Efficacy of rhizobacteria for growth promotion and biocontrol of *Pythium ultimum* and *Fusarium oxysporum* on sorghum in Ethiopia and South Africa

By

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Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

In the Faculty of Natural and Agricultural Sciences

Department of Microbiology and Plant Pathology

University of Pretoria

Pretoria

July 2007

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DECLARATION

I, the undersigned, declare that the PhD thesis entitled: ‘’Efficacy of rhizobacteria for growth promotion and biological control of *Pythium ultimum* and *Fusarium oxysporum* on sorghum in Ethiopia and 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.

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ACKNOWLEDGEMENTS

I would like to express my heartfelt and sincere gratitude to my supervisors Prof. Nico Labuschagne and Prof. Lise Korsten for their close supervision and scientific advice throughout the course of this study. I am also so grateful for the encouragement and support made by Prof. N. Labuschagne without whom this thesis couldn't have achieved its success.

My due thanks and appreciations also go to the following people and institutions:

- Alemaya University of Ethiopia, particularly the Agricultural Research Training Project (ARTP) for giving me this opportunity and funding to complete the project.
- Dr. Brook Lemma of the Faculty of Education, Alemaya University in Ethiopia for allowing me to use the facilities in the Microbiology Laboratory during my stay in Ethiopia.
- FABI (Forestry and Agricultural Biotechnology Institute) for providing me a Laboratory space and access to the facilities in the entire FABI complex.
- Mrs Amelita Lombard of Microbiology and Plant Pathology, University of Pretoria for her unreserved technical and material assistance throughout the course of this study.
- Mrs Sonya September of the Department of Microbiology and Plant pathology, University of Pretoria for her assistance during PCR amplification and 16S rDNA sequencing.
- Mrs Melita of the Telematic education centre (photographic section) for her skilled ability to take very nice pictures of my project.
- My colleague Mrs Misrak Kebede of the Department of Biology, Alemaya University, Ethiopia for representing me back home in all aspects of social and financial matters by devoting her precious time.
- All my friends (Alemaya and EARO staff studying at UP) who in one or another way provided me with their unreserved support.
- My brother Nunu (Elias Siraj) who was always besides my family whenever needed.
- May the souls of my late mother Desta Hawas, my sister Zeytuna Seid and my nephew Hannan Mohammod rest in peace.
- Last but not least, I am so grateful to my beloved family, my wife Yalemzerf Yimenu and my son Frezer Ahmed, for accompanying me here in South Africa and for their patience and endurance to share the ups and downs ever since this project was started.

CONTENTS

LIST OF ABBREVIATIONS

PGPR = Plant growth promoting rhizobacteria
cfu = colony forming units
RBGU = Rose-bengal-glycerol-urea medium
API = Analytical profile index
ANOVA = Analysis of variance
DMR = Duncan's Multiple Range
ISR = Induced systemic resistance
IAA = Indole 3- acetic acid
ACC deaminase = 1-amino cyclopropane 1-carboxylic acid deaminase
KB medium = Kings B medium
PDA = Potato dextrose agar
PVK = Pikovskaya agar
CAS = Chrome-azurol-sulphur
NBRIY = National botanical research institute (modified PVK) medium.
2, 4- DAPG = 2-4-diacetyl phloroglucinol.
PCR = Polymerase chain reaction
DNA = Deoxyrhibonucliec acid
dNTP = deoxy nucleotide triphosphate.
et al. = and others
sp = species (single)
spp. = species (plural)
µg = microgram
µl = microlitre

SUMMARY

In-vitro and greenhouse screening of 78 bacterial isolates from sorghum rhizosphere in Ethiopia and 86 isolates from the rhizosphere of grasses at Nylsvlei Nature Reserve in South Africa was conducted in terms of inhibition of *Fusarium oxysporum* that causes root rot in sorghum. Among the Ethiopian isolates KBE5-7, KBE5-1, KBE2-5 and NAE5-5 resulted in 100% disease suppression while disease suppressions ranging from 85.6% - 95.8% were rendered by South African isolates KBS9-H, KBS9-B, KFP9-A, NAS6-B and KBS5-F. According to identification by means of API and 16S rDNA sequencing, the majority of the effective isolates belong to members of the genus *Bacillus*. Other Gram negative isolates effective in this study have been identified as *Serratia marcescens*, *Chryseomonas luteola*, *Stenotrophomonas maltophilia* and *Enterobacter sakazaki*.

Screening of rhizobacterial isolates was also conducted in terms of *in-vitro* and *in-vivo* antagonistic activity against *Pythium ultimum* Trow, a common soilborne pathogen causing yield reductions in a wide variety of crops including sorghum. Statistically significant disease suppression was achieved by a number of isolates both from Ethiopia and South Africa. Most of the effective isolates maintained themselves in the rhizosphere at a level of $\geq 10^{5}$ cfu/g four weeks after inoculation. While *Bacillus cereus* was the predominant isolates from both sites, *Brevilbacterium laterosporus*, *Serratia marcescens* and *Pseudomonas fluorescens* were among the most effective isolates with the potential to suppress *Pythium ultimum in-vitro* and *in-vivo*.

Modes of action studies assessing production of antibiotics, siderophores, chitinolytic activity and induction of systemic resistance in sorghum were conducted for rhizobacterial isolates effective against *F. oxysporum* and *P. ultimum*. The antibiotic substances produced in the culture filtrates of many of these effective bacteria resulted in strong antifungal activity against both pathogens. The antibiotics from *Bacillus cereus* (KBS5-H) and *Bacillus subtilis* (KBS6-3) resulted in an efficient antagonistic activity against *F. oxysporum* and *Pythium ultimum* respectively. Siderophore production was evident in the Gram-negative strains *Serratia marcescens* (KBS9-R), *C. violaceum* (KBE9-1) and *E. sakazaki* (NAS6-B) with prominent yellow/orange halo development on CAS-agar plates demonstrating the potential by these isolates to produce siderophores under iron stressed conditions. Chitinolytic activity on chitin-agar plates was shown by isolates which mostly (83 %) belonged to strains of *B. cereus* The split root system has also demonstrated that *B. cereus* (KBS5-H), *C. violaceum* (KBE9-1) and *S. marcescens* (KBS9-R) were capable of rendering significant induction of systemic resistance against *F. oxysporum* in sorghum. The successful *in-vitro* and *in-vivo* suppression of *F. oxysporum* and *P. ultimum* by the effective rhizobacterial isolates and the

presence of various modes of action provide useful information on the potential of these isolates as biocontrol agents against soilborne fungal pathogens.

The isolation and screening of rhizobacteria for growth promotion of sorghum has also been conducted under greenhouse condition in pathogen free soils. Three isolates from Ethiopia and 10 isolates from South Africa have been identified as the most effective growth promoting isolates in these studies. The isolates also tested positive for the production of siderophores, production of indoleacetic acid and phosphate solubilization, the direct modes of actions through which bacteria promote plant growth in the rhizosphere of several plants. Of the most effective isolates 44 % were identified as *Bacillus cereus,* 19 % as *Chryseomonas luteola,* 13 % as *Serratia marcescens*, 13 % as *Sphingomonas paucimobilis*, and 6% each as *Stenotrophomonas maltophilia* and *Brevibacterium laterosporus* respectively.

The best biocontrol agents were selected out of a total of 24 isolates both from Ethiopia and South Africa. The selection procedure was conducted by using criteria such as the *in-vitro* and *in-vivo* suppression of *Fusarium oxysporum* and *Pythium ultimum,* the root colonization ability of the bacterial isolates and selected modes of action including production of antibiotic substances and siderophores, chitinolytic activity and induction of systemic resistance in sorghum. According to this procedure five isolates from Ethiopia (KBE5-7, KBE5-1, KBE9-1, NAE1-7 and NAE5-7) and six isolates from South Africa (KBS5-F, KBS9-R, KBS6-H, KBS5-H, KFP9-K and KBE6-17) have been selected as the most efficient biocontrol isolates. The selection of the best performing growth promoting isolates was conducted out of 12 efficient isolates using the following criteria: root colonization, siderophores and indoleacetic acid (IAA) production, phosphate solubilization and bacterial growth profiles in liquid cultures. Two isolates from Ethiopia (KBE7-8 and KBE9-1) and five isolates from South Africa (KBS5-H, KBS5-F, KBS6-H, KBS9-B and NAS4-3) have been selected as the best growth promoting isolates. As the screening and selection of this study are based on laboratory and greenhouse studies, further evaluation of the best isolates under field conditions and additional modes of action studies are warranted to ascertain their full potential as biocontrol and growth promoting agents.

CHAPTER 1– Literature Review

1. 1. *Fusarium* and *Pythium* spp. as pathogens of sorghum

One of the major concerns to agricultural food production worldwide is diseases caused by phytopathogenic fungi of which *Fusarium* and *Pythium* attack most of the economically important crop plants (Gohel *et al.,* 2006). Sorghum (*Sorghum bicolour* (L) Moench) is ranked second among the five most important cereal crops in Eastern Africa (FAO, 1999). It is believed that sorghum was probably initially domesticated in central Africa in the region of Ethiopia and Sudan. From its initial cultivation in Africa, the crop was introduced into Asia, America and Australia (Forbes *et al*., 1986). Sorghum is an economically important crop providing food and fodder in the semi arid tropics of the World. In Ethiopia, it is widely grown in the Southern part of the country especially in the dry land areas with high temperature and low rainfall. The grain yield currently estimated in the continent is relatively lower than those in other parts of the World (FAO, 1999). Low sorghum yields are mainly due to insect pests and diseases caused by phytopathogenic fungi and bacteria. Most of the fungal pathogens reported on sorghum worldwide occur in Eastern Africa including Ethiopia (Huluka and Esele, 1992).

Several species of fungi are known to cause various types of diseases in sorghum. *Pythium* and *Fusarium* spp. are among the most common phytopathogenic fungi that cause seedling and root rot diseases in sorghum (Forbes *et al*., 1986; Horne and Frederickson, 2003). *Pythium* spp. survives in the soil as oospores and germinates in response to seed and root exudates in wet soil. They germinate either directly by producing germ tubes or indirectly by producing zoospores. The pathogens can then rapidly penetrate host cells and tissues that lack secondary wall thickenings (Forbes *et al.,* 1986). Evidence has been presented that *Pythium ultimum* Trow var. *ultimum* causes a chronic root and seed rot of grain sorghum and negatively affects grain yield in a continuous sorghum production system (Davis and Bockus, 2001). *Pythium graminicola* Subramanian is also reported to cause root rot in sorghum (Horne and Frederickson, 2003). *Pythium* root rot in sorghum is characterized by various symptoms including necrosis of seedling leaf tip and blade, collar rot root rot and streaking of the vascular system leading to the death of the plant (McLean and Lawrence, 2001).

Sorghum is also attacked by root and stalk diseases caused by a number of *Fusarium* spp. The primary inoculum of *Fusarium* spp. consists of conidia and mycelia that have over seasoned in crop debris and the propagules are not capable of surviving more than three months in the absence of

plant debris (Claffin, 1986). The fungi are widely distributed in host root tissues under field conditions and respond to stress in the plant by taking advantage of preferential growth conditions to incite diseases (Leslie, 1990). In one experiment for example (Leslie, 1990), *F. moniliforme* and *F. proliferatum* have been recovered from root tissues in 71 % sorghum samples.

In tropical and temperate regions, *F. moniliforme* is the major causative agent of seed rot; seedling blight; root and stalk rot (Claffin, 1986; Horne and Frederickson, 2003). *F. moniliforme* is also involved in causing grain mold of sorghum, a serious disease which became a major constraint to sorghum improvement and production worldwide (Navi *et al.,* 2005). Prominent in the root and stalk rot of sorghum are also other *Fusarium* spp. such as *F. oxysporum*, *F. graminearum*, *F. tricinctum*, *F.solani* and *F. equiseti* (Claffin, 1986). Mahalinga *et al.* (1988), reported that *F. oxysporum* and *F. pallidoroseum* are potentially pathogenic on some sorghum genotypes causing a negative effect on seed germination and seedling growth. *Fusarium* spp. from sorghum, millet and maize have recently become the subject of in depth research due to serious production losses in these crops as a result of stalk rot, ear rots and grain mold infections by these fungi (Leslie *et al.,* 2005). According to this report, *Fusarium* isolates recovered from sorghum and millet are identified as *F. moniliforme.* This is an indication that sorghum is attacked by a wide spectrum of *Fusarium* spp. leading to a serious loss in yield. Losses due to seedling blight and root and stalk rot caused by *Fusarium* spp. vary from 5-10% and may approach 100% in localized areas (Claffin, 1986).

Although many fungicides can be applied to sorghum seeds, they do not provide effective control of seedling and root diseases caused by *Pythium* and *Fusarium* spp. In the past, seed treatment with systemic oomycetes fungicide metalaxyl to prevent *Pythium* spp. has been found effective. However, as these fungicides are specific to oomycetes only and with the reported development of fungicide resistant species, the use of fungicides has become restrictive. In the light of global chemophobia, alternative disease control strategies such as biological control have become important.

1. 2. Plant growth promoting rhizobacteria (PGPR)

Bacteria that can improve plant growth through various mechanisms have been known for decades and have been introduced into soil, on seeds or roots to improve plant growth and health (Raaijmakers *et al.,* 2002). The Genus *Rhizobium*, an example of a growth-promoting organism, is the most widely known group. It has been successfully commercialized with many practical applications in agriculture by developing symbiosis with plants.

Early in this century, many bacterial species associated with plants but without symbiotic association were discovered (Bashan and Holguin, 1998). Although many of these bacteria were able to promote plant growth, they were not widely recognized until the mid 1970's, with the discovery that some bacteria mainly *Pseudomonads* are capable of controlling soil borne pathogens and indirectly enhance plant growth (Kloepper *et al.,* 1980). The discovery of *Azosprillum* species, a diazotrophic free living bacterium that proliferates in the rhizosphere of many tropical grasses (Maria *et al.,* 2002), is the other break through in the study of plant- microbe- interaction in the rhizosphere.

The term plant growth promoting rhizobacteria (PGPR) was originally used to describe this unique biocontrol group (Kloepper *et al.,* 1980). As this term does not encompass all the beneficial bacteria associated with plants in the rhizosphere, generally the plant growth promoting rhizobacteria are classified into two major group viz. Biocontrol Plant Growth Promoting Bacteria (Biocontrol PGPR) and Plant Growth Promoting Bacteria (PGPB) (Bashan and Holguin, 1998).

1. 2. 1. Biocontrol PGPR

The application of chemical inputs such as fertilizers and pesticides has long been used to improve productivity in conventional agriculture. However, there is now a growing desire for alternatives to this system (Mark et al., 2006). The use of bacteria as biocontrol agents of soilborne plant pathogens has been investigated for several decades (Landa *et al.,* 2004). However, suppression of soilborne root pathogens in soil has drawn considerable attention only recently as alternative farming method to maintain the productivity of agro ecosystems (Hu *et al.,* 1997). Rhizobacteria designated as biocontrol PGPB are those that suppress plant pathogens by producing various types of inhibitory substances, or by increasing the natural resistance of the plant (Jetiyanun and Kloepper, 2002; Gardner *et al.,* 2001; Bashan and de Bashan, 2002) or by displacing (out competing) the pathogen (O'Sullivan and O'Gara, 1992). Such biocontrol PGPBs have the capacity to rapidly colonize the rhizosphere, and compete with deleterious microorganisms as well as soilborne pathogens at the root surface (Rangarajan *et al.,* 2003). Some of these modes of action used by biocontrol plant growth promoting bacteria are discussed below.

1. 2. 1. 1. Antibiosis

Recent advances in the understanding of genetics and the regulation of synthesis of bacterial metabolites especially antibiotics have contributed significantly to the advancement of plant protection. The biocontrol PGPBs are mainly endowed with the capacity to produce antibiotics

against a number of phytopathogenic fungi and bacteria. Such biocontrol PGPB produce one or more of the antibiotics 2, 4-diacetylphloroglucinol (2, 4-DAPG), phenazine compounds (*Phz*), pyrrolnutrine (*Prn*), and pyluteorin (*Plt*) (Mazzola *et al.,* 1992; Raaijmakers *et al.,* 1997). These antibiotics are currently the major focus of research in biological control in soil ecosystems (Raaijmakers *et al.,* 1997). Hydrogen cyanide (HCN) is also reported to be one of the anti-fungal secondary metabolites produced by such biocontrol plant growth promoting bacteria (Cheryl *et al.,* 1998). More recently the production of new antifungal metabolites belonging to the class of cyclic lipopeptides such as visconsinamide and tensin has been reported (Bloemberg and Lugtenberg, 2001).

Screening of microorganisms to identify biocontrol agents, which are active against many phytopathogenic fungi and bacteria, has been carried out in the past. The mode of action of many bacteria has been ascribed to the antibiotics they produce. Among the potential biocontrol agents, which are active in the rhizosphere, *Pseudomonas* and *Bacillus* spp. have been widely investigated (Williams and Asher, 1996). Several strains of *Pseudomonas* spp. are used to control diseases in a variety of crops and other non-crop plants (Commare *et al.,* 2002). During their stationary growth phase, biocontrol strains of *Pseudomonas* synthesize the antibiotics phenazine carboxylic acid (PCA), 2, 4-DAPG, pyoluterin and pyrrolnitrin (Schnider *et al.,* 1995). Many of these antibiotics produced by *Pseudomona*s spp. *in-situ* contributed to the suppression of many plant diseases. Such antibiotic producing *Pseudomonas* spp. have been isolated from the rhizosphere soils that are naturally suppressive to diseases (Keel *et al.,* 1996). Plant diseases caused by the fungal pathogens *P. ultimum* and *Rhizoctonia solani* Kühn are, for instance, suppressed by different strain of *Pseudomonas fluorescens* (Cheryl *et al.,* 1998). *P. ultimum* mediated damping-off in sugar beet has been inhibited due to the production of 2, 4-diacetylphloroglucinol (2, 4-DAPG) by *P. fluorescens* F111 biocontrol strain. This product is also produced by other *P. fluorescens* strains and has been found effective against *Fusarium oxysporum*. attacking tomatoes. Recently, it has been demonstrated that fluorescent *Pseudomonas* spp. producing the antibiotic 2, 4-DAPG play a key role in the suppressiveness of take-all decline (TAD) in soils (de Souza *et al.,* 2003a) and the amount of 2, 4-DAPG produced *in-vitro* by these strains correlated with disease inhibition.

Phenazine antibiotics are another group of secondary metabolites effective against phytopathogenic fungi. *Pseudomonas* strains which produce phenazine antibiotics are reported for their suppression of take all of wheat caused by *Gaeumanomyces graminis* var. *tritici* (Mazzola *et al.,* 1992). To determine the importance of this antibiotic in suppression of take all, experiment with phenazine deficient mutants (*Phz*$^{-}$) generated by Tn 5 mutagenesis failed to inhibit *G. graminis* var tritici on media supportive of antibiotic production (Thomashow and Weller, 1990).

Antibiotics of the group cyclic lipopeptides such as visconsinamide produced by *P. fluorescens* have been shown to have an impact on the control of *Pythium* spp. and *R. solani* (de Souza *et al.,* 2003b). These cyclic lipopeptides induce encystment of *Pythium* zoospores and adversely affect the mycelia of *R. solani* and *P. ultimum* by causing reduced growth and intracellular activity, hyphal swelling and increased branching (Thrane *et al.,* 2000; de Souza *et al.,* 2003b). Nielson and Sorensen (2003), screened *P. fluorescens* strains capable of antagonizing *P. ultimum* and *R. solani* on agar plates. Further investigation during the early seed germination and root development of sugar beet revealed that the cyclic lipopeptide antibiotics were responsible for the antagonistic activity *in-vitro* (Nielson *et al.,* 1999; Nielson *et al.,* 2000; Nielson *et al.,* 2002).

The next most widely researched and commercialised bacteria for biocontrol activity in soil ecosystems are the endospore forming genus *Bacillus*. Most of the antibiotics produced by *Bacillus* spp. *in-vitro* were found to be peptide antibiotics and are responsible for biocontrol *in-vivo* (Leiferat *et al.,* 1995). *Bacillus cereus* UW85 that produce the antibiotics zwittermycin A and antibiotic B tend to suppress damping off disease more effectively than do *Bacillus* strains that do not produce antibiotics (Stabb *et al.,* 1994). This strain was initially identified from a collection of rhizosphere isolates by its ability to suppress alfalfa damping off consistently (Handelsman *et al.,* 1990). Since then, *B. cereus* UW85 has proven an effective biocontrol agent against Phytophthora damping-off and root rot of soy beans (Emmert and Handelsman, 1999). In general, the antibiotic zwittermycin A produced by this strain has been reported to adversely affect the growth and activity of a wide range of plant pathogenic fungi (Silo-Suh *et al.,* 1998).

Several other members of the genus have been shown to produce antibiotics of which the most important species is *Bacillus subtilis* (Foldes *et al.,* 2000). *B. subtilis* is one of the most widely distributed bacterial species in agricultural systems. The most commercially successful strains among this group is *B. subtilis* GBO3. This strain which effectively colonizes plant roots and produce antifungal compounds is the active ingredient in one of the widely distributed biofungicide (Kodiac, Guftafson LLC) (McSpadden and Fravel, 2002). Another best known biocontrol strain of this species isolated 25 years ago in Australia is *B. subtilis A13* (Kim *et al.,* 1997). This strain, in addition to inhibiting all the nine pathogens tested in an *in-vitro* test, subsequently promoted the growth of cereals, sweet corn and carrots when applied as seed inoculants (Kim *et al.,* 1997). *Bacillus* spp. are therefore considered ideal candidates for use as biocontrol agents in seed treatment programs against soilborne pathogens (Walker *et al.,* 1998).

1. 2. 1. 2. Siderophore production

Iron is one of the most abundant minerals on earth, yet in the soil, it is unavailable for direct assimilation by plants or microorganisms. This is because ferric iron (Fe^{+3}), the most common form of iron in nature, is only sparingly soluble (10^{-18} M at pH 7). Therefore, the amount of soluble iron in the soil barely supports microbial growth (Glick and Bashan, 1997). To overcome this problem, soil microorganisms secrete siderophores, iron-binding proteins of low molecular mass (400-1000 daltons) which bind Fe^{+3} with a very high affinity (KD = 10^{-20} to 10^{-15}). Most aerobic and facultative anaerobic microorganisms produce Fe^{+3} chelating siderophores which bind and transport ferric iron back to the microbial cells, where it is taken up by means of cellular receptors (Brait, 1992; Glick and Bashan, 1997; Bultreys *et al.,* 2001).

Biocontrol PGPBs prevent the proliferation of soilborne pathogens and facilitate plant growth through the production and secretion of such siderophores. The siderophores bind most of the Fe^{+3} available in the rhizosphere thereby effectively preventing any fungal pathogen in the immediate vicinity from proliferating due to a lack of iron (O'Sullivan and O'Gara, 1992). Siderophores produced by fungal pathogens have a much lower affinity for iron than those of biocontrol PGPB. Thus, biocontrol PGPB out-compete fungal pathogens for the available iron in the rhizosphere (Glick and Bashan, 1997). Siderophores also indirectly stimulate the biosynthesis of other anti-microbial compounds by making these minerals easily available to the bacteria (Duffy and Defago, 1999).

The major types of siderophores produced by biocontrol PGPB include pyoverdin, pyochelin and salicylic acid (Lemanceau *et al.,* 1992; Duffy and Defago, 1999; Bultreys and Gheysen, 2000). Numerous studies indicate that among the biocontrol PGPB in the rhizosphere, the fluorescent *Pseudomonas* species are efficient competitors for ferric iron (Fe^{+3}). The most commonly detected siderophores in these species are called pyoverdins or pseudobactins (Lemanceau *et al.,* 1993). Many potential biocontrol strains of this species produce pyoverdines. They are generally peptide siderophores all containing the same quinoline chromophore which is responsible for the colour of the molecule, a peptide chain and a dicarboxylic acid connected to the chromophore (Bultreys and Gheyson, 2002; Bultreys *et al*., 2003). The characteristic fluorescent pigments of fluorescent *Pseudomonas* are due to the pyoverdins (Budzikiwicz, 1993). Apart from this taxonomic importance and most importantly, pyoverdins produced *in-situ* chelate iron and make iron unavailable to pathogens in the rhizosphere (Looper and Henkels, 1999). Some fluorescent *Pseudomonas* species

also produce a non-fluorescent siderophore called pyochelin, a salicylic substitute cystein peptide (Leeman *et al.,* 1996).

Many workers have reported the suppression of disease development of several soilborne pathogens by different strains of biocontrol *Pseudomonas* spp. producing siderphores. A pyoverdin siderophore called pseudobactin 358, for instance, produced by a strain of *Pseudomonas putida* was reported as an effective biocontrol agent against *Fusarium* wilt (Lemanceau *et al.,* 1993.). *Fusarium* wilt diseases are currently responsible for important yield loses on a variety of crops (de-Boer *et al.,* 1999). Many strains belonging to the *Fusarium* genus often cause severe diseases such as vascular wilt, root rot and abnormal growth in various agricultural crops (Kurek and Jaroszuk-Scisel, 2003). Although many inputs of agrochemicals are used to protect the crops against this pathogen, they are adversely affecting the quality of the food product and that of the environment (Lemanceau *et al.,* 1992).

More sophisticated techniques are currently being used to evaluate the importance of siderophore mediated competition for iron by biocontrol rhizobacteria. Recent studies using a well defined mutant (Pvd^-) has indicated the involvement of pyoverdin siderophores in the control of *Fusarium* wilt of radish and carnations (Lemanceau *et al.,* 1992; Lemanceau *et al.,* 1993; Raaijmakers *et al*., 1995; Thomashow, 1996). Similarly, *Pythium* induced post emergence damping-off has also been suppressed in hydroponically grown tomato using strains which produce pyoverdins and pyochelins (Buysens *et al.,* 1996).

In many other studies, the efficacy of siderophores of biocontrol PGPB strains of *Pseudomonas* spp. has been proven to be very promising. In this respect, for instance, a mutant strain of *Pseudomonas aerogenosa* that lacks the ability to produce siderophore no longer had the ability to protect tomato plants from damping off (Glick and Bashan, 1997). Normally, siderophores are produced by bacteria under iron limiting conditions in the rhizosphere. In an effort to prove this, researchers (Elsheriff and Grosmen, 1994) conducted an experiment in which the amount of iron present in the soil was increased to 40 μmol Fe^{+3} / lit. The result obtained indicated a concomitant decrease in both the amount of siderophores produced and the inhibitory effect against the wheat pathogen *G. graminis* var *tritici*.

Whether biocontrol PGPB in the rhizosphere actually synthesizes siderophores in response to iron limiting conditions can be detected by means of a more advanced technique, namely an ELISA assay

using monoclonal antibodies. With this method it is possible to quantify the amount of siderophores produced in an ecosystem (Buyer *et al.*, 1993).

1. 2. 1. 3. Induction of Systemic Resistance

Under normal conditions all plants possess active defence mechanisms against pathogens' attack, which sometimes fails upon infection by a virulent pathogen. This happens as a result of the pathogen suppressing the resistance reactions (van Loon *et al.*,1998). If, however, defence mechanisms are triggered by stimulus before infection by the pathogen, the disease can be minimized i.e. the plants will have enhanced defensive capacity. This systemic protection of a plant by an inducing agent when applied to a single part of the plant is known as *Induced Systemic Resistance* (ISR). (Liu *et al.,* 1995; Nandakumar *et al.,* 2001; Ramamoorthy *et al.,* 2002). In nature induced resistance occurs as a result of limited infection by a pathogen and the subsequent development of a hypersensitive reaction (van Loon *et al.,* 1998).

Induced resistance brought about by the inducing agent is systemic as the defensive capacity is increased not only in the primary infected tissue, but also in the non-infected tissue. According to Sticher *et al.* (1997), induced resistance is commonly referred to as *Systemic Acquired Resistance* (SAR) due to its systemic character. In some cases however, localized acquired resistance occur when only those tissue exposed to the primary invader become more resistant (van Loon *et al.,* 1988).

Different biotic and abiotic inducers are involved in induction of systemic induced resistance in plants against various pathogens. These include pathogens, chemical plant products and PGPR (Liu *et al*., 1995; Leeman *et al*., 1995; Nandakumar *et al.,* 2001). The mechanism by which these inducing agents stimulate resistance is that they activate defence genes encoding chitinase, peroxidase, β-1, 4-glucanase and enzymes involved in the synthesis of phytoalexins (van Per *et al*., 1991; Maurhoef *et al*., 1994).

Induced systemic resistance against plant pathogens by biocontrol PGPR is a relatively new topic in disease suppression. It is mediated by effective biocontrol agents such as *Pseudomonas* spp. (Leeman *et al,* 1996). In most of the investigations so far conducted, several strains of *P. fluorescens* are rendering promising results by ISR in many crops. Biocontrol PGPRs elicit ISR in plants through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological

and biochemical reaction of the host plant. This leads to the synthesis of defence chemicals against the challenge pathogen (Ramamoorthy *et al.,* 2001).

Physical and mechanical strength of the cell wall was induced for instance by a biocontrol PGPR in peas (Benhamou *et al.,* 1996a). According to these researchers, treatment of pea plants with a strain of *P. fluorescens* resulted in the formation of structural barriers, i.e. cell wall papillae and deposition of phenolic compounds at the site of the penetration of the invading hyphae of *P. ultimum* and *F. oxysporum*. Similar experiments in potato resulted in the deposition of phenolic compounds, which inhibited the growth of *F. oxysporum* f. sp.radicis Lycopersici in the epidermal cell wall and outer cortex of the root system (Ramamoorthy *et al.,* 2001).

The other mechanism of ISR mediated by biocontrol PGPR is through development of biochemical or physiological changes in the plant. These include the production of PR-proteins (Pathogenesis related proteins) such as chitinase, peroxidase, sysnthesis of phytoalexins and other secondary metabolites (Zdor and Anderson, 1992; van Per *et al.,* 1999). Increased expression of plant peroxidase and chitinase enzymes in rice using strains of *P. fluorescens* was efficient enough to inhibit mycelial growth of the sheath blight fungus *R. solani* (Nandakumar *et al.,* 2001). In another experiment, seed treatment of pea by one strain of *P. fluorescens* resulted in the production of hydrolytic enzymes such as β-1, 4- glucanase, and chitinase (Benhamou *et al.,* 1996b). In all cases the host lytic enzymes accumulate at the site of penetration of the fungus.

Induction of systemic resistance by biocontrol PGPB is not confined to the aforementioned plant species. *Pseudomonas* spp. mediated ISR was also observed in carnation against *F.* oxysporum f.sp.dianth (van Per *et al.,* 1991), in cucumber against *Colletotrichum orbiculare* (Berk. and Mont.) (Wei *et al.,* 1996) and *Pythium aphanidermatum* (Edson) Fitzp (Chen *et al.,* 2000).

Elicitation of ISR in plants has been reported to be mediated by specific strains *Bacillus* spp. such as *B. amyloliquifaciens, B. subtilis*, *B. cereus*, *B. mycoides*, and *B. pumilis* with significant reductions in the incidence or severity of various diseases (Kloepper *et al.,* 2004). Certain strains of *Bacillus pumilus* have been reported to be involved in SIR in plants by inducing the accumulation of phenolic compounds in the newly formed wall appositions in pea roosts in response to attack by *F.oxysporum* f. sp. *pisi* (Benhamou *et al.,* 1998; Jetiyanun and Kloepper, 2002). The phenolic compounds contribute to enhance mechanical strength of the host cell wall and may also inhibit fungal growth as phenolics are toxic to fungi in nature (Ramamoorthy, 2002). This is an indication for the potential of *Bacillus* spp. to be used as bio-control PGPR similar to *Pseudomonas* spp. in the soil rhizosphere.

The major bacterial determinants that are claimed to produce ISR in plants by the aforementioned mechanisms are the O' antigen of cell wall lipopolysacharides, siderophores and salicylic acid (Leeman *et al.,* 1996; Bloemberg and Lugtenberg, 2001). For instance, the development of ISR in carnation against *Fusarium* wilts by *F. oxysporum*. f. sp. *dianthi* is associated with the lipopolysacharide present in the outer membrane of PGPR *P. fluorescens* strain (Van Per and Shippers, 1992). Similarly in rice, the increased activity of chitinase peroxidase has been reported to be due to the release of these signal molecules by *P. fluorescens* (Ramamoorthy *et al.,* 2001).

The fact that bacterial cell wall lipopolysacharides are involved in ISR was proved in one experiment conducted using a mutant strain of *P. fluorescens* lacking the O' antigen side chain of lipopolysacharides (Leeman *et al.,* 1995). This mutant, unlike the wild strain, failed to induce resistance in radish, showing that the O' antigen side chain of LPS serves as a signal in the induction of systemic resistance. Lipopolysacchride is not the only trait in determining the ISR, because in another study a mutant strain lacking the O' antigen side chain has been reported to elicit defence mechanisms in Arabidopsis (Van Wees *et al.,* 1997). In this respect, while LPS of some *P. fluorescens* strains are the major determinants of ISR under iron replete conditions, siderophores of the same bacterial strain are responsible for ISR in radish against Fusarium wilt under iron limited conditions (Van Loon *et al.,* 1998). How these siderophores trigger ISR is however unclear.

As mentioned before, salicylic acid is also involved in the induction of ISR in plants. Treatment of plants with salicylic acid decreased disease development in tobacco due to tobacco mosaic virus (Kessmann *et al.,* 1994). According to these researchers, certain PGPR strains are endowed with the capacity to produce this compound and induce systemic resistance in plants. Mutant strains lacking the ability to produce salicylic acid production lost their ability to induce systemic resistance in bean as opposed to the wild strain (De Meyer and Hofte, 1997).

Various experiments indicated that ISR by bacterial determinants varies with many factors. These factors include iron limiting conditions, bacterial strains, host plants and their cultivars (Leeman *et al.,* 1996; Van Loon *et al.,* 1998). Although ISR has been studied mainly under laboratory and green house conditions, reports indicate that ISR can protect plants under field conditions (Tuzun *et al.,* 1992; Zhang *et al.,* 2002).

Advantages of ISR over other mechanisms of biological control systems include, once expressed, ISR activate multiple potential defence mechanisms such as increasing the activities of chitinase, β-

1,3 glucanase, peroxidase, pathogenesis related proteins and accumulation of phytoalexins (Wei *et al.,* 1996). Another important aspect of ISR is that, apart from protecting plants against a wide spectrum of pathogens once induced, it also protects plants systematically following application of an inducing agent. Contrary to this, other mechanisms of biological control are generally not systemic (Wei *et al.,* 1996; Zhang *et al.,* 2002).

In conclusion, although plants have their own defence genes, these are quiescent in normal healthy plants i.e. they are inducible genes (Nandakumar *et al.,* 2001). When these endogenous defence mechanisms are induced by appropriate stimuli or signals, the plants own defence mechanisms will be activated. The use of biocontrol plant growth promoting *Pseudomonas* spp. and more recently the application of *Bacillus* spp. to develop ISR in plants is now becoming a novel plant protection strategy.

1.2. 1. 4. Competition in the rhizosphere.

