani* infected seedlings treated with isolate S7 and seedlings grown in *R. solani* inoculated soil. The disease severity rating of seedlings planted in soil that was inoculated with *R. solani* as well as rhizobacteria isolate S7 was 4.

Application of rhizobacterial isolate S6 to seedlings grown in *R. solani* inoculated soil did not have any significant effect on dry mass compared to the *R. solani* inoculated control and the seedlings were scored a disease severity rating of 4. Individual application of isolate T22 and S6 in the absence of *R. solani* did not have any growth promoting effects on maize seedlings.

Despite the presence of *R. solani* in the soil, dry root mass of maize seedlings individually treated with three rhizobacterial isolates viz. A08, A07 and T19 was increased significantly by 53.2%, 54.1% and 68.2%, respectively, compared to the *R. solani* inoculated control. Furthermore, these isolates had a tendency to enhance dry root mass of seedlings planted in *R. solani* infected soil above that of the untreated control, although, these differences were not statistically significant. Disease symptoms of *R. solani* were visible on seedling roots even though isolate A08, A07 and T19 were applied and a disease severity rating of 3 was scored to these seedlings. Significant growth promotion was observed when isolates A08, A07 and T19 were applied to maize in the absence of *R. solani*. Rhizobacterial isolate T19 enhanced dry root mass by 30.3%, isolate A07 by 35.1% and isolate A08 by 32.6% compared to the untreated control. In the absence of *R. solani* application of isolate T19 also enhanced dry shoot mass by 29.5% compared to the untreated control.

5.3.4 Biocontrol activity of selected rhizobacterial isolates as a soil drench and seed treatment against *R. solani* in a maize pot trial

The results indicating the biocontrol activity of rhizobacterial isolates A08, A07 and T19 applied as a soil drench and seed treatment against *R. solani* are shown in Table 5.5. The untreated control and control plants treated only with rhizobacterial isolates appeared healthy and had a disease severity rating of zero. Conversely, severe root rot symptoms were observed on the seedlings of the *R. solani* inoculated control and pre-emergence damping-off drastically reduced seedling emergence compared to the untreated control. Although similar disease symptoms were observed in the pot trial as in the seedling tray trial the pathogen was even more aggressive in the pot trial and a disease severity value of 5 was recorded for the *R. solani* inoculated control seedlings. The total fresh and dry mass of seedlings grown in *R. solani* inoculated soil was significantly reduced by 96.7% and 91.2%, respectively, compared to the untreated control (Table 5.5). The root, shoot and total mass of seedlings treated with the commercial control was significantly higher in comparison with the *R. solani* inoculated control but was however lower than the untreated control. The disease severity rating of the commercial control was 4.

The application of isolate A07 and A08 as a soil drench or seed treatment to maize seedlings planted in *R. solani* infected soil did not have a significant effect on any of the growth parameters measured when compared to the *R. solani* inoculated control (Table 5.5).

Table 5.5: Biological control activity of selected rhizobacterial isolates against *R. solani* 10375 in maize pot trials

	Seedling mass in grams					
	Fresh mass*			Dry mass*		
Treatment**	**Root**	**Shoot**	**Total**	**Root**	**Shoot**	**Total**
Untreated control	1.90 cd	5.08 bc	6.98 bc	0.23 de	0.37 d	0.6 de
Commercial control	0.88 b	2.38 b	3.26 b	0.15 b	0.19 c	0.33 c
R. solani 10375 inoculated control	0.04 a	0.19 a	0.23 a	0.01 a	0.05 a	0.05 a
T19 SD only	2.31 de	5.60 cd	7.91 cd	0.3 f	0.4 de	0.7 ef
T19 ST only	1.65 c	4.45 b	6.10 b	0.25 def	0.33 d	0.58 de
T19 SD & *R. solani* 10375	0.45 ab	0.82 a	1.27 a	0.03 a	0.14 bc	0.17 bc
T19 ST & *R. solani* 10375	0.15 a	0.52 a	0.67 a	0.04 a	0.05 ab	0.09 a
A08 SD only	2.27 de	5.43 bcd	7.70 cd	0.23 cd	0.40 de	0.63 def
A08 ST only	1.89 cd	4.75 bc	6.64 bc	0.17 bc	0.35 d	0.52 d
A08 SD & *R. solani* 10375	0.19 a	0.55 a	0.74 a	0.03 a	0.06 ab	0.1 ab
A08 ST & *R. solani* 10375	0.01 a	0.02 a	0.03 a	0.01 a	0.02 a	0.02 a
A07 SD only	2.54 e	6.23 d	8.77 d	0.29 ef	0.47 e	0.76 f
A07 ST only	2.05 cde	4.91 bc	6.96 bc	0.2 bcd	0.38 de	0.58 de
A07 SD & *R. solani* 10375	0.03 a	0.07 a	0.10 a	0.01 a	0.01 a	0.01 a
A07 ST & *R. solani* 10375	0.12 a	0.33 a	0.45 a	0.01 a	0.05 ab	0.06 a
LSD	0.538	1.106	1.504	0.059	0.098	0.138
CV (%)	17.4	20.5	17.8	18.7	17.8	14.9

*In each column, mean values followed by the same letters do not differ significantly according to Fisher's LSD test at P = 0.05.
** SD = The particular rhizobacterial isolate was applied to the soil as a soil drench; ST = The particular rhizobacterial isolate was applied as a seed treatment before planting.
Values are means from two repeated experiments with five replications each consisting of two plants per pot.

Both of these isolates (A07 and A08) were unable to perform as well as the commercial control. The disease symptoms on seedlings planted in soil infected with *R. solani* and treated with a soil drench of A07 and a seed treatment of A07 and A08, respectively, was rated 5 on the disease severity scale. The disease severity for seedlings that received a soil drench of isolate A08 was 4. In the absence of *R. solani* however, isolate A07 was the only treatment to increase all plant parameters except dry root mass significantly, compared to the untreated control when applied as a seed drench. Also, in pathogen free soil the soil drench application of isolate A07 performed statistically better than the seed treatment application for all parameters measured except root fresh mass and shoot dry mass. The

A08 soil drench and seed treatment which was applied to maize planted in pathogen free soil did not enhance plant growth compared to the untreated control.

The results showed that there was no significant difference between the *R. solani* inoculated control and the T19 seed treatment which was applied to seeds before planting in *R. solani* infected soil (Table 5.5). A disease severity rating of 5 was scored to seedlings grown in soil inoculated with *R. solani* and treated with the T19 seed treatment. In contrast shoot dry mass and total dry mass of seedlings planted in *R. solani* infected soil was significantly increased by more than 100% when drenched with isolate T19 as compared to the *R. solani* inoculated control which received no rhizobacterial treatment. According to statistical analysis, the T19 soil drench treatment had a similar effect on root fresh mass, shoot dry mass and total dry mass of seedlings planted in *R. solani* infected soil as the commercial control. However, root evaluation of seedlings treated with a soil drench of isolate T19 revealed that more than 75% of the root surface was brown and rotten and a disease rating of 4 was scored. When the seed treatment consisting of isolate T19 was applied to maize and seeds were planted in *R. solani* free soil, results were statistically similar to the untreated control whereas the seed drench application of isolate T19 was the only treatment that performed significantly better than the untreated control with respect to dry root mass.

5.4 Discussion

Results from the pathogenicity trial showed that *R. solani* 10375 had a more severe effect on seedlings than *R. solani* 10376 since root, shoot and total seedling mass was significantly lower compared with seedlings infected by *R. solani* 10376. The higher disease severity rating of seedlings infected with *R. solani* 10375 also supported these results. Most seedlings infected with *R. solani* 10375 had severe root rot which limited shoot growth significantly and pre-emergence damping-off was more prevalent than post-emergence damping-off. Since *R. solani* 10375 was the more pathogenic of the two fungal isolates, the pathogen was used to determine the biocontrol activity of various rhizobacteria against *R. solani*.

