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Efficacy of rhizobacteria for growth promotion and biocontrol of *Fusarium oxysporum* and *Rhizoctonia solani* on wheat in South Africa

by

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DECLARATION

I, the undersigned, declare that the MSc thesis entitled: “Efficacy of Rhizobacteria for Growth Promotion and Biocontrol of *Fusarium oxysporum* and *Rhizoctonia solani* on Wheat in South Africa” submitted to the University of Pretoria is my own original work and it has not formed previously the basis for the award of any degree.

Theresa Pretorius

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To the Lord God Almighty, who holds me in His hand.

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Summary

Plant growth promoting rhizobacteria (PGPR) are found in the rhizosphere and directly contribute to nutrient availability and plant growth. Due to the global move away form reliance on chemical fertilisers and crop protection products, plant growth promoting rhizobacteria (PGPR) and their application for improving crop production is receiving much attention. In the current work the selected PGPR isolates from the University of Pretoria PGPR Culture Collection were evaluated on wheat seedlings for their ability to promote seedling growth and control of selected root diseases.

The ability of the PGPR to control root and crown rot of wheat caused by *Fusarium oxysporum*, *F. graminearum* and *Rhizoctonia solani* was evaluated in greenhouse pot trials. In addition the seedling tray assay was also evaluated as a means to screen PGPR for biocontrol. Under the test conditions, the seedling tray assay was unsuccessful as a method to screen the PGPR for biocontrol of wheat root pathogens. In the greenhouse pot trials several isolates were able to improve the growth of wheat seedlings cultivated in pathogen inoculated soil.

For growth promotion the bacterial isolates were assessed in greenhouse trials. Following initial selection trials further analyses were conducted with selected isolates to determine what effect the PGPR inoculum dose and fertiliser application has on growth promotion efficacy. The isolates T06, T07, T11, T13, T19, T21, T23 and T24 were most effective in promoting the growth of wheat seedlings in the greenhouse. The dose response and fertiliser trials indicated that these factors do affect isolate activity.

To facilitate understanding of the isolates and their activities, the isolates that performed well in the greenhouse were identified through 16S rRNA sequence data and selected modes of action determined. The isolates were predominantly Bacilli, from the genera *Bacillus, Lysinibacillus* and *Paenibacillus*. *In vitro* assessments revealed that the isolates have a wide spectrum of activity, including phosphate solubilisation, indole acetic acid production, growth in nitrogen free media, inhibition of pathogens in dual culture, chitinase production and siderophore production.

A selection protocol was developed to assist with the selection of the best isolates. Based on ratings given for the greenhouse and *in vitro* assay the top performing isolate were selected. Spider diagrams were then constructed to visually represent each isolate's performance. The following best performing isolates were selected as a) T10 (*Bacillus cereus*) for control of *R. solani*, b) T28 (*Bacillus cereus*) for control of *F. oxysporum*, c) T31 (*Bacillus cereus*) for control of *F. graminearum*, d) T13 (*Bacillus sp. / Bacillus pumilus*) for growth promotion of wheat and e) T29 (*Paenibacillus alvei*) as the most versatile isolate.

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CHAPTER 1
General Introduction

1.1 Background

Wheat is an important staple food in many regions of the world. In South Africa it is the second most important grain crop after maize, and is cultivated on approximately 4000 commercial wheat farms, planting between 74 8000 and 1 382300 ha of wheat annually (Figure 1.1). In 2007 the annual production was around 197000 tons, equalling a bruto income of approximately R30 000 million (NDA 2007, ERSA 2008). Wheat is mainly cultivated in the Western Cape, Free State and Northern Cape (Figure 1.1), where the climate is most suitable to wheat production. An ideal climate for wheat production is a cool moist winter period followed by a warm dry season for harvesting. In the summer rainfall region only 20% of the wheat is irrigated. Most of the wheat produced is dryland wheat where the crop relying on residual moisture from summer rains. Planting occurs mainly between mid-April and mid-June in winter rainfall areas and mid-May to end July in summer rainfall areas. The South African wheat production rate for dryland wheat is 2 to 2.5 t/h and 5t/ha for irrigated wheat areas (DAFF 2010, Wheat production guide).

Though this is such an important crop, the yield of dryland wheat production in South Africa is on average less than that of other areas, such as America and Europe, where wheat is produced on a large scale. Several factors contribute to the low yield, including unpredictable weather and drought, low soil fertility and the occurrence of pests and diseases (Scott 1990, Pannar Produksiehandleidingsreeks). Furthermore the soil fertility in the South African wheat production regions is low. All wheat planted in South Africa is fertilised. Fertiliser and lime can contribute between 17 and 30% of the total variable input cost of wheat production in some areas (Anonymous 2008, DAFF 2010).

In addition to the mentioned abiotic factors, disease also restricts yield. Wheat is affected by a wide range of organisms including bacteria, viruses, nematodes, and fungi. Important fungal diseases include the rusts such as stem, leaf, stripe and crown rust, mildew, spots and blotches, Septoria, loose smut, karnal blunt and eyespot (DAFF 2010, Wheat production guide, March 2010). In South Africa several fungal diseases affect wheat. Important diseases include basal stem rot caused by *Sclerotium rolfsii*, crater disease and bare patch disease caused by *Rhizoctonia solani* Kuhn, Pythium root rot and take-all caused by *Gaeumannomyces graminis* var. *tritici*.

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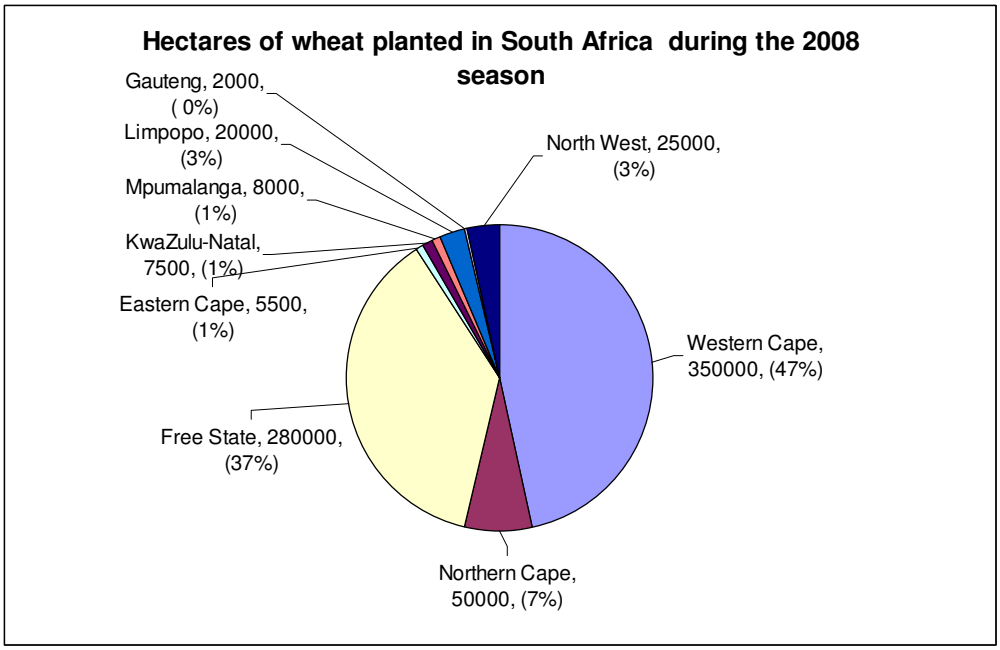

Figure 1.1: Wheat production areas in South Africa (Crop estimate committee, www.doa.agric.za).

Root and crown rot, caused by species of *Rhizoctonia* and *Fusarium* are especially important with regard to reduced yield in wheat (Scott 1990). Van Wyk *et al*. (1988), states that root and crown rots of wheat can be considered the most important disease of wheat in the Orange Free State area. In many other production regions the importance of these diseases are often underestimated.

Fungal disease of the foliage, stem, ears and grain are routinely controlled with fungicides and chemical seed treatments (DAFF, Wheat production guide, March 2010, Draper, 2000). Soilborne diseases including crown and root rots are considered a challenge to effectively manage. All cultivars are susceptible to these diseases to different levels of severity, and chemical application is mostly not effective. Seed treatments are generally applied but the level of control achieved is inconsistent due to the influence of temperature and the long period of time that the wheat plants are exposed to pathogens. High dose rates are also required for effective chemical control. These diseases are therefore managed by crop rotation and management practices that reduce the loss of soil moisture and plant stress (Wheat production guide, Pannar seed, Draper, 2000). There is therefore a great need for new innovations that would contribute to improving the wheat yield. One such possible option is the application of plant growth promoting microbes

The term “plant growth promoting rhizobacteria” or PGPR was first used by Kloepper and co workers in the 1970’s (Vessey, 2003) and is a collective term for free living rhizobacteria whose presence benefits plant growth. These direct benefits can be due to the microbes influencing plant growth or through pathogen control. For over seventy years soils that are

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suppressive to or develop suppression to root disease as a result of microbial action have been observed. Since that time, these beneficial microbes have been considered potential agents to control plant diseases in an environmentally friendly way (Hass and Defago 2005, and Alabouvette *et al*. 2006). More recently rhizobacteria have also been investigated for their direct plant growth promotion effects (Bashan and Holguin 1998). In the literature there are numerous reports and reviews of the beneficial effect PGPR have on the growth of various crops including canola, lettuce, wheat, maize, tomatoes, pearl millet, chick-pea, banana, rice, peppers, and squash (Singh *et al.* 2011).

With specific reference to wheat, there are various examples where PGPR improved the growth of the crop and controlled soilborne disease. *Azospirillum* isolates were able to improve the growth of wheat that received suboptimal levels of fertiliser (Ozturek *et al.* 2003 and Diaz-Zorita and Fernandez-Canigia, 2009). Various PGPR, including *Azospirillum* and *Pseudomonas* strains were able to help the plants to better withstand environmental stresses such as salt and water deficiency (Bashan *et al*. 2006). With regards to disease control research done by Dal Bello *et al.* (2002) and Khan *et al.* (2005) demonstrates that PGPR have the potential to control important soilborne pathogens such as *Fusarium graminearum* and *Gaeumannomyces graminis*. PGPR therefore have the potential to improve the yield of wheat in South Africa through enhanced plant growth and control of soilborne diseases. Further more PGPR can also contribute to improved fertiliser use and therefore reduced input costs, thereby making the crop more profitable.

1.2 Aim

Against this background, the aim of this study is to evaluate selected rhizobacteria from the University of Pretoria's-PGPR culture collection for growth promotion and biocontrol of root and crown rot caused by *Fusarium oxysporum*, *F. graminearum* and *Rhizoctonia solani* on wheat.

1.3 General objectives

To achieve the abovementioned aim, the following specific objectives were addressed.

- Determine the ability of selected PGPR isolates from the UP-PGPR Culture Collection to control root and crown rot of wheat caused by *Fusarium oxysporum*, *Fusarium graminearum* and *Rhizoctonia solani* in the greenhouse (Chapter 3).
- Assess the ability of selected PGPR isolates to promote the growth of wheat seedlings in greenhouse trials (Chapter 4).
- Determine certain modes of action and characteristics of the PGPR (Chapter 5).
- Develop a selection protocol for the best PGPR isolates (Chapter 6)
- Assemble information regarding biocontrol, growth promotion and MOA and select the most promising isolates for further work (Chapter 6).

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CHAPTER 2

Literature review of root and crown rot of wheat caused by Fusarium spp. and Rhizoctonia solani on wheat and a general overview of plant growth promoting rhizobacteria

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2.1 Introduction

Soil is home to a large host of microbes which affect plant growth in various ways (Whipps 2001). Most microbes found in the soil do not affect the growth of plants but the few that do are however of great importance and of commercial interest in agriculture. Understanding these microbes and their interactions with each other and plants is an important aspect of plant health management. Microbes such as the nitrogen-fixing bacteria, mycchorrhizal fungi, and plant growth promoting fungi and bacteria have a positive impact on plant growth. Unfortunately there are also several fungi, bacteria and nematodes that cause disease in plants (Whipps 2001).

Reducing the impact of negative plant microbe interactions is one of the challenges faced in crop production. Root and crown rots of wheat are examples of such challenges. Various pathogens are associated with these diseases, including *Fusarium graminearum* Schwabe*, F. oxysporum* Schlectend and *Rhizoctonia solani* Kuhn. These pathogens are important contributors to low yields in many wheat production areas. Though chemical disease control is available and commercially used for many fungi there are many challenges associated with its use such as fungicide resistance, human health and environmental contamination concerns. These challenges have led to the search for alternative control measures.

Since the current trend in agriculture is to adopt more green technologies and reducing the use of chemicals, the role of beneficial soil microbes, and in particular the “plant growth promoting rhizobacteria” (PGPR) has received much attention (Singh *et al.* 2011). The presence of these organisms improves plant growth and the general health of plants. These positive effects can be attributed to a variety of modes of action, broadly categorised as direct growth promotion or biological control.

Plant growth is increased via mechanisms such as increasing nutrient availability, phytohormone production, increasing plant stress tolerance and altering the microbial community. Biocontrol is achieved by parasitism, antibiosis, competition, or inducing resistance in the plant. When developing and using PGPR various aspects such as their sourcing, assessment and application need to be considered to insure that PGPR inoculants are effective. In order to manage and utilise these beneficial microbes to improve crop yields

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it is important to have a thorough understanding of the organisms involved and their interactions with the host.

2.2 Fusarium: the disease and species of Fusarium causing root rot of wheat

Fusarium species are generally associated with both healthy and diseased wheat roots (Klaasen *et al.* 1992) and some species are also well known plant pathogens of wheat (Wiese, 1987). Root and crown rot caused by *Fusarium* is a chronic disease found globally, in all production regions at different severity levels (Liu and Griffiths 2009). Fusaria generally severely affect plants growing in areas that receive a low annual rainfall and exposed to drought stress (Klaasens *et al.* 1992). Recent surveys in the Victoria region of Australia indicate that annual yield loss as a result of Fusarium root and crown rot is at least 2-3%. Some areas within that region experience losses of up to a 9.5% reduction in yield. However, these estimates are considered conservative (Hollaway and Exell 2010). The monetary loss to Australian growers due to Fusarium root and crown rot is estimated to amount to $79 million per year and poses a potential total loss of $434 million per year (Liu and Griffiths 2009).

Several species of *Fusarium* cause crown and root rot of wheat. *Fusarium graminearum* and *F. pseudograminearum* are generally considered the most pathogenic and widespread species responsible for these diseases. Other species are also known to cause crown and root rot, but their economic importance is related to the area in which they occur. Van Wyk *et al.* (1987), for instance, showed that *Fusarium avenaceum, F. culmorum* and *F. graminearum* are important pathogens of wheat roots in specific areas of the south Western Cape. In Australia the virulence of *Fusarium* species is also related to the area in which they occur. The distribution and pathogenicity of *Fusarium pseudograminearum* and *F. culmorum* were associated with the climatic conditions that prevail in the different areas. Rainfall, specifically at the end of the season, influences the *Fusarium* species responsible for causing disease. *F. culmorum* was most abundant in high rainfall regions and *F. pseudograminearum* dominant in areas with low rainfall (Backhouse *et al.* 2004).

In addition to the abovementioned species, several other *Fusarium* species cause wheat root and crown rot in South Africa. *Fusarium culmorum*, *Fusarium crookwellense, Fusarium pseudograminearum (Fusarium graminearum* Schwabe group 1), *Gibberella avenacea* (*F. avenaceum, F. anguioides, F. granulosum, F. viticola*), and *Gibberella intricans (F. equiseti, F. roseum, F. scripti*) are listed as causal agents of wheat root and crown rots in South Africa by Crous *et al.* 2000. Additionally Van Wyk *et al.* (1987) reported *F. compactum, F. equiseti, F. oxysporum, F. proliferatum, F. sambucinum, F. serpi and F. solani* in association with diseased wheat crowns.

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The symptoms most commonly associated with soilborne infections of wheat by *Fusarium* are plants that have white heads at the end of the season. The white heads produce less grain and the kernels can be shrivelled (Klaasen *et al.* 1992). Severely diseased plants become obvious near the end of the season when mature plants show poor growth, yellowed leaves and tip scorch in patches. Upon closer inspection of the whitehead plants little rotting is evident. A brown discolouration may be visible on the stem bases and to a lesser extent the root system. Such plants are easily knocked over or pulled up. Fusaria also cause seedling blight of wheat seedlings. Infected seedlings die before or soon after emergence. Early signs of infection are brown lesions on coleoptiles, subcrown internodes, roots, and culms of seedlings (Wiese, 1987). Seedlings that survive the infection suffer from brown root rot and have brown marks on their roots and stem bases. Under many farming conditions the seedling loss is negligible as the remaining plants compensate for the lost plants by increased tillering of healthy plants (Gair *et al.* 1972).

Disease symptoms are often seen in a pattern following the most severely water stressed plants. In areas where plants experience severe water stress the crop losses can be severe. In many cases *Fusarium* can be regarded as a secondary infection, with drought being the main trigger for stress induced diseases (Gair *et al.* 1972). Moisture or high humidity is needed for root infections. Thereafter disease development is highly dependant on warm temperatures and moisture stress. Growth of most Fusaria causing root rot is optimal in culture media with a low water potential. *F. graminearum* and *F. culmorum* are more prevalent in warm soils (Wiese, 1987). When low rainfall occurred, Klaasen *et al.* (1992) isolated *F. graminearum* more frequently from plants with white heads than healthy plants. These results support the theory that water stress is a key factor in the development of root and crown rot caused by Fusaria. A few species do not follow this pattern such as *Fusarium culmorum* that was isolated more frequently in cool areas and *F. avenaceum* during wet conditions. Excessive nitrogen fertilisation also contributes to disease as the larger plants utilise water at a faster rate and are therefore more sensitive to dry conditions (Cook 1981).

The primary inoculum source is from host debris that is infected with *Fusarium*. Contaminated wheat seeds can also be a source of inoculum. *Fusarium* mycelia, chlamydospores, and ascospores are all infectious. Perithicia are important over-wintering structures. The chlamydospores can remain viable in the soil for months and usually remaining inactive if a host is not available. The threshold population necessary for *F. culmorum* to produce a detectable effect on wheat is about 100 propagules (chlamydospores) per gram soil (Wiese, 1987). Van Wyk *et al.* (1987) however found that the commonly accepted wheat root rot pathogens *Fusarium graminearum, F. crookwellense* and *F. culmorum* do not survive well in plant debris in the soil.

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Infection occurs principally on coleoptiles, primary and secondary roots and subcrown internodes. Root rotting is tolerated as long as the plant is able to generate new supportive roots. Favourable environmental conditions are therefore important for symptom development. Secondary *Fusarium* infection occurs when the infection progresses above the soil level and conidia are produced. These conidia are dispersed by the wind and initiate lesions on the leaves and culms later in the season (Wiese, 1987).

Soilborne diseases caused by *Fusarium* are difficult to control. Management is mainly through production practices, such as stubble burning and crop rotation that reduce the exposure of new plants to pathogenic *Fusarium* residing in plant residues (Liu and Griffiths 2009). For winter wheat the planting dates can be delayed and nitrogen fertilisation adjusted in accordance with the anticipated water availability in the late growing stages (Paulitz *et al.* 2002). These practices are however of limited efficacy. In some instances rotation crops can favour the build-up of inoculum in the soil (Gair *et al.* 1972). Seed treatments partially control seed borne infection and give some protection against the seedling phase of the disease. Resistant varieties are the most effective strategy for disease control but there are no highly resistant varieties available (Liu and Griffiths 2009). In South Africa, the situation is similar. Farmers are thus advised to practice crop rotation, control weeds that can harbour inoculum, control the stubble, avoid excessive nitrogen fertilisation, insure that there is not a zinc shortage, use tillage practices that conserve water and avoid high plant densities (Paul and Lamprecht 2009).

2.3 Rhizoctonia: the disease and species of Rhizoctonia causing root rot of wheat

Rhizoctonia root rot, also known as Rhizoctonia patch disease or bare patch disease is caused by *Rhizoctonia solani*, anastomosis group 8 (AG8). The disease is prevalent in most temperate regions of the world (Wiese, 1987). This root rot is commonly associated with light sandy soils. Plants that are infected by *R. solani* are usually not killed but struggle to grow.

Affected plants are usually found in distinct patches and show a purple discolouration. Diseased plants mature later and are therefore less mature at harvest (Gair *et al.* 1972). The most damage done by *Rhizoctonia* is during the seedling phase. Roots of infected seedlings become rotted and girdled and are eventually severed. This results in the characteristic “spear tip” symptom of *Rhizoctonia* infections. Roots also have small brown lesions that are about two to three millimetres long. Plants are generally able to tolerate the infection by producing new roots. The root damage results in delayed maturity of the affected plants (Wiese, 1987).

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In South Africa a unique strain of *Rhizoctonia solani* AG-6 causes crater disease of wheat. Root symptoms allow the differentiation between bare patch and crater disease. Bare patch caused by *R. solani* AG8 causes girdling and rotting of roots. In contrast the symptoms of crater disease caused by *R. solani* AG-6 are nodulose swellings on roots and formation of sclerotial sheaths on the seminal roots (Meyer *et al.* 1998).

Rhizoctonia solani is readily recovered from the soil and different isolates can infect the root, the culm or both the root and culm. *R. solani* can be divided into anastomosis groups (AG) based on hyphal fusion between compatible strains, AG8 is the group mostly responsible for bare patch disease of wheat. *R. solani* grows on plant residues in the soil and on many artificial media on which the mycelia can appear white to a deep brown. The hyphae are 4 to 15 µm wide and branch at right angles. A septum near the branch and a slight constriction near the base are diagnostic of *R. solani*. The mycelium differentiates into black- brown sclerotia in culture and on the plant. These sclerotia are of an irregular shape and germinate to form mycelium (Wiese, 1987).

Rhizoctonia can be classified using classical, biochemical and molecular methods. The classical methods include differentiation by the dolipore or parenthesome septum by electron microscopy, classification by hyphal anastomosis reactions, and classification by the teleomorph reproductive structures. The biochemical methods used to distinguish between *Rhizoctonia* include soluble protein analysis, isozyme analysis, lectin analysis, fatty acid profiling (FAME) and serological differentiation. Various molecular methods are used to differentiate between *Rhizoctonia* including the use of DNA sequence analysis, DNA hybridizations and ribosomal RNA analysis. An integrated approach, which includes molecular, biochemical and cultural methods, can also be used to identify and differentiate between *Rhizoctonia* species (Sneh *et al.* 1996).

Rhizoctonia solani is an efficient saprophyte and is omnipresent in the soil as mycelia and sclerotia. Since *R. solani* does not produce vegetative spores the only sources of inoculum are mycelia, sclerotia and basidiospores. Sclerotia are the primary survival structures. Basidiospores are fragile and short-lived and therefore not an important inoculum source. Infection is initiated when mycelium, already present in the soil or from a germinating sclerotium or basidiospore, grows over the root surface. Once the mycelium has grown over the root surface infectious structures are formed followed by penetration of the host cells and tissue colonisation (Figure 2.1) (Sneh *et al.* 1996). Root infection can occur any time during the growing season and is most damaging when initiated on seedlings. These infections often result in bare patches. *R. solani* is also an important opportunistic pathogen on roots that are injured by nematode attack.

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Damage caused by *Rhizoctonia* is highly dependant on the environment (Wiese, 1987). *R. solani* growth and disease is influenced by the soil texture. Disease is more severe in sandy soil than clay soil. The soil texture is more important for disease development than the nutrient status (Gill *et al.* 2000). Soil moisture is also an important factor influencing the severity of disease caused by *Rhizoctonia* on wheat. Gill *et al.* (2001) found that the disease severity decreased by 69% as the soil moisture was increased from 15% to 75%. Root colonisation by *Rhizoctonia* also decreased as the soil moisture increased (Gill *et al.* 2001).

Tillage practices also influence disease. Numerous reports indicate that *Rhizoctonia* associated root disease increases when there is a switch from conventional tillage to minimal or no-till practices (Bockus and Shroyer 1998; Schroeder and Paulitz 2006). The disease tends to return to the same level observed before the transition in approximately ten years. It is hypothesised that this is due to the change in microflora and subsequent establishment of a new equilibrium (Schroeder and Paulitz, 2006).

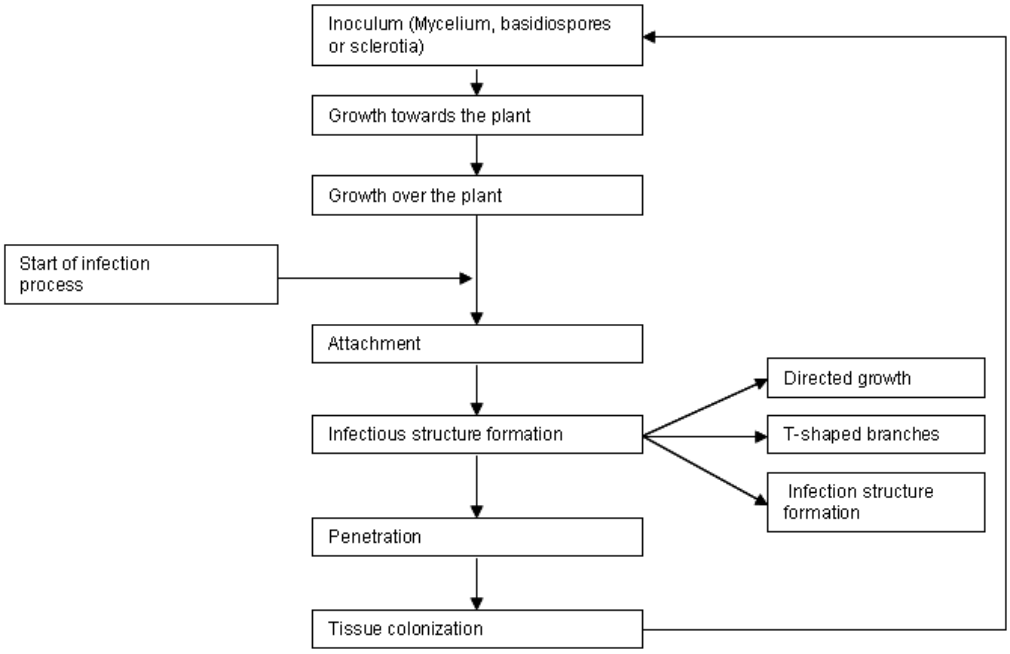

Figure 2.1: The disease cycle and infection process of *Rhizoctonia solani* (Adapted from Sneh *et al* 1996).

There are no truly resistant wheat cultivars to *R. solani* root rot. Management practices are therefore important to control the disease. It is advantageous if the seeds are sown shallowly and root growth is promoted with fertilizers (Wiese, 1987). Other practices such as killing weeds and volunteer plants a few weeks before sowing can reduce the inoculum in the soil. Tillage also reduces the disease severity. It is thought that tillage decreases the inoculum

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potential by breaking up the hyphal networks, increasing soil drying and enhancement of microbial activity (Paulitz 2006). Crop rotation is of little benefit because of the large host range of *Rhizoctonia* and its ability to survive in the soil. Some chemicals, such as Tolclofos-methyl, flutolanil (for *R. solani* AG8 and *R. oryzae*) and difenoconazole are used for control of the disease (Wiese, 1987). *Streptomyces Bacillus* spp. and *Pseudomonas fluorescence* Pf-5 and *P. chloraphilis 30-84* provide limited biological control. They are however more active against *R. oryzae* and *R. cerealis* than *R. solani* AG8 (Sneh *et al.* 1996).

2.4 Plant growth promotion by rhizobacteria

Microbes have an important effect on soil health and quality and also greatly influence plants (Wellbaum, 2004). Soil microbes are abundant and especially active in and around plant roots. The rhizosphere is the area immediately surrounded by and influenced by plant roots and is a site of high microbial activity. The main reason for this activity in the rhizosphere is the nutrients released by the roots. Bacterial root coverage is reported to be in the range of 5-10% with proportionately more Gram negative bacteria (e.g. *Pseudomonas, Achromobacter*) and denitrifiers, and fewer Gram positive and Gram variable forms (e.g. *Bacillus, Arthrobacter*) (Paul and Clark 1989).

Among the root colonising microbes several beneficial isolates and strains have been discovered. The possibility to exploit these strains for improved plant production has received much attention in recent years. This is driven by a large demand for reducing chemical pesticide usage. The main reasons for reducing chemical use are due to agricultural and public concerns. The agricultural concern regards the increase in disease resistance to the available chemical controls. Public concerns are focused on the negative effects that chemicals have on the environments and human health (Horrigan *et al.* 2002). The concept of applying microbial inoculants to the soil for disease control was proposed about fifty years ago (Alabouvette *et al.* 2006).With this need for sustainable production innovations and the existence of beneficial soil microbes the concept of using soil microbial inoculants to increase plant productivity has received much attention lately.

Literature regarding bacterial isolates that have potential to be used as soil inoculants to improve plant growth and health is voluminous (Table 2.1). Commercially available inoculants are also available in several countries throughout the world (Table 2.2). Ryder *et al.* 1999 mentions China as an example of a country where beneficial soil microbial inoculants have been used for many years on several million hectares of agricultural land. In scientific and agricultural literature and in the agricultural industry one will find several terms for these beneficial microbes namely biocontrol agents, bioinoculants, biopesticides, microbial pest control agents, biofertilizers and PGPR (Labuschagne *et al.* 2010). In general these terms are

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not specifically defined and the definition usually depends on the context in which they are used. The PGPR group for example are generally understood to be bacteria, found in the rhizosphere, that exert a beneficial effect on plant growth. However PGPR are considered by some to be only those isolates that promote growth and by others isolates that promote growth and control pathogens (Bashan and Holguin 1998; Pal and McSpadden 2006; Eilenberg *et al.* 2001).

The differences in terminology can be attributed to the historical existence of two main strains of research on beneficial soil bacteria, one focusing on biocontrol the other on growth promotion. Though research has generally focused on either one of the two i.e. biocontrol or growth promotion, a plant beneficial bacterium can be both (Avis *et al.* 2008). In this context PGPR are defined as "*rhizobacteria that exert beneficial effects (any) on plant growth and development*" (Zahir *et al.* 2003). PGPR can subsequently be divided into two groups based on their main mode of action i.e. growth promoting PGPR that enhance plant growth by directly affecting plant growth and biocontrol PGPR that indirectly enhance plant growth by reducing diseases associated with the plant (Figure 2.2). In this review PGPR's are discussed with a focus assessment, screening and factors that affect activity and commercial use.

2.4.1 Modes of action for direct growth promotion

Direct growth promotion occurs mainly by mechanisms that enhance the plant's nutrient status such as increased nutrient availability and altered root morphology. Plant growth promoting rhizobacteria can have several direct effects on plant health. The general effect is to improve the plant's nutrient status (Vessey 2003) which can be affected through nitrogen fixation and increase of available nutrients or causing an increase in root growth. Other less common modes include vitamin production, stress alleviation and the creation of a more suitable microbial environment through enhanced host symbioses and altered soil microflora (Figure 2.2).

Nitrogen (N) is an important plant nutrient, and one of the major yield limiting factors in crop production systems. Annually large amounts of N are applied using fertilizers. The conventional practice of fertiliser applications is however associated with run-off and leaching that result in water contamination. An attractive alternative to conventional fertiliser application practices is biological nitrogen fixation (BNF) (Mantelin and Touraine 2004). Biological nitrogen fixation is defined as "*the conversion of atmospheric nitrogen by living organisms or combination of organisms into molecular more complex compounds at normal pressure and temperature*" (Staphorst 2009). Microbes can fix nitrogen symbiotically or asymbiotically. Symbiotic nitrogen fixation is well known to greatly increase plant growth in areas where nitrogen is deficient. The most common example of symbiotic nitrogen fixation is that of the legume – rhizobium symbioses. Several free living soil bacteria such as the *Azospirillums'* are

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able to fix atmospheric nitrogen asymbiotically and may contribute to the nitrogen status of plants (Dobbelaere *et al.* 2003).

Some researchers are of the opinion that asymbiotic nitrogen fixation is not suitable for agricultural exploitation (Mantelin and Touraine 2004, Staphorst 2009, Dobblarier *et al.* 2003). One of the main arguments supporting this view is that agricultural soils usually contain large amounts of nitrogen. These combined forms of N such as nitrate or ammonia inhibit the nitrogenase enzyme responsible for conversion of atmospheric N to ammonia (Dobbelaere *et al.* 2003). Furthermore the cost of N-assimilation is high. The free living N-fixers therefore generally do not excrete the fixed N but utilize it mainly for cellular processes. The N is therefore only released once the bacterium dies. With this mechanism of nitrogen release in mind, the rhizosphere population of asymbiotic N-fixers renders them insignificant nitrogen contributors. This is highlighted when we consider that asymbiotic N-fixers are normally found at 10^3 of 10^5 cfu/g plant root, in comparison to endophytic rhizobia that are present at about 10^7 to 10^8 cfu/gram root.

There are selected examples where asymbiotic nitrogen fixation does significantly contribute to the plant's nitrogen needs. An example is the endophytic diazotrophs that are associated with sugarcane plants (Vessey *et al*. 2003). Rodriguez et al. (2008) demonstrated that *Azospirillum amazonense* isolates contributed to the nitrogen used by rice plants. Using nitrogen isotopes they showed that some isolates supplied up to 27% of the nitrogen assimilated by the plants. They also noted that the majority of the isolates that they screened had a low nitrogenase activity. To find isolates that effectively contribute nitrogen to plants, numerous isolates need to be screened. Though asymbiotic N-fixation may not contribute significantly to the plant's health it is still an important ability for a PGPR to have. Research suggests that the ability to fix nitrogen gives the bacteria a competitive advantage. This advantage can help the PGPR to reach populations high enough to influence plant growth by other MOA (Dobbelaere et al. 2003).

Plant growth promoting rhizobacteria can also increase the nitrogen status of plants through mechanisms other than nitrogen fixation. Research has shown that some PGPR can stimulate nitrogen uptake by plants. Nitrogen uptake is stimulated by the activation of plant genes. This gene activation can occur as a result of PGPR's producing ammonia or altering the NO_3^- concentration in the rhizosphere. Many PGPR also cause the plant's root area to increase, which will lead to an increase in nutrient acquisition (Mantelin and Touraine 2004). Thus these inoculants may not directly increase the nitrogen available to plants, but they can be employed to improve the utilisation of available nitrogen by plants and in this way reduce fertiliser inputs. These occurrences should be studied since a better understanding may lead to the ability to manipulate these systems to improve the nitrogen status of soils.

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2.4.1.1 *Increased nutrient availability*

Plant nutrients, though present in the soil, may not be available to the plants. The main reasons for low nutrient availability include nutrient scarcity and the inability of plants to utilise certain forms of a nutrient. Plant growth promoting rhizobacteria can help to make these nutrients more available to the plant. The most common examples of increased nutrient availability by PGPR include phosphate solubilisation and enhancement of iron acquisition by production of siderophores (Vessey 2003).

Phosphate (P) solubilisation is known to improve plant growth. Though agricultural soils contain large amounts of P, it is second to nitrogen as the mineral that most commonly limits plant growth. Phosphate availability is often a limiting factor to plant growth because P reserves are frequently in a form that plants can not utilize. Phosphates in fertilisers are rapidly immobilised in the soil, and inorganic P is found in large molecular weight molecules that must be degraded for plants to utilise. Microbes that solubilise P are commonly found in soils and the rhizosphere. These microbes solubilise inorganic P by the production of organic acids. Organic P is hydrolysed by microbial phosphatases to inorganic P that plants can utilise. *Rhizobium Pseudomonas* and *Bacillus* spp. are among the most effective P solubilizers. These microbes are usually not present in the soil in large populations and need to be inoculated to substantially contribute to plant nutrition. Though many bacteria are able to solubilise P they are not necessarily growth promoters, and P solubilising growth promoters do not necessarily increase plant P levels. Phosphate solubilization may also influence the mycorrhizal associations with plants, enhancing these associations and thereby enhancing plant growth (Vessey 2003; Rodriguez and Fraga 1999).

PGPR can also increase nutrient availability to plants through the production of molecules that increase the plants' nutrient uptake efficiency. Siderophores are the best known examples of such molecules that are produced by PGPR. Siderophores are relatively low molecular weight, ferric iron-specific chelating agents. These compounds scavenge iron form the environment and make this essential mineral available to the microbial cells. Plants prefer to utilise reduced ferrous iron (Fe^{2+}). In well aerated soils, the ferric (Fe^{3+}) ion is more commonly available and is easily precipitated to iron-oxide. To obtain iron, plants thus excrete cheaters and phytosiderophores to bind Fe^{3+} and maintain it in solution. There is evidence that plants can absorb bacterial siderophores in addition to their own (Rosas 2007; Vessey 2003).

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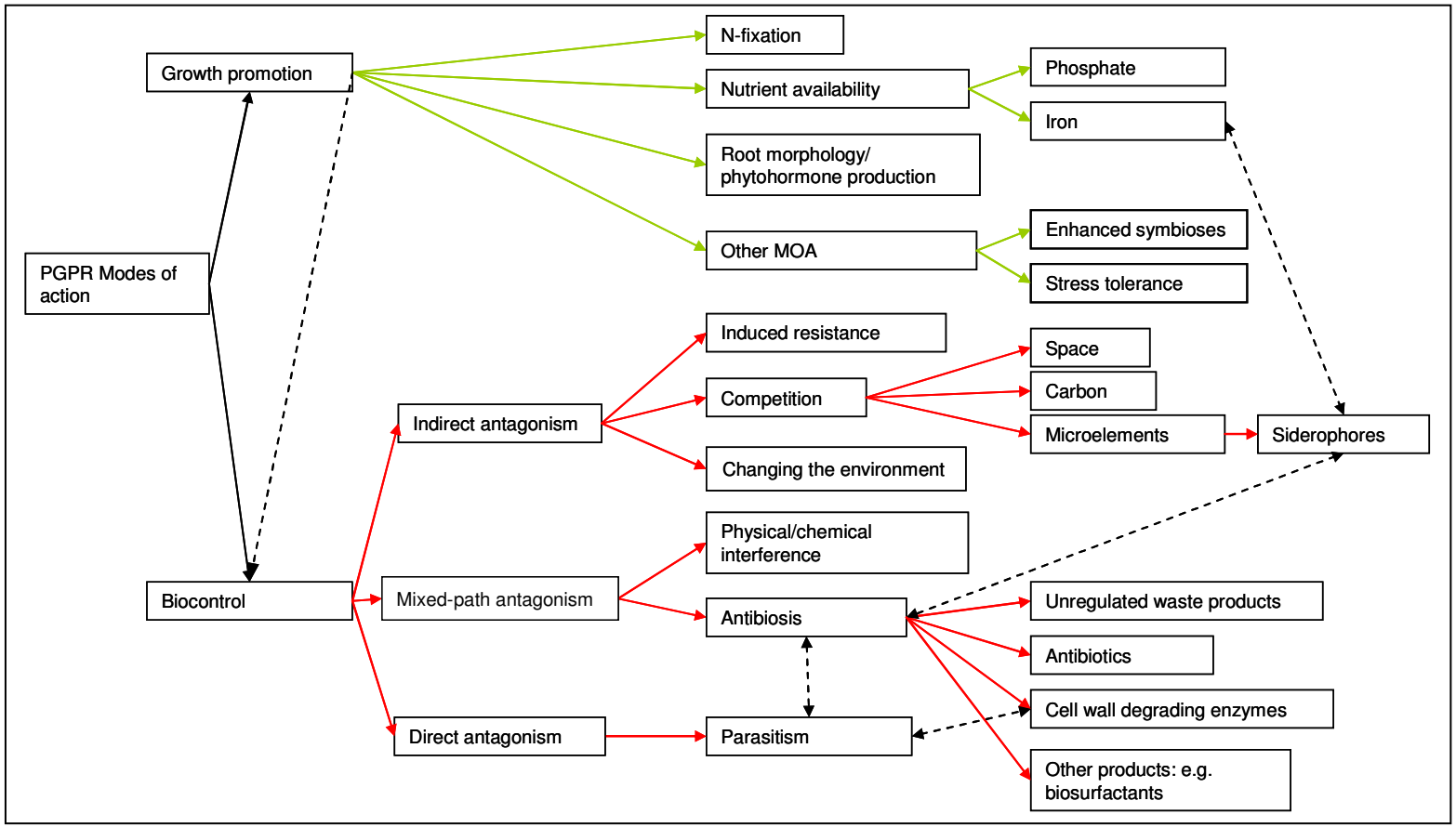

Figure 2.2; Diagram summarising the different modes of action of plant growth promoting rhizobacteria (compiled from different literature sources referred to in text). Dashed lines indicate where modes of action classification overlap, for example, biocontrol can be considered as modes of action contributing to growth promotion.