Biocontrol plant growth promoting bacteria also inhibit phytopathogens by other mechanisms than those mentioned before. An example is competition for nutrients and suitable niche on the root surface (O'Sullivan and O'Gara, 1992). The ability to compete for nutrients with indigenous microbial populations within the rhizosphere is an important trait for effective bio-control of soilborne pathogens (Walsh *et al.,* 2001). Strains of *Pseudomonas* spp. have been reported to have the ability to metabolize the constituents of seed exudates in order to produce compounds inhibitory to *Pythium ultimum* (Glick and Bashan, 1997). There is no relationship observed between the ability of these bacteria to inhibit the fungal pathogen by the production of siderophores or antibiotics (Stephens *et al.,* 1993). This was detected by growing the bacterium on a medium that favoured the production of either antibiotics or siderophores.

Due to competition, biocontrol agents have the ability displace some bacterial plant pathogens. The pathogenic *Pseudomonas syringae*, which increases frost susceptibility in tomato and soybean and causes ice nucleation, is reported to have been out competed by an antagonistic ice nucleation deficient medium (Wilson and Lindow, 1994). In one greenhouse experiment (Cooksey, 1990), a non-pathogenic copper resistant Tn 5 mutant of *P. syringae* pv. tomato, the casual agent of bacterial speck of tomato, was co-inoculated with a pathogenic strain. The result was that the non-pathogenic strain decreased the disease incidence significantly by competing with the pathogen for the same niche

In another experiment, protection of tomato seedlings against infection by *P. syringae* pv. tomato was made possible using the plant growth promoting bacterium *Azosprillum brasilense* (Bashan and de-Bashan, 2002). *Azosprillum* spp. are not known as typical biocontrol PGPBs as they lack the ability to produce significant amounts of antimicrobial substances, nor do they induce systemic resistance in plants (Shah *et al.,* 1992). However, because of their rhizo-competent ability and the capacity to form large populations on leaves, *A. brasilense* displaces leaf pathogens and in the process reduce disease severity. The displacement of *P. syringae* pv.tomato by *A.brasilense* was demonstrated by the reduced colonization of the pathogen in the rhizosphere and on the leaf surfaces in the presence of *A. brasilense* (Shah *et al.,* 1992).

1. 2. 2. Plant growth promoting PGPR

The second important division of beneficial bacteria in the rhizosphere are those referred to as plant growth promoting bacteria (PGPB), which promote growth via production of phytohormones and improvement of plant nutrition status (Bai *et al.,* 2002). Because of these properties, the co-inoculation of these PGPB with the symbiotic rhizobia is currently becoming a valuable technique in the development of sustainable agriculture. Among the major groups of plant growth promoting bacteria, the most widely studied and efficient group include *Azosprillum* spp. (Bertrand *et al.,* 2001), *Pseudomonas* spp. (Amy *et al.,* 2002) and *Bacillus* spp. (Bai *et al.,* 2002).

1. 2. 2. 1. Synthesis of phytohormones

The ability of rhizobacteria, particularly the plant growth promoting bacteria (PGPB) to synthesize various metabolites, influences plants as well as the availability of mineral nutrients for plants and the soil structure. A great proportion of microorganisms capable of producing *in-vitro* phytohormones are found to survive in the rhizosphere (Vancura and Jander, 1986). According to this finding, 20 % of the bacteria produced phytohormones. Moreover, from 50 bacterial strains isolated from the rhizosphere of agriculturally important plants, 43 strains produced auxins (IAA), 29 gibberilins, 45 kinetin like substances and 20 strains all three types of phytohormones. All of these strains were able to solubilize poorly soluble phosphates and thus enable phosphorous up take (Vancura and Jander, 1986). The auxin type phytohormone known as indole-3-acetic acid (IAA) is the main type of phytohormone produced by plant growth promoting bacteria (Patten and Glick, 1996; Gonzalez and Bashan, 2000; Patten and Glick, 2002).

One mechanism by which PGPB affect plant growth in the rhizosphere is by contributing to the host plant endogenous pool of phytohormones such as IAA (Patten and Glick, 1996). Beneficial bacteria synthesize IAA through the indole-pyruvic acid pathway. In this pathway, the amino acid trypthophan is first transformed into indole-3 pyruvic acid by oxidative deamination, which is then decarboxylated to indole-3-acetaldehyde. Indole-3-acetaldehyde is finally oxidized to IAA (Vancura and Jander, 1986; Patten and Glick, 2002).

Among the most efficient PGPB studied for their capacity to produce phytohormones are *P. putida*, *P. fluorescens*, *Azosprillum* spp. and *Bacillus* spp. In all these bacteria the formation of IAA and other auxins has been proved using HPLC and mass spectrometers (Vancura and Jander, 1986).

Recently, the role of many such rhizobacterial IAA in the development of the host plant root system has been studied. In one experiment, canola seeds treated with a wild type of *P. putida* strain that produce IAA and another IAA deficient mutant constructed by insertional mutagenesis responded differently (Patten and Glick, 2002). The canola seeds primary roots from seeds treated with the wild type strain were on the average longer than the roots from seeds treated with the mutant strain and the roots from un-inoculated seeds. It was previously indicated by other studies that, while low levels of IAA stimulate primary root elongation, high levels of IAA stimulates the formation of lateral and adventitious roots (Sawar and Kremer, 1995; Xie *et al.,* 1998).

Bacterial IAA promotes root growth either directly by stimulating plant cell elongation or cell division or indirectly by its influence on the ACC deaminase activity. 1-Amino cyclopropane-1-carboxylic acid deaminase (ACC deaminase) is an enzyme produced by many plant growth promoting bacteria (Glick *et al.,* 1998) and is involved in the stimulation of root elongation in seedlings (Glick and Bashan, 1997; Lie *et al.,* 2000). The ACC deaminase hydrolyses plant ACC, the immediate precursor of the phytohormone ethylene. Ethylene in plants acts as a secondary messenger stimulating leaf or fruit abscission, disease development and inhibition of growth (Glick and Bashan, 1997). Mutants of plant growth promoting bacteria that do not produce ACC deaminase, for instance, have lost the ability to stimulate root elongation (Lie *et al.,* 2000). There are several other reports of the role of IAA produced by plant growth promoting rhizobacteria in enhancement of growth and yield of many crops (Ayyadurai *et al.,* 2006).

Another key member of the plant growth promoting bacteria, *Azosprillum brasilense* promotes the growth of many terrestrial plants upon seed or root inoculation (Bloemberg and Lugtenberg, 2001; Bashan and de Bashan, 2002). All the known *Azosprillum* species produce IAA (Gonzalez and

Bashan, 2000) and it is reported that this is the most abundant phytohormone secreted by *Azosprillum*. It is also generally agreed that in most *Azosprillum* species, it is the production of IAA rather than nitrogen fixation that contributes to stimulation of rooting and enhancement of plant growth (Bloemberg and Lugtenberg, 2001). The auxin type phytohormone produced by *Azosprillum* spp. affect root morphology and thereby improve nutrient uptake from soil (Barea *et al.,* 2005). Apart from increasing the density and length of legume root hairs, IAA secreted by *Azosprillum* increases the amount of flavinoids that are exuded and act as signals for initiations of root nodulation by rhizobial strains (Glick *et al.,* 2001).

To summarize, although IAA does not apparently function as a hormone in the bacterial cells, it is important in the microbial-plant relationship, particularly when it comes to stimulating the development of the host plant root system.

1. 2. 2. 2. Assymbiotic nitrogen fixation

In order to sustain sufficient crop production, a reliable source of nitrogen is vital. Microbial oxidation of soil organic matter may thus provide plants with potentially available nitrogen. However in soils with poor soil organic matter, biological fixation of nitrogen is that which fills the deficiency in the soil organic nitrogen pool (Chote *et al.,* 2002). In the rhizosphere, free living nitrogen-fixing PGPR affect plant growth directly by non-symbiotic nitrogen fixation.

Many non-legume plants have been shown to be associated with the free living diazotrophic nitrogen-fixing bacteria. With the advent and the application of the acetylene reduction assay, it has now become a common practice to screen plants and microorganisms for the presence of the nitrogenase activity (Malik *et al.,* 1997). In ecosystems where legumes are sparse or absent, nitrogen fixation by free living diazotrophic bacteria is the mechanism to meet part of the nitrogen requirement of the plants (Brejda *et al.,* 1994). Most of this nitrogen fixation by free-living diazotrophic bacteria in the rhizosphere is associated with the roots of grasses and is regarded as an important component of the nitrogen cycle in many ecosystems.

Rhizosphere bacteria commonly known for such non-symbiotic nitrogen fixation include *Azosprillum, Herbasprillum and Beijerinkia* (Anonymous, 2003). These bacteria are commonly microaerophilic and can be best recovered from tissues by growth in semi solid media with malate as energy source. These free living nitrogen fixers in the rhizosphere are nowadays given attention

(Chotte *et al.,* 2002) as they are known for the utilization of plant exudates as a source of energy to support the fixation process.

Azosprillum spp. proliferates in the rhizosphere of many tropical grasses, fixing nitrogen and transferring it to the plant (Maria *et al.,* 2002). Field inoculation with *Azosprillum* in many investigations revealed that these bacteria are capable of promoting the yield of many important agricultural crops (Okon and Gonzalez, 1994). In wheat, for example, a non-tropical cereal, *Azosprillum* has been assayed widely for field inoculation and resulted in significant yield increase (Maria *et al.,* 2002). *Azosprillum* are also involved in pronounced nitrogen fixation in several other crops such as rice (Malik *et al.,* 1997), corn (*Zea mays*), sorghum (*Sorghum bicolor*) and switch grass (*Pinatum virgutum*) (Bredjda *et al.,* 1994). Although the main emphasis in the search for nitrogen fixing plant growth promoting rhizobacteria in the soil rhizosphere focused on the isolation of *Azosprillum* (Berge *et al.,* 1991), other nitrogen fixing strains such as *Bacillus* spp. have also been found in association with grass roots.

1. 2. 2. 3. Solubilization and mineralization of organic and inorganic phosphates

Phosphate is the second most critical plant nutrient after nitrogen. In the soil rhizosphere, although the total phoshorous pool is high, only a part of this is available to plants. Thus it can be inferred that many soils throughout the world are P-deficient as the free P-concentration even in fertile soils is not higher than 10μM at pH 6.5 (Rodriguez and Fraga, 1999; Gyanshewar *et al.,* 2002).

Soluble phosphorous has a high level of reactivity with calcium, iron or aluminium. This leads to phosphorous precipitation resulting in low levels of P (Gyanshewar *et al.,* 2002). The type of the soil and pH affects the fixation and precipitation of 'P' in soil. Thus, in acidic soils, P is fixed by free oxides and hydroxides of 'Al' and 'Fe', while in alkaline soils it is fixed by 'Ca' (Jones *et al.,* 1991). To overcome the problem of P-deficiency, chemical fertilizers are added. However, the production of chemical phosphatic fertilizers is such an energy intensive process that it requires energy worth $4 billion per annum so as to meet the global need (Goldstain *et al.,* 1993). Despite the fact that most agricultural soils contain large reserves of 'P' due to regular application of phosphorous, a large part of this applied inorganic 'P' is rapidly immobilized and become unavailable to plants (Rodriguez and Frag, 1999).

Because of the aforementioned problems of 'P' availability to plants, there is now a growing need in the selection and manipulation of biofertilizers in plant nutrition. In terms of phosphate

solubilization, the arbuscular mycorrhizae belong to the former category. In recent years the ability of different bacterial species to solubilize inorganic phosphate compounds has been detected and proved to be beneficial in agriculture (Rodriguez and Fraga, 1999; Gyanshewar *et al*., 2002). The higher proportion of these phosphate-solubilizing bacteria is commonly found in the rhizosphere (Baya *et al.,* 1981). The mechanism by which these microorganisms solubilze Ca-P complexes is by their ability to reduce the pH of their surroundings either by the release of organic acids or protons (Gyanshewar *et al.,* 2002). Once the organic acids are secreted, they dissolve the mineral phosphate as a result of anion exchange of $PO_4^{=}$ or they chelate both Fe and Al ions associated with phosphates (Gyanshewar *et al.,* 2002).

Pseudomonas and *Bacillus* spp. are reported as the most important phosphate solubilizers among the PGPR (Baya *et al.,* 1981). At first the production of antibiotics, siderophores and phytohormones has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Kloepper *et al.,* 1989). However, at present there is evidence supporting the role of this mechanism in plant growth enhancement. A strain of *P. putida* for example, stimulated the growth of roots and shoots and increased 'P' labelled phosphate uptake in canola (Lifshitz, 1987). Inoculation of crops with *Bacillus firmis* (Datta *et al.,* 1982) and *Bacillus polymyxa* also resulted in phosphate uptake and yield increase. Rice seeds inoculated with *Azosprillum lipoferum* strain 34H, a known rhizobacteria, increased phosphate ion content and resulted in significant improvement in root length and shoot weights (Murty and Ladha, 1988).

A second major source of plant available phosphorous is that derived from the mineralization of organic matter. Soil contains a wide range of organic substances. Particularly in tropical soils a large part of 'P' is found in organic forms (Rodriguez and Fraga, 1999; Kwabiah *et al.,* 2002). This organic phosphate (Po) is so complex that plants can not directly utilize it but only utilize 'P' in its inorganic form. It is therefore necessary that to make the organic phosphate available to plants, it must be first hydrolyzed to inorganic 'P'. This is called mineralization of organic phosphorous and it is achieved by the activity of phosphatase enzyme, which hydrolyses Po to inorganic forms (George *et al.,* 2002). Plant growth promoting bacteria in the rhizosphere show a significant phosphatase activity (Dinkelager and Marshner, 1992). Plants inoculated with PSMs showed growth enhancement and increased 'P' content as a result of mineralization of organic phosphates. Among these, *Bacillus megaterium* is regarded as the most effective PSM in many field experiments releasing 'P' from organic phosphate, but does not solubilze mineral phosphate (Gyanshewar, 2002).

Phosphate solubilizing bacteria are also reported to function as mycorrhizal helper bacteria (Kraus and Loper, 1995). When such bacteria are associated with mycorrhizal fungi, they promote root colonization. The principle is that, their association with mycorrhizal fungi contribute to the biogeochemical cycle of nutrients by more than just providing a greater surface area for scavenging nutrients that may be relatively immobile in soil (Toro *et al.,* 1997). Generally the role of microorganisms, especially of the growth promoting rhizobacteria in 'P' solubilization and mineralization is very crucial to make 'P' easily available to plants.

1. 3. Bio-formulations and application of rhizobacteria as biocontrol agents

The economic feasibility of any given biocontrol agent is affected by many factors of which formulations of these agents and their delivery system are very important. It has been a common practice to use seed treatment with cell suspensions of many PGPR to control several diseases. This methodology is however becoming impractical due to difficulty in handling, transport and storage of bacterial suspensions (Trapero-Cascas, 1990; Parke *et al.,* 1991). It is very difficult to use bacterial cell suspension for large scale field use. Therefore the need arises to device techniques for the development of formulations in which the biocontrol agents can survive in a carrier material for longer periods of time (Rabindran and Vidhyasekaran, 1996).

Formulation can be considered as the industrial art of converting a promising laboratory proven bacterium into a commercial field product (Bashan, 1998). Such microbial inoculum formulations not only overcome loss of viability during storage in the growers' warehouse, they have also longer shelf life and stability over a range of temperatures between -5 - 30 ^{o}C while in the marketing distribution chain (Bashan, 1998). Formulations are in general composed of the active ingredients i. e. microorganisms or spores which are carried by an inert material used to support deliver the active ingredients to the target (Hynes and Boyetchko, 2005).

Bacterial formulations can be prepared either in liquid or dry forms. Liquid formulations may be oil-based, aqueous based, polymer based or combinations while dry formulations include an inert carrier such as fine clay, peat, vermiculite alginate or polyacrilamide beads (Boyetchko *et al.,* 1999). Among the dry formulations, peat based formulations have been widely investigated and used giving significant result in yield increase and bio-control efficiency. Bacteria can survive well in peat-based formulations for longer periods and PGPR have been reported to survive in such types of dry formulations (Vidhyasekeran and Muthamilan, 1995). Peat-based or talc-base dry formulations allow

the antagonists to be supplied to the farmers for seed treatment or to the seed producers to supply treated seeds to the farmers.

Formulations of PGPR are in general used to promote growth and health of crop plants. Treatment with rhizobacterial formulations for instance enhanced the growth of pearl millet plants and reduced the percentage of downy mildew incidence (Nirajan-Raj *et al.,* 2003). Control of rice sheath blight caused by *R. solani* has been achieved using peat-based formulation of *P. fluorescens* (Rabindran and Vidhyasekaran, 1996). In another experiment, field emergence of chickpea plants was improved by seed treatment with talc based *P. fluorescens* formulation (Vidhyasekaran and Muthamilan, 1995). These results were obtained as the formulated products suppressed pre-emergence damping off caused by various pathogens. Powdered formulations of PGPR in an organic carrier mixed into soilless media provide seeding growth promotion and induce systemic disease protection (Reddy *et al.,* 1999). The practical applications of these PGPR formulations were supported due to the fact that the growth promotion detected was highly significant in comparison with the non-treated controls in various experiments.

Peat formulation has been the carrier of choice and the most commonly used in the rhizobia inoculation industry (Bashan, 1998). It has been common to use peat-based formulations to introduce *Azosprillum*, a biofertilizer, into the rhizosphere. There are however some drawbacks of the peat-based formulations. Peat, as it is an undefined complex organic material, affects the final product and causes difficulties in inoculants dosage and storage condition (Bashan, 1998). Moreover, peat formulations are susceptible to contaminations reducing the shelf life of the inoculants. Due to such problems in peat-based formulations, new trends to use unconventional synthetic materials as PGPR formulations are now becoming more practical and proved more advantageous than peat based formulations. These formulations are based on polymers, which encapsulate the living cells thereby protecting the microorganisms against many environmental stresses.

Alginate is the material most commonly used for encapsulation of microorganisms and the resulting inocula are used as biological control agents and in bacterial chemotaxis research (Bashan and Holguin, 1994). It is a naturally occurring polymer composed of β-1, 4 linked D-mannuronic acid and L-glucuronic acid and it is extracted from different microalgae as well as several bacteria (Smidsrod and Skjac-Break, 1990). Compared with peat-based formulations, alginate-based PGPR formulations have such advantages as being non-toxic, biodegradable and slow release of microorganisms into the soil (Kitamikado *et al.,* 1990).

In general, the selection of appropriate formulations not only improves product stability and viability, but also reduces inconsistency of field performance of many potential biocontrol and growth promoting agents (Boyetichko, 1999). Moreover, irrespective of the type of formulation used, it must be born in mind that effective control and yield increase also depends on the method of treatment and the concentration of the microbial inoculum used (Rabindran and Vidhyasekaran, 1996). As formulation of microorganisms or their spores determines efficient delivery, shelf life and stability of its effectiveness against plant pathogens, it can generally be regarded as a key to bio-product success (EL-Hassan and Gowen, 2006).

1. 4. Current status and future prospects of using rhizobacteria as biocontrol and growth-promoting agents

In the preceding sections of this chapter, an overview was given of PGPR in biocontrol, biofertilization and phytostimulation. Plant growth promoting bacteria interact with their biotic environments in a complex pattern. Due to this, substantial advance is being made in understanding the genetic basis of the beneficial effects of these PGPRs on plants (Thomashow, 1996; Bloemberg and Lugtenberg, 2001).

1. 4. 1. Biocontrol PGPR

It has previously been mentioned that most of the biocontrol PGPB such as *Pseudomonas* produce various anti-fungal metabolites (AFMs). The genetic basis of the biosynthesis of the more frequently detected AFMs such as pyoluteorin in *P. fluorescens* Pf5 (Nowak *et al.,* 1999) and 2, 4-DAPG in *P. fluorescens* Q-2-87 (Bangera *et al.,* 1999; Delany *et al.,* 2000) has been elucidated. Various such great advances in the molecular basis of biocontrol agents have been achieved. Recently for instance, the biocontrol efficacy of *P. fluorescens* F113 has been enhanced by altering the regulation and production of 2, 4-DAPG (Delany *et al.,* 2001).

In many studies, it has been demonstrated that antibiotic negative mutants of *Pseudomonas* strains have reduced ability to suppress root diseases compared with the wild strains (Schnider *et al.,* 1995). A phenazine negative mutant of *P. fluorescens* for instance was shown to lack part of its ability to suppress take-all of wheat (Pierson, 1994). Similarly, pyrrolnitrin defective mutant of *P. fluorescens* BL915 failed to suppress *R. solani* induced damping off in cotton (Hill *et al.,* 1994). But when

antibiotic production is restored in these mutants by complementation or recombination, their biocontrol efficiency is also restored.

Promising results are being obtained in improving the biocontrol performance of soilborne *Pseudomonas* by the introduction of antibiotic biosynthetic genes (Dowling and O' Gara, 1994). Vincent *et al*. (1991) transferred a recombinant cosmid expressing the *Phl* structural genes of *P. aurofacience* Q 2-87 to *P. fluorescens* that naturally produces phenazine carboxylic acid. The resulting recombinant strain had increased anti-fungal activity *in-vitro* against *Gaeumannomyces graminis* (Sacc.) var. tritici, *P. ultimum* and *R. solani*. In a related experiment, by introducing the PCA biosynthetic genes of *P. fluorescens* 2-79 into different PCA non-producing strains, it was possible to develop recombinant strains. The recombinant strains proved to be more inhibitory to *G. graminis* var. tritici *in-vitro* and *in-vivo* than the wild types (Schnider *et al.,* 1995). It is thus reasonable to speculate that these and a number of other related advances will lead to more efficient use of these biocontrol strains through their improvement by genetic modifications.

The question still remains, however, why is biological control of soilborne diseases achieved by most biocontrol agents including *Pseudomonas* and *Bacillus* spp. still so inconsistent. One factor particularly associated with this inconsistency is insufficient root colonization by the introduced bacteria (Latour *et al.,* 1996; Bloemberg and Lugtenberg, 2001). Lack of knowledge about the bacterial traits that promote root colonization is another contributing factor. For a biocontrol inoculant to perform effectively, its root colonization ability and hence its rhizosphere competence is very important. In this regard the inoculant bacteria must be able to establish themselves in the rhizosphere at a threshold population density (10^5 cfu/gm) sufficient to produce a beneficial effect (Raaijmarks and Weller, 2001). Rhizobacteria have a superior ability to establish and maintain high rhizosphere population densities over an extended period of time. Saravanan *et al*.(2004), also reported that *Pseudomonas fluorescens* strains that inhibited the growth of *Fusarium oxysporum* f. sp. *cubensis* causing wilt in banana do so by aggressively colonizing roots and establishing themselves at the root environment.

During the last two decades however, the population densities of many *Pseudomonas* strains has declined substantially (Mazzola and Cook, 1991). If a biocontrol agent can not adequately compete within the rhizosphere and colonize the root surface, it will not have an efficient biocontrol activity (Walsh *et al.,* 2001). An efficient inoculum strain must be capable of competing with the indigenous soil bacteria. Among many approaches used to overcome this problem include inoculation at higher concentration than the indigenous population, repeated inoculation and the use of genetically

engineered strain with enhanced competitiveness (Nautiyal, 1997). Thus, the selection of strains that are rhizosphere competent will contribute to improve the efficacy of biocontrol agents.

It is important therefore to investigate bacterial colonization and gene expression *in-situ* in the rhizosphere. In recent years, the green fluorescent protein (GFP) and bioluminescence techniques have been employed to study bacterial root colonization and rhizosphere competence (Walsh *et al.,* 2001; Bloemberg and Lugtenberg, 2001). The GFP technique together with the confocal laser scanning microscopy has facilitated the detection of a single bacterial cell. The technique revealed that *Pseudomonas* biocontrol agent often form microcolonies on the roots of crop plants (Normander *et al.,* 1999; Tombolini *et al.,* 1999).

The identification of genes and traits involved in the process of inoculation and root colonization is therefore an important strategy to improve the inadequate biocontrol activity and inconsistency in field experiments. In this regard, *P. fluorescens* genes that are specifically expressed in the rhizosphere *(rhi* genes) have been identified using the *in-vivo* expression technology (Bloemberg and Lugtenberg, 2001). Many other root colonization genes and traits have been identified from *Pseudomonas* biocontrol species (Lugtenberg *et al.,* 2001).

There are, however, certain instances in which root colonization may play a less significant or even minimal role in determining the level of disease control obtained in response to rhizobacteria that suppress disease via induced systemic resistance mechanisms. Liu *et al*. (1995), for instance demonstrated that ISR activity mediated by PGPR strains did not depend on high root colonization ability and high populations. The study, conducted to determine ISR activities of *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166 on cucumber revealed that ISR increased over time whereas the bacterial populations decreased. There was hence no relationship between ISR activity and populations of the two strains on roots.

1. 4. 2. Plant growth promoting PGPR

Although inoculation with PGPR especially with non-symbiotic associative rhizosphere bacteria is not a new technology, many of the attempts failed. Inoculation trials with *Azotobacter* on a large scale in Russia in the late 1930s and an attempt to use *Bacillus megaterium* for phosphate solubilization in the 1930's also failed (Bashan, 1998). It is only in the late 1970's that a major breakthrough in plant inoculation technology was made. One of the major breakthroughs is the finding of the plant growth promoting, free living *Azosprillum spp.* These bacteria enhance the

growth of non-legume plants by directly affecting the metabolism of the plants (Bashan and Holguin, 1997). In later years after the discovery of *Azosprillum* as PGPR, many other bacteria such as *Bacillus*, *Flavobacterium* and *Acetobacter* have been evaluated for their potential in plant growth promotion (Tang, 1994). The biocontrol agents, mainly *P. fluorescens and P. putida* are also regarded as agents of plant growth promotion.

An important feature of these plant growth-promoting bacteria is their ability to colonize roots and promote plant growth (Sharma *et al.,* 2003; Patten and Glick, 2002). The potential of rhizosphere colonization by PGPB is very crucial for what is known as soil biofertilization (Villacieros *et al.,* 2003). The term 'biofertilizer', though misleading is a widely used term to describe bacterial inoculants. It refers to preparation of microorganisms that may be a partial or complete substitute for chemical fertilization like rhizobial inoculants (Bashan, 1998).

Improving plant growth by biofertilization is a crucial mechanism by which iron acquisition in most agricultural crops is achieved. Normally the total iron in the soil is by far much higher than most crops require. However, the concentration of free Fe^{+3} in most soils is far below that required for optimum growth (10^{-9} and 10^{-4}M Fe^{+3}) in the soil solution (Masahla *et al.,* 2000). In the decades before, many studies have indicated that the production of siderophores by plant growth promoting bacteria, particularly by the biocontrol *Pseudomonas* spp. increases plant iron acquisition (Masahla *et al.,* 2000). The high binding affinity and specificity for iron facilitates the transport of iron into the bacterial cells. Plants make use of this ferric-siderophore complex in their systems through the action of enzymes like ferric reductase (Sharma *et al.,* 2003). According to many reports, the possible role of plant growth promoting bacteria in iron uptake by plants in the rhizosphere is indicated by the fact that, under non sterile soil system plants show no iron deficiency symptoms in contrast to plants grown in sterile system (Walter *et al.,* 1994).

Another important aspect of biofertilization is that it accounts for approximately 60 % of the nitrogen supply to crops worldwide. This is achieved both by the symbiotic and free-living nitrogen fixers. To date the genes involved in nitrogen fixation and nitrogen assimilation have been described for *Azosprillum* (Bloemberg and Lugtenberg, 2001).

A promising trend in the field of inoculation technology with plant growth promoting bacteria is, the finding that co-inoculation of growth promoting bacteria with other microorganisms increased growth and yield (Bashan, 1998). Mixed inoculations allow the bacteria to interact synergistically and provide nutrients, remove inhibitory products and enhance some beneficial aspects of their

physiology such as nitrogen fixation. *Azosprillum* spp. co-inoculated with phosphate solubilizing bacteria for instance frequently increased plant growth by providing the plant with more balanced nutrition, improved absorption of nitrogen, phosphorous and iron (Bashan and Holguin, 1997).

At present, the discovery of many traits and genes involved in the beneficial effects of PGPR has resulted in a better understanding of the performance of these growth promoting agents in the field. This also provided the opportunity to enhance the beneficial effects of PGPR strains by genetic modification for future use.

1. 5. Objectives of the study

Sorghum is one of the most important crops and a staple food crop in arid and semi arid areas in Ethiopia. However production is very low in this country because of, amongst other factors, infection by soilborne pathogenic fungi including *F. oxysporum* and *P. ultimum*. Moreover quite a large group of the fungal pathogens reported on sorghum are predominantly recorded in Ethiopia. Repeated attempts to control these pathogens using fungicides were not successful. In addition, it is also believed that the growing cost of chemical pesticides is unaffordable by the poor farmers in the less-affluent countries such as Ethiopia. Attempts to control the disease using biological control are totally lacking. This study was therefore undertaken to asses the biocontrol and growth promoting abilities of rhizobacteria isolated from the rhizosphere of sorghum and the rhizosphere and rhizoplane of several species of grasses.

The main objectives of the current study were to:

- isolate and screen rhizobacteria for *in-vitro* mycelial inhibition of *Fusarium* and *Pythium.*
- evaluate the isolates in terms of biocontrol of *Fusarium* and *Pythium* root rot in sorghum under greenhouse conditions.
- evaluate the isolates in terms of the growth promotion of sorghum in pathogen free soil under greenhouse conditions.
- determine the modes of action used by the most effective antagonistic and PGPR isolates.
- identify the most effective isolates using biochemical and molecular techniques.
- Set out selection criteria for the best performing isolates for future use.

1. 6. References

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CHAPTER 2

In-vitro and greenhouse screening of rhizobacteria for biological control of *Fusarium oxysporum* that causes root and crown rot of sorghum*

Abstract

Fusarium oxysporum causes root and crown rot in several crops including sorghum and results in low grain yield in Ethiopia and other East African countries. Seventy-eight bacterial isolates from the rhizosphere of sorghum in Ethiopia and 86 isolates from the rhizosphere and rhizoplane of grasses in South Africa were obtained and subsequently tested both *in-vitro* and in the greenhouse. Of the Ethiopian isolates tested, 23 displayed between 30 and 66 % *in-vitro* inhibition of mycelial growth of *F. oxysporum* and showed significant root colonization ability on sorghum seedlings. The maximum *in-vitro* inhibition by isolates from South Africa was 37.6 % by isolate KBS5-F. The isolates were further tested for their biocontrol ability against *F. oxysporum* in the greenhouse. Four Ethiopian isolates viz. KBE2-5, KBE5-1, KBE5-7, and NAE5-5 resulted in 100 % disease suppression and no symptoms of root and crown rot were observed compared to the control. Five isolates from South Africa viz. KBS5-F, KBS9-B, KBS9-H, KFP9-A, NAS6-B and resulted in *in-vivo* disease suppression ranging from 85.6 - 95.8 %. Identification of the most effective isolates using the API system and sequencing of the bacterial 16 S rDNA indicated that the majority of the isolates from sorghum rhizosphere in Ethiopia belong to members of the Genus *Bacillus* including *B. cereus*, *B. circulans, B. licheniformis*, *B. stearothermophilus* and *B. subtilis*. The most effective isolates from the rhizosphere of grasses in South Africa contained, in addition to *Bacillus cereus*, Gram negative isolates such as *Chryseomonas luteola*, *Enterobacter sakazaki, Serratia marcescens* and *Stenotrophomonas maltophilia*. The study demonstrated effective biological control by the rhizobacterial isolates tested, thereby indicating the possibility of application of rhizobacteria for control of soilborne diseases of sorghum in Ethiopia and other countries.

*Part of this chapter has been included in the following publication: H. Ahmed Idris, N. Labuschagne and L. Korsten (2007). Screening rhizobacteria for biological control of fusarium root and crown rot of sorghum in Ethiopia. *Biological Control*, 40: 96 – 107.

2. 1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is ranked second among the five most important crops in East Africa (FAO, 1999) and it is a staple food crop in arid and semi-arid areas in Ethiopia. The crop grows under a wide range of ecological conditions and is drought tolerant. Production is, however, very low in this country because of, amongst other factors, diseases caused by phytopathogenic fungi. Symptoms such as seedling death and root rot a few weeks after planting are commonly observed in the major sorghum fields. Several members of the Genus *Fusarium* cause root diseases in sorghum leading to serious yield losses. Among the major pathogens in this group are *Fusarium oxysporum* Schlectend, *F. moniliforme* J. Sheld, *F. graminearum* Schwabe and *F. tricinctum* (Corda) Sacc. (Forbes *et al*., 1986) of which *F. oxysporum* and *F. moniliforme* are predominantly found in Ethiopia. Moreover, most of the fungal pathogens reported on sorghum are found predominantly in Ethiopia and other East African countries (Huluka and Esele, 1992).

In Ethiopia, repeated attempts have been made to control *Fusarium* root rot in sorghum with fungicidal treatments using, amongst others, benomyl. However none of the fungicides used have been successful mainly because of their phytotoxicity (Benhamou, 1992). Chemical control of sorghum diseases is also unaffordable in most developing countries. The use of broad-spectrum fungicides further results in imbalances within the microbial community creating unfavourable conditions for the activity of beneficial organisms (Villajuan-Abgona *et al.,* 1996).

Soilborne diseases have been controlled more recently by means of certain beneficial bacteria that are indigenous to the rhizosphere of plants (Thomashow, 1996). The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large and metabolically active groups of bacteria (Villacieros *et al*., 2003) known as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper *et al.,* 1980). The PGPR group are known to rapidly colonize the rhizosphere and suppress deleterious microorganisms as well as soilborne pathogens at the root surface (Rangarajan *et al.,* 2003). These organisms can also be beneficial to the plant by stimulating growth (Bloemberg and Lugtenberg, 2001).

Currently there is very limited knowledge regarding the biological suppression of *Fusarium* root and crown rot in sorghum by the application of PGPR in Ethiopia and South Africa. The aim of this study is therefore to isolate bacteria from the sorghum rhizosphere and the rhizosphere and rhizoplane of grasses in South Africa and screen the organisms for *in-vitro* and *in-vivo* antagonistic

activity against *F. oxysporum,* one of the major causes of root rot in Ethiopia (unpublished data). This study may contribute to the introduction of PGPR systems alongside biological control of phytopathogenic fungi in sorghum and other crops in Ethiopia and South Africa.