The biocontrol properties of Gram positive bacteria belonging to the Bacilli class have often been reported (Jung *et al*. 2003; Jacobsen *et al*. 2004; Kloepper *et al*. 2004; Cardinale *et al*. 2006; Muis and Quimio, 2006; Pérez-García *et al*. 2011). Ugoji and Laing (2007) demonstrated in rhizotrons that *Bacillus subtilis* isolate B81 effectively controlled *R. solani* on maize by 35 and 48% based on dry shoot and root mass increase, respectively. In the current study, three of the rhizobacterial isolates belonging to the Bacilli class viz.

Lysinibacillus sphaericus isolate T19 and *Bacillus aryabhattai* isolates A08 and A07 showed biocontrol activity towards the pathogen in the seedling tray trial. *Lysinibacillus sphaericus* isolate T19 achieved the best control of 68.2% based on dry root mass increase whereas isolate A07 and A08 increased dry root mass by 54.1% and 53.2%, respectively. Isolates T19, A07 and A08 performed better than the commercial control where seeds were treated with the fungicide Celest XL. Furthermore isolate T19, A07 and A08 controlled the pathogen to such an extent that dry root mass of seedlings was higher than that of the untreated control, although not statistically significant. When studying the biocontrol potential of *B. subtillis*, Ugoji and Laing (2007) also reported that the most apparent effect of the bacterium in controlling *R. solani* was observed on the roots of maize seedlings. They suggested that pathogen suppression specifically on the roots could be due to the mechanisms of biocontrol utilized by bacteria functioning in the root area (Ugoji and Laing, 2007).

In the dual culture study it was demonstrated that isolate A07, A08, T22 and S7 inhibited *R. solani,* indicating antibiotic production. Two of these isolates i.e. A07 and A08, as mentioned above, supressed *R. solani* in seedling trays. According to Ongena and Jacques (2008), production of antibiotic compounds such as lipopeptides are most frequently involved in pathogen suppression mechanisms of *Bacillus*. The iturin and fengycin lipopeptides have strong antifungal activity (Ongena and Jacques, 2008) and the role of iturin A produced by *Bacillus subtilis* RB14 in controlling *R. solani* damping-off of tomatoes has previously been demonstrated (Asaka and Shoda, 1996). Isolate S7 and T22 which inhibited *R. solani* by 77.3 and 58.4% *in vitro* had no effect on the pathogen in seedling trays. Other studies have also reported poor correlation between *in vivo* and *in vitro* results (Pengoo *et al*. 2000; Smyth *et al*. 2011; Pretorius, 2012). Knudson *et al*. (1997) suggested that the antibiotics synthesized by the bacteria may become inactivate when released into the natural soil environment where conditions are more erratic than in the laboratory. Rhizobacterial survival in the soil may also be affected adversely in the stressful soil environment where nutrients may be limited and competition with other microorganisms may occur, thereby limiting the biocontrol effects exerted by the bacteria (Van Veen *et al.* 1997; Roy, 2010). It is also possible that other mechanisms of action are utilised by the bacteria for biocontrol *in vitro* compared to *in vivo* where bacteria are directly associated with the plant (Smyth *et al*. 2011). *In vitro* results are thus not always indicative of *in vivo* performance and should be interpreted cautiously by taking into account that conditions in the natural environment vary from those in the laboratory.

In the dual culture trial, isolate T19 showed no antibiosis towards *R. solani* although significant control of the pathogen was achieved in seedling trays. This may indicate that

mechanisms other than the production of antibiotics may have been utilized by the bacterium. Alternative mechanisms by which the rhizobacteria reduced disease was not determined in the present study. However, Hassen (2007) and Pretorius (2012) found that isolate T19 had chitinolytic activity on chitin minimal media and produced halos on chrome azurol S media which indicated siderophore production. Indeed, the biocontrol effect of *Bacillus* spp. is generally ascribed to their secretion of extracellular lytic enzymes (Pérez-García *et al*. 2011), competition (Ugoji and Laing, 2007) or siderophore production (Zahir *et al.* 2004). Extracellular enzymes such as chitinases, glucanases and proteases contribute to pathogen suppression by degrading the pathogen cell wall. Jung *et al*. (2003) demonstrated that a chitinase-producing bacterium *Paenbacillus illinoisensis* KJA-424 caused mycelial lysis of *R. solani* during *in vitro* co-culture studies and significantly reduced the incidence of damping-off in cucumber seedlings *in vivo* (Jung *et al*. 2003).

Of interest is the finding that the rhizobacterial isolates, T19, A07 and A08, which had biocontrol ability against *R. solani,* also showed good root growth promoting effects when applied to maize planted in pathogen free soil. The isolates treatments (S6, S7, T29 and T22) which were unable to improve the growth parameters of maize planted in *R. solani* inoculated soil were also deficient in root growth promoting effects when applied to the seedlings in the absence of *R. solani.* These isolates did however reduce the disease severity of *R. solani* compared to the *R. solani* inoculated control treatment. Similarly, Pereira *et al.* (2009) found that maize seed treatment with *Bacillus amyloliquefaciens* or *Microbacterium oleovorans* reduced disease severity of *Fusarium verticillioides,* however, did not significantly affect any of the seedling growth parameters. They proposed that the results were an indication that the biocontrol agents *B. amyloliquefaciens* and *M. oleovorans* had biocontrol activity but lacked growth promoting abilities. The current study supports their hypothesis since isolates S6, S7, T29 and T22 did not have any significant effect on root growth however reduced *R. solani* disease symptoms. The ability of isolates T19, A07 and A08 to suppress *R. solani* may thus be attributed to their root growth promoting effect that may have enabled the seedlings to outgrow and escape pathogen infection.

Considering the potential of isolates T19, A07 and A08 to control *R. solani* on infected maize seedlings in seedling trays, further evaluation of their efficacy as biocontrol agents in pot trials was done. The isolates were applied as seed treatments prior to planting in *R. solani* infected soil and as soil drenches after planting. The only treatment that showed biocontrol towards *R. solani* was isolate T19 applied as a soil drench. The T19 soil drench treatment enhanced dry shoot mass and total dry mass above that of the *R. solani* inoculated control. This treatment also reduced disease symptoms of *R. solani* and gave similar control of

R. solani as the commercial control. The effectiveness of rhizobacteria to control *R. solani* on maize (Hebbar et al. 1998; Muis and Quimio, 2006) and various other crops has been reported by several authors (Howell and Stipanovic, 1978; Jung *et al*. 2003; Pretorius, 2012). Research by Muis and Quimio (2006) showed that *Bacillus subtilis* increased seed germination and plant height of maize significantly compared to the *R. solani* inoculated control and decreased disease incidence and severity of banded leaf and sheath blight caused by the pathogen. Similar to the findings in the current study, Muis and Quimio (2006) found that a *B. subtilis* treatment did not perform significantly better, regarding aforementioned parameters, than the commercial control which consisted of a fungicide (captan) seed treatment. The bacterial treatment did, however, improve grain yield above that of the commercial control (Muis and Quimio, 2006). Isolate T19 used in the current study may also have potential to increase grain yield in future field trials.

Application of rhizobacterial isolates as a seed treatment did not result in any biocontrol of *R. solani*. Callan *et al*. (1997) investigated the biocontrol activity of *Pseudomonas aureofaciens* as a seed treatment against Pythium root rot on *sh2* sweet corn (*Zea mays* L.) and demonstrated that the minimum concentration of bacterium required for suppression of Pythium was 10^7 CFU/seed if normal seed coating was done. However, when seed bio-priming was the method used to treat seeds an initial concentration of 10^5 CFU/ml was enough to ensure rapid multiplication of *P. aureofaciens* up to 10^7 CFU/seed (Callan *et al*. 1997). In the current study, the concentration of rhizobacterial inoculum on seeds was 10^5 CFU/seed which may have been too low to result in seedling protection from *R. solani*. Methods such as seed bio-priming which involve coating of the seed with the biocontrol agent and then incubating in warm, moist conditions until just before emergence should be investigated. Other strategies such as those discussed in chapter 4 should also be considered to enhance the inoculum concentration on seeds.