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Siderophores produced by bacteria can therefore sequester iron for use by plants. There is however a controversy as to the significance of bacterial siderophores in the iron nutrition of plants. Some scientists hypothesise that the iron acquired from bacterial siderophores has an important or even vital role, while others maintain that the overall iron contribution is small. The main contribution of siderophores is usually attributed to biocontrol through competing with phytopathogenic fungi for the available iron (Rosas 2007).

PGPR can also increase the plant's nutrient status by other mechanisms. Recent research indicates that PGPR can also influence the plant's own nutrient uptake mechanisms. *Bacillus subtilis* has for example been shown to improve the iron uptake efficiency of *Arabidopsis* plants. The *Bacillus* enhances the plant's own iron acquisition mechanism causing an increase in iron assimilation. The bacteria can also enhance release of protons from roots causing the rhizosphere to acidify. The higher acidity increases iron mobility (Zhang *et al.* 2009). Another mechanism whereby PGPR can contribute to the plant nutrient status is the increase of plant root area.

2.4.1.2 Inducing increases in root surface area, positive effects on root growth and morphology and phytohormone production

PGPR have the ability to affect the morphology of plant roots and root systems. This is a very important mechanism for growth promotion since the root system is the limiting factor for nutrient uptake when nutrients are unlimited and in an available form. Large numbers of PGPR's are also not necessary to produce the host changes in root morphology, since the main catalysts for these changes are hormones produced by PGPR, or other molecules that are similarly active at very low concentrations. This advantage makes PGPR with this MOA highly exploitable since it is difficult to inoculate and maintain PGPR numbers at a high level (Vessey 2003, Dobbelaere *et al.* 2003).

Phytohormones produced by PGPR include auxins (e.g. Indole acetic acid (IAA)), Indole-3-butyric acid, cytokinins, and gibberellins (gibberellic acid) (Tsavkelova 2006, Spaepen *et al.* 2008). The most important hormone produced by PGPR seems to be IAA, which stimulates root elongation, cell division and cell enlargement. Experiments with PGPR that have mutations in their IAA production pathway have shown that PGPR possessing this mechanism are able to increase the formation of root hairs by the host plant. In addition some levels of IAA produced by the PGPR were shown to decrease the root length. This dose related response shows the necessity to understand the MOA clearly. Production of IAA is also influenced by the host plant. This is because IAA production is dependant on tryptophan, and the main source of tryptophan is from root exudates. Macronutrients (carbon, nitrogen and phosphorous) as well as stress also affect IAA production by enhancing the utilisation of tryptophan by the PGPR. The increase in tryptophan production results in enhanced IAA production and therefore root hair development (Malhotra and Sivestava 2009).

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Ethylene is also an important plant hormone that PGPR's affect. Ethylene is a phytoregulator that is especially associated with the plant stress response. Levels of ethylene are decreased by the 1-aminocyclopropane-1-carboxylate (ACC)-deaminase enzyme that many PGPR produce. The ACC-deaminase breaks down ACC, an important precursor in the ethylene synthesis pathway. Rhizobacteria thereby act as a sink for plant produced ACC. The reduced levels of ACC in turn decrease the effects stress has on plants (Glick *et al.* 2007, Ribaudo *et al.* 2009).

The interactions between plant hormones produced by PGPR are complex since their effects are interrelated. Ribaudo *et al.* (2009) found that there is an interaction between IAA and ethylene production. Furthermore PGPR can affect plant root systems by other mechanisms such as increasing the ion uptake, specifically NO^-_3. This influences root branching and NO^-_3 uptake itself (Mantelin and Touraine 2004). PGPR can also affect the root respiration and thereby increase root length (Vessey 2003). The total hormone balance in the plant also influences the way in which the plant reacts to hormones produced by PGPR.

2.4.1.3 Lesser known / other modes of action for growth promotion

Beside the well known MOA, there are several other ways by which PGPR can enhance plant growth; including increasing plants stress tolerance, vitamin production, enhanced symbioses and changing the rhizosphere environment. The ability of PGPR to enhance the stress tolerance of plants is becoming an increased focus for researchers. In this regard Yang *et al.* proposed the term "induced systemic tolerance" (IST). Induced systemic tolerance refers to PGPR induced changes in plants that enhance their resistance to abiotic stress. Such abiotic stress includes drought, salinity, and heavy metals. Plant stress related to nutrients is also included in this definition (Yang *et al.* 2008). Studies with Arabidopsis have shown that PGPR can enhance drought tolerance through interaction with a plant drought response gene. PGPR can also affect the plant homeostasis by affecting the Na^+ balance and thereby reducing the stress imposed on plants growing in saline environments. Another mechanism whereby PGPR can increase plant stress tolerance may involve the reduction of damage caused by reactive oxygen spp. that plants produce in response to stress. Some PGPR also produce vitamins that may improve plant growth and plant stress tolerance (Yang *et al.* 2008, Dobbelaere *et al.* 2003). Besides increasing stress tolerance and vitamin production PGPR can influence plants growth indirectly through enhancing beneficial plant-microbe symbioses.

Plant growth promoting rhizobacteria that enhance beneficial plant microbe symbioses are known as "helper bacteria". Most reports of helper bacteria focus on the legume-rhizobia symbioses and arbuscular mycorrhizae (AM) colonisation of forest trees. Through enhancing these beneficial relationships the helper bacteria indirectly enhance the plant nutrient status (Vessey 2003). Plant growth promoting rhizobacteria may also influence the rhizosphere

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environment by increasing the root adhering soil. This occurs through the production of exopolysaccharides (EPS) that aggregate the soil. This can benefit the plant by providing a niche for other beneficial microbes or due to a beneficial effect on the water and air relationship (Dobbelaere *et al.* 2003). More recent studies are also showing that quorum sensing molecules produced by the PGPR's can influence plants. Some PGPR's produce volatile compounds that enhance plant growth. For example, Nitric oxide produced by the PGPR may also play a part in plant growth promotion. As more is published about these effects bacteria have on plants it becomes apparent that the interactions are complex and not adequately understood.

2.4.2 MOA for biocontrol

The MOA for PGPR that control plant pathogens have been a topic of research since the discovery of this beneficial interaction. This voluminous body of research has resulted in a large amount of literature and knowledge on the subject (Santoyo I. 2012; Weller 2007; Whipps 2001) Some mechanisms suggested for this interaction include the production of antibiotics, parasitism, production of lytic enzymes, competition, and induced resistance. Using the framework of Pal and McSpadden (2006) these interactions can be grouped together in a way that facilitates understanding of the mechanisms and their role in the disease cycle and the environment. According to this structure the MOA are grouped into three main categories based on the amount of interspecies interaction involved. These categories are direct antagonism, mixed-path antagonism and indirect antagonism.

Direct antagonism occurs when the PGPR specifically targets and attacks the pathogen. This MOA involves physical contact and a high degree of selectivity for the pathogen. Hyperparasitism and predation fall into this category. The second category of antagonism is mixed-path antagonism. It is similar to direct antagonism in that the pathogen is directly affected, however a high degree of specificity and targeting of the pathogen is not involved. Modes of action in this category include antibiosis, production of toxic waste products, and interference with the plant pathogen functions. Lastly indirect antagonism includes MOA such as competition and induced resistance. The pathogen is thus influenced via a secondary effect produced as a result of the action of the PGPR.

2.4.2.1 Direct antagonism: Parasitism

In the context of biological control of fungal plant pathogens parasitism is the directed attack of the beneficial microbe (antagonist) on the plant pathogenic fungus resulting in destruction or lysis of the pathogen's hyphae or propagules. This interaction is distinguished from others that adversely affect the plant pathogen in that the antagonist senses the fungal pathogens and then targets the fungus directly (Larkin *et al.* 1998). This MOA is difficult to distinguish

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from the mixed-path MOA such as antibiosis because they share several common mechanisms and it is difficult to prove a targeted and specific relationship with the plant pathogen. Such overlapping mechanisms include production of antibiotics, lytic enzymes and other toxic compounds.

Parasitism is most commonly associated with fungal-fungal interactions, such as *Coniothyrium minitans* parasitizing *Sclerotinia* (Smith *et al.* 2008) and ascribed as a mode of action of the well known biocontrol agents belonging to the genus *Trichoderma* (Howell 2003). Leveau and Preston (2008) provided a comprehensive review of bacterial mycophage, and defined it as the practice of purposefully consuming fungal tissue. Examples of bacteria with mycophagous traits include *Bacillus, Paenibacillus, Pseudomonas* and *Streptomyces* spp. The three main strategies of bacterial mycophages are extracellular nectrotrophy, extracellular biotrophy and endocellular biotrophy. Extracellular necrotrophs permealise and lyse fungal hyphae resulting in the death of the fungal cells. The extracellular biotrophs live in close proximity of the hyphae, without killing them, and use the nutrients exudating from the fungal cells. These bacteria are able to tolerate or suppress the antibacterial metabolites produced by the fungi and may even alter the fungi metabolism to increase nutrient release. Lastly the endocellular biotrophs are found living inside the live fungal hyphae and absorb nutrients directly from the host cytoplasm. The strategies can overlap and usually progress from biotrophy to necrotrophy as the bacterial numbers increase. The primary causal agent of fungal inhibition and cell death is associated with low molecular weight toxins. Induction of apoptosis may also be a mechanism for lysis of fungi. For the lysis of fungal cell walls the bacteria need to produce a large spectrum of cell wall degrading exoenzymes. With regards to utilising this mechanism for biocontrol it should be noted that the interaction is regulated by positive feedback. This means that the bacteria need the fungus to multiply (Leveau and Preston 2008).

2.4.2.2 Mixed path antagonism: Antibiosis

Antibiosis is a term that is often used in biocontrol discussions. In contrast to parasitism where the main purpose is to obtain nutrients from fungal hyphae, the main role of antibiosis is to colonise, retain and proliferate in a niche. Antibiosis is mediated by several allelochemicals such as antibiotics, lytic enzymes, volatiles and detoxification enzymes that are produced by the PGPR. The production of waste products in an unregulated way can also contribute to antibioses in the rhizosphere. These products inhibit the pathogen or destroy its propagules (Compant *et al.* 2005, Larkin *et al.* 1998).

Many biological control strains of PGPR are known antibiotic producers and this MOA is probably one of the best studied biocontrol mechanisms. Antibiotics are organic molecules of a low molecular weight that have a harmful effect on other microbes. The antibiotics produced

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by PGPR vary in their spectrum of activity and in action, most having a broad spectrum of activity against both bacteria and fungi (Raaijmakers *et al.* 2002).

Many soil inhabiting bacteria produce antibiotics that are active against soilborne plant pathogens. The fluorescent pseudomonads are probably the best known antibiotic producers and most widely studied PGPR group in this regard. A well known example of effective disease suppression in which antibiotic production is clearly implicated is the suppression of take-all disease of wheat (caused by *Gaeumannomyces graminis)* by Pseudomonads. Antifungal metabolites implicated in disease control include phenazine, 2,4-diacetylphloroglucinol, butylactones, HCN, ammonia, pyrrolnitrin, pyoluterin, xanthobaccin viscosinamide, and zwittermycin A, as well as several other uncharacterised compounds (Handelsman and Stabb, 1996; Rosas 2007). Several bacilli also produce antibiotics for example a *Bacillus subtilis* isolate produces the antibiotics bacilysin and iturin, that are implicated in biocontrol of disease caused by *Fusarium oxysporum* f. sp. *cucumerinum* on cucumber (Chung *et al.* 2008). Most of the research regarding antibiotics has been *in vitro* studies. The role of antibiotics in biocontrol has been studied *in situ* using culture filtrates and antibiotic mutants. The use of mutant strains has been important in assessing the actual role that antibiotics have in biocontrol. Mutants include strains that lack the ability to produce specific antibiotics, have an improved ability to produce antibiotics and naturally occurring non-producer strains into which antibiotic production genes have been introduced (Chin-A-Woeng 2003).

There are many difficulties associated with detection of antibiotics in the soil. Strategies to overcome them include the use of reporter genes and high performance liquid chromatography (HPLC). Reporter genes are however not very accurate and HPLC, though currently the best method, is complicated and difficult to perform. The question still remains whether these compounds are active *in situ* and produced in large enough quantities to affect disease control. New technologies such as *in situ*-PCR may help to answer these questions. This information is important since many factors influence the production and activity of antibiotics. These factors include the bacterium itself, regulatory pathways such as quorum sensing, temperature, soil moisture, pH, host plant exudates, and the availability of carbon sources and minerals. Understanding the ecology of antibiotic production is important to manipulate the BCA and achieve consistent and effective biocontrol (Raaijmakers *et al.* 2002).

Lytic enzymes are considered to play an important role in biological control and are involved in both parasitism and antibiosis. Lytic enzymes are enzymes produced by microbes that degrade fungal cell wall components and cause the lysis of fungal hyphae. Examples include chitinases, cellulase, glucanases, proteases and amylases (Whipps 2001). In literature there are several studies that demonstrate the involvement of lytic enzymes in biological control.

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Studies include experiments showing that the antagonistic bacteria are able to grow using the cell wall components of fungi as a food source (Chet *et al.* 1990). For example Arora and co workers (2007) showed that both chitinase and B-1,3-glucanases have detrimental effects on *Rhizoctonia solani* and *Phytophtora* growth *in vitro*. Even thought lytic enzymes are implicated in biocontrol there is little direct evidence for their action in the rhizosphere and application of mycolytic strains for biocontrol is usually unsuccessful (Rosas 2007). The probable reason for the failure of mycolytic strains that have been introduced in the rhizosphere is that the main function of these enzymes are to degrade non-living organic matter as a food source (Pan and McSpadden-Gardener 1999). This means that the enzymes are most likely not produced in response to the pathogens. Also, as with their role in parasitism, the enzymes act synergistically with other antifungal compounds (Chin-A-Woeng 2003).To effectively use strains producing lytic enzymes, a better understanding of the function and interaction with the environment is necessary (Rosas *et al.* 2007).

Volatile compounds produced by microbes can inhibit fungal germination and mycelial growth. Three types of volatiles, the amines, aldehydes and alkenes, are known to be involved in soil fungastasis (Zou *et al.* 2007). Several plant pathogens including *Rhizoctonia, Aspergillus, Fusarium, Microdochium, Neurospora, Paecilomyces, Penicillium, Phoma, Sclerotinia, Trichoderma* and *Verticillium* are inhibited *in vitro* by volatiles produced by bacteria. In soil the bacilli are important contributors to soil fungistasis. Bacterial isolates from the genera *Strenotrophomonas, Serratia, Pseudomonas,* and *Burcholderia* also produce volatiles that inhibit fungal mycelial growth. Studies have shown that a wide range of volatiles are produced by PGPR's. The antifungal compounds included aldehydes, alcohols, ketones and sulphides (Vesperman *et al.* 2007, Kai *et al.* 2007). Many fungistatic volatiles, such as ammonia, carbon dioxide and hydrogen cyanide are produced as waste products of bacterial growth (Arora *et al.* 2007) that contribute to biological control.

Biosurfactants are compounds (produced by bacteria) that are amphiphilic in nature and therefore have emulsifying and foaming properties. Biosurfactants can cause lipid membrane destabilisation that may result in lysis of the fungal structures (Hultberg 2008, Onega *et al.* 2007). The use of inoculants producing biosurfactants are of special interest for the control of zoosporic pathogens such as *Phytophtora* and *Pythium* (Stanghellini and Miller 1997). Biosurfactants have received much attention because synthetic surfactants successfully control zoosporic pathogens in hydroponic growing systems. In soilless cultivation systems 18.5 % of the indigenous bacterial population produces biosurfactants. Studies have also shown that biosurfactants have promise to control pathogens both in hydroponic systems and on foliage. Research suggests that biosurfactants are produced by bacteria to improve their swarming motility. The main biocontrol action seems to be the disruption of the zoospore membrane causing the zoospore to lyse. Biosurfactant producing strains are promising agents to use in an integrated control system. For such a system to be effective the

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biodegradation and optimal temperature of the biosurfactants needs to be taken in to account. Furthermore bacterial strains that produce both antibiotics and biosurfactants are more effective indicating a synergistic effect. (Stranghellini and Miller 1997; Hultberg *et al.* 2008)

2.4.2.3 *Mixed-path antagonism: Physical or chemical interference with the pathogen*

The interference of PGPR with pathogen activities is a MOA that has been considered more recently. PGPR can interfere with fungal actions, both on the physical and chemical level. Interference occurs through the detoxification of pathogen virulence factors, and quenching of the pathogen quorum sensing communication by degrading the autoinducer signals (Compant *et al.* 2005). Specific examples include the degradation of fusaric acid, a virulence factor produced by *Fusarium oxysporum*, by biocontrol strains of *Bacillus cepacia* and *Burcholderia* strains. *Pythium ultimum* sporangia germination is retarded by bacteria that degrade linolenic acid and other exudates that stimulate sporangia germination. These new pathways of biocontrol have great potential for disease control and may even have the potential to be developed as plant disease cures (Hass and Defago 2005).

2.4.2.4 *Indirect antagonism: Competition*

Soils with a high microbial biomass can have a general suppressive effect on pathogens due to the intense competition that exists in such a situation. Competition is important as a MOA of biocontrol and for the establishment of introduced antagonists in the rhizosphere. Via competition the pathogens are deprived of nutrients, this inhibits their growth since they are therefore in a constant state of starvation (Brussaard *et al.* 2007). Competition as a MOA is effective when the antagonists are present in sufficient numbers, at the correct time and location to deprive the pathogen of nutrients and other resources. The pathogen is thereby excluded from the site where the antagonists are present such as areas on the root where infection occurs. Antagonists with competition as a MOA usually have a versatile metabolism and are able to utilise a wide range of compounds. This characteristic enables the antagonists to effectively colonise sites and scavenge nutrients away from the pathogens, thus effectively excluding the pathogen. Competition is also important since the efficacy of an antagonist is determined to a large extent by its colonisation and survival ability regardless of the MOA (Raajmakers *et al.* 2008, Brussaard *et al.* 2007). There are several examples where competition is considered to have an important role in biocontrol. Control of *Fusarium oxysporum* by *Collimonas fungivorans* was attributed to competition for nutrients and space on the roots (Kamilova *et al.* 2007). On maize the control of *Fusarium* by *Streptomyces* isolates is related to competition for carbon sources. Siderophore production is probably the most well known example of biocontrol due to competition.

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Iron is important for microbial growth and under aerobic condition is needed for several metabolic functions including ATP production and the formation of DNA precursors. Iron is generally found in complexes making it essentially unavailable for use by microbes. The ferric-siderophore complexes are recognised and bound by specific outer membrane receptor proteins on the microbial cells that import the iron into the cell. Bacterial siderophores are thought to sequester the limited iron available in the rhizosphere, making it unavailable for pathogenic fungi, thereby restricting their growth. The role of pyroverdine, produced by *Pseudomonas* spp. has been clearly demonstrated in the control of *Pythium* and *Fusarium*. Some siderophores can only be used by the bacteria that produce them whereas others can be used by many different bacteria. Biotic and abiotic factors influence the amount of siderophores produced (Rosas 2007).

Competition as a MOA has some shortcomings, the main restriction being that large numbers of the antagonist need to be present. Naturally occurring biocontrol pseudomonads are usually present at around 10^5 – 10^6 cfu/gram root. Assuming that there are 10^8 – 10^9 cfu/gram of culturable aerobic bacteria present per gram of root, the biocontrol agents represent 0.1 - 1% of the cultural population. Though artificially inoculated bacteria can reach levels of about 10^7 – 10^8 cfu/gram root they always decline after a few weeks. It is thus unlikely that PGPR inoculants are able to outcompete pathogens in the soil (Hass and Defago, 2005). Furthermore pathogens are able to reduce the competitive ability of antagonists by various mechanisms. An example is the production of molecules that alter the gene regulation of the antagonist to be more favourable to the pathogen (Duffy *et al.* 2003).

2.4.2.5 Indirect antagonism: Induced systemic resistance

Induced resistance is a general response of plants that results in increased broad spectrum systemic resistance to pathogen attack and other stress factors. Two main pathways are involved in induced resistance namely systemic acquired resistance (SAR) and induced systemic resistance (ISR). Systemic acquired resistance is mediated by salicylic acid and occurs in response to the primary attack from a pathogen, generally when the hypersensitive response is activated, or through application of chemicals. Induced systemic resistance is mediated by jasmonic acid and ethylene and develops amongst others as a result of root colonisation by certain PGPR strains. Both pathways stimulate the defence response and result in expression of defence genes, phytoalexin accumulation and cell wall strengthening. Induction of systemic resistance may involve activation of multiple potential defence mechanisms, including increased activity of chitinases, B-1-3-glucnases, peroxidases, and other pathogenesis related (PR) proteins, accumulation of antimicrobial compounds such as phytoalexins, and formation of protective biopolymers, such as lignin, callose, and hydroxyproline-rich glycoproteins (Walters 2009, Larkin *et al.*1998).

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Induced systemic resistance and SAR may also result in priming or immunisation of plants, usually in response to a low dose of the inducing agent. Plants that are primed typically show no detectable change in resistance traits in response to the priming agent but respond more rapidly to pathogen attack. Priming agents cause plants to rapidly activate multiple mechanisms of disease resistance, which in susceptible plants, are latent or expressed too late to control disease. The mechanisms include accumulation of antimicrobial low molecular weight chemicals, and protective biopolymers, as well as the increase in the production and activity of chitinases, b-1-3-glucanase, peroxidases, and other pathogenesis related proteins (Tuzun and Kloepper, 1995, Walters 2009). The significance is that primers, in contrast to inducing agents, do not lead to the expression of the defence related proteins, the production of these protein are held in check until the pathogen infects. This means that the plant growth is not limited due to the cost of producing defence proteins unnecessarily (Buensanteai and Prathuangwong 2009).

Induction of resistance by PGPR is an active area of research. Examples include the induction of defence in wheat roots by PGPR inoculants. Wheat plants inoculated with *Pseudomonas fluorescens* had enhanced levels of glucanases and several other defence enzymes and the total phenolic content of the roots increased dramatically. This correlated with a significant reduction in take-all caused by *Gaeumannomyces graminis* var. tritici (Sari *et al.* 2006). In split root trials *Bacillus cereus* strains and *Pseudomonas aeruginosa* induced systemic resistance in pigeon pea against *Fusarium udum*. Reduction in disease was related to the production of increased levels of defence related enzymes in the plants including the levels of L-phenylalanine ammonia lyase (PAL), peroxidase (POX), and polyphenol oxidase (PPO) (Dutta *et al.* 2008). This observation has also been reported for the cucumber root and crown rot pathogen *Pythium aphanidermatum*. Cucumber roots treated with a *Pseudomonas* isolate showed an increase in PAL, PPO and peroxidase activity which was correlated with reduced disease (Chen *et al.* 2000). Induced resistance has many benefits because it is broad spectrum and systemic and can increase plant resistance without drastically affecting growth and production. As a result the use of PGPR to induce disease resistance holds great promise for agricultural application

2.4.3 Sourcing and assessment of PGPR isolates

One of the most challenging aspects of developing PGPR inoculants is to obtain isolates that have the potential to be applied commercially. It is easy to obtain isolates, but very difficult to find isolates that will be successful. No universal protocol exists for the screening and assessment of PGPR. This is due to the fact that all screening methods are to a certain degree selective. The methods that are used during screening must therefore be selected carefully. The aims of the investigation and the intended use of the PGPR should be used as

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a framework for selecting the screening protocol. The protocol will be influenced by aspects such as the target crop, the environment into which the PGPR will be deployed, and for biocontrol, the disease control strategy needs to be taken into account. Preferences and requirements such as a specific organism's mode of action, or characteristic such as formation of spores of growth requirement will also influence the choice of assessment methods (Knudsen, 1997).

2.4.3.1 Sourcing

Bacteria can be isolated from the rhizosphere of almost all plants and PGPR's, even antagonists against soilborne disease, can be isolated from virtually all soils (Adesina *et al.* 2007). Even though this is the case the proportion of PGPR isolates in soils that will be commercially effective is very small. Special attention should therefore be given to the sourcing of these isolates. Aspects that will influence the range and efficacy of isolates obtained include the sampling site, host plant isolated from and the isolation procedure and conditions.

The site where the sample for bacterial isolation is taken from will influence the range of isolates obtained. The microbial composition and diversity of a soil is influenced by a range of factors such as the soil type (Ryder *et al.* 1999), and soil management practices (Hiddink *et al.* 2005). For biological control it is often suggested that isolates should be sought in areas where the disease does not occur, has declined, or cannot develop, despite the presence of a susceptible host rather than where the disease occurs (Baker and Cook 1974). For growth promotion isolates are often obtained from areas where the particular traits will be required, such as isolating from saline soils to increase the chance of obtaining isolates that enhance plant growth under saline conditions (Principe *et al.* 2007). An interesting example where the area was specifically selected for a particular trait is that of Hamaldid *et al.* 2008 where microbes were isolated from phosphate mines in order to obtain phosphate solubilising isolates that can be applied as PGPR. Using this strategy, isolates that not only increased plant growth but also controlled *Pythium* on wheat, were successfully obtained.

The plants that are growing at the isolation site or from which the isolates are obtained can influence the abundance and characteristics of the PGPR's. For instance some antagonists seem to be host specific and in some cases even cultivar specific, whereas other isolates have a broad plant host range on which they effectively control disease. The effect that the crop type has on PGPR populations was investigated by Lawongsa *et al.* (2008). Their research showed that the composition of pseudomonad population differed between maize and wheat fields. PGPR strains may also be cultivar specific, as shown by Khan *et al.* (2006). Fifteen PGPR isolates were evaluated for their ability to ameliorate the effect of *Fusarium culmorum* on coleoptile growth of six wheat cultivars. The results showed that only four of the isolates performance was consistent on all the wheat cultivars. Johanson and Wright (2003)

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furthermore showed that some plants such as those of the Brassicaceae family have a higher percentage of effective PGPR's on their roots. This indicates that certain plants are able to attract antagonistic rhizobacterial populations.

The type of sample and sampling procedures affect the range of PGPR's obtained. Different bacterial populations are found in the soil, rhizosphere (soil adhering to the roots), and on the roots (Johansson and Wright 2003). The spatial location on the roots (root tip or near the stem base) also influences the microbial population and it is well known that microbes are not equally distributed along roots. Generally there is more microbial activity at the root tips and root junctions and less activity near the stem base. Lesions caused by pathogens also influence the microbial population at those sites (Barnett 1998). Endophytes have recently started to receive more attention as potential biocontrol agents. Since endophytes are present inside the roots they are selected for by isolating from surface sterilised roots (Liu *et al.* 2009). Environmental factors are also important determinants of microbial composition and should be taken into account when sampling. Preferably samples should be taken during conditions similar to the conditions that will prevail when the PGPR's are used. As an example, Johansson and Wright (2003) collected samples during cold temperatures to increase the chances of selecting a cold tolerant PGPR. Their aim was to obtain a PGPR that would protect wheat seedlings against *Microconidium nivale*, a pathogen that kills snow covered seedlings.

2.4.3.2 Isolation and initial isolate selection

Once samples have been collected, potential PGPR's need to be isolated and selected for further studies. Due to the large number of bacteria present in the soil and rhizosphere an appropriate procedure for isolating and selecting isolates for further screening needs to be followed. General or selective isolation and selection procedures can be followed. For general isolations non-selective microbiological media is used and a representative pool of colonies is selected. Alternatively selective media and selective isolation can be used to choose isolates with specific characteristics and MOA.

The media used for isolating is important since all media is somewhat selective. Even the media generally considered to be non-selective is selective to some degree, since slow growing microbes are excluded through competition and microbes with specific requirements are not detected. The isolation strategy can also focus on specific groups of bacteria. For instance Pseudomonads are often targeted and King's B media used for their isolation (Das 2008). Bacilli are also routinely selected through treating the sample with heat to kill all non-spore forming isolates before isolation (Cavagleti *et al.* 2004, Liu *et al.* 2009, Kim *et al.* 1997). Other specialised media is also available to select microbes with specific modes of action, such as the chrome-azurol-S agar used for siderophore detection, and Nitrogen-Free media for the isolation of nitrogen fixing isolates.

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The importance of the media type and niche (root region) isolated from is illustrated by Barnett (1998). In his work the suppression of take all of wheat by Pseudomonads was investigated. It was found that healthy roots, and not lesions, selected for a larger population of take all suppressive bacteria. Furthermore the effective biocontrol isolates were not fluorescent and therefore not recovered on Tryptic soy agar, traditionally the agar of choice for isolating biocontrol pseudomonad strains. This suggests that previous studies may have overemphasised the role of the fluorescent pseudomonads in control of take-all. Also special attention should be given to the niche from which isolations are made. Barnett showed that the culture conditions, namely the media and incubation conditions, selected variant phenotypes, with regards to colony morphology. These variant colonies had different biocontrol abilities. Johansson and Wright (2003) also found that specific colony characteristics can be used to select effective isolates. This conclusion was based on a screening of over 400 isolates. Pseudomonads which had crystal like inclusions generally had excellent biocontrol activities. During isolation other factors that may impose selectivity on the isolation should also be taken into account. These factors include the incubation conditions, for example selecting for cold tolerant isolates by incubation at low temperatures (Kim *et al.* 1997).

2.4.3.3 Screening

Once the rhizosphere isolates have been isolated and selected, screening commences. The aim of screening is to determine which isolates have the most potential as PGPR's. Conventionally screening can be divided into three types, *in vitro* screening, greenhouse screening and field trials. The aim of screening is to systematically determine which isolate have the most potential.

Traditionally *in vitro* screening has been used to select isolates for greenhouse or field assessment. These laboratory assays include screening for well known modes of action such as mycolitic enzymes, production of plant hormones, inhibition of pathogens (by dual culture tests) and more recently screening for genes known to be involved in growth promotion or biocontrol. The advantage of this type of screen is that clear, visible and quantifiable results are generated by screens that are relatively quick and easy to perform. Many effective isolates have been obtained using this approach (Das *et al.* 2008).

Recently the usefulness of these *in vitro* methods has been questioned by many researches. This is due to the inconsistency between *in vitro* and *in vivo* results as well as the possibility of missing possible PGPR's due to the selectivity of the approach. This is especially true for selection of effective biological control antagonists. There are many reports that highlight this concern including the work of Sari *et al.* (2006) and Milus and Rothrock (1997).Their research showed that though antibiosis was a mode of action, in some instances there is no

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relationship between levels of inhibition, measured by inhibition zones, and efficacy in the field. Furthermore, the growth media used for the *in vitro* screens also affected the isolate ratings.

There are several factors that can contribute to the inconsistency between laboratory results and the results in the greenhouse or field. Reasons include the variable production of the antibiotic compounds in the soil. This can be due to different conditions between the agar and soil environments with regards to parameter such as temperature, nutrient availability and competition. As a result the bacteria produce different metabolites in the two environments. Also the antibiotics can be absorbed by the soil particles (Barker and Cook 1974; Campbell 1989).

An important concern regarding the use of *in vitro* screens is that highly effective PGPR isolates can be excluded. Exclusion of effective isolates occurs because these methods are selective and do not take into account all the MOA that exist. Agar based screens select for a particular mode of action, such as the dual-culture assay that selects for the production of antibiotic compounds or selection for specific mycolitic enzymes or particular plant growth hormones. Potential antagonists are excluded in molecular based screens because they select for a specific gene or genes and do not include those that have not yet been implicated with PGPR MOA. Non-related genes coding for new or different modes of biocontrol are therefore excluded (Barker and Cook 1974; Campbell 1989).

Though *in vitro* screens have limitations they can still be very useful and there are numerous ways to improve the chances of selecting effective isolates using these assays. The assays that are used for screening should be selected and designed with their limitations in mind. Some of the strategies employed to make the assays more representative include adjusting the media and incubation conditions to be more representative (Das *et al.* 2008). New assays are also being developed that incorporate the host plant and pathogen (for biocontrol) (Khan *et al.* 2006).

Greenhouse and field trials are essential to biocontrol screening and are the best way to select effective PGPR isolates. Greenhouse trials are usually more representative of the results that are obtained in field trials than *in vito* trials. Not all greenhouse trials are however successful and many result in insignificant field results (Johnson *et al.* 2003, Cook *et al.* 2002). There are many reasons for inconsistent results in the greenhouse and field. Several factors influence the PGPR activities and their effect on plant growth. These include formulation and delivery, plant type, pathogen presence and other influences in the environment.

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Isolate production and formulation is an important facet in the process of screening and utilising PGPR's. The outcome of a treatment depends on the method of inoculation/application, the antagonist's physiological state, the concentration and dosage, the presence or absence of nutrients, and the presence or absence of adjuvants such as adhering or protective agents (Knudsen 1997). The plant type and even cultivar of the same species influence the PGPR activity. PGPR can exhibit host genotype and/or tissue specificity (Khan *et al.* 2006). The presence of pathogens and level of pathogen infestation also influences both growth promotion and biocontrol. For growth promotion it was found that pathogens such as *Rhizoctonia* can inhibit the growth promotion effect because the root tips are severely damaged. This root tip damage results in a decreased number of receptors for the growth enhancing molecules produced by the PGPR inoculants (Ryder *et al.* 1999). In the case of biocontrol, the level of pathogen infestation or the occurrence of other pathogens and disease replacement, where one root disease is controlled but another takes its' place also influences the perceived results (Kim *et al.* 1997). Other factors that may also be important are mostly related to the effect of the environment on the isolate function and survival. These factors include the soil microbial composition, temperature, soil properties, cultivation and tillage practices.

2.4.3.4 Characterisation

Characterisation of PGPR involves determining the isolate characteristics which are of relevance to the growth promotion/ biocontrol effect. These include determination of the MOA (Okubara *et al*. 2008), growth characteristics of the isolates, influence on other microbes (Scherwinski *et al*. 2008), and factors affecting formulation and delivery. This occurs, to varying degrees, throughout the process of assessing PGPR isolates. During isolate screening characterisation is used as a method of selecting isolates of interest, for instance those that grow at a specific temperature (Johansson and Wright 2003), those that produce spores (Sadfi *et al.* 2001), or exhibit specific MOA (Ahmad *et al.* 2008).

Usually intensive characterisation of the isolates takes place in the final stages of selection and is only done with the few isolates that show the most potential. Isolates are characterised to choose those that are commercially viable and are suited to the particular requirements where they will be used (Kohl *et al*. 2011). For non-commercial research isolates are characterised to broaden the knowledge of growth promotion and biocontrol and deepen the understanding of the interaction leading to improved plant growth. Such knowledge enables the improvement of the PGPR application (Avis *et al.* 2008).

Characterisation of PGPR can be divided into two main areas namely, determination of the MOA whereby plant growth promotion is achieved and the isolate ecology. MOA studies are usually done in two phases, a preliminary assessment to help select isolates (Kim *et al.* 2008) and a detailed study of the isolate MOA with the aim of understanding the interaction (Ugoji *et*

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al. 2008). With regards to the isolate ecology the focus is on factors that influence the isolate efficacy and activity (Avis *et al.* 2008). The degree to which an isolate is studied is determined by the aims of the study. General characteristics that are determined include the isolate identity, basic metabolism and traits and growth requirements. Nakkeeran *et al*. (2005) mentions eight characteristics of a successful PGPR. These are: 1) a high rhizosphere competence, 2) a saprophyte that is highly competitive, 3) easy to multiply in bulk, 4) having a broad spectrum of activity, 5) having a reliable effect, 6) not a risk to humans or the environment, 7) compatibility with other rhizobacteria, 8) tolerant to adverse environmental factors such as UV radiation, heat, desiccation, and oxidising agents.

The aim of characterisation is therefore to increase the knowledge of the PGPR agent as well as factors affecting the interaction, so that the PGPR can be effectively implemented and used to its full potential. In the following section the factors that affect PGPR activity are discussed.

2.4.4 Factors affecting PGPR activity

One of the main hurdles to the widespread use of PGPR in commercial agriculture is the fact that they tend to give inconsistent results (Bashan, 1998).This inconsistency is related to PGPR being living organisms in contrast to traditional chemical products. PGPR and their activities are influenced by several factors. Larkin *et al.* (1998) identified five main areas of knowledge necessary to develop and implement effective biocontrol. These concepts can be applied to PGPR in general. The areas that need to be researched are 1) to identify requirements for specific MOA to function optimally, 2) the use of mixtures of PGPR with several MOA, 3) the dynamics between the host plant and microbial communities, 4) integration of PGPR with other agricultural practices and 5) improving formulation and delivery systems. Additional points that need to be considered are the factors that affect colonisation, survival and persistence of the introduced PGPR and the interaction of the PGPR with resident microbes including pathogens.

2.4.4.1 Identification of traits, conditions and requirements necessary for colonisation, persistence and survival of plant growth promoting rhizobacteria

Effective root colonisation is generally considered an important trait of a successful PGPR. In this regard, poor field performance of antagonists is usually attributed to poor rhizosphere competence. Rhizosphere competence comprises of effective root colonisation combined with the ability to survive and proliferate along growing roots over a considerable time period, in the presence of indigenous microflora (Compant *et al.* 2005). The importance of colonization

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efficacy is related to the MOA. For example if the MOA is antibiotic production, the antagonist needs to be a very effective coloniser to ensure that the antibiotic is spread along the whole root. Effective colonisation is even more important where competition for niches and nutrients is the MOA. When ISR or plant hormone production is the MOA, fewer PGPR are required to produce the effect, a less efficient coloniser will therefore still be effective. To improve the efficacy of biocontrol agents, understanding of the root-microbe communication, as affected by the genetic and environmental determinants in a spatial and temporal context needs to be gained (Chin-A-Woeng 2003; Benizri *et al.* 2001).

The contribution of effective root colonisation to successful PGPR activity is indicated in the following examples. Streptomycetes that were effective root colonisers were able to produce a greater effect than isolates that produced larger amounts of plant growth inducer molecules such as IAA (El-Tariby 2008). With regard to biocontrol Piliego *et al.* (2008) showed that difference in the root colonisation ability and strategy of two Pseudomonas strains affected their ability to protect avocado roots against white rot (*Rosellinia necatrix*). Colonisation ability and level of persistence in the rhizosphere is related to consistent results. One of the most consistent inhibitors of Pythium root rot of wheat in the growth chamber and field was also among the best colonisers of wheat rhizosphere. These results demonstrated that there is a positive relationship between the population size of *Pseudomonas fluorescens* on seminal roots of wheat and suppression of root lesions caused by *Gaeumannomyces graminis* var *tritici* during the early phase of pathogenesis. Poor colonisation ability can therefore be the reason for failure of introduced PGPR to control diseases (Milus and Rothrock, 1997).

Typically root colonisation is very variable and in general introduced microbes only have a temporary effect on the microbial composition of the rhizosphere (Milus and Rothrock, 1997, Bull *et al.* 1991). Root colonisation can be divided into three stages namely attachment, dispersion and multiplication, and survival. During each stage the PGPR properties, host plant properties, biotic factors and abiotic factors have an impact on the root colonisation process. Traits of the PGPR inoculant that are important to root colonisation include the efficiency of nutrient uptake and use, chemotaxis and ability to move around, attachment to plant surfaces, the ability to utilise plant exudates, production of vitamins and amino-acids, osmotolerance, growth rate and resistance to predators. Root colonisation is also influenced by factors such as the host plant cultivar, the composition of root exudates, soil properties and conditions and water percolation (Benizri *et al.* 2001; Chin-A-Woeng 2003).

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2.4.4.2 *Identification of traits, conditions and requirements necessary for optimal performance in terms of specific biocontrol mechanisms and interactions*

Even if a PGPR is present on the roots this does not assure the efficacy of the inoculant. There are several factors that influence the PGPR actions and activity and the efficacy of the MOA. PGPR metabolic behaviour and secondary metabolite production is influenced by a large range of ecological factors and signals. An excess of iron, for instance, suppresses the production of siderophores, and several nutrients and quorum sensing molecules affect the levels of antibiotics produced. Other factors that influence gene expression include soil properties, nutrient status, oxygen status, soil pH, soil moisture and temperature and pollutants (Verma *et al.* 2007; Pielach *et al.* 2008; Saleh-Lakha *et al.* 2005).