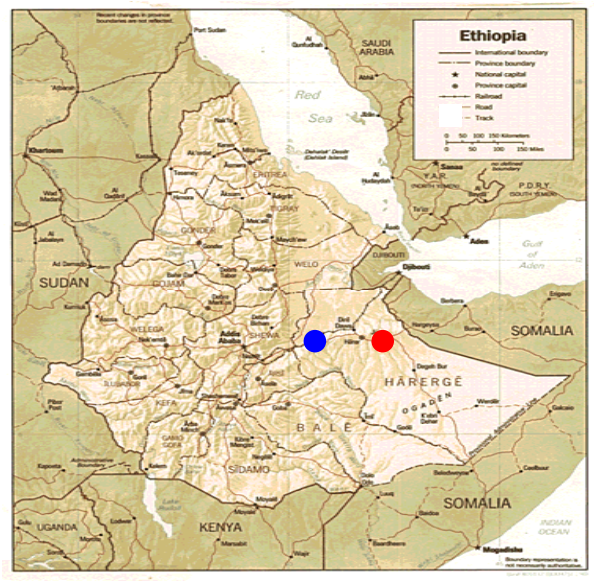
2. 2. MATERIALS AND METHODS

2. 2. 1. Soil sample collection and analysis

In December 2003, a total of nine soil samples were collected from the rhizosphere of sorghum in two fields in Ethiopia, namely Meeson and Jijiga. Both areas are located in the Eastern part of the country where sorghum is commonly produced as a staple crop. These sites were selected because the soils have previously been cropped for at least 20 years with sorghum, maize (*Zea mays*) and teff (*Eragrostis teff*), an indigenous crop of the grass family commonly used as a staple crop in Northern and Central Ethiopia. Farming practices in these fields include fungicide sprays with benomyl and metalaxyl. After harvest, the remaining stubble is ploughed into the soil. Five collection sites in Meeson and four collection sites in Jijiga were selected and carefully marked before sample collection. Sorghum roots were uprooted and 1 kg rhizosphere soil, the thin layer of soil about 1-2 mm thick surrounding the root, was carefully transferred by shaking into sterile plastic bags and placed in cool boxes. They were then transported to the Microbiology Laboratory, Alemaya University, Ethiopia for isolation of bacteria.

Samples were also collected in September 2003 from ten selected sites of virgin soil from the rhizosphere of grasses in the Nylsvlei Nature Reserve in South Africa (Plate1).The 4000ha reserve lies east of the Waterberg Mountains between Modi Molle and Mookgophong possessing a unique biodiversity of plant and animal communities. The soil samples were collected from the rhizosphere of seven grass species of the typical bushveld savannah surrounding the grassveld flood plain. The seven grass species along with their sample numbers are: *Sporobolus fimbriatus* (1), *Stipagrotis zeyheri* subsp. Zericans (2, 3), *Themeda triandra* (4) *Eragrostis biflora* (5), *Arstida canescens* subsp. canescens (6, 7), *Eragrostis* spp. (8) and *Cyprus esculantus* L. (9). The samples were transferred into plastic bags in cool boxes and then transported to the Laboratory at the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa. Each sample of rhizosphere soil was sieved to remove plant debris before being processed for isolation of bacteria.

A



B

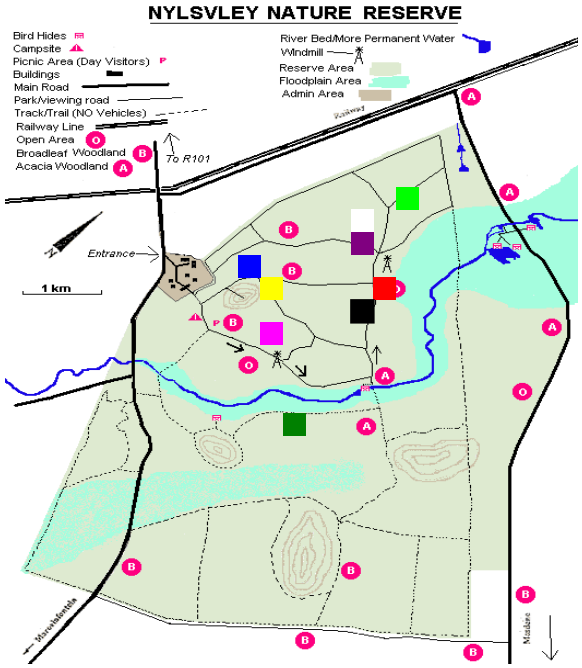


Plate 1. Maps illustrating sampling points in Ethiopia (A) and Nylsvlei Nature Reserve in South Africa (B). ● = Meeson & ● = Jijiga sampling points in Ethiopia. The nine sampling sites at the Nylsvlei are represented on the map using coloured squares: sample no.1= red, 2= black, 3= violet, 4 = white, 5= bright green, 6= yellow, 7=blue, 8= pink, 9 & 10= green.

2. 2. 2. Isolation of bacteria

The initial isolation of bacteria from sorghum rhizosphere samples was conducted in the Microbiology Laboratory at the Department of Biology, Alemaya University, Ethiopia. Each soil sample was mixed and one gram was transferred to 9 ml quarter strength sterile Ringer's (Merck, Halfway house, South Africa) solution and was serially diluted. A 0.1ml aliquot of the serially diluted suspension was spread-plated on King's B medium (Oxoid, London, UK) (King *et al.,* 1954) and nutrient agar (NA) medium (Biolab, Wadesville, South Africa) in triplicate. The spread-plate cultures were incubated for 24 hr. at 28 ^{o}C. Representative colonies, with different morphological appearances, were selected from the countable plates and re-streaked on a new plate of the same media to obtain pure colonies. A total of 100 isolates originally obtained in this manner were maintained on agar slants and transported to the Plant Pathology Laboratory at the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa (Permit No. 0010826). The samples were handled according to the stated protocols/conditions in respect of the importation of samples for laboratory and/or glasshouse studies.

To isolate bacteria from soil samples obtained from the rhizosphere of grasses in South Africa, the samples were not directly used but baited with sorghum seeds in the greenhouse. The soils (400 g/pot) were deposited in 500 ml plastic pots and planted with five sorghum seeds. Three weeks later, emerged sorghum seedlings were removed from the pot and their roots were gently shaken to collect the adhered soil. Serial dilution, plating and incubation were conducted as described for Ethiopian samples.

Bacteria were also isolated from the rhizoplane of grasses by shaking off all the adhered soil from the root surface and sonicating the soil-free roots (1g) in 9 ml diluent (1 M $MgSO_4$) for one minute and making ten fold serial dilutions. Initially as many as 160 colonies from the rhizosphere and rhizoplane of grasses in South Africa were selected. Because many isolates were morphologically similar, preliminary characterization procedures, for both Ethiopian and South African isolates, included the following tests: Gregorson's KOH (Gregorson, 1978), cytochrome oxidase (Kovacs, 1956), oxidation fermentation (Hugh and Leifson, 1953), catalase and motility tests. A total of 78 isolates from the sorghum rhizosphere and 86 isolates from the rhizosphere of grasses were selected. For short term use, pure cultures of these isolates were stored at -70^{o}C in nutrient broth supplemented with 15 % glycerol. Replicate stocks of the cultures were also lyophilized for long term storage. Soil particle size and chemical analysis for both Ethiopian and South African samples

was conducted in the soil analysis laboratory of the department of Plant Production and Soil Sciences, University of Pretoria, South Africa.

2. 2. 3. Bacterial inoculum preparation

Isolates were grown in nutrient broth (BioLab, Wadesville, South Africa) on a rotary shaker (LABOTECH) at 28 ^{o}C and 180 rpm for 24 hrs. The suspension was centrifuged (Avanti TM J-25 Beckman centrifuge) in 50 ml capacity sterile plastic tubes at 3000 x *g* for 10 min. The pellets were re-suspended in quarter strength sterile Ringer's (Merck) physiological saline solution to give a final concentration of 10^8 cfu/ml (OD = 0.5) at 550 nm using the viable plate count method and optical density measurement.

2. 2. 4. In-vitro antagonistic activity

The *in-vitro* inhibition of mycelial growth of *Fusarium oxysporum* by the bacterial isolates was tested using the dual culture technique as described by Paulitz *et al.* (1992) and Landa *et al.* (1997). Three 50 µl drops from the 10^8cfu/ml suspension were equidistantly placed on the margins of potato dextrose agar (PDA) (BioLab) plates and incubated at 28 ^{o}C for 24 hr. A 4mm agar disc from fresh PDA cultures of *F. oxysporum* was placed at the centre of the PDA plate for each bacterial isolate and incubated at 27 ± 1 ^{o}C for seven days. The radii of the fungal colony towards and away from the bacterial colony were measured. The percentage growth inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [(R - r)] / R \times 100$$

Where, r = the radius of the fungal colony opposite the bacterial colony and,

R = the maximum radius of the fungal colony away from the bacterial colony.

All isolates, which resulted in more than 30 % mycelial growth inhibition against the two selected pathogens and/or which colonized the sorghum roots at a level higher than 10^5 cfu/cm roots in the *in-vitro* experiments were stored in nutrient broth supplemented with 15 % glycerol at –70 ^{o}C.

2. 2. 5. In-vitro root colonization

The isolates, which showed antagonistic activity in the dual culture assays, were tested for their ability to colonize sorghum roots *in-vitro,* using a modification of the methods by Patten and Glick (2002) and Montealegre *et al.* (2003). Sorghum seeds (South African variety Gewoon) were surface sterilized with 70 % ethanol for 5 min and subsequently with 1 % sodium hypochlorite for 1 min and

rinsed three times in sterile distilled water. For each treatment, 15 seeds were transferred to a sterile moist chamber i.e. discs of filter paper placed in 90 mm diameter plastic Petri dishes and moistened with sterile distilled water. For inoculation, the bacterial inoculum was prepared as described above. A 1 ml aliquot of each inoculum was added to the seeds in the moist chamber and the plates were incubated at room temperature for one hour to allow binding of the bacteria to the seed coat. Both treated seeds and controls were then incubated at 30 ^{o}C for 4-5 days in the dark for root development. One centimetre of root from each treatment was aseptically excised, one seed per treatment, and transferred to 0.1M $MgSO_4$ solution and serially diluted. From each dilution, a 0.1 ml aliquot was plated on King's B and NA media and the plates were incubated at 30 ^{o}C for colony counts. The number of bacteria colonizing the root was calculated as colony forming units/cm root (cfu/cm root) as one centimetre of the bacterial treated root was used in the serial dilution procedure. In the subsequent chapters involving *in-vivo* experiments however, results will be reported as cfu/g soil.

2. 2. 6. Greenhouse evaluation of rhizobacterial isolates

2. 2. 6. 1. Pathogenecity tests and fungal inoculum preparation

Fusarium oxysporum isolate RC331, *F. oxysporum* isolate SC314, *F. verticilloides* isolate RA145 and *F. equiseti* isolate RB125 all obtained from infected sorghum roots from the University of the Free State, Bloemfontein, South Africa were re-tested for pathogenecity on sorghum under greenhouse conditions. Based on the observation of visual stunting, root and crown rot and death of young seedlings, *F.oxysporum* isolate RC3B1 was selected as the most virulent and was used as the target pathogen in this experiment. Millet seed (*Panicum miliaceum* L.) inoculum was prepared as follows: 150 g millet seed, together with 200 ml distilled water was deposited in an autoclavable polyethylene bag and autoclaved at 121^{o}C for 15 min. Each bag was subsequently inoculated with five 4 mm agar discs cut from a fresh PDA culture of *F. oxysporum*. The inoculum was incubated at 27 ± 1 ^{o} C for seven days.

2. 2 .6. 2. Planting of sorghum and inoculation of fungal and bacterial isolates

All bacterial isolates which rendered more than 30 % inhibition of *F. oxysporum* mycelial growth in the *in-vitro* assay and which showed effective root colonization (beyond 10^4 cfu/cm root) were selected for the *in-vivo* evaluation in the greenhouse. Sorghum seeds were surface sterilized with 70 % ethanol for 5 min, 1 % sodium hypochlorite for 1min and rinsed five times with sterile distilled

water. The seeds were then pre-germinated in sterile vermiculite for four days at 30 ^{o}C in a growth cabinet. Prior to seedling transplanting, the millet seed inoculum of *F. oxysporum* was mixed into steam-pasteurized soil in a 12 cm by 10.5 cm diameter plastic pots at the rate of 30 g/kg. For the control treatments, sterile millet seed was mixed into the soil at the same rate. Eight sorghum seedlings were transplanted into each pot and the pots were maintained in the greenhouse at 30 ± 1 ^{o}C. One day after transplanting of seedlings, each pot was drenched with 30 ml of the bacterial inocula (10 ^{8} cfu/ml). Two successive bacterial applications were made a week apart. The treatments in the *in-vivo* biocontrol experiment were: Plants inoculated with *F. oxysporum* and bacteria, Plants inoculated with *F. oxysporum* on its own (control a) and a non-inoculated control (control b). The non-inoculated control was treated with sterile millet seed without fungal and bacterial inoculum. The plants were irrigated twice daily with tap water by means of an automatic watering system. All the *in-vitro* and *in-vivo* experiments were arranged in a randomized block design in three replications and each experiment was repeated twice.

2. 2. 6. 3. Disease assessment

Four weeks later, plants were removed from the soil and the roots washed with sterile distilled water. Roots were excised from the plant and data collected for analysis. Data included root and crown rot severity assessed on a rating scale of 0- 4 (Brien *et al.,* 1991). 0 = no infection, 1= 1-25% infection, 2= 26-50% infection, 3= 51-75% infection and 4= 76- 100% infection in the root and crown regions. Based on the disease severity index, the percentage suppression of root and crown rot was calculated (Villajuan-Abgona *et al.,* 1996) as follows:

% Suppression = [(A – B)/A] x 100 where;

A = Disease severity exhibited in the root/crown region due to *F. oxysporum* alone and

B= Disease severity exhibited on the root/crown region after inoculation with both the pathogen and bacterial antagonists). Roots from eight plants per treatment were excised and the fresh and dry weights were determined by taking four roots per treatment. The remaining four roots were subsequently used to determine the incidence of *F. oxysporum* as described next.

2. 2. 6. 4. Incidence of Fusarium oxysporum in roots

For determination of the incidence of *F. oxysporum* in roots, RBGU (Rose Bengal Glycerol Urea) medium selective for *Fusarium* was used (Van Wyk *et al.,* 1986). RBGU medium contains: glycerol 10 ml/L, urea 1.0 g/L, L-alanine 0.5 g/L, PCNB 1 g/L, Rose Bengal 0.5 g/L, chloramphenicol 0.25

g/L, and bacteriological agar 12 g/L. The agar was dissolved in 1 L capacity Erlenmeyer flask containing 800 ml distilled water. The other ingredients were dissolved in small quantity of absolute ethanol and mixed with sterile 200 ml distilled water in a separate flask. This was then added to the sterile agar medium. The resulting RBGU medium was poured into sterile plastic Petri dishes.

Roots from both infected and non-infected plants were excised, surface sterilized with 0.5% sodium hypochlorite and rinsed five times with sterile water. The roots from all three replications in each treatment were pooled and a sub sample (10 root pieces per plate) aseptically plated in triplicate on RBGU medium. The plates were incubated at 27 ± 1 ^{o}C for seven days. The resulting fungal colonies were examined microscopically and the number of root pieces rendering *F. oxysporum* recorded.

2. 2. 6. 5. Rhizosphere colonization

The survival of the bacterial isolates in the rhizosphere of the sorghum plants was determined according to a modification of the procedure described by Landa *et al.* (2004). Plants were carefully removed from the pots and roots were gently shaken to remove all but the tightly adhering soil. One gram of the adhering rhizosphere soil was collected and placed into 9ml. sterile 0.1 M $MgSO_4$ solution. Serial dilutions of the suspension were vortexed and plated onto King's B or NA medium as described before. The plates were then incubated at 30 ^{o}C for 24 hr after which the developed colonies were counted and the number of CFU/gm of soil calculated.

2. 2. 7. Identification of bacterial isolates

2. 2. 7. 1. Identification by the API system

Based on the results of the preliminary characterization described above, the most promising isolates were selected and further identified to the species level by means of the Analytical Profile Index (API) identification system assisted by the API-PLUS computer software program (bioMèrieux Inc, Durham, USA). Gram positive, endospore forming rods were identified to the species level using API® 50 CH test strips. Gram negative rod isolates with fermentative reaction in the Hugh and Leifsons' oxidation-fermentation test were identified using the API® 20 E test strips while those with oxidative reaction were identified by the API 20 NE test strips.

2. 2. 7. 2. 16S rDNA sequencing

The identity of the isolates rendering unsatisfactory results (low probability %) with the API system was further confirmed by means of PCR amplification of the bacterial 16 S rDNA.

2. 2. 7. 2. 1. Extraction of DNA

DNA was extracted from Gram-positive isolates using the DNeasy Tissue Kit (Quagen) according to the manufacturers' instructions. For extraction of DNA from Gram-negative isolates, the boiling method described by Mohran *et.al*.(1998), was used. Briefly, for each isolate a loopful of the bacterial colony was suspended in 100 µl sterile water and boiled for 10 min. The suspension was then placed on ice for 5 min. and centrifuged in a microcentrifuge at 10, 000 x *g* for 5 min to extract the DNA.

2. 2. 7. 2. 2. PCR amplification and DNA sequencing

A portion of the 16S rRNA (corresponding to positions 8-1541 in the *Escherichia coli* numbering system) of each isolate was amplified using forward primer pA (5'AGAGTTTGATCCTGGCTG-AG3') and reverse primer pH (5'AAGGAGGTGATC CAGCCGCA3') (Coenye *et.al.,* 1999). The reaction mix for the PCR contained: 5 µl 10x buffer; 1 µl of 100 mM of each primer; 2.0 µl mix of dNTP's (1:1: 1:1); 0.2 µl *Taq DNA Polymerase*; 31.8 µl nuclease free water (NFW); 5 µl of the extracted DNA sample. The PCR was conducted by first incubating the reaction mixture at 94 ^{o}C for 5min. followed by 30 cycles consisting of denaturing at 94 ^{o}C for 1 min; annealing at 58 ^{o}C for 1 min; and elongation at 72 ^{o}C for 1 min. The PCR reaction was terminated with a final extension at 72 ^{o}C for 5min. The PCR products (5 µl) were run on a 1 % agarose gel and sequencing of the amplified 16S rDNA was performed using primer *pD (5'-CAG CAG CCG CGG TAA TAC-3') (Inqaba Biotech, South Africa). The resulting sequences were blasted (NCBI Blast Search).

2. 2. 8. Statistical analysis

The data were subjected to Analysis of Variance using SAS-9.1 software (SAS Institute, 2003). Data on the *in-vitro* root colonization and the survival of the bacterial isolates in the rhizosphere were log transformed before subjecting to Analysis of Variance (ANOVA). Mean values among treatments

were compared by the least significant difference (LSD) test and Duncan's Multiple Range test at 5 % ($p = 0.05$) level of significance.

2. 3. Results

2. 3. 1. Soil particle size analysis and chemical composition

Soil samples from the sorghum rhizosphere and the rhizosphere of grasses showed variation in terms of particle size composition and inorganic contents. Generally the soil from the rhizosphere of sorghum in Ethiopia had an alkaline pH (8.0 – 8.7) with the exception of two samples with slightly neutral pH (6.7 – 6.9). The soil particle size varied among the nine samples ranging from 18 % - 61.2 % coarse sand, 11.5 % - 37.7 % silt and 21.8 % - 61.1 % clay. All the soil samples had a larger proportion of Calcium (Ca) ranging from 838 mg/kg to 8830 mg/kg followed by Magnesium (Mg), Potassium (K) and Sodium (Na) ranging from 191 mg/kg to 1079 mg/kg, 88 mg/kg to 583 mg/kg, and 20 mg/kg to 88 mg/kg respectively. The inorganic component which occurred at the lowest level was Phosphorous (P) ranging from 0.1 mg/kg – 1 mg/kg.

Soils from the rhizosphere of grasses in South Africa had slightly acidic characteristics (pH = 5.0-6.3). Soil particle size, composition and inorganic content were determined as described above. Coarse sand ranged from 40 % to 86 %, silt from 4.3 % to 30 % and clay particles ranged from 12 % to 35 %. A greater portion of the soil inorganic content was Calcium (Ca) ranging from 189 mg/kg to 928 mg/kg followed by Potassium (K) ranging from 129 mg/kg to 392 mg/kg, Sodium (Na) from 29 mg/kg to 203 mg/kg and Phosphorous (P) from 1.1 mg/kg to 4.1mg/kg.

2. 3. 2. In-vitro assays

2. 3. 2. 1. Ethiopian isolates

Of the 78 isolates tested, 15 originally isolated on the King's B medium resulted in ≥ 30 % inhibition of mycelial growth of *F. oxysporum* (Table 2.1). Of those isolated on NA, seven isolates resulted in ≥ 30 % growth inhibition of *F. oxysporum* (Table 2. 2). The maximum inhibition achieved by any isolate was 66.3 % (KBE9-1) (Table 2. 1). Control plates not treated with the bacterial isolates were completely covered by the phytopathogens showing no inhibition (Table 2.1 and Table 2. 2). Fungal growth was not inhibited by isolates NAE4-4, NAE5-3, NAE5-8, and KBE9-3 (Fig. 2.1). In contrast, KBE7-6, KBE9-1, NAE2-4, and NAE7-1 were amongst the most effective

isolates against *F. oxysporum* displaying 56 %, 63 %, 39 % and 40 % inhibition of mycelial growth respectively (Fig.2.1; Table 2. 1 & Table 2. 2). The mean mycelial growth inhibition of the target pathogen revealed that the inhibition was highly significant.

The *in-vitro* root colonization study demonstrated that some of the isolates are more effective root colonizers than others. After four days of germination, the bacterial cell counts obtained from the roots have increased by 1.28 log cfu/cm root for isolate KBE8-3 and by 1.22 log cfu/cm root for isolate KBE9-1 (Table 2. 1) as compared to control where counting was of 10^4 cfu/cm root length from the initial inoculum level of 1 x 10^8 cfu/ml (Table 2.1). Similarly, isolate NAE6-2 colonized the roots and the count, compared to the initial inoculum level increased by 0.98 log cfu/cm root length (Table 2. 2.). For most isolates however, the count decreased from the initial inoculum level to up to 10^4 cfu/cm root length. Control plates inoculated with sterile distilled water rendered less than 30 colonies per plate indicating a low number of bacterial cells which presumably originated from the seed.

2. 3. 2. 2. South African isolates

Twenty two isolates, out of the 86 tested resulted in an *in-vitro* inhibition ranging from 10.2 % to 37.6 % of which the best performing isolates were KBS5-F, KBS6-H and KBS9-R. More than 50 isolates tested did not show any inhibition against *F. oxysporum* being entirely overgrown by the fungal mycelia (data not shown). Only two isolates resulted in ≥ 30 % inhibition of mycelial growth of *F. oxysporum* viz. isolate KBS5-F (37.6 %) and isolate KBS6-H (35.7 %) and one isolate, KBS9-R resulted in 24.8 % inhibition in the *in-vitro* experiment. The least inhibition observed was by isolate KBS1-A rendering only 8.74 % mycelial growth inhibition. Control plates not treated with bacterial isolates were completely covered by the mycelial growth of the fungus (Table 2. 4).

2. 3. 3. Greenhouse experiments

2. 3. 3. 1. Ethiopian isolates

Results from the greenhouse pot experiment demonstrated that isolates KBE 2-5, KBE5-1, KBE5-7, and NAE5-5 significantly inhibited root rot of sorghum caused by *Fusarium oxysporum*. (Table 2.3) These treated plants looked healthy showing no symptoms of root or crown rot (Fig. 2.2). Isolates KBE4-3, KBE5-4, KBE9-1, and NAE5-7 resulted in more than 80 % suppression of root rot whilst isolates KBE5-2, KBE8-3, and NAE6-2 resulted in disease reduction of more than 75 % (Table 2.3). Control plants not treated with bacteria but inoculated with *F. oxysporum* alone rendered up to 100

% root rot incidence with the majority of plants completely stunted or dead (Fig.2. 2, control a). Plants inoculated with neither the pathogen nor the bacterial isolates also survived but rendered some infection presumably from low level of cross contamination (Fig. 2. 2, Table 2. 3).

All infected roots were characterized by dark red to black discoloration and rotting. The leaves of infected seedlings were pale green and plants were stunted (Fig. 2. 2). The fungus resulted in a pronounced decrease in the fresh and dry weight of the roots compared to the non inoculated control and to some of the treatments with the most effective bacterial isolates. The reduction in fresh weight of roots amounted to 94.4 % in the control treatment inoculated with *F. oxysporum* alone, whereas 33 % reduction in fresh root weight was recorded for the treatments inoculated with both the pathogen and isolates KBE2-5 and KBE5-7. The least reduction in fresh root weight (5.5 %) was recorded for the treatment inoculated with KBE8-3. Interestingly however, a 5.2 % increase in fresh root weight was recorded with isolate KBE5-1, one of the effective isolates that prevented root and crown rot in this study (Table 2. 3).

Root dry weight of the control treatment inoculated with only *F. oxysporum* decreased by 97.8 % in relation to the non-inoculated control. Among the potential biological control agents in this study, isolates KBE5-1 and KBE9-1 resulted in 34.8 % and 65.2 % reduction in root dry weight respectively compared to the 97.8 % reduction recorded for the control inoculated with *F. oxysporum* alone. Table 2. 3. shows that 100 % disease suppression was rendered by isolate KBE5-1 while this same isolate resulted in the reduction of root biomass by 34.8 %. This could have resulted from errors while recording root rot severity by visual measurement. If this isolate rendered 34.8 % reduction in root biomass, there could be some level of root rot which could not be easily detected visually. This is also reflected by the fact that some level of incidence of *F. oxysporum* in the roots of plants treated by isolate KBE5-1 has been recorded.

Percentage of roots of sorghum plants showing presence of *F. oxysporum* in the various treatments showed a reduction of the fungus by some of the bacterial isolates ranging from 60 % to 87 % (Fig. 2. 4 A). Almost 100 % of the roots from the control treatment (*F. oxysporum* only) rendered growth of *F. oxysporum* compared to an incidence ranging from 13.3 % to 53.3 % for plants treated with isolates KBE5-7, NAE5-5, KBE4-3, KBE9-1, KBE5-1, NAE5-7 and KBE2-5, KBE7-8 and KBE8-3 (Fig. 2. 4A).

2. 3. 3. 2. South African isolates

Five isolates from the Nylsvlei Nature Reserve in South Africa viz. KFP5-F, KFP9-A, KBS9-B, KBS9-H and NAS6-B resulted in significant suppression of root rot caused by *F. oxysporum*. The percentage disease suppression by these isolates ranged from 85.57 % by KBS9-B to 95.83 % by isolate KBS5-F. Although no significant increase in the dry and fresh weights of roots was rendered by these isolates compared to the healthy control, significant reduction of root biomass as a result of inoculation with *F. oxysporum* was alleviated in the presence of these bacterial isolates.

A 100 % reduction of fresh and dry weight of roots was recorded for control plants inoculated with *F. oxysporum* only, compared to the un-inoculated plants. It was not possible to retrieve roots from these pots due to the death of all the plants caused by severe root rot. The least reduction in fresh weight of roots as a result of co-inoculation with the bacterial antagonists was achieved by isolate KBS5-F (6.7 % reduction in fresh weight) followed by isolate KFP9-A (7 % reduction) and isolates KBS2-12 and KBS9-H, each 9 % reduction (Table 2. 4). Similarly significant alleviation in the reduction of root dry weight due to *F. oxysporum* was achieved by inoculation with isolates KBS5-F, KFP9-A, NAS4-3, KBS9-B, NAS2-F, KBS2-12 and KBS5-H. The least reduction recorded was again by KBS5-F (0.7 %), KFP9-A (1.4 %), NAS4-3 and KBS9-B, each with 3 % reduction in the dry weight of roots (Table 2. 4).

The percentage incidence of *F. oxysporum* in the roots of sorghum was also reduced by isolates KBS5-H, KBS9-R, KBS5-F, KBS6-H, KFP9-A and KBS9-B with an incidence ranging between 7.3 % - 26.7 % compared to 93.3 % incidence of *F. oxysporum* in the control treatment which was not treated with any bacterial isolates (Fig. 2. 4B).

2. 3. 4. Identification of bacterial isolates

According to the API tests and sequencing of the bacterial 16S rDNA gene, 80 % of the bacteria from the rhizosphere of sorghum in Ethiopia belong to members of the Genus *Bacillus* with 45 % corresponding to *Bacillus cereus*. The rest of the *Bacillus* species were identified as *B. subtilis, B. licheniformis*, *B. circulans* and *B. stearothermophilus*. Two Gram negative isolates were identified as *Chromobacterium violaceum*. On the other hand, of the 15 South African isolates that were effective in promoting sorghum growth, 12 isolates were identified to species level as *B. cereus* (6

isolates), *Serratia marcescens* (2 isolates), *Chryseomonas luteola* (2 isolates), *Stenotrophomonas maltophilia* (1 isolate) *and Brevibacterium laterosporus* (1 isolate). The identity of three isolates could not be determined.

2. 4. Discussion

The aim of this study was the isolation, screening and selection of rhizobacteria inoquous to sorghum plants with antagonistic activity against *F. oxysporum* associated with root and crown rot. The selected strategy was to screen the bacterial isolates for their *in-vitro* biocontrol activity against *F. oxysporum* and determine *in-vitro* root colonization. The bacterial isolates selected were initially obtained from the rhizosphere of sorghum plants from the major sorghum growing fields in Ethiopia as well as from the rhizosphere and rhizoplane of different grass species in a pristine environment of the Nylsvlei in South Africa. The approach provided an opportunity to select effective biocontrol strains capable of antagonizing soilborne pathogens in the same environment where they will be used commercially (Landa *et al.,* 1997). Moreover, isolation of bacteria from within the rhizosphere of the target crop is essential for successful identification of potential biocontrol agents (Williams and Asher, 1996).

It is known that *in-vitro* assays have certain limitations in that the biocontrol efficiencies may not be equally expressed under gnotobiotic (axenic) and *in-vivo* conditions (Inam-ul-Haq *et al.,* 2003). However, the *in- vitro* assays conducted in our study (pathogen inhibition and root colonizing ability) were used to screen and select potential biocontrol agents and subsequently test their ability to suppress *Fusarium* root rot of sorghum under greenhouse conditions.

A bigger range of inhibition (i.e. 5.8 – 66.3 %) was obtained for Ethiopian compared to South African isolates (8.9 – 37.6 %). Such variation in the extent of mycelial growth inhibition can be influenced by the nature of bacterial isolates. Landa *et al.* (1997), for example indicated that the ability of four bacterial isolates to inhibit different races of *F. oxysporum* differed significantly. Some bacterial isolates were found to be highly inhibitory of *F. oxysporum* growth whereas others showed only mild activity or no activity at all. This suggests that the mode of action exerted and /or the type of antifungal metabolite produced by the isolates may vary and that the bacterial isolates are taxonomically different from each other (Williams and Asher, 1996).

The isolates that most effectively inhibited fungal growth in the dual culture experiment resulted in such a major inhibition zone that there was no physical contact with the pathogens suggesting that the rhizobacteria could be producing certain antifungal metabolites (Montealegre *et al.,* 2003). Moreover, as the PDA medium used for the dual culture assay is rich in nutrients, competition might be excluded as the mode of action for these isolates (Landa *et al.,* 1997). The antifungal metabolites produced seem to vary among the isolates tested in this study. In the dual culture assay, some of the isolates not only inhibited the mycelial growth but also changed the appearance of the mycelia from white to reddish brown and red as was evident for isolates KBE7-6, KBE9-1, NAE2-4 and NAE7-1 (Ethiopian isolates) and isolates KBS5-F, KBS6-H, KBS9-R (South African isolates). This suggests that the fungal mycelia might be inhibited not only by antibiosis but also by other antifungal metabolites such as siderophores, hydrogen ions and gaseous products including ethylene, hydrogen cyanide and ammonia (Williams and Asher, 1996; Kumar *et al.,* 2002; Saravan *et al.,* 2004). Furthermore, the efficacy of a given biological control agent mostly result, not only from a single mechanism but from a combination of different modes of actions (Alabouvette *et al.,* 1993).

In most biocontrol investigations, a large number of antagonists are commonly isolated over a short period of time and screened *in-vitro* for antagonistic activity. However, tests based on *in-vitro* mycelial inhibition and root colonization do not always correlate with biocontrol efficacy under natural conditions (Paulitz *et al.,* 1992; Williams and Asher, 1996). All promising isolates from the current study were therefore further evaluated under greenhouse conditions.

The bacterial isolates which showed significant *in-vitro* root colonization also colonized the sorghum rhizosphere effectively in the greenhouse experiment. Isolates from the sorghum rhizosphere in Ethiopia such as KBE6-3, KBE7-8, and KBE8-3 maintained their initial population level up to 30 days after inoculation. Six isolates from the rhizosphere of grasses in South Africa similarly maintained high population level in the sorghum rhizosphere with a decrease of only one log unit from the initial inoculum level. However, as these assays were conducted in a pasteurized soil system, the isolates established populations that may not be obtained in a native soil system in the field. Hence for the bacteria to provide a similar level of disease suppression, it is recommended that a mixture of the effective isolates be used under field condition by applying at a higher concentration repeatedly.

The effective colonization of sorghum roots by isolates such as KBE5-1, KBE5-7, KBE9-1, and NAE5-5 from Ethiopia and isolates KBS5-F, KBS5-H , KBS6-H, KBS9-R, and KFP9-A from South

Africa might have contributed to their capability to inhibit infection of sorghum roots by *F. oxysporum* and reduce root and crown rot. All four bacterial isolates inhibited *F. oxysporum* both in the dual culture assay and in the greenhouse experiments.

Nevertheless, some isolates showed inconsistency in the *in-vitro* and *in-vivo* experiments. This was particularly true for Ethiopian isolates, NAE2-4, NAE7-1 and KBE9-8 which rendered a 40% mycelial inhibition of *F. oxysporum* in the dual culture experiment. However, under greenhouse conditions in this study these isolates achieved relatively low population levels of less than 10^5 cfu/gm in the rhizosphere. This population was also found to be below the effective threshold value for a biological control agent to successfully compete in the rhizosphere (Raaijmakers and Weller, 2001). On the other hand, South African isolates KBS9-B, KBS9-H, KFP9-A, and NAS6-B which rendered a relatively lower level of *in-vitro* inhibition resulted in significant disease suppression against *F. oxysporum* under greenhouse conditions. Owenly *et al*. (2003), have indicated that such differences result from variability in the physical and chemical properties within niches occupied by biocontrol agents which in turn affect both colonization and expression of bio-control mechanisms.

Among the potential biocontrol agents active in the rhizosphere, several members of the Genus *Bacillus* are reported to be effective in controlling a variety of fungal plant diseases (Williams and Asher 1996; Landa *et al.,* 1997; Commare *et al.,* 2002). Most of these agents were able to inhibit the *in-vitro* mycelial growth of *F. oxysporum* effectively. A diversity of pathogenic *F. oxysporum* isolates including *F. o. ciciris, F. o. phasioli* and *F. o. melonies* have been successfully suppressed by *Bacillus* spp. isolated from chickpea rhizosphere (Landa *et al.,* 1997).