No biocontrol of *R. solani* was observed when isolates A07 or A08 were applied as a soil drench. The soil drench with isolate A08 did however reduce the disease severity of *R. solani* from a rating of 5 to a rating of 4. The reduced ability of the isolates to control *R. solani* in pots as compared to in seedling trays may be due to the larger volume of soil in pots providing more nutrients and space for pathogen development and growth than in the seedling tray compartments. A higher concentration of *R. solani* may therefore have been present within the pots. Indeed, the ability of *R. solani* to rapidly increase inoculum levels and infect seeds prior to and during germination is well known (Cummings, 2009a). This may also explain why the disease severity of *R. solani* was higher in the pots, where seedlings had a disease rating of 5, as compared to in the seedling trays, where seedlings had a rating

of 4. In addition, it is well known that root exudates such as organic acids, amino acids and certain sugars create attractive nutrient-rich niches for both rhizobacteria and plant pathogens. Competition between the pathogen and rhizobacteria for niches and nutrients is thus highly likely and may be an important mechanism of biological control (Compant *et al*. 2005). The larger volume of soil available in pots would thus provide more space and nutrients encouraging less competition between rhizobacteria and the pathogen and thus reduced disease control.

The amount of rhizobacteria applied per volume of soil was also much smaller in the pots than in the seedling tray compartments and thus the concentration of rhizobacteria within pots was much lower. This may explain why a reduction in plant growth promoting effects of rhizobacteria was observed in the pot trial as compared to the seedling tray trial when rhizobacterial isolates were applied to seedlings grown in pathogen free soil. Various studies have demonstrated that the inoculum dose and frequency of application significantly influence the efficacy of rhizobacteria in soil (Zang *et al*. 2004; Kifle and Laing, 2007; Manikandan *et al*. 2010). In general, rhizobacterial inoculum concentrations of between 10^8-10^9 CFU/ml are more effective than lower concentrations and applying a treatment several times delivers better results than a single application (Kumar *et al*. 2012; Pretorius, 2012). The strategy of repeated applications of rhizobacterial treatments will however only be practical if the advantages conferred justify the additional expense associated with several applications.

The results demonstrated that *L*. *sphaericus* isolate T19 was the only treatment that reduced the detrimental effects of *R. solani* in both the seedling tray and pot trial. Isolate A07 and A08 also have potential to control *R. solani,* as indicated in the seedling tray trial, however more research regarding the modes of action used and inoculum rates to be applied needs to be done before effective pathogen control can be achieved. Results also demonstrated that a soil drench was more effective in controlling *R. solani* disease than a seed treatment, however, the true potential of the formulations will only be known once the optimum rhizobacterial inoculum concentration is achieved.

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CHAPTER 6

Survival of selected plant growth promoting rhizobacterial strains as a powder and liquid formulation on composted chicken manure pellets

Abstract

Agricultural waste products such as chicken manure contain essential nutrients that can be utilized by plants and microbes. Studies have shown that organic waste such as dehydrated sludge, vegetable waste and sugarcane bagasse can maintain cell viability and stability of rhizobia and can thus be used as microbial inoculant carriers. These organic carriers are cheaper and safer than inorganic inoculants and simultaneously provide a means for waste disposal. In this present study, the survival of three PGPR isolates viz. T19 (*Lysinibacillus sphaericus*), S7 (*Bacillus cereus*) and T29 (*Paenibacillus alvei*) applied to composted chicken manure pellets was evaluated. The isolates were formulated into a powder and liquid formulation, respectively, before inoculation onto the pellets and the viability of the isolates was recorded over a period of six months at two storage temperatures of 25 and 35˚C. The powder formulation supported isolate survival significantly better than the liquid formulation and the viable microbial population decreased faster at the higher temperature. It was also shown that viable cell numbers of isolate T19 remained the most stable throughout the shelf-life study and that isolate T29 was the least stable. The use of composted chicken manure pellets therefore offers a new exciting approach for PGPR inoculant carriers and future aspects involving quality control, field performance and reliability of the product are worth further investigation.

6.1 Introduction

Phosphorus (P) deficiency in soil often limits the use of potentially good land for crop production in South Africa (Materechera and Morutse, 2009; Yazdani 2011). In some of the soils in the country approximately 65 kg/ha of P is required to restore P levels to be able to obtain average maize yields of 4 tons/ha (FSSA, 1989). Although synthetic fertilizers can be used to add the required amount of P, extensive use of inorganic chemicals damages the soil physical, chemical and biological properties (Ahmad *et al*. 2006). Furthermore, the majority of small-scale maize farmers in South Africa are unable to afford addition of costly fertilizers (Materechera and Morutse, 2009; Harman *et al.* 2010).

An alternative source of P to chemical fertilizers is organic compost. Compost such as chicken manure is readily available in South Africa since numerous small scale farmers and large scale commercial poultry production units exist. The composted organic material is available at a fraction of the cost of chemical fertilizers (Materechera and Morutse, 2009), is a rich source of plant nutrients (Ahmad *et al*. 2006) and can improve the physical properties of soil (Al-Moshileh and Motawei, 2007). Furthermore, composts are known to support the growth of microbial populations in the soil (Marcos *et al*. 1995). A novel approach is thus to enrich composts with microorganisms such as plant growth promoting rhizobacteria (PGPR) to produce a value-added product (Naveed *et al*. 2008). For example, Naveed *et al*. (2008) studied the effect of a biofertilizer product consisting of fruit and vegetable waste amended with *Pseudomonas fluorescens* biotype G on maize yield. The biofertilizer significantly increased maize yield in comparison with an individual application of synthetic N-fertilizer or organic waste respectively (Naveed *et al*. 2008). Other agricultural waste products that have been used as carriers for microbial inoculants include dehydrated wastewater sludge, plant compost and filtermud (Rebah *et al*. 2007).

The use of microbial products has much potential in organic farming, which has recently become a priority worldwide as a consequence of the increased consumer demand for safer, healthier food production (Mahdi *et al.* 2010). Farmers are, however, concerned about the shelf-life of microbial products which generally do not last as long as synthetic chemicals under storage conditions (Vendan and Thangaraju, 2007). Fortunately, researchers have been focusing on producing formulations or carriers that guarantee the long term survival of the microorganisms in a good physiological state until delivery (Ramamoorthy *et al*. 2001; Spadaro and Gullino, 2005; Sangeetha and Stella, 2012). Few, if any of these studies have however pertained to the shelf-life of plant growth promoting bacteria (PGPR) on composted chicken manure as carrier material. Organic material is generally rich in essential nutrients that can ensure the survival of microbes for a long period of time. For example, Ngampimol and Kunathigan, (2008) illustrated that vegetable waste supported the survival of a high amount of microbes for up to four months at room temperature. The purpose of the current study was therefore to determine the survival of three PGPR isolates as powder or liquid formulations on composted chicken manure pellets stored at 25 and 35˚C.

6.2 Materials and Methods

6.2.1 Preparation of liquid and powder inoculum

The bacteria used in the study were *Lysinibacillus spaericus* isolate T19, *Bacillus cereus* isolate S7 and *Paenibacillus alvei* isolate T29. All isolates were obtained from the University of Pretoria's PGPR culture collection at the Department of Microbiology and Plant Pathology. Bacterial suspensions of each isolate were prepared in nutrient broth (Biolab, Wadeville, South Africa) as described in chapter 3 section 3.2.2.1. The concentration of the isolate suspensions was 10^8 CFU/ml as determined by serial dilutions. The bacterial suspensions in nutrient broth served as the liquid inoculum.