The rhizosphere community is influenced by plant age, fertilizer input, and inoculant type (Roetsi et. al 2006). The change in the pH of the soil was shown to influence the suppression of *Fusarium* wilt of carnations by antagonistic inoculants. Other factors that also influence antagonism are clay types, mineral-ion content, humidity, temperature and fertilizer inputs (Hass and Defago 2005). Currently researchers are starting to focus on the activities of PGPR in the rhizosphere in order to gain a better understanding of how PGPR function in the environment. An example of such a study is the quantification of the antibiotics produced by *Bacillus subtilis* in the rhizosphere (Kinsella *et al.* 2009). Such studies will allow a better evaluation of the biocontrol MOA and application.

In conclusion the ecophysiological parameters need to be taken into account and researched more in depth to enable the effective use of PGPR inoculants. Regardless of the mode of action the microbe is useless if it does not survive or express the required characteristics in the soil (Avis *et al.* 2008).

2.4.4.3 *Influence of host plant on PGPR and vice versa*

Plants vary in their ability to attract and support antagonistic populations. This variation has been observed among different plant species as well as cultivars (Larkin *et al.* 1998). Plants all vary in their abilities to support and respond to beneficial microorganisms. This variation exists between different species and different cultivars within species. The host plant influence the PGPR activity directly by affecting the microbe population on the roots or indirectly by its response to the PGPR or through influencing the PGPR activities. Plants can increase biocontrol by producing root exudates that support the growth of the biocontrol agent, induce the expression of genes involved in biocontrol and enhance attachment of the agent to the infection sites (Handelsman & Stabb 1996).

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Biedrzycki and Bais (2009) showed that plants are able to selectively modify root excretions to change the microbial population in the rhizosphere. Studies with Arabidopsis have shown that the plants selectively secrete malic acid to recruit PGPR's (Rudrappa *et al.* 2008). The affinity of *Pseudomonas* to colonise maize and mugbean has also been related to the crop genotype (Singh *et al.* 2009). The host plant can also influence the production of active molecules by the PGPR. Levels of the antifungal molecule 2,4-diacetylphloroglucinol (2,4-DAPG) produced by Pseudomonads in the wheat rhizosphere are dependant on a host cultivar-bacterial strain interaction (Okubara and Bonsall 2008). Host plants also differ in their reaction to PGPR. All bean cultivars for instance do not respond in the same way to auxins produced by PGPR (Remas *et al.* 2008).These examples show the importance of understanding the PGPR-host interaction.

2.4.4.4 Resident microbe influence

In general PGPR's are not applied to sterile plants but to a system where a resident microbial population already exists. To utilise PGPR effectively and sustainably a detailed knowledge of the interrelationship between the PGPR added to the system and microbes already present in the soil should be gained (Naiman *et al.* 2009). The interrelationship has two components namely the effect that the resident microbes has on the PGPR and its activities and the effect that the PGPR exerts on the resident microbial balance.

The surrounding microbial community can influence the PGPR either positively or negatively. Positive interactions include for example stimulation of root colonisation by PGPR by the symbiotic fungus *Piriformospora indica* (Singh *et al.* 2009). Soluble carbon from arbuscular mycorrhizal fungi can also stimulate the production of antifungal compounds by Pseudomonads (Siasou *et al.* 2009). The resident microbial community can also negatively influence the inoculated PGPR. PGPR inoculation was more effective in soils poor in microorganisms than soils rich in microbes. This can be explained by the buffering effect that a diverse population has to invading species (Fliessbach *et al.* 2009). This is linked to the ability of introduced microbes to competitively displace the indigenous microbes on the root niche (Avis *et al.* 2008). The antibiotics produced by the PGPR's may also be degraded by the indigenous microbes. For example Soymeya and Akutsu (2009) found that there are bacteria in the phylloplane of tomato and cyclamen that can break down the antibiotic prodigiosin produced by *Serratia marcescens*. In this way the biocontrol activity of the *Serratia* isolate was reduced.

In the case of biocontrol various studies have shown that the presence of pathogens can have a pronounced effect on the biocontrol activity of antagonists. Pathogens can change the colonisation behaviour of bacteria. *Gaeumannomyces graminis and Rhizoctonia solani* were found to increase the numbers of *Pseudomonas* strains whereas *Pythium* spp. decreased the

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Pseudomonas populations on wheat roots. This occurrence was attributed to the leakage of nutrients from lesions caused by *G. graminis* and *R. solani*. In contrast *Pythium* does not usually cause lesions but reduces the root hairs which are important colonisation sites of PGPR inoculants (Mazzola and Cook 1991). Bacterial isolates are influenced by pathogens in differing ways as was shown with two *Bacillus* strains. In the presence of the wheat pathogen, *G. graminis,* the growth of one bacterial isolate was stimulated and the other inhibited (Maplesone and Campbell 1989). Plant pathogens are also able to influence the gene expression of PGPR. A study of the effect of *Gaeumannomyces graminis* on the biocontrol agent *Pseudomonas fluorescens* with regard to gene regulation of the bacterium showed that *G. graminis* increased the growth of the bacterium and induced several bacterial genes involved in mycelial colonisation (Barret *et al.* 2009).

PGPR can also have a large influence on the resident microbial population and there is an increased awareness of the effect introduced inoculants may have on the resident population. A special concern is the effect introduced isolates may have on the microbial community, structure, and function, especially the effect that antibiotic producing strains may create. (Castro-Sowinski *et al.* 2007; Felici *et al.* 2008). PGPR inoculations may have no lasting effect on the microbial populations (Scherwinsky *et al.* 2008) or the inoculants can change the rhizosphere microbial populations. This change can be beneficial or negative for the crops. Beneficial changes include enhancement of beneficial microbial symbioses (Vessey 2003), or altering the rhizosphere populations to have a greater disease suppressive or growth promotion effect (Halverson *et al.* 1993; Gilbert *et al.* 1993). Unfortunately PGPR can also negatively impact the rhizosphere populations, for example a biocontrol strain of *Bacillus* was found to reduce mycorrhizal formation on maize roots. Due to the possibility of such negative interactions PGPR compatibility with natural microflora should be taken into account when they are used (Xiao *et al.* 2008).

2.4.4.5 Use of multiple antagonists and multiple mechanisms of action

The use of mixtures of several PGPR strains is often mentioned as a way to improve the reliability of PGPR inoculation. As shown by natural disease suppression, which is usually due to a consortium of organisms, mixtures are more ecologically sound. The reason is that a single organism is not able to be active in all soil environments and at all times (Larkin *et al.* 1998; Rosas 2007). Combinations of growth promoting PGPR with different modes of action, including phosphate solubilisation and nitrogen fixation, were found to be more effective than single inoculants (Elkoca 2008). Co-inoculation of the diazotrophs *Azospirillum* and *Herbaspirillum* on sugar cane increased the root colonisation of the isolates (Oliveira *et al.* 2009). An interesting example is the co-inoculation of *Azospirillum* strains with *Rhizobium*. *Rhizobium* inoculants are widely used to fix nitrogen for in legumes. Inoculation of wheat with a mixture of *Azospirillum* and *Rhizobium* caused a greater growth promotion effect than either inoculant separately, and increased yield with up to 53% (Askary *et al.* 2009).

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Mixtures of PGPR also enhanced the efficacy of disease control. A three-way mixture of PGPR isolates resulted in better disease control of cucumber pathogens than applications of the isolates singly (Raupach and Kloepper 1998). An advantage of using biocontrol mixtures is also that they are able to control diverse diseases of several crops. Jetiyanon and Kloepper (2002) studied the ability of PGPR mixtures to induce systemic resistance in cayenne pepper, tomato, green kuang futsoi (*Brassica*) and cucumber, against bacterial wilt (*Ralstonia solanacearum*), anthracnose (*Colletotrichum gloesporioides*), damping off (*Rhizoctonia solani*) and cucumber mosaic virus respectively. Of the seven strains screened, only one isolate was effective against all the diseases. In contrast four antagonist combinations could successfully control all the diseases. Mixtures are not only limited to PGPR's. Other beneficial organism such as Rhizobia and mycorrhizal fungi can also be incorporated to improve disease control and growth promotion (Akhtar and Siddiqui 2007). Mixtures therefore can improve the efficacy of PGPR inoculation due to improved ecological adaptation, a larger range of activity and by controlling several pathogens (Spadaro and Gullino 2005).

2.4.4.6 Integration of PGPR with other strategies

PGPR can be integrated with other agricultural practices, both current and new (Brussard *et al.* 2007; Perez *et al.* 2008). An example is the proposal of "integrated nutrient management strategies". In such a strategy PGPR inoculants are incorporated to reduce the amount of chemical fertiliser used for crop production. In a study with tomatoes, plants that received 75% of the recommended fertiliser rate and were inoculated with PGPR gave the same yield as plants that received the recommended amount of fertiliser (Adesemoye *et al.* 2009). PGPR inoculants can also be integrated with other farming practices such as traditional crop rotations, green manuring and organic amendments (Campbell 1994; Markakis *et al.* 2008; Welbaum *et al.* 2004).

For disease control PGPR treatments can be combined with chemical disease control agents or practices such as soil solarisation (Spadaro 2005; Duffy 2000; Larkin *et al.* 1998). Principles learnt form PGPR inoculants can also be used to manage the soil microbial community as a whole to improve crop production and enhance the actions of naturally occurring PGPR's (Struz *et al.* 2003). Integration of PGPR with agricultural practices is important to ensure efficacy and consistency of the inoculants. Furthermore, integration with current agricultural production methods may help to improve the acceptance and widespread use of PGPR inoculants.

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2.4.4.7 Improved formulation and delivery systems

The efficacy and consistency of PGPR inoculants is greatly influenced by the formulation and delivery methods used. Formulation and delivery is important because the method of formulation can affect the isolate efficacy as well as the plant response (Bashan 1998). Various examples of the effect of application method and formulation on the PGPR efficacy are to be found in literature. *Bacillus subtilis* was more effective against *Sclerotium rolfsii* on chilli when the plants were treated with a root dip before transplanting than if the PGPR were applied as a seed coat or soil drench (Abeyssinghe 2009).In a trial with lettuce seedlings the germination was reduced by alginate and broth formulation of the isolates (Amer and Utkhede 2000). A comparison of alginate bead application of *Streptomyces* isolates to seed application showed that seed coating was more effective. A 90% reduction in disease was obtained for the seed inoculation compared to the alginate bead inoculation that only achieved 22 – 30% reduction in damping off of tomato (Sabaratnam and Traquair 2002). In contrast Rekha *et al.* (2007) found that alginate encapsulation of Pseudomonads and Bacilli was a feasible technique for application of antagonists in the rhizosphere. Some bacterial traits also make the isolate more suitable for easy formulation and storage. Bacilli, for instance, produce endospores and are therefore more suitable for large-scale production methods (Liu *et al.* 2009; Brannen and Kenney 1997; Kim *et al.* 1997).

Other factors regarding application of PGPR should also be taken into consideration. These aspects include the concentration of inoculant as well as the timing of inoculation. On wheat for example *Azospirillum* inoculants were found to be most effective when applied in the range of 10^5-10^6 cells/ml. Successive inculcations did not increase the efficacy. Timing of inoculation however was important for colonisation and effect on the plant. Treatments may therefore fail in the field where it is very difficult to optimise timing of application for all plants, resulting in suboptimal root colonisation and thereby also inefficacy (Bashan 1986). Knowledge of the precise MOA and bacterial actions are needed to effectively adjust timing of PGPR applications and management practices to ensure effective utilisation of the PGPR (Avis *et al.* 2008).

2.4.5 Conclusion and new developments

The focus of PGPR research is shifting from the discovery phase to in depth ecology, MOA and traits that affect their efficacy type of studies. Endophytes have been receiving more attention as PGPR's since they are less influenced by the surrounding environment and therefore survive better and are more consistent in their growth promotion actions. For biocontrol this characteristic is advantageous because the antagonist is able to inhibit the pathogen inside the plant (Ryan 2007; Compant *et al.* 2005; Liu *et al.* 2009; Gray *et al.* 2005).

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With regards to MOA there is a general increase in detailed studies of how the MOA function and discovery of novel genes and gene products involved in growth promotion. Such studies include the characterisation of the genes of the ACC-deaminase enzyme of new strains to increase the general understanding of bacterial interactions and to improve screening effectiveness (Govindasamy *et al.* 2008). Research is also more directed with a focus on topics such as determining the specific details of auxin production and export systems of molecules important for PGPR activity (Da Mota *et al.* 2008), and the effect of quorum sensing on the production of antibiosis related compounds (Muller *et al.* 2009).New tools, such as metagenomics, functional genomics and proteonomics are being employed to develop a precise understanding of the MOA (Avis *et al.* 2008; Leveau *et al.* 2007).

New concepts regarding PGPR are also being introduced. For instance, Jousset *et al.* (2008) suggest that biocontrol is understood incorrectly. They propose that the toxic metabolites produced by soil bacteria are not related to plant protection or niche competition, but rather function as a protectant against predators. Production of these metabolites can potentially shift the predator pressure to other bacteria competing for the same resources. The concept of how PGPR are used is also expanding from direct inoculation of isolates to more advanced systems such as a “microbial precision production system”, based on management of microbial communities to benefit plant health. In such a system probes and nanosensors that monitor the soil are coupled to a computerised system. It would then be possible to enhance the population of beneficial microbes through adjustment of factors such as the soil pH, water content, minerals, nutrients, PGPR and plant signal molecules and inoculant applications (Welbaum *et al.* 2004). Concepts such as this expand our understanding of PGPR in crop production systems and are helpful to utilise our knowledge of PGPR to its full potential.

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2.6 Tables

Table 2.1: A selection of plant growth promoting rhizobacteria (PGPR) reported in the literature

Name of PGPR	Crop benefiting	Note	Reference/s
Azospirillum brasilense, Rhizobium meliloti	Wheat	Co-inoculation of the two microbes increased grain yield up to 53 % and also shows increases in N, P and K content. Results varied with cultivar, strain and microbe combinations.	Askary *et al,* 2009
Azospirillum brasilense, Bradyrhizobium japonicum	Corn and soybean	Inoculants of both bacteria alone and in combination promoted seedling germination and growth in corn and soybean. Possible modes of action include indole 3-acetic acid, gibberellic acid and zeatin production.	Cassan *et al.* 2009
Azospirillum brasilense	Rice	*Azospirillum* isolates increased N-content and grain yield; Some strain variation was seen among the isolates.	Pedraza *et al.* 2009
Rhizobium, Bacillus subtilis, Bacillus megaterium	Chickpea	Inoculations with bacteria resulted in equal, or higher yields than fertilisation, a combination of the isolates resulted in the largest effect, Bacilli tend to increase nodulation by Rhizobia, moa N-fixation (B. subtilis) and P-solubilisation (B. megaterium)	Elcoa *et al.* 2008
Azospirillum amozonense	Rice	Inoculation with the *Azospirillum* strain increased yield and growth of rice, the MOA was shown to be nitrogen fixation (with isotope labelling).	Rodrigues *et al.* 2008.
Bacillus isolates	Sugar beet and barley	Strains of Bacillus with N-fixing or P-solubilising capabilities increased plant growth in both sugar beet and barley, mixtures of the strains showed the most pronounced growth promotion.	Sain *et al.* 2004
Pseudomonas fluorescens	Snow mould of wheat	MOA the control of Microconidium nivale (Fusarium nivale) and growth promotion	Amien *et al.* 2008

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Table 2.1: A selection of plant growth promoting rhizobacteria (PGPR) reported in the literature

Name of PGPR	Crop benefiting	Note	Reference/s
Acinetobacter, Pseudomonas, Staphylococcus, Bacillus, Enterobacter, Pantoae, and Alcaligenes	Wheat	Antagonistic control of *Fusarium oxysporum, F. culmorum, F. solani, Pythium ultimum, Alternaria alternate, Botrytis cinerea,* and *Phytophtora cryptogea*, and plant growth promotion	Egamberdieva *et al.* 2008
Pseudomonas fluorescens	Rye	*Fusarium culmorum*, Fe(III) chelating compounds (including siderophores)	Kurek and Jaroszuk-Scisel, 2003
Bacillus sp. (L324-92)	Wheat	*Gaeumannomyces graminis* var *tritici*, Rhizoctonia root rot, *R. solani* AG8, Pythium root rot, *Pythium irregulare* and *P. ultimum*.	Kim *et al.* 1997
Streptomyces griseus (2-A24 and 3) *Bacillus subtilis*	Wheat	Growth promotion	Merriman *et al.* 1974
Bacillus subtilis and *B. cereus*	Wheat	Take all (G. *gramins* var *tritici*) and Rhizoctonia root rot (R*hizoctonia solani* AG8), Growth promotion	Ryder *et al.* 1999
Bacillus spp, *Pseudomonas fluorescens*	Wheat	*Gaeumannomyces graminis*, *Rhizoctonia solani*, *Rhizoctonia oryzae*, and *Pythium ultimum*,	Cook *et al.* 2002
Bacillus subtilis (CE1)	Maize	*Fusarium verticilloides*	Cavaglieri *et al.* 2005
Pseudomonas chlororaphis	Sorghum	*Macrophomina phaseolina*, charcoal rot of sorghum, extracellular antibiotics, volatile production, siderophores, effective root colonisation	Das *et al.* 2008
Bacillus stearothermophilus, *B. cereus, B. licheniformis, B. circulans, Chromobacterium violaceum*	Sorghum	*Fusarium oxysporum, antibiotic production, chitinolytic enzymes, efficient root colonisation*	Idris *et al.* 2007

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Table 2.1: A selection of plant growth promoting rhizobacteria (PGPR) reported in the literature

Name of PGPR	Crop benefiting	Note	Reference/s
Bacillus cereus, Brevibacterium laterosporus, Pseudomonas fluorescens, Serratia marcescens	Sorghum	*Pythium ultimum, antibiotic production, siderophores, induction of systemic resistance*	Idris *et al.* 2008
Pseudomonas fluorescens (MKB 100 and MKB 249), *P. frederiksbergensis* (202), *Pseudomonas* spp. (MKB 158)	Wheat and barley	*Fusarium culmorum, induced resistance, antibiotic production,*	Khan *et al.* 2006
Bacillus amyloliquefaciens Microbacterium *oleovorans*	Maize	*Fusarium verticillioides*	Pereira, 2009
Bacillus subtilis (ME488)	Cucumber and pepper	*Fusarium oxysporum* f. sp. *cucumerinum Phytophtora capsici, Antibiotics, bacilysin, iturin, mersscidin*	Chung *et al.* 2008
Bacillus subtilis	Red pepper	*Phytophtora capsici, antibiotics, siderophores, HCN, IAA, phosphatase, ACC-deaminase*	Lee *et al.* 2008
Pseudomonas corugata Chryseobacterium *indologenes* Lysobacter *enzymogenes* Flavobacterium sp.	Pepper	*Phytophtora capsici*	Sang *et al.* 2008.
Bacillus subtilis (RB14-C)	Tomato	*Rhizoctonia solani, antibiotic, (iturin A)*	Szchech and Shoda, 2006

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Table 2.2: Some commercially available plant growth promoting rhizobacteria (PGPR)

Name of PGPR (isolate number)	Commercial use	Crop benefitting	Additional information	Reference/s
Bacillus subtilis (GB03)	Used as seed treatment for the control of a range of seedling fungal pathogens including *Fusarium* spp. *Pythium* spp. and *Rhizoctonia* sp.	Wide range including soybeans, peanuts, wheat, barley, leguminous food crops and , particularly, cotton	Commercially available as Kodiac® (Gustafson), Quantum 400® (Gustafson) , System 3® (Uniroyal), Rotor® (Applied Chemicals, Thailand), or Companion® (Growth Products)	Copping, 2001, Brannen and Kenney 1997
Bacillus subtilis (MBI 600)	Wilts, crown rot, root rot and other seed-borne diseases caused by *Fusarium, Aspergillus, Pythium* and *Rhizoctonia*. Also target as a foliar formulation for the control of *Botrytis* and mildew.	Soybeans, cotton, dry beans, barley, wheat, maize, peas and peanuts	Commercially available as Subtilex® (Formerly 'Epic®")(MicroBio), Integral® (MicroBio), and Stimulex® (Scotts)	Copping, 2001
Breviabacillus brevis	A wide range of pathogens, especially *Botrytis cinerea*, *Pythium* spp. and *Sphaerothecia fulginea*. Also under evaluation against other foliar, stem base and soil-borne pathogens.	Vegetable crops, under evaluation for use in cereals, potatoes and post-harvest disease control		Copping, 2001
Burkholderia cepacia (several strains)	Soil-colonizing fungal pathogens and nematodes.	Many outdoor crops, including alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetable crops and wheat. Also used to treat transplanted crops.	Commercially available as Deny® (CCT and Stine Microbial Products), Intercet® (Soil Technologies), and Blue Circle Liquid Biological Fungicide® (Stine Microbial Products).	Copping, 2001
Rhizosphere bacterial mixture, includes five Gram negative cocci, seven Gram-positive rods and three Gram negative rods. One of the	Growth promotion and antibiotic production	Wide range, including vegetable crops, maize and cotton	Commercially available as Vitazyme® (Vital Earth Resources)	Copping, 2001

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Table 2.2: Some commercially available plant growth promoting rhizobacteria (PGPR)

Name of PGPR (isolate number)	Commercial use	Crop benefitting	Additional information	Reference/s
Gram-negative rods is *Bacillus macerans*.				
Pseudomonas chlororaphis	Soil and seed-borne fungal pathogens.	Cereals (Cedamon) and glasshouse ornamentals, nursery crops and vegetable transplants (AtEze)	Commercially available as Cedmon® (BioAgri), AtEze® (Eco Soil Systems).	Copping, 2001 and Johansson and Wright, 2003b
Streptomyces lydicus		Greenhouse, nursery, turf	Available in the USA	McSpadden *et al.* 2002
Bacillus subtilis (GB03), other *B. subtilis, B. lichenformis, B. megaterium*	*Rhizoctonia, Pythium, Fusarium, Phytophtora*	Greenhouse and nursery	Available in the USA	McSpadden *et al.* 2002
Burkholderia cepacia	*Rhizoctonia solani, Fusarium, Pythium*	Maize, vegetables, cotton	Available in the USA	McSpadden *et al.* 2002
Bacillus subtilis (GB03)	*Rhizocotnia solani, Fusarium, Alternaria, Aspergillus*	Cotton, legumes	Available in the USA	McSpadden *et al.* 2002
Gliocladium catenulatum	Soil borne pathogens	Ornamentals, vegetables, tree crops	Available in the USA	McSpadden *et al.* 2002
Trichoderma harzianum Rifai strain (ERL-G2 (T-22)),	*Pythium, Rhizoctonia solani, Fusarium*,	Trees, shrubs, ornamentals, cabbage, cucumber, tomato	Available in the USA	McSpadden *et al.* 2002

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Table 2.2: Some commercially available plant growth promoting rhizobacteria (PGPR)

Name of PGPR (isolate number)	Commercial use	Crop benefitting	Additional information	Reference/s
Gliocladium virens (Trichoderma virens) (GL-21)	Root rot pathogens, esp. *Rhizoctonia solani, Pythium*	Greenhouse, nursery home crop and ornamentals	Available in the USA	McSpadden *et al.* 2002
Bacillus pumilus	Soil fungal pathogens	soybean	Available in the USA	McSpadden *et al.* 2002
3 *Bacillus* strains	Growth promotion and pest suppression especially Nematodes, *Fusarium oxysporum*	All crops	Available in the RSA, as Biostart®, Waterbac®, and Landbac® produced by Microbial solutions.	Microbial solutions, South Africa
Bacillus strain	Plant growth promoter and control of disease caused by fungi including Phytophtora*, Pythium, Rhizoctonia, Fusarium as well as nematodes.*	All crops	Available in the RSA as Dpress®, produced by Microbial solutions	Microbial solutions, South Africa
Bacterial inoculant	Opportunistic pathogens on roots or leaves of plants	All crops	Available in the RSA, as Defender® produced by Biological Control Products.	Biological Control Products, South Africa
Bacterial inoculant	Compete with pathogens for root space and nutrients	All crops	Available in the RSA as Bac-Up® and produced by Biological Control Products	Biological Control Products, South Africa

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CHAPTER 3

Assessment of rhizobacterial isolates for biological control of root and crown rot of wheat caused by *Fusarium* spp. and *Rhizoctonia solani*

Abstract

In recent years there has been a trend in agriculture to reduce the use of chemical plant disease control products and much focus has been placed on the development of biological control agents. Plant growth promoting rhizobacteria (PGPR) have shown potential in this regard and are widely investigated as a means to control soilborne disease. In the current work selected rhizobacterial isolates were assessed in the greenhouse for control of root disease of wheat seedlings caused by *Fusarium oxysporum*, *F. graminearum* and *Rhizoctonia solani*. The use of the seedling tray assay screening PGPR for control of these diseases was also evaluated. The results indicated that under test conditions, the seedling tray assay is not an effective method to screen rhizobacterial isolates for biological control of wheat pathogens. This can be attributed to inconsistent disease development in the seedling tray cells as a result of varying environmental factors such as soil moisture, which were difficult to control. Pot trials indicated that several PGPR isolates have the potential to be developed as disease control agents of root and crown rots of wheat caused by *F. oxysporum*, *F. graminearum* and *R. solani*. It was also shown that the inoculum dose and number of PGPR applications influence the efficacy of the PGPR treatments.

3.1 Introduction

Biological control is the "*use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be*" (Eilenberg 2001). This strategy of disease control has been proposed for control of soilborne diseases of plants more than 50 years ago (Hass and Defago, 2005). Investigation of naturally occurring suppressive soils has shown that a wide variety of fungi and bacteria contribute to biological control of soilborne pathogens (Weller *et al*. 2002). The use of these microbes is appealing because they can suppress disease without lasting effects on the microbial community and ecosystem (Emmert and Handelsman 1999).

Plant growth promoting rhizobacteria (PGPR) is the term given to bacteria found on the roots of plants whose presence enhances plant vitality through mechanisms including the biological control of soilborne pathogens (Alabouvette *et al.* 2006). Rhizosphere isolates from various genera such as the *Pseudomonas*, *Bacillus* and *Streptomyces* have been identified as

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promising biocontrol agents of soilborne diseases. Some strains have proven effective in the field and in commercial greenhouses (Rosas 2007).

There are several reports where PGPR have been evaluated for the control of root diseases of wheat. These include using *Bacillus* spp to control take all caused by *Gaeumannomyces graminis* var *tritici*, Rhizoctonia root rot and Pythium root rot (Kim *et al* 1997; Ryder *et al.* 1999). *Pseudomonas* spp. have also been implicated in the development of suppressive soils against take all of wheat (Barnett *et al.* 1998), and *Pseudomonas fluorescens* controlled take all and root rot caused by *Rhizoctonia solani*, *R oryzae*, and *Pythium ultimum* (Cook *et al.* 2002).

There are several challenges associated with developing PGPR as biocontrol agents. One of these is to select a PGPR that would be effective in field conditions, reliable and commercially viable. Greenhouse disease control trials are generally considered the best way to select effective biocontrol isolates (Campbell, 1989; Shoda, 2000). Plant related assays are however lengthy and require a large amount of space (Das *et al*. 2008). To overcome these constraints several researchers have screened the PGPR isolates in smaller containers such as test tubes or seedling trays as an alternative to the larger pot trials.

Examples of tube or tray assays include the test tube assay developed by Handelsman *et al.* 1990. The assay was developed with the aim of providing a “rapid bioassay” to identify antagonists for the control of Phytophtora damping off of alfalfa. Development criteria were that the assay should be rapid and simple while having some relevance to natural infection. The test was considered to be better than *in vitro* tests that may have no relationship to natural conditions since the field conditions could be approximated in the assay. Specifically wheat tube assays were used to determine the pathogenicity of several fungi (Carter *et al.* 1999), and evaluate bacteria for the control of root rot caused by pathogens including *Rhizoctonia* (Kim *et al.* 1997; Milus and Rothrock 1997). Other applications where the concept was applied include determining the ability of bacterial inoculants to colonise roots (Shivanna *et al.* 1996; Duffy *et a.* 2000) and promote plant growth (Adhikari *et al.* 2001).

The aim of the work reported in this chapter was to evaluate the efficacy of selected isolates from the University of Pretoria’s PGPR culture collection (UP-PGPR) for control of root disease caused by *F. oxysporum*, *F. graminearum* and *R. solani* on wheat and determine whether the seedling tray assay is a reliable method for screening of bacterial isolates against these diseases.

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3.2 Materials and Methods

3.2.1 Cultures

All bacterial cultures were obtained from the UP-PGPR culture collection. The isolates were originally part of a PhD study by Hassen (2007). Appendix 1 gives further details regarding the isolate selection, maintenance and inoculum preparation.

The fungal cultures *F. oxysporum* UPGH 132, *F. graminearum* WP4F and *R. solani* UPGH 122 were maintained on half strength PDA (Biolab, Wadeville, South Africa). Appendix 2 gives details regarding culture origin, maintenance and millet seed inoculum preparation.

3.2.2 Host and greenhouse conditions

Wheat seedlings, variety SST822 (Sensako) were used throughout the trials and prepared, germinated, grown and harvested as described in appendix 3. In short, the seeds were surface sterilised, germinated in sterile vermiculite and transferred to seedling trays or pots containing soil. Greenhouse temperatures were maintained at 16 - 25℃ for all trials.

3.2.3 Evaluation of the seedling tray assay as a method to screen PGPR isolates for biocontrol of disease on wheat caused by Fusarium and Rhizoctonia

Two seedling tray trials were conducted with *Rhizoctonia solani* and *Fusarium oxysporum* respectively to confirm that the fungal isolates caused disease and determine the best inoculum dose for further biocontrol trials in seedling trays. The first trial received daily watering and the second received water every second day. In both trials millet inoculum was incorporated into steam pasteurised soil using two methods. Different concentrations, from 10 to 50g millet inoculum per litre soil (tables 2 and 3) were added to the soil followed by thorough mixing. Polystyrene seedling trays (comprising 40mm x 40mm cells) were then filled with the infected soil and the seedlings transferred to the trays (one seedling per cell). Alternatively the seedlings were transplanted into seedling tray cells containing clean soil and millet seed inoculum was placed at the base of the seedling stem. The plants were harvested and assessed four weeks after transplanting into the seedling trays.

Based on visual root rot symptoms and decrease in plant mass, the inoculum level of 40g millet seed treatment per litre soil was selected for further biocontrol trials in seedling trays. A total of 31 bacterial isolates were screened in two trials in seedling trays. In the first trial 15 of the 31 isolates were evaluated for biocontrol activity against *Rhizoctonia solani*. In the second

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trial the remaining 16 isolates were assessed for biocontrol of both *R. solani* and *Fusarium oxysporum*.

Ten days old seedlings were transferred to seedling trays containing sandy-loam soil augmented with millet seed inoculum of the pathogen. Directly after transplanting the seedlings were treated with 5 ml of a single bacterial suspension (10^8 cfu/ml). The bacterial treatment was repeated thrice at weekly intervals. Controls included were; 1) the untreated healthy control, 2) the second healthy control in which the soil was inoculated with sterile millet and treated with sterile Ringers solution, and 3) the pathogen inoculated control in which plants were inoculated with millet seed inoculum of the pathogen. The trials were harvested and assessed four weeks after the seedlings were transplanted into the seedling trays.

3.2.4 Pot trials for assessment of biological control activity of PGPR isolates

Bacterial isolates were tested for biocontrol of *F. oxysporum* UPGH134 and *R. solani* UPGH122. Surface sterilised wheat seeds were germinated in seedling trays containing sterile vermiculite. One week after planting the seeds the seedlings were given a bacterial pre-treatment of 5ml bacterial suspension per seedling tray cell. One week after the bacterial pre-treatment (two weeks after planting the seeds) the seedlings were transplanted into 12 cm by 10.5cm diameter plastic pots containing steam pasteurised sandy loam soil augmented with millet seed inoculum of either *R. solani* UPGH122 (20g millet/kg soil) or *F. oxysporum* UPGH 132 (30g millet /kg soil). The seedlings were drenched with sterile water after transplanting. Directly after watering the bacterial isolates (10^8cfu/ml) were applied at a rate of 50ml per pot as a drench treatment. The bacterial inoculation was repeated three times a week apart. Due to the loss of virulence of the *R. solani* and *F. oxysporum* only 16 of the 31 bacterial isolates were screened against these pathogens.

Thirty one bacteria isolates, including the previously tested 16, were tested for biocontrol of Fusarium *graminearum* (UP isolate WP4F). The following changes to the above mentioned protocol were made. After germination the wheat seedlings were transferred to 500ml plastic pots, five seedlings per pot. The rhizobacteria were applied directly after transplanting as a 25ml soil drench per pot. Plants were harvested four weeks after being transplanted.

The treatments for each pathogen were as follows, 1) plants inoculated with the pathogen alone, 2) plants inoculated with the pathogen and single bacterial isolate, 3) plants that were not inoculated with the pathogen or bacteria. As previously described the optimal millet seed dose of the pathogens was determined in the greenhouse prior to the biocontrol trials. Plants

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were harvested four weeks after transplanting and their fresh and dry mass recorded. The incidence of pathogen in the roots was also determined as follows: five root segments (about 7mm long) from each replicate were removed. For *Rhizoctonia the* root segments were placed on water agar plates and incubated at 25℃. After 24 hours the cultures were examined using a light microscope. For *Fusarium* the root segments were plated onto rose-bengal-glycerol-urea (RBGU) medium selective for *Fusarium*. The RBGU plates were incubated for about a week and then the resultant colonies counted. The RBGU media was prepared by dissolving 10ml glycerol, 1.0 g urea, 0.5g L-alanine, 1.0g PCNB, 0,25g chloramphenicol, 0.5g rose-bengal in a small amount of ethanol. This mixture was then added to 200ml sterile distilled water and then to 800ml prepared sterile bacteriological agar (12 g bacteriological agar in 800 ml water) (Hassen *et al.* 2007 as modified from Van Wyk *et al*. 1985).

3.2.5 Statistical analysis

Treatments for the seedling tray trials were arranged according to a completely randomised (block less) design. A treatment unit consisted of three adjacent cells, each cell containing one plant and each treatment was replicated thrice. For the pot trials the treatments were arranged in a randomised block design with four replicate pots per treatment. Each experimental unit consisted of one pot with five wheat seedlings, and treatments were replicated four times. Data was subjected to analysis of variance (ANOVA) using SAS-9.2 software. Means values in each treatment were compared using the least significant difference (LSD) test at 5% (p = 0.05) level of significance.

3.3 Results

3.3.1 Pathogenicity seedling tray trials with *Rhizoctonia solani*

Results for the seedling tray assays are given in tables 3.1 and 3.2. For the *Rhizoctonia solani* inoculum dose assay in seedling trays where the seedlings received daily watering no significant decrease in fresh or dry root mass was caused recorded. In the treatment where two pathogen infected millet seeds were placed at the base of the seedlings a growth promotion effect of 46% and 67% was observed in terms of fresh and dry root mass respectively. For all the doses of inoculum both the fresh and dry foliage mass showed a trend of decreasing mass. The inoculum doses of 15g, 25g, 30g, 35g, 40g, and 50g millet inoculum per litre soil caused a significant decrease in dry seedling foliage mass. The 50g millet inoculum per litre soil resulted in the most disease and caused a 44% reduction in dry foliage.

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In the trial where seedlings only received water every second day some treatments caused a significant increase in the fresh root mass. The four treatments comprising the highest doses of millet inoculum (40g, 50g, 75g and 100g millet per litre soil), significantly decreased the fresh foliage mass of wheat seedlings. These treatments as well as the treatments comprising 10 infected seeds and 2g millet seed placed at the plant base respectively significantly reduced dry foliage mass of the seedlings. The treatment where 30g millet inoculum was applied was the only treatment that caused a decrease in dry root mass. Some plants had stunted roots with brown lesions and spear tips. Sclerotia were also observed on the roots, especially at the higher doses of millet inoculum.

3.3.2 Pathogenicity seedling tray trials with *Fusarium oxysporum*

In the seedling tray assay to determine the effect of different levels of *Fusarium oxysporum* inoculum on the growth of wheat seedlings that received daily watering, no treatments significantly decreased the fresh or dry foliage mass (table 3.3). However all the doses, except 10g millet per litre soil, caused a significant decrease in dry root mass. The greatest decrease in root mass was observed with the treatments where one or two millet seeds were placed at the plant base.

Where the seedlings received water only every second day, two of the treatments caused a significant reduction of fresh root mass. The treatments were 2g millet seed inoculum applied per plant and 100g millet seed inoculum applied per litre of soil. The results in terms of dry mass are indicted in table 3.4. Several doses of millet seed inoculum decreased the dry foliage mass. The treatment where 30g millet seed inoculum was mixed into a litre of soil caused the largest decrease in both root and foliage mass (table 3.4). In both trials some treatments resulted in discoloured lesions on the roots and blackened crowns. These symptoms were however not consistent or clear enough to use in a disease assessment key.

3.3.3 Biocontrol screening against Fusarium oxysporum and Rhizoctonia solani in seedling trays

Results for the biocontrol trials conducted in seedling trays are shown in tables 4.1 and 4.2. The results were inconclusive since the pathogen inoculated disease controls were not sufficiently diseased at the end of the experiment. In the first trial, with only *Rhizoctonia*, the dry root mass of the pathogen inoculated control plants was 53% greater than that of the sterile millet control plants (calculated % according to the formula [treatment – disease control)/ disease control x100]). In the second trial with both *Rhizoctonia* and *Fusarium* the disease control and sterile millet controls did not differ significantly. Due to the latter result the effect of the bacterial inoculants on disease could not be appropriately assessed.

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Based on dry mass of plants that were inoculated with bacteria compared to those of the pathogen inoculated controls no improved growth was seen in either the first or second trial with *Rhizoctonia*. In the second trial some bacterial treated plants showed a decreased mass when compared to the pathogen inoculated control. For the trial with *Fusarium*, some bacterial treatments such as T01 showed a significant improvement of mass when compared to the disease control (table 4.3)

3.3.4 Screening of rhizobacterial isolates for biocontrol of Fusarium oxysporum, Rhizoctonia solani and Fusarium graminearum in pot trials.

The results of the greenhouse pot experiments in which the PGPR isolates were tested for their ability to control *Fusarium oxysporum* are shown in table 4.4. In terms of root mass no difference was observed between the healthy and pathogen inoculated control. The isolates T15, T28 and T30 however significantly increased root growth when compared to the uninoculated millet control. *Fusarium* was isolated on RBGU from all the treatments except the pathogen free control. There was a large significant difference between the average shoot mass of the pathogen inoculated and uninoculated experimental controls. All the isolates, except T16, improved the shoot mass of plants compared to the disease control. Isolates T10, T11, T22, T26, T28, T29 and T30 were the most effective in increasing shoot mass of plants.

Of the 16 bacterial isolates tested in the greenhouse for biocontrol of *Rhizoctonia solani* several significantly improved plant mass compared to the diseased control (table 4.5). All the isolates except T08 and T09 significantly improved dry shoot mass of plants. The isolates T16, T22 and T26 were the most effective and in comparison with the pathogen inoculated control caused an increase in dry shoot mass of 172%, 200% and 173% respectively.

Results of the assessment of biocontrol ability of rhizobacterial isolates against *F. graminearum* WP4F is shown in table 4.6 and figure 4.7. The dry root mass data showed that only three isolates, namely T23, T24, and T31, significantly improved dry root mass compared to the pathogen inoculated control. Four isolates namely, T17, T18, T27 and T31, caused a significant increase in dry shoot mass ranging from 31 to 44%.

3.4 Discussion

The ability of PGPR isolates from the UP-culture collection was evaluated for their ability to control root and crown rot of wheat caused by *Fusarium oxysporum*, *F. graminearum* and *Rhizoctonia solani*. The results indicate that the seedling tray trial is not an effective method to screen PGPR for control of *F. oxysporum* and *R. solani.* In the pot trials several PGPR

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improved the growth of wheat plants in the presence *of F. oxysporum, F. graminearum* and *R. solani* demonstrating effective biocontrol of these pathogens.