Similarly, in the current study, the majority of isolates from the sorghum rhizosphere in Ethiopia showing significant biocontrol activity belong to members of the genus *Bacillus* of which 45 % are *B. cereus*. The *B. cereus* strains KBE4-3, KBE5-1, KBE7-8, KBE8-3 and NAE5-5 have dominated the natural biocontrol population in the rhizosphere of sorghum in this study. All five isolates have significantly inhibited *F. oxysporum in-vitro* and in the greenhouse and have potential as biocontrol agents in the sorghum rhizosphere. Likewise 40 % of the effective bacterial isolates such as KBS2-6, KBS5-H, KBS9-H, KFP9-A, and NAS4-3, from the rhizosphere and rhizoplane of grasses (South African isolates) were identified as *B. cereus*. A strain of *B. cereus* UW85 has previously been reported to suppress plant diseases caused by oomycetes (Silo-suh *et al.,* 1994; Handelsman and Stabb, 1996) due to the production of the antibiotics Zwittermicine (Milner *et al.,* 1996a) and Kanosamine (Milner *et al.,* 1996b). Indirect promotion of plant growth occurs when PGPR lessen or

prevent the deleterious effects of phytopathogens through mechanisms such as antibiosis against the pathogens (Asghar *et al.,* 2004). Elizabeth and Handelsman (1999), suggested that the effect of *B. cereus* on the microbial community in the rhizosphere is perhaps exerted by stimulating growth of other bacteria that stimulate root growth, antagonize the pathogen or induce resistance in the host.

Isolate KBE6-3 which resulted in 68 % suppression of *Fusarium* root rot (Table 3) was identified as *B. subtilis*. Ii is also known that *B. subtilis* also occurs in the soil surrounding the root and has often been reported as an antifungal agent against plant pathogens (Marten *et al.,* 2000 and Bais *et al.,* 2004). *Fusarium*s wilt of chickpea caused by *F. oxysporum* f.sp. *ciceris* has been suppressed by *B. subtilis* isolate GBO3 (Hervas *et al.,* 1998). The same *B. subtilis* isolate has also been reported to activate an ISR pathway in Arabidopsis by the production of some volatiles (Compant *et al.,* 2005). Bochow *et al.* (1995), demonstrated that certain root colonizing strains of *B. subtilis* play a role as biocontrol agents through induced tolerance of treated seedlings against attack by *F. oxysporum*. In another experiment (Basha and Ulaganathan, 2002) lysis and dissolution of fungal mycelium of *Aspergillus niger* strain have been associated with the chitinolytic property of *B. subtilis* strain AF1.

Among the other promising isolates identified as members of the Genus *Bacillus* in this study *are* isolates KBE5-7, KBE7-6, and NAE5-7 which were identified as *B. stearothermophilus*, *B. licheniformis*, and *B. circulans* respectively. One of the mechanisms by which *Bacillus* species exert their antagonistic activity against fungal pathogens is parasitism which operates by degradation of cell walls of pathogenic fungi. In this regard, both *B. circulans* (Watanabe *et al.,* 1990) and *B. licheniformis* (Trachuk *et al.,* 1996) produce the enzyme chitinase that degrade chitin. Because of their ability to degrade chitin, the major structural component of the cell walls of phytopathogenic fungi (Someya *et al.,* 2004), chitinolytic enzymes are considered important in biological control of soilborne pathogens (Singh *et al.,* 1999).

In the current study, isolates KBE9-1 and KBE8-2 which were characterized by the production of a violet to reddish pigment on agar media were identified using the API system as *Chromobacterium violaceum*. Both isolates were able to colonize sorghum roots at a concentration higher than the required threshold level and were also able to inhibit *F. oxysporum in-vitro* and *in-vivo*. Chitinolytic enzymes produced by *C. violaceum* have been shown to be involved in the biological control of *F. oxysporum* (Park *et al.,* 1995). A strain of *C. violaceum* ATCC 12472 (Chernin *et al.,* 1995) selected from a variety of chitin utilizing bacterial species has previously been reported as the most active chitin degrading isolate. Although *C. violaceum* usually constitute only a small proportion of the

total micro-flora in soil, the ability of certain strains to produce antibiotics, hydrogen cyanide, proteases and a number of chitinolytic enzymes (Chernin *et al.,* 1995) indicates that *C. violaceum* have the potential as biological control agents against phytopathogenic fungi.

The Gram negative isolates from the rhizosphere and rhizoplane of grasses from South Africa which resulted in significant disease suppression in the greenhouse experiment were identified as *Chryseomonas luteola* (KBS5-F), *Stenotrophomonas maltophilia* (KBS9-B), *Serratia marcescens* (KBS9-R and KBS6-H) and *Enterobacter sakazaki* (NAS6B). Of all the isolates obtained from the Nylsvlei Nature Reserve in South Africa, *Chryseomonas luteola* (KBS5-F) rendered the highest *in-vitro* and *in-vivo* inhibition against *F. oxysporum*. Similar screening study for plant growth promoting rhizobacteria by Donate-Correa *et al.* (2004), resulted in the isolation of *C. luteola* having the capacity to inhibit the growth of *F. oxysporum*. Earlier, Gyung-Hyun *et al.* (2001), isolated *C. luteola* with a strong antagonistic activity against fungal pathogens due to its ability to produce antibiotics, siderophores and the enzyme cellulose.

Stenotrophomonas maltophilia were found to be common inhabitants of the wheat rhizosphere (Tilak *et al.,* 2005) representing a species of agronomic importance. In our study we have isolated one strain, *S. maltophilia* KBS9-B, from the rhizoplane of grasses with the potential to inhibit *F. oxysporum* under greenhouse conditions. Traits of *S. maltophilia* associated with biocontrol mechanisms include antibiotic production, extracellular enzyme activity and rhizosphere colonization potential (Kobayashi *et al.,* 2002). This isolate rendered a percentage *in-vivo* disease suppression of 87.5 %. In the *in-vitro* tests however, this strain did not perform well rendering a percentage inhibition of only 13.5 %. There is a likelihood that the PDA medium employed in the *in-vitro* assay has contributed to the lack of expression of the biosynthetic genes by *S. maltophilia* responsible for antibiotic production. Mazolla and Cook (1991), for instance reported that *P. fluorescens* Q27a-80 inhibited hyphal growth of *P. ultimum* by 51 % on NBY medium whereas 0% on PDA.

Two other Gram-negative isolates, KBS9-R and KBS6-H which were identified with 16S r DNA sequencing as *Serratia marcescens*, resulted in ≥ 60 % disease suppression against *F. oxysporum* in the greenhouse experiment. These strains were also very efficient in the *in-vitro* mycelial inhibition test against *F. oxysporum.* Other reports on the involvement of *S. marcescens* as an efficient biocontrol agent include suppression of Fusarium wilt (Liu *et al.,* 1995; Bora *et al.,* 2004), rice

sheath blight by *Rhizoctonia solani* (Someya *et al.,* 2005), damping-off in beans caused by *Rhizoctonia solani* and *Sclerotium rolfsii* (Chet *et al.,* 1990).

On the basis of this study it is concluded that the rhizosphere of sorghum and grasses harbour beneficial microorganisms with the potential to suppress soilborne diseases against *Fusarium oxysporum* and possibly other soilborne pathogens. In order to develop these promising strains into commercial inoculants, it is suggested that their modes of action as well as their biocontrol efficacy under field conditions be determined.

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Table 2. 1. Inhibition of *Fusarium oxysporum* mycelial growth on potato dextrose agar and *in- vitro* root colonization of sorghum roots by rhizobacterial isolates originally isolated on King's B medium from sorghum rhizosphere in Ethiopia

Bacterial Isolates	Dual culture assay (% mycelial inhibition[x])*	*In- vitro* root colonization (Log cfu/cm roots ± SE [y])*
KBE5-7	40.73^{c}	5.72 ± 0.47^{d}
KBE2-5	38.43^{c}	5.90 ±0.30^{d}
KBE4-3	37.43^{cd}	6.96 ± 0.28^{c}
KBE4-4	35.66^{cd}	4.88 ±0.19^{fgh}
KBE5-1	33.83^{cd}	6.92 ± 0.28^{c}
KBE5-2	56.53^{ab}	7.09 ±0.07^{c}
KBE5-3	12.36^{ef}	4.32 ± 0.44^{j}
KBE5-4	48.86^{bc}	4.98± 0.07^{fg}
KBE6-1	17.40^{bc}	6.99± 0.07^{c}
KBE6-2	7.06^{f}	4.80± 0.21^{fghi}
KBE6-3	42.73^{bc}	8.25±0.12^{b}
KBE6-5	42.30^{bc}	5.15±0.18^{ef}
KBE6-8	18.06^{ef}	4.60 ±0.13^{hij}
KBE7-6	56.36^{ab}	4.58±0.12 ^{hij}
KBE7-8	38.53^{c}	8.09±0.11^{b}
KBE8-2	46.70^{bc}	6.89±0.19 ^{c}
KBE8-3	46.80^{bc}	9.28±0.25^{a}
KBE9-1	66.33^{a}	9.22±0.11^{a}
KBE9-4	22.70^{de}	7.09±0.08^{c}
KBE9-5	15.56^{ef}	5.34±0.10^{e}
KBE9-8	41.00^{c}	4.69±0.26^{ghi}
Control	0.00 ^{g}	4.47±0.10^{ij}

[x] % Mycelial inhibition was calculated as [(R – r)] / R x 100, where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia), r is mycelial growth towards the bacteria.

[y] Root colonization expressed as Log cfu/cm root after dilution plating of roots on solid media.

*Means followed by the same letters do not differ significantly (*P=0.05*) according to the Least Significant Difference (LSD) tests using the GLM procedure.

Table 2. 2. Inhibition of *Fusarium oxysporum* mycelial growth on potato dextrose agar and *in-vitro* colonization of sorghum roots by rhizobacterial isolates initially isolated on nutrient agar medium from sorghum rhizosphere in Ethiopia

Bacterial Isolates	Dual culture assay (% mycelial inhibition[x]) *	*In-vitro* root colonization (Log cfu/cm roots ± SE) *
NAE2-1	19.30^{def}	7.37±0.18^{b}
NAE2-2	19.73^{def}	7.16 ±0.20^{bc}
NAE2-4	39.06^{ab}	6.75 ±0.13^{cd}
NAE2-6	22.96^{cdef}	7.30 ±0.21^{b}
NAE2-8	35.23^{abc}	4.890.17^{g}
NAE3-1	14.00^{f}	4.86±0.10 ^{g}
NAE3-3	26.00$^{b\text{ -f}}$	6.99±0.16^{bcd}
NAE3-6	27.6$^{a\text{ -f}}$	4.64±0.16^{gh}
NAE3-8	22.03^{cdef}	4.64 ±0.29^{gh}
NAE4-1	29.56$^{a–\text{ f}}$	5.52 ±0.31^{f}
NAE4-2	21.0^{cdef}	7.27±0.22 ^{b}
NAE4-4	31.33$^{a-\text{ e}}$	6.05±0.06^{e}
NAE5-5	37.46^{ab}	5.900.13^{ef}
NAE5-7	26.86$^{a-\text{ f}}$	7.13±0.27^{bcd}
NAE5-8	28.20^{a-f}	4.70 ±0.12^{gh}
NAE6-2	30.00$^{a-\text{ e}}$	8.98 ±0.09^{a}
NAE6-3	21.56^{cdef}	4.98±±0.61 ^{g}
NAE7-1	40.93^{a}	6.66 ±0.43 ^{d}
NAE7-2	20.43^{def}	5.92 ±0.12^{ef}
NAE7-3	18.76^{def}	7.13±0.02^{bcd}
NAE9-3	29.33$^{a-\text{ e}}$	4.76 ±0.29^{gh}
NAE9-5	28.93$^{a\text{ -e}}$	5.93 ±0.19^{ef}
Control	0.00^{g}	4.37±0.26 ^{h}

[x] % Mycelial inhibition was calculated as [(R – r)] / R x 100 where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia), r is mycelial growth towards the bacteria.

*Means followed by the same letters do not differ significantly (*P=0.05*) according to the Least Significant Difference (LSD) tests using the GLM pr

Table 2. 3. Effect of inoculated rhizobacteria isolated from the rhizosphere of sorghum in Ethiopia on suppression of *Fusarium oxysporum* root and crown rot under greenhouse conditions

Treatment	Disease suppression (%)†*		Root biomass (g)*	
	Root	crown	Fresh weight	Dry weight
KBE2-5	100^{a}	100^{a}	0.126^{a-f}	0.033^{b}
KBE4-3	95.53^{ab}	86.74^{abc}	0.043^{gh}	0.016^{defg}
KBE5-2	77.57^{abcd}	25.55^{fg}	0.060^{fgh}	0.010^{fgh}
KBE5-4	86.22^{ab}	78.99^{abc}	0.083^{d-h}	0.013^{efgh}
KBE5-1	100^{a}	100^{a}	0.190^{a}	0.030^{bc}
KBE5-2	77.57^{abcd}	25.55^{fg}	0.060^{fgh}	0.010^{fgh}
KBE5-4	86.22^{ab}	78.99^{abc}	0.083^{d-h}	0.013^{efgh}
KBE5-7	100^{a}	100^{a}	0.120^{a-g}	0.030^{bc}
KBE6-3	68.00^{abcd}	45.45^{def}	0.100^{c-g}	0.015^{defg}
KBE7-6	57.25^{bcd}	45.56^{def}	0.083^{d-h}	0.010^{fgh}
KBE7-8	71.71^{abcd}	59.86^{bcde}	0.096^{c-g}	0.023^{bcde}
KBE8-2	71.08^{abcd}	94.47^{a}	0.153^{abcd}	0.023^{bcde}
KBE8-3	76.34^{abcd}	56.97^{cde}	0.173^{abc}	0.026^{bcd}
KBE9-1	84.45^{abc}	90.77^{ab}	0.053^{fgh}	0.016^{defg}
KBE9-8	60.43^{abcd}	44.53^{def}	0.096^{c-g}	0.016^{defg}
NAE1-7	59.93^{abcd}	44.99^{def}	0.053^{fgh}	0.010^{fgh}
NAE2-4	61.15^{abcd}	57.11^{cde}	0.106^{b-g}	0.030^{bc}
NAE5-5	100^{a}	100^{a}	0.140^{a-e}	0.020^{cdef}
NAE5-7	95.83^{ab}	95.83^{a}	0.130^{a-f}	0.026^{bcd}
NAE6-2	78.32^{abcd}	81.07^{abc}	0.110^{b-g}	0.016^{defg}
NAE9-3	42.05^{de}	42.35^{def}	0.043^{gh}	0.010^{fgh}
NAE9-5	13.68^{ef*}	16.33^{fg*}	0.046^{gh*}	0.007^{gh*}
Control a§	0.00^{f}	0.00^{g}	0.010^{h}	0.001^{h}
Control b	71.19^{abcd}	69.06^{abcd}	0.183^{ab}	0.046^{a}
LSD $_{0.05}$	*40.33*	*32.29*	*0.079*	*0.012*

† Percent disease suppression was determined as [A-B/A] x 100, where A is disease severity index in the root/crown of control plants inoculated with only *Fusarium oxysporum* without bacterial antagonists, B is disease severity index in the root/crown of plants treated with both the pathogen and bacterial antagonists.

§ Control a = pathogen only inoculated (disease control) ; Control b = uninoculated (healthy control).

* Means followed by different letters are significantly different at *P=0.05* according to the Least Significance Difference (LSD) t test using the GLM procedure in SAS-9.1 software.

Table 2. 4. Inhibition of *Fusarium oxysporum* on potato dextrose agar and suppression of root rot by this pathogen in sorghum by rhizobacteria isolated from the rhizosphere and rhizoplane of grasses within the Nylsvlei Nature Reserve in South Africa

Bacterial Isolates	*In-vitro* mycelial inhibition (%)	Root fresh weight (g)	Root dry weight(g)	Root rot severity index (0-4)	Disease† Suppression (%)
KBS1A	8.74^{g}	0.010^{g}	0.000^{f}	3.850^{a}	2.00^{h}
KBS2-6	19.28^{b-d}	0.036^{c-f}	0.013^{c-f}	1.530^{c-f}	61.10^{a-e}
KBS2-12	17.62^{b-f}	0.050^{bcd}	0.026^{cd}	2.760^{abc}	51.30^{def}
KBS5-F	37.60^{a}	0.073^{a}	0.053^{a}	0.160^{g-h}	95.83^{a}
KBS5-H	16.32^{c-g}	0.020^{d-g}	0.010^{def}	1.600^{c-f}	59.13^{cde}
KBS6-H	35.70^{a}	0.010^{g}	0.001^{f}	1.560^{c-f}	60.00^{b-e}
KBS6-11	13.85^{c-g}	0.016^{d-g}	0.004^{f}	2.500^{bcd}	37.87^{efg}
KBS8-7	15.83^{d-g}	0.016^{d-g}	0.007^{ef}	3.360^{ab}	14.83^{gh}
KBS9-B	13.50^{c-g}	0.040^{b-f}	0.030^{bc}	0.500^{e-h}	87.50^{abc}
KBS6-H	35.70^{a}	0.010^{g}	0.001^{f}	1.560^{c-f}	60.00^{b-e}
KBS9-R	24.80^{b}	0.013^{efg}	0.004^{f}	1.400^{d-g}	64.50^{a-e}
KBS10-9	8.88^{g}	0.020^{d-g}	0.010^{def}	1.630^{c-f}	58.10^{cde}
KFP9-A	11.48^{d-g}	0.070^{bc}	0.046^{ab}	0.200^{g-h}	94.77^{ab}
NAS2-B	18.60^{c-g}	0.020^{d-g}	0.010^{def}	3.360^{ab}	15.83^{fgh}
NAS2-F	15.28^{d-g}	0.047^{b-e}	0.026^{cd}	1.960^{cd}	49.77^{efg}
NAS4-3	15.46^{d-g}	0.047^{b-e}	0.030^{bc}	1.630^{c-f}	58.50^{cde}
NAS6-B	18.60^{c-g}	0.020^{d-g}	0.007^{ef}	0.330^{fgh}	91.43^{abc}
NAS6-2	10.40^{c-f}	0.023^{d-g}	0.013^{c-f}	1.660^{cde}	57.27^{cde}
Control a	0.00^{h}	0.000^{g}	0.000^{f}	3.930^{a}	0.00^{h}
Control b	-	0.140^{a}	0.060^{a}	0.200^{gh}	94.87^{ab}
Pr > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
$LSD_{0.05}$	0.034	0.034	0.0167	1.3265	35.47

[X] % Mycelial inhibition was calculated as [(R – r)] / R x 100 where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia), r is mycelial growth towards the bacteria.

† Percent disease suppression was determined as [A-B/A] x 100, where A is disease severity index in the root/crown of control plants inoculated with only *Fusarium oxysporum* without bacterial antagonists, B is disease severity index in the root/crown of plants treated with both the pathogen and bacterial antagonists.

§ Control a = pathogen only inoculated (disease control) ; Control b = uninoculated (healthy control).

*Means followed by different letters are significantly different at *P = 0.05* according to the Least Significance Difference (LSD) t test using the GLM procedure.

Table 2. 5. Identification to species level of isolates from Ethiopia and South Africa effective in the *in-vitro* and *in-vivo* inhibition of *Fusarium oxysporum*

Bacterial Isolates	Gram reaction	Endo-spore#	Catalase test	Cytochrome oxidase	Motility* test	O/F test	Bacterial species	Means of identification
KBE2-5	+	+	+	+	+	Nd	*Bacillus* spp.	API 50 CHB
KBE4-3	+	+	+	+	+	Nd	*Bacillus cereus*	API 50 CHB
KBE5-1	+	+	+	+	+	Nd	*B. cereus*	16S rDNA Sequencing
KBE5-7	+	+	+	-	+	Nd	*B. stearothermophilus*	API 50 CHB
KBE6-3	+	+	+	+	-	Nd	*B. subtilis*	API 50 CHB
KBE7-6	+	+	+	-	+	Nd	*B. licheniformis*	API 50 CHB
KBE7-8	+	+	+	+	+	Nd	*B. cereus*	16S rDNA Sequencing
KBE8-2	-	-	+	+	+	O	*Chromobacterium violaceum*	API 20 NE
KBE8-3	+	+	+	-	+	Nd	*B. cereus*	API 50 CHB
KBE9-1	-	-	+	+	+	O	*C. violaceum*	API 20 NE
KBS2-6	+	+	-	+	+	Nd	*B. cereus*	16S rDNA Sequencing
KBS2-12	+	-	+	-	-	Nd	*Brevibacterium laterosporus*	API 50 CHB
KBS5-H	+	-	+	_	+	Nd	*B. cereus*	API 50 CHB
KBS5-F	-	-	-	+	+	O	*Chryseomonas luteola*	API 20 NE
KBS6-H	-	-	+	+	+	O	*Serratia marcescens*	16S rDNA Sequencing
KBS6-11	-	-	-	+	+	O	*C. luteola*	API 20 NE
KBS9-B	-	-	+	-	+	O	*Stenotrophomonas maltophilia*	API 20 NE
KBS9-H	+	+	+	-	-	Nd	*B. cereus*	16S rDNA Sequencing
KBS9-R	-	-	+	-	+	O	*S. marcescens*	16S rDNA Sequencing

KBS10-9							*not known*	-
KFP9-A	+	+	+	+	-	Nd	*B. cereus*	16S rDNA Sequencing
NAE5-5	+	+	+	+	+	Nd	*B. cereus*	API 50 CHB
NAE5-7	+	+	+	+	+	Nd	*B. circulans*	API 50 CHB
NAS2-F							*not known*	-
NAS4-3	+	+	+	+	+	Nd	*B. cereus*	API 50 CHB
NAS6-B	-	-	-	-	+	F	*Enterobacter sakazaki*	API 20 E
NAS6-2							*not known*	-

[#] += endospore present, - endospore absent, * + = motile, - = non motile, Nd = not done for Gram positives.

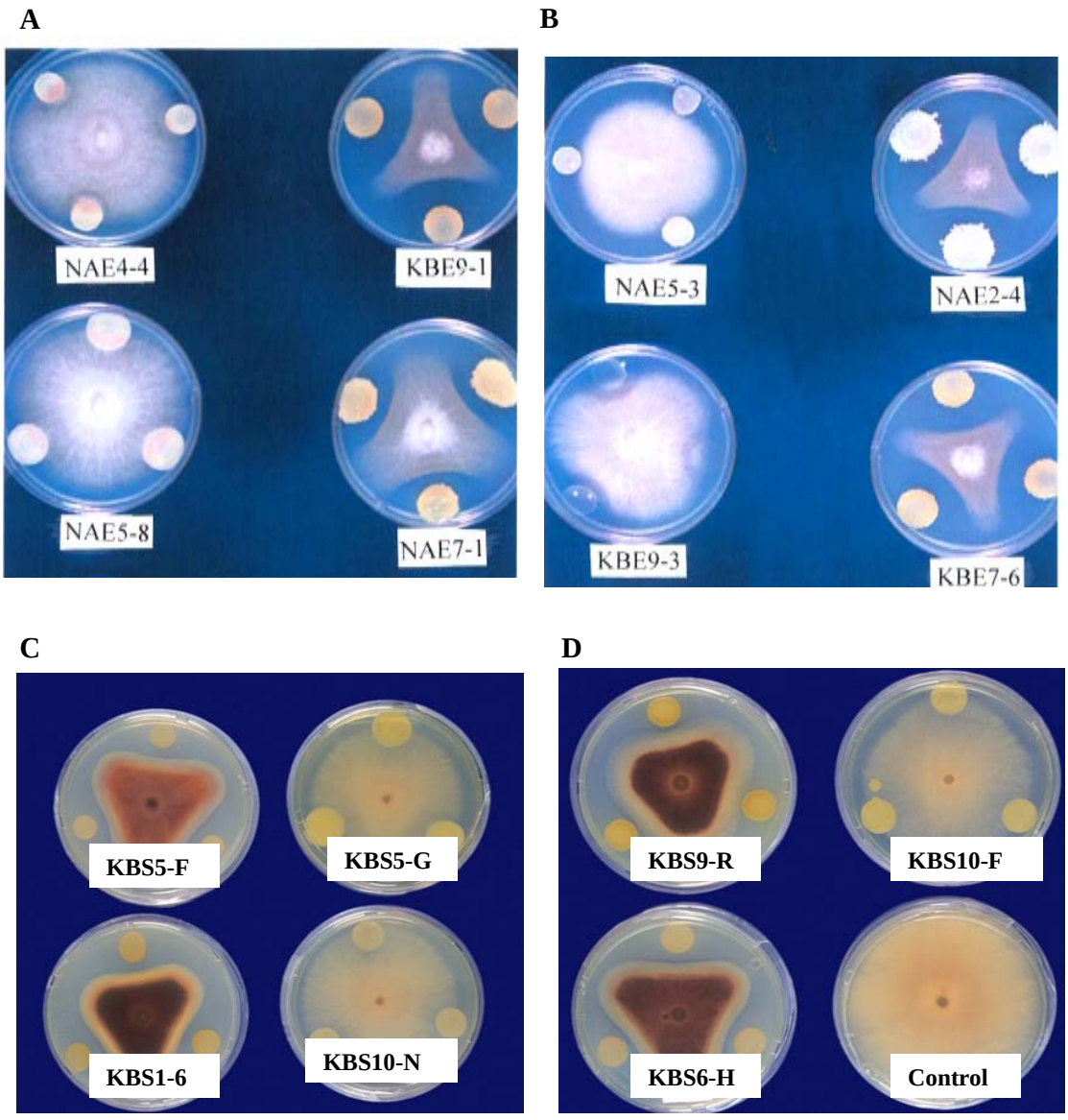

Figure 2. 1. Dual culture assay for *in-vitro* inhibition of mycelial growth of *Fusarium oxysporum* by selected bacterial isolates from sorghum rhizosphere in Ethiopia (**A & B**) and from the rhizosphere and rhizoplane of grasses in South Africa (**C & D**). Note the formation of clearly visible inhibition zones on the plates with bacterial isolates KBE9-1, KBE7-6, NAE7-1, NAE2-4 (Ethiopian isolates) and KBS5-F, KBS6-H and KBS9-R (South African isolates) compared to the plates with isolates NAE4-4, NAE5-8, NAE5-3, KBE9-3 (A) and isolates KBS5-G, KBS10-F, KBS10-N (B) which resulted in little or no inhibition zones.

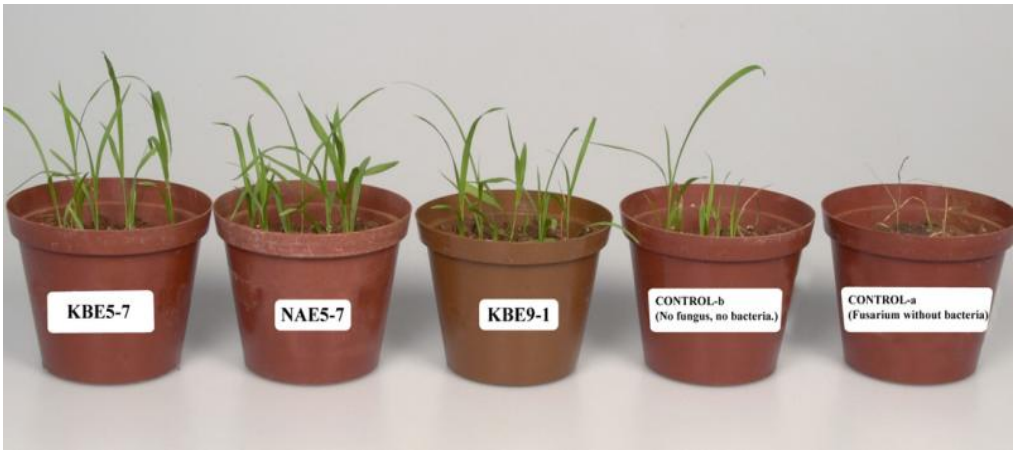

Figure 2. 2. Greenhouse pot experiment illustrating the efficacy of some of the bacterial isolates from Ethiopia in the suppression of root and crown rot diseases by *F. oxysporum* in four weeks old sorghum seedlings. All plants treated with *F. oxysporum* alone died (Control a, far right) whereas 100 % of the plants inoculated with both the pathogen and isolates KBE5-7, NAE5-7 and KBE9-1 survived with no symptoms of infection.

Figure 2. 3. Biological control of *Fusarium oxysporum* in four-weeks-old sorghum seedlings by bacterial isolates KBS5-F, KFP9-A and KBS2-6 all from the rhizosphere of grasses at the Nylsvlei Nature Reserve in South Africa. Inoculation of plants with *Fusarium oxysporum* only in the control (right) resulted in 100% infection causing the death of all plants

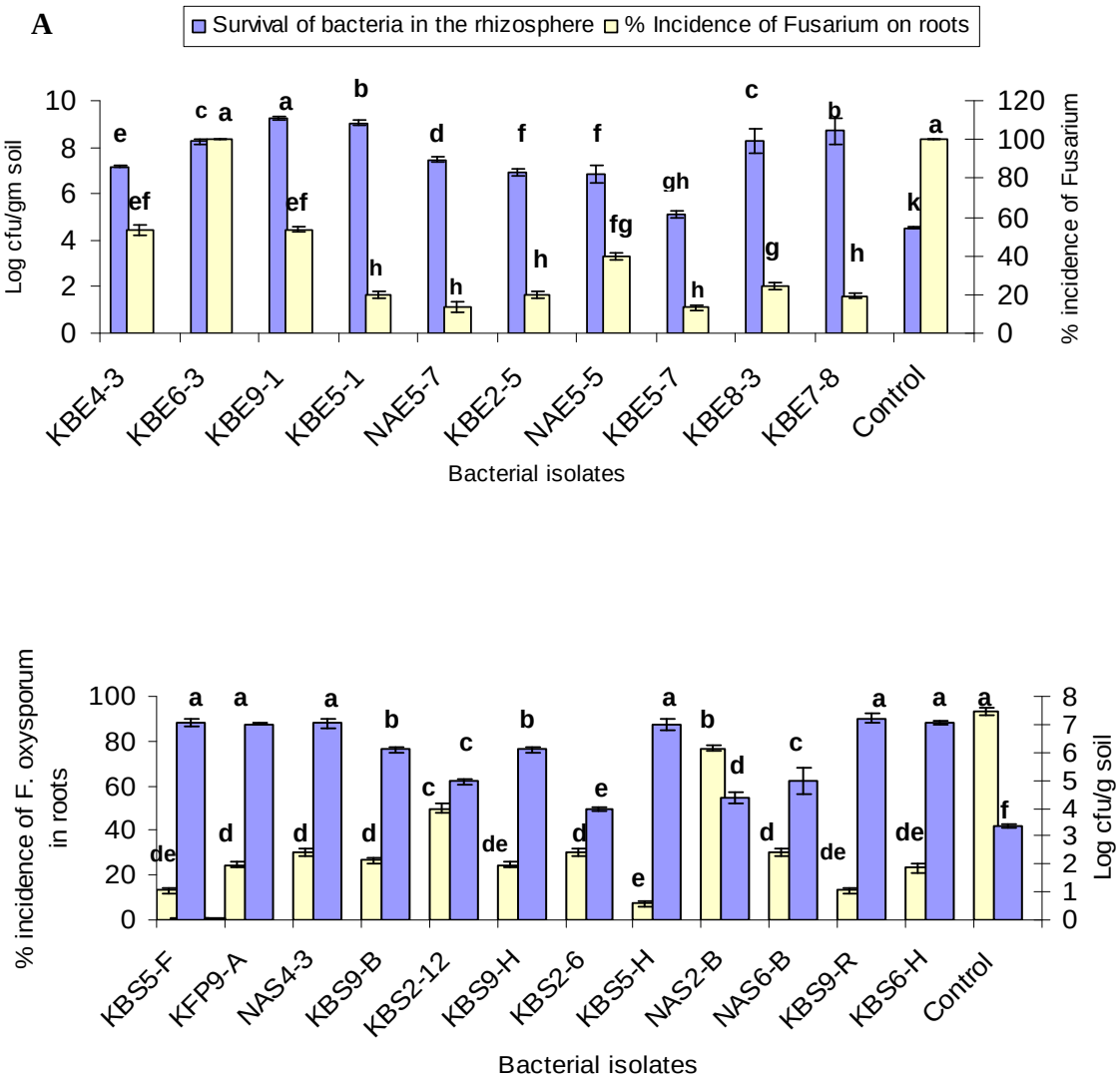

Figure 2. 4. Efficacy of the bacterial isolates obtained from the sorghum rhizosphere in Ethiopia (**A**) and from the rhizosphere of grasses in South Africa (**B**) as biological control agents as illustrated by their high rate of survival in the rhizosphere and low incidence of *F. oxysporum* in roots four weeks after inoculation with both the bacterial isolates and the target pathogen. Bars represent standard errors of means. Means with the same letter are not significantly different according to Duncan's Multiple Range Test ($p = 0.05$) using the GLM procedure. In both cases control plants not treated with bacterial isolates (control a = disease control) rendered a significantly higher incidence of *F. oxysporum*.

CHAPTER- 3

Suppression of *Pythium ultimum* root rot in sorghum by rhizobacterial isolates from Ethiopia and South Africa*

Abstract

Bacteria isolated from the rhizosphere of sorghum in Ethiopia and from different grass species within the Nylsvlei Nature Reserve in South Africa were evaluated for their *in-vitro* and *in-vivo* antagonistic activity against *Pythium ultimum* that causes root rot in sorghum. More than 50 % disease suppression was achieved with 15 Ethiopian isolates obtained from the sorghum rhizosphere, whereas eight isolates from the rhizosphere of grasses at Nylsvlei Nature Reserve in South Africa resulted in more than 80 % root rot suppression. The isolates maintained themselves at a level of more than 10^5 cfu/g indicating that they can effectively survive in the sorghum rhizosphere and maintain their population. Isolates which rendered the best overall *in-vitro* and *in-vivo* performance were identified by means of the API system and sequencing of the bacterial 16S rDNA genes. The majority of these isolates were identified as members of the Genus *Bacillus* of which *Bacillus cereus* comprised 56 %. Three other isolates were identified as *Brevibacterium laterosporus*, *Pseudomonas fluorescens* and *Serratia marcescens*. The results of this study offer a significant impetus to the application of plant growth promoting rhizobacteria for use as biological control agents against *P. ultimum* in sorghum.

*Submitted for publication to *Biological Control Journal*

3. 1. Introduction

Pythium ultimum Trow is a common soilborne pathogen and attacks a large number of plant species (Green and Jenson, 2000). Infection by *P. ultimum* results in yield reductions in a wide range of greenhouse grown crops (Ghaouth *et al.,* 1994) and a number of field crops including sorghum (Davis and Bockus, 2001). The pathogen survives in soil as oospores and when soils are wet, seed and root exudates stimulate germination of resting spores after which the fungus attacks young succulent tissue and increases its inoculum level (Forbes *et al.,* 1986; Windels and Jones, 1989).

In a field survey conducted as part of this study in 2003 in Ethiopia, sorghum (*Sorghum bicolor* (L.) Moench) plants showing any disease symptoms were described and the pathogens isolated.

From the predominant root rot, leaf tip and leaf blade necrosis symptoms, more than 90 % of the isolates were *P. ultimum* (unpublished data).