The same procedure as above was repeated to prepare bacterial suspensions in nutrient broth for use as inoculants in the dry powder formulation. The powder formulation was prepared as follows: Filtraflo® perlite (200g) (INFIGRO Natural Technologies (Pty) Ltd, 4 Turk street, Clayville, Olifantsfontein 1666, Johannesburg, South Africa) with a particle size of 15µm was autoclaved three times at 121˚C for 30 minutes in an autoclavable bag. A sterile syringe was used to inoculate 21ml of the above prepared bacterial suspension in nutrient broth into the sterile perlite bag (Stimuplant, Pretoria, South Africa). To prevent contamination of the inoculated perlite, the point of inoculation was re-sealed using masking tape. The bag was manually kneaded to mix the inoculum with the perlite after which it was incubated at 25˚C for four days in the dark. After incubation serial dilutions were performed by dissolving 1g of perlite in 9ml sterile ringers (Merck, 64271 Darmstadt, Germany) and diluting to 10^{-8} in test tubes. Dilutions (0.1ml) were spread plated onto nutrient agar (NA) (Biolab, Wadeville) and Petri dishes were incubated for two days at 25˚C in the dark before colonies were counted.

6.2.2 Compost pellet treatment with liquid and dry powder formulation

The composted chicken manure pellets (Rescue®) was obtained from Ag-chem Africa, 288 Mundt St, Silverton, Pretoria, South Africa. The nutrient content of the pellets was as reported in chapter 4 section 4.2.2.1b.

The compost pellets treated with the liquid formulation was prepared by coating the pellets with the bacterial cell suspensions of each isolate (T19, S7 and T29), respectively, by spraying 15ml onto 500g pellets in a 5L plastic bowl using a pressure gun connected to an air compressor (ABAC, Pietermaritzburg, South Africa). The bowl was constantly rotated while the pellets were sprayed to ensure even coating of the pellets. The dry powder

formulation was applied to the compost pellets by mixing perlite inoculum (1g containing the population load of 10^7 cells) with the pellets at a concentration of 3g/500g pellets in a 5L plastic mixing bowl using a wooden spoon. Untreated pellets served as the control. Three replications consisting of 500g pellets each were treated per isolate for every formulation and treatments were prepared in duplicate since the experiment was to be conducted at two temperatures (25 and 35˚C).

The pellet samples were packaged into woven polypropylene bags and labelled accordingly. The bags were incubated in incubators in the dark at 25˚C and 35˚C, respectively, for a period of six months. Within each incubator the bags were arranged in a completely randomized design. Serial dilutions of the compost pellets was prepared as described in chapter 4 section 4.2.2.1b and plated onto semi-selective media (see next section), directly after pellet treatment, as well as on a monthly basis for six months to determine viable bacterial counts. Since colony counts were performed on semi-selective media a low concentration of resident pellet microflora or contaminants was also able to grow on the media. Therefore, the CFU/g pellets was determined by subtracting the number of colonies occurring on the control plate of untreated pellets (resident pellet microflora) from the number of colonies present on the treatment plate (PGPR isolates plus resident pellet microflora).

6.2.3 Semi-selective media for enumeration of PGPR isolates on compost pellets

Since the composted chicken manure pellets were not sterile, resident bacteria would also be obtained on NA when performing colony counts of PGPR isolates during the longevity study. Therefore, to obtain more accurate counts of PGPR isolates on the non-sterile pelletised compost, a semi-selective medium was developed to reduce the number of resident bacterial colonies on agar plates.

6.2.3.1 Antibiotic disc assay

A disc assay was performed to screen for a single antibiotic which would inhibit resident pellet bacteria on NA while all three PGPR isolates (T19, S7 and T29) would remain viable. A suspension of the native microorganisms present on the compost pellets was prepared by adding 10g of pellets to 90ml sterile ringers in an Erlenmeyer flask and vortexing for 30 seconds to loosen surface colonizing bacteria. A bacterial suspension of each PGPR isolate was also prepared as described in chapter 3 section 3.2.2.1. The isolate and resident pellet microflora suspensions (100µl) were separately spread plated onto NA plates ensuring that

the entire surface was evenly covered. A sterile antibiotic ring (Mastring-S M13, Mast Diagnostics) was placed on the NA surface and the Petri dishes were incubated at 25˚C in the dark. The ring consisted of eight antibiotic discs containing chloramphenicol, erythromycin, fusidic acid, oxacillin (OX), novobiocon, penicillin G (PG), streptomycin and polymyxin B (PB). The control was a sterile filter disc soaked in sterile water. After two days of incubation the agar plates were viewed for the presence/absence of inhibition zones surrounding the antibiotic discs. The antibiotics that created an inhibition zone on the agar plates with the resident pellet microflora but failed to form an inhibition zone on the plates with the PGPR isolate suspensions were selected for the next step. Three repeats for every PGPR isolate and resident pellet microflora were done.

6.2.3.2 Antibiotic sensitivity assay

In the antibiotic disc assay, all rhizobacterial isolates showed resistance towards two antibiotics that inhibited resident pellet microflora. To select the most applicable antibiotic for PGPR isolate enumeration in the shelf-life study, the degree of sensitivity of rhizobacterial isolates to specific antibiotics was determined by plating onto antibiotic amended media. A stock solution of each antibiotic [10mg/ml twice distilled water] was prepared, filter sterilized (Sartorius, Ministar, 0.2µm) and stored at 5˚C in sterile eppendorf tubes. Each antibiotic solution was added individually to autoclaved NA at a concentration of 10mg/L using a pipette before pouring the media into the Petri dishes to solidify. Suspensions of the PGPR isolates were prepared (as in 3.2.2.1) and a dilution series up to 10^{-8} was made for each isolate suspension. The dilutions (100µl) were pipetted onto the various antibiotic containing media and spread evenly over the surface using a sterile L-shaped spreader. Non-amended NA was used as the control. After two days at 25˚C in the dark the number of bacterial colonies that developed on each amended medium was compared to the control and the degree of resistance of PGPR isolates towards the antibiotics was assessed.

The antibiotics to which the isolates showed complete resistance were selected and the sensitivity of resident pellet microflora towards these antibiotics was evaluated. A suspension of the resident pellet microorganisms was prepared as for the antibiotic disc assay and an 8-fold dilution range was prepared. Aliquots of 100µl were spread plated onto both antibiotic amended media and nutrient agar (control). The Petri dishes were incubated at 25˚C for 24h after which bacterial colonies were counted and CFU/g determined. Three replications were done. The antibiotic which inhibited pellet microflora the most was selected and combined with NA at a concentration of 10mg/ml to serve as a semi-selective media for enumeration of PGPR isolates in the shelf-life study.

6.2.4 Experimental design and statistical analysis

For the longevity trial three replications of each isolate (T19, S7 and T29) in a liquid- and powder form were applied to composted pellets, deposited into woven polypropylene bags and placed within a 25 or 35˚C incubator in a completely randomized pattern. The CFU/g pellets obtained over the six months period were $\log_{10}$ transformed prior to analysis of variance using the Statistical Analysis System (SAS) software package. The repeated measurements over time were included as a sub-plot factor. The means of significant source effect were compared using the protected Student's t LSD (least significant difference) test at a 5% significance level. There was strong evidence for a four factor interaction which was best demonstrated by six line graphs (Figure 6.1 -6.3).

6.3 Results

6.3.1 Antibiotic disc assay

Results from the antibiotic disc assay showed that oxacillin (OX) and polymyxin B (PB) were the only two antibiotics to which all three PGPR isolates were resistant, since no inhibition zones were present (Table 6.1). The pellet microflora was however inhibited by these antibiotics as indicated by the presence of inhibition zones. Although the PGPR isolates also showed resistance towards other antibiotics the purpose of the disc assay was to obtain a single antibiotic to which all three PGPR isolates showed resistance. Thus, the degree of sensitivity of PGPR isolates and pellet microflora towards these antibiotics were studied.