In the trails with Rhizoctonia and Fusarium in the greenhouse a large majority of the isolates improved plant growth. Some of the bacterial treatments resulted in increased mass over and above that of the healthy (sterile millet) control. This occurrence has not often been reported in literature. Generally only a small portion of the bacterial treatments are expected to improve the growth of plants affected by disease (Berg, 2009), as was the case with the *F. graminearum* experiments in this work. The fact that the isolates were selected based on their growth promoting abilities on other crops (Hassen 2007) could explain why such a large percentage of the isolates were effective in the current study.

In the particular biocontrol trials where the PGPR isolates showed an exceptional growth promotion the bacterial isolates were applied several times. For the trials with *R. solani* and *F. oxysporum* seedlings received a bacterial application before transplanting to infected soil, and four additional bacterial applications in the pots. Various sources in literature show that the dose and repetition of inoculation greatly influences the efficacy of PGPR since the numbers of bacteria introduced into the soil usually decline rapidly (Milus and Rothrock, 1997; Bull *et al.* 1991; Georgakopoulos 2002). The additional bacterial applications therefore could contribute to replenish most of the cell numbers of those isolates that are ineffective root colonisers and competitors.

The isolate history could additionally contribute to the large portion of isolates that had biological control activity. As with chemical treatments, PGPR can have a wide or narrow range of pathogens which they inhibit (Weller *et al.* 2002). The isolates were selected based on good performance with regards to growth promotion and biocontrol on other crops including sorghum (Hassen 2007). Similar findings have been reported in literature. Research by Recep *et al.* (2009) showed that *Bacillus cepacia* strain OSU-7 was able to control dry storage rot caused by three different Fusarium spp. In addition this strain also inhibited other pathogenic fungi including quince brown rot and fruit and foliar rots of tomato. On wheat specifically Kim *et al.* (2007) reported a Bacillus strain that control *Gaeumannomyces graminis* var *tritici*, *Rhizoctonia* and *Pythium* in growth chambers. Johansson *et al.* (2003) also mentioned that they were able to obtain effective isolates for the control of Microdochium nivale (snow mould) in wheat field trials even though the isolates were selected by greenhouse screening against *Fusarium culmorum*.

The assay for screening the biocontrol activity of PGPR in seedling trays was shown to be ineffective because consistent development of disease was not observed for the trials that were conducted. During the development of the assay pathogenicity trials were conducted to establish the appropriate conditions for disease development. These initial results were in

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agreement with findings such as that of Kim *et al* (2008) where seedling trays were successfully utilised as a preliminary screen to evaluate the biocontrol ability of rhizobacteria isolates against Phytophtora blight of pepper. In the current study during subsequent seedling tray trials with the PGPR, under the same conditions, the pathogen inoculated control was not sufficiently diseased compared to the healthy plants. A large variation in the appearance and mass of plants within the same replicate, which consisted of three adjacent cells of the seedling tray containing one seedling each, was observed. A possible explanation for these results is the fact that the soil in the seedling tray cells dried at different rates even though they received the same amount of water. High humidity resulting from summer weather conditions (summer rainfall region) at the time during the second seedling tray trial, probably influenced the results since most plants only required watering once a week. These conditions could influence the disease expression since it is known that in general these pathogens require relatively dry soils to cause disease. Burnett *et al.* (2004) mentions similar problems encountered while assessing techniques to test the resistance of sugar beet to rhizoctonia root rot. In their trials a definite influence of the seasons on disease development was observed even though the greenhouse conditions were constant for all trials.

The characteristics that contribute to successful greenhouse screening are that the pathogen should not only infect the plants but symptoms should develop under the screening conditions. Symptoms should also develop reasonably quickly. The disease severity should be sufficiently high without significant variability between replicates and trials. The pathogen isolates should also maintain it's pathogenicity during culture since large amounts of inoculum must be prepared for the screening trials (Johansson *et al.* 2003).

The results presented here indicate that although the conditions most suitable for an appropriate level of disease development in the seedling trays were established in pilot trials, factors such as humidity, that are difficult to control greatly influence the outcome of the seedling tray screening. As such the seedling tray trial is not an effective screening method when the growing conditions can not be meticulously controlled to ensure conditions that favour disease development by these pathogens. With regards to the pot trials the results indicate that there are several PGPR isolates which are effective biocontrol agents for control of root and crown rot of wheat caused by Fusarium oxysporum, F. graminearum and *Rhizoctonia solani.* Important aspects to consider when screening and using the PGPR are the inoculum dose and timing of inoculation.

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3.5 Tables and figures

3.5.1 Pathogenicity trials in seedling trays

Table 3.1. The first assessment of *Rhizoctonia solani* pathogenicity on wheat seedlings planted in seedling trays

	Seedling mass in grams				% change in seedling mass*			
	Fresh mass**		Dry mass**		Wet mass**		Dry mass**	
Treatment	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated control	1.17 bc	0.70 bc	0.27 bcd	0.20 a	3 bc	-32 bc	6 bcd	0 a
Sterile millet control	1.13 bc	1.03 a	0.25 bcd	0.20 a	0 bc	0 a	0 bcd	0 a
One *R. solani* infected millet seed***	1.17 bc	0.93 ab	0.31 ab	0.20 a	3 bc	-10 ab	23 ab	-1 a
Two *R. solani* infected millet seeds***	1.65 a	0.85 abc	0.42 a	0.17 ab	46 a	-18 abc	67 a	-15 ab
10g *R. solani* inoculum****	1.17 bc	0.70 bc	0.28 bc	0.17 ab	3 bc	-32 bc	12 bc	-14 ab
15g *R. solani* inoculum****	1.27 ab	0.80 abc	0.27 bcd	0.16 bc	12 ab	-23 abc	8 bcd	-22 bc
20g *R. solani* inoculum****	0.90 bcd	0.80 abc	0.22 bcd	0.18 ab	-21 bcd	-23 abc	-12 bcd	-10 ab
25g *R. solani* inoculum****	0.77 cd	0.77 bc	0.22 bcd	0.16 b	-32 cd	-26 bc	-12 bcd	-19 b
30g *R. solani* inoculum****	0.87 bcd	0.83 abc	0.24 bcd	0.17 b	-24 bcd	-19 abc	-3 bcd	-17 b
35g *R. solani* inoculum****	0.90 bcd	0.67 c	0.19 cd	0.13 cd	-21 bcd	-35 c	-25 cd	-34 cd
40g *R. solani* inoculum****	0.50 d	0.73 bc	0.17 cd	0.12 d	-56 d	-29 bc	-34 cd	-38 d
50g *R. solani* inoculum****	0.83 cd	0.60 c	0.20 cd	0.11 d	-26 cd	-42 c	-22 cd	-44 d

Rhizoctonia solani UPGH 122 was obtained from the UP-PGPR culture collection.
*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure.
*** Treatment where one or two pathogen infected millet seeds were placed next to the crown of the seedlings. Untreated control = no inoculum or millet was added to the soil, Sterile millet control = sterile millet mixed evenly throughout the soil at a rate of 30g millet per litre soil; for all other treatments the grams millet inoculum of *Rhizoctonia solani* that was added per litre soil is indicated.
**** *R. solani* millet seed inoculum per litre soil

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Table 3.2. The second assessment of *Rhizoctonia solani* pathogenicity on wheat seedlings planted in seedling trays

	Seedlings mass in grams				% change in seedling mass*			
	Fresh mass **		Dry mass **		Fresh mass **		Dry mass **	
	Root	Shoot	Root w	Shoot	Root	Shoot	Root	Shoot
Untreated control	0.53 ab	0.70 ab	0.19 bc	0.18 ab	30 ab	12 ab	-3 bc	-3 ab
Sterile millet control	0.41 bc	0.62 abc	0.19 bc	0.18 a	0 bc	0 abc	0 bc	0 a
Two infected millet seeds***	0.62 a	0.60 a	0.17 bcd	0.16 ab	50 a	-3 a	-13 bcd	-12 ab
Five infected millet seeds***	0.64 a	0.55 abcd	0.20 ab	0.16 ab	55 a	-11 abcd	4 ab	-12 ab
Ten infected millet seeds***	0.53 ab	0.48 bcde	0.19 bc	0.12 bc	30 ab	-23 bcde	-1 bc	-32 bc
0.25 g/plant *R. solani* inoculum****	0.61 a	0.64 abc	0.20 b	0.15 ab	47 a	2 abc	2 b	-18 ab
0.5 g/plant *R. solani* inoculum****	0.60 a	0.58 abc	0.20 ab	0.15 ab	46 a	-7 abc	4 ab	-15 ab
1.0 g/plant *R. solani* inoculum****	0.53 ab	0.62 abcd	0.21 ab	0.15 ab	30 ab	-1 abcd	9 ab	-19 ab
1.5 g/plant *R. solani* inoculum****	0.48 ab	0.52 e cdde	0.18 bcd	0.13 abc	18 ab	-17 e cdde	-7 bcd	-30 abc
30 g/litre *R. solani* inoculum****	0.65 a	0.44 abc	0.27 a	0.12 bc	57 a	-29 abc	42 a	-34 bc
2 g/plant *R. solani* inoculum****	0.49 ab	0.57 def	0.17 bcd	0.12 bc	18 ab	-9 def	-9 bcd	-33 bc
40 g/litre *R. solani* inoculum****	0.44 ab	0.35 ef	0.11 de	0.09 dc	6 ab	-44 ef	-45 de	-52 dc
50 g/litre *R. solani* inoculum****	0.33 bc	0.32 def	0.12 cde	0.08 dc	-20 bc	-49 def	-38 cde	-53 dc
75 g/litre *R. solani* inoculum****	0.37 bc	0.33 f	0.11 de	0.07 dc	-10 bc	-47 f	-45 de	-61 dc
100 g/litre *R. solani* inoculum****	0.18 c	0.14	0.06 e	0.05 d	-57 c	-77	-70 e	-74 d

Rhizoctonia solani UPGH 122 was obtained from the UP-PGPR culture collection.
*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
** Values are means of three replications and means within columns followed by the same letters do not differ significantly (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure.
*** Treatment where two, five or ten pathogen infected millet seeds were placed next to the crown of the seedlings. Untreated control = no inoculum or millet was added to the soil. Sterile millet control = sterile millet mixed evenly throughout the soil at a rate of 30g millet per litre soil.
**** Treatments marked per litre indicate the grams millet inoculum of *Rhizoctonia solani* that was added per litre soil, and those marked per plant the grams millet applied at the base of each plant.

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Table 3.3.The first assessment of *Fusarium oxysporum* pathogenicity on wheat seedlings planted in seedling trays

	Seedling mass in grams				% change in mass*			
	Wet mass **		Dry mass **		Wet mass **		Dry mass **	
Treatment	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Untreated control	0.73 cd	0.67 bc	0.18 e	0.17 bc	-51 cd	-13 bc	-51 e	-12 bc
Sterile millet control	1.50 a	0.77 abc	0.36 a	0.19 abc	0 a	0 abc	0 a	0 abc
One *F. oxysporum* infected millet seed***	0.60 d	0.67 bc	0.18 de	0.16 c	-60 d	-13 bc	-50 de	-17 c
Two *F. oxysporum* infected millet seeds***	0.67 d	0.53 c	0.18 de	0.17 bc	-56 d	-30 c	-50 de	-15 bc
10g *F. oxysporum* inoculum******	1.33 a	0.67 bc	0.32 ab	0.16 c	-11 a	-13 bc	-12 ab	-16 c
15g *F. oxysporum* inoculum******	1.13 abc	0.87 ab	0.24 cde	0.19 abc	-24 abc	13 ab	-32 cde	-3 abc
20g *F. oxysporum* inoculum******	1.20 ab	0.80 ab	0.25 bcde	0.20 ab	-20 ab	4 ab	-31 bcde	4 ab
25g *F. oxysporum* inoculum******	0.67 d	0.83 ab	0.22 cde	0.19 abc	-56 d	9 ab	-39 cde	-3 abc
30g *F. oxysporum* inoculum******	0.60 d	0.83 ab	0.21 cde	0.18 abc	-60 d	9 ab	-42 cde	-9 abc
35g *F. oxysporum* inoculum******	0.83 bcd	0.87 ab	0.26 bcd	0.19 abc	-44 bcd	13 ab	-27 bcd	-1 abc
40g *F. oxysporum* inoculum******	0.83 bcd	0.80 ab	0.22 cde	0.18 abc	-44 bcd	4 ab	-39 cde	-5 abc
50g *F. oxysporum* inoculum******	0.87 bcd	0.93 a	0.27 bc	0.21 a	-42 bcd	22 a	-26 bc	8 a

Fusarium oxysporum UPGH 132 was obtained from the UP-PGPR culture collection.

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

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

*** Treatment where one or two pathogen infected millet seeds were placed next to the crown of the seedlings. Untreated control = no inoculum or millet was added to the soil, Sterile millet control = sterile millet mixed evenly throughout the soil at a rate of 30g millet per litre soil; for all other treatments the grams millet inoculum of *Rhizoctonia solani* that was added per litre soil is indicated.

****** *R. solani* millet seed inoculum per litre soil

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Table 3.4. The second assessment of *Fusarium oxysporum* pathogenicity on wheat seedlings planted in seedling trays

	Seedling mass in grams**				% change in mass*			
	Wet mass **		Dry mass **		Wet mass **		Dry mass **	
Treatment	Foliage	Root	Foliage	Root	Foliage	Root	Foliage	Root
Untreated control,	0.820 dcb	1.023 abc	0.183 bcd	0.257 abcd	-17 dcb	-7 abc	-18 bcd	-12 abcd
Sterile millet control	0.987 a	1.098 abc	0.222 a	0.292 abc	0 a	0 abc	0 a	0 abc
One *F. oxysporum* infected millet seed***	0.841 bcd	0.964 abc	0.184 bcd	0.252 abcd	-15 bcd	-12 abc	-17 bcd	-14 abcd
Two *F. oxysporum* infected millet seeds***	0.847 bc	0.816 bcde	0.186 bcd	0.237 bcde	-14 bc	-26 bcde	-16 bcd	-19 bcde
10g/ litre *F. oxysporum* inoculum****	0.780 bcde	0.899 abcd	0.178 bcd	0.245 abcde	-21 bcde	-18 abcd	-20 bcd	-16 abcde
15g/ litre *F. oxysporum* inoculum****	0.862 ab	1.137 ab	0.198 abc	0.261 abcd	-13 ab	4 ab	-11 abc	-10 abcd
20g/ litre *F. oxysporum* inoculum****	0.754 bcde	0.992 abc	0.174 bcd	0.302 ab	-24 bcde	-10 abc	-21 bcd	3 ab
25g/ litre *F. oxysporum* inoculum****	0.845 bc	0.789 bcde	0.187 bcd	0.193 de	-14 bc	-28 bcde	-16 bcd	-34 de
30g/ litre *F. oxysporum* inoculum****	0.830 bcd	1.242 a	0.201 ab	0.338 a	-16 bcd	13 a	-9 ab	16 a
35g/ litre *F. oxysporum* inoculum****	0.678 ef	0.737 cde	0.159 d	0.155 e	-31 ef	-33 cde	-28 d	-47 e
40g/ litre *F. oxysporum* inoculum****	0.719 cdef	0.530 de	0.198 abc	0.290 abc	-27 cdef	-52 de	-11 abc	-1 abc
50g/ litre *F. oxysporum* inoculum****	0.655 ef	0.874 abcd	0.167 d	0.202 cde	-34 ef	-20 abcd	-25 d	-31 cde
50 g/litre *F. oxysporum* inoculum****	0.713 def	0.776 bcde	0.177 bcd	0.171 de	-28 def	-29 bcde	-20 bcd	-41 de
75 g/litre *F. oxysporum* inoculum****	0.713 def	0.810 bcde	0.186 bcd	0.216 bcde	-28 def	-26 bcde	-16 bcd	-26 bcde
100 g/litre *F. oxysporum* inoculum****	0.597 f	0.482 e	0.170 dc	0.175 de	-40 f	-56 e	-23 dc	-40 de

Fusarium oxysporum UPGH 132 was obtained from the UP-PGPR culture collection.
*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Values are means of three replications and means within columns with the same letters do not differ significantly (P=0.05) according to the Least Significant Difference (LSD) test using the GLM procedure
*** Treatment where one or two pathogen infected millet seeds were placed next to the crown of the seedlings. Untreated control = no inoculum or millet was added to the soil. Sterile millet control = sterile millet mixed evenly throughout the soil at a rate of 30g millet per litre soil.
****Treatments marked per litre indicate the grams millet inoculum of *Fusarium oxysporum* that was added per litre soil, and those marked per plant the grams millet applied at the base of each plant.

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Table 3.5. Assessment of rhizobacterial isolates for biocontrol of *Rhizoctonia solani* UPGH 122 on wheat seedlings planted in seedling trays (Batch 1)

	Seedling mass in grams				% Change in mass*			
	Wet mass**		Dry mass**		Wet mass**		Dry mass**	
Treatment	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated control	0.22 a	0.60 a	0.17 ab	0.24 ab	-51 a	-16 a	-32 ab	29 ab
Sterile millet control	0.27 a	0.76 a	0.12 b	0.17 abc	-41 a	5 a	-53 b	-6 abc
Pathogen inoculated control***	0.45 a	0.72 a	0.25 a	0.19 abc	0 a	0 a	0 a	0 abc
T04 + pathogen***	0.17 a	0.72 a	0.09 b	0.15 bc	-62 a	-1 a	-65 b	-18 bc
T05 + pathogen***	0.17 a	0.57 a	0.11 b	0.15 bc	-63 a	-21 a	-54 b	-20 bc
T06 + pathogen***	0.16 a	0.77 a	0.06 b	0.14 c	-64 a	7 a	-77 b	-23 c
T07 + pathogen***	0.19 a	0.72 a	0.09 b	0.16 bc	-59 a	0 a	-66 b	-14 bc
T10 + pathogen***	0.26 a	0.66 a	0.13 b	0.16 bc	-42 a	-9 a	-49 b	-13 bc
T11 + pathogen***	0.28 a	0.66 a	0.15 ab	0.17 bc	-37 a	-9 a	-42 ab	-11 bc
T12 + pathogen***	0.28 a	0.76 a	0.14 ab	0.19 abc	-37 a	6 a	-46 ab	0 abc
T13 + pathogen***	0.17 a	0.61 a	0.07 b	0.14 bc	-62 a	-15 a	-73 b	-26 bc
T14 + pathogen***	0.21 a	0.66 a	0.10 b	0.16 bc	-52 a	-8 a	-61 b	-15 bc
T15 + pathogen***	0.29 a	0.62 a	0.13 b	0.13 c	-36 a	-14 a	-47 b	-32 c
T16 + pathogen***	0.22 a	0.70 a	0.09 b	0.12 c	-50 a	-3 a	-64 b	-35 c
T18 + pathogen***	0.24 a	0.66 a	0.16 ab	0.18 abc	-47 a	-9 a	-37 ab	-1 abc
T19 + pathogen***	0.26 a	0.73 a	0.14 ab	0.21 ab	-42 a	1 a	-44 ab	14 ab
T25 + pathogen***	0.33 a	0.86 a	0.12 b	0.16 bc	-26 a	19 a	-52 b	-12 bc
T26 + pathogen***	0.26 a	0.68 a	0.09 b	0.15 bc	-43 a	-6 a	-65 b	-20 bc

*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Means within columns followed by the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure
*** *R. solani* millet seed inoculum was added to the soil at 40 g millet per litre soil.

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Table 3.6 Assessment of rhizobacterial isolates for biocontrol of *Rhizoctonia solani* UPGH 122 in seedling trays (Batch 2)

	Seedling mass in grams				% Change in mass			
	Wet mass		Dry mass		Wet mass		Dry mass	
Treatments	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Untreated control	0.68 [a]	0.54 [a]	0.182 [a]	0.174 [a]	-35 [a]	-35 [a]	-50 [a]	-22 [a]
Sterile millet	0.77 [a]	0.84 [a]	0.238 [a]	0.231 [a]	-26 [a]	1 [a]	-34 [a]	4 [a]
Pathogen inoculated control***	1.04 [a]	0.84 [a]	0.361 [a]	0.222 [a]	0 [a]	0 [a]	0 [a]	0 [a]
T01 + pathogen***	0.94 [a]	0.60 [a]	0.282 [a]	0.183 [a]	-9 [a]	-28 [a]	-22 [a]	-17 [a]
T02 + pathogen***	1.01 [a]	0.83 [a]	0.226 [a]	0.179 [a]	-3 [a]	0 [a]	-38 [a]	-19 [a]
T03 + pathogen***	1.09 [a]	0.88 [a]	0.403 [a]	0.243 [a]	4 [a]	5 [a]	12 [a]	10 [a]
T08 + pathogen***	0.97 [a]	0.82 [a]	0.342 [a]	0.247 [a]	-7 [a]	-2 [a]	-5 [a]	11 [a]
T09 + pathogen***	1.12 [a]	1.01 [a]	0.307 [a]	0.273 [a]	7 [a]	21 [a]	-15 [a]	23 [a]
T17 + pathogen***	0.80 [a]	0.90 [a]	0.300 [a]	0.255 [a]	-24 [a]	7 [a]	-17 [a]	15 [a]
T20 + pathogen***	0.87 [a]	0.85 [a]	0.294 [a]	0.239 [a]	-17 [a]	2 [a]	-19 [a]	8 [a]
T21 + pathogen***	1.02 [a]	0.94 [a]	0.338 [a]	0.247 [a]	-2 [a]	13 [a]	-6 [a]	11 [a]
T22 + pathogen***	0.98 [a]	1.05 [a]	0.379 [a]	0.241 [a]	-6 [a]	26 [a]	5 [a]	9 [a]
T23 + pathogen***	1.05 [a]	0.88 [a]	0.292 [a]	0.269 [a]	0 [a]	5 [a]	-19 [a]	21 [a]
T24 + pathogen***	0.99 [a]	0.78 [a]	0.354 [a]	0.233 [a]	-5 [a]	-6 [a]	-2 [a]	5 [a]
T27 + pathogen***	0.98 [a]	0.78 [a]	0.356 [a]	0.291 [a]	-6 [a]	-6 [a]	-1 [a]	31 [a]
T28 + pathogen***	0.96 [a]	0.91 [a]	0.304 [a]	0.246 [a]	-8 [a]	9 [a]	-16 [a]	11 [a]
T29 + pathogen***	0.88 [a]	0.90 [a]	0.297 [a]	0.260 [a]	-15 [a]	7 [a]	-18 [a]	17 [a]
T30 + pathogen***	0.92 [a]	0.88 [a]	0.292 [a]	0.250 [a]	-12 [a]	5 [a]	-19 [a]	13 [a]
T31 + pathogen***	1.02 [a]	0.71 [a]	0.322 [a]	0.203 [a]	-2 [a]	-15 [a]	-11 [a]	-9 [a]

*Means within columns with the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure

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

*** *R. solani* millet seed inoculum was added to the soil at 40 g millet per litre soil.

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Table 3.7 Assessment of rhizobacterial isolates for biocontrol of *Fusarium oxysporum* UPGH132 on wheat in seedling trays

	Seedling mass in grams				% Change in mass*			
	Wet mass **		Dry mass **		Wet mass **		Dry mass **	
Treatments	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated control	0.34 e	0.32 f	0.10 d	0.10 e	-54 e	-67 f	-42 d	-52 e
Sterile millet	0.68 e	0.76 e	0.22 abc	0.19 d	-7 e	-21 e	30 abc	-5 d
Pathogen inoculated control***	0.74 de	0.97 e	0.17 bcd	0.20 cd	0.00 de	0 e	0 bcd	0 cd
T01 + pathogen***	1.02 cde	1.34 de	0.30 a	0.30 a	39 cde	39 de	76 a	49 a
T02 + pathogen***	0.97 bcd	0.95 abc	0.26 ab	0.20 bcd	32 bcd	-2 abc	53 ab	2 bcd
T03 + pathogen***	1.18 bcd	1.22 de	0.24 abc	0.27 abc	60 bcd	26 de	42 abc	37 abc
T08 + pathogen***	0.89 b	1.00 abcd	0.26 ab	0.21 bcd	21 b	4 abcd	52 ab	5 bcd
T09 + pathogen***	1.17 b	1.43 cde	0.28 ab	0.29 a	58 b	48 cde	63 ab	43 a
T17 + pathogen***	0.93 bcd	1.23 a	0.19 abcd	0.28 ab	27 bcd	27 a	14 abcd	39 ab
T20 + pathogen***	0.74 bcde	1.13 abcd	0.14 cd	0.24 abcd	1 bcde	18 abcd	-19 cd	19 abcd
T21 + pathogen***	0.94 bcd	1.27 abcd	0.19 abcd	0.27 abc	28 bcd	31 abcd	12 abcd	35 abc
T22 + pathogen***	1.96 a	1.11 abcd	0.26 ab	0.25 abcd	167 a	15 abcd	51 ab	27 abcd
T23 + pathogen***	0.54 e	1.07 abcde	0.24 abc	0.24 abcd	-27 e	11 abcde	38 abc	22 abcd
T24 + pathogen***	0.85 bcd	0.97 bcde	0.26 ab	0.22 abcd	15 bcd	0 bcde	49 ab	12 abcd
T27 + pathogen***	0.88 bdc	1.12 de	0.22 abc	0.24 abcd	20 bdc	16 de	30 abc	22 abcd
T28 + pathogen***	0.89 bcd	1.17 abcd	0.24 abc	0.25 abcd	21 bcd	22 abcd	39 abc	27 abcd
T29 + pathogen***	0.84 bcd	1.29 abcd	0.22 abc	0.27 ab	15 bcd	33 abcd	29 abc	38 ab
T30 + pathogen***	0.80 bcde	1.21 abcd	0.19 abcd	0.28 ab	9 bcde	25 abcd	13 abcd	38 ab
T31 + pathogen***	1.15 dc	1.37 ab	0.29 ab	0.27 abcd	56 dc	42 ab	67 ab	33 abcd

*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Means within columns with the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure
*** *F. oxysporum* millet seed inoculum was added to the soil at 40 g millet per litre soil.

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Table 3.8 Assessment of rhizobacterial isolates for biocontrol of *Fusarium oxysporum* UPGH 132 on wheat seedlings grown in pots

	Seedling mass in grams				% change in mass*			
	Fresh mass**		Dry mass**		Fresh mass**		Dry mass**	
Treatment	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Pathogen inoculated millet***	2.20 c	1.20 f	0.53 e	0.25 e	0 c	0 f	0 e	0 e
Sterile millet	2.75 bc	3.35 a	0.65 cde	0.63 ab	25 bc	179 a	24 cde	150 ab
T08 + pathogen***	3.25 abc	2.50 cde	0.73 bcde	0.45 cd	48 abc	108 cde	38 bcde	80 cd
T09 + pathogen***	3.15 abc	2.08 cde	0.83 abcd	0.48 bcd	43 abc	73 cde	57 abcd	90 bcd
T10 + pathogen***	3.10 abc	2.35 cde	0.68 cde	0.55 abc	41 abc	96 cde	29 cde	120 abc
T11 + pathogen***	3.00 abc	2.28 cde	0.63 de	0.53 abcd	36 abc	90 cde	19 de	110 abcd
T15 + pathogen***	4.13 a	1.93 de	0.98 ab	0.45 cd	88 a	60 de	86 ab	80 cd
T16 + pathogen***	3.10 acb	1.70 ef	0.73 bcde	0.38 de	41 acb	42 ef	38 bcde	50 de
T22 + pathogen***	3.50 ab	2.55 bcd	0.70 bcde	0.58 abc	59 ab	113 bcd	33 bcde	130 abc
T26 + pathogen***	3.68 ab	2.33 e	0.90 abcd	0.53 abcd	67 ab	94 e	71 abcd	110 abcd
T28 + pathogen***	4.08 a	2.65 bc	1.08 a	0.55 abc	85 a	121 bc	105 a	120 abc
T29 + pathogen***	4.03 a	3.15 ab	0.93 abc	0.68 a	83 a	163 ab	76 abc	170 a
T30 + pathogen***	3.78 ab	2.33 cde	0.98 ab	0.55 abc	72 ab	94 cde	86 ab	120 abc

*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Means within columns with the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure
*** *F. oxysporum* millet seed inoculum was added to the soil at 30 g millet per litre soil.

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Table 3.9 Assessment of rhizobacterial isolates for biocontrol of *Rhizoctonia solani* UPGH 122 on wheat seedlings grown in pots

	Seedling mass in grams				% Change in mass			
	Fresh mass		Dry mass		Fresh mass		Dry mass	
Treatments	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Inoculated control***	1.30 e	1.25 f	0.30 c	0.38 e	0 e	0 f	0 c	0 e
Uninoculated millet	2.08 abcde	4.43 bcd	0.48 abc	0.98 ab	60 abcde	254 bcd	58 abc	160 ab
T08 + pathogen***	1.78 bcde	2.50 e	0.53 abc	0.58 de	37 bcde	100 e	75 abc	5 de
T09 + pathogen***	2.18 abcde	3.10 de	0.55 abc	0.63 de	67 abcde	148 de	83 abc	67 de
T10 + pathogen***	2.75 a	3.23 de	0.68 a	0.70 cd	112 a	158 de	125 a	87 cd
T11 + pathogen***	2.73 a	3.00 de	0.58 abc	0.70 cd	110 a	140 de	92 abc	87 cd
T15 + pathogen***	1.88 abcde	4.40 bcd	0.53 abc	0.90 abc	44 abcde	252 bcd	75 abc	140 abc
T16 + pathogen***	1.53 de	5.33 abc	0.35 bc	1.03 a	17 de	326 abc	17 bc	173 a
T22 + pathogen***	2.45 abc	5.68 ab	0.60 ab	1.13 a	88 abc	354 ab	100 ab	20 a
T26 + pathogen***	2.28 abcd	6.18 a	0.43 abc	1.03 a	75 abcd	394 a	42 abc	173 a
T28 + pathogen***	1.58 cde	4.73 abc	0.53 abc	0.90 abc	21 cde	278 abc	75 abc	140 abc
T29 + pathogen***	2.13 abcde	4.30 bcd	0.70 a	0.98 ab	63 abcde	244 bcd	133 a	160 ab
T30 + pathogen***	2.53 ab	3.95 cde	0.53 abc	0.73 bcd	94 ab	216 cde	75 abc	93 bcd

*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Means within columns with the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure
*** *R. solani* millet seed inoculum was added to the soil at 20 g millet per litre soil.

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Table 3.10 Effect of rhizobacterial isolates on growth of wheat seedlings grown in soil inoculated with *Fusarium graminearum* WP4F in the greenhouse

		Seedling mass in grams				% Change in mass*			
		Fresh Mass**		Dry Mass**		Wet mass **		Dry mass**	
Treatment	Root rot rating	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated control	0.0	1.556 abcdefg	2.522 a	0.180 bcde	0.364 a	36 abcdefg	89 a	11 bcde	60 a
Uninoculated millet	0.4	1.781 abcd	2.378 ab	0.215 abcd	0.345 ab	55 abcd	78 ab	32 abcd	51 ab
Inoculated control***	2.5	1.146 hijkl	1.337 lm	0.162 cdefgh	0.228 ghijk	0 hijkl	0 lm	0 cdefgh	0 ghijk
T01 + pathogen***	2.8	1.151 hijkl	1.955 cdefg	0.128 efgh	0.285 cdefg	0 hijkl	46 cdefg	-21 efgh	25 cdefg
T02 + pathogen***	2.8	1.058 kl	1.647 efghijkl	0.123 fgh	0.240 fghijk	-8 kl	23 efghijkl	-24 fgh	5 fghijk
T03 + pathogen***	3.0	1.314 fghijkl	1.767 cdefghi	0.154 efgh	0.267 defghi	15 fghijkl	32 cdefghi	-5 efgh	17 defghi
T04 + pathogen***	2.5	1.509 abcdefghi	1.979 cdef	0.162 cdefgh	0.284 cdefgh	32 abcdefghi	48 cdef	0 cdefgh	24 cdefgh
T05 + pathogen***	3.0	1.356 efghijkl	1.481 hijklm	0.159 defgh	0.220 ijkl	18 efghijkl	11 hijklm	-2 defgh	-4 ijkl
T06 + pathogen***	3.0	1.350 efghijkl	1.783 cdefghi	0.158 efgh	0.266 defghi	18 efghijkl	33 cdefghi	-3 efgh	17 defghi
T07 + pathogen***	2.8	1.201 ghijkl	1.593 fghijkl	0.129 efgh	0.226 hijkl	5 ghijkl	19 fghijkl	-20 efgh	-1 hijkl
T08 + pathogen***	2.8	1.100 jkl	1.199 m	0.116 gh	0.180 l	-4 jkl	-10 m	-28 gh	-21 l
T09 + pathogen***	3.0	1.429 cdefghijk	1.463 hijklm	0.159 defgh	0.207 jkl	25 cdefghijk	9 hijklm	-2 defgh	-9 jkl
T10 + pathogen***	1.8	1.883 a	1.671 defghijkl	0.150 efgh	0.285 cdefg	64 a	25 defghijkl	-8 efgh	25 cdefg
T11 + pathogen***	1.0	1.787 abc	1.774 cdefghij	0.156 efgh	0.247 efghijk	56 abc	33 cdefghij	-4 efgh	9 efghijk
T12 + pathogen***	2.0	1.206 ghijkl	1.383 jklm	0.112 h	0.210 ijkl	5 ghijkl	3 jklm	-31 h	-8 ijkl
T13 + pathogen***	1.3	1.605 abcdef	1.581 ghijklm	0.134 efgh	0.242 efghijk	40 abcdef	18 ghijklm	-18 efgh	6 efghijk
T14 + pathogen***	3.0	1.359 efghijkl	1.410 ijklm	0.128 efgh	0.226 hijkl	19 efghijkl	5 ijklm	-21 efgh	-1 hijkl
T15 + pathogen***	3.0	1.683 abcdef	1.875 cdefg	0.148 efgh	0.262 defghij	47 abcdef	40 cdefg	-9 efgh	15 defghij
T16 + pathogen***	2.5	1.826 ab	1.369 klm	0.133 efgh	0.201 kl	59 ab	2 klm	-18 efgh	-12 kl
T17 + pathogen***	2.5	1.127 ijkl	1.848 cdefgh	0.152 efgh	0.300 bcde	-2 ijkl	38 cdefgh	-6 efgh	31 bcde
T18 + pathogen***	2.3	1.392 defghijk	2.115 bc	0.172 bcdefg	0.328 abc	21 defghijk	58 bc	6 bcdefg	44 abc
T19 + pathogen***	2.0	1.101 jkl	1.745 cdefghijk	0.143 efgh	0.251 efghijk	-4 jkl	30 cdefghijk	-12 efgh	10 efghijk

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Table 3.10 Effect of rhizobacterial isolates on growth of wheat seedlings grown in soil inoculated with *Fusarium graminearum* WP4F in the greenhouse

		Seedling mass in grams				% Change in mass*			
		Fresh Mass**		Dry Mass**		Wet mass **		Dry mass**	
Treatment	Root rot rating	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
T20 + pathogen***	2.5	0.965 l	1.681 defghijkl	0.124 fgh	0.264 defghij	-16 l	26 defghijkl	-24 fgh	16 defghij
T22 + pathogen***	2.5	1.291 fghijkl	1.800 cdefghi	0.162 cdefgh	0.262 defghij	13 fghijkl	35 cdefghi	0 cdefgh	15 defghij
T23 + pathogen***	2.5	1.537 abcdefgh	1.758 cdefghijk	0.221 ab	0.258 defghijk	34 abcdefgh	31 cdefghijk	36 ab	13 defghijk
T24 + pathogen***	2.5	1.683 abcdef	1.915 cdefg	0.238 a	0.258 defghijk	47 abcdef	43 cdefg	47 a	13 defghijk
T25 + pathogen***	2.0	1.593 abcdefg	1.891 cdefg	0.172 bcdefg	0.262 defghij	39 abcdefg	41 cdefg	6 bcdefg	15 defghij
T26 + pathogen***	2.0	1.660 abcdef	2.062 bcd	0.154 efgh	0.263 defghij	45 abcdef	54 bcd	-5 efgh	15 defghij
T27 + pathogen***	2.5	1.682 abcdef	2.026 bcde	0.215 abc	0.310 abcd	47 abcdef	52 bcde	33 abc	36 abcd
T28 + pathogen***	2.5	1.461 bcdefghij	1.888 cdefg	0.172 bcdefg	0.278 cdefgh	27 bcdefghij	41 cdefg	6 bcdefg	22 cdefgh
T29 + pathogen***	2.0	1.533 abcdefgh	1.724 cdefghijkl	0.178 bcdef	0.260 defghij	34 abcdefgh	29 cdefghijkl	9 bcdef	14 defghij
T30 + pathogen***	2.0	1.445 bcdefghijk	1.706 defghijkl	0.140 efgh	0.251 efghijk	26 bcdefghijk	28 defghijkl	-14 efgh	10 efghijk
T31 + pathogen***	2.0	1.738 abcde	2.030 bcde	0.239 a	0.298 bcdef	52 abcde	52 bcde	47 a	31 bcdef

*% change in mass [(treatment - disease control)/ disease control x 100] therefore negative values are treatments that are less than the disease control and positive values are treatments with a higher mass than the disease control.
**Means within columns with the same letters do not differ significantly (p = 0.05) according to the least significance difference (LSD) tests using the GLM procedure
*** *F. graminearum* millet seed inoculum was added to the soil at 30 g millet per litre soil.

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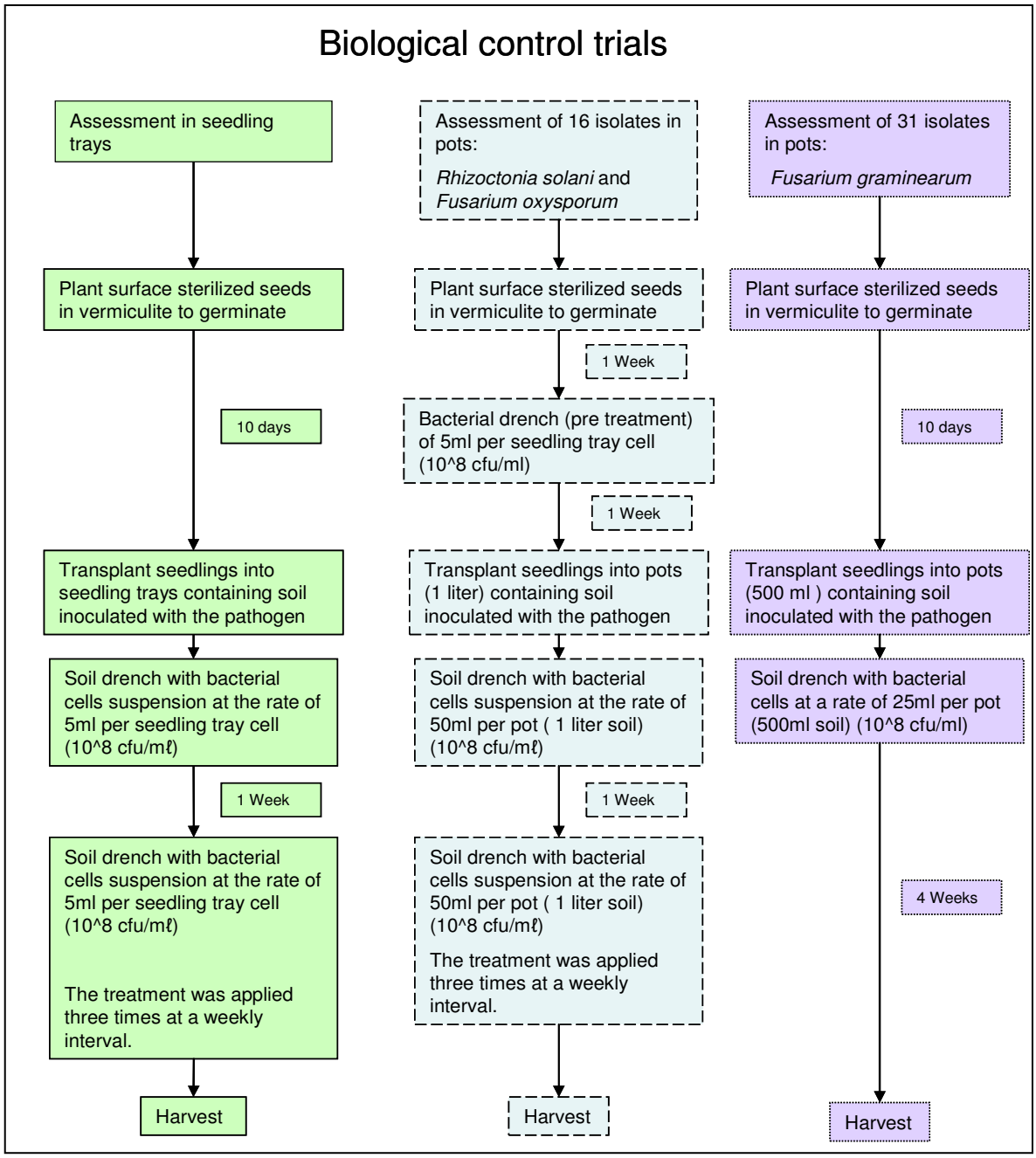

3.6 Figures

Figure 3.1. An overview of the procedures whereby the rhizobacteria isolates were screened for biocontrol activity. Harvesting and assessment criteria were the same for all trials, temperature and watering as described in text.