Sorghum is known to become more susceptible to *Pythium* species in cool, wet soils because of slower seed germination rates, delayed emergence and reduced growth of primary roots (Forbes *et al.,* 1986). Alemaya is located at a higher altitude (2000 m.a.s.l). The rainy wet season extending from April to September has a monthly rainfall more than 100 mm, the highest being 144 mm in August (Muleta *et al.,* 2006). In addition, the influence of the nearby Lake Alemaya makes the soils in these fields relatively cooler and moist compared to other sorghum growing areas such as Meeson and Jijiga. This increased soil moisture might have created a favourable condition for the occurrence of *P. ultimum* causing root rot of sorghum in Alemaya areas as infections by *Pythium* spp. are favoured by wet soil conditions. Mundel *et al*. (1995), for instance indicated that the severity of disease in safflower infected by *P. ultimum* under greenhouse conditions increases with increasing soil moisture.

Pythium ultimum can be successfully controlled using metalaxyl (Hwang *et al.,* 2001; Taylor *et al.,* 2002). However, efforts made to reduce the disease severity caused by *P. ultimum* Trow in Alemaya areas in Ethiopia using drenches of metalaxyl have been unsuccessful. Some reduction in disease severity were achieved at higher dosages but the efficacy of the fungicide declined quickly (unpublished data). Moreover in countries like Ethiopia where subsistence farming prevails, the cost of fungicides such as metalaxyl is unaffordable. An alternative, less expensive and environmentally friendly method to control soilborne fungal pathogens is biological control by means of a group of bacteria known as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.,* 1980; deWeger *et al.,* 1995; Marten *et al.,* 2000; Arora *et al.,* 2001; Rangarajan *et.al.,* 2003).

Plant growth promoting rhizobacteria exert their biological control activity directly through the production of metabolites such as antibiotics, hydrogen cyanide, siderophores and cell wall degrading enzymes which inhibit the pathogen (Kloepper *et al.,* 1999). Moreover, PGPR also mediate biological control indirectly by eliciting induced systemic resistance (ISR) against a number of plant diseases (Jetiyanon and Kloepper, 2002).

There is currently a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops (Thakuria *et al.,* 2004). However, little is known about

the use of beneficial rhizosphere bacteria for use in agriculture in Ethiopia. The objective of this study is therefore to evaluate bacterial isolates obtained from sorghum rhizosphere in Ethiopia and from the rhizosphere of grasses in South Africa, for their ability to inhibit root rot of sorghum caused by *P. ultimum,* under *in-vitro* and greenhouse conditions.

3. 2. Materials and methods

3. 2. 1. Rhizosphere soil sample collection and analysis

See section 2. 2. 1. of the previous chapter for soil sample collection from the rhizosphere of sorghum in Ethiopia. Unlike the soils in Alemaya, the soils in the sorghum growing fields of Meeson and Jijiga areas are suppressive to *P. ultimum* probably due to their drier nature (personal communication). The samples were placed in plastic bags and transferred to the Microbiology Laboratory of the Department of Biology, Alemaya University. The properties of the soil samples collected were assessed in terms of particle size, composition and inorganic component.

Rhizosphere soil samples were also collected from ten selected sites of the rhizosphere of grasses within the Nylsvlei Nature Reserve in South Africa, representing a typical bushveld savannah. The samples were transported to the Plant Pathology Laboratory at the Department of Microbiology and Plant Pathology, University of Pretoria. Each rhizosphere soil sample was sieved to remove plant debris.

3. 2. 2. Isolation of bacteria

The approach used to isolate bacteria from soil samples collected from Ethiopia and South Africa followed the same protocol described in the previous chapter section 2. 2. 2.

3. 2. 3. Bacterial inoculum preparation and in-vitro antagonistic activity

Preparation of the bacterial inoculum used in this study was conducted as described in 2. 2. 3. The *in-vitro* mycelial inhibition of the bacterial isolates against *P. ultimum* was conducted by means of the dual culture method (Paulitz *et al.*, 1992).

3. 2. 4. In- vivo inhibition of Pythium ultimum by rhizobacterial isolates

3. 2. 4. 1. Fungal inoculum preparation and pathogenecity test

Four *Pythium* isolates viz. *P. ultimum* M1256G from wheat, *P. ultimum* PPRI from sorghum, *P. graminicola* M2794AD and *P. irregulare* M1255AA from barley all obtained from the University of the Free State, Bloemfontein, South Africa were re-tested for their pathogenecity on sorghum in the greenhouse. Millet seed (50 g) together with 200ml-distilled water was deposited in an autoclavable polyethylene bag and autoclaved at 121 ^{o}C for 15 min. Each bag was subsequently inoculated with five 4 mm diameter agar discs aseptically cut from the edge of an actively growing mycelium of each isolate. The inoculated bags were subsequently incubated at 25 ^{o}C for five days until the millet seeds were fully covered by the mycelial growth. For the control experiment, sterile un-inoculated millet seed was used.

The fungal inocula (30 g/kg soil) were applied into 12 cm x 10 cm plastic pots filled with steam-pasteurized soil. Sorghum seeds (South African variety Gewoon) were surface sterilized with 70 % ethanol for 3 min, 1 % sodium hypochlorite for 1 min and rinsed with sterile distilled water five times. The seeds were then pre-germinated in sterile vermiculite for five days and eight seedlings were transplanted into each pot. The pots were regularly watered and the temperature of the greenhouse was maintained at 26 ± 1^{o}C. The experiment was arranged in a randomized block design with three replications and was repeated twice. Pathogenecity of the four *Pythium* isolates were assessed by recording leaf necrosis, root rot and incidence of *Pythium* in roots plated on BNPRA medium (Massago *et al.,* 1997) selective for *Pythium* spp. BNPRA medium contains: Benomyl 0.01 g/L, Nystatin 0.025 g/L, Pentachloronitrobenzene 0.05 g/L, Rhizolex 0.2 g/L, Rifampicin 0.025 g/L, Ampicilline 0.25 g/L, bacteriological agar 12 g/L. The agar was dissolved in 800 ml distilled water and autoclaved. The other ingredients were first dissolved in small quantity of absolute ethanol and mixed with sterile 200 ml distilled water in a separate flask. This was then added to the sterile agar medium and poured onto sterile Petri dishes to solidify.

3. 2. 4. 2. Planting of sorghum and inoculation of fungal and bacterial isolates

Sorghum seeds prepared and pre-germinated as described in 2.4.1 were used in this experiment. Prior to seedling transplanting, the millet seed inoculum of *P. ultimum* M1256G was mixed into steam-pasteurized soil in 12 cm x 10 cm diameter plastic pots at the rate of 30 g/kg. For the

control treatments, sterile millet seed was mixed into the soil at the same rate. Eight sorghum seedlings were transplanted into each pot and the pots were maintained in the greenhouse at 26 ± 1 ^{o}C. One day after transplanting seedlings, each pot was drenched with 30ml of one of the bacterial inocula (10 ^{8} cfu/ml). Two successive bacterial applications were made a week apart. The treatments in the *in-vivo* biocontrol experiment were: Plants inoculated with *P. ultimum* and the different selected bacteria, Plants inoculated with *P. ultimum* only (control a) and a non-inoculated control (control b). The non-inoculated control was treated with sterile millet seed without fungal and bacterial inoculum. The plants were irrigated twice daily with tap water by means of an automatic watering system. All the *in-vitro* and *in-vivo* experiments were arranged in a randomized block design with three replications and the experiment was conducted twice.

3. 2. 4. 3. Disease assessment

Plants were removed from the soil, the roots washed with sterile distilled water and the severity of root rot was determined visually using a 0 – 4 rating scale (Brien *et al.,* 1991; Harveson and Rush, 2002) where, 0 = no disease symptoms, 1= 1-25 %, 2= 26-50 %, 3= 51-75 %, and 4= 76-100 % root rot.. The disease severity index was used to calculate the percentage suppression of root rot (Villajuan-Abgona *et al.,* 1996).

% Suppression = [(A-B)/A] x 100 where,

A = Disease severity exhibited in the root region due to *P. ultimum* alone and,

B= Disease severity exhibited in the root region after inoculation with both the pathogen and bacterial antagonists.

Four plants per treatment were removed, the roots excised and fresh and dry weights of each individual roots were determined. The remaining four roots were subsequently used to determine the incidence of *P. ultimum* infection as described below.

3. 2. 4. 4. Incidence of Pythium ultimum in roots

The incidence of *P. ultimum* in roots was determined by making re-isolations of the fungus on BNPRA. Roots from both infected and non-infected plants were excised, surface sterilized with 1 % sodium hypochlorite for 3 min and rinsed five times with sterile water. The roots from all three replications in each treatment were pooled and a sub sample of 10 root pieces (3 mm long) per plate aseptically plated in triplicate on BNPRA medium. The plates were incubated at 26 ± 1

^{o}C for seven days. The resulting fungal colonies were examined microscopically and the number of root pieces rendering *P. ultimum* was recorded.

3. 2. 4. 5. Root colonization of bacterial isolates

In order to assess the survival of the bacterial isolates in the rhizosphere of the sorghum plants, a modification of the procedure described by Landa *et al.* (2004) was used. Four weeks after inoculation, plants were carefully removed from the pots and roots were gently shaken to remove all but the tightly adhering soil. One gram of the adhering rhizosphere soil was collected and placed into 9 ml sterile 0.1 M $MgSO_4$ solution. Serial dilutions of the suspension were vortexed and plated on King's B or NA medium as described before. The plates were incubated at 30 ^{o}C for 24 hr after which the developed colonies were counted and the number of CFU/g of soil calculated.

3. 2. 5. Identification of the bacterial isolates

Identification of the bacterial isolates effective in the suppression of *P. ultimum* in this study was conducted by means of the API system and sequencing of the bacterial 16S rDNA genes as described in 2. 2. 7.

3. 2. 6. Statistical analysis.

The data were subjected to analysis of variance (ANOVA) using SAS-9.1 software (SAS Institute, 2003). Data on the survival of the biological control isolates in the rhizosphere of roots were log transformed before subjecting to ANOVA. Mean values in each treatment were compared by the least significant difference (LSD)t test and Duncan's Multiple Range test at 5 % ($p = 0.05$) level of significance. Pearson's linear correlation coefficient was used to evaluate the relationship between root rot severity index and the number of plants with necrotic leaves.

3. 3. Results

3. 3. 1. Pathogenecity test

All four *Pythium* species resulted in significant disease symptoms (root rot and leaf necrosis) on sorghum (Table 3. 1; Fig. 3. 2). Both *P. ultimum* M1256GA and *P. irregulare* M1255AA

resulted in a significantly higher number of plants with necrotic leaves and both isolates were also re-isolated from the roots more frequently compared to the other isolates and the control treatment. The pathogenecity test showed *P. ultimum* M1256GA resulted in significantly higher root rot in sorghum (Table 3. 1) and was therefore selected as the target pathogen in subsequent trials. The 20 % Pythium root infection observed in the control treatment might have resulted from a low level of cross contamination probably caused by the automatic watering system in the greenhouse.

3. 3. 2. In-vitro antagonistic activity

3. 3. 2. 1. Ethiopian isolates

Of the bacterial isolates used in the *in-vitro* test against *P. ultimum* M1256GA, 20 isolates resulted in suppression of *P. ultimum* ranging from 30 % to 57.6 % mycelial inhibition (Table 3. 2). Prominent inhibition zones were visible on the agar plates (Fig. 3. 1). On the other hand, 36 isolates were found to be totally overgrown by fungal mycelium and 12 other isolates did not result in a significant inhibition zone ($P = 0.05$) on the agar plate compared to the control. The percentage inhibition achieved by these isolates ranged from 5.7 % (NAE3-3) to 16.76 % (NAE9-5) (data not shown).

3. 3. 2. 2. South African isolates

Eighty six bacterial isolates obtained from the rhizosphere of grasses at Nylsvlei Nature Reserve were used in the *in-vitro* test for antagonistic activity. Only 27 of these showed some degree of mycelial inhibition of *P. ultimum* mycelial growth. With the exception of seven isolates, all other bacteria originally isolated on NA agar medium were completely overgrown by the fungal mycelium. The inhibition exhibited by the seven isolates ranged from as little as 11.03 % by isolate NAE2-8 (data not shown) to 34.8 % by isolate NAS2-D (Table 3. 3). Twenty two bacterial isolates showed some inhibition ranging from 9.1 % by isolate KBS10-E (data not shown) to 56 % by isolate KBS5-H (Table 3. 3). In both cases results are included only for those isolates which resulted in at least 30 % suppression of *P. ultimum*. For most of these isolates, the inhibition achieved was highly significant ($P = 0.05$) compared to the control (Table 3. 3).

3. 3. 3. In-vivo inhibition of Pythium ultimum by rhizobacterial isolates

3. 3. 3. 1. Ethiopian isolates

Out of the 20 bacterial isolates tested, three (NAE1-7, NAE7-3, and KBE5-2) resulted in a significant increase in fresh and dry weight of roots compared to the control (Table 3. 2). Isolate NAE1-7 resulted in 84.7 % increase in fresh weight of roots, whereas no significant increase in root dry weight was observed. Isolates KBE5-2 and NAE7-3 resulted in 50 and 53.5 % increase in the dry weight of the roots respectively (Table 3. 2).

Yellowing and necrosis of the leaf tip and blades were commonly observed in plants severely affected by the pathogen. Six isolates significantly decreased the percentage of plants showing leaf necrosis in comparison with the control plants which showed 100 % incidence of necrotic leaves (Table 3. 2). The number of plants with leaf tip necrosis was decreased by 50 % by isolate KBE5-8, 70 % by KBE6-1, 62.5 % by KBE8-4, 75 % by NAE1-7, and 79 % by NAE7-1. Compared to the *P. ultimum* inoculated control, the untreated control showed leaf tip necrosis less by 83.4 % (Table 3. 2). The rest of the bacterial isolates however did not result in a significant reduction in the number of plants showing leaf tip/blade necrosis compared to the control treated with only *P. ultimum* (Table 3. 2; Fig. 3. 3). When root rot severity was correlated with leaf necrosis, a significantly positive relationship was recorded with ($r = 0.696$, $P = 0.005$) (Fig. 3. 4A). The final disease suppression by the rhizobacterial isolates was determined by measuring root rot severity in comparison to the control. The highest level of disease suppression was exhibited by isolate NAE1-7 (86.23 %) followed by isolate NAE7-1 (78.33 %) (Table 3. 2). However, for isolate KBS6-17 that rendered 83.83 % disease suppression (Table 3. 3), root rot severity was low although a high incidence of leaf necrosis was recorded.

3. 3. 3. 2. South African isolates.

A statistically significant increase in the fresh weight of the roots was achieved by isolates KBS2-6 (56.95 %), KBS6-3 (54.9 %), KBS9-H (57.5 %), and KFP9-K (55.5 %) (Table3.3.). Although there seem to be some increases in the fresh weight of the roots by other isolates, none have been found to be statistically significant in relation to the un-inoculated control. Likewise none of the rhizobacterial isolates resulted in an increase in dry weight of the roots. In terms of leaf necrosis, nine isolates significantly reduced the number of plants with leaf tip/blade necrosis. Of these, four isolates viz. KBS2-12, KBS5-H, KFP9-K, and KBS9-R, reduced leaf tip necrosis

by 87.5 % and one isolate (KBS6-3) reduced the symptom by 83.3 % (Table 3. 3). A significant positive correlation ($r = 0.719$, $P = 0.005$) was recorded between root rot severity and leaf necrosis (Fig. 3. 4B). In general eight isolates significantly suppressed the disease symptom. The highest percentage disease suppression was achieved by isolate KBS5-H (100%) followed by KBS6-3 (99.12 %), KFP9-K (96.5 %), and KBS2-12 (90.7 %) and KBS9-H (80 %).

3. 3. 4. Incidence of Pythium ultimum in roots

Re-isolation of *P. ultimum* from roots of infected plants was recorded upon plating root pieces on BNPRA agar medium. Roots from control plants not treated with bacterial isolates rendered the highest percentage of *P. ultimum* (Fig. 3. 4A; B). Very low incidence (15 - 20 %) of *P. ultimum* was recorded in roots of plants treated with Ethiopian rhizobacterial isolates KBE8-4, KBE9-1, NAE1-7, and NAE7-1 (Fig. 3. 4. A). Likewise, treatment with South African bacterial isolates KBS2-6, KBS2-12, KBS5-H, KBS9-H, and KBS9-R resulted in significantly lower levels of disease incidence compared to the untreated control (Fig. 3. 4B). The root systems in these instances were dense and white in colour. In contrast, root systems in the control treatment inoculated with *P. ultimum* M1256GA were blackened and dark in appearance (Fig.3.3.) with characteristic white mycelial growth of the pathogen visible on the root surface.

3. 3. 5. Root colonization of bacterial isolates

When the survival of the inoculated bacterial isolates in the rhizosphere was assessed four weeks after planting and inoculation, some isolates rendered a count equal to or greater than the initial inoculum level of 10^8 cfu/ml. Isolate KBE9-1 increased from the initial level by 1.25 log units isolate KBE6-1 by 1.00 log units and isolate NAE6-2 by 0.99 log units (Fig. 3. 5. A). In contrast, NAE7-1, one of the effective isolates, decreased by 0.55 log units (Fig. 3. 5. A).

Similar results were also observed for the South African isolates except that NAS2-D, KBS3-1, and KBS2-4 rendered a counted below that considered to be the threshold level of competency (10^5cfu/g) needed for a bacterial isolate in the rhizosphere. The count for isolate KBS2-4 for instance decreased by 4. 48 log units from the initial inoculum concentration. On the other hand, isolates KBS2-6, KBS2-12, KBS5-H, KBS9-H, and KBS9-R maintained their initial inoculum level of log 8.0 CFU/ml (Fig. 3. 5. B).

3. 3. 6. Identification of bacterial isolates

Identification of the bacterial isolates from both the rhizosphere of sorghum and grasses indicated that most of the isolates effective in suppressing *P. ultimum* in the current study were dominated by members of the Genus *Bacillus* of which *B. cereus* comprised 56 % of the total bacterial isolates (Table 3. 4). Two Gram-negative isolates KBS9-R and KBS6-17 were identified as *Serratia marcescens and Pseudomonas fluorescens* respectively.

3. 4. Discussion

The current study demonstrated that certain groups of bacteria isolated from the rhizosphere of sorghum in Ethiopia and from the rhizosphere and rhizoplane of grasses in South Africa suppressed *P. ultimum* M1256GA *in-vitro* and inhibited *Pythium* root rot of sorghum under greenhouse conditions. Paulitz and Loper (1991) and Loper and Buyer (1991) have warned against associating *in-vitro* inhibition by biocontrol agents with *in-vivo* activity.

However, in the current study most of the bacterial strains that resulted in more than 30 % *in-vitro* mycelial growth inhibition of *P. ultimum* significantly suppressed *P. ultimum* root rot in the greenhouse. These results agree with previous reports (Milus and Rothrock, 1997) that antibiosis (*in-vitro* inhibition) may be one mechanism of biological control. Conversely, isolates which were less effective in the dual culture experiment (KBE2-5, KBE6-3, KBE8-3 and KBE4-2, KBS9-N) have displayed significant disease suppression under greenhouse conditions. This result indicates that modes of actions other than antibiosis such as secretion of siderophores, and/ or induction of systemic resistance might have been used by these isolates.

In the current experiment, only four Ethiopian isolates from the sorghum rhizosphere, all of which were identified as members of the Genus *Bacillus*, rendered a disease suppression of ≥ 75 %. On the other hand isolates from the rhizosphere of grasses (South African isolates) contained, in addition to *Bacillus* species, some Gram negative isolates effective in suppressing *P. ultimum* root rot under greenhouse condition. Most of these isolates rendered a disease suppression of ≥ 80 % against *P. ultimum*. The difference in the type of bacterial species and efficacy of the isolates between the sorghum rhizosphere and the rhizosphere of grasses may be due to differences in the nature of the soil and types of plant species. In this regard, Gryston *et al.* (1998), have indicated that the abundance and activities of soil microorganisms are influenced

by, among various other factors, the types of plant species. Plants influence the types of bacteria in soil because bacteria respond differently to differences in the composition and release of root exudates (Lemanceau *et al.,* 1995; Dunfield and Germida, 2001).

Identification of the bacterial isolates by means of the API system and sequencing of the bacterial 16S rDNA indicated that about 80 % of the bacterial isolates, which rendered suppression of *P. ultimum* root rot in the current study belong to members of the Genus *Bacillus* of which 56 % are *B. cereus.* Other *Bacillus* species include *B. subtilis*, *B. pumilus* and *B. mycoides*. Similar results have been reported by other researchers that strains of *Bacillus species* are able to colonize the roots and prevent root diseases in many plants. Among the growing list of *Bacillus* species capable of successful rhizosphere colonization and root disease control (Kim *et al.,* 1997) are *B. cereus* strain UW85 (Handelsman *et al.,* 1990) for the control of damping off of alfalfa; *B. mycoides* for the control of wheat take all (Maplestone, 1989) and *B. subtilis* GBO3 for the control of damping-off of cotton (Mahaffee and Beckman, 1993).

Moreover, Handelsman and Stabb (1996) and Kloepper *et al.* (2004), have indicated that although bacilli have received less attention as potential biocontrol agents than their counterpart pseudomonads, they are involved in promoting effective disease suppression on a diversity of hosts. *Bacilli* spp. as a group offer several advantages over fluorescent Pseudomonas and other Gram negative bacteria as protectant against root pathogens because of their ability to form endospores and a broad spectrum of antibiotics (Kim *et al.,* 1997).

Isolate NAE1-7 which successfully inhibited *P. ultimum* both in the dual culture experiment and in the *in-vivo* study (Fig.2; Table 2) has been identified as *B. subtilis*. Although not a typical rhizobacterium, *Bacillus subtilis* has been reported as an antifungal agent against various plant pathogens (Marten *et al.,* 2000). Earlier, Kloepper *et al.* (1989) have categorized a strain of *B. subtilis* A13 as a plant growth promoting rhizobacterium (PGPR). Recently strains of *B. subtilis* were isolated from the rhizosphere as potential antagonistic bacteria against *P. ultimum* var. *ultimum* causing root rot of cauliflower (Abdelzaher, 2003).

Two other Gram positive isolates that resulted in significant suppression of *P. ultimum* were identified as *Brevibacterium laterosporus*. No reports were found on *B. lateroporus* as a potential biocontrol agent against *P. ultimum* causing soilborne diseases in many plants. Further investigations including modes of action studies need to be conducted to confirm these results.

Isolates KBS9-R and KBS6-17 which resulted in significant disease suppression in the current study have been identified as *Serratia marcescens* and *Pseudomonas fluorescens* respectively. Both of these bacteria have been isolated from the rhizosphere of grasses at Nylsvlei Nature Reserve. These strains were also among the best colonizers of the sorghum rhizosphere when assessed four weeks after inoculation. Although several strains of *P. fluorescens* have been reported as having biocontrol activity against several phytopathogenic fungi due to their root colonizing and antibiotic producing capacity (Raaijmakers *et al.,* 1997; Ongena *et al;* 1999; Han *et al.,* 2000; Villacieros *et al.,* 2003), they were not isolated as frequently as the *Bacillus* species from the sorghum rhizosphere in our study.

The other Gram negative isolate, KBS9-R, identified as *S. marcescens* is one of the most effective bacterial isolates both in terms of disease suppression and rhizosphere competence in the current study. In a separate study we conducted, this isolate has been observed to exhibit various modes of action such as chitinolytic activity, siderophore production and antibiosis (unpublished data). This result concurs with that of Kishore *et al.* (2005) that *S. marcescens* possesses a high level of chitinolytic activity against fungal pathogens. *S. marcescens* GPS 5 resulted in improved biological control of *Cercosperidium personatum*, the causative agent of late leaf spot in groundnut due to its chitinolytic activity (Kishore *et al.,* 2005). The presence of more than one modes of action is important for a biological control agent to be effective against several pathogens. In a previous study by Someya *et al.* (2000), *S. marcescens* strain B2 suppressed fungal diseases of cyclamen plants caused by *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *cyclaminis* in the greenhouse. In other studies, *S. marcescens* strain 90-166 has been reported as a potential biocontrol agent by inducing systemic resistance against multiple pathogens in cucumber including anthracnose (Wei *et al.,* 1991), angular leaf spot (Liu *et al.,* 1995a) and Fusarium wilt (Liu *et al.,* 1995b). In our study, *S. marcescens* strain KBS9-R also displayed consistency in repeated in-*vitro* and *in-vivo* trials indicating that this strain has the potential to be used as an antagonist against *P. ultimum* and possibly also against other phytopathogenic fungi.

A significantly positive correlation between root rot severity and leaf necrosis with the effective Ethiopian and South African isolates is indicative of the biocontrol potential by these bacterial isolates against *P. ultimum*. The high incidence of leaf necrosis for the plants treated with isolate

KBS6-17 could have resulted from nitrogen deficiency which causes yellowing of leaves in both broad leaved plants and grasses (Kucharec *et al.,* 2000).

Some of the rhizobacterial isolates that suppressed *P. ultimum* in the current study viz. KBE4-3, KBE2-5 and KBE6-3 have previously been evaluated for their ability to suppress root and crown rot of sorghum caused by *F. oxysporum* (Idris *et al.,* 2007). The results of the present study indicate that the rhizobacterial isolates have the potential to suppress two or possibly more pathogens.

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Table 3. 1. Pathogenecity test results of four isolates of *Pythium* species on sorghum under greenhouse condition

Treatment †	% incidence of *Pythium* on roots * ¶	% leaf necrosis* ‡	Root rot severity index* §
P. ultimum M1256GA	80.0a	91.6a	2.580a
P. irregulare M1255AA	73.3a	95.8a	1.120bc
P. ultimum PPRI	50.0b	62.5b	0.710bc
P. graminicola M2794	43.3b	58.3b	1.290 b
Control	20.0c	16.6c	0.120e
Pr > F	0.001	0.0004	0.0064
$LSD_{0.05}$	0.20	0.23	1.04

†*P. ultimum* M1256G was isolated from wheat, *P. ultimum* PPRI from sorghum, *P. irregulare* and *P. graminicola* both from barley and all were obtained from the Free State in South Africa.

¶ Percentage incidence of *Pythium* in roots was determined from the ratio of the number of roots rendering *P. ultimum* to the total number of root pieces plated on BNPRA agar medium.

‡ Percentage leaf necrosis was determined from the ratio of the number of plants with leaf tip/blade necrosis per treatment to the total number of plants.

§ Root rot severity was determined using a rating scale of 0-4 (0= no infection; 1= 1-25%; 2= 26-50%, 3= 51-75%; 4= 100 % root rot).

*Values are means of three replications and means within columns with the same letters are not significantly (*P=0.05*) different from each other according to the Least Significant Difference (LSD) test using the GLM procedure.

Table 3. 2. Suppression of *Pythium ultimum* root rot of sorghum by rhizobacterial isolates from Ethiopia

Bacterial Isolates	*in-vitro* mycelial [p] inhibition (%)	Root* fresh weight (g)	Root dry weight(g)	% leaf [q] necrosis	Root rot [r] severity index	% disease [s] Suppression
KBE2-5	20.13^{ef}	0.05^{bcd}	0.02^{bc}	95.83^{a}	1.90^{bc}	50.43^{cde}
KBE4-3	30.33^{cd}	0.08^{bcd}	0.03^{bc}	87.50^{a}	1.73^{bc}	54.21^{bcde}
KBE5-2	33.40^{bcd}	0.17^{bc}	0.08^{a}	95.83^{a}	2.50^{b}	35.26^{e}
KBE5-7	21.63^{ef}	0.18^{b}	0.03^{bc}	95.83^{a}	2.20^{bc}	42.98^{de}
KBE5-8	5.86^{bc}	0.02^{bcd}	0.01^{bc}	50.00^{bc}	1.83^{bc}	52.89^{bcde}
KBE6-1	35.30^{bcd}	0.08^{bcd}	0.02^{bc}	29.17^{cd}	0.86^{def}	77.56^{abc}
KBE6-2	31.63^{cd}	0.07^{bcd}	0.03^{bc}	91.67^{a}	1.86^{bc}	51.71^{bcde}
KBE6-3	18.13^{f}	0.06^{bcd}	0.02^{bc}	87.50^{a}	1.70^{bcd}	55.96^{bcde}
KBE7-6	47.36^{a}	0.09^{bcd}	0.03^{bc}	33.33^{cd}	0.86^{def}	48.21^{cde}
KBE8-3	27.50^{de}	0.08^{bcd}	0.05^{b}	75.00^{ab}	2.26^{bc}	41.36^{de}
KBE8-4	40.70^{ab}	0.01^{bcd}	0.001^{c}	37.50^{cd}	1.43^{cde}	62.98^{a-e}
KBE9-4	40.46^{ab}	0.05^{bcd}	0.02^{bc}	70.83^{ab}	0.30^{f}	50.04^{cde}
KBE9-5	32.50^{cd}	0.046^{bcd}	0.02^{bc}	91.67^{a}	1.63^{bcd}	56.80^{a-e}
KBE9-8	45.73^{a}	0.03^{bcd}	0.02^{bc}	87.50^{a}	1.80^{bc}	53.51^{bcde}
NAE1-7	33.20^{bcd}	0.360^{a}	0.02^{bc}	25.00^{cd}	0.53^{ef}	86.23^{a}
NAE2-1	19.30^{f}	0.12^{bcd}	0.03^{bc}	87.50^{a}	2.00^{bc}	48.24^{cde}
NAE3-1	14.00^{f}	0.03^{bcd}	0.02^{bc}	83.33^{a}	1.90^{bc}	50.61^{cde}
NAE6-2	30.00^{cd}	0.02^{bcd}	0.03^{bc}	100.00^{a}	2.13^{bc}	45.17^{de}
NAE7-1	40.93^{ab}	0.04^{bcd}	0.02^{bc}	20.83^{cd}	0.83^{def}	78.33^{abc}
NAE7-3	18.76^{f}	0.13^{bcd}	0.09^{a}	100.00^{a}	1.90^{bc}	50.70^{cde}
Control a[t]	0	0.01^{d}	0.001^{c}	100.00^{a}	3.80^{a}	3.33^{f}
Control b[u]	-	0.06^{bcd}	0.04^{b}	16.67^{d}	0.70^{ef}	81.84^{ab}

[p] *in-vitro* mycelial inhibition (%) = [(R – r)/ R] x 100 where, R = mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia, r = mycelial growth towards the bacteria.

[q] % leaf necrosis = [(No. of plants with yellowing leaf tip (blade) / Total no. plants)] x 100.

[r] Root rot severity was rated on a scale of 0 - 4 where, 0 = no symptom, 1 = 1- 25 %, 2 = 26 – 50 %, 3 = 51-75% and 4 = 76 – 100 % root rot.

[s] Percent diseases suppression was calculated using [(A – B) / A] x 100 where A = root rot severity index exhibited by the control treatment inoculated with the pathogen alone and, B = root rot severity index exhibited by plants treated with both the pathogen and the bacterial antagonists.

[t] Control a = treatment inoculated only with *Pythium ultimum* , [U]Control b = un-inoculated control

* Means within columns followed by the same letters do not differ significantly (*P=0.05*) according to Duncan's Multiple Range test using the GLM procedure in SAS-9.1 software.

Table 3. 3. Suppression of *Pythium ultimum* root rot of sorghum by rhizobacterial isolates from South Africa

Bacterial Isolates	*in-vitro* mycelial [p] inhibition (%)	Root* fresh weight (g)	Root dry weight (g)	% leaf [q] necrosis	Root rot [r] severity index	Disease [s] Suppression (%)
KBS1-T	47.50^{b}	0.186^{abc}	0.023^{b}	79.16^{bc}	1.83^{bcd}	40.82^{c-g}
KBS2-4	34.03^{c-f}	0.090^{bcde}	0.020^{bc}	100.00^{a}	2.23^{ab}	23.81^{efg}
KBS2-6	30.00^{fg}	0.223^{a}	0.026^{b}	62.50^{c}	1.00^{cdef}	69.14^{abcd}
KBS2-12	52.60^{ab}	0.130^{a-e}	0.019^{bc}	12.50^{d}	0.30^{f}	90.70^{a}
KBS5-H	55.80^{a}	0.186^{abc}	0.023^{b}	12.50^{d}	0.00^{f}	100.0^{a}
KBS6-3	31.66^{efg}	0.216^{a}	0.026^{b}	16.66^{d}	0.03^{f}	99.12^{a}
KBS6-11	25.63^{gh}	0.090^{b-e}	0.013^{bc}	95.83^{ab}	2.63^{ab}	15.46^{g}
KBS6-17	37.23^{cde}	0.123^{a-e}	0.013^{bc}	95.83^{ab}	0.56^{def}	83.83^{abc}
KBS9-H	55.73^{a}	0.226^{a}	0.030^{b}	29.16^{d}	0.60d^{ef}	80.40^{abc}
KFP9-K	38.66^{cd}	0.213^{a}	0.020^{bc}	12.50^{d}	0.10^{f}	96.55^{a}
KBS9-R	52.36^{ab}	0.126^{a-e}	0.030^{b}	12.50^{d}	0.43^{ef}	86.25^{ab}
KFP9-A	39.76^{c}	0.200^{ab}	0.020^{bc}	66.66^{c}	0.04^{f}	98.7^{a}
NAS2-D	34.83^{c-f}	0.116^{a-e}	0.014^{bc}	87.50^{ab}	2.20^{abc}	33.13^{defg}
NAS6-N	30.00^{fg}	0.156^{abcd}	0.030^{b}	91.66^{ab}	1.76^{bcde}	43.53^{b-g}
NAS7-L	31.20^{efg}	0.053^{de}	0.012^{bc}	100.00^{a}	1.26^{cdef}	57.63^{a-g}
Control a[t]	0.00^{i}	0.020^{e}	0.001^{c}	100.00^{a}	3.16^{a}	20.83f^{g}
Control b[u]	-	0.096^{bcde}	0.053^{a}	25.00^{d}	1.23^{cdef}	78.2^{abc}

[p] *in-vitro* mycelial inhibition (%) = [(R – r)/ R] x 100 where, R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia), r is mycelial growth towards the bacteria.

[q] Percent leaf necrosis = [(No. of plants with yellowing leaf tip (blade) / Total no. plants)] x 100.

[r] Root rot severity was rated on a scale of 0 - 4 where, 0 = no symptom, 1 = 1- 25 %, 2 = 26 – 50 %, 3 = 51 – 75% and 4 = 76 – 100 % root rot.

[s] Percent disease suppression was calculated using [(A – B) / A] x 100 where A is root rot severity index exhibited by the control treatment inoculated with the pathogen alone and, B is root rot severity index exhibited by plants treated with both the pathogen and the bacterial antagonists.