Table 6.1: Results of *in vitro* antibiotic disc assay to determine the sensitivity of PGPR (plant growth promoting rhizobacteria) isolates and resident pellet microflora to various antibiotics

	PGPR isolates			Resident pellet microflora
Antibiotic discs	**S7**	**T29**	**T19**	
Chloramphenicol	Y	N	N	Y
Erythromycin	Y	N	Y	N
Fusidic Acid	Y	Y	Y	Y
Oxacillin	N	N	N	Y
Novobiocin	Y	N	Y	N
Penicillin G	Y	N	Y	Y
Polymyxin B	N	N	N	Y
Streptomycin	Y	N	N	N
Control	N	N	N	N

*N = No inhibition zone, Y= an inhibition zone of at least 7mm diameter was present

6.3.2 Antibiotic sensitivity assay

The results showed that the number of colonies of isolate S7 on OX amended media was similar to the number of colonies obtained on the control plate with non-amended media, indicating that isolate S7 was not significantly affected by OX. However, isolates T29 and T19 were inhibited by OX by 23.7 and 61.9% respectively. The use of OX amended media for enumeration of isolate T29 and T19 in the longevity trial would therefore not represent the true number of viable bacteria on the pellets since OX inhibited the growth of these bacteria.

Colony counts of the three PGPR isolates respectively on PB amended media were similar to the number of bacterial colonies obtained on the control plate. These results indicated that isolate S7, T19 and T29 were resistant to PB and that bacterial cell counts of these isolates were not significantly affected by this antibiotic. Furthermore, the antibiotic proved to be effective in reducing the concentration of resident pellet microflora by 90.51% when grown on PB amended media (2.6×10^3 CFU/ml) vs. on non-amended NA (2.74×10^4 CFU/ml). Polymyxin B in NA media was thus used as a semi-selective media to determine the viability of PGPR isolates T19, S7 and T29 on compost pellets.

6.3.3 Survival of rhizobacterial isolates on compost pellets at 25 and 35˚C

Viable cell counts for three PGPR isolates singly inoculated onto composted chicken manure pellets in a powder and liquid form respectively were monitored over a six month period at 25 and 35˚C (Figure 6.1-6.3). The initial concentration of isolate T19 (*L. sphaericus*) in liquid suspension on the compost pellets incubated at 25˚C (Figure 6.1 A) and 35˚C (Figure 6.1B) was 6.93×10^4 CFU/g and 1.57×10^5 CFU/g, respectively. A gradual decline in bacterial numbers was observed for isolate T19 applied in liquid susupension on the pellet carrier at 25˚C so that after four months of incubation, cell counts were significantly lower compared to the initial concentration (Figure 6.1A). Although cell counts increased slightly between four and five months of storage at 25˚C the cell concentration declined between five and six months to 1.02×10^4 CFU/g, which was significantly lower than the initial count. A rapid decrease in cell density of isolate T19 in liquid form was recorded for the first four months of incubation at 35˚C. Between four and five months of incubation however, cell numbers rapidly increased. Survivability of isolate T19 applied to pellets in liquid suspension form was however significantly reduced between five and six months of storage at 35˚C resulting in a final count of 1.57×10^3 CFU/g which was two orders of magnitude lower than the initial cell count.

The population of isolate T19 applied to the compost pellet in powder form was 5.92 x 10^3 CFU/g before incubation at 25˚C (Figure 6.1). The survival of isolate T19 applied to the pellet in the powder formulation remained relatively constant with no significant changes in cell counts obtained during the six month period of storage at 25˚C. The final cell count was 4.33 x 10^3 CFU/g. The concentration recorded for isolate T19 powder inoculum on compost pellets before incubation at 35˚C was 8.9 x 10^3 CFU/g. During the first month of incubation at 35˚C, isolate T19 showed a significant increase in the bacterial population; however, viable cells gradually decreased over the next three months of incubation so that cell numbers were similar to the initial count at day zero. After four to five months of storage, cell numbers increased sharply but declined again according to colony counts performed after 6 months of storage. Nevertheless, the concentration of isolate T19 applied to the pellets in powder form after six months of storage at 35˚C was 4.13 x 10^3 CFU/g and did not differ significantly compared to the initial cell numbers counted before incubation.

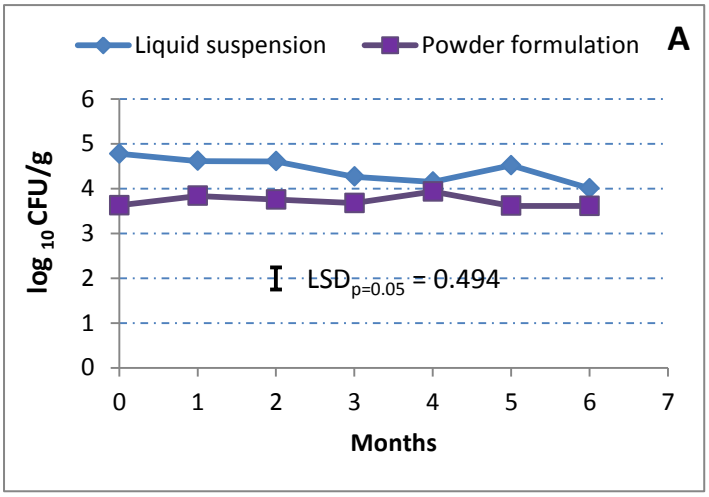
The survival of isolate S7 applied to the pellets as a powder or liquid suspension and subsequently stored for six months at 25 and 35˚C, is illustrated in Figure 6.2. Viable cell counts of isolate S7 (liquid suspension) decreased significantly after inoculation onto the compost pellets and incubation for one month at both 25 (Figure 6.2A) and 35˚C (Figure 6.2B). After one to five months of incubation at 25˚C the cell numbers remained fairly constant. At six months of storage however, colony counts were found to have increased significantly so that the final concentration (5.33 x 10^3 CFU/g) of viable cells was not statistically different to the original count (1.04 x10^4 CFU/g) at zero days of storage. In contrast to these results, at 35˚C the cell counts of isolate S7 (liquid suspension) continued to decrease significantly after two months of incubation so that no viable cells were obtained after a three month storage period.

The initial population of isolate S7 applied as a powder inoculum on pellets at day zero at 25 and 35˚C was 7.26 x 10^4 and 1.13 x 10^5 CFU/g, respectively. At 25˚C, a significant increase in viable cells was observed after three months of storage (Figure 6.2 A). Cell numbers however declined rapidly after three to four months incubation. Nevertheless, the initial concentration of cells applied to pellets was maintained for the six month period as indicated by the final count of 8.53 x 10^4 CFU/g. Incubation at an elevated temperature of 35˚C resulted in the cell density of isolate S7 being significantly lower after three months incubation compared to the initial inoculum concentration (Figure 6.2 B). However, cell numbers increased significantly between three and four months of storage so that the final count at six months storage was 6.67 x 10^4 CFU/g. No statistical difference was detected in

the survivability of isolate S7 at six months of storage at 35˚C and the initial cell count before incubation.

The initial concentration of isolate T29 (*P. alvei*) applied to the pellets in a liquid suspension and stored at 25 and 35˚C was found to be 5.83 x 10^2 CFU/g and 2.27 x 10^3 CFU/g, respectively (Figure 6.3 A and B). After inoculation of the isolate onto the pellets, cell numbers declined significantly at both storage temperatures. Isolate T29 was unable to survive on the pellet at either temperature for longer than two months when applied as a liquid suspension.

Colony counts of isolate T29 applied to the pellets as a powder formulation fluctuated considerably during the six months of storage at 25˚C (Figure 6.3A). The initial cell count was 1.37 x 10^3 CFU/g. After the first month of incubation cell density increased significantly to 6.8 x 10^3 CFU/g, remained constant for another month and then decreased to 1.5 x 10^3 between months two and three. A sharp increase in cell numbers to 8.67 x 10^3 CFU/g was observed between months three and four followed by a rapid decline in concentration according to colony counts performed at five months of incubation. At six months of storage the cell numbers however did not differ significantly from those recorded at six months of storage at 25˚C. The concentration of isolate T29 applied to the pellets in powder form before incubation at 35˚C was 4.08 x 10^4 CFU/g (Figure 6.3B). Cell counts decreased significantly during the first few months of storage so that no viable cells were detected at three months of incubation at 35˚C in the powder formulation.