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CHAPTER 4

Assessment of rhizobacterial isolates for growth promotion of wheat

Abstract

The ability of plant growth promoting rhizobacteria (PGPR) to promote the growth and yield of wheat is widely documented. The beneficial effect of PGPR on wheat can greatly benefit the South African wheat industry, since wheat production is in general low compared to other wheat producing countries. In this study 31 rhizobacterial isolates that improved the growth of sorghum in previous studies, were screened in the greenhouse for their ability to promote the growth of wheat seedlings. Isolate were initially screened in batches, and the best isolates selected for further trials to evaluate the effect of inoculum dose and fertilization on PGPR activity. Isolates T06, T07, T11, T13, T19, T21, T23 and T24 were the best performing in the screening trials. Results for inoculum dose response and fertiliser trials indicate that these factors have an effect on PGPR activity. Inconsistent efficacy of the PGPR was also noted. The results indicate that rhizobacterial can improve the growth of wheat seedlings. For PGPR application to be effective in practice, attention should be given to consistent growth promotion by the isolate, the effective inoculum dose and conducive levels of fertiliser.

4.1 Introduction

Plant growth promoting rhizobacteria (PGPR) are known to affect the growth of plants in two ways, by directly impacting the plant and indirectly, through the effect they have on pathogens. Direct growth promotion is an important aspect of PGPR-plant interactions. This direct interaction of the PGPR with plants results in increased root and shoot growth, increased germination rate of seeds, increased leaf area, chlorophyll, and protein content, tolerance to drought, and delayed leaf senescence (Lucy *et al.* 2004). Plant growth can additionally be stimulated directly by PGPR mediated processes involved in nutrient cycling, such as nutrient solubilization, nitrogen fixation and by the production of phytohormones (Hameeda *et al.* 2006; Barea *et al.* 2005) or by decreasing heavy metal toxicity (Whipps 2001).

Plant growth promoting rhizobacteria that directly promote plant growth are found in various bacterial general including the Bacilli, Pseudomonads and *Azospirillum* (Barea *et al.* 2005). PGPR have been shown to improve the growth and yield of a variety of crop plants as diverse as peas, tomatoes, wheat, and turf. There are also a number of plant growth promoting bacteria commercially available (Coping 2001). These interactions are of agricultural

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importance since the PGPR can be harnessed to improve the efficacy of fertiliser applications thereby reducing fertiliser inputs and benefiting the farmers and the environment (Perez *et al*. 2008).

In the literature, examples such as the work of Adesemoye and Kloepper (2009), and Ozturek *et al.* (2003) showed that inoculation of wheat and barley with *Azospirillum brasilense* enhanced growth of plants that received suboptimal fertilisation. In field trials in Argentina a liquid formulation of *Azospirillum brasilense* was able to increase the productivity of dryland wheat grain yield by up to 8% and positive responses to inoculation was recorded in 70% of the locations (Diaz-Zorita and Fernandez-Canigia, 2009). Similar findings on other crops such as tomato (Adesemoye, 2009) demonstrated the versatility of PGPR with regards to improving plant growth and crop yield.

These beneficial growth stimulant effects would be important in the South African wheat industry, since wheat production is in general low compared to other wheat producing countries and large amounts of fertiliser are applied (Scott 1990). The aim of this study is to evaluate selected PGPR isolates for their ability to promote the growth of wheat seedlings.

4.2 Materials and Methods

Rhizobacterial isolates from the UP-PGPR collection were assessed in the greenhouse for their ability to promote the growth of wheat seedlings. Appendix 2 gives further details regarding their origin, preservation and preparation. Due to space and time constraints the isolates were screened in two successive trials/ batches. Figure 4.1 gives an outline of the screening procedure.

As a first screening the isolates were assessed in two batches in the greenhouse. The four best performing isolates from each batch were selected for subsequent trials. These eight isolates were re-assessed for growth promotion at two different inoculum doses. The dose response interaction trials were repeated in a trial that included the assessment of a fertiliser-PGPR- dose interaction.

For the first screening phase the bacterial isolates were applied to ten day old wheat seedlings that had been transplanted into 500ml (10cm diameter) plastic pots, containing steam pasteurized sandy loam soil, at the rate of 5 seedlings per pot. Apart from the rhizobacterial isolates from the UP collection the product QCM-360, was also applied for comparison. QCM-360 is marketed by Agrilibrium (Pretoria, South Africa), and contains various bacterial and fungal cultures. (Label information: *Bacillus subtilis, B. thuringiensis, Azotobacter chrococcum, Pseudomonas fluorescens, Saccharomyces cerivisiae, Trichoderma harzianum*, compost cultures, *Streptococcus* sp. *Lactobacillus* sp., oligosaccharides, fulvic acids, micro elements. Application rate is recommended to be 20l/

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hectare). Details of seed preparation, seedling germination and greenhouse conditions are given in appendix 3. The trial was harvested four weeks after the plants were transplanted in to the pots.

The respective treatments were: 1) untreated control, 2) 25 ml bacterial suspension (10^8 cfu/ml.) 3) 25 ml QCM-360 solution prepared by mixing 2ml QCM-360 with 23ml Ringer's solution (equivalent to 25L / ha). Treatments were replicated four times, each treatment unit consisting of a pot containing five seedlings. The pots were arranged in a randomised block design.

4.2.1 Re-evaluation of eight selected isolates for growth promotion at different doses

Eight isolates which performed best during the initial screening were re-assessed for growth promotion in two trials. The trials differed from the previous screening trials with regards to the bacterial dose applied. The trials were preformed as described for the screening trials with the following changes. One litre capacity pots with six seedlings each constituted a replicate, and the trial was harvested after five weeks. Treatments were 1) untreated control, 2) plants inoculated with one of the eight rhizobacterial isolates respectively, 3) plants inoculated with the commercial product BacUp (Biological Control Products, Ashwood, South Africa). The latter commercial biological product was included as a standard for comparison. BacUp was applied by dilution with an equal amount of Ringers solution (resulting in a concentration of 10^8cfu/ml), and the eight selected isolates applied separately as a 25ml drench (trial 1) or 50 ml drench (trial 2) treatment per pot.

4.2.2 Fertiliser-PGPR-dose response interaction

To establish whether fertiliser has an effect on the growth promoting activity of the PGPR isolates a greenhouse pot trial was performed. The eight isolates (T06, T07, T11, T13, T19, T21, T23 and T24) were grown and prepared as described in appendix 3. Each isolate was applied separately at two concentrations (12.5ml and 25ml per pot) and with or without fertilisation. The fertiliser treatment comprised superphosphate that was ground to a fine powder with a mortar and pestle and mixed into the soil by means of a concrete mixer at a rate of 0.125g per 500 ml soil, (1g per 4kg soil). Nitrogen fertiliser was applied in the form of ammonium nitrate (NH_4NO_3) applied, directly after the seedlings were transplanted, at a rate of 3.5ml (1% solution) per 500ml pot. The ammonia solution was prepared by dissolving 10g NH_4NO_3 in one litre water.

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4.2.3 Statistical analysis

For all trials each experimental unit consisted of one pot with five wheat seedlings. Treatments were replicated four times (four pots) and arranged in a randomised block design. Data was subjected to analysis of variance (ANOVA) using SAS-9.2 software. Means values in each treatment were compared using the least significant difference (LSD) test at 5% ($p = 0.05$) level of significance.

4.3 Results

4.3.1 Initial screening of the 31 bacterial isolates for growth promotion of wheat in the greenhouse

For the initial assessment of growth promotion by the rhizobacteria the isolates were screened in two batches. The results are given in tables 4.1 and 4.2. In the first batch no isolate significantly increased the fresh or dry root mass compared to the untreated control. The fresh foliage mass was significantly increased by isolates T01, T05, T06, T07, T10, T11 and T13. Dry foliage mass was however only increased by isolates T06, T07, T11 and T13. These isolates caused an increase in dry shoot mass of 18, 16, 19 and 18% respectively. These isolates were therefore chosen for subsequent trials.

In the second batch, plants treated with the bacterial isolates T19, T0, T21, T23, and T24, had significantly heavier fresh root masses than the untreated control. All the plants treated with rhizobacteria had significantly increased foliage mass. The dry foliage mass of all the plants inoculated with bacteria was significantly heavier than that of the control. The increase in dry foliage mass ranged from 42% to 66% (table 4.2). Four isolates were subsequently selected from each batch for further evaluation of their growth promotion activities.

4.3.2 Re-evaluation of growth promotion activity of the four selected best performing isolates at two inoculum doses

During the initial greenhouse screenings a high bacterial dose (25ml of a 10^{8} cfu/ml bacterial suspension per 500ml soil) was applied. Because this inoculum dose was high the isolates were subsequently screened in a pot trial at a lower dose of 25ml of a 10^{8} cfu/ml bacterial suspension per 1 litre soil, as a more stringent test for the isolates growth promoting abilities. The results for this trial are presented in table 4.3. There were no isolates that increased plant dry mass at this inoculum dose, however all the bacterial treatments except BacUP and T24 increased the fresh shoot mass.

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Because the isolates did not promote the growth of the plants at the lower inoculum dose, the trial was repeated at a higher dose (50 ml of a 10^{8} cfu/ml bacterial suspension per litre soil). As shown in table 4. 3 the isolates T07, T19, T21, T23 and T24 significantly increased the fresh root mass. Only isolate T07 and BacUp however increased dry root mass. BacUp increased dry root mass by 20.6% and T07 by 25.6%. The only treatments to increase fresh shoot mass were BacUp, T13, T19, and T23. Dry shoot mass was increased by 12.2% (T13) and 13.4 % (T19). Surprisingly BacUp caused a decrease in the dry shoot mass of 16% (table 4.3).

4.3.3 Fertilizer-dose response

The expected trend that fertilised plants perform better than non fertilised plants was observed for most treatments. When the bacterial treatments were compared within their groups on fertilized vs. non-fertilised plants, few treatments differed from the untreated control (Tables 4.4 and 4.5). There was also no clear trend showing that the inoculum dosage affected the outcome. The only isolate showing a dose-related response was T19. This isolate also caused an increase in mass on the unfertilised plants, but not on the fertilised plants. The 25ml inoculum dose of isolate T19 resulted in a 31% increase in the dry foliage mass whereas the 12ml did not differ significantly form the control treatment (table 4.5).

Three bacterial treatments caused a significant decrease of the plant mass. The 25ml drench treatment of isolate T07 in conjunction with fertilisation resulted in a 33% decrease in dry root mass. The dry shoot mass of fertilised plants decreased with the 12ml inoculum dose of isolate T23. A 40% decrease in dry root mass was observed for the treatment of unfertilised plants with isolate T07 at the 12ml inoculum dose (table 4.5).

4.4 Discussion

In the current study 31 rhizobacteria isolates were selected based on results from trials on other crops including tomatoes and sorghum (Hassen 2007) and screened in the greenhouse for their ability to promote the growth of wheat seedlings. The isolates were initially screened in two batches and the four best performing isolates selected from each batch for further greenhouse trials involving the response of PGPR to different doses and fertiliser application. The results of this study demonstrate that PGPR can significantly promote the growth of wheat under certain conditions, a large impediment to the screening and use of PGPR is however their inconsistent efficacy.

A comparison of the first and second screening trials revealed that the second trial rendered a much larger percentage effective inoculants and the growth promotion effect was much

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greater. This finding is probably due to variation in trial conditions since the commercial inoculant QCM360, which was included in both trials also performed much better during the second batch. Various factors such as environmental conditions, fertiliser inputs and the host plant responses are known to influence the metabolic behaviour of the bacteria and hence their effect on the plant (Pielach *et al.* 2008, Hass and Defago 2005). As the trial conditions were kept as similar as possible, it is difficult to explain why this occurred. These results however highlight the difficulties associated with screening and using biological products such as PGPR's for increasing plant yields.

From the initial screening eight isolates were selected for further trials. These trials were to determine the effect of PGPR dose and fertilisation on plant growth promotion. It is well known that the effect PGPR have on the plants is dose-related. In general, the plant growth promotion caused by the PGPR will decrease as the PGPR inoculum dose decreases (Benizri *et al.*2001). In the greenhouse trials that were conducted this trend was also observed. Fewer isolates consistently increased the growth at the lower inoculum doses than at the higher inoculum doses. The most probable reason for this is related to the number of PGPR that survive and colonise the plant roots. There is a threshold population level that the PGPR must reach to cause plant growth promotion (Paulitz, 2000).

Plant growth promoting rhizobacteria have been shown to increase the efficacy of fertiliser applications. For instance Shaharoona *et al.* (2008) showed that when wheat that received 75% the recommended rate of fertiliser was inoculated with PGPR *Pseudomonas* strains the yield was comparable to wheat that received the recommended fertiliser application. When the eight PGPR isolates, which showed good growth promotion in selection trials, were applied in conjunction with fertilisation they had no effect on the growth of the seedlings. This variation between trials can be attributed to the inconsistency associated with PGPR applications in general (Rosas 2007), or fertiliser levels that were too high for the PGPR to contribute noticeably to plant growth.

A well known hurdle to the commercial use of PGPR is their inconsistency and various factors contribute to this unpredictability, including environmental conditions (Lugtenburg and Kamilova 2009) and the occurrence of genetic variation in the strain (e.g. phase variation etc.) (Van den Broek 2003). The nutrient status of the soil also plays an important role in the efficacy of PGPR application. In trials where tomato plants received different levels of fertiliser in conjunction with PGPR inoculation it was shown that PGPR do not results in significant increase in tomato plant growth at optimal fertiliser levels (Adesemoye and Kloepper, 2009). Similar results were obtained at UP in trials with maize (Labuschagne, unpublished). The effect of the PGPR inoculation may also become apparent if the plants are grown for a longer period of time. This is because several nutrients such as P decrease over time and the MOA

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of these microbes is to make these unavailable nutrients more accessible to the plants (Adesemoye and Kloepper 2009).

These results confirm that rhizobacteria can be used to improve the growth of wheat. Obstacles associated with screening and utilising PGPR strains were also highlighted in the results. A principal obstacle is the inconsistency of PGPR performance under various conditions and the effects that external factors have on the PGPR action.

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4.5 Tables

Table 4.1. Efficacy of rhizobacterial isolates for promoting growth of wheat seedlings in the greenhouse in pots (Batch 1)

	Seedling mass in grams				% Change in mass*			
	Fresh mass**		Dry mass**		Fresh mass**		Dry mass**	
Treatments	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated control	2.187 abcde	3.361 def	0.425 abc	0.403 d	0 abcde	0 def	0 abc	0 d
QCM360****	1.740 de	3.642 bcdef	0.331 c	0.433 abcd	-20 de	8 bcdef	-22 c	7
T01***	2.204 abcd	4.000 abc	0.417 abc	0.437 abcd	1 abcd	19 abc	-2 abc	8 abcd
T02***	2.115 abcde	3.328 def	0.364 bc	0.412 cd	-3 abcde	-1 def	-14 bc	2 cd
T03***	1.844 bcde	3.743 abdce	0.298 c	0.435 abcd	-16 bcde	11 abdce	-30 c	8 abcd
T04***	2.322 abc	3.648 bcdef	0.416 abc	0.414 cd	6 abc	9 bcdef	-2 abc	3 cd
T05***	1.977 abcde	4.006 abc	0.399 abc	0.449 abcd	-10 abcde	19 abc	-6 abc	11 abcd
T06***	2.308 abc	4.043 abc	0.480 ab	0.476 ab	6 abc	20 abc	13 ab	18 ab
T07***	2.316 abc	4.119 ab	0.371 bc	0.467 abc	6 abc	23 ab	-13 bc	16 abc
T08***	1.637 e	3.269 f	0.313 c	0.417 cd	-25 e	-3 f	-26 c	3 cd
T09***	2.504 a	3.857 abcd	0.511 a	0.460 abcd	15 a	15 abcd	20 a	14 abcd
T10***	2.225 abcd	3.906 abc	0.398 abc	0.439 abcd	2 abcd	16 abc	-6 abc	9 abcd
T11***	1.999 abcde	4.210 a	0.396 abc	0.478 a	-9 abcde	25 a	-7 abc	19 a
T12***	1.811 cde	3.768 abdce	0.355 bc	0.455 abcd	-17 cde	12 abdce	-16 bc	13 abcd
T13***	1.970 abcde	4.177 a	0.337 c	0.477 ab	-10 abcde	24 a	-21 c	18 ab
T14***	1.695 de	3.619 bcdef	0.312 c	0.438 abcd	-23 de	8 bcdef	-27 c	9 abcd
T15***	2.023 abcde	3.850 abdce	0.352 bc	0.447 abcd	-7 abcde	15 abdce	-17 bc	11 abcd

* % change in mass [(treatment - control)/ control x 100] therefore negative values are treatments that are less than the untreated control and positive values are treatments with a higher mass than the untreated control.
** Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure.
***Rhizobacterial isolates from the UP-PGPR culture collection.
****Commercial product.

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Table 4.2. Efficacy of rhizobacterial isolates for promoting growth of wheat seedlings in the greenhouse in pots (Batch 2)

	Seedling mass in grams				% Change in mass*			
	Wet mass**		Dry mass**		Wet mass**		Dry mass**	
Isolates	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Untreated Control	3.935 c	2.795 e	0.412 ab	0.239 b	0 c	0 e	0 ab	0 b
QCM360****	4.708 abc	3.373 cd	0.548 ab	0.376 a	20 abc	21 cd	-1 ab	57 a
T16***	4.896 abc	3.283 cd	0.540 ab	0.349 a	24 abc	17 cd	10 ab	46 a
T17***	5.030 abc	3.553 abc	0.562 ab	0.386 a	28 abc	27 abc	1 ab	61 a
T18***	5.077 abc	3.537 abc	0.517 ab	0.359 a	29 abc	27 abc	-9 ab	50 a
T19***	5.154 a	3.404 bcd	0.577 ab	0.361 a	31 a	22 bcd	24 ab	51 a
T20***	4.926 ab	3.293 cd	0.530 ab	0.346 a	25 ab	18 cd	6 ab	45 a
T21***	5.569 a	3.835 ab	0.595 ab	0.397 a	42 a	37 ab	14 ab	66 a
T22***	4.920 abc	3.513 abc	0.551 ab	0.365 a	25 abc	26 abc	7 ab	53 a
T23***	5.146 a	3.443 bc	0.580 ab	0.389 a	31 a	23 bc	10 ab	63 a
T24***	5.241 a	3.527 abc	0.566 ab	0.376 a	33 a	26 abc	9 ab	57 a
T25***	4.880 abc	3.520 abc	0.511 ab	0.360 a	24 abc	26 abc	-13 ab	51 a
T26***	5.006 abc	3.520 abc	0.592 a	0.373 a	27 abc	26 abc	26 a	56 a
T27***	4.725 c	3.571 abc	0.500 b	0.367 a	20 c	28 abc	-24 b	54 a
T28***	5.171 bc	3.950 a	0.538 ab	0.385 a	31 bc	41 a	-12 ab	61 a
T29***	5.032 abc	3.690 abc	0.593 ab	0.394 a	28 abc	32 abc	15 ab	65 a
T30***	4.927 abc	3.546 abc	0.553 ab	0.388 a	25 abc	27 abc	-5 ab	62 a
T31***	4.814 abc	3.425 bcd	0.553 ab	0.359 a	22 abc	23 bcd	12 ab	50 a

* % change in mass [(treatment - control)/ control x 100] therefore negative values are treatments that are less than the untreated control and positive values are treatments with a higher mass than the untreated control.
** Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure.
***Rhizobacterial isolates from the UP-PGPR culture collection.
****Commercial product.

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Table 4.3. The effect of different inoculum doses of the 8 selected PGPR isolates on mass of wheat plants in the greenhouse

	50 ml bacterial drench treatment / pot				25 ml bacterial drench treatment /pot			
	Fresh mass (g) *		Dry mass (g) *		Fresh mass (g) *		Dry mass (g) *	
Isolates	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Untreated control	3.36 de	5.53 bcd	0.41 bcd	0.72 bcd	5.01 ab	5.76 b	1.02 a	0.54 abc
BacUP***	4.44 bcd	4.60 e	0.52 ab	0.62 e	4.69 ab	6.30 ab	1.04 a	0.51 abc
T06**	3.84 cde	5.01 de	0.36 cd	0.66 de	4.74 ab	6.64 a	1.02 a	0.43 abc
T07**	5.30 ab	6.03 ab	0.55 a	0.79 ab	4.17 ab	6.75 a	1.02 a	0.48 abc
T11**	3.80 cde	5.58 bc	0.45 abc	0.76 abc	5.22 ab	6.67 a	0.98 a	0.56 ab
T13**	4.23 bcd	6.26 a	0.41 bcd	0.82 a	4.70 ab	6.72 a	1.01 a	0.53 abc
T19**	4.72 abc	6.32 a	0.36 cd	0.83 a	5.10 ab	6.72 a	0.98 a	0.51 abc
T21**	4.26 bcd	5.90 ab	0.43 abc	0.79 ab	3.98 b	6.86 a	0.94 a	0.38 c
T23**	5.06 ab	6.21 a	0.46 abc	0.80 ab	4.74 ab	6.64 a	0.95 a	0.43 abc
T24**	5.61 a	5.56 bcd	0.36 cd	0.72 bcd	4.49 ab	6.41 ab	0.97 a	0.41 bc

*Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure.
**Rhizobacterial isolates from the UP-PGPR culture collection.
***Commercial product.

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Table 4.4 The effect of two different inoculum doses of the 8 selected PGPR isolates on fresh mass of fertilised and unfertilised wheat seedlings grown in the greenhouse

	Fertilised plants		Non-fertilised plants	
	25 ml drench		25 ml drench	
Isolates	**Root mass (g)***	**Shoot mass (g) ***	**Root mass (g) ***	**Shoot mass (g)***
Untreated control	2.602 CDE	3.892 a	1.785 ND	2.534 ND
T06**	3.056 BCDE	4.111 a	1.775 ND	2.607 ND
T07**	2.813 BCDE	3.777 a	1.778 ND	2.501 ND
T11**	2.347 DE	3.796 a	1.721 ND	2.650 ND
T13**	2.865 BCDE	4.120 a	2.374 ND	2.668 ND
T19**	3.719 B	4.324 a	2.325 ND	3.230 ND
T21**	2.521 CDE	3.691 a	1.689 ND	2.563 ND
T23**	4.768 A	4.016 a	-	-
T24**	2.467 CDE	3.851 a	2.031 ND	2.443 ND
	12ml drench		**12ml drench**	
Isolates	**Root mass (g)**	**Shoot mass (g)**	**Root mass (g)**	**Shoot mass (g)**
Untreated control	2.602 CDE	3.892 a	1.785 ND	2.534 ND
T06**	2.441 CDE	3.664 a	1.843 ND	2.467 ND
T07**	2.858 BCDE	4.045 a	1.685 ND	2.326 ND
T11**	3.247 BCDE	3.932 a	2.216 ND	2.452 ND
T13**	3.298 BCD	3.895 a	2.289 ND	2.336 ND
T19**	2.995 BCDE	4.115 a	2.098 ND	2.456 ND
T21**	3.087 BCDE	3.742 a	2.214 ND	2.516 ND
T23**	2.273 E	2.947 b	2.828 ND	2.742 ND
T24**	3.384 BC	3.616 ab	2.074 ND	2.545 ND

ND = No significant difference
* Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure
Note, T23 non-fertilised at 25 ml no value due to experimental fault.
**Rhizobacterial isolates from the UP-PGPR culture collection.

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Table 4.5 The effect of two different inoculum doses of the 8 selected PGPR isolates on dry mass of fertilised and unfertilised wheat seedlings grown in the greenhouse

	Fertilised plants		Non-fertilised plants	
	25 ml drench*		25 ml drench*	
Isolates	**Root mass (g)**	**Shoot mass (g)**	**Root mass (g)**	**Shoot mass (g)**
Untreated control	0.304 ab	0.453 a	0.243 abcd	0.327 bcd
T06**	0.249 bc	0.491 a	0.205 bcde	0.331 abcd
T07**	0.205 c	0.435 ab	0.196 bcde	0.334 abcd
T11**	0.230 bc	0.455 a	0.177 cde	0.346 abcd
T13**	0.277 bc	0.466 a	0.254 ab	0.352 abcd
T19**	0.255 ab	0.481 a	0.250 abc	0.427 a
T21**	0.305 abc	0.475 a	0.167 de	0.340 abcd
T23**	0.357 a	0.483 a	-	-
T24**	0.247 bc	0.469 a	0.191 bcde	0.408 abc
	12ml drench*		12ml drench*	
Isolates	**Root mass (g)**	**Shoot mass (g)**	**Root mass (g)**	**Shoot mass (g)**
Untreated control	0.304 ab	0.453 a	0.243 abcd	0.327 bcd
T06**	0.235 bc	0.432 ab	0.195 bcde	0.323 cd
T07**	0.285 abc	0.492 a	0.146 e	0.297 d
T11**	0.291 abc	0.477 a	0.205 bcde	0.316 cd
T13**	0.295 ab	0.462 a	0.235 abcd	0.317 cd
T19**	0.261 bc	0.487 a	0.238 abcd	0.317 cd
T21**	0.273 abc	0.448 a	0.216 bcde	0.327 bcd
T23**	0.241 bc	0.366 b	0.301 a	0.424 ab
T24**	0.268 bc	0.440 ab	0.227 abcd	0.347 abcd

*Treatment means within the same column followed by the same letter do not differ significantly, (P=0.05) according to the Least Significant Difference (LSD) tests using the GLM procedure
Note, T23 non-fertilised at 25 ml no value due to experimental fault.
**Rhizobacterial isolates from the UP-PGPR culture collection.

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4.6 Figures

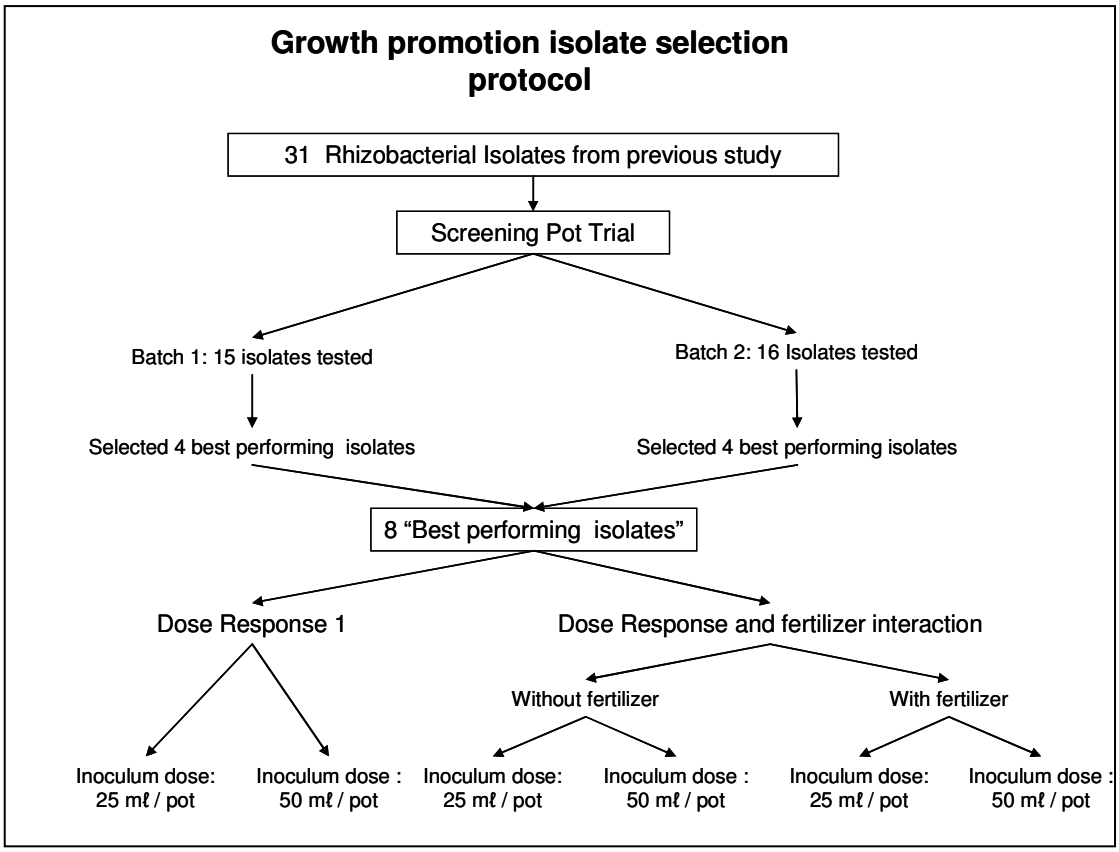

Figure 4.1; A diagrammatic representation of the trials and selection of the best growth promoting rhizobacterial isolates

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CHAPTER 5

Characterisation of plant growth promoting rhizobacteria isolates

Abstract

Characterisation of a plant growth promoting rhizobacteria (PGPR) isolate involves amongst others the study of traits that are important for the plant beneficial activities of the bacterium. In this study rhizobacterial isolates that showed promise in greenhouse trials were identified and selected modes of action (MOA) for growth promoting and biocontrol assessed. The isolates were identified based on the sequence data of the 16S rRNA region. For growth promotion the MOA that were assessed included *in vitro* assessment of phosphate solubilisation, indole acetic acid production and growth in nitrogen free media. For biological control the inhibition of selected wheat pathogens were tested in dual culture, chitinase and siderophore production assessed. The *in vitro* root colonisation ability of selected isolates was also determined. The majority of the isolates belong to the Bacilli group, with species from the genera *Bacillus, Lysinibacillus* and *Paenibacillus*. As expected, the isolates possess several of the MOA tested for. Information gained from the identification and MOA will assist with selection of specific isolates and tailoring the use of the PGPR to particular applications.

5.1 Introduction

Characterisation of the plant growth promoting rhizobacteria (PGPR) is of both scientific and commercial value and involves aspects such as the identification of the PGPR, determining of the modes of action, factors that affect the activity, and traits of commercial importance (Handelsman and Stabb 1996; Avis *et al.* 2008; Larkin *et al.* 1998). In essence characterisation of PGPR endeavours to understand their ecology and interactions with their surroundings better. This understanding of the isolate that is gained is important to overcome practical problems associated with the use of PGPR such as the inconsistency of performance in the field (Mercado-Blanko and Bakker 2007).

It is important to establish the identity of the PGPR isolates (Hirsch *et al.* 2010) because the identity allows comparison with known traits of the species or those of relatives. Such comparison can give valuable information and direction to further studies. From a commercial context the identity is also important. If the product requires registration, the identity would reveal negative aspects associated with the species such as pathogenicity to humans or animals and possible harmful effects on the environment. Isolates perceived as having a “low risk” can therefore be chosen to avoid excessive registration costs due to toxicological and environmental impact studies (Montesinos, 2003; Kohl *et al*. 2011).

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There are various modes of action (MOA) whereby PGPR can benefit plant growth. They can broadly be categorised into those causing a direct growth promoting effect and those indirectly benefiting the plant through the control of soilborne pathogens (Avis *et al.* 2008). MOA involved in direct growth promotion include nitrogen fixation, increased nutrient availability and production of compounds such as phytohormones that affect the plant's growth.

Plant growth promoting rhizobacteria contribute to increased nutrient availability by mechanisms such as asymbiotic nitrogen fixation (Vessey 2003) and siderophore production (Rosas 2007). Another way in which PGPR is able to increase nutrient availability is by solubilising nutrients such as phosphate which are present in the soil in a form that the plant is unable to utilise. Several bacterial genera including *Bacillus* and *Pseudomonas* are able to solubilise organic phosphate (Rodríguez & Fraga 1999).

Another important MOA whereby PGPR can improve plant growth is through the production of phytostimulatory molecules such as plant growth hormones. The most notable effect is usually an increase in the root surface area which results in better growth of the plant resulting from the production of indole acetic acid (IAA) (Malhotra and Sivestava 2009).. Several other hormones including cytokinins and gibberelins are also produced by PGPR. Furthermore PGPR can indirectly regulate the amount of ethylene present in the rhizosphere. By the production of the enzyme ACC-deaminase PGPR are able to lower the ethylene concentration, thereby decreasing the inhibitory effect ethylene has on root growth (Glick *et al.* 2007). Lastly PGPR are able to improve plant growth by facilitating other beneficial symbioses and by improving the tolerance of plants to stress (Avis *et al.* 2008).

PGPR are able to control soilborne pathogens in various ways. The most direct mechanism of control is through parasitism, whereby the bacterium directly targets the fungi (Leveau & Preston 2008). Related to parasitism are antibiosis and the production of lytic enzymes. Antibiosis is one of the most common MOA attributed to biocontrol PGPR (Raaijmakers *et al.* 2002). Antifungal metabolites produced by PGPR include kanosamine, pyrrolnitrin, viscosinamide, xanthobaccin, and zwittermycin A (Rosas 2007). PGPR also produce a range of volatiles that are fungistatic. The volatiles produced include ammonia, carbon dioxide and hydrogen cyanide (Kai & Piechulla 2009; Arora *et al.* 2007). Plant growth promoting rhizobacteria are able to produce various lytic enzymes that degrade fungal structures. Enzymes produced include chitinases, cellulases, glucanases, proteases and amylase (Whipps 2001, Rosas *et al.* 2007).

The competition for space and nutrients can also be an important MOA whereby PGPR exclude pathogens from the rhizosphere (Brussaard *et al.* 2007). One of the best studied examples of nutrient competition between PGPR and fungal pathogens is competition for iron,

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the availability of which is limited by the PGPR through the production of siderophores (Handelsman & Stabb 1996).

Root colonisation is also an important characteristic of a PGPR. Many authors including have stressed the importance of effective root colonisation if the isolate is to perform effectively and consistently in the field (Berenzi *et al.* 2001, Whipps 2001; Raaijmakers *et al.* 2008). Regardless of the mode of action, the isolate will not be effective if it is not able to survive and proliferate on the plant roots.

The aim of the current study was to identify, characterise and determine the MOA of selected PGPR isolates that were screened for plant growth promotion and biocontrol of root disease on wheat in previous trials. The isolates were identified by 16s rRNA sequencing, and assessed for MOA including dual culture antibiosis, chitinase production, siderophore production, phosphate solubilisation, growth in nitrogen free media, Indole acetic acid production, and root colonisation of wheat seedlings.

5.2 Materials and Methods

5.2.1 Cultures

All bacterial cultures were obtained from the UP-PGPR culture collection. Isolates were maintained using Microbank™ beads (Pro-Lab Diagnostics) stored at -70℃ and streaked onto nutrient agar (Biolab, Wadeville) as needed. Fungal cultures were obtained from the UP culture collection. *Rhizoctonia solani* UPGH122 was grown on potato dextrose agar (Biolab, Wadeville) and *Fusarium oxysporum* UPGH 132 and *Fusarium graminearum* WP4F on half-strength potato dextrose agar (Biolab). Further details are given in appendices 2 and 3.

5.2.2 Identification

For identification a pure culture of each isolate was 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).

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5.2.3 Modes of action determination

5.2.3.1 Dual culture assay to determine antibiosis

Inhibition of fungal pathogens by the bacterial isolates was determined by means of the dual culture method on Water-yeast agar (WYA) and Potato dextrose agar (PDA). Water yeast agar was used because it is a minimal medium that mimics the carbon-limiting environment of soil. The WYA contained 20g agar, 5g NaCl, 1g KH_2PO_4, 0.1g yeast extract in1L distilled water. A single isolate of the bacterium to be tested was stab inoculated in three places equidistant from the centre and each other onto a WYA plate. A 5mm diameter fungal plug was then placed in the centre of the plate between the bacterial spots (De Boer *et al.* 2007). Three replicate plates were used for each isolate. When the fungal colonies on the control plate without bacteria reached the edge of the plate (after approximately four days), the growth towards and away form the bacterial colonies was recorded and the % inhibition of mycelial growth calculated. The percentage inhibition of mycelial growth was calculated by means of the formula [(R2-R1)/R2] x100, with R1 being the distance of mycelial growth towards the bacterial colonies and R2 the maximum mycelial growth on the control plate. During the dual culture assay with *Rhizoctonia* abnormal mycelial growth was observed and recorded as well.

5.2.3.2 Chitinase activity

Chitinase production by the bacterial isolates was determined on two media, water agar with chitin (WAC) (Ajit *et al.* 2006) media and Chitin minimal media (CMM) (Felse & Panda 2000). Colloidal chitin for the media was prepared by grinding crab shell chitin (Sigma, Johannesburg South Africa) to a fine powder with a coffee grinder. Forty grams of the powdered chitin was dissolved in 400 ml concentrated hydrochloric acid and left overnight at 4℃ to digest. The colloidal chitin was precipitated from the acid by adding the chitin-acid solution to 5 litres cold water and recovered by filtration. To determine the chitin concentration of the filter cake a sample was weighed and dried in the oven overnight (adapted from Berger and Reynolds 1985; Hsu and Lockwood 1975). The WAC media was prepared by mixing 20g bacteriological agar (Biolab) with 0.4% colloidal chitin in one litre distilled water. The media pH was adjusted to 7 and autoclaved at 121℃ for 15 minutes (Ajit *et al.* 2006). Chitin minimal media was prepared by dissolving 1g $(NH_4)_2SO_4$, 1g K_2HPO_4, 1g NaCl, 0.1g $MgSO_4.7H_2$, 0.5g yeast extract, 0.4% colloidal chitin and 20g bacteriological agar in one litre agar. The pH was adjusted to 7 by the addition of NaOH and autoclaved at 121℃ for 15 minutes.

To determine whether the bacterial isolates produced chitin, each isolate was stab inoculated in triplicate onto water agar with chitin (WAC) (Ajit *et al.* 2006) media and Chitin minimal media (CMM) (Felse & Panda 2000). The plates were incubated at 25℃ and after seven days

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the presence of halos in the media was recorded. Both media are opaque and the formation of a clear halo around the bacterial colony indicates the production of chitinase.

5.2.3.3 Assessment of siderophore production

Siderophore production by the isolates was determined on the chrome azurol S (CAS) –agar. Chrome azurol S -agar plates were prepared as described by Hassen 2007. This method is a modification of Schwyn and Nielands (1987) through the substitution of piperazine-1,4-bis(2-ethane sulfonic acid) (PIPES) with 3-(N-morpholino propane sulphonic acid) (MOPS). The CAS media was prepared by separately making CAS indicator and basal solutions and 20ml sterile 50% glucose solution. The CAS Indicator solution consisted of 60.5mg chrome azurol S in 50ml ddH_2O mixed with 10ml Fe^{3+}. A solution of 72.0mg HDTMA in 40ml ddH_2O was slowly added to the above mixture. The result is a dark blue solution with a total volume of 100ml. CAS basal medium was prepared by dissolving 30g MOPS, 0.1g NH_4Cl, 0.5g NaCl, and 0.3 KH_2PO_4 in 880 ml ddH_2O and adjusting the pH to 6.8 with 6M NaOH. Fifteen grams agar was added to the solution while stirring. The solutions were autoclaved separately. After autoclaving the solutions were cooled to 50℃ and the glucose solution added to the basal medium. The 100ml CAS indicator solution was then added to the basal medium by carefully pouring it in along the flask wall. The resulting blue-green agar solution was poured into Petri dishes. The CAS-agar plates were overlaid with a thin layer (5 mm) of Nutrient agar. These plates were then stab inoculated with the bacterial isolates from NA in triplicate and incubated at 25℃. Halo formation was recorded after three days (modified from Adesina *et al.* 2007).