[t] Control a = treatment inoculated only with *Pythium ultimum*, [u] Control b = un-inoculated control

* Means within columns followed by the same letters do not differ significantly (*P=0.05*) according to Duncan's Multiple Range test using the GLM procedure

Table 3. 4. Identification to species level of the bacterial isolates from Ethiopia and South Africa that suppressed *P. ultimum in-vitro* and *in-vivo*

Bacterial Isolates	*Gram reaction*	*Endo-spore*[§]	*Catalase Test*	*Cytochrome oxidase*	*Motility Test*[f]	*O/F Test*[†]	*Identification used*	*Bacterial species*
KBE4-3	+	+	+	+	Motile	Nd	*16S rDNA sequencing*	*Bacillus cereus*
KBE5-8	+	+	+	+	Motile	Nd	*16S rDNA sequencing*	*B. mycoides*
KBE6-1	+	+	+	+	Motile	Nd	*API 50 CHB*	*B. pumilus*
KBE6-3	+	-	+	+	N-m	Nd	*API 50 CHB*	*B. subtilis*
KBE8-4	+	+	+	+	Motile	Nd	*API 50 CHB*	*B. cereus.*
KBS2-6	-	-	-	+	Motile	O	*16S rDNA sequencing*	*B. cereus*
KBS2-12	+	-	+	-	Motile	Nd	*API 50 CHB*	*Brevibacterium laterosporus*
KBS5-H	+	-	+	-	Motile	Nd	*16S rDNA sequencing*	*B. cereus*
KBS6-17	-	-	-	+	Motile	O	*API 20 NE*	*Pseudomonas fluorescens*
KBS9-H	+	+	+	+	N. m	Nd	*16S rDNA sequencing*	*B. cereus*
KBS9-R	-	-	+	-	Motile	O	*16S rDNA sequencing*	*Serratia marcescens*
KFP9-A	+	+	+	-	Motile	Nd	*16S rDNA sequencing*	*B. cereus*
KFP9-K	+	+	+	+	Motile	Nd	*16S rDNA sequencing*	*B. cereus*
NAE1-7	+	+	+	+	Motile	Nd	*API 50 CHB*	*B. subtilis.*
NAE7-1	+	+	+	+	Motile	Nd	*API 50 CHB*	*B. cereus*

[†]O/F, O = Oxidative metabolism, F = Fermentative metabolism, Nd = not done for Gram-positives.

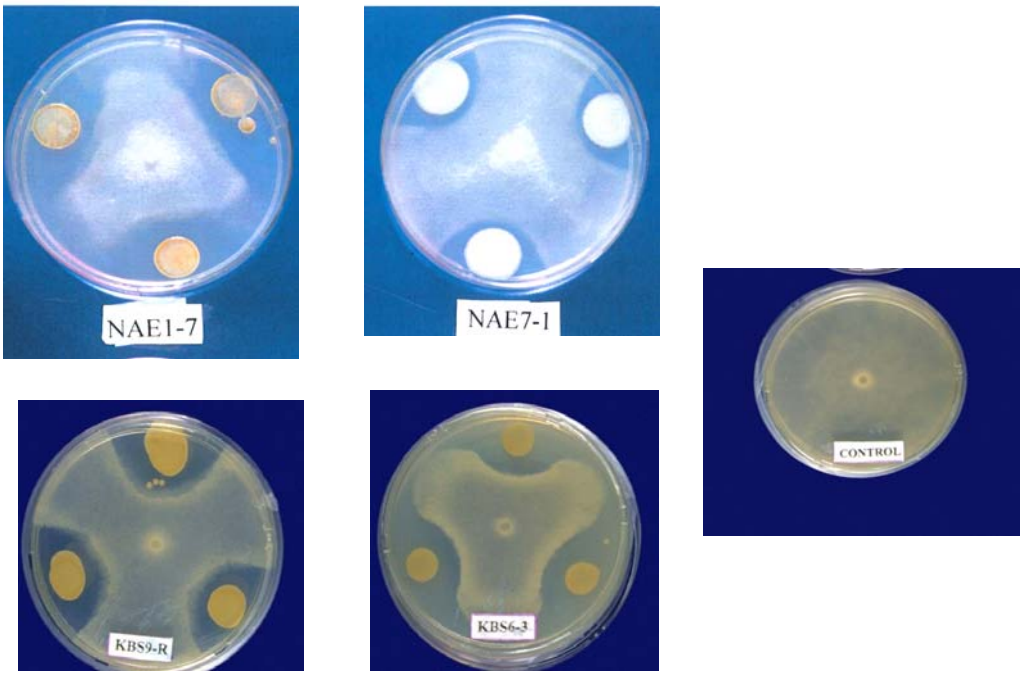


Figure 3. 1. *In-vitro* inhibition of *Pythium ultimum* by selected rhizobacterial isolates from Ethiopia (top) and South Africa (bottom). Note that control plates inoculated with sterile nutrient broth were entirely overgrown by the fungal pathogen with no inhibition zones (right).

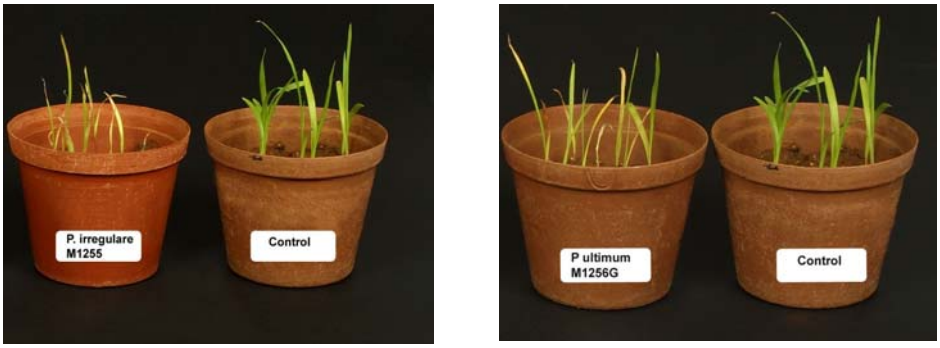


Figure 3. 2. Effect of *Pythium irregulare* M1225 and *Pythium ultimum* M1256G on sorghum seedlings two weeks after inoculation. Yellowing (necrosis of the leaf tip and blade) in the seedlings treated with the two pathogens was evident in comparison with the un-inoculated controls.

Figure 3. 3. Suppression of *Pythium ultimum* root rot in four-weeks-old sorghum by rhizobacterial isolates from the rhizosphere of grasses at Nylsvlei nature reserve in South Africa (A). Control plants that were treated only with *Pythium ultimum* developed visible root rot with necrotic leaves (B).

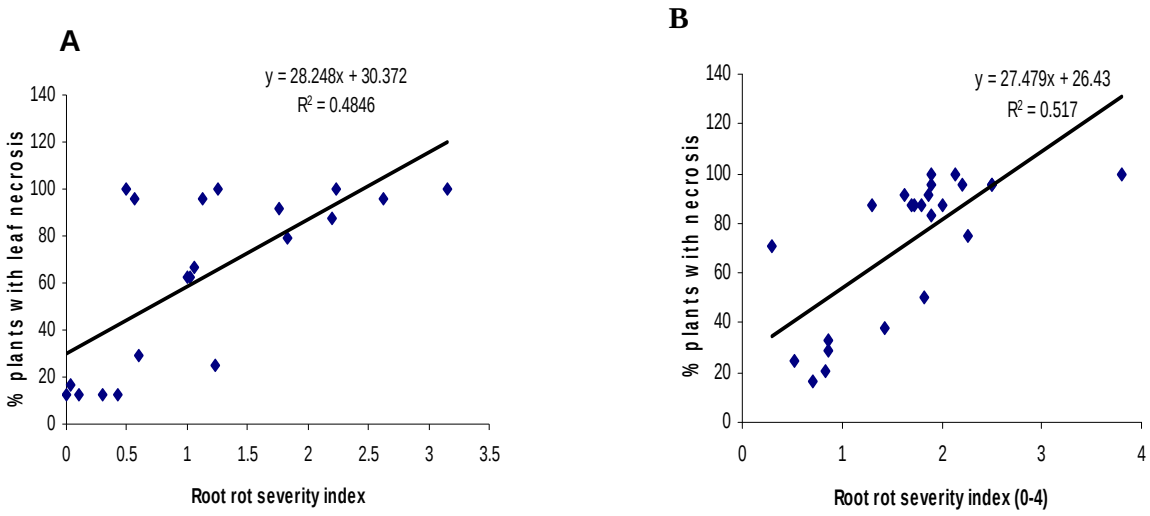
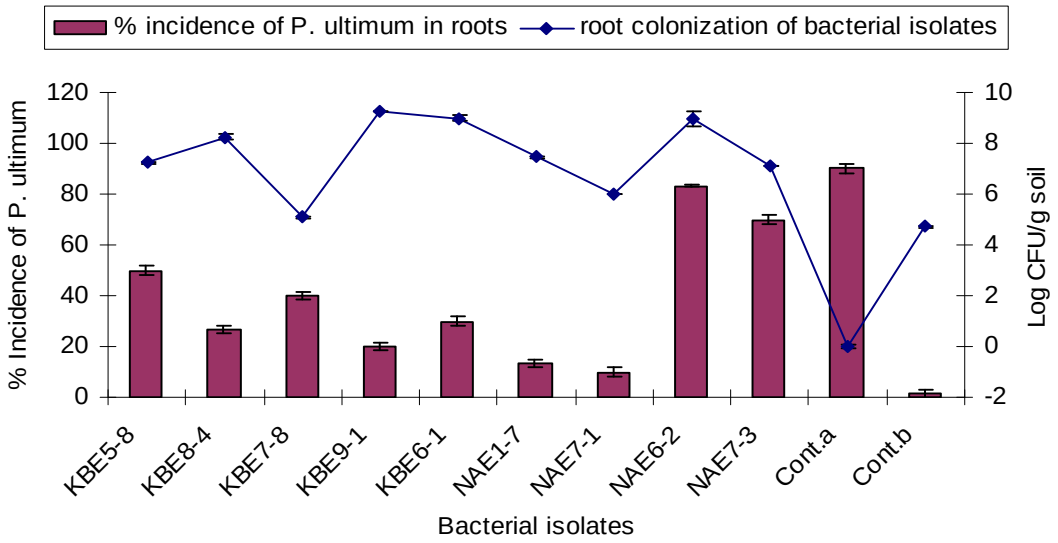


Figure 3. 4. Linear correlation coefficient representing positive relationship (*P= 0.005*) between root rot severity index and the number of plants with leaf tip and blade necrosis in sorghum seedlings after treatment with *Pythium ultimum* and rhizobacterial isolates from Ethiopia (A) and South Africa (B).

A



B

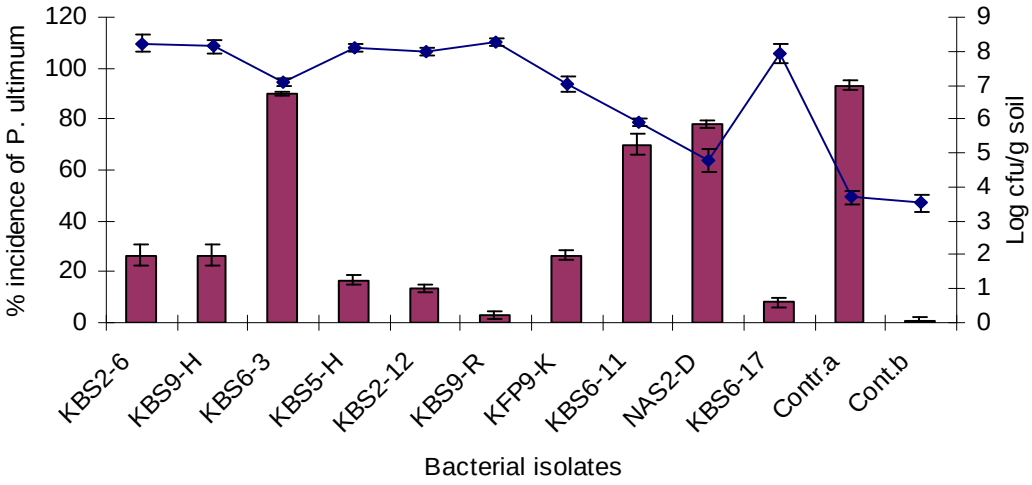


Figure 3. 5. Efficacy of selected rhizobacterial isolates from Ethiopia (A) and South Africa (B) in the biological control of *Pythium ultimum* root rot in sorghum as illustrated by incidence of the pathogen in roots (%) and the survival of bacteria in the rhizosphere (Log cfu/g soil) four weeks after inoculation. Values are means of three replications whereas bars indicate standard errors of the means.

CHAPTER 4

Study of the modes of action of rhizobacterial isolates effective in the suppression of *Fusarium oxysporum* and *Pythium ultimum*

Abstract

Rhizobacterial isolates effective in suppressing root rot of sorghum caused by *Fusarium oxysporum* and *Pythium ultimum* under greenhouse conditions were tested for selected modes of action including production of antibiotics, siderophores, chitinolytic activity and induction of systemic resistance in sorghum. The antibiotic substances produced in the culture filtrates of many of these effective bacteria resulted in strong antifungal activity against both pathogens. The antibiotics from *Bacillus cereus* (KBS5-H) and *Bacillus subtilis* (KBS6-3) resulted in the strongest antagonistic activity against *F. oxysporum* and *Pythium ultimum* respectively. The Gram-positive *Bacillus* species effective in the suppression of these pathogens fail to produce siderophores whereas the Gram-negative strains *Serratia marcescens* (KBS9-R), *Chromobacterium violaceum* (KBE9-1) and *Enterobacter sakazaki* (NAS6-B) produced prominent yellow/orange halos on CAS-agar plates demonstrating the ability of these isolates to produce siderophores under iron stressed conditions. On the other hand, degradation of chitin on chitin-agar plates was shown by isolates most of which (83%) were strains of *Bacillus cereus*. In addition to these modes of action results, the split root assay also indicated that *B. cereus* (KBS5-H), *C. violaceum* (KBE9-1) and *S. marcescens* (KBS9-R) were capable of inducing systemic resistance against *F. oxysporum* in sorghum. The successful *in-vitro* and *in-vivo* suppression of *F. oxysporum* and *P. ultimum*, the causative agents of root rot in various crops, and the presence of various modes of action provide useful information on the potential of these bacterial isolates as biocontrol agents against soilborne fungal pathogens.

4. 1. Introduction

Soilborne non-pathogenic bacteria with the ability to antagonize fungal phytopathogens and thus prevent plant diseases represent a realistic alternative to chemical fungicides (Walsh *et al.,* 2001). This is because in most cases, the use of synthetic fungicides was unsuccessful in eradicating these pathogens and also resulted in environmental hazards and chronic health problem (Bashan and Ulangathan, 2002). The search for alternatives to chemical control of pathogens has gained momentum in recent years (Emmeret, 1999; Whipps, 2001; Rangarajan *et al.,* 2003) and using

plant growth promoting rhizobacteria is an example of a valid alternative to chemicals (Cicillio *et al.,* 2002).

It is known that only those microorganisms that can grow in the rhizosphere are suitable for use as biocontrol agents against root infecting pathogens, as the rhizosphere provides the first line of defence to the roots of a plant against attack by soilborne pathogens (Singh *et al.,* 2003). Such rhizosphere bacterial isolates as *Bacillus, Pseudomonas and Serratia* have been repeatedly reported as excellent biocontrol agents of soilborne fungal diseases (Bashan and Ulangatan, 2002). Several mechanisms have been proposed to be involved in the suppression of fungal pathogens by rhizobacteria. Many species of the Genus *Bacillus,* for instance, produce various antibiotics and other antifungal metabolites (Foldes *et al*., 2000). *Pseudomonas* spp. also produce a number of antifungal metabolites and provide effective biocontrol mechanisms (Hass and Keel, 2003). Antifungal metabolites other than antibiotics include siderophores that chelate iron, making it unavailable to the pathogens (Compant *et al*., 2005), cell wall-lysing enzymes, or hydrogen cyanide (Nelson, 2004), which suppresses the growth of fungal pathogens.

Certain bacteria in the genera *Pseudomonas, Bacillus, Arthrobacter, Rodococcus, Acinetobacter* and *Corynebacterium* have been reported to produce rhamnolipid biosurfactants, non ionic surfactants that destroy zoospores of fungi (Stanghellini and Miller, 1997). Biosurfactants disrupt the plasma membrane and result in lysis of zoospores. The identification of biosurfactants by Stanghellini and Miller, (1997) has significant implications for the biological control of zoosporic plant pathogens.

In addition to direct antagonism, treatment of plants with efficient biocontrol PGPR elicits induction of systemic resistance in plants. Elicitation of this host defence is indicated by reduction in disease severity caused by the pathogens that are spatially separated from the inducing agent (Kloepper *et al*., 2004). For expression of all these biocontrol activities, the introduced microorganisms need to colonize the plant roots and should have rhizosphere competence (Whipps, 2001). Once the plant growth promoting rhizobacteria colonized the rhizosphere and the plant root system, they exert their antagonistic activity against soilborne pathogens by means of several modes of actions.

The current study was undertaken to determine the major mechanisms by which the rhizobacterial isolates in this study exerted their *in-vitro* and *in-vivo* antagonistic activities against *Fusarium oxysporum* and *Pythium ultimum* associated with root diseases of sorghum.

4. 2. Materials and Methods

4. 2. 1. Detection of antibiotic substances

All bacterial isolates which showed prominent inhibition of *F. oxysporum* in the greenhouse experiment were streaked on NA medium and incubated at 28^{o}C for 24 h. A loopful of inoculum from the overnight culture was transferred into 100 ml of minimal defined medium to initiate the production of antibiotic substances (Sadfi *et al.,* 2001). The minimal medium contained per litre: 20 g glucose; 5g glutamic acid; 1.02g $MgSO_4$; 1.0g K_2HPO_4; 0.5g KCl and 1ml of trace element solution (0.5g $MnSO_4$ H_2O; 0.16g $CUSO_4$. $5H_2O$ and 0.015g $FeSO_4$. $7H_2O$ in 100 ml of water). The pH was adjusted to 6.2 with 5N NaOH. The inoculated media was incubated for 72 h at 28 ^{o}C and 180 rpm on a rotary shaker. The bacterial suspension was then centrifuged at 12, 000 x *g* for 10min at 4^{o}C. The cell-free filtrate was used to detect the production of antibiotic substances by means of the agar well diffusion method (Tagg and McGiven, 1977).

Five ml of molten PDA medium kept at 45^{o}C was seeded with conidia of *F. oxysporum* and spread uniformly over solidified NA medium. Once the seeded layer solidified, 3 wells were made using a no. 2 cork borer. Each well was filled with 60µl of the cell-free filtrate. The control treatment received the same volume of sterile medium not inoculated with bacteria. The plates were incubated at 28 ^{o}C for 48 h and any zones of inhibition of the mycelial growth of *F. oxysporum* recorded.

4. 2. 2. Detection of siderophore production

The production of siderophores as the mode of action of the bacterial isolates against *F. oxysporum* and *P. ultimum* was assessed by the universal chemical assay using Chrome Azurol S (CAS) agar medium (Schwyn and Neilands, 1987). The CAS agar plates were used to detect the presence of siderophores in the culture supernatants of the bacterial isolates.

The CAS agar plate contains two major components: CAS indicator solution and Basal agar medium. The *CAS indicator solution* consisted of the following ingredients: 60.5 mg chrome azurol S in 50 ml ddH_2O; 10 ml Fe^{+3} (27 mg FeCl .6H2O and 83 µl conc. HCl in 100 ml ddH_2O); 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) in 40 ml ddH_2O. The HDTMA was added slowly while stirring to give a dark blue solution of 100 ml total volume. The solution was autoclaved before use. The *basal agar medium* contained: 30 g 3-(N-morpholino propane sulphonic acid (MOPS) (0.1M); 0.1g NH3Cl; 0.5g NaCl; 0.3g KH_2PO_4 all dissolved in 830 ml ddH_2O. The pH was adjusted to 6.8 using 6M NaOH. After adjusting the total volume to 880 ml using distilled water, 15g agar was added while stirring. The resulting solution was autoclaved before use.

To prepare the final CAS agar plates, the autoclaved basal agar medium and the CAS indicator solution were cooled to 50 ^oC in a water bath along with 50 % glucose solution. To the basal agar medium was added 20 ml of the 50 % glucose solution while stirring, bringing the total volume to 900 ml. To this was added 100 ml of the CAS indicator solution slowly with constant stirring. The resulting blue-green solution was poured into sterile plastic Petri dishes.

The selected bacteria were grown in modified minimal medium. This medium contained per litre: 5.03 g KH2PO4; 5.03 g L-asparagine; 5 g glucose; 40 mg MgSO4; 100 µg MnSO4 and 500 µg ZnCl. A high iron modified minimal medium was prepared by adding 5.56 mg/L $FeSO_4$ (20 µM) whereas 0.5 µM $FeSO_4$ was added to prepare iron restricted modified minimal medium. A loopful of the bacteria was inoculated into the medium and incubated at 28 ^oC and 180 rpm on a rotary shaker for 24 hr. The suspension was centrifuged at 12,000 x *g* for 5 min and the supernatant collected. Using a no.2 cork borer, wells were prepared in the CAS agar plates and 60 µl of the supernatant was applied into the wells. The plates were incubated for 8 hr at room temperature and any yellow-orange halos produced from the supernatants of the cultures grown in an iron-restricted media were recorded.

4. 2. 3. Detection of bacterial Chitinolytic activity

The production of chitinase enzymes by the bacterial isolates was assessed using the chitin agar plate method of Felse and Panda (2000). For the chitin agar plate, chitin (practical grade, Sigma) was used. Size reduction of chitin was performed by pre-treatment involving deproteinization at pH = 11 and 30 ^oC for 60 min followed by demineralization at the same temperature with 8 % HCl

for 8 hr. Through milling and sieving, a 20-60 mesh particle of chitin was obtained. The bacterial isolates were grown in an Erlenmeyer flask containing 100ml modified mineral medium (Rodriguez-Kabana, 1983). This medium contained per litre of distilled water: 7g $(NH_4)_2SO_4$; 1g K_2HPO_4; 1g NaCl; 0.1g $MgSO_4$ $7H_2O$; 0.5 g yeast extract and 0.5 % of the pre-treated chitin. The pH was adjusted to 7.65. The inoculated cultures were incubated at 27 ± 2 ^oC on a rotary shaker at 200 rpm for 3 days and centrifuged at 12, 000 x *g* for 10min. Portion of the clear supernatant (50 µl) was added onto wells made using no. 2 cork borer at the margins of chitin agar prepared from the same mineral medium described above. For the control experiment un-inoculated (50 µl) sterile mineral medium was used. The plates were incubated at 28 ^oC for 4 days and any clear zones which developed on the plates were recorded.

4. 2. 4. Induction of systemic resistance in sorghum

To detect whether the rhizobacterial isolates that inhibited *F. oxysporum* and *P. ultimum* in this study induce systemic resistance in sorghum against these pathogens, a slight modification of the split-root system (Liu *et al.,* 1995) which spatially separates the pathogens and the PGPR was used.

4. 2. 4. 1. Bacterial and fungal inoculum preparation

Rhizobacterial isolates that resulted in significant inhibition of both *F. oxysporum* and *Pythium ultimum* were selected for the induced systemic resistance experiment. Fresh, 24-h-old cultures in nutrient broth were centrifuged at 12,000 x *g* for 10min. The supernatant was discarded and the pellets were resuspended in quarter strength Ringer's solution (Merck) and the suspensions were adjusted to $\log_{10}$ 8 to 9 cfu/ml for inoculation of the roots.

For pathogen inoculum preparation, *F. oxysporum* isolate RC331 was used. The isolate was cultured on potato-dextrose-agar (PDA) (Biolab, South Africa) for seven days. Microconidia were collected by pouring sterile water on the surface of the medium stroking the agar surface with sterile glass rod. The suspension was then filtered through cheesecloth to remove mycelial fragments and the concentration was adjusted to $\log_{10}$ 5.3 conidia per ml with a haemocytometer.

4. 2. 4. 2. Inoculation of sorghum roots in the split root system

Roots of two-weeks old sorghum seedlings germinated in vermiculite were uprooted and washed with tap water. Plants with root systems which could be divided into two portions (i. e. two strong roots originating from the base of the plant) were selected and the roots divided into two portions. One half of the root was immersed in 20 ml of the spore suspension of *F. oxysporum* and the other half in 50 ml of bacterial suspension. The two halves were kept separated during and after treatments. The plants were transplanted into Styrofoam seedling trays (96 cells) in such a way that the two root portions were located in two separate (adjacent) cells. There were 11 treatments and 10 replications (one plant per treatment) in a completely randomized design and the experiment was conducted twice. The treatments included 9 rhizobacterial isolates; non-bacterized, pathogen inoculated (diseased) control (DC) and un-inoculated healthy control (HC). For the diseased control, one half of the split root system was inoculated with the pathogen and the other half was treated with sterile water. No treatment was applied to the roots in the healthy control.

Data collected included the number of dead plants one to three weeks after pathogen inoculation and bacterial treatments, and root rot rating Root rot symptoms on each plant was rated according to the scale used by Liu *et al.* (1995) as follows: 5 = plant dead, 4 = 76-100 % of the root length with symptoms, 3 = 51-75 % root length with symptoms, 2 = 26-50 % of root length with symptom, 1 = < 25 % of the root length with symptoms, 0 = no symptoms. A root rot severity index was calculated from the rating by the following formula (Liu *et al.*, 1995).

$$\text{Root rot index} = \frac{\sum (\text{rating no. x no. of plants in the rating})}{\text{Total no. plants x highest rating}} \text{ x } 100\%$$

The root rot indices data were analysed by Analysis of variance (ANOVA) and the means were compared by the least significance difference (LSD) t test using the GLM procedure (SAS Institute, 2003).

4. 3. Results

4. 3. 1. Production of antibiotic substances

During the agar diffusion assay, 75 % of the effective isolates tested positive for antibiotic substances as evidenced by the formation of inhibition zones. Antibiotic substances produced by *B. cereus* KBS5-H and *S. marcescens* KBS9-R have shown the capacity to inhibit both *F. oxysporum* and *P. ultimum*. The antibiotics produced by some isolates were found to be more inhibitory to either *F. oxysporum* or *P. ultimum* respectively. Antibiotic substances more effective against *F. oxysporum* than *P. ultimum* were produced by *B. cereus* (KBS5-H, NAS4-3, KBE5-1, NAE5-5, KFP9-A), *C. violaceum* KBE8-2, and *S. marcescens* KBS9-R with the formation of an inhibition zone ranging from 6 mm to more than 10 mm in diameter (Table 4.1). On the other hand an antibiotic more effective against *P. ultimum* was produced by *B. cereus* (KBS6-3, KBS9-H), *B. subtilis* NAE1-7, *C. violaceum* KBE9-1 and *B. laterosporus* KBS2-12 (Table 4.1).

4. 3. 2. Production of siderophores

Six rhizobacterial isolates have been found to produce a yellow/orange halo on CAS agar plates as a result of siderophore production which sequesters iron from the medium. The isolates which produced the most siderophores (based on the diameter of the yellow halo) were found to be *P. fluorescens* KBS6-17, *S. marcescens* KBS9-R and *B. cereus* KBE9-1 each of which resulted in a yellow/orange halo with ≥ 5 mm in diameter. The halo formation was more evident in the iron restricted medium than in the medium rich in iron (Fig. 4. 1.).

4. 3. 3. Chitinolytic activity

Out of the 25 isolates tested for the production of chitinase enzyme, 12 (48 %) produced clear zones on chitin agar medium as a result of degradation of chitin. The majority of the isolates (83%) which showed chitinolytic activity were *B. cereus* isolates producing a clear zone ranging from as little as 1mm to more than 6mm in diameter. Among the Gram-negatives, only *Chromobacterium violaceum* KBE8-2 and *Serratia marcescens* KBS6-H tested positive. The highest chitinolytic activity was rendered by *C. violaceum* KBE8-2, *B. cereus* KBE8-3 and *B. cereus* KFP9-K. (Table 4.1; Fig. 4.1).

4. 3. 4. Induction of systemic resistance

During the split-root experiments, induction of systemic resistance was demonstrated in sorghum by rhizobacterial isolates *C. violaceum* (KBE9-1), *B. cereus* (KBS5-H) and *S. marcescence* (KBS9-R). The average number of dead plants in these treatments in two experiments was 1, 0.5, and 1 respectively compared to the non-bacterized, pathogen inoculated control where the mean number of dead plants was 7 (Table 4. 2.). While the average root rot index was 0.82 in the diseased control, this was significantly reduced to 0.26 (KBS9-R and KBE9-1), and 0.27(KBS5-H). Root rot indices similar to the non-bacterized diseased control were recorded with isolates KFP9-K and KBE5-7 in which no sign of induction of systemic resistance was observed based on the number of dead plants. When pieces of root segments were plated on PDA supplemented with antibiotics, *F. oxysporum* RC331 was isolated from the samples from the non-bacterized diseased control as well as the other treatments with high root rot indices (Table 4. 2).

4. 4. Discussion

Twenty five rhizobacterial isolates (from Ethiopia and South Africa) with antagonistic activity against *F. oxysporum* and/or *P. ultimum* (Chapters 2 and 3) have been tested for selected modes of action commonly exhibited by most biocontrol PGPR. The major mechanisms by which most PGPR exert their antagonistic effect against fungal pathogens include antibiosis, competition, parasitism and induction of systemic resistance (Sadfi *et al.*, 2001; Liu *et al.*, 1995). We have, in the current study, detected the existence of several modes of action from the tested rhizobacterial isolates from the rhizosphere of sorghum and from the rhizosphere and rhizoplane of grasses.

One of the effective means of control of soilborne pathogens by biocontrol organisms in a natural ecosystem is by means of production of antibiotics (Glick and Bashan, 1997; Raaijmakers and Weller, 1998). Culture filtrates of *B. cereus* NAE5-5, *B. cereus* KBE5-1 and *C. violaceum* KBE8-2 from Ethiopia showed strong antibiotic activity against *F. oxysporum* with prominent inhibition zones in the agar well diffusion assay. Similarly culture filtrates of South African isolates *S. marcescens* KBS9-R, and *B. cereus* strains KBS5-H, KFP9-A, NAS4-3 also showed strong antibiotic activity against *F. oxysporum*. These results correlate with the *in-vitro* antagonistic activity of the isolates against *F. oxysporum* in dual culture assays for all isolates. Suppression *of F. oxysporum* by these bacterial isolates in the *in-vitro* assay and the formation of inhibition zones

were presumably due to the antibiotics being released into the culture medium. Antibiotic substances effective against *P. ultimum* were produced in the culture filtrates of *S. marcescens* KBS9-R, *B. laterosporus* KBS2-12, *C. violaceum* KBE9-1, *B. cereus* KBS9-H, and *B. subtilis* strains KBS6-3 and NAE1-7. The occurrence of this varied group of antibiotic producing bacteria in the rhizosphere of sorghum and grasses is anticipated due to the fact that antibiotic-producing bacteria are common constituents of the indigenous microflora in soil and plant-associated environments (Raaijmakers *et al.,* 2002).

The antibiotics produced *in-vitro* were generally assumed to be the compounds responsible for biocontrol *in-vivo* (Leifert *et al.,* 1995). In addition, there are numerous reports of the production of antibiotics by bacteria *in-vitro* that may also have activity *in-vivo* (Whipps, 2001). The *Bacilli* species comprising mainly of *B. cereus* and *B. subtilis* in the current study have displayed the potential for antibiotic production as well as biocontrol activity under *in-vivo* conditions. In other studies for instance, an antibiotic producing *B. cereus* UW85 suppressed alfalfa damping off consistently (Handelaman *et al.,* 1990), and Phytophtora damping off and root rot of soybean under diverse field conditions (Osburn *et al.,* 1995). It has also been reported that there existed a significant quantitative relationship between disease suppressiveness and antibiotic productions by this strain (Handelsman and Stabb, 1996).

Of all the isolates tested for antibiotic production, only *S. marcescens* (KBS9-R) produced antibiotics in the culture filtrates capable of inhibiting both *F. oxysporum* and *P. ultimum.* A strain of *S. marcescens* has previously been reported to produce antibiotics and has once proven to be a useful biocontrol agent against *Sclerotium rolfsi* and *F. oxysporum* (Someya *et al.,* 2000; Someya *et al.,* 2002). There is thus a possibility that *S. marcescens* (KBS9-R) isolated in our study can be used as biocontrol agent against several soilborne plant pathogens. Although we did not do a qualitative and quantitative analysis of antibiotic production by the effective isolates in this study, there is an observable relationship between *in-vitro* antibiotic production and *in-vivo* disease suppression by most of these isolates.

Several *Bacillus* spp. are reported to have the ability of producing enzymes that degrade chitin, an insoluble linear polymer of β-1-4-N-acetylglucoseamine, the major component of most fungal cell walls (Sadfi *et al.,* 2001). In this study, seven isolates of *Bacillus* spp. have shown the ability to degrade chitin on chitin agar medium with the formation of a clearing zone, the most degrading isolates being *B. cereus* (KBE8-3) and *B. cereus* (KFP9-K). *B. cereus* (KBE8-3) inhibited both *F.*

oxysporum and *P. ultimum in-vivo*. This isolate tested negative for both the production of siderophores and antibiotic substances. The *in-vivo* inhibition of *P. ultimum* could have resulted from modes of action other than chitinolytic activity as these oomycetes do not possess chitin in their cell walls. Although Chang *et al.* (2003), reported inhibition of hyphal extension of *P. ultimum* by purified chitinase from *B. cereus*, the role of chitinase in the inhibition of oomycetes with cellulose-glucan cell wall is not clear. Of the Gram-negative isolates, *C. violaceum* (KBE8-2) showed strong chitinolytic activity which, together with its potential to produce antibiotic substances, suppressed *F. oxysporum* under greenhouse conditions. The existence of a number of chitinolytic enzymes has been described in *C. violaceum* ATTC 3152 (Chernin *et al.,* 1998) and these chitinolytic enzymes have been shown to be involved in the biological control of *F. oxysporum* (Park *et al.,* 1995).

Although strains of *S. marcescens* have been reported to posses a highly active chitinolytic system (Kishore *et al.,* 2005) and their chitinase enzymes have been found to be highly antifungal (Ordentlich, 1988), both of our most effective Serratia strains i. e. *S. marcescens* (KBS9-R) and *S. marcescens* (KBS6-H) tested negative or showed very little chitinolytic activity. Culture filtrates from these two strains also tested negative for antibiotic production against *F. oxysporum* and *P. ultimum*. The antagonistic activities of *S. marcescens* strains KBS9-R and KBS6-H against *F. oxysporum* and *P. ultimum* in the *in-vitro* and *in-vivo* experiments could be due to either their ability to produce siderophores or production of other hydrolytic enzymes not examined in this study.

Seven isolates have been shown in the current study to have the ability to produce siderophores on CAS agar medium. PGPR prevent the proliferation of phytopathogenic fungi through their ability to produce siderophores for sequestering iron. These siderophores bind to Fe^{+3} that is available in the rhizosphere and effectively prevent the growth of pathogens in that region (Kumar *et al.,* 2002). Siderophore mediated iron acquisition is very important because by establishing a critical competition for iron in the rhizosphere, it creates iron starvation conditions for phytopathogens and effectively prevent any fungal pathogens in the immediate vicinity from proliferating because of a lack of iron (O'Sulivan and O'Gara, 1992).