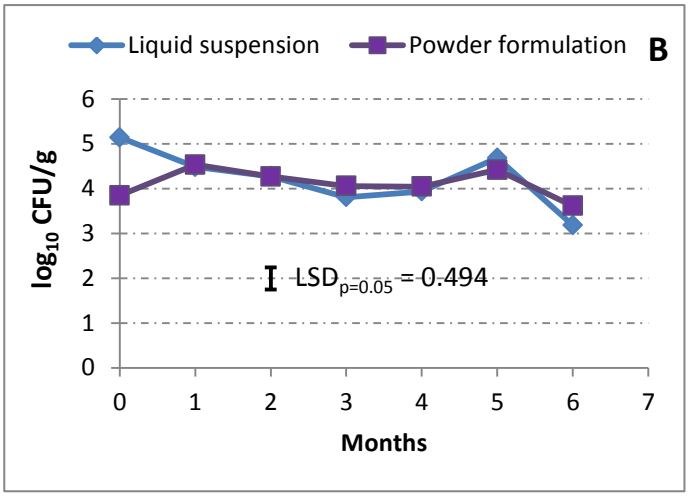
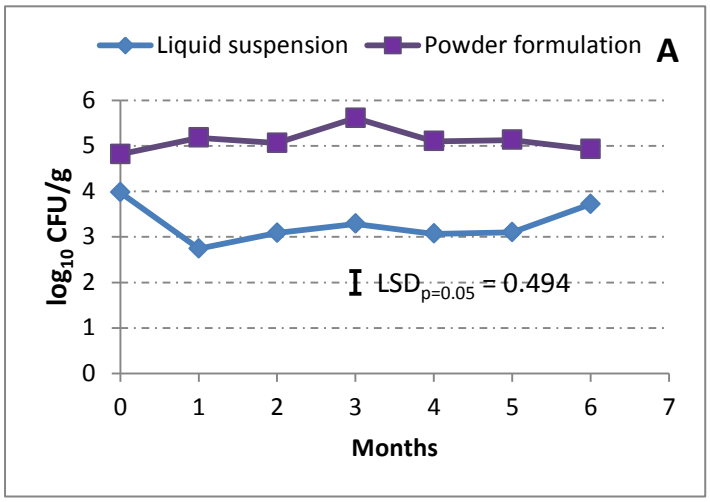


Figure 6.1: Survival of isolate T19 in a powder and liquid suspension formulation on compost pellets and incubated at 25 (A) and 35˚C (B) for six months.



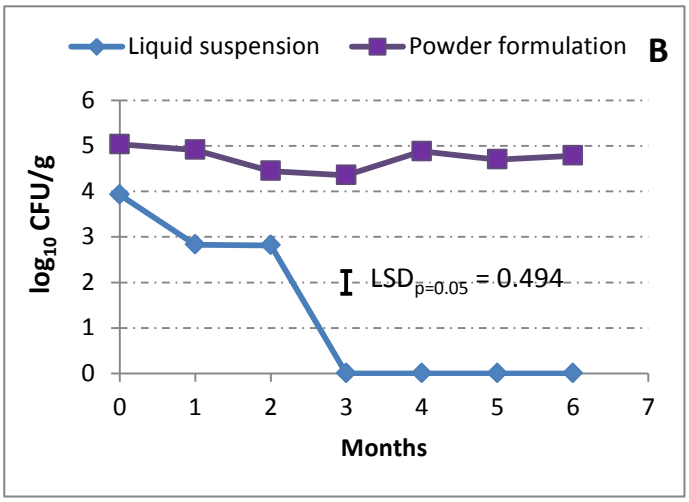
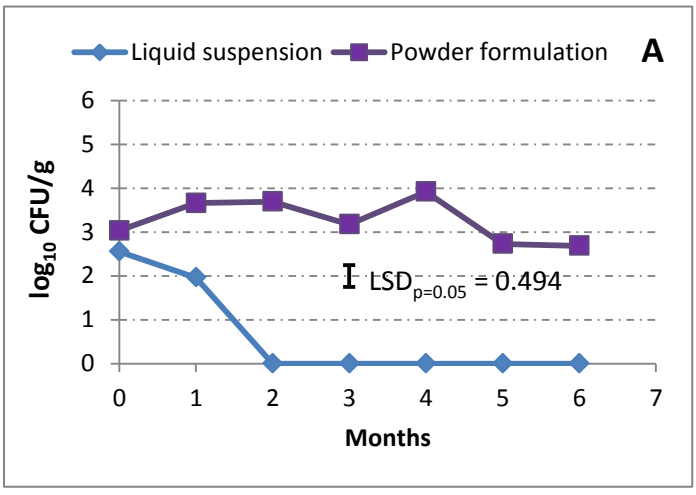


Figure 6.2: Survival of isolate S7 as a powder and liquid suspension formulation on compost pellets and incubated at 25 (A) and 35˚C (B) for six months.



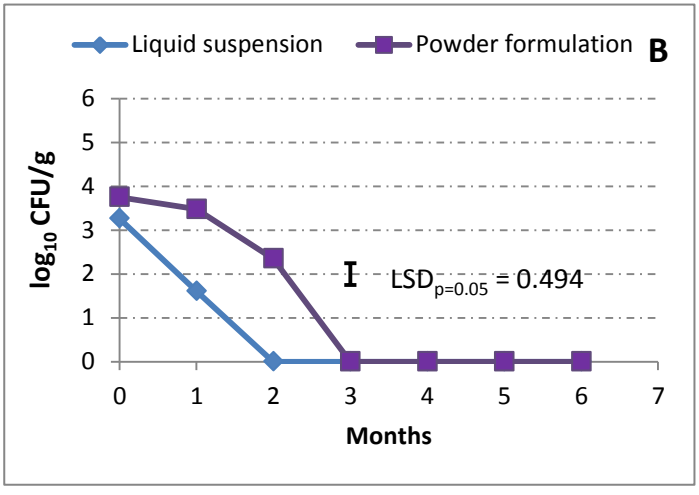


Figure 6.3: Survival of isolate T29 applied as a powder and liquid suspension formulation on compost pellets and incubated at 25 (A) and 35˚C (B) for six months.

6.4 Discussion

A microbial product that is commercially feasible and desired in the marketplace is one which is formulated in such a manner that it is safe to use, easy to handle, consistently delivers satisfactory results and has a long shelf-life preferably at room temperature (Mathre *et al*. 1999; Ramamoorthy *et al*. 2001; Nakkeeran *et al.* 2005). Obtaining a suitable formulation and carrier that is able to maintain viable microbial cells for extended periods during storage is one of the most challenging aspects of biological product development. In general, the majority of microbial products that are known to have long shelf lives contain spore forming bacteria such as the Bacilli (Mathre *et al*. 1999; Abeysinghe, 2009). In the current study the survival of three Bacilli bacteria viz. *Lysinibacillus sphaericus*, *Bacillus cereus* and *Paenibacillus alvei* inoculated singly onto a compost pellet carrier as a liquid suspension and powder formulation, respectively, was evaluated over a period of six months. The shelf-life study was performed at room temperature (25˚C) and an elevated temperature of 35˚C.

In general, the powder formulation was more effective than the liquid suspension formulation in maintaining viable cells of PGPR isolates on composted chicken manure pellets. Remarkably the powder formulation supported the survival of isolate T19 and S7 for six months without any significant decrease in cell numbers at both 25 and 35˚C. The initial inoculant cell load for isolate T29 powder inoculum was also maintained for six months at 25˚C. The use of a perlite powder formulation as a suitable substrate for microbial survival has also been reported in numerous other studies (Daza *et al*. 2000; Temprano *et al*. 2002; Khavazi *et al*. 2007; Albareda *et al*. 2008). However, this is the first report indicating the survival of PGPR isolates in powder form on an organic carrier consisting of composted chicken manure. Khavazi *et al*. (2007) investigated the survival of *Bradyrhizobium japonicum* strain CB1809 in a 1:4 mixture of perlite and sugarcane bagasse or malt residue, respectively. Their results showed that after six months storage at 4˚C cell numbers were sufficiently high in both the carrier mixtures tested and no decrease in inoculant concentration occurred. The authors suggested that the ability of the carriers to support the survival of *B. japonicum* for as long as six months was likely attributed to the presence of high levels of organic matter and nutrients in the sugarcane bagasse and malt residue (Khavazi *et al*. 2007). Indeed one of the main characteristics of a good inoculant carrier is the presence of high levels of organic material (Smith, 1992; Rebah *et al*. 2007). Organic matter such as carbohydrates is utilized by microorganisms for maintenance and growth (Wu *et al*. 2005). The compost pellets used as carrier in this study consisted of high amounts of organic material and macro elements such as nitrogen, potassium, phosphorous,

magnesium and calcium which could be utilized by the PGPR isolates for survival over the six months period. Other properties such as the neutral pH of the compost pellets also created a suitable environment for survival of bacterial cells.