5.2.3.4 Phosphate solubilisation

The ability of bacterial isolates to solubilise phosphate was determined on Pikovskaya medium (PVK) and the National Botanical Research Institute's phosphate growth medium (NBRIY) (Nautiyal 1999). The PVK media contained per litre dH_2O 10g glucose, 5g $Ca_3(PO_4)_2$, 0.5g $(NH_4)_2SO_4$, 0.2g NaCl, 0.1g $MgSO_4.7H_2O$, 0.2g KCl, 0.5g yeast extract, 0.002g $MnSO_4.H_2O$, 0.002g $FeSO_4.7H_2O$ and 15g bacteriological agar. The pH was adjusted to 7 before autoclaving. The NBRIY media was prepared as described for the PVK with the omission of the yeast extract. Bacterial isolates were stab inoculated onto the agar using a sterile inoculation needle. Four isolates were inoculated on each plate using three replicate plates. After 14 days incubation at 25℃ the colonies and halos' (if present) diameters were measured. The halo size was calculated by subtracting the colony diameter from the halo diameter.

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5.2.3.5 Assessment of atmospheric Nitrogen fixing ability

The ability of bacterial isolates to fix atmospheric nitrogen was determined through a preliminary assay that determined the ability of the bacteria to grow in nitrogen free media (Baldani and Dobereiner 1980, Caceres 1982). Nitrogen-free semisolid media (NFb) contained 5g D-L malic acid, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 4g KOH, 0.1g NaCl, 0.02g $CaCl_2$, 2ml 5% Bromothymol blue (in ethanol), 4ml FeEDTA, 1ml vitamin solution, 2ml trace element solution and 1.75g agar in 1 litre dH_2O. The vitamin solution contained 10mg biotin and 20 mg pyridoxine in 100 ml dH_2O and the trace element solution consisted of $Na_2MoO_4.2H_2O$ (200mg), $MnSO_4.H_2O$ (235 mg), H_3BO_3 (280 mg), $CuSO_4.5H_2O$ (8 mg), $ZnSO_4.7H_2O$ (24 mg) in 200 mlH_2O. The media was prepared and then 10 ml thereof dispensed into a 20 ml vial and autoclaved. A loop full of a single bacterial isolate from a single colony was transferred to vials containing NFb. The vials were incubated at 25℃ and observed for the formation of a white halo or pellicle indicating bacterial growth after 4 and 7 days. Vials were incubated for a further three days after the first observation because it was difficult to determine the presence or absence of growth in some vials.

5.2.3.6 IAA production

IAA production by rhizobacteria was assessed by the S2/1 method of Glickman *et al.* (1995). Isolates were inoculated into 100 ml nutrient broth and incubated in a rotary shaker for 48 hours at 25℃ and 150rpm. The bacterial culture was centrifuged in 50ml conical plastic tubes at 3000 x g for 10 minutes. One ml of the supernatant was transferred to a test tube and two ml Salkowski's reagent was added. After 20 minutes the absorbance was measured at 530nm in a spectrophotometer. One ml sterile broth with 2ml Salkowski's reagent was used as a blank. Salkowski's reagent was prepared by carefully adding 4.5g $FeCl_3$ to one litre 10.8 M (67%) H_2SO_4. A standard IAA curve was prepared by means of a series of IAA concentrations ranging from 0 to 200 µl in sterile nutrient broth.

5.2.4 Root colonisation

Three methods were evaluated to determine the ability of selected bacterial isolates to colonise wheat roots. These were the *in vitro* root colonisation assay performed in Petri dishes (Hassen 2007), the *in vitro* root colonisation bioassay performed in test tubes (Silva *et al.* 2003) and a modified paper doll assay (International Seed Testing Association (ISTA), www.seedtest.org). For all the assays the wheat seeds were surface sterilised by placing them in 70% ethanol for 5 min, then in 1% sodium hypochlorite for 1min followed by rinsing three times in sterile tap water. Bacterial inoculum was prepared as described for the growth promotion trials in appendix 3.

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The *in vitro* root colonisation in Petri-dishes was performed as described by Hassen (2007). For each bacterial treatment 15 surface sterilised seeds were placed in a 90mm Petri dish containing a filter paper disk. The filter disks were moistened with sterile water. One ml bacterial inoculum was added to the seeds in each Petri dish. The seeds were then incubated at 25℃ for 5 days in the dark for root development.

For the *in vitro* root colonisation bioassay in test tubes a single bacterized seed was transferred to a test tube containing 0.6% water agar. Each treatment was replicated 5 times. Daily visual inspections were performed to determine bacterial growth around the roots visible as a turbid zone in the rhizosphere area, indicating root colonisation. A second trail was conducted in which the seeds were pre-germinated and then treated with the bacteria before placing them in the tubes.

For the modified paper doll root colonisation assay bacterized seeds were placed on seedling germination paper in a single line 2cm from the upper edge of the paper and then rolled into a paper doll (Fig 6.3). The paper dolls were then placed upright into an incubator and incubated at 25℃. After 7 days the roots were excised and 0.5 g roots placed in 45ml sterile Ringer's solution in a beaker. The beakers were then sonicated for 30 seconds and a dilution series prepared from the resultant solution. After counting the number of resultant colonies which developed the number of colony forming units was calculated.

5.2.5 In vitro compatibility assay

To assess which of the selected 8 best performing growth promotion isolates would be compatible with each other in mixtures, an *in vitro* inhibition assay was performed. The method used was adapted from the antimicrobial susceptibility test using the disk diffusion method (Ortez, 2005). Isolates were grown in 100 ml Nutrient broth on a rotary shaker at 160 Rpm at 30℃ for approximately 24 hours. The broth culture was diluted ten times by placing 100 µ l broth cultures in 900µl sterile broth in a sterile Eppendorf tube. One hundred µl of the diluted culture was spread onto NA plates. Four 5 mm diameter filter disks (Watman no 1), each soaked for 5 minutes in a different undiluted broth culture of one of the respective test isolates were placed on the seeded plates. Three replicate plates were prepared for each treatment and incubated at 25℃. After 24 hours the plates were visually assessed for the presence of inhibition zones around the filter disks. Isolate combinations were scored as incompatible if a there was a clear zone larger than 1mm surrounding the filterpaper disk.

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5.3 Results

5.3.1 Identification

The sequencing results are given in table 5.1. The majority of the isolates were identified as *Bacillus* species of which 8 were *B. cereus*. Seven isolate were *Paenibacillus* sp, mainly *P. alvei*. Two isolates were identified as *Lysinibacillus sphaericus* or *fusiformis*.

5.3.2 Modes of action

The dual culture results are shown in table 5.2. In general the largest mycelial inhibition zones were observed with the *Fusarium* isolates grown on PDA. *F. oxysporum* UPGH 132 was only inhibited by isolate T29 on PDA where a 24% inhibition was observed. No other isolates inhibited this pathogen on either PDA or WYA. *Fusarium graminearum* WP4F was inhibited by several isolate on both PDA and WYA. Of the 31 isolates tested only 7 did not inhibit this pathogen on PDA. On WYA 10 isolates were not inhibitory. The greatest inhibition against *F. graminearum* WP4F was observed with isolates T10 (49%), T27 (48%), T29 (46%) and T25 (46%) on PDA. On WYA isolates T01 (32%), T04 (32%), T09 (34%) and T18 (32%) caused the most inhibition of mycelial growth.

The degree of inhibition of the *R. solani* by the bacterial isolates was low (10% or less) on both the PDA and WYA. The largest inhibition zones on PDA was caused by isolates T08 and T09 resulting in 8% and 9% inhibition respectively. Fifteen other isolates also inhibited *R. solani* on PDA. On the WYA 13 isolates inhibited the growth of *R. solani.* Isolates T21 and T31 resulted in the largest zones of inhibition. In addition to inhibition of the mycelial growth of *R. solani*, the mycelia surrounding some bacterial isolates had an altered appearance. The mycelium grew less densely and was more highly branched. In general there was no discrepancy between the productions of inhibition zones by the bacteria on the two media.

The results for siderophore production, chitinase production, phosphate solubilisation, IAA production and growth in N-free media are presented in table 5.3. Chitinase production by the isolates was assessed on two different media namely CCM and WAC. The CCM contained nutrients whereas the WCA only consisted of colloidal chitin, agar and water. Of the 31 isolates tested 14 gave a positive reaction for chitinase on WCA and 19 on CCM. The isolates with the greatest chitinolytic activity were T06, T10, T17, T23, T25, and T28 on WCA and T02, T04, T15, T23, and T27 on CCM. A large number of isolates produced clear halos on the CCM media rather than on the WCA media. Two isolates T16 and T22 produced chitinase on the WCA and not on the CCM media.

Several isolates showed an ability to solubilise phosphate. However the two media tested gave different results. On the yeast free media the colonies were much smaller and fewer

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isolates produced clear halos. On PVK (the media containing yeast extract) clear halos were observed with 22 of the 31 isolates. The isolates T11, T12, T13, T14, T23 and T26, produced the largest clear zones indicating phosphate solubilisation on PVK. On NBRIY (the media without yeast extract) only 10 isolates tested positive for phosphate solubilisation. On the NBRIY isolate T12, T13, and T14 were the most efficient phosphate solubilizers. Several isolates that solubilised P in the PVK did not solubilise P in the NYBR.

Siderophore production was assessed using the CAS media overlay method of Adesina *et al.* (2007). Fifteen of the 31 isolates caused a change in the colour of the CAS media indicating that they produce siderophores. The ability of isolates to grow on nitrogen free media was assessed by pellicle formation in Nitrogen-free semisolid media (NFB-free) (Baldani and Dobereiner 1980). Sixteen isolates were able to grow and produce a pellicle in the NFB-free media. The isolates were T04, T05, T06, T07, T11, T12, T13, T14, T15, T18, T19, T20, T21, T22, 28, T29.

IAA produced by the bacteria growing in nutrient media was assessed using regent S2/1 as described by Glickman *et al.* (1995). With this method, only IAA concentrations below 10 µl/ml were detected for all the bacteria. Isolates T03, T12, T14, T13 and T16 produced the most IAA in broth culture (Table 5.3).

5.3.3 Root colonisation

Various challenges were encountered with the assays used to determine root colonisation ability of the isolates. In the test tube assay the seeds did not germinate uniformly. As a result the test tube assay was repeated by selecting pre-germinated seeds and inoculating them with the bacterial suspensions. With the test tube assay root colonisation is determined by visual assessment of the absence or presence of a turbid zone around the roots. No turbid zone was observed for any of the isolates. A modified paper doll assay was also used to assess root colonisation. The results are shown in table 5.4. The counts are however estimates since a problem with bacterial contamination were encountered.

5.3.4 In vitro compatibility

Results of the *in vitro* compatibility assay are presented in the form of a matrix (table 5.5). Isolates were rated as incompatible if there was a clear zone, larger than 1mm, around the filter paper disk. Most of the bacterial combinations were compatible. Isolate T13 inhibited the growth of isolate T07 causing an inhibition zone of about 3mm. The isolate T24 had a wide inhibition range, causing inhibition zones toward isolates T06, T07, T13, T21 and T23. Some isolate combinations did not show definite inhibition zones, but their colonies did not grow

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over or touch each other. These isolate combinations were T06 and T07, isolates T21 and T13 and isolates T23 and T21.

5.4 Discussion

Selected isolates with growth promotion and biocontrol activity were identified by their 16S rRNA sequences and found to be from the Bacillaceae family. The results of the assay conducted to determine the modes of action of the isolates indicate that these PGPR have a wide range of activities that contribute to their biocontrol and growth promoting effects.

The identity of the PGPR can reveal important information about the PGPR strain (Mathre 1999).The isolates were identified as bacilli characteristically produce endospores (Slepecky and Hemphill, 2006), and are well known growth promoters and biocontrol agents (Lugtenberg and Kamilova, 2009). The production of endospores by this group is an advantage since spore formers are usually easier to formulate and have a longer shelf life than non-spore forming bacteria (Berg, 2009). Labuschagne *et al.* (2010) reported that *Paenibacillus alvei* was effective as a control agent of root and crown rot of wheat. The Bacilli are also not restricted to growth promotion and control of fungal pathogens. For example *B thuringiensis* is a well known insecticidal bacterium (Jacobsen *et al.* 2004) and *Bacillus sphaericus* was reported as being a particularly active biocontrol agent of nematodes (Tian *et al.* 2007).

Unfortunately the Bacilli are also well known for producing a range of secondary metabolites, many of which are toxic. Some strains of *Bacillus cereus* for instance produce emetic and enterotoxins that are responsible for food poisoning (Granum and Lud 1997). The isolates should therefore be thoroughly investigated to ensure that they are safe to use. In the soilborne setting this is of importance because large quantities of this product will be used. It would be unwise to increase possible pathogens beyond the numbers that occur naturally. Furthermore, registration of a PGPR that is associated with harmful organisms is difficult. It is therefore better to develop a PGPR for commercialisation that is not a potential harmful species (Montesinos, 2003; Kohl *et al.* 2011).

Literature has also shown that the Bacilli produce a range of compounds and have several MOA that make them effective PGPR. The assays for MOA showed that the isolates in this study possess a wide range of activities that contribute to their beneficial effects. There was in general no clear distinction between the biocontrol and growth promoting PGPR. Within literature it is apparent that the PGPR are versatile organisms capable of influencing plant health in a variety of ways (Avis *et al.* 2008).Though the assays were *in vitro* assays indicating that they should be interpreted with caution since the bacteria do not always

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produce the same metabolites *in vitro* as they do in the soil (Campbell 1989; Smyth *et al*. 2011), insight can be gained from the results to guide further selection and application of the PGPR.

A general trend that was observed among the assays, whether phosphate solubilisation, chitinase production or the dual culture assay, was that on the nutrient poor media the effect was less than on nutrient rich media. For chitinase production research has shown that the genes regulating chitinase production is affected by low nutrient availability (Pal and McSpadden-Gardener 2006; Felese and Panda, 2000). Environmental conditions beside nutrient availability are able to influence the production of antifungal metabolites (Raaijmakers *et al*. 2002) and other plant growth promoting activities of the PGPR (Bashan 1998). Since soil is generally a nutrient limiting environment (De Boer *et al.* 2001), such information is important to the understanding and use of PGPR for increasing crop productivity.

The activity and consistency of PGPR may be enhanced by careful management of the soil. Amendment of soil with chitin for instance has been shown to increase the numbers of chitinolytic bacteria in the soil and improve biocontrol of root diseases (Sarravanakumar *et al.* 2007). Welbaum *et al.* (2004) furthermore suggests specific nutrients can be added to the soil that would stimulate or "feed" a targeted group of beneficial PGPR, whether already present or introduced. The use of mineral amendments to enhance the biocontrol of fungi has also been proposed as a way to enhance biocontrol (Duffy and Defago, 1999).

In the current study, determining the root colonisation ability of selected isolates posed some challenges in that the wheat seeds did not germinate uniformly. The test tube assay was described as a rapid assay to determine root colonisation of tomato by PGPR isolates by Silva *et al.* (2003), in which root colonisation is visually assessed for the presence of a turbid zone surrounding the roots. In our work no turbid zones were observed. The probable reason for this is that the wheat seedlings grow faster than tomato seedlings. The roots of the wheat seedlings reached the bottom of the tube within three days. Due to this rapid growth it is unlikely that the bacteria on the roots would reach populations high enough to be visible within that time (Compant *et al.* 2005).

A modified paper doll assay was also used to determine root colonisation of selected isolates. Determination of root colonisation using the modified paper doll assay was more successful than the other colonisation methods. A problem that was however encountered with this method was a high incidence of contamination with bacteria. The reason for this is difficult to explain since all materials and the seeds were thoroughly sterilised before use. An alternative method for initial screening of effective root colonisers is that of Simons *et al.* (1996). In their protocol effective root colonising rhizobacteria are selected for by inoculating the bacterial isolate on plants grown in tubes containing sterile quartz sand. The system was successful for

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determining root colonisation on tomato, radish, wheat and potato plants. An alternative would be to use mutant isolates that have antibiotic resistance. The inoculated strain can then be selected for by planting onto antibiotic containing media (Hassen and Labuschagne 2010).

Despite the problems encountered, the method gives a good indication of the root colonisation abilities of the isolates. Generally 10^7-10^9 cfu culturable bacteria are found per gram rhizosphere soil (Benizri *et al.* 2001). It is well known that successful rhizosphere colonisation is important for the success of a PGPR (Raaijmakers *et al.*2008). However the threshold colonisation value should be established for each individual strain or application (Somers *et al.* 2004). For instance it has been shown that the threshold level for DAPG producing pseudomonads to protect against take-all of wheat is 10^5 cfu/g root (Mercado-Blanco *et al*. 2007) and that the population of *P. fluorescence* strain WCS365, known to be an effective root colonising PGPR, ranges form 10^6 near root base to 10^2 near root tips. The isolates tested in this study can therefore be considered average (10^4 cfu/gram root) to good (10^7cfu/gram root) root colonisers (Table 5.3).

The characterisation of the PGPR showed that the isolates belonged to the Bacilli group, including genera of *Bacillus, Lysinibacillus* and *Paenibacillus*. These groups have been reported before in literature as PGPR, and as expected they show activity of several of the common MOA. The results show that caution should be taken to insure that harmful isolate are not used for further trials since many *Bacillus* strains are known to produce toxic metabolites. The MOA results give valuable insight regarding the PGPR and can be utilised to enhance the efficacy of the PGPR in the filed. These results are valuable for selection of PGPR for further and more detailed studies.

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5.5 Figures and tables

Table 5.1. The identification of selected rhizobacterial isolates from this study by means of 16s rRNA sequence analysis

Isolate code	UP-culture collection code	16S Identification
T06	KBS 1F2	*Paenibacillus sp. / P. alvei*
T07	KBS 1F3	*Paenibacillus sp. / P. alvei*
T11	KBS 5K(T)	*Bacillus thuringiensis / cereus*
T13	KBS 9-0	*Bacillus sp. / B. pumilus*
T16	KFP 9H(T)	*Paenibacillus sp. / P. alvei*
T17	KFP 9I	*Bacillus thuringiensis / cereus*
T18	NAS 10B	*Bacillus cereus*
T19	NAS 10E	*Lysinibacillus sphaericus / L. fusiformis*
T20	NAS 3D	*Lysinibacillus fusiformis / L. sphaericus*
T21	NAS 3E	*Bacillus cereus*
T22	NAS 3J(T)	*Paenibacillus alvei*
T23	NAS 6G5	*Bacillus cereus*
T24	NAS 6G6	*Paenibacillus alvei*
T25	NAS 6M	*Bacillus cereus*
T26	NAS 3J II(A)	*Bacillus cereus*
T27	NFP 9(A)	*Bacillus cereus*
T28	NFP 9D(T)	*Bacillus cereus*
T29	NFP 9D I(A)	*Paenibacillus alvei*
T30	NFP 9D II(A)	*Paenibacillus alvei*
T31	NFP 9E	*Bacillus cereus*

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Table 5.2. Inhibition of *Rhizoctonia solani*, *Fusarium oxysporum and Fusarium graminearum* mycelial growth by rhizobacterial isolates on potato dextrose agar and water yeast agar

	% mycelial growth inhibition*						
	R. solani			*F. oxysporum*		F. graminearum	
Isolates	PDA[a]	WYA[b]	% changed zone on WYA**	PDA	WYA	PDA	WYA
T01	4	3	9	0	NG[c]	41	32
T02	4	2	20	0	G[c]	42	20
T03	0	0	0	0	NG	0	0
T04	2	0	0	0	NG	22	32
T05	0	0	0	0	NG	0	21
T06	4	4	22	0	G	41	28
T07	0	0	0	0	NG	0	5
T08	8	4	18	0	G	30	32
T09	9	5	18	0	G	32	34
T10	2	3	16	0	G	49	23
T11	5	5	19	0	G	43	0
T12	0	0	0	0	NG	13	3
T13	0	0	0	0	NG	13	-1
T14	0	0	0	0	NG	7	0
T15	0	0	0	0	NG	0	18
T16	0	0	0	0	NG	0	0
T17	0	4	19	0	G	13	16
T18	3	3	15	0	G	41	32
T19	4	0	0	0	NG	0	0
T20	0	0	0	0	NG	6	1
T21	5	10	19	0	G	14	30
T22	0	0	0	0	NG	0	0
T23	4	3	18	0	G	0	2
T24	0	0	0	0	NG	0	g
T25	3	1	17	0	G	46	2
T26	4	0	16	0	G	37	22
T27	2	1	10	0	NG	48	26
T28	1	2	15	0	G	7	22
T29	0	0	0	45	NG	46	0
T30	0	0	0	0	NG	0	0
T31	2	6	20	0	G	24	29

*Mycelial growth inhibition was calculated as [(R – r)/ R] x 100 where R is mycelial growth away from the bacterial colony (the maximum growth of fungal mycelia) and r is the mycelia growth towards the bacteria.
**% changed zone on WYA calculated as for mycelial inhibition representing the area of *R. solani* mycelia that showed an altered growth being less dense and more branched.
[a] PDA = Potato dextrose agar
[b] WYA = Water yeast agar
[c] For interactions where no inhibition zone was present, the bacterial isolates were rated by whether the fungal mycelium grew over the bacterial colony or not. G = overgrown; N G= not overgrown.

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Table 5.3. Specific modes of action exhibited by the rhizobacterial isolates

Isolates	Siderophore production[a]	Phosphate solubilisation[b]		Chitinolytic activity[c]		N-fixation[d]	IAA production[e]
		PVK	NBRIYR	WAC	CMM	N-free media	
T01	+	+	-	-	+	-	0.06
T02	+	-	-	+	++	-	0.04
T03	+	+	+	-	+	-	0.16
T04	-	+	+	-	++	+	0.03
T05	-	-	-	-	-	+	0.05
T06	-	+	-	++	+	+	0.00
T07	-	-	-	-	-	+	0.05
T08	+	+	-	+	+	-	0.03
T09	+	+	-	+	+	-	0.03
T10	-	++	-	++	+	-	0.01
T11	-	+++	-	+	+	+	0.01
T12	+	+++	+++	-	-	+	0.14
T13	-	+++	+++	-	-	+	0.10
T14	+	+++	+++	-	-	+	0.11
T15	-	-	-	-	++	+	0.00
T16	+	+	+	+	-	-	0.10
T17	-	+	-	++	+	-	-0.03
T18	+	-	-	-	+	+	0.00
T19	-	-	-	-	-	+	0.09
T20	-	-	-	-	-	+	0.00
T21	-	-	-	-	+	+	-0.02
T22	+	+	+	+	-	-	0.04
T23	-	+++	-	++	++	-	-0.01
T24	+	+	+	-	-	-	0.08
T25	-	+	-	++	+	-	-0.01
T26	+	+++	-	+	+	-	0.02
T27	+	+	-	+	++	-	0.02
T28	-	+	-	++	+	+	0.02
T29	+	++	++	-	-	+	0.04
T30	+	+	+	-	-	-	0.09
T31	-	-	-	-	+	-	0.01

[a]The presence of a halo on CAS agar plates due to the presence of siderophores, + = the presence of a halo, - = no halo.
[b] Phosphate solubilisation was assessed on Pikovskaya (PVK) and the National Botanical Research Institute's phosphate growth medium (NBRIY) agar: – = no clearing zone, + = 0-1 mm zone, ++ = 1-2 mm zone, +++ = 2-3mm zone.
[c] Chitinolytic activity was determined by measuring the diameter of the clearing zone on chitin minimal medium (CMM) and water agar containing chitin (WCA): 0 = no clearing zone; + = 1-2 mm zone, ++ = 2-4 mm zone surrounding the bacterial colony.
[d] Nitrogen fixation was determined by pellicle formation by bacterial isolates inoculated in N-free media prepared as described by Baldani and Dobereiner (1980) and Caceres (1982).
- = no pellicle; + = presence of pellicle.
[e] optical density readings, at 530nm, relating to the amount of IAA produced, amounts could not be determined as the values were less than could be reliably determined from the standard curve (concentration 10 µl IAA = absorbance value of 0.18, 20 µl IAA = absorbance 0.24).

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Table 5.4: Ability of selected rhizobacterial isolates to colonise the roots of wheat seedling germinated in paper dolls

isolates	cfu/gram root	log cfu/gram root
T06	1.9×10^4	4.28
T07	2.85×10^5	5.45
T10	3.0×10^4	4.48
T11	1.05×10^6	6.02
T13	1.95×10^5	5.29
T15	2.75×10^5	5.44
T21	1.2×10^6	6.06
T22	2.0×10^6	6.30
T23	1.0×10^7	7.01
T24	Spreader, on plates up to 10^{6*}	
T25	2.0×10^6	6.30
T27	3.8×10^6	6.58
T28	1.5×10^6	6.18
T29	2.0×10^4	4.30
T31	5.0×10^4	4.70

*Colony forming units for isolate T24 could not be determined since this isolate does not form distinct colonies but spreads over the whole Petri dish.

Table 5.5. *In vitro* compatibility of eight rhizobacterial isolates selected as effective plant growth promoters

Isolates*	TO6	T07	T11	T13	T19	T21	T23	T24
T06	-	✓	✓	✓	✓	✓	✓	x
T07	✓	-	✓	x	✓	✓	✓	x
T11	✓	✓	-	✓	✓	✓	✓	✓
T13	✓	✓	✓	-	✓	✓	✓	x
T19	✓	✓	✓	✓	-	✓	✓	✓
T21	✓	✓	✓	✓	✓	-	✓	x
T23	✓	✓	✓	✓	✓	✓	-	x
T24	✓	✓	✓	✓	✓	✓	✓	-

✓ isolate that were compatible, no zone of inhibition was present.
X, isolate combinations that were incompatible, a zone of inhibition larger that 1mm was observed.
* The isolates listed vertically in the column are those that were spread on the agar, isolates in the horizontal row are those applied to the filterpaper disk.

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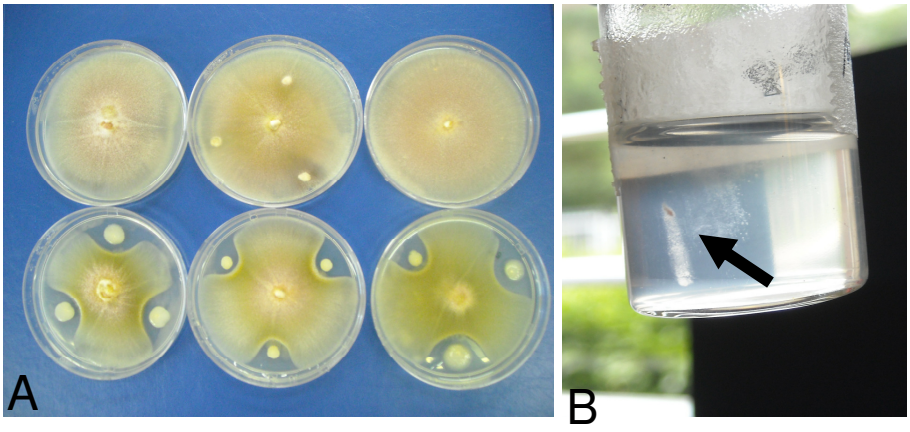

Figure 6.1 A) Inhibition zones indicating antibiosis activity of plant growth promoting rhizobacterial isolates against *Fusarium graminearum* WP4F grown on potato dextrose agar. B): Pellicle formation indicated by arrow in N-free media.

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CHAPTER 6

Selection of the best performing plant growth promoting rhizobacteria isolates

Abstract

The large quantity of data generated during evaluation of plant growth promoting rhizobacteria (PGPR) poses a challenge with regards to selecting the most effective isolates. Such data includes results from various *in vitro* assays as well as greenhouse trials. In previous trials (Chapters 3, 4, and 5), 31 PGPR isolates were assessed for their biocontrol and growth promoting activities on wheat. Described in this chapter is the development of a protocol for selection and comparison of those PGPR isolates and subsequent selection procedure of the best isolates. The isolates were rated based on the results obtained from *in vitro* and greenhouse assessment. This rating was used to select the top performing isolates. For each of the best isolates a spider diagram was constructed to visually represent the isolate characteristics. The best performing isolates selected were a) T10 (*Bacillus cereus*) for control of *R. solani*, b) T28 (*Bacillus cereus*) for control of *Fusarium oxysporum*, c) T31 (*Bacillus cereus*) for control of *F. graminearum*, d) T13 (*Bacillus sp. / Bacillus pumilus*) for growth promotion of wheat and e) T29 (*Paenibacillus alvei*) as the most versatile isolate. During the protocol development stage it was demonstrated that care should be taken to ensure that effective isolate are not excluded due to bias within the selection process. The spider diagrams proved to be a valuable tool for selection and comparison of the rhizobacterial isolates.

6.1 Introduction

Which one or two isolates of those that have been screened is the best? This is the final question of a project involving screening and selection of plant growth promoting rhizobacteria (PGPR), whether for research or commercial purposes. The importance of this question is highlighted by the fact that few PGPR are persistently effective when applied in the field (Bashan 1998). Literature abounds with work regarding PGPR, especially the isolation of PGPR for improving the growth of various crops. Evaluation of the research has shown that there is no universal approach to obtain an effective PGPR. Each project should be carefully planed and the steps considered in detail. Special attention should be given to the end goal or intended application (Schisler and Slininger 1997). Recently Kohl *et al.* (2011) and Pliego *et al.* (2001) have highlighted the importance of appropriate screening and selection of potential PGPR to ensure reliable, effective and economically viable PGPR products.

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Most PGPR screening protocols involve a hierarchal screening whereby isolates considered ineffective are eliminated at each step (Knudsen *et al.* 1997).The decision of which isolates to discard or retain for further screens depends on the type of assay selected as well as the desired outcome. Whereas most published papers addresses the importance of correctly selecting the assay, few mention in detail how the results were evaluated and the isolate selected. The question thus remains: how, based on the assay results, will the best PGPR isolate be chosen? Examples of protocols in which the detail of selection is described are those of Faltin *et al.* (2004) and Berg *et al*. (2001).

To select PGPR for the control of *Rhizoctonia solani* Faltin *et al.* (2004) used a protocol whereby rhizosphere isolates received points based on the results of specifically selected assays. The screening included *in vitro* assays such as the dual culture inhibition assay, and detection of siderophores and cell wall degrading enzymes. Plant growth promotion assays and *in planta* biocontrol experiments were also included. For each *in vitro* assay a positive result awarded the corresponding isolate one point whereas two points were awarded for efficacy in greenhouse assays. Berg *et al.* (2001) described a similar procedure for the selection of PGPR to control *Verticillium dahliae* in strawberry. In their assessment the effect of the inoculants on the growth of strawberry plants also were awarded more points than *in vitro* assays.

In the previous chapters PGPR isolates from the University of Pretoria PGPR culture collection were assessed in the greenhouse for growth promotion of wheat seedlings and biocontrol of root rot. Furthermore selected isolates were identified and various modes of action (MOA) and characteristics were also determined *in vitro*. This chapter describes the development of a PGPR evaluation protocol and the subsequent selection of PGPR for growth promotion and biocontrol of root disease on wheat based on the results of the greenhouse screening and *in vitro* characterisation.

6.2 Methods

Isolates were selected in a stepwise manner using a rating procedure similar to that of Berg *et al.* (2001). The best isolates for biological control of *R. solani*, *F. oxysporum*, *F. graminearum* and growth promoting on wheat were selected based on their performance in greenhouse trials (chapters 3 and 4). Isolates were then rated using a scoring system and spider diagrams constructed for each isolate to visually represent these scores. Appendix 5 shows in detail how the ratings were awarded.

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6.3 Results

Based on efficacy in the greenhouse (chapters 3 and 4) and score received (Appendix 5) isolates T10, T29, T22, T25, T16 were selected as the best isolates for control of *R. solani*, T28, T15, T19, T29, for control of F. oxysporum and T31, T23, T24, T28 for control of F. graminearum. For growth promoting the isolates T06, T07, T11, T13, T19, T21, T23 and T24 were selected. Spider diagrams were constructed for these isolates to give a visual representation of their attributes. The spider diagrams were constructed using in Microsoft Excel®, using the "Radar" chart function, and the ratings presented in Appendix 5.

Evaluation of the scores (Appendix 5 and 6) and spider diagrams (Figures 6.1 to 6.5) indicated the following isolates to be the best performing: a) for control of *R. solani* T10 (*Bacillus cereus*), b) for control of *F. oxysporum* T28 (*Bacillus cereus*), c) for control of *F. graminearum* T31 (*Bacillus cereus*), d) for growth promotion of wheat T13 (*Bacillus sp. / Bacillus pumilus*) and e) the most versatile isolate, T29 (*Paenibacillus alvei*).

6.4 Discussion

The aim of this chapter was to select the most effective isolate for growth promoting of wheat and biocontrol of root and crown rot caused by *R. solani, F. oxysporum*, and *F. graminearum*. The selection strategy was to select the top isolates from the greenhouse trials and apply a rating to these isolates followed by construction of spider diagrams based on the results obtained in the various assays to assist with selection of the most effective isolates. Fifteen isolates were selected based on greenhouse performance. Of these the following isolates were identified as being the most promising; T10 (*Bacillus cereus*), for control of R. solani, T28 (*Bacillus cereus*), for control of *F. oxysporum*, T31 (*Bacillus cereus*), for control of *F. graminearum*, T13 (*Bacillus sp. / Bacillus pumilus*) as best growth promoter and T29 (*Paenibacillus alvei*) as the most versatile isolate.

The best biocontrol isolates (T10, T28, T31 and T13) all inhibited the growth of both *F. graminearum* and *R. solani* in dual culture. Though *in vitro* inhibition results should be viewed with caution (Vessey 2003), it indicates that the mode of action for these isolates is probably the production of antifungal metabolites. The PGPR were also selected because they tested positive for the production of chitinase. The ability to produce chitinase is important since it has been associated with biocontrol of several soilborne pathogens such as *Fusarium oxysporum*, and *Rhizoctonia solani* (Ajit *et al.* 2006).

The effective biocontrol isolates also possessed several MOA associated with growth promotion such as phosphate solubilisation. Though not directly related to biocontrol, these attributes can help to improve the performance of the isolate (Avis *et al.* 2008). Growth in

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Nitrogen free media for instance, is hypothesized to confer a competitive advantage to the isolate that improves rhizosphere colonisation and persistence (Dobbelaere *et al.* 2003).

Isolate T31 was chosen as the best growth promotion isolate since it was a good performer in the greenhouse and has the important MOA of phosphate solubilisation and Indole acetic acid (IAA) production. Phosphate solubilisation is an important trait for a PGPR to have since although farmers apply phosphate to crops, it quickly becomes unavailable for use by the plant (Rodríguez and Fraga, 1999). As such this trait is especially important for farmers (F. Denner, personal communication). Indole acetic acid production is a good trait since this hormone stimulates increased root growth. Furthermore lower levels of PGPR are needed to cause an effect upon plant growth since the hormone is active in small amounts (Vessey 2003). IAA production has also been found by other researchers to be an important MOA contributing to increased growth of wheat plants (Dobbelaere *et al.* 1999).

Isolate T29 (*Paenibacillus alvei*) was identified as the most versatile isolate. The greenhouse results showed that the isolate has growth promotion activities as well as some control of diseased caused by *R. solani* and *F. oxysporum*. In addition the isolate has a range of MOA including *in vitro* inhibition of the pathogens, siderophore production, phosphate solubilisation and growth in nitrogen free media. This combination of properties makes the isolate a good versatile or “all-round” performer. Although this isolate is not a strong performer in all areas, activity may be enhanced by improving the formulation (Schisler *et al.* 2004), biomass production protocols (Ashofteh *et al.* 2009), manipulation of soil properties (Ownley *et al.* 2003) or adding amendments such as minerals (Saikia *et al.* 2009).

During selection of the isolates several important limitations regarding the selection protocol used in the current study were revealed. Depending on the design of the selection protocol many potentially effective isolates could be excluded. Comparison of the isolate scores and actual greenhouse data confirmed that ineffective isolates would be selected if based on in vitro tests only. Isolates T01, T08, T09, and T28 received the highest scores for *in vitro* MOA (appendix 5, Table3), however only T28 was effective in the greenhouse (for the control of *F. oxysporum*). These results are in agreement with the work of (Sari *et al.* 2006 and Milus and Rothrock, 1997) who showed that isolates that appeared to be non antagonistic towards pathogens in *in vitro* assays were effective in greenhouse trials.

The importance of assignment and combination of categories for scoring became apparent during isolate selection. For instance if a combined score of growth promoting and biocontrol ability is used several isolates, such as T15 which increases both the root and shoot mass of plants infected with *R. solani,* would be excluded because it does not show growth promotion. The exclusion of more effective isolates was also encountered when each attribute is scored using a yes/no system and the magnitude of the effect is excluded. For the control of *R. solani*

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for instance only two isolates (T10 and T29) would be selected using this type of scoring system, because they increased both the root and shoot mass of diseased plants. Other isolates such as T22, T25 and T16 which caused a large increase in shoot mass, but not root mass, would be excluded since the degree of increase is not indicated in the score (appendix 5). The publications of Berg *et al.* (2001), and Faltin *et al.* (2004), which used such schemes to assess their isolates, do not mention these shortcomings.

These results show the importance of careful evaluation of the data and selection of criteria whereby the PGPR are selected. The scoring system should therefore be assessed carefully (Nakkeeran *et al.* 2005; Knudsen *et al.* 1997). To overcome these constraints care should be taken in selecting the assessment steps and consideration should be given to the limitations of the selection process. Each category in the rating can be awarded points based on the strength of the isolate in that category. For instance, instead of giving one point for phosphates solubilisation, points for that category can be awarded based on the halo size, e.g. 0 = no halo, 1= halo 1-3 mm, 2=halo 3-5 mm, as indicated in the MOA assessment. Isolates can thereby be assessed based on the number of characters as well as combined "strength" of the characters

The spider diagrams indicate that PGPR isolates with diverse characteristics contribute to control of root disease and growth promotion of wheat. The spider diagrams clearly indicted that for some isolates the *in vitro* MOA did not correlate with greenhouse activity. An example is isolate T07, which showed growth promotion but had only one of the growth promotion MOA screened for. These results are in agreement with Smyth *et al.* (2011), who showed that for a range of bacteria including *Bacilli* and *Pseudomonas, in vitro* modes of action tests do not correspond with results obtained in the greenhouse. In contrast, Idris *et al.* (2007) shows that PGPR can be successfully isolated based on *in vitro* MOA. In the current study a similar tendency was observed for some isolates, such as T31 which showed high inhibition of *F. graminearum* in dual culture and in the pot trials. Interestingly this trend was not observed for control of *R. solani* and *F. oxysporum*. Two isolates namely T24 and T23 showed both good growth promotion and some biocontrol activity of *F. graminearum*.

For a more complete indication of the isolates' suitability as a PGPR, more characteristics can be assessed. These could include attributes such as survival, repeatability and consistency. Isolate T19 (*Lysinibacillus fusiformis / L. sphaericus*) for instance, was the only isolate of the eight selected growth promotion that consistently improved the growth of wheat plants in subsequent dose response and fertiliser interaction trials. An isolate such as this would be more valuable in a commercial setting than an isolate that sporadically causes a larger increase in growth (Stewart 2001).

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In conclusion, five isolates namely T10, T28 (*Bacillus cereus*), T31 (*Bacillus cereus*), T13 (*Bacillus sp. / Bacillus pumilus*) and T29 (*Paenibacillus alvei*) were selected as the isolate with the most potential for improving growth of wheat seedlings. During the selection process it was found that care should be taken to insure that potentially effective PGPR are not excluded due to bias in the selection protocol. Spider diagrams were a valuable tool to aid in the selection of PGPR.