Based on the formation of yellow-orange halos around the colonies on CAS agar plates, the isolates which produced siderophores in this study were *S. marcescens* (KBS9-R), *Enterobacter sakazaki* (NAS6-B), *C. violaceum* (KBE9-1), *P. fluorescens* (KBS6-17), *C. luteola* (KBS5-F) and

B. circulans (NAE5-7). In each case, larger halos were formed in the plates with iron restricted conditions and the production of siderophore was reduced in the plates with high iron conditions. In one greenhouse experiment for example (Kloepper *et al.,* 1980), siderophore producing *Pseudomonas putida* strain B10 suppressed *Fusarium* wilt and take-all, but this suppression was lost when the soil was amended with iron, which consequently suppressed siderophore production in this strain.

With the exception of *S. marcescens* (KBS9-R), the other six isolates which rendered between 50 – 100 % disease suppression against *F. oxysporum* had no chitinolytic activity and very little or no antibiotic activity against *F. oxysporum*. Therefore the inhibitory activity of these isolates against *F. oxysporum* is presumably due to their siderophore producing ability. Since these strains have a high rhizosphere competence (chapters 2 and 3) there might be an intense competition for iron (Fe^{+3}) at the rhizoplane when the rhizobacteria produce siderophores. Therefore iron is bound in such a way that it is unavailable to *F. oxysporum* (Bora *et al.,* 2004). These competitions for iron may contribute to the inhibition of *F. oxysporum* as iron is necessary for germ tube elongation of microconidia of *F. oxysporum* (Scher and Baker, 1982). The role of siderophores in the suppression of various phytopathogenic fungi including *Fusarium* and *Pythium* has been extensively reported (Kloepper *et al.,* 1980; Kloepper and Schroth, 1981; Elad and Baker, 1985; Becker and Cook, 1988; Lopper, 1988).

Three of the most effective isolates in this study (KBE9-1, KBS9-R and KBS5-H) which previously tested positive for one or more of the modes of action including the production of antibiotics, siderophores or chitinolytic activity also induced systemic resistance in sorghum against *F. oxysporum* RC331 in the split root system. All three isolates rendered the lowest number of dead plants, whilst the root rot index in the three treatments was significantly lower than the non-bacterized diseased control. Such a reduction in the disease development could be due to delayed movement of the pathogen within PGPR plants (Liu *et al.,* 1995). The actual mechanism by which ISR was initiated in the sorghum seedlings by the selected isolates has not been examined in this study. However, such defence chemicals as pathogenesis related proteins including β-1, 3 glucanase and endochitinases (Maurhofer *et al.,* 1994), synthesis of phytoalexins and other metabolites (Zdor and Anderson, 1992) have been reported to be associated with ISR by PGPR.

In order for a PGPR to induce resistance by these mechanisms, sufficient colonization of the root system must be achieved by the bacterial isolates (Zehnder *et al.,* 2001). The three isolates in this study showed good root colonization capacity in repeated trials throughout the study. It is likely that, although not tested in this study, the bacterial isolates that induced resistance against *F. oxysporum* can do the same against *P. ultimum* and possibly other soilborne pathogens, because once ISR is expressed by a single inducing agent, a wide spectrum of pathogens can be controlled (Wei *et al.,* 1996).

In the split root system in this study, it was also found that the part of the root system treated only with the bacterial suspension developed many and longer roots compared to the other half of the root system treated with the pathogen alone. This enhanced survival of the plants treated with isolates such as KBE5-1 and KBS9-B is not simply a function of enhanced root growth than disease control. The root rot severity in the pathogen inoculated control which received the same level of *F. oxysporum* as the rest of the treatments was significantly higher than the treatments which received both the pathogen and the bacterial isolates. Once suppression of root rot infection is achieved, the bacterial isolates might have exerted their growth promoting activity resulting in enhanced root growth. This observation corresponds with our data on the growth promoting ability of the rhizobacterial isolates (chapter 5).

4. 5. References

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Table 4. 1. Specific modes of action exhibited by the most effective rhizobacterial isolates from Ethiopia and South Africa against *Fusarium oxysporum* and *Pythium ultimum*

Bacterial isolates	Antibiosis (Inhibition zone in mm) [a]		Siderophore production (halo zone) [b]		Chitinolytic activity [c] (Clear zone in mm)	Disease suppression [d]	
	F. oxysporum	*P. ultimum*	Low iron	High iron		*F. oxysporum*	*P. ultimum*
B. stearothermophilus KBE5-7	+	-	-	-	++	++++	+
B. laterosporus KBS2-12	-	+++	-	-	-	++	++++
C. violaceum KBE8-2	++ +	-	-	-	+++	++	-
S. maltophila KBS9-B	+ +	-	-	-	-	++++	-
S. marcescens KBS9-R	+ + +	+++	+++	++	-	++	++++
S. marcescens KBS6-H	-	-	++	+	+	++	-
Enterobacter Sakazaki NAS6-B	-	-	+++	+	-	++++	-
B. cereus KBS5-H	+ + + +	-	-	-	+	++	-
P. fluorescens KBS6-17	-	++	++	+	-	-	+++
B. subtilis NAE1-7	+	+++	-	-	-	++	++++
B. cereus NAE7-1	+	++	-	-	++	+	+++
B. circulans NAE5-7	+	-	++	+	-	++++	-
C. luteola KBS5-F	+	-	++	+	-	++++	-
B. cereus NAS4-3	+ + +	-	-	-	-	++	-
B. cereus KBE8-3	+ +	-	-	-	+++	+++	+
B. cereus KBE5-1	+ + +	-	-	-	++	++++	-
C. violaceum KBE9-1	+	+++	+++	++	-	+++	++
B. cereus KFP9-K	++	-	-	-	+++	-	++++
B. cereus KBE7-8	+	-	-	-	++	++	-

B. cereus NAE5-5	+ + +	-	-	-	-	++++	-
B. cereus KFP9-A	+ + +	-	-	-	++	++++	++++
B. cereus KBE4-3	-	++	-	-	+	++++	+
B. cereus KBS2-6	-	++	-	-	+	++	++
B. cereus KBS9-H	-	+ + +	-	-	-	++++	+++
B. subtilis KBS6-3	-	+ + + +	-	-	-	-	++++
Control	-	-	-	-	-	-	-

[a] Inhibition zone due to the presence of antibiotic substances in the culture filtrate: - = no inhibition, + = 1-3mm, + + = 4 – 5 mm, + + + = 6 -9 mm, + + + + = ≥ 10mm

[b] The diameter of yellow/orange halo on CAS agar plates as a result of siderophores sequestering iron from the medium : - = no halo formation, + = halo diameter ≤ 3mm, ++ = 4 – 5 mm, +++ = > 5mm.

[c] Chitinolytic activity was determined by measuring the diameter of the clearing zone on chitin agar: - = no clear zone, + = clear zone diameter 1 – 3mm, ++ = 4 – 5 mm, +++ = ≥ 6mm.

[d] Disease suppression (%) against *F. oxysporum* and *P. ultimum* under greenhouse conditions (Data adopted from chapter 2, Table 2.4.and Chapter 3, Table 3.3.) - = no disease suppression, + = < 50 %, ++ = 50 – 75 %, +++ = 75 – 85 %, ++++ = 85 – 100 % disease suppression in the i*n-vivo* experiments.

Table 4. 2. Evaluation of selected rhizobacterial isolates for induction of systemic resistance in sorghum against *Fusarium oxysporum* RC331 in a split root system under greenhouse condition

Treatment†	No. of dead plants			Presence or absence of *F. oxysporum*‡	Root rot index *
	Exp. 1	Exp. 2	Mean		
KBE9-1 + Foc	1	0	1	+	0.26^{e}
KBE5-7 + Foc	5	4	4.5	++	0.71^{ab}
KBE5-1+ Foc	2	2	2	++	0.58^{cd}
KBS9-B + Foc	4	2	3	++	0.51^{d}
KFP9-A + Foc	2	3	2.5	++	0.61^{bcd}
KBS9-R + Foc	1	1	1	+	0.26^{e}
KBS9-H + Foc	2	2	2	++	0.66^{bc}
KBS5-H + Foc	0	1	0.5	+	0.27^{e}
KFP9-K + Foc	3	4	3.5	++	0.80^{a}
Disease control	6	8	7	++	0.82^{a}
No PGPR, no Foc	0	0	0	-	0.10^{f}

† There were ten replicates per treatment (i. e. a single plant representing one replicate)

‡ Ten pieces of root segments from each treatment were plated on antibiotic containing PDA agar to check the presence or absence of *F. oxysporum*. + = ≤ 4 root pieces rendering *F. oxysporum* growth, ++ = ≥ 5 root pieces rendering *F. oxysporum* growth, - = no *Foc* growth observed.

* Means with the same letters are not significantly different at $P = 0.05$, $LSD_{0.05} = 0.12$

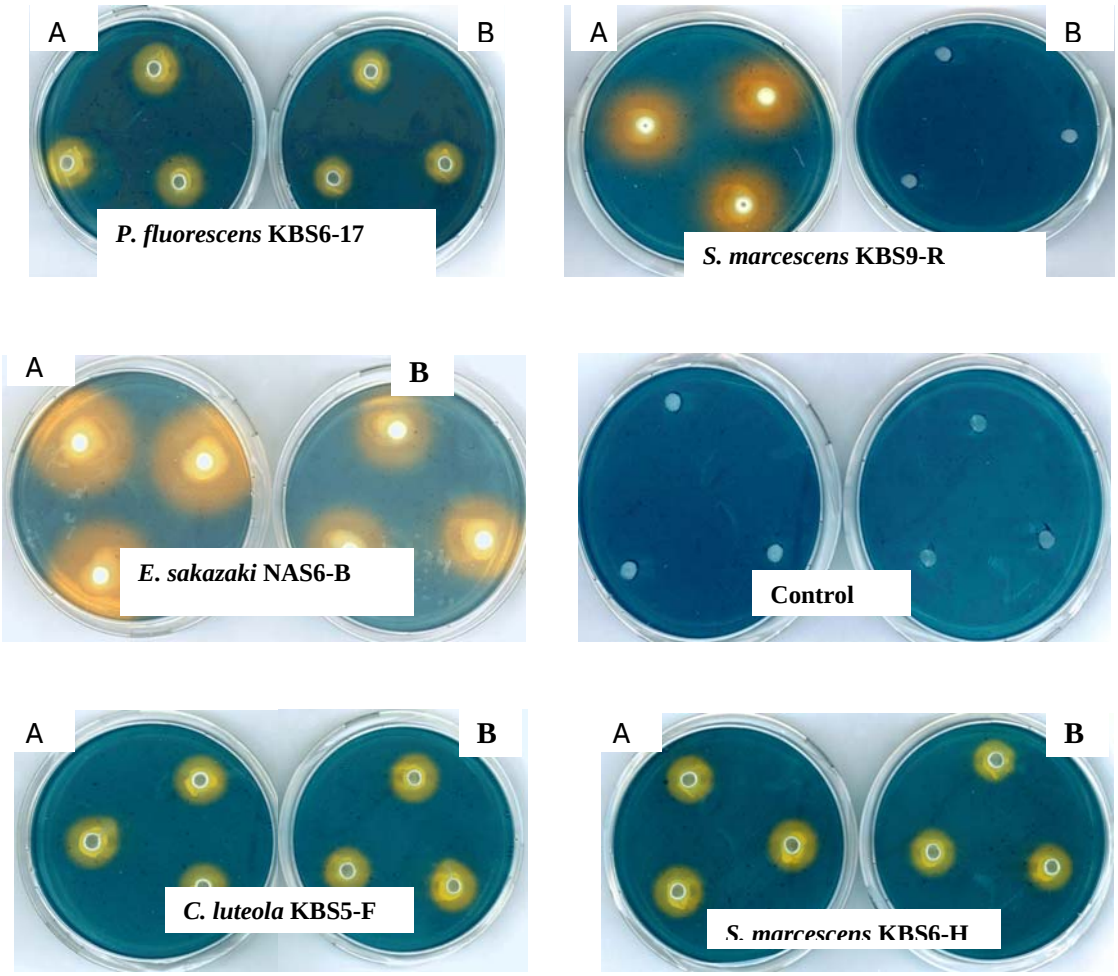

Figure 4. 1. Assay for the production of siderophores in culture supernatants of some of the most effective rhizobacterial isolates under iron restricted (A) and high iron (B) conditions on CAS agar plates. The production of siderophores is confirmed by a change in the colour of the CAS- agar medium from blue to orange/yellow as a result of the siderophores sequestering and binding iron from the medium. Note that in each case the yellow/orange halo produced was larger in size in the iron restricted medium (A) than in the medium with high iron concentration (B). Control plates which received sterile culture medium without bacterial inoculum rendered no halo in the CAS agar plate.

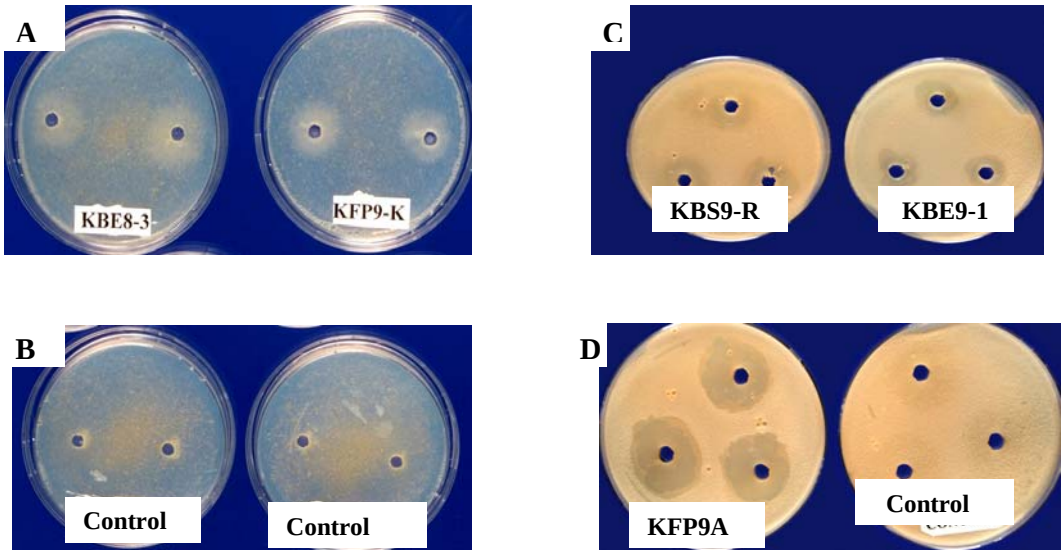

Figure 4. 2. A & B: Chitinolytic activity by bacterial isolates on chitin agar plate as evidenced by the formation of clearing zones. C & D: Inhibition of *F. oxysporum* by antibiotic substances from culture filtrates of bacterial isolates in the agar well diffusion assay.

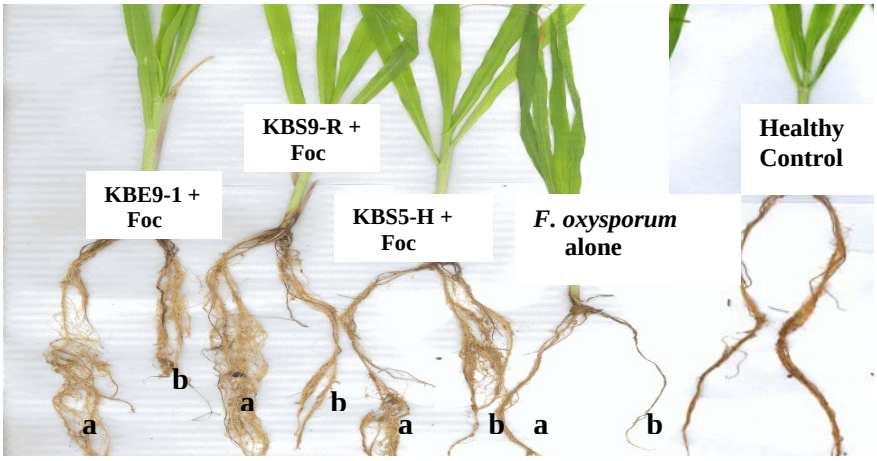

Figure 4. 3. Induction of systemic resistance in sorghum by rhizobacterial isolates KBE9-1, KBS9-R and KBS5-H in the split root experiment in which half of the root was treated with bacterial isolates (a) and the other half with *Fusarium oxysporum* RC331 (b). Half of the root system in the diseased control was treated with sterile water (a) and the other half with *F. oxysporum* only (b). The healthy control was treated only with sterile water

CHAPTER- 5

Growth promotion in sorghum (*Sorghum bicolor* (L.) Moench) by rhizobacterial isolates from the rhizosphere of sorghum and grasses in Ethiopia and South Africa

Abstract

Mineral fertilizers have long been used as the quickest way of improving crop productivity. However, due to their cost and associated environmental problems, continues use of fertilizers has resulted in a search for alternative approaches such as the use of plant growth promoting rhizobacteria. In most developing countries including Ethiopia and South Africa, the application of this approach in agriculture is scanty. This paper presents the isolation and screening of rhizobacteria for growth promotion of sorghum under greenhouse conditions. A total of 78 bacteria isolated from the rhizosphere of sorghum in Ethiopia and 86 isolates from the rhizosphere and rhizoplane of grasses in South Africa were screened for their growth promoting ability in sorghum. Statistically significant growth promotion was achieved with three isolates from Ethiopia and ten isolates from South Africa.. The effective isolates colonized the sorghum roots at a concentration of $\geq 10^{5}$ cfu g^{-1} root and were further tested (*in-vitro*) for the major/direct modes of action for plant growth promotion. Six isolates changed the colour of CAS-agar medium from blue to yellow/orange due to their ability to produce siderophores. The phytohormone indoleacetic acid was detected in the culture filtrate of 11 isolates with concentrations ranging between 4.2 µg – 22.8µg in the presence of trypthophan. Thirteen effective isolates solubilized tricalcium phosphate on Pikovskaya (PVK) agar medium which was evident from the formation of clear zones of varying diameter. Of the effective isolates identified by means of the API and / or sequencing of the bacterial 16S rDNA genes, 44 % were *Bacillus cereus*, 19% *Chrysoemonas luteola*, 13% *Serratia marcescens,* 13% *Sphingomonas paucimobilis* and 6% each of *Stenotrophomonas maltophila* and *Brevibacterium laterosporus*

5. 1. Introduction

Sorghum (*Sorghum bicolour* (L.) Moench) ranks fifth among the World's major cereals following wheat, maize, rice and barley (House, 1995; FAO, 1999). It is one of the dry land summer cereals and is a staple crop in arid and semi-arid areas in Ethiopia. It is also a staple food for more than 500

million people in more than 30 countries, although maize has to some extent replaced its use in Southern Africa. In countries like Ethiopia where traditional agriculture predominates, the average yield is very low, ranging between 200 to 1500 kg/ha compared to developed countries such as the USA where commercial production yielded between 3775-4400 kg/ha in the 1980's (House, 1995). Diseases caused by fungal pathogens (Huluka and Esele, 1992; Davis and Bockus 2001) and unavailability of essential nutrients such as phosphorous and iron (Rodriguez and Fraga, 1999; Igual *et al.,* 2001) are among some of the major causes for the low sorghum yield in these developing countries.

Bacteria are constantly involved in interactions with plant roots. By benefiting from the nutrients secreted by plant roots within the rhizosphere, the bacteria influence the plants in a direct or indirect way and result in stimulation of plant growth (Bloemberg and Lugtenberg, 2001). Bacteria inhabiting the rhizosphere and positively influencing plant growth are referred to as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper *et al.,* 1986). Significant yield increases have been achieved in crops such as maize, rice, potato, wheat and canola after inoculation with PGPR (Khalid *et al.,* 1997; Zahir *et al.,*. 1998; Bertrand *et al.,* 2001; Thakuria *et al*.2004) which resulted in increased interest in this group of organisms (Asghar *et al.,*. 2004; Thakuria *et al.,*. 2004).

Data on the application of PGPR as growth promoting agents ultimately resulting in yield increases in sorghum is lacking in Ethiopia and South Africa. Although there is a growing interest in replacing chemical fertilizers and pesticides with bacterial inoculants (Mayak *et al.,* 2001), little effort has been made in terms of laboratory and field studies concerning the potential role of PGPR as plant growth promoting agents. This study is therefore aimed at isolating bacteria from the rhizosphere of sorghum and other grasses in Ethiopia and South Africa and evaluating them for growth promotion of sorghum under greenhouse conditions. It is anticipated that the study will provide important information toward application of PGPR as inoculants in agriculture.

5. 2. Materials and Methods

5. 2. 1. Soil sample collection and Isolation of bacteria

Soil sample collection and isolation of bacteria followed the same procedure as described in Chapters 2 and 3.

5. 2. 2. Bacterial inoculum preparation

Bacterial isolates were grown in nutrient broth (Biolab, South Africa) on a rotary shaker (Labotech) at 28 ^{o}C for 24 hrs. The suspension was centrifuged in 50 ml capacity sterile plastic tubes at 3000 x *g* for 10 min using an Avanti TM J-25 centrifuge (Beckman, Ireland). The pellets were re-suspended in quarter strength sterile Ringer's (Merck, South Africa) solution and the suspension was adjusted to give a final concentration of 10^{8}-10^{9}cfu/ml (OD = 0.1- 0.5) at 550 nm using the viable plate count method and optical density measurement.

5. 2. 3. Evaluation of bacterial isolates for growth promotion

Prior to the greenhouse pot experiment, a preliminary screening of 78 bacterial isolates from Ethiopia and 86 isolates from South Africa was conducted for early root and shoot development in sorghum in 96 cell styrofoam seedling trays. The trays were filled with steam-pasteurized sandy loam topsoil. Sorghum seeds (Ethiopian variety, Meko) were surface sterilized with 70% ethanol for 5 min, 1% sodium hypochlorite for 1 min and rinsed five times in sterile water. Three cells in each tray received four sorghum seeds and constituted one replicate and three replications were used in a randomized block design in the tray. The bacterial inoculum (10^{8} – 10^{9} cfu/ml) was applied in the form of soil drenching twice a week apart. The temperature of the greenhouse was maintained at 28 ^{o}C and watering was done twice daily. The plants were harvested three weeks after the first inoculation. Shoot and root length as well as fresh and dry weight measurements were compared with the un-inoculated control (data not shown).

Plate 2. Experiment on 96 Styrofoam seedling trays for the preliminary screening of bacterial isolates for growth promotion of sorghum in the greenhouse.

Based on the results from the screening experiment in the seedling tray, a total of 20 Ethiopian and 39 South African isolates were selected for the greenhouse pot trial. For the pot trial, eight surface sterilized seeds were sown in a 12cm x 10 cm diameter pot containing steam-pasteurized topsoil. The seeds were watered regularly until the emergence of the first shoot. Five days later, 30ml of the bacterial inoculum (10^8-10^9 cfu/ml) was applied to the pots as a soil drench and a second application was made one week later. The pots were watered twice daily with an automatic watering system. The experiment was arranged in a randomized block design with three replications and was repeated twice. Plants were harvested 35 days after planting and 30 days after inoculation. Growth promoting ability of the bacterial isolates was determined based on the data collected on shoot height, shoot dry and fresh weight, root length and root dry weight, leaf width and leaf chlorophyll content as measured with a Minolta SPAD 502 chlorophyll meter (Minolta, Japan) and expressed in spad units.

5. 2. 4. Root colonization

Root colonization by the bacterial isolates was assessed according to the procedure described by Han *et al.* (2000). Briefly, surface sterilized roots of two plants per pot in each replication were macerated in 3 ml 0.1M Phosphate buffer (pH = 7) using a homogenizer. The suspension was serially diluted in quarter strength Ringer's solution (Merck, South Africa). Aliquots of 0.1ml were plated on Nutrient agar (Biolab, South Africa) amended with 50 µg rifampicin per ml. The plates were incubated at 28 ± 2 ^{o}C for 24h. The resulting colonies were counted and root colonization by each bacterial isolate was expressed as CFU/g root.

5. 2. 5. Modes of action for growth promotion

5. 2. 5. 1. Siderophore production

Siderophore production was detected by the universal chemical assay using chrome-azurol S (CAS) agar (Schwyn and Neilands, 1987). Cultures of selected isolates that resulted in significant growth promotion in the greenhouse pot experiment were grown in a modified minimal medium (under iron restricted and high iron condition) at 27 ± 1 ^{o}C for 48 hr and at 180 rpm on a rotary shaker. Each culture was centrifuged at 12,000 x *g* for 10 min and the supernatant was collected. Three wells were made equidistantly on the CAS agar plate using # 1 cork borer after which 30 µl of the culture supernatant was deposited into the wells. Control plates received sterile broth media without bacteria

under both low and high iron conditions. The plates were incubated at room temperature for 8 hrs after which any colour change in the medium was recorded.

5. 2. 5. 2. Indole acetic acid production

The production of the hormone indoleacetic acid (IAA) was detected from the culture supernatants of the bacterial isolates selected based on their growth promoting efficiency following the procedure described by Thakuria *et al.* (2004). Briefly, pure colonies from a 24 hr. culture were inoculated into nutrient broth with 2 % trypthophan and in the absence of trypthophan, and were incubated at 28 ± 2 ^{o}C for 48 hrs. Five ml culture was removed from each tube and centrifuged at 12,000 x *g* for 15 min. Two millilitre aliquot of the supernatant was transferred to a fresh tube and washed with ethyl acetate to extract free IAA like substance. This was then treated with 4 ml salkowsky reagent (1ml 0.5 M FeCl in 50ml $HClO_4$) and incubated at room temperature for 25 min. The absorbance of the solution (pink colour developed) was read at 530 nm. For the control experiment sterile nutrient broth was used. The concentration of IAA in the culture supernatants was determined using a calibration curve of pure IAA as a standard.

5. 2. 5. 3. Phosphate solubilization

Phosphate solubilization activity of the selected rhizobacterial isolates was detected by means of a plate assay using Pikovskaya (PVK) agar (Pikovskaya, 1948) which results in a clear halo formation. To compare the reproducibility of the halo formation, isolates were also tested on PVK agar supplemented with 0.1 % bromophenol blue (Gupta *et al.,* 1994) and a modified PVK medium devoid of yeast extract i.e. NBRIY medium (Nautiyal, 1999). A pure colony from a fresh culture of each isolate was stab inoculated in duplicate into each of the three-agar media using a sterile needle. The diameter of the resulting clear halo was measured after 14 days of incubation of plates at 28 ^{o}C. Control plates were inoculated with sterile nutrient broth.

5. 2. 6. Identification of bacterial isolates

Identification of the bacterial isolates effective in the growth promotion of sorghum in the current study was conducted by means of the API system and sequencing of the bacterial 16S rDNA genes as described in chapter 2 section 2. 2. 7.

5. 2. 7. Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SAS-9.1 software (SAS Institute, 2003). Data on root colonization of the bacterial isolates were log transformed before subjecting to ANOVA. Mean values in each treatment were compared by the least significant difference (LSD) and Duncan's Multiple Range tests at 5 % ($P = 0.05$) level of significance. Pearson's linear correlation coefficient was used to evaluate the relationship between phosphate solubilization and root/ shoot dry weight as well as the relationship between IAA concentration and root/shoot length.

5. 3. Results

5. 3. 1. Greenhouse pot trials

5. 3. 1. 1. Ethiopian isolates

Of all the isolates obtained from the rhizosphere of sorghum, three isolates resulted in a significant increase ($P = 0.05$) in at least two growth parameters. Two isolates (KBE5-1 and KBE7-8) resulted in a significant increase in at least five parameters (Table 5. 1). Isolate KBE5-1 displayed increases in all parameters tested except chlorophyll content and root length. This isolate increased shoot and root length by 53 and 93 % respectively, increased shoot and root biomass by more than 90 %, increased leaf width by 68 %. Isolate KBE7-8, on the other hand resulted in a significant increase in all seven parameters (Table 5. 1; Fig. 5. 1). Likewise, the increase in the shoot and root biomass by this isolate was > 90 % while the increase in shoot and root length was 51 % and 77 % respectively. Another isolate, KBE9-1 resulted in a 95 % and 64 % increase in the length and dry weight of the roots respectively (Table 5.1). This isolate however rendered no significant increase in shoot length, shoot biomass, chlorophyll content as well as shoot width of the leaves over the control by this isolate (Table 5. 1).

5. 3. 1. 2. South African isolates

Isolates KBS9-B and NAS4-3 resulted in significant increases in all seven parameters measured (Table 5. 2; Fig.5. 2). The percentage increases in all parameters were very similar for the two isolates. The increase in both shoot fresh weight and root dry weight was 92 % by isolate KBS9-B and 93 % by isolate NAS4-3 (Table 5. 2) whereas both isolates resulted in ca. 64 and 73 % increase

in shoot and root length respectively. Another isolate (KBS5-H) also rendered significant increases in shoot and root length (Table 5. 2; Fig.5. 2). This isolate increased shoot and root length by 66 and 76 % respectively but it had less effect on shoot and root dry weights than isolates KBS9-B and NAS4-3. Other isolates which stimulated growth in sorghum were isolates KBS6-H, KBS5-F, KBS2-12 and KFP9-E all of which resulted in significant increases in five parameters. The rest of the isolates resulted in significant increases in at least three parameters except for isolates KBS1-T and KBS10E which resulted in increases in only two parameters. Most of the isolates that resulted in significant growth promotion mainly affected plant biomass (fresh and dry weights of shoots and roots), root length and chlorophyll content. Compared to the control, these isolates had no effect on shoot length and leaf width by.

5. 3. 2. Root colonization

Both Ethiopian and South African isolates, which resulted in significant growth promotion in sorghum, were able to colonize the roots successfully. Six isolates from the rhizosphere of grasses in South Africa and five isolates from sorghum rhizosphere in Ethiopia colonized sorghum roots with a concentration of $\geq 10^5$ cfu /g root. The count of bacterial colonies similar to the inoculated ones for each of the isolates KBS9-B, KFP9-K, KBS9-H, KBS6-H, KBS5-H and NAS4-3 was 8 log units compared to the un-inoculated control which rendered a count of only 4 log units (Table 5. 3). Similarly Ethiopian isolates KBE7-8, KBE5-1, KBE9-1, KBE5-7 and KBE5-8 were detected at levels $\geq 10^8$ cfu /g root. Most of the other isolates were however detected at a lower level than the initial inoculum level of $10^8 – 10^9$ cfu /ml and the decrease from the initial inoculum level ranged between 1 – 4 log units.

5. 3. 3. Modes of action for growth promotion

5. 3. 3. 1. Siderophore production

Six bacterial isolates, two from sorghum rhizosphere in Ethiopia and four from the rhizosphere of grasses in South Africa, were able to produce siderophores on CAS agar plates (Table 5. 4). This was confirmed by a change in the colour of the CAS agar plates from orange/yellow to blue as a result of the siderophores sequestering and binding iron from the medium. Only one of the two isolates from the rhizosphere of sorghum in Ethiopia that produced siderophores in culture enhanced growth in sorghum (KBE9-1) reflected in a significant increase in root length and root dry weight.

On the other hand, of all the isolates from the rhizosphere of grasses obtained within South Africa and only isolates KBS6-H, KBS5-F and KBS6-17 tested positive for the production of siderophores on CAS agar plates (Table 5. 4). The production of siderophores in culture by isolates NAE5-7 from Ethiopia and isolates KBS9-R and KBS6-17 from South Africa did not result in any significant growth promoting effect in sorghum (Tables 5. 1 and 5. 2).

5. 3. 3. 2. Indole acetic acid (IAA) production

Of the 15 isolates tested for the production of the hormone IAA, 11 (73 %) were capable of producing the hormone in liquid culture with concentrations ranging between 4.2 - 22.8 µg/ml in the presence of 0.2 % trypthophan (Table 5. 4). This concentration however decreased significantly in the absence of trypthophan ranging between 1.82 - 5.43 µg/ml indoleacetic acid. The highest amount of IAA was produced by isolate KBS5-H (22.8 µg/ml) followed by isolates KBS9-H (22.6 µg/ml), KBS6-H (21.4 µg/ml), KBE9-1 (20.8 µg/ml) and NAS4-3 (20.5 µg/m) in the presence of 0.2 % trypthophan. In the absence of trypthophan the amount produced by these isolates decreased to 2.24, 5.21, 5.43, 2.17, 3.4 µg/ml respectively. Isolates KBS5-F, KFP9-K and KBS1-T all of which affected some aspect of sorghum growth under greenhouse condition (Table 5. 2) were unable to produce IAA in culture (Table 5. 4).

5. 3. 3. 3. Phosphate solubilization

Thirteen isolates (86 %) were able to solubilize tri-calcium phosphate on Pikovskaya (PVK) agar medium. Nine of these isolates were also capable of solubilizing phosphate on a modified PVK medium devoid of yeast extract (NBRIY medium). Isolates KBS5-F, KBE9-1 and KBS6-H resulted in the greatest level of phosphate solubilization, rendering a 10 mm diameter clear zone followed by isolate KBS5-H that rendered a clear zone of 7mm in diameter (Table 5. 4). Eight other isolates showed some ability to solubilize phosphate on PVK medium rendering a clear zone ranging between 0.5 - 5 mm in diameter. The same isolates, which effectively solubilized phosphate on PVK medium, displayed improved phosphate solubilization ability on NBRIY medium with clear zone diameters of 12 mm (KBS6-H, KBS9-1) and 14.5 mm (KBS5-F). Similarly isolates KBS9-R and KBE7-8, both of which tested positive for phosphate solubilization on PVK medium (clear zone of 5mm in diameter each) solubilized phosphate better on NBRIY medium (clear zone of 8.5 and 10 mm in diameter respectively) (Table 5. 4).

5. 3. 4. Identification of bacterial isolates

The three isolates obtained from the sorghum rhizosphere in Ethiopia and which resulted in significant growth promotion in sorghum in the present study have been identified as *Bacillus cereus*. Identification of effective isolates obtained from the rhizosphere of grasses and rhizoplane of roots (South African isolates) resulted in 30 % *B. cereus*, 23 % *Chryseomonas luteola*, 15% each of *Serratia marcescens* and *Sphingomonas paucimobilis* and 8 % each of *Stenotrophomonas maltophilia* and *Brevibacterium laterosporus* (Table 5.5).

5. 4. Discussion

The present study demonstrated that rhizobacteria isolated from the rhizosphere and rhizoplane of grasses in South Africa and from the rhizosphere and of sorghum in Ethiopia have the ability to promote growth of sorghum under greenhouse conditions.

According to our knowledge, this is the first report of PGPR associated with enhancement of growth in sorghum. Other similar studies have been done on the role of plant growth promoting bacteria in increasing the growth and yield of wheat (Khalid *et al.,* 2004), rice (Thakuria *et al.,* 2004), maize (Berg *et al.,* 1991) and several other crops. However, there are no reports on the occurrence of PGPR in the sorghum rhizosphere, and their beneficial effect on the growth of this crop.