In contrast to the results obtained with the isolates in the powder form, inoculum levels in the liquid suspension formulation generally declined significantly on the compost pellets during long term storage. Cell numbers had decreased significantly at both storage temperatures for isolate T19 at six months incubation. Also, the liquid suspension formulation failed to support isolate T29 for more than two months at either temperature and zero viable cells were recorded at three months storage of isolate S7 at 35˚C. A possible reason for the increased sustainability of the isolates in the perlite as compared to the liquid suspension formulation is that the bacterial cells were in a different physiological state in each formulation (Albareda *et al*. 2008). In the current study, the perlite inoculum was incubated for four days before application to the compost pellets whereas the liquid suspension formulation was applied immediately after preparation. Feng *et al.* (2002) demonstrated that storage of rhizobia in broth for a few days before inoculation onto peat caused the cells to enter the stationary phase, leading to morphological changes such as cell wall thickening. The authors suggested that the morphological changes enabled the rhizobia to adapt to the peat carrier allowing for long-term survival (Feng *et al.* 2002). Furthermore, it has been demonstrated that a period of nutrient starvation of bacteria such as *Pseudomonas fluorescens, Pseudomonas putida* (Van Overbeek *et al*. 1995) and *Rhizobium leguminosarum* bv. *phaseoli* can increase cell tolerance to stresses such as pH, heat, or osmotic shock (Thorne and Williams, 1997). A decrease in bacterial cell division has also been associated with conditions of nutrient starvation (Tittabutr *et al*. 2007). Since perlite does not contain any essential nutrients the cells most likely experienced a period of starvation during the four day incubation period before application to the pellets, which may have increased their tolerance to stressful conditions, allowing for long term survival.

Incubation at an elevated temperature of 35˚C was more detrimental for isolate survival compared to storage at 25˚C, especially in the case of inoculant application in the liquid suspension formulation. After six months incubation at 35˚C, viable cell counts were two orders of magnitude lower than the starting population in the case of isolate T19 in the liquid suspension form, whereas at 25˚C the concentration only decreased by one order of magnitude. Isolate S7 in the liquid formulation was negatively affected by the elevated temperature since no viable cells were detected at 3 months of storage whereas at 25˚C no significant change in concentration occurred over the six month storage period. Isolate T29 was perhaps the most sensitive to the elevated temperature as indicated by the rapid

decrease in cell numbers from day zero of incubation at 35˚C in both the liquid and powder formulation. Temprano *et al.* (2002) also observed a significant reduction in Rhizobia cell counts in peat and vermiculite formulations incubated at 28˚C vs. refrigeration at 4˚C. Similarly, Sangeetha and Stella (2012) found that the concentration of PGPR strains of *Azospirillum, Bacillus, Pseudomonas* and *Azotobacter* in a variety of carrier materials decreased with increasing storage temperatures. They concluded that the temperature increase was associated with a loss of moisture from the carrier materials which adversely affected the survival of the bacterial populations (Sangeetha and Stella, 2012).

Considering the results obtained, it is evident that the survival of cells also depend on the bacterial species used as inoculant. Survival of isolate T19 (*L. sphaericus*) remained the most consistent during the shelf-life study at both storage temperatures and in both formulations compared to the other isolates. Isolate S7 (*B. cereus*) performed well in the powder formulation surviving for six months at both temperatures but failed to survive in the liquid suspension formulation at 35˚C. The performance of isolate T29 was the least promising compared to the other two isolates since the bacterium was unable to survive in the liquid suspension formulation at 25˚C and could not endure incubation at 35˚C in either formulation. It has been shown that the growth rate of different bacterial species varies and that slower growing species generally survive longer than fast-growing bacteria. The slow metabolism of slow-growing bacteria enable them to maintain viability under stressful conditions for longer than fast-growing bacteria since energy reserves will be utilized at a slower rate (Tittabutr *et al*. 2007).

In the current study, the concentration (10^3-10^5 CFU/g) of bacterial isolates, initially inoculated onto the carrier pellet, was lower compared to the inoculation dose (10^7-10^9 CFU/g) applied to carriers in other studies (Albareda *et al*. 2008; Ngampimol and Kunathigan, 2008; Al-Taweil *et al*. 2010). To date, no legal standard for the concentration of PGPR required on inoculants has yet been established. This is primarily because the level of bacteria required to perform sufficiently varies between different bacterial species and conditions (Brahmaprakash and Sahu, 2012). However, most studies have reported that for an inoculant to sufficiently perform its intended role in the soil or on the plant the minimum recommended number of viable cells needed on the carrier is 10^7 CFU/g (Khavazi *et al*. 2007; Brahmaprakash and Sahu, 2012). Future work should therefore focus on increasing the concentration of PGPR isolates applied to the compost pellets. This could involve centrifuging the bacterial suspensions to collect and concentrate bacterial cells before application to the perlite or pellets. Also, as discussed in chapter 4, the addition of certain additives and adhesives to bacteria growth media (Manikandan *et al*. 2010; Kumaresan and

Reetha, 2011) could enhance the concentration of the isolates on the compost pellets. The use of adhesives is especially important, since loose perlite was observed at the bottom of every bag containing the pellets treated with the perlite inoculum.

One of the concerns in the use of organic material such as composted chicken manure as carrier for PGPR isolates is that the nutrient status of the material varies from batch to batch. The variation is however expected to be small, as found in the study of Materechera and Morutse (2009), where the chemical properties of chicken manure remained fairly consistent in batches from two different years. Also, even though some variation will occur between pellet batches the concentration of essential nutrients will still be high enough to support the growth of the inoculant bacteria.

Perhaps more of a concern is the variation in concentration and species of resident pellet microflora or contaminants that are present on different pellet batches. The two most important characteristics that should be considered when establishing a quality standard of a microbial inoculant is firstly the number of viable cells and secondly the concentration of contaminants on the carrier. Although most countries do not have regulations set in place for the acceptable number of contaminants allowed on non-sterile carriers the India Ministry of Agriculture and Cooperation has developed some standards. They require that no contamination should be detected at the 10^5 dilution of bacterial inoculants (Brahmaprakash and Sahu, 2012). In the current study the level of contamination was found to be 2.74×10^4 CFU/g which is therefore acceptable according to India standards. It is important that carriers have as low a level of contaminants as possible since they may have an antagonistic effect on the bacterial inoculants thereby reducing their shelf-life (Khavazi *et al*. 2007). Furthermore, the bacterial contaminants may be hazardous to human, animal or plant health. However, throughout the many years of use of non-sterile carriers there have been no reports associated with any health hazards (Bashan, 1998). Nevertheless, the potential risk should not be overlooked and future work should involve identification of the pellet contaminants from different batches over multiple production seasons. Thereby a general indication of the population of contaminants can be obtained and the presence of potentially harmful pathogens can be detected.

This study demonstrated that composted chicken manure can support the growth of PGPR isolates over the long term. Improvements to the liquid suspension and powder formulation should focus on amongst other aspects, the increase in the initial concentration of bacterial inoculum to improve the effectiveness of the pellet inoculant. Future work should focus on

setting standards for quality control of the product and ultimately the growth promoting effect of the pellet inoculant should be evaluated under greenhouse and field conditions.