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6.5 References

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6.6 Figures and diagrams

Figures 6.1 to 6.2:
Spider diagrams of the most effective PGPR isolates. After each assessment character BC indicates biological control, GP indicates growth promotion, GH that the assay as conducted in the greenhouse (GH) and/or and IV *in vitro*

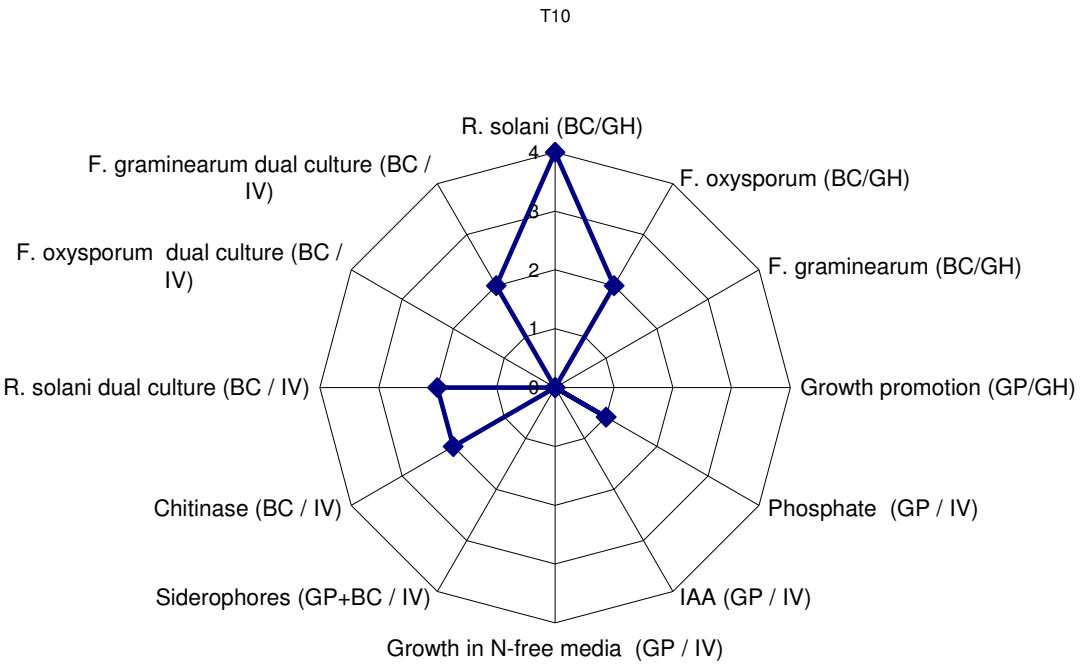

Figure 6.1: Isolate T10 (*Bacillus cereus*)

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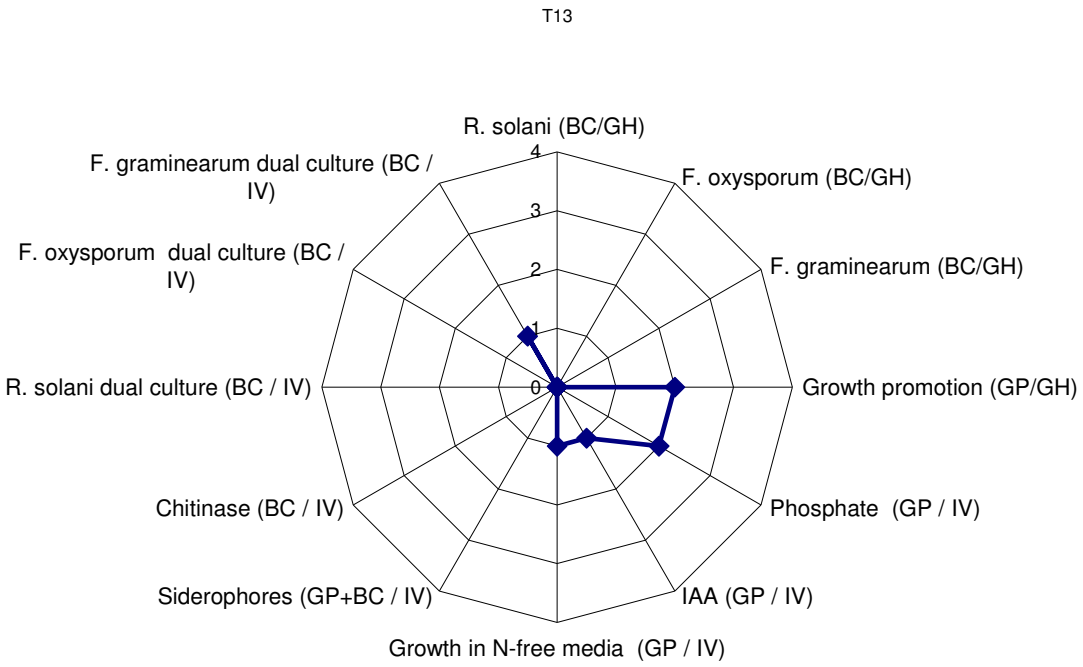

Figure 6.2: Isolate T13 (*Bacillus sp. / B. pumilus*)

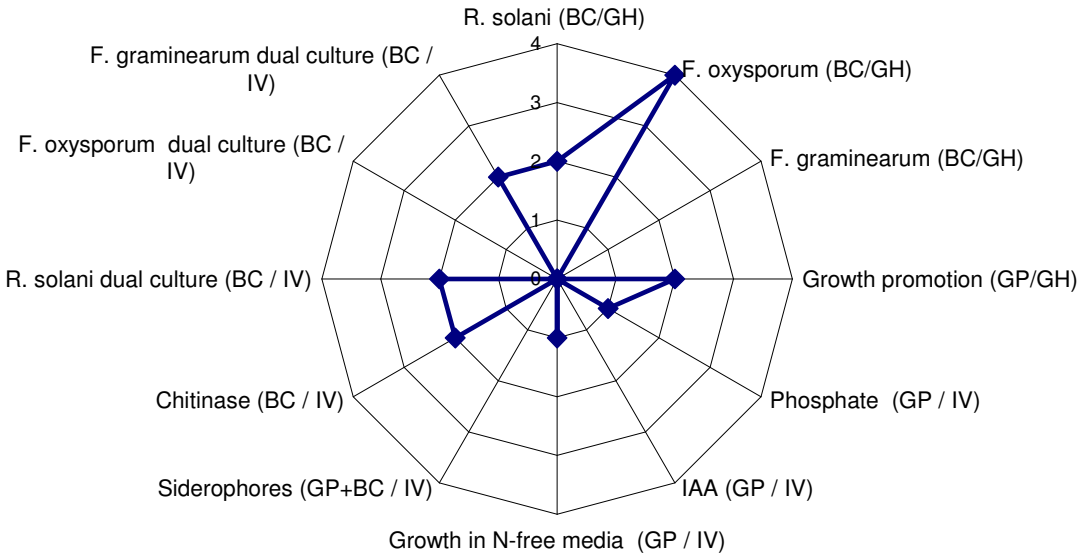

Figure 6.3: Isolate T28 (*Bacillus cereus*)

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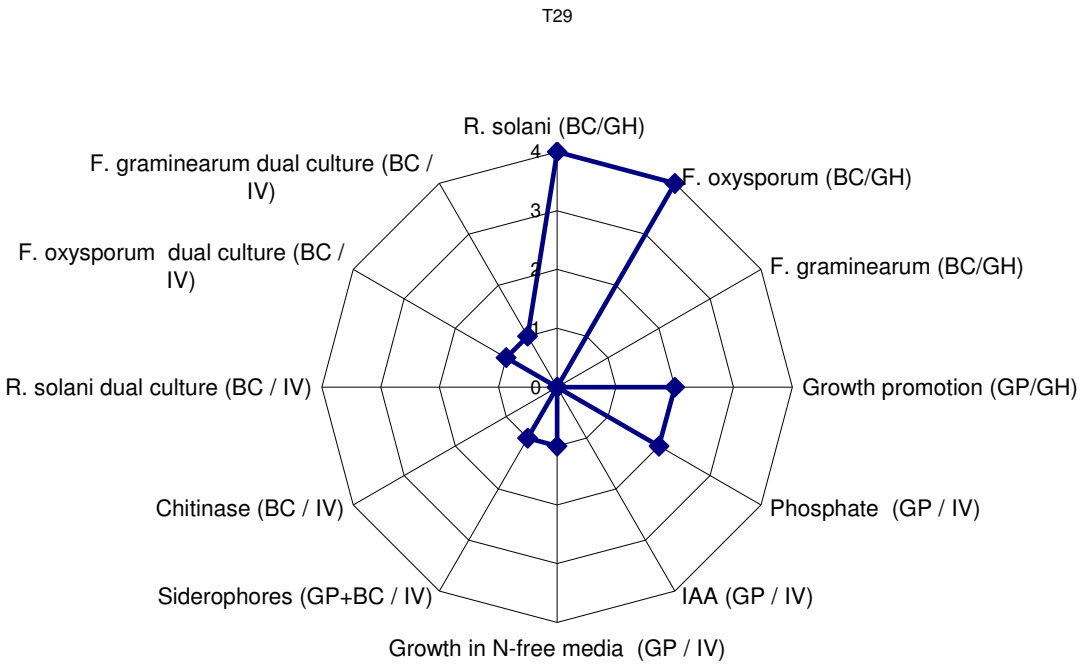

Figure 6.4: Isolate T29 (*Paenibacillus alvei*)

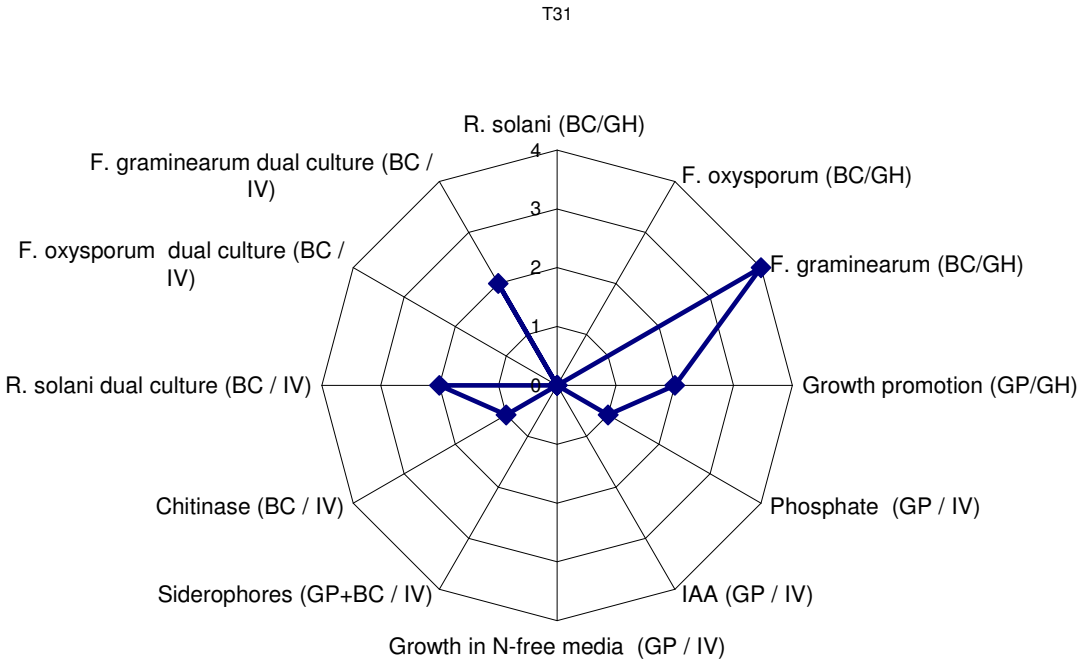

Figure 6.5: Isolate T31 (*Bacillus cereus*)

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Appendix 1: Bacterial isolates and culture preparation

Bacterial culture origin and maintenance

The plant growth promoting rhizobacteria isolates were originally isolated from the rhizosphere of indigenous grasses by Hassen (2007). The bacterial isolates used in this study were selected based on their performance in trials with sorghum. Bacterial cultures were obtained from the UP PGPR culture collection in lyophilised form. The isolates were revived on Nutrient agar (Bioloab, Wadeville) and checked for purity. Pure cultures were preserved by freeze drying and a working culture stock was prepared by storage at -70℃ using Microbeads® system from Davies diagnostics. When needed the cultures were streaked onto Nutrient agar (Biolab) from the microbeads.

Bacterial inoculum preparation

Bacterial inoculum was prepared by inoculating 100 ml Nutrient broth (NB) (Biolab) with one frozen bead containing the bacteria isolate. The cultures were incubated on a rotary shaker for 24 hours at 25℃ and 180 RPM. After incubation the cell concentration was determined by spectrophotometer at 550nm. The cultures were transferred to 50 ml centrifuge tubes and centrifuged at 3000 x g for 10 minutes. The pellet was resuspended in sterile Ringer's solution to a concentration of approximately 10^8 cfu/ml (Idris *et al.*2007).

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Appendix 2: Fungal cultures and preparation of millet inoculum

Rhizoctonia solani UPGH122

Rhizoctonia solani Kuhn isolate UPGH 122 (PPRI 2405) was obtained from the University of Pretoria's PGPR culture collection (UP-PGPR). The cultures were stored in the culture collection on Potato Dextrose Agar (PDA) (Biolab, Wadeville) slants at room temperature. Working cultures were grown on PDA or half strength PDA.

Fusarium oxysporum UPGH 132

Fusarium oxysporum Schlectend isolate UPGH 132 was obtained from the University of Pretoria's PGPR culture collection (UP-PGPR). The cultures were stored in the culture collection on PDA (Biolab) slants at room temperature. Working cultures grown on half strength PDA.

Fusarium graminearum WP4F

Fusarium graminearum Schwabe isolate WP4F was isolated at the UP laboratories from diseased root material of wheat in March 2010. The wheat plant from which the isolate was obtained originated from the Swartland region of the Western Cape, South Africa. The plant symptoms were white head symptoms and the root had pink-purple discolouration. Greenhouse trials were conducted to confirm that the isolate was pathogenic to wheat.

The isolate was initially identified at the UP laboratories as a *Fusarium* spp. and sent to the Agricultural Research Council, Identification Services Biosystematics Division, Mycology Unit, Pretoria, South Africa for identification (ARC accession number M43/128).

Millet seed inoculum of fungal cultures

The millet seed inoculum was prepared by placing 250ml red millet seed and 200ml distilled water in autoclavable polyethylene bags. The bags with millet were then autoclaved thrice at 121℃ for 30 minutes. Thereafter each bag was inoculated with five 5mm diameter agar discs cut from a fresh fungal culture grown on PDA or ½PDA. The inoculum was incubated at room temperature (approximately 25℃) for two to three weeks (Adapted from Hassen 2007).

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Appendix 3: Wheat plants, seedling germination, growth conditions and harvesting

Wheat seeds and seed preparation

All trials were conducted with wheat seeds variety SST822 from Sensako (Randburg, South Africa) Batch number: 20025A07VV. The seeds were commercially treated with Vitavax Plus, and K-Orbitol by Sensako. During the current study the fungicides were removed and the seeds surface sterilised before experiments were conducted by immersion in 70% ethanol for 5 minutes followed by immersion in 1% sodium hypochlorite for one minute thereafter the seeds were rinsed five times with sterile water.

Seedling growing conditions

All experiments were conducted at the University of Pretoria's Plant Pathology Greenhouses. Unless otherwise specified the seeds were geminated and plants grown in the following manner: The seeds were germinated in sterile vermiculite. One week after sowing in the vermiculite the seedlings were transferred to pots containing sandy loam soil. Greenhouse temperatures were maintained at 16 - 25℃ and the seeds and plants received daily watering with municipal tap water.

Harvesting

Plants were harvested by carefully removing them from the pots and washing the soil from the roots under running tap water. The fresh mass of the roots and foliages was then determined by weighing. For trails involving a pathogen symptoms were recorded. The plants were then dried in an oven at approximately 75℃ for three days and the dry root and foliage masses determined by weighing.

Plants were harvested and their roots rinsed in tap water. The roots were then excised and the root and foliage mass determined separately. After drying the plants in an oven at approximately 80℃ for two days the dry mass was determined. Symptoms were also recorded.

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Appendix 4: Summary of pot trial results

Table A4.1. Summary of results for the assessment of PGPR to promote the growth of wheat seedlings in the greenhouse shown as % change in wheat seedling mass

	Growth promotion				Dose response (1 liter pots)				Dose response (500 ml pots)				Fertiliser response (500 ml pots)			
					50 ml PGPR dose / pot		25 ml PGPR dose / pot		25ml PGPR dose / pot		12.5 ml PGPR dose / pot		25ml PGPR dose / pot		12.5 ml PGPR dose / pot	
	Wet mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*	
Isolate	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
T01	1	19	-2	8	-	-	-	-	-	-	-	-	-	-	-	-
T02	-3	-1	-14	2	-	-	-	-	-	-	-	-	-	-	-	-
T03	-16	11	-30	8	-	-	-	-	-	-	-	-	-	-	-	-
T04	6	9	-2	3	-	-	-	-	-	-	-	-	-	-	-	-
T05	-10	19	-6	11	-	-	-	-	-	-	-	-	-	-	-	-
T06	6	20	13	18	-14.31	-9.73	0.45	-20.75	-16	1	-20	-1	-18	8	-23	-5
T07	6	23	-13	16	25.61	8.95	0.06	-11.63	-19	2	-40	-9	-33	-4	-6	8
T08	-25	-3	-26	3	-	-	-	-	-	-	-	-	-	-	-	-
T09	15	15	20	14	-	-	-	-	-	-	-	-	-	-	-	-
T10	2	16	-6	9	-	-	-	-	-	-	-	-	-	-	-	-
T11	-9	25	-7	19	9.05	4.50	-3.72	3.57	-27	6	-16	-4	-24	0	-4	5
T12	-17	12	-16	13	-	-	-	-	-	-	-	-	-	-	-	-
T13	-10	24	-21	18	0.36	12.17	-1.36	-2.04	4	8	-4	-3	-9	3	-3	2
T14	-23	8	-27	9	-	-	-	-	-	-	-	-	-	-	-	-
T15	-7	15	-17	11	-	-	-	-	-	-	-	-	-	-	-	-
T16	24	17	10	46	-	-	-	-	-	-	-	-	-	-	-	-
T17	28	27	1	61	-	-	-	-	-	-	-	-	-	-	-	-
T18	29	27	-9	50	-	-	-	-	-	-	-	-	-	-	-	-
T19	31	22	24	51	-12.81	13.43	-3.67	-5.70	3	31	-2	-3	-16	6	-14	8
T20	25	18	6	45	-	-	-	-	-	-	-	-	-	-	-	-
T21	42	37	14	66	5.59	8.38	-7.45	-30.43	-31	4	-11	0	0	5	-10	-1

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Table A4.1. Summary of results for the assessment of PGPR to promote the growth of wheat seedlings in the greenhouse shown as % change in wheat seedling mass

	Growth promotion				Dose response (1 liter pots)				Dose response (500 ml pots)				Fertiliser response (500 ml pots)			
					50 ml PGPR dose / pot		25 ml PGPR dose / pot		25ml PGPR dose / pot		12.5 ml PGPR dose / pot		25ml PGPR dose / pot		12.5 ml PGPR dose / pot	
	Wet mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*		Dry mass*	
T22	25	26	7	53	-	-	-	-	-	-	-	-	-	-	-	-
T23	31	23	10	63	11.79	9.86	-7.20	-19.87	-	-	24	30	18	6	-20	-19
T24	33	26	9	57	-14.87	-0.42	-5.29	-24.32	-22	25	-7	6	-19	3	-12	-3
T25	24	26	-13	51	-	-	-	-	-	-	-	-	-	-	-	-
T26	27	26	26	56	-	-	-	-	-	-	-	-	-	-	-	-
T27	20	28	-24	54	-	-	-	-	-	-	-	-	-	-	-	-
T28	31	41	-12	61	-	-	-	-	-	-	-	-	-	-	-	-
T29	28	32	15	65	-	-	-	-	-	-	-	-	-	-	-	-
T30	25	27	-5	62	-	-	-	-	-	-	-	-	-	-	-	-
T31	22	23	12	50	-	-	-	-	-	-	-	-	-	-	-	-

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

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Table A4.2. Summary of results for assessment of the biological control ability of PGPR isolates in the greenhouse

	% change in the dry mass of wheat seedlings					
	Rhizoctonia solani		*Fusarium oxysporum*		*Fusarium graminearum*	
Isolate	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
T01	-	-	-	-	-21	25
T02	-	-	-	-	-24	5
T03	-	-	-	-	-5	17
T04	-	-	-	-	0	24
T05	-	-	-	-	-2	-4
T06	-	-	-	-	-3	17
T07	-	-	-	-	-20	-1
T08	75	53	38	80	-28	-21
T09	83	67	57	90	-2	-9
T10	125	87	29	120	-8	25
T11	92	87	19	110	-4	9
T12	-	-	-	-	-31	-8
T13	-	-	-	-	-18	6
T14	-	-	-	-	-21	-1
T15	75	140	86	80	-9	15
T16	17	173	38	50	-18	-12
T17	-	-	-	-	-6	31
T18	-	-	-	-	6	44
T19	-	-	-	-	-12	10
T20	-	-	-	-	-24	16
T21	-	-	-	-	-	-
T22	100	200	33	130	0	15
T23	-	-	-	-	36	13
T24	-	-	-	-	47	13
T25	42	173	71	110	6	15
T26	-	-	-	-	-5	15
T27	-	-	-	-	33	36
T28	75	140	105	120	6	22
T29	133	160	76	170	9	14
T30	75	93	86	120	-14	10
T31	-	-	-	-	47	31

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

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Appendix 5: Scores awarded for selection of plant growth promoting rhizobacterial isolates

Table A5.1: Scoring system whereby rhizobacterial isolates were evaluated and spider diagrams constructed.

	Points awarded for each assay*	Total points per category
Greenhouse assays		
Biocontrol of *R. solani* in pot trials		4
Increase in dry root mass	2	
Increase in dry shoot mass	2	
Biocontrol of *F. oxysporum* in pot trials		4
Increase in dry root mass	2	
Increase in dry shoot mass	2	
Biocontrol of *F. graminearum* in pot trials		4
Increase in dry root mass	2	
Increase in dry shoot mass	2	
Growth promotion in the greenhouse		4
Increase in dry root mass	2	
Increase in dry shoot mass	2	
In vitro modes of action		
In vitro phosphate solubilisation assay		2
Pikovskaya medium (PVK)	1	
National Botanical Research Institute's phosphate growth medium (NYBR)	1	
Siderophore production assay		1
Chrome azurol S agar	1	
IAA production assay		
Salkowski's reagent		
Chitinase production assay		2
Water agar with chitin (WAC)	1	
Chitin minimal medium (CMM)	1	
Growth in Nitrogen free media		1
Dual culture assay (evaluated separately for *R. solani*, *F. oxysporum* and *F. graminearum*)		2
Potato dextrose agar (PDA)	1	
Water yeast agar (WYA)	1	

*Isolates were rated using a scoring system similar to that used by Berg *et al.* (2001)

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Table A5. 2. Scoring of plant growth promoting rhizobacteria isolates based on their performance in greenhouse trials to assess growth promotion and biological control of *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium graminearum* on wheat seedlings

Isolate	Biological control									Growth promotion			Total score for greenhouse trials
	R. solani			*F. oxysporum*			*F. graminearum*						
	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total	
T01	-	-	0	-	-	0	0	0	0	0	0	0	0
T02	-	-	0	-	-	0	0	0	0	0	0	0	0
T03	-	-	0	-	-	0	0	0	0	0	0	0	0
T04	-	-	0	-	-	0	0	0	0	0	0	0	0
T05	-	-	0	-	-	0	0	0	0	0	0	0	0
T06	-	-	0	-	-	0	0	0	0	0	2	2	2
T07	-	-	0	-	-	0	0	0	0	0	2	2	2
T08	0	0	0	0	2	2	0	0	0	0	0	0	2
T09	0	0	0	2	2	4	0	0	0	0	0	0	4
T10	2	2	4	0	2	2	0	0	0	0	0	0	6
T11	0	2	2	0	2	2	0	0	0	0	2	2	6
T12	-	-	0	-	-	0	0	0	0	0	0	0	0
T13	-	-	0	-	-	0	0	0	0	0	2	2	2
T14	-	-	0	-	-	0	0	0	0	0	0	0	0
T15	0	2	2	2	2	4	0	0	0	0	0	0	6
T16	0	2	2	0	0	0	0	0	0	0	2	2	4
T17	-	-	0	-	-	0	0	2	2	0	2	2	4
T18	-	-	0	-	-	0	0	2	2	0	2	2	4
T19	-	-	0	-	-	0	0	0	0	0	2	2	2
T20	-	-	0	-	-	0	0	0	0	0	2	2	2
T21	-	-	0	-	-	0	-	-	0	0	2	2	2

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Table A5. 2. Scoring of plant growth promoting rhizobacteria isolates based on their performance in greenhouse trials to assess growth promotion and biological control of *Rhizoctonia solani*, *Fusarium oxysporum* and *Fusarium graminearum* on wheat seedlings

Isolate	Biological control									Growth promotion			Total score for greenhouse trials
	R. solani			*F. oxysporum*			*F. graminearum*						
	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total	Root	Shoot	Total	
T22	0	2	2	2	2	4	0	0	0	0	2	2	8
T23	-	-	0	-	-	0	2	0	2	0	2	2	4
T24	-	-	0	-	-	0	2	0	2	0	2	2	4
T25	0	2	2	2	2	4	0	0	0	0	2	2	8
T26	-	-	0	-	-	0	0	0	0	0	2	2	2
T27	-	-	0	-	-	0	0	2	2	0	2	2	4
T28	0	2	2	2	2	4	0	0	0	0	2	2	8
T29	2	2	4	2	2	4	0	0	0	0	2	2	10
T30	0	2	2	2	2	4	0	0	0	0	2	2	8
T31	-	-	-	-	-	-	2	2	4	0	2	2	6

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Table A5.3. Scoring of plant growth promoting rhizobacteria isolates based on results from selected modes of action assays

Isolate	Dual culture						Siderophore production	Phosphate solubilisation		Chitinolytic activity		Nitrogen-fixation (growth in N-free medium)	Indole acetic acid production	Total score awarded
	R. solani		*F. oxysporum*		*F. graminearum*			PVK[c]	NBRIY[d]	WAC[e]	CMM[f]			
	PDA[a]	WYA[b]	PDA[a]	WYA[b]	PDA[a]	WYA[b]								
T01	1	1	0	0	1	1	1	1	0	0	1	0	1	8
T02	1	1	0	0	1	1	1	0	0	1	1	0	0	7
T03	0	0	0	0	0	0	1	1	1	0	1	0	1	5
T04	1	0	0	0	1	1	0	1	1	0	1	1	0	7
T05	0	0	0	0	0	1	0	0	0	0	0	1	1	3
T06	1	1	0	0	1	1	0	1	0	1	1	1	0	8
T07	0	0	0	0	0	1	0	0	0	0	0	1	0	2
T08	1	1	0	0	1	1	1	1	0	1	1	0	0	8
T09	1	1	0	0	1	1	1	1	0	1	1	0	0	8
T10	1	1	0	0	1	1	0	1	0	1	1	0	0	7
T11	1	1	0	0	1	0	0	1	0	1	1	1	0	7
T12	0	0	0	0	1	1	1	1	1	0	0	1	1	8
T13	0	0	0	0	1	0	0	1	1	0	0	1	1	6
T14	0	0	0	0	1	0	1	1	1	0	0	1	1	7
T15	0	0	0	0	0	1	0	0	0	0	1	1	0	4
T16	0	0	0	0	0	0	1	1	1	1	0	0	1	4
T17	0	1	0	0	1	1	0	1	0	1	1	0	0	6
T18	1	1	0	0	1	1	1	0	0	0	1	1	0	7
T19	1	0	0	0	0	0	0	0	0	0	0	1	1	3
T20	0	0	0	0	1	1	0	0	0	0	0	1	0	3
T21	1	1	0	0	1	1	0	0	0	0	1	1	0	6
T22	0	0	0	0	0	0	1	1	1	1	0	0	0	4
T23	1	1	0	0	0	1	0	1	0	1	1	0	0	7

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Table A5.3. Scoring of plant growth promoting rhizobacteria isolates based on results from selected modes of action assays

Isolate	Dual culture						Siderophore production	Phosphate solubilisation		Chitinolytic activity		Nitrogen-fixation (growth in N-free medium)	Indole acetic acid production	Total score awarded
	R. solani		*F. oxysporum*		*F. graminearum*			PVK[c]	NBRIY[d]	WAC[e]	CMM[f]			
	PDA[a]	WYA[b]	PDA[a]	WYA[b]	PDA[a]	WYA[b]								
T24	0	0	0	0	0	0	1	1	1	0	0	0	1	4
T25	1	1	0	0	1	1	0	1	0	1	1	0	0	7
T26	1	0	0	0	1	1	1	1	0	1	1	0	0	8
T27	1	1	0	0	1	1	1	1	0	1	1	0	0	8
T28	1	1	0	0	1	1	0	1	0	1	1	1	0	8
T29	0	0	1	0	1	0	1	1	1	0	0	1	0	6
T30	0	0	0	0	0	0	1	1	1	0	0	0	1	4
T31	1	1	0	0	1	1	0	0	0	0	1	0	0	5

[a] PDA = Potato dextrose agar
[b] WYA = Water yeast agar
[c] Pikovskaya agar (PVK)
[d] National Botanical Research Institute's phosphate growth medium (NBRIY) agar
[e] WAC = Water agar amended with chitin
[f] CMM = Chitin minimal medium

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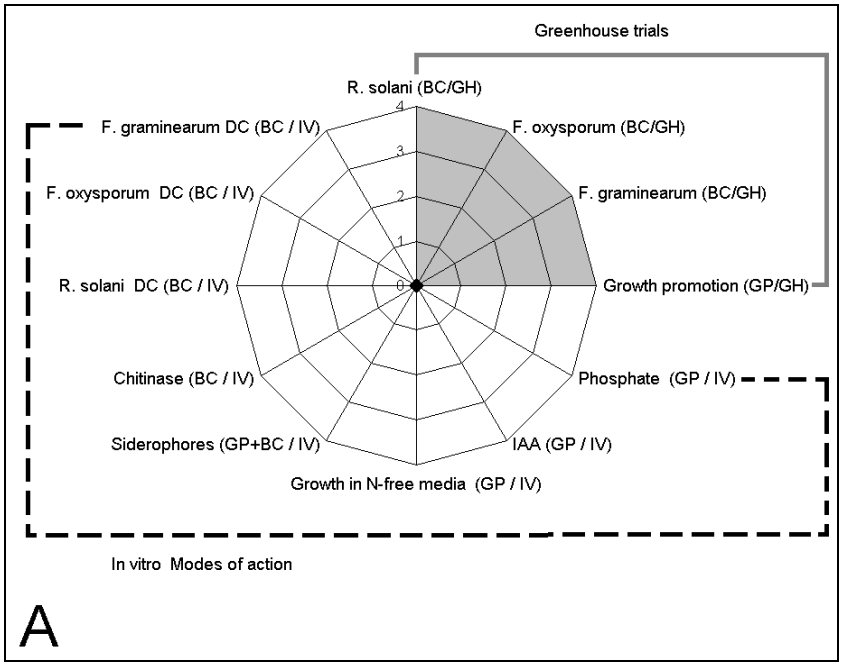
Appendix 6: Spider diagrams of high scoring isolates with, the exception of the five best isolates for which the diagrams are shown in chapter 6



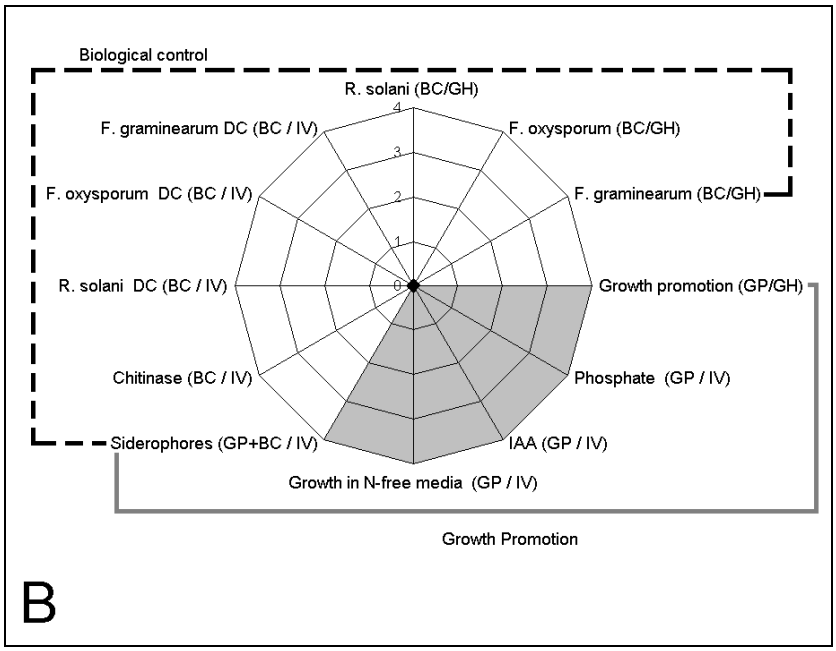


Figure A6.1: A) Description of the spider diagram indicating which categories are greenhouse trials and which are *in vitro* trials, coloured areas and solid line indicate tests conducted in the greenhouse, uncoloured areas and dashed line indicates *in vitro* modes of action. B) Description of the spider diagrams indicating which categories represent biological control and which represent growth promotion, coloured areas and solid line indicate growth promotion traits and white areas and dashed line indicate growth promotion traits.
Behind each assessment character is shown whether it is biological control (BC) or growth promotion (GP) and whether the assays were conducted in the greenhouse (GH) or *in vitro* (IV) assessment

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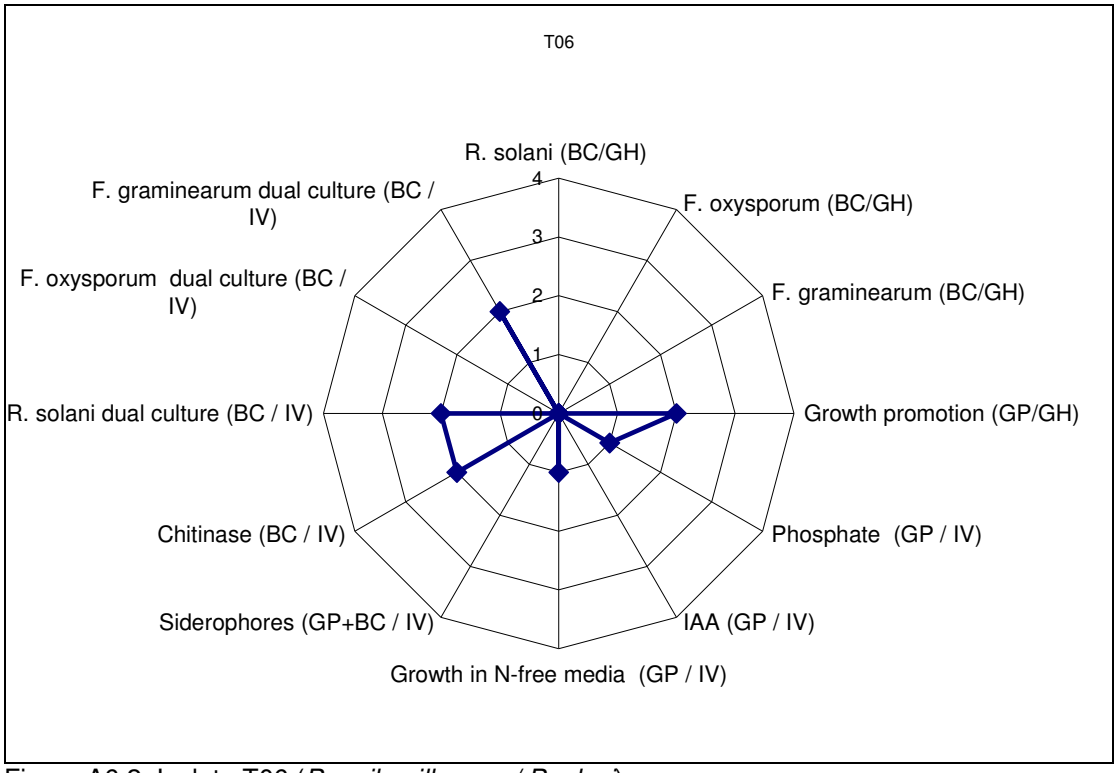

Figure A6.2: Isolate T06 (*Paenibacillus sp. / P. alvei*)

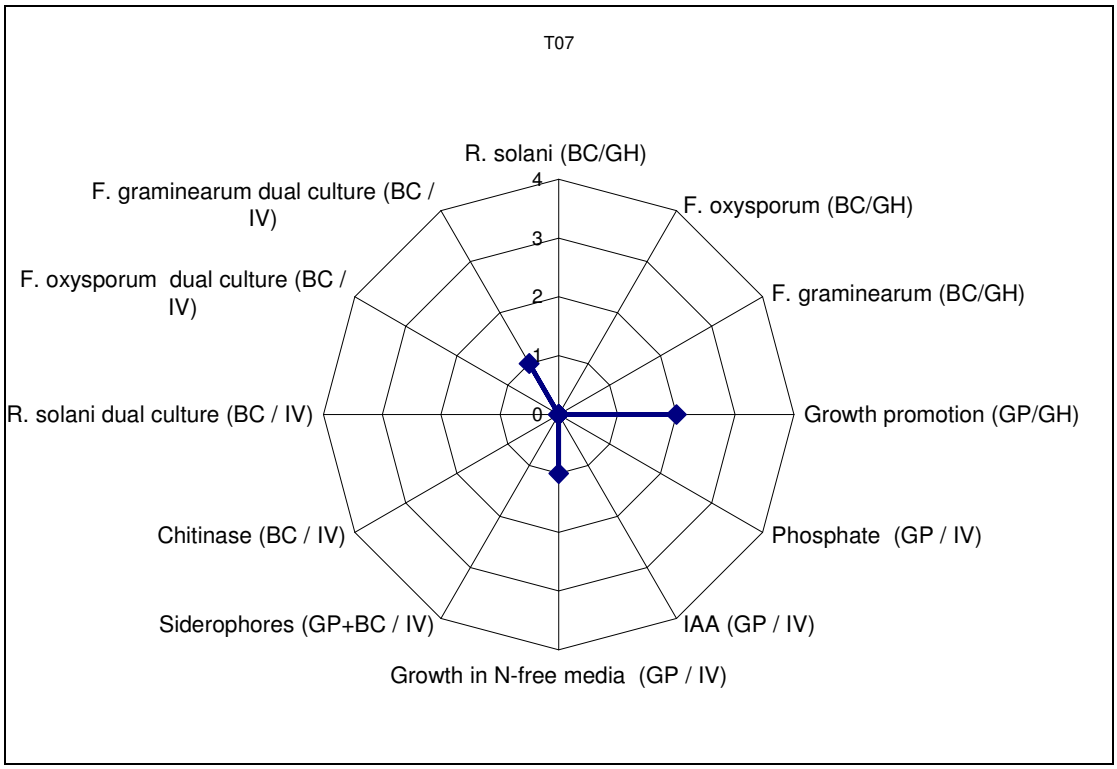

Figure A6.3: Isolate T07 (*Paenibacillus sp. / P. alvei*)

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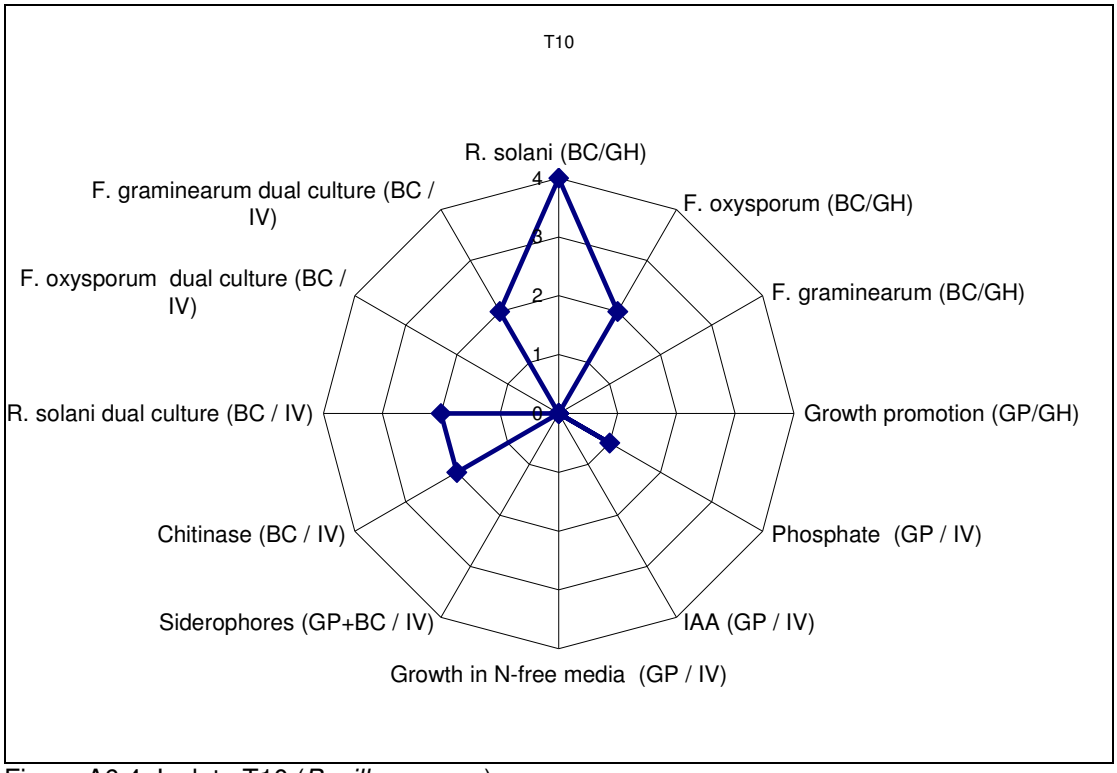

Figure A6.4: Isolate T10 (*Bacillus cereus*)

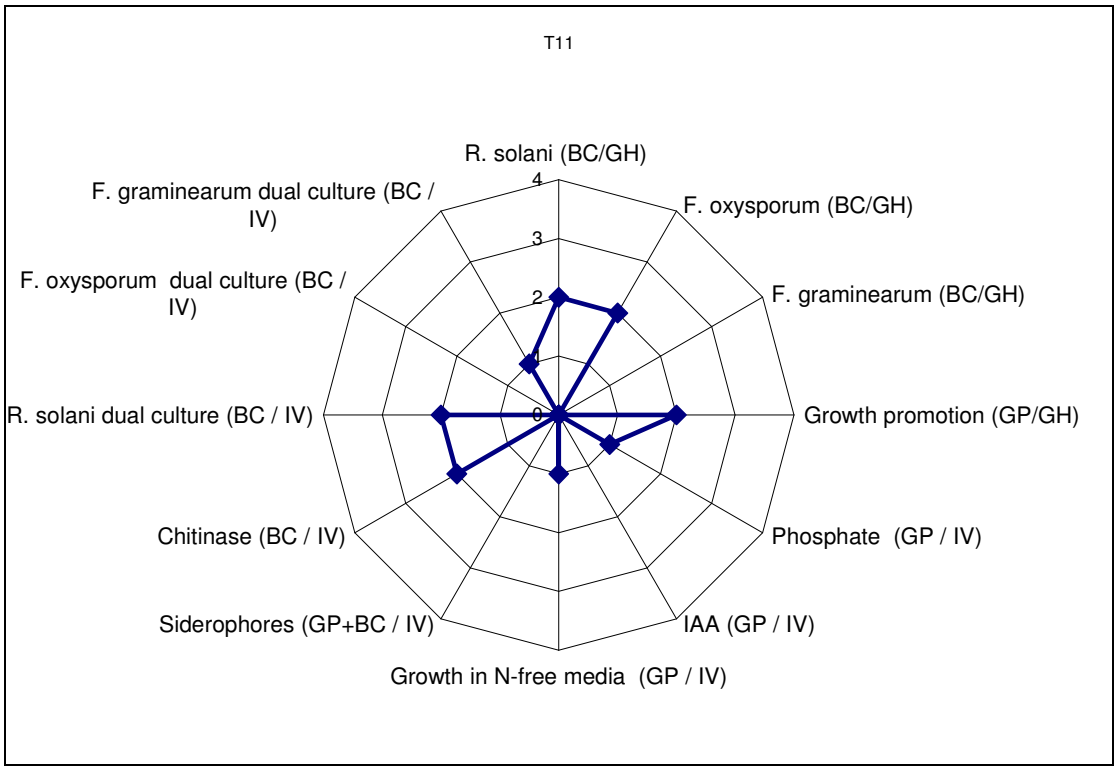

Figure A6.5: Isolate T11 (*Bacillus thuringiensis / B. cereus*)

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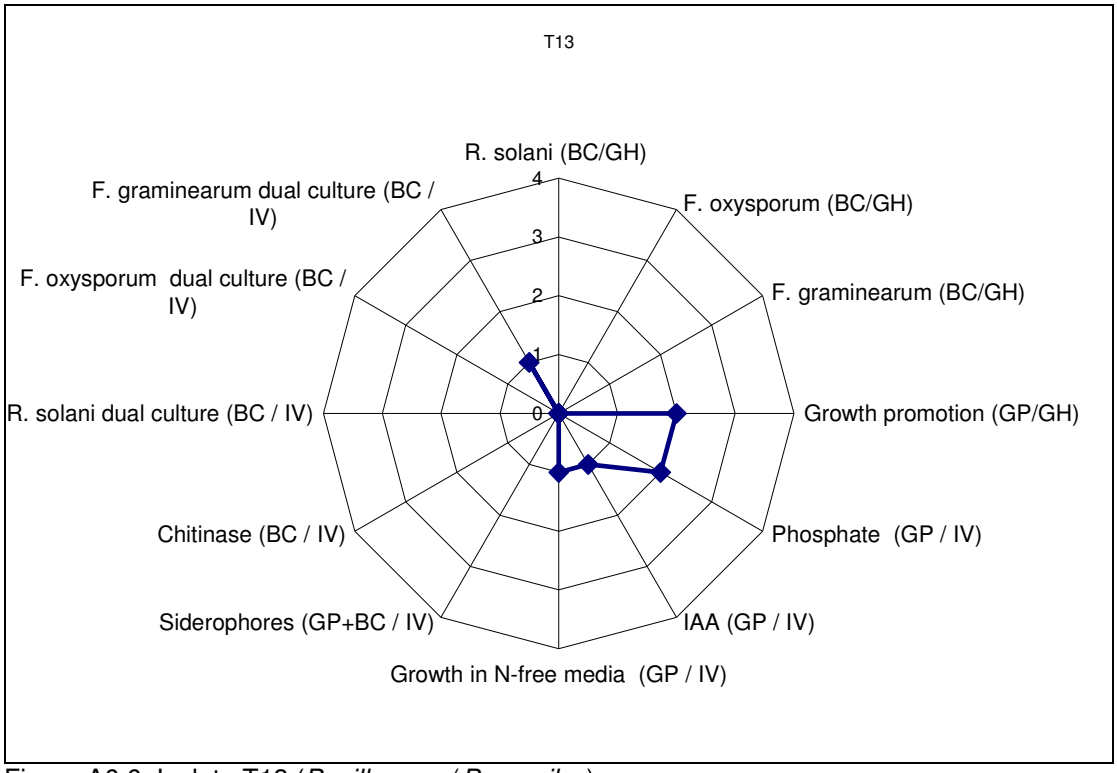

Figure A6.6: Isolate T13 (*Bacillus sp. / B. pumilus*)

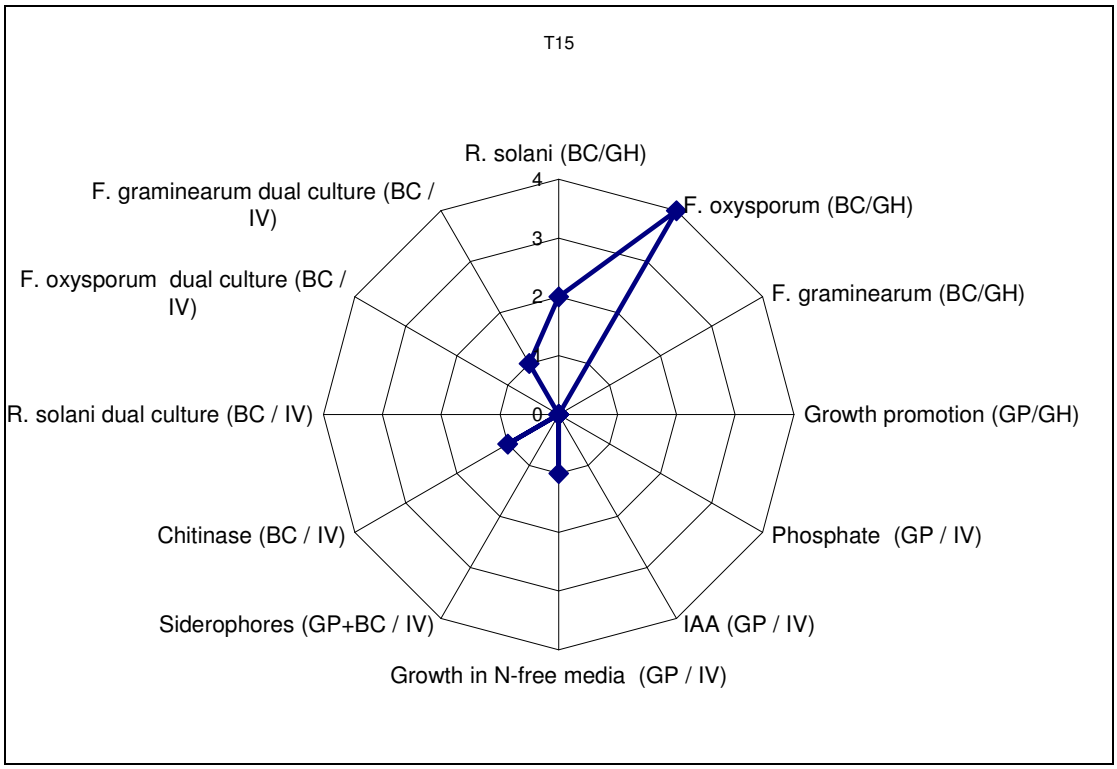

Figure A6.7: Isolate T15 (*Bacillus sp. / B. cereus*)

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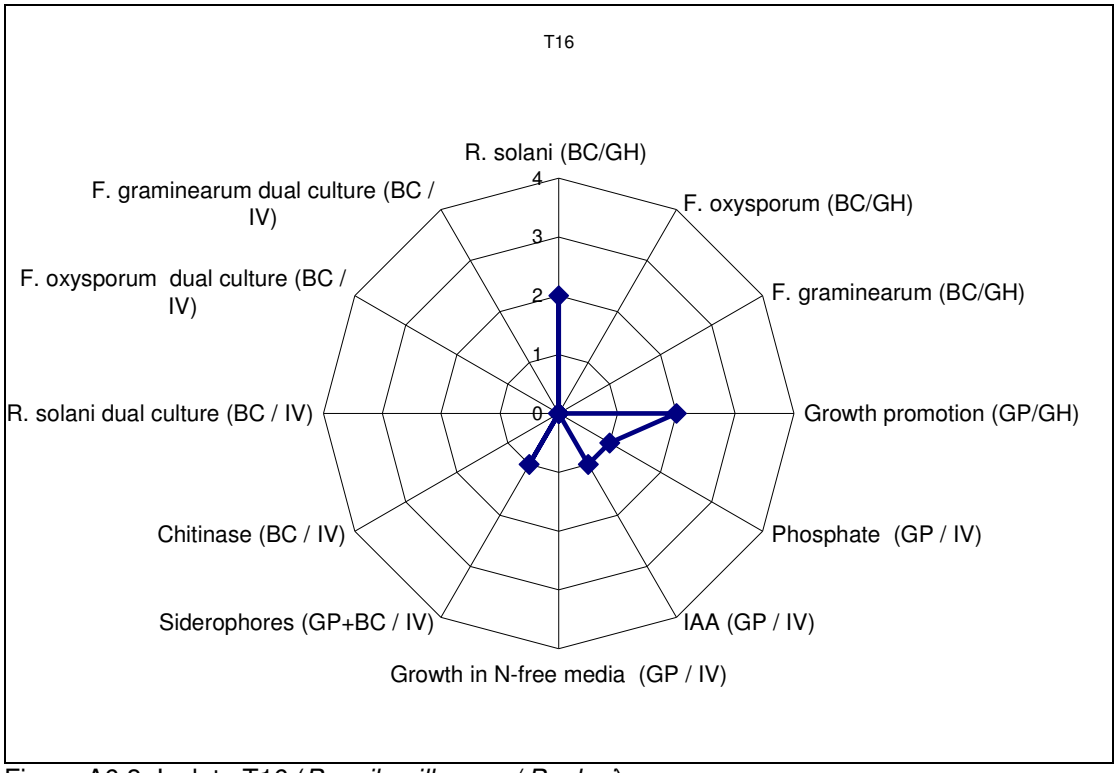

Figure A6.8: Isolate T16 (*Paenibacillus sp. / P. alvei*)

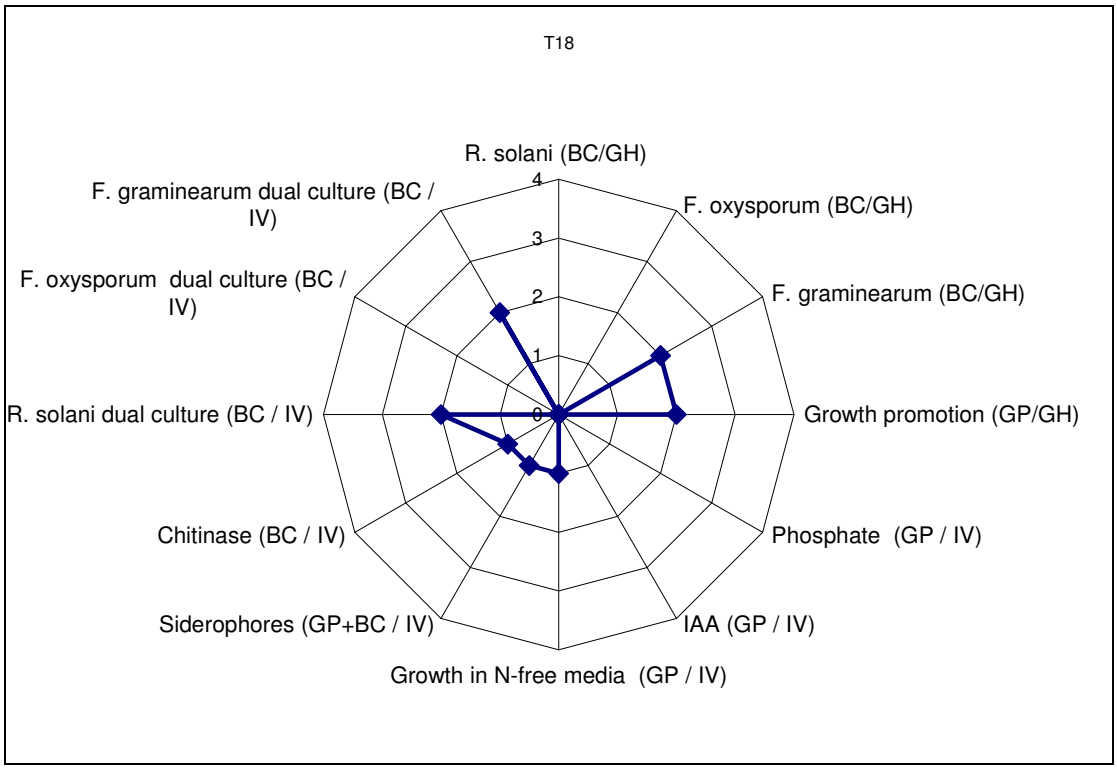

Figure A6.9: Isolate T18 (*Bacillus cereus*)

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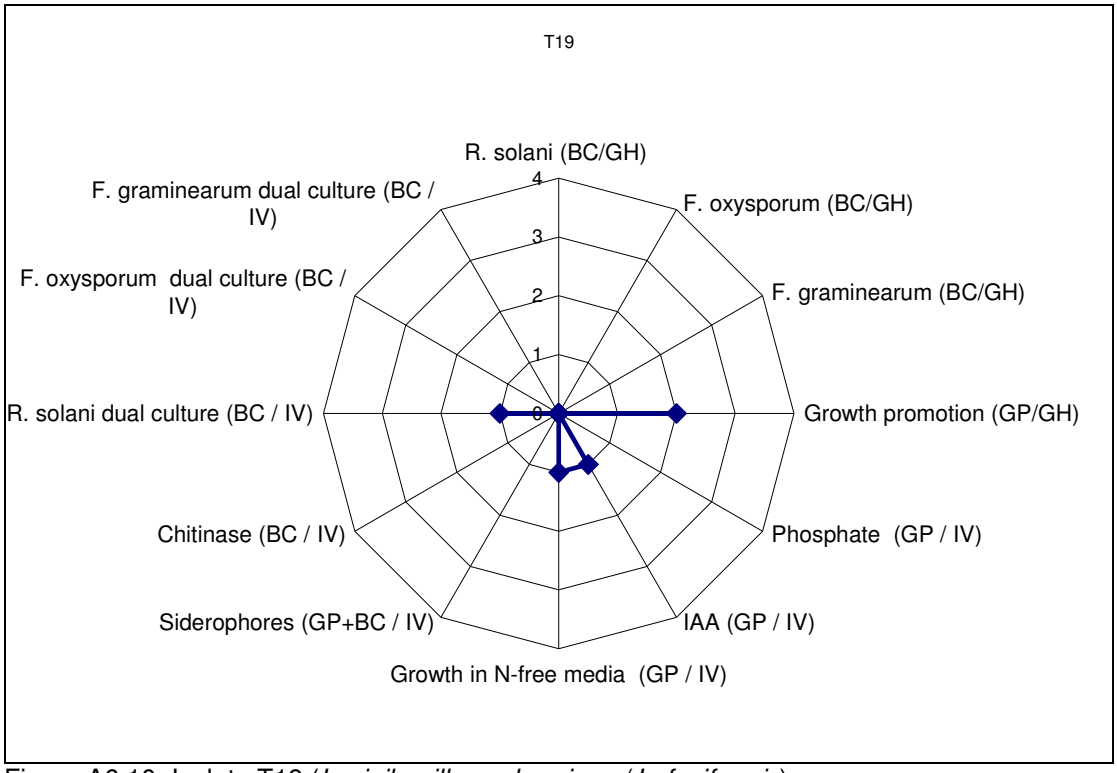

Figure A6.10: Isolate T19 (*Lysinibacillus sphaericus / L. fusiformis*)

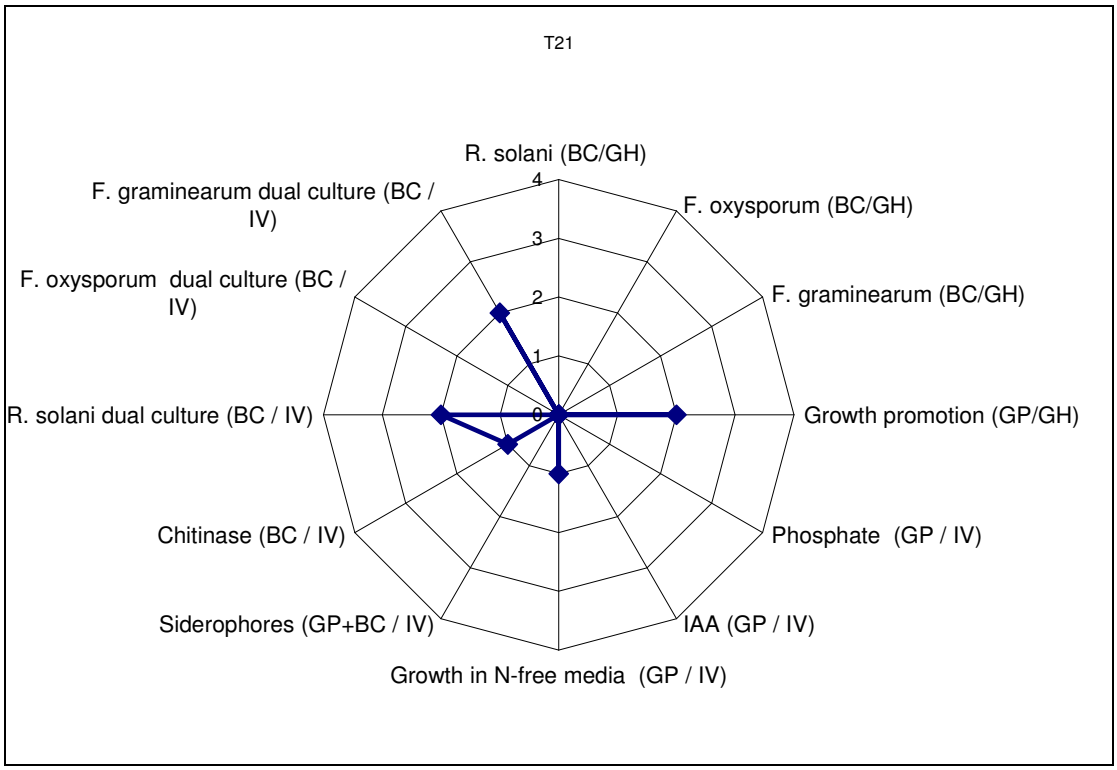

Figure A6.11: Isolate T21 (*Bacillus cereus*)

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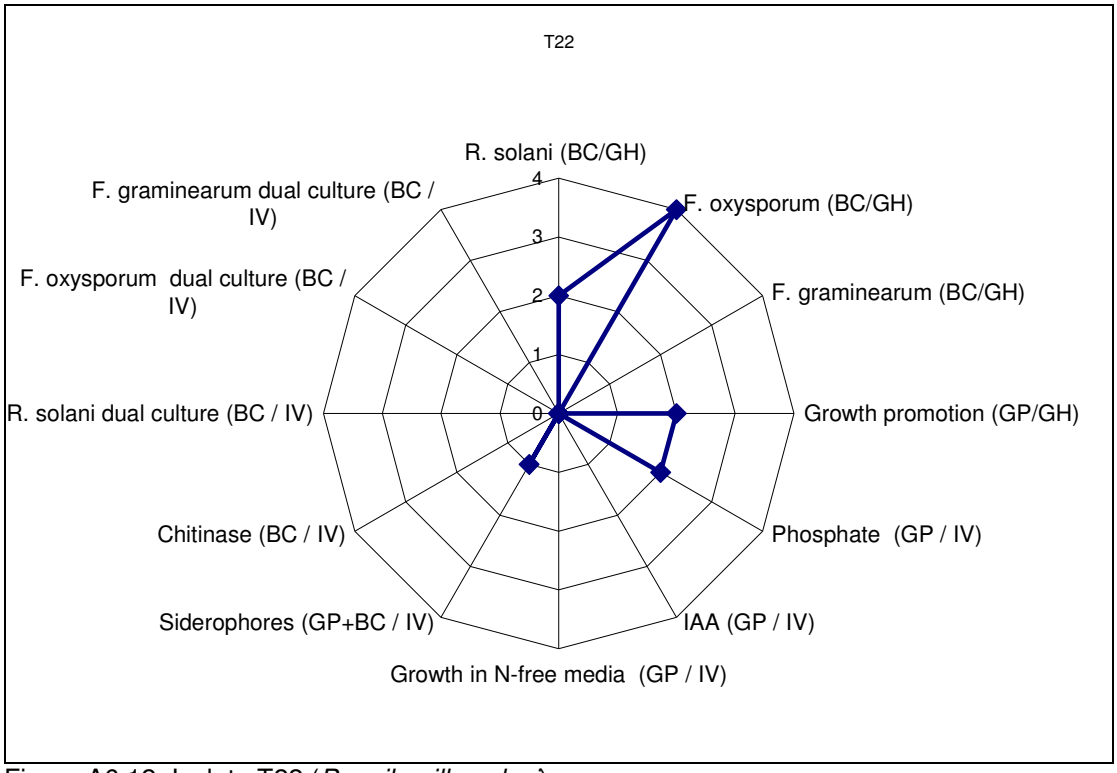

Figure A6.12: Isolate T22 (*Paenibacillus alvei*)

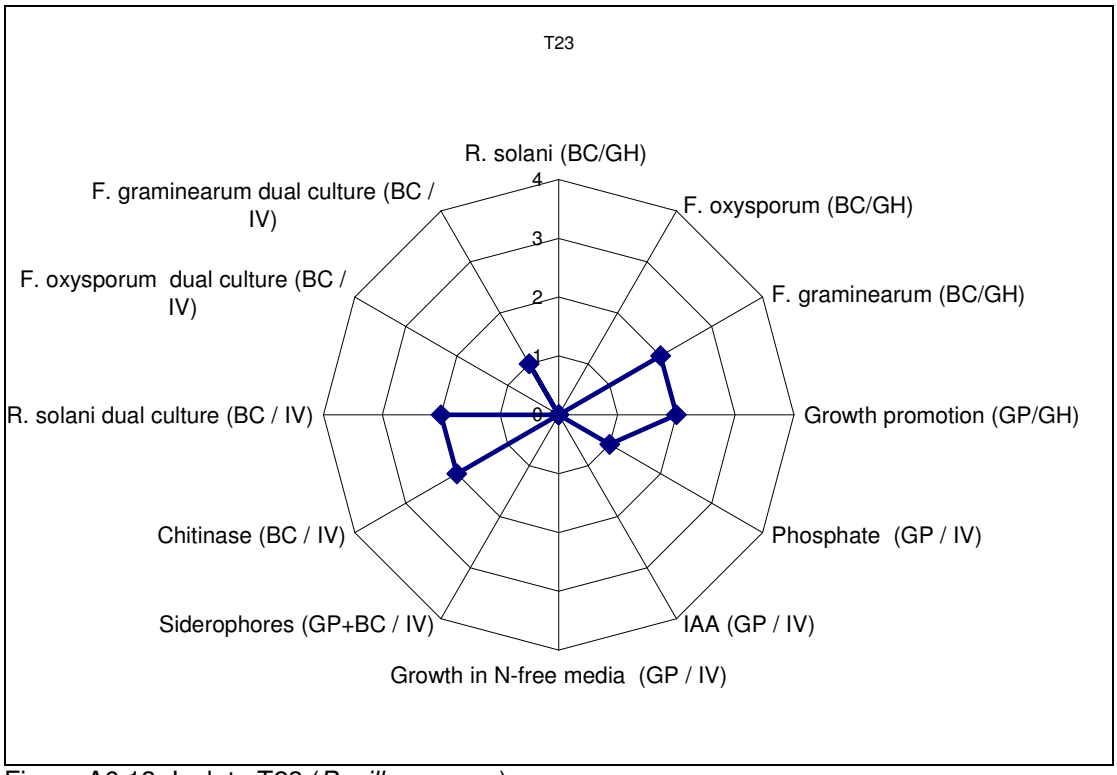

Figure A6.13: Isolate T23 (*Bacillus cereus*)

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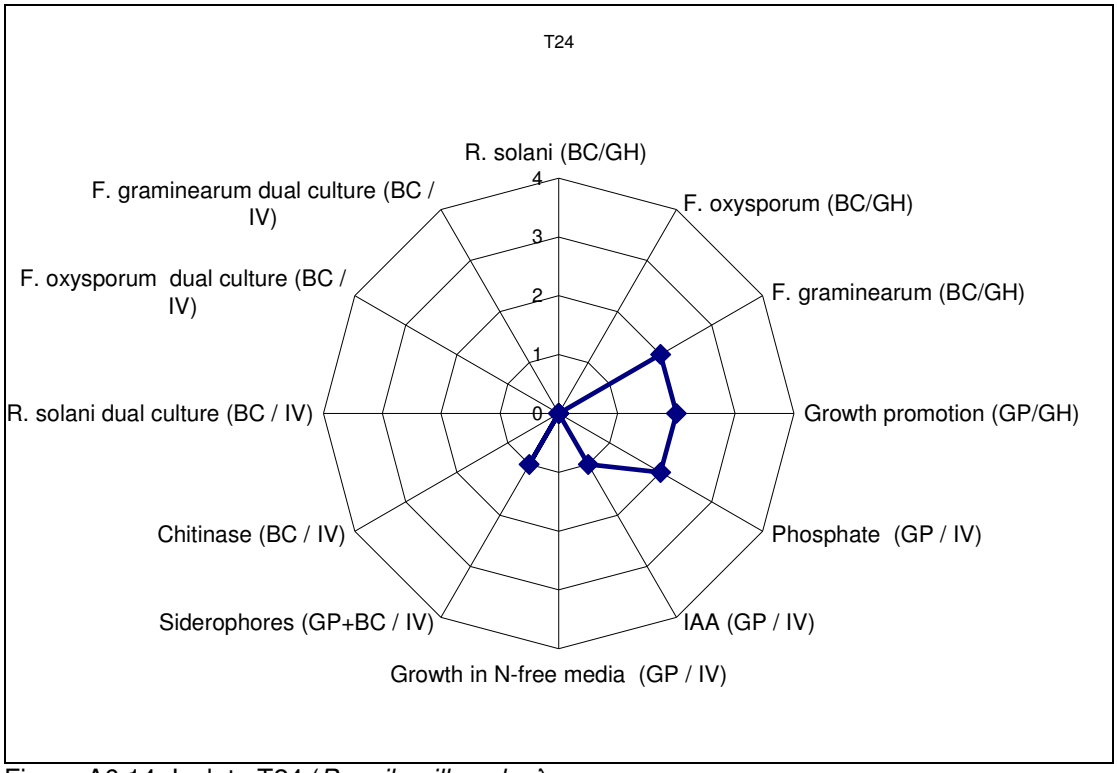

Figure A6.14: Isolate T24 (*Paenibacillus alvei*)

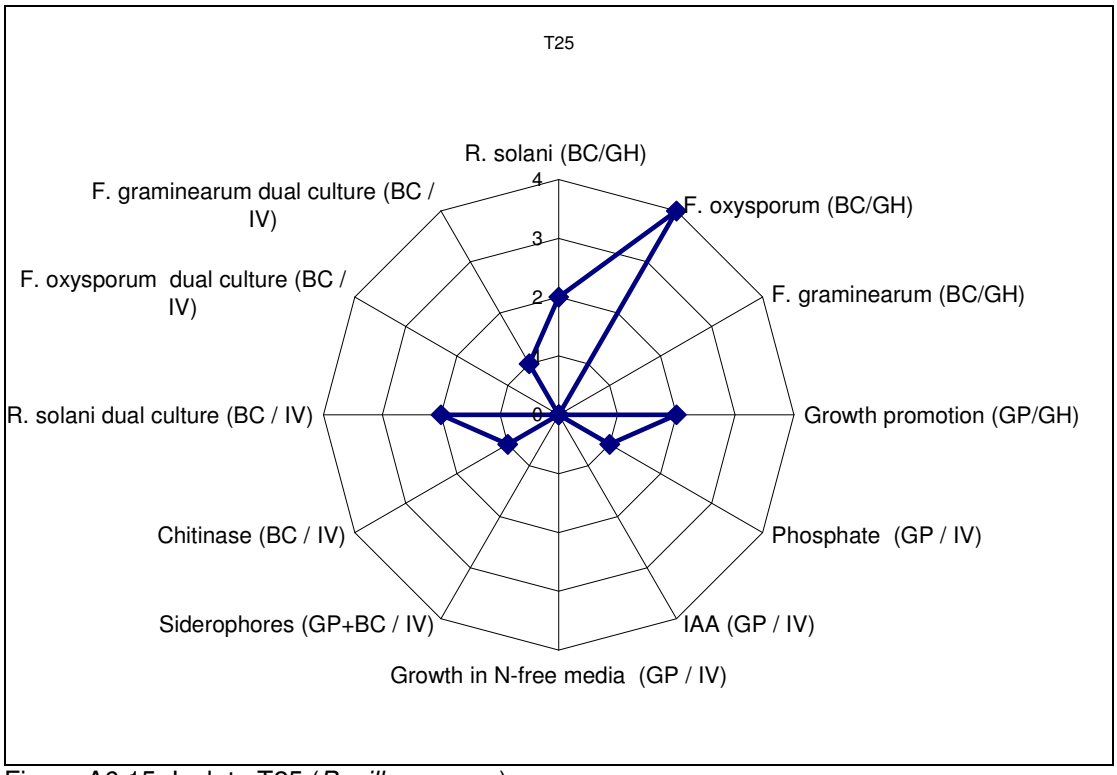

Figure A6.15: Isolate T25 (*Bacillus cereus*)

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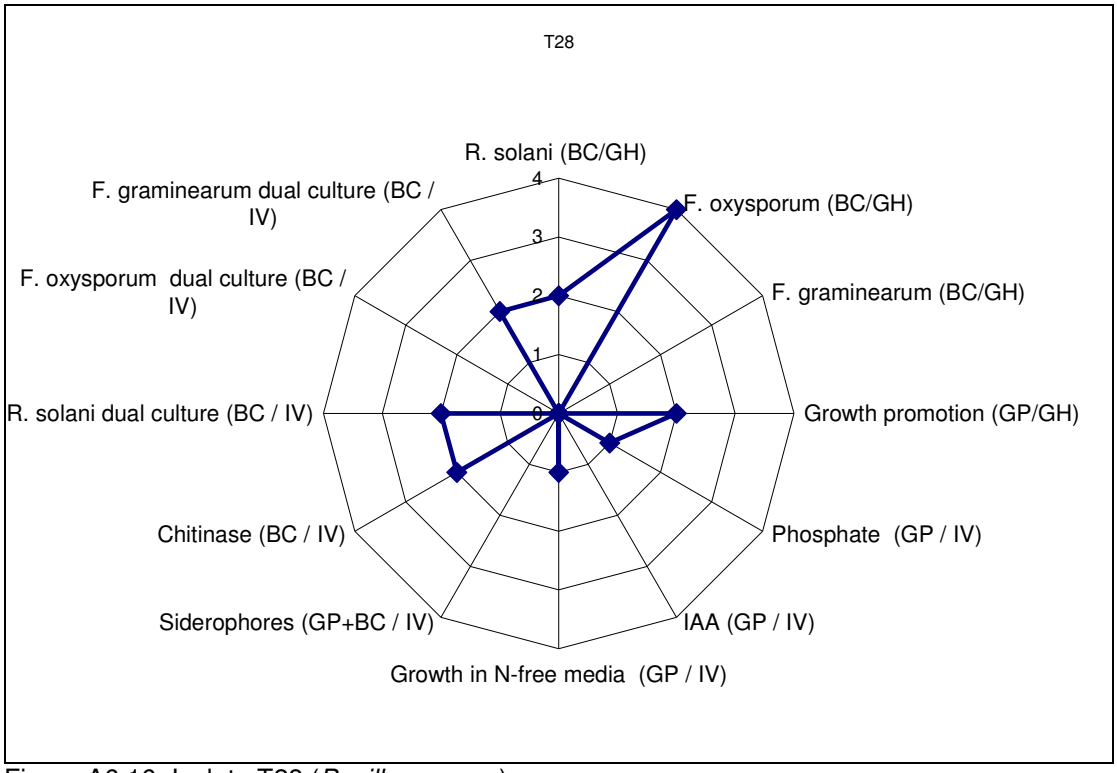

Figure A6.16: Isolate T28 (*Bacillus cereus*)

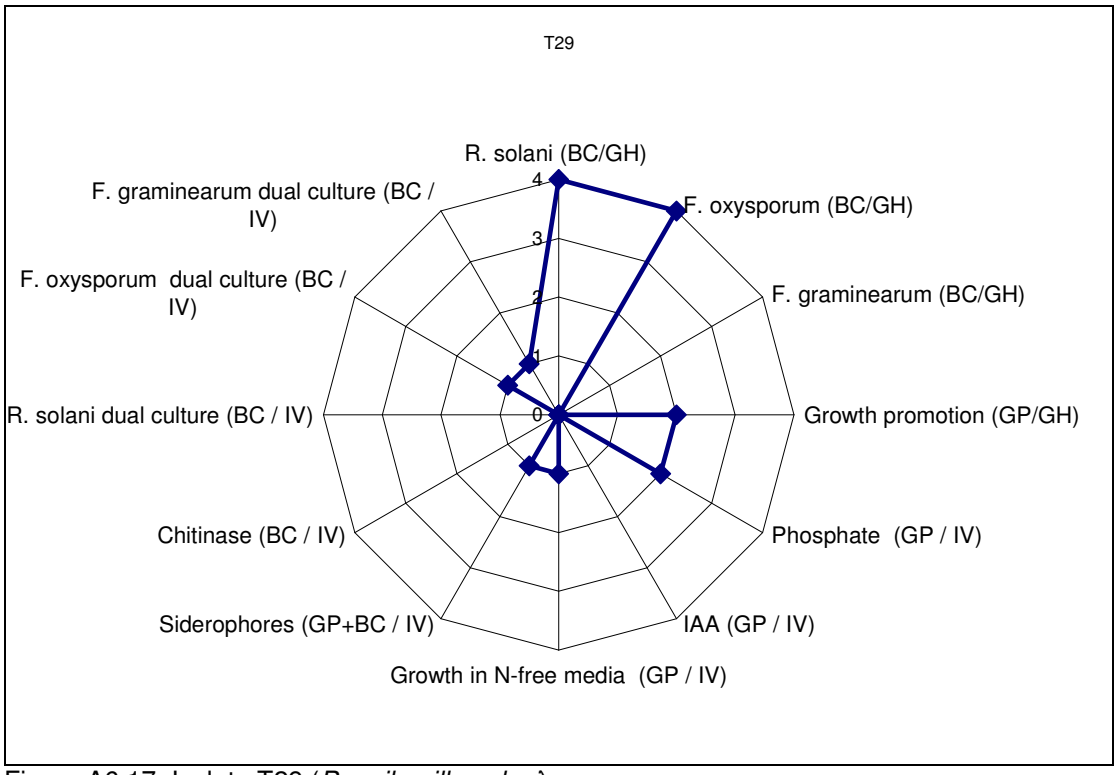

Figure A6.17: Isolate T29 (*Paenibacillus alvei*)

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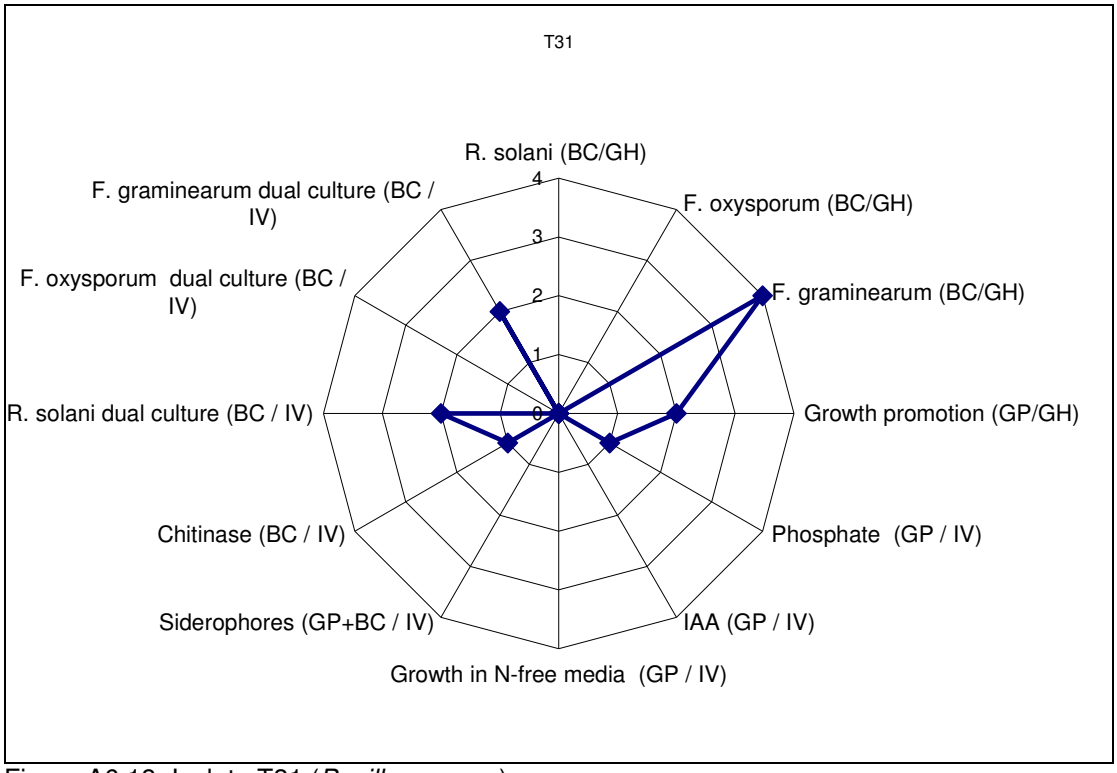

Figure A6.18: Isolate T31 (*Bacillus cereus*)

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