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CHAPTER 7
General conclusion

The beneficial effects of PGPR on various agricultural crops have been demonstrated in numerous studies (Bhattacharyya and Jha, 2012; Mishra *et al*. 2013; Noumavo *et al*. 2013). The extensive and systematic research in these studies has emphasized the important role that PGPR play as plant growth stimulators and disease control agents in the era of sustainable crop production. In 2004, the global economic value for biocontrol products was around R5.8 billion with most sales (43.5%) occurring in North American Free Trade Agreement countries (including Mexico) and the least sales in Africa (3.9%) (Berg, 2009). The interest in microbial products continues to grow as organic farming becomes more popular (Berg, 2009; Babalola, 2010) and regulations regarding chemical use become stricter (Pal and Gardener, 2006). Considering the future market potential and need for microbial products in developing countries (Thakore, 2006) the current study focused on PGPR and their application to the most important crop in South Africa i.e. maize. The main conclusions of this study are summarized in this chapter.

In chapter 3 a modified seed germination bioassay was conducted by applying eight PGPR isolates viz. T19 (*Lysinibacillus sphaericus*), T29 (*Paenibacillus alvei*), S6 (*Bacillus cereus*), S7 (*B.cereus*), A32 (*Stenotrophomonas* sp.), T22 (*Paenibacillus alvei*), A07 and A08 (*Bacillus aryabhattai*) as individual seed treatments to a low vigour maize cultivar. Seed germination, seedling growth and vigour index of the maize cultivar was significantly enhanced by the PGPR treatments. The seed germination was increased by up to 7.8% by isolates S6, A32, and T29 and seedling dry mass was 20.1 to 29.7% higher than the untreated control as a result of seed treatment with isolates S6, S7, A32, T29 and T22. Isolate S6 improved the vigour index of maize seeds by a significant 22% followed by isolates A32, T19, T29 and S7 which enhanced vigour index by up to 21.2%. The positive effect of the PGPR treatments on the early growth parameters of maize seedlings was possibly attributed to the production of phytohormones such as gibberellic acid and indole 3-acetic acid (Zahir *et al*. 2000; Boiero *et al*. 2007; Shahab *et al*. 2009); however, further tests determining the modes of action of these isolates would have to be conducted to confirm this. Although the modified seed germination bioassay was a quick test that enabled the selection of PGPR isolates with promising traits, this method did not take into account those PGPR that stimulate plant growth by means of other modes of action such as phosphate solubilisation, which would only be evident in other growth media such as soil, as opposed to germination paper.

In chapter 4 the plant growth promoting abilities of isolates A32, S6, T19, T29 and S7 were evaluated in the greenhouse in a pot trial. Isolates T19, S7 and T29 significantly enhanced the growth of maize, as indicated by the increase in total dry mass after four weeks of growth by 31.8, 32.5 and 34.3%, respectively, compared to the untreated control. The beneficial effects of these PGPR observed in this study may have been due to modes of action that have been documented in previous studies by Hassen, (2007) and Pretorius, (2012). The modes of action detected in their studies included nitrogen fixation, phosphate solubilisation and the production of antibiotics, siderophores, chitinase and indole acetic acid (Hassen, 2007; Pretorius, 2012).

The formulation efficacy trial demonstrated that application of rhizobacterial isolates T19, T29 and S7 individually as a soil drench, as opposed to a seed treatment or an inoculant on a pelletised compost carrier, resulted in the best growth promotion of maize (chapter 4). After six weeks in the greenhouse the dry root mass of maize inoculated with a soil drench of isolates T19 and T29, respectively, was 65.6 and 82.7% higher than the untreated control. Also, the combined application of isolates T19, T29 and S7 as a soil drench enhanced the dry root and shoot mass by 82.7 and 38.2%, respectively, compared to the control treatment consisting of uninoculated nutrient broth. The only compost pellet containing treatment that caused a significant increase in root dry mass (65.6%) was where isolate T29 was applied onto the composted chicken manure pellets in a perlite powder form. The results did however, indicate there was a tendency towards increased root dry mass of maize planted in soil inoculated with compost pellets treated with isolates S7, T19 and the consortium of isolates. With reference to the seed treatment, none of the isolates applied individually or as a combination had a significant effect on any of the growth parameters measured. The formulation in which the rhizobacterial isolates were applied was thus of vital importance affecting their ability to enhance plant growth. This was primarily suspected to be due to the variation in final inoculant concentration (i.e. bacterial cell concentration) delivered to the seed by the different types of formulations tested during this study.

In chapter 5 the ability of seven rhizobacterial isolates viz. T19, T29, T22, S6, S7, A08 and A07 to supress the soil-borne pathogen *R. solani* was demonstrated. Results from the seedling tray trial showed that isolate A07, A08 and T19 had the ability to control *R. solani* pre- and post-emergence damping-off of maize seedlings. In the seedling tray trial individual soil drench applications of isolates A08, A07 and T19 reduced disease severity and enhanced dry root mass by 53.2, 54.1 and 68.2%, respectively, compared to the control seedlings planted in *R. solani* inoculated soil. When these isolates were applied as seed treatments and soil drenches in a greenhouse pot trial (chapter 5) the only treatment that

was able to control *R. solani* was the soil drench with isolate T19. The primary reason suggested for the inability of any of the seed treatments to control *R. solani* was that the concentration of rhizobacterial cells on the seeds was too low to effectively protect the seedlings. It was suggested that the reduced ability of isolate A07 and A08 to control *R. solani* as soil drench treatments in the pot trial was due to the volume of inoculum applied being too low. Although increasing the volume of inoculum applied would most likely result in better biocontrol of *R. solani*, the increased cost associated with the higher volume might render the product commercially uneconomical. Therefore, future research should focus on increasing the initial inoculum concentration within the various formulations.

The shelf-life of a microbial formulation is an important characteristic which determines the commercial viability of the product (Rebah *et al*. 2007). Therefore the shelf-life of isolates T19, S7 and T29 on a novel pelletized compost carrier was determined in chapter 6. Overall, the results demonstrated that the powder formulation was better suited for survival of isolate T19, S7 and T29 on the compost pellets than the liquid suspension formulation. All three isolates remained viable on the compost pellets for six months as a perlite powder inoculum at 25˚C. The concentration of isolate T19, S7 and T29 powder inoculum on compost pellets after the six month period was 4.33×10^3, 8.53×10^4 and 7.27×10^2 CFU/g, respectively. Incubation at 35˚C generally decreased survival of PGPR isolates at a faster rate than at 25˚C except for isolate T19 and S7 in powder form which remained stable over the six months incubation at the elevated temperature. In general, isolate T19 performed the best in terms of survival in both the powder and liquid suspension formulation at both storage temperatures compared to isolates S7 and T29. As demonstrated in chapter 4 the low inoculum concentration on the compost pellets was a concern when the compost pellet treatments were evaluated for their growth promoting effect on maize. However, if the concentration of PGPR on the pellets could be increased to achieve improved plant growth the novel carrier would have great potential as a microbial product since the PGPR isolates were able to survive in the powder form on the pellets for six months.

In general, the results of this study have demonstrated that rhizobacterial isolates T19, S7 and T29 have significant potential in agriculture being able to increase the vigour index and improve the growth of maize. Isolate T19 has also shown potential as a biocontrol agent of *R. solani* on maize and isolate T29 was effective in enhancing dry root mass when applied as a powder formulation on a novel compost pellet carrier. The compost pellets showed great potential as a novel carrier being able to support the survival of isolates T29, T19 and S7 in a powder form for six months at 25˚C. Further research is needed to improve the effectiveness of the formulations tested in this study i.e. the soil drench, seed treatment and

PGPR fortified compost pellet carrier by increasing the inoculum concentration within the formulations. Determining the synergistic effect of the isolates could also improve the success of the formulations containing consortia of isolates. Ultimately, there is a need to assess the performance of the PGPR isolates in their various formulations in field trials under natural environmental conditions.

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