Three isolates from the rhizosphere of sorghum in Ethiopia and 16 from the rhizosphere of grasses within the Nylsvlei Nature Reserve in South Africa resulted in significant growth promotion of sorghum under greenhouse conditions. The most frequently isolated species with growth promoting abilities in the current study were *B. cereus*, *C. luteola* followed by *S. marcescens, Sphmon. paucimobilis, S. maltophilia, and B. laterosporus.*

Three isolates from sorghum rhizosphere in Ethiopia viz. isolates KBE7-8, KBE5-1 and KBE9-1 and four isolates from the rhizosphere and rhizoplane of grasses in South Africa viz. NAS4-3, KBS5-H, KBS9-H and KFP9-K, all of which resulted in significant growth promotion, were identified as *B. cereus*. Several reports on the growth promoting activity of *B. cereus* support the results obtained in the current study. Chen *et al.* (1994), reported that inoculation with *B. cereus* increased grain yield in rapeseed. In a similar study, Xia *et al.* (1990), reported that *B. cereus* strain 83 -10 promoted growth and increased grain yield of rapeseed in a repeated field trial. In the current study the *B. cereus*

isolates significantly promoted early growth of sorghum by mechanisms involving the production of the auxin IAA, siderophores and by their ability to solubilize phosphate.

All seven strains of *B. cereus* were highly efficient in colonizing sorghum roots and significantly increased root length. The stimulation of root growth may be attributed to the production of IAA in culture at a concentration ranging between 4.2 - 22.8 µg/ml in the presence of 0.2% trypthophan. Patten and Glick (2002), reported the production of IAA by wild type *Pseudomonas putida* from as little as 0.5 µg/ml in the absence of trypthophan to as much as 32.7 µg/ml in the presence of 500 µg/ml trypthophan which resulted in the development of the host plant root system. Bacterial secreted indoleacetic acid may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylic acid deaminase (ACC-deaminase) activity (Patten and Glick, 2002). ACC- deaminase produced by plant growth promoting bacteria is involved in the stimulation of root elongation in seedlings (Li *et al.*, 2000). This enzyme hydrolyses plant ACC, the immediate precursor of the plant growth inhibiting phytohormone ethylene (Penrose *et al.,* 2001). In our study however, no attempt was made to determine the ACC deaminase activity of the rhizobacterial isolates. Suzuki *et al.* (2003), observed that IAA producing strains of *Psedomonas fluorescens* stimulated root development in black currant.

Many root-associated bacteria have been reported to produce IAA in culture media (Patten and Glick, 1996; Patten and Glick, 2002). In the present study we have observed a positive linear relationship with significant r value (r = 0.57, P = 0.05) between the *in-vitro* IAA production and increase in root length. Asghar *et al.* (2002) and Khalid *et al.* (2004) have previously reported a positive correlation between *in-vitro* auxin production and growth promoting activities of PGPR. The ability to produce IAA by the most effective isolates in our study might have contributed to the successful root colonization by these isolates (Suzuki *et al.,* 2003) and resulted in significant increase in root growth.

Glick (1995) and Rodriguez and Fraga (1999) indicated that many PGPR promote plant growth by increasing the availability of nutrients in the rhizosphere by means of solubilization of unavailable forms of nutrients and /or siderophore production. The solubilization of phosphate in the rhizosphere is the most common mode of action implicated in PGPR by increasing nutrient availability to host plants (Vessey, 2003). Of the *B. cereus* strains isolated in this study, *B. cereus* strains KBE7-8 and KBE9-1 solubilized phosphate on Pikovskaya (PVK) medium with a clear zone formation of 5 and

10 mm in diameter respectively while clear zone formation by *B. cereus* strains KBS9-H and KBS5-H was 5 and 7mm respectively.

Although we did not conduct a comparative study of the influence of soil type and plant species on the microbial flora in the rhizosphere, the occurrence of only a specific group of bacteria *(Bacillus* spp.) from the rhizosphere of the sorghum fields from two separate sites in Ethiopia is noticeable and could be related to the nature of the soil and plant type. In this regard, Lemanceau *et al.* (1995), reported that non-leguminous crops select specific groups of bacteria in the rhizosphere. The colonization of the maize rhizosphere, for example, by specific groups of bacteria was consistently found at two distinct geographical locations (Tilak *et al.*, 2005). Earlier, Gryston *et al.* (1998), indicated that the abundance and activities of soil microorganisms are influenced by, among other factors, the types of plant species.

Unlike the rhizosphere of sorghum where the majority of isolates were found to be *Bacillus* spp., the rhizosphere of grasses from the Nylsvlei Nature Reserve was found to be colonized by Gram negative isolates such as *S. marcescens, C. luteola, S. maltophila* and *Sphingom. paucimobilis* which exhibited one or more of the properties for growth promotion. *S. maltophilia* and *Serratia* spp. have been reported as members of the naturally occurring rhizosphere community (Lottman *et al.,* 1999).

The Gram-negative isolates that resulted in significant growth promotion of sorghum in this study showed the ability to solubilize phosphate on PVK medium by producing clear zones ranging from 3 - 10 mm in diameter. Clear zone formation was improved by *C. luteola* KBS5-F and *S. marcescens* KBS6-H when these isolates were inoculated on a modified PVK medium devoid of yeast extract (NBRIY) medium. The diameter of the clear zone was increased by 4mm and 2mm for the two isolates respectively. This was also true for the Gram-positives *B. cereus* KBE7-8 and KBE9-1. These results are in agreement with the findings of Nautiyal (1999), who demonstrated that by omitting yeast extract from PVK medium, higher phosphate solubilization was achieved by *Pseudomonas* sp. For some isolates such as *S. maltophila* KBS9-B, phosphate solubilization was similar with and without yeast extract. For other isolates like *B. cereus* KBS5-H and *Sphmon. paucimobilis* KBS1-T, the extent of solubilization (observed from the clear zone formation) decreased or was not observed when yeast extract was removed from the medium. The main effect of phosphate solubilization on plant growth is an increase in biomass and P content (Bashan and de-Bashan, 2005) and in this study a positive linear correlation (significant r-value) was obtained

between phosphate solubilization and root dry weight (r = 0.92, P = 0.005) with the most effective bacterial isolates.

Serratia marcescens strain KBS6-H resulted in growth promotion of sorghum by increasing shoot fresh and dry weights, chlorophyll content as well as root length. The growth promoting effect of some *Serratia* strains in soybean was previously studied under controlled condition where growth promotion was achieved through the production of plant growth regulating compounds (Zhang *et al.*, 1996). Ryu (2005), evaluated the efficacy of *S. marcescens* 90-166 for of *in-vitro* and *in-vivo* growth promotion in Arabidopsis. The results indicated that the bacteria increased foliar fresh weight of Arabidopsis by means of signal transduction of IAA, salicylic acid, and gibberellins. In another study in soybean Dashti *et al.* (1997), observed an increase in grain and protein yield as a result of co-inoculation with *Bradirhizobium japonicum* and *Serratia* strains. In the current study, *S. marcescens* (KBS6-H) colonized the roots at a higher level (8. 16 log cfu/g) than the control and other non effective isolates and tested positive for the production of siderophores, indoleacetic acid and phosphate solubilization. Interestingly, the other *Serratia marcescens* strain (KBS9-R) did not stimulate plant growth (i.e. root and shoot weight, leaf diameter and chlorophyll content) although it tested positive for the production of siderophores, indoleacetic acid and phosphate solubilization. This probably indicates that the ability to manifest these modes of actions by a given bacterium may not necessarily mean that the bacterium is a PGPR (Vessey, 2003). Cattelan *et al.* (1999), for instance found that out of five rhizosphere isolates which tested positive for P solubilization, only two had a positive effect on soybean seedling growth. In chapters two and three of this study however, *S. marcescens* (KBS9-R) has been proven to be very effective in the suppression of *F. oxysporum* and *P. ultimum* associated with soilborne diseases of sorghum.

Previous reports on *S. maltophila* focussed mainly on their antagonistic activity due to traits associated with biocontrol mechanisms including production of antibiotics, production of the enzyme chitinase and rhizosphere colonization (Kobayashi *et al.,* 1995; Kobayashi *et al.,* 2002; Zhang *et al.,* 2000). Sturz *et al.* (2001), recovered plant growth promoting strains of *S. maltophila* from the root zones of quack grass (*Agropyron repens* (L.) Beauv. that significantly increased the biomass of shoots and roots in an *in-vitro* bacterization study. In the current study, *S. maltophilia* strain KBS9-B resulted in a significant increase in all the seven parameters used to evaluate growth promotion in sorghum. The growth promoting ability of this strain was probably due to its high level of root colonization (8.3 Log cfu/g), phosphate solubilization and production of indoleacetic acid. Our result partly concurs with that of Donnate-Correa *et al.* (2004), who previously isolated IAA

producing strains of *S. maltophilia, S. paucimobilis* and *C. luteola* from the rhizosphere of the perennial legume tagasate. However, *C. luteola* KBS5-F and *S. paucimobilis* KBS1-T did not show any signs of indoleacetic acid production in this study and their growth promoting ability is probably due to their ability to solubilize phosphate and produce siderophores.

Enhanced iron nutrition resulting in increased plant growth can also be achieved due to the ability of some plants to bind and release iron from bacterial iron-siderophore complexes and utilizing the iron for growth (Bashan and de-Bashan, 2005). Of all the isolates that resulted in significant growth promotion in the current study, only *S. marcescens* KBS6-H, *C. luteola* KBS5-F and *B. cereus* KBE9-1 produced siderophores.

The present study offers a significant impetus to the application of PGPR for use in agriculture in Ethiopia and South Africa. However, in order to develop the best performing PGPR strains for commercial applications, further selection and screening in field trials will be required and additional studies on modes of action need to be conducted.

5. 5. References

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Table 5. 1. Effect of rhizobacterial isolates from sorghum rhizosphere in Ethiopia on shoot and root growth enhancement of sorghum under greenhouse conditions

Bacterial Isolates	Shoot height(cm)*	Shoot fresh weight (g)*	Shoot dry Weight (g)*	Chlorophyll* (spad units)	Leaf* Width(cm)	Root* Length(cm)	Root dry mass (g)*
KBE4-3	19.50^{cd}	1.50^{d}	0.16^{b}	21.40^{abc}	0.39^{bc}	12.80^{de}	0.07^{abc}
KBE5-3	22.93^{bcd}	2.17^{cd}	0.27^{b}	21.56^{abc}	0.44^{bc}	16.00^{de}	0.08^{abc}
KBE5-7	23.53^{bcd}	2.25^{cd}	0.33^{b}	21.76^{abc}	0.42^{bc}	14.33^{de}	0.01^{abc}
NAE4-1	21.56^{cd}	1.73^{d}	0.23^{b}	21.80^{abc}	0.44^{bc}	19.33^{bcd}	0.06^{abc}
NAE7-1	21.00^{cd}	2.08^{d}	0.30^{b}	22.46^{abc}	0.42^{bc}	17.33^{cde}	0.06^{abc}
KBE7-6	21.06^{cd}	1.97^{d}	0.22^{b}	22.73^{abc}	0.42^{bc}	16.93^{cde}	0.05^{abc}
KBE5-2	19.46^{cd}	1.17^{d}	0.17^{b}	22.93^{abc}	0.48^{bc}	11.93^{de}	0.04^{bc}
KBE9-4	28.80^{abcd}	4.52^{cd}	0.66^{b}	23.83^{abc}	0.82^{abc}	13.80^{de}	0.10^{abc}
KBE9-1	35.26^{ab}	4.09^{cd}	0.50^{b}	29.43^{a}	0.70^{abc}	26.26^{ab}	0.15^{a}
NAE2-8	23.20^{bcd}	1.71^{d}	0.21^{b}	24.36^{abc}	0.65^{bc}	14.86^{de}	0.03^{bc}
KBE8-2	21.66^{cd}	1.33^{d}	0.23^{b}	24.40^{abc}	0.52^{bc}	17.40^{cde}	0.06^{abc}
KBE6-3	30.60^{abc}	1.55^{d}	0.13^{b}	25.30^{abc}	0.59^{bc}	13.40^{cde}	0.05^{bc}
NAE9-5	23.20^{bcd}	1.65^{d}	0.26^{b}	25.33^{abc}	0.54^{bc}	18.73^{bcd}	0.04^{bc}
KBE5-1	38.40^{a}	12.40^{ab}	1.33^{a}	26.16^{abc}	0.93^{ab}	24.40^{abc}	0.11^{ab}
KBE5-8	25.00^{abcd}	7.70^{bc}	0.59^{b}	28.36^{ab}	0.92^{ab}	12.80^{de}	0.08^{abc}
KBE7-8	36.86^{ab}	14.84^{a}	1.41^{a}	29.83^{a}	1.28^{a}	29.56^{a}	0.13^{ab}
NAE5-7	17.73^{cd}	1.78^{d}	0.22^{b}	19.40^{bc}	0.39^{bc}	14.53^{de}	0.07^{abc}
KBE1-7	20.26^{cd}	1.39^{d}	0.19^{b}	18.53^{c}	0.40^{bc}	14.73^{de}	0.06^{abc}
KBE8-3	15.43^{d}	1.43^{d}	0.17^{b}	18.23^{c}	0.40^{bc}	17.93^{cd}	0.04^{bc}
KBE6-1	22.33^{cd}	2.08^{d}	0.27^{b}	20.36^{abc}	0.42^{bc}	19.33^{bcd}	0.09^{abc}
Control	18.13^{cd}	0.63^{d}	0.10^{b}	19.93^{bc}	0.30^{c}	9.43^{e}	0.008^{c}
Pr > F	< 0.0001	< 0.0001	< 0.001	0.0021	< 0.0001	< 0.0001	0.0008
$LSD_{0.05}$	14.14	5.54	0.62	9.58	0.59	7.98	0.1024

* Means within each column followed by the same letter/s are not significantly different according to the least significant difference (LSD) test at *P = 0.05* using the GLM procedure

Table 5. 2. Effect of rhizobacterial isolates from the rhizosphere and rhizoplane of grasses at Nylsvlei Nature Reserve in South Africa on shoot and root growth enhancement of sorghum under greenhouse conditions

Bacterial Isolates	Shoot height(m)	Shoot fresh weight (g)	Shoot dry Weight (g)	Chlorophyll (spad units)	Leaf Width(cm)	Root Length(c)	Root dry Weight(g)
KBS9-B	41.53^{a*}	7.36^{ab}	1.79^{a}	29.96^{ab}	1.27^{a}	21.56^{b}	0.25^{ab}
NAS4-3	40.58^{a}	9.37^{a}	1.36b	28.26^{ab}	1.14^{ab}	20.93^{b}	0.28^{ab}
KBS1-F	30.50^{b}	3.93^{def}	0.68^{cd}	20.23^{fgh}	0.66^{def}	5.76^{g}	0.16^{a-d}
KBS5-F	30.47^{b}	6.80^{a-d}	0.99^{c}	24.76^{cd}	0.65^{def}	19.80^{bc}	0.20^{abcd}
KBS2-12	30.27^{b}	3.90^{ef}	0.74^{cd}	26.80^{bc}	0.72^{d}	21.23^{b}	0.25^{ab}
KBS9-H	29.67^{b}	4.27^{c-f}	0.49^{de}	29.16^{ab}	0.73^{d}	21.50^{b}	0.10^{cde}
KFP9-K	28.90^{b}	2.93^{efg}	0.56^{de}	28.36^{ab}	0.75^{d}	21.00^{b}	0.10^{cde}
KBS5-H	43.50^{a}	11.85^{a}	0.58^{de}	30.33^{a}	0.78^{cd}	24.13^{a}	0.08de
KFP9-E	28.53^{b}	5.50^{b-e}	0.99^{c}	24.30^{cd}	0.45^{f}	11.50^{e}	0.21^{abc}
KBS6-1	28.27^{b}	2.97^{efg}	0.77^{de}	23.40^{c-f}	0.61^{def}	15.46^{d}	0.10^{cde}
KBS6-H	28.17^{b}	6.97^{abc}	1.72^{a}	30.46^{a}	1.00^{bc}	20.63^{bc}	0.13^{b-e}
NAS2-B	26.93^{b}	4.07^{def}	0.48^{de}	24.50^{cd}	0.60^{def}	8.26^{f}	0.08^{de}
NAS1-6	25.77^{b}	3.90^{ef}	0.72^{cd}	20.16^{fgh}	0.70^{de}	8.26^{f}	0.09^{cde}
NAS6-N	25.60^{b}	4.23^{c-f}	0.56^{de}	19.70^{gh}	0.63^{def}	19.76^{bc}	0.10^{cde}
KBS1-J	25.20^{b}	3.70^{ef}	0.51^{de}	23.36^{def}	0.63^{def}	7.53^{fg}	0.09^{cde}
KBS10-E	24.83^{b}	1.53^{g}	0.35^{ef}	23.30^{def}	0.47^{ef}	8.66^{f}	0.10^{cde}
KBS6-11	23.80^{b}	2.83^{efg}	0.60^{de}	20.90^{efg}	0.63^{def}	18.30^{c}	0.13^{b-e}
KBS1-T	23.70^{b}	3.23^{efg}	0.45^{de}	18.63^{gh}	0.63^{def}	19.96^{bc}	0.09^{cde}
KBS9-R	23.50^{b}	3.10^{efg}	0.38^{f}	19.80^{gh}	0.70^{de}	7.65^{fg}	0.08^{de}
Control	14.70^{b}	0.62^{g}	0.46^{f}	16.83^{h}	0.56^{def}	5.73^{g}	0.02^{e}
Pr > F	0.0001	< 0.0001	<0.0001	< 0.0001	<0.0001	< 0.0001	0.0111
$LSD_{0.05}$	8.07	2.89	0.32	3.42	0.24	2.41	0.128

* Means within each column followed by the same letter/s are not significantly different according to the least significant difference (LSD) test at *P = 0.05* using the GLM procedure.

Table 5.3 Origin of rhizobacterial isolates from Ethiopia and South Africa and their ability to colonize sorghum roots under greenhouse conditions

South African isolates				*Ethiopian isolates(sorghum rhizosphere)*		
Isolate	*Log cfu g $^{-1}$ ± SE*†	*Origin*	*Host grass*	*Isolate*	*Log cfu g $^{-1}$ ± SE*†	*Origin*
KBS9-B	8.30 ± 0.17^{a}	rhizoplane	*Cyprus esculentus* L.	KBE7-8	8.19 ± 0.11^{a}	rhizosphere
KFP9-K	8.19 ± 0.57^{ab}	rhizoplane	*Cyprus esculentus* L.	KBE5-1	8.09 ± 0.11^{a}	,, ,, ,, ,,
KBS9-H	8.17 ± 0.16^{ab}	rhizoplane	*Cyprus esculentus* L.	KBE9-1	8.08 ± 0.15^{a}	,, ,, ,, ,,
KBS6-H	8.16 ± 0.17^{ab}	rhizoplane	*Arstidia canescens* subsp. canescens	KBE5-7	8.07 ± 0.21^{a}	,, ,, ,, ,,
KBS5-H	8.08 ± 0.20^{ab}	rhizoplane	*Eragrostis biflora*	KBE5-8	8.06 ± 0.12^{a}	,, ,, ,, ,,
NAS4-3	8.03 ± 0.12^{b}	rhizosphere	*Themeda triandra*	KBE7-6	7.03 ± 0.13^{b}	,, ,, ,, ,,
KBS6-11	7.22 ± 0.22^{c}	rhizosphere	*Arstidia canescens* subsp. canescens	KBE4-3	6.56 ± 0.64^{bc}	,, ,, ,, ,,
KBS2-12	7.06 ± 0.09^{cd}	rhizosphere	*Stipagrotis zeyheri* subsp. sericans	NAE7-1	6.06 ± 0.14^{cd}	,, ,, ,, ,,
KBS6-1	6.98 ± 0.08^{d}	rhizosphere	*Arstidia canescens* subsp. canescens	KBE6-1	5.99 ± 0.06^{d}	,, ,, ,, ,,
KBS1-T	6.61 ± 0.42^{e}	rhizoplane	*Sporobolus fimbriatus*	KBE6-3	5.97 ± 0.10^{d}	,, ,, ,, ,,
NAS6-N	5.94 ± 0.13^{f}	rhizoplane	*Arstidia canescens* subsp. canescens	NAE4-1	5.93 ± 0.22^{d}	,, ,, ,, ,,
KFP9-E	5.83 ± 0.20^{f}	rhizoplane	*Cyprus esculentus* L.	KBE5-2	5.90 ± 0.03^{d}	,, ,, ,, ,,
NAS2-B	5.74 ± 0.21^{f}	rhizoplane	*Stipagrotis zeyheri* subsp. sericans	KBE8-2	5.84 ± 0.15^{d}	,, ,, ,, ,,
KBS2-3	4.97 ± 0.73^{g}	rhizosphere	*Stipagrotis zeyheri* subsp. sericans	NAE2-8	4.88 ± 0.08^{e}	,, ,, ,, ,,
NAS1-6	4.92 ± 0.07^{g}	rhizosphere	*Sporobolus fimbriatus*	KBE9-4	4.88 ± 0.03^{e}	,, ,, ,, ,,
KBS1-F	4.88 ± 0.32^{g}	rhizoplane	*Sporobolus fimbriatus*	Control	4.32 ± 0.26^{f}	
KBS1-J	4.84 ± 0.02^{g}	rhizoplane	*Sporobolus fimbriatus*	*Pr > F*	*< 0.0001*	
KBS10-E	4.77 ± 0.09^{g}	rhizoplane	*Cyprus esculentus* L.	*LSD$_{(0.05)}$*	*0.53*	
Control	4.44 ± 0.15 ^{h}					
Pr > F	*< 0.0001*					
LSD $_{(0.05)}$	*0.24*					

† Values represent means of three replications plus or minus standard errors of the means and means with the same letter are not significantly different (*P = 0.05*) according to the least significant difference (LSD)t test using the GLM procedure.

Table 5. 4. Modes of action of rhizobacterial isolates in the growth promotion of sorghum under greenhouse conditions

Bacterial isolate	Siderophore production†		Phosphate solubilization		Indoleacetic acid (µg/ml)*	
	High iron	Low iron	Pikovskaya agar	NBRIY medium	0.2 % trypthophan	no trypthophan
KBS6-11	-	-	-	-	10.4^{cd}	2.62^{abcd}
KBS6-H	+	+ +	++++	+++++	21.4^{a}	5.43^{a}
KFP9-K	-	-	+	+	0.0^{f}	0.00^{d}
KBS5-H	-	-	+++	+	22.8^{a}	2.24^{abcd}
KBE5-1	-	-	-	-	10.6^{cd}	2.71^{abcd}
NAS4-3	-	-	+	-	20.5^{a}	3.42^{abc}
KBS9-H	-	-	++	-	22.6^{a}	5.21^{ab}
KBS9-R	++	+ + +	++	+++	4.2^{e}	2.13^{bcd}
KBE7-8	-	-	++	++++	12.4^{bc}	2.10^{bcd}
KBS9-B	-	-	+	+	15.5^{b}	2.30^{abcd}
KBS1-T	-	-	++	-	0.0^{f}	0.00^{d}
KBE9-1	++	+ + +	++++	+++++	20.8^{a}	2.17^{abcd}
KBS5-F	+	+ +	++++	+++++	0.0^{f}	0.00^{d}
NAE5-7	+	+ +	-	-	7.5^{de}	1.82^{cd}
Control	-	-	-	-	0.0^{f}	0.00^{d}

† Siderophore production was compared among the bacterial isolates by measuring the diameter of yellow/orange halo on CAS agar plates: + = halo diameter ≤ 3mm, ++ = 4 - 5mm, +++ = > 5 mm.

ƒ Diameter of clear zone formed (mm) around the colony as a result of solubilization of tri-calcium phosphate on Pikovskaya and NBRIYmedium was measured: + = ≤ 3mm clear zone, ++ = 3 – 5mm clear zone, +++ = 5 – 8 mm clear zone, ++++ = 8 – 10mm clear zone, +++++ = > 10mm clear zone formation.

* Values are means of three separate experiments. Means followed by the same letter/s are not significantly different according to Duncan's Multiple Range test ($P = 0.05$).

Table 5. 5. Identification of the most effective South African and Ethiopian rhizobacterial isolates based on the API system and 16 S rDNA sequencing

Bacterial Isolates	Gram reaction	Endo-spore#	Catalase test	Cytochrome oxidase	Motility test	O/F reaction*	Bacterial species	Type of identification†
KBE7-8	+	+	+	+	Motile	Nd	*Bacillus cereus*	*16S rDNA sequencing*
KBE5-1	+	+	+	+	Motile	Nd	*B. cereus*	*16S rDNA sequencing*
KBS9-H	+	+	+	-	Motile	Nd	*B. cereus*	*16S rDNA sequencing*
NAS4-3	+	+	+	+	Motile	Nd	*B. cereus*	*API-50CHB*
KFP9-K	+	+	+	+	Motile	Nd	*B. cereus*	*16S rDNA sequencing*
KBE9-1	+	+	+	+	Motile	Nd	*B. cereus*	*16S rDNA sequencing*
KBS5-H	+	-	+	-	Motile	Nd	*B. cereus*	*16S rDNA sequencing*
KBS6-H	-	-	+	+	Motile	Oxidative	*Serratia marcescens*	*16S rDNA sequencing*
KBS9-R	-	-	+	-	Motile	Oxidative	*S. marcescens*	*16S rDNA sequencing*
KBS5-F	-	-	-	+	Motile	,, ,, ,, ,,	*Chryseomonas luteola*	*API 20 NE*
NAS1-6	-	-	+	+	Motile	,, ,, ,, ,,	*C. luteola*	*API 20 NE*
KBS6-11	-	-	+	+	N. motile	Oxidative	*C.luteola*	*API 20 NE*
NAS2-B	-	-	+	+	N. motile	,, ,, ,,	*Sphingomonas puacimobilis*	*API 20 NE*
KBS1-T	-	-	+	+	N. motile	,, ,, ,,	*S. puacimobilis*	*API 20 NE*
KBS2-12	+	-	+	-	N. motile	Nd	*Brevibacterium.laterospors*	*API 20 NE*
KBS9-B	-	-	+	-	Motile	Oxidative	*Stenotrophomonas maltophila*	*API 20 NE*

* Nd = oxidation fermentation test not conducted, # += endospore present, - endospore absent.

† Sequencing of the bacterial 16S r DNA was performed for those isolates identified by the API system with less than 80 % identity with the isolates on the data base.

Figure 5. 1. Growth promotion in five-weeks- old sorghum plants in the greenhouse by *Bacillus cereus* KBE5-1, *B. cereus* KBE7-8 and *B. cereus* KBE9-1 isolated from sorghum rhizosphere in Ethiopia. Note that the un-inoculated control resulted in retarded growth as compared to the treatments inoculated with bacterial isolates.

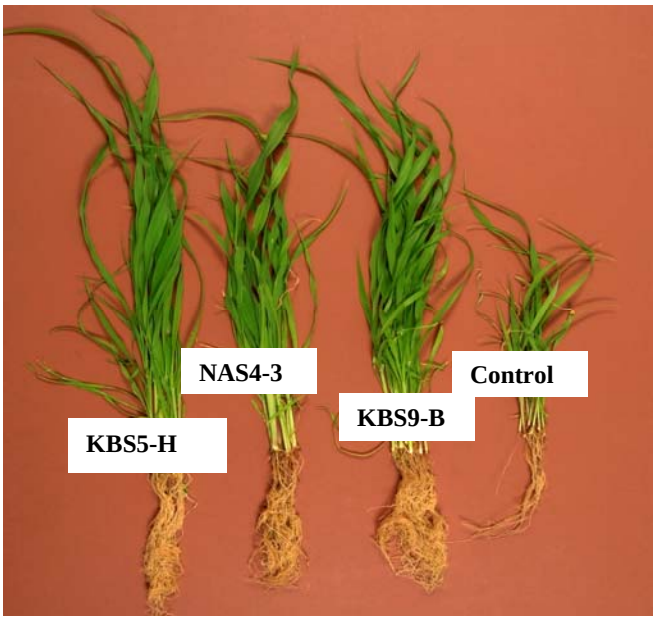

Figure 5. 2. Shoot and root growth enhancement in 4-weeks-old sorghum plants by bacterial isolates *Bacillus cereus* KBS5-H, *B. cereus* NAS4-3 and *Stenotrophomonas maltophilia* KBS9-H all isolated from the rhizosphere of grasses within the Nylsvlei Nature Reserve in South Africa in comparison with the un-inoculated control.

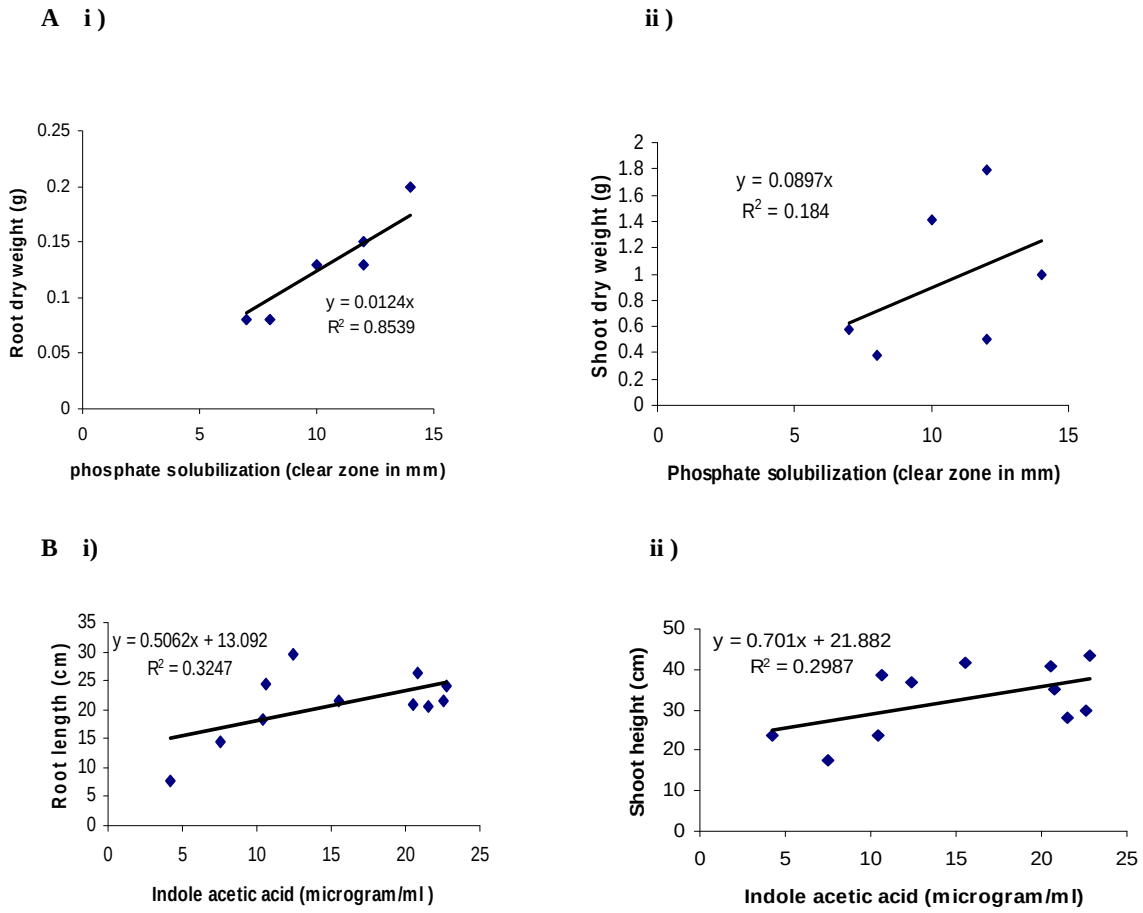

Figure 5. 3. Relationship between P- solubilization and root/shoot dry weight: i) r is significant (*P = 0.005*) for root dry weight and P- solubilization. ii) r is not significant (*P = 0.05*) for shoot dry weight and P- solubilization **(A)**. Relationship between concentration of IAA and root/shoot length: i) r is significant (*P = 0.05*) for root length and IAA concentration. ii) r is significant (*P = 0.05*) for shoot growth and IAA concentration according to Pearson's linear correlation coefficient test **(B).**

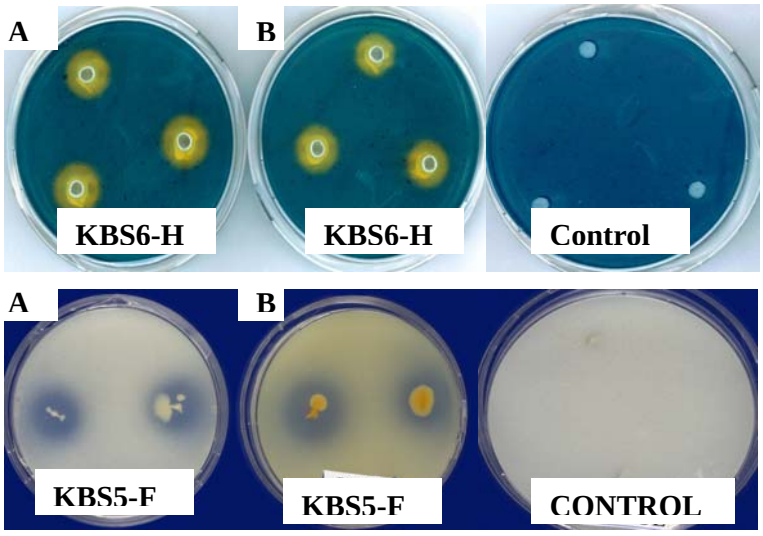

Figure. 5. 4. Siderophores production by *Serratia marcescens* (KBS6-H) as shown by the yellow halo on CAS agar plates under low iron (A) and high iron (B) conditions with the controls inoculated with sterile broth only (Top). Phosphate solubilization by *Chrysoemonas luteola* KBS5-F stab inoculated on NBRIY medium (A) and PVK medium containing bromophenol blue (B) (bottom)

CHAPTER 6

Selection of the most effective rhizobacterial isolates as biocontrol and growth promoting agents in the sorghum rhizosphere

Abstract

The screening of biocontrol and growth promoting rhizobacterial (PGPR) isolates originally obtained from the rhizosphere of sorghum in Ethiopia and from the rhizosphere and rhizoplane of grasses in South Africa rendered a number of bacterial isolates that showed the potential to be used as biocontrol and growth promoting agents. However due to the large number of efficient isolates, a need arose to further select the best performing isolates. The best biocontrol agents were selected out of a total of 24 effective isolates both from Ethiopia and South Africa. The screening procedure was based on the *in-vitro* and *in-vivo* suppression of *Fusarium oxysporum* and *Pythium ultimum,* the root colonization ability of the bacterial isolates and selected modes of action including production of antibiotic substances and siderophores, chitinolytic activity and induction of systemic resistance in sorghum. According to these procedures five isolates from Ethiopia (KBE5-7, KBE5-1, KBE9-1, NAE1-7 and NAE5-7) and six isolates from South Africa (KBS5-F, KBS9-R, KBS6-H, KBS5-H, KFP9-K and KBE6-17) have been selected as the most efficient biocontrol isolates. Selection of the best performing growth promoting isolates was conducted on 12 efficient isolates based on root colonization efficiency, siderophore and indoleacetic acid (IAA) production, phosphate solubilization activity and bacterial growth profiles in liquid cultures. Two isolates from Ethiopia (KBE7-8 and KBE9-1) and five isolates from South Africa (KBS5-H, KBS5-F, KBS6-H, KBS9-B and NAS4-3) have been selected as the best growth promoting isolates. As the screening and selection results are entirely based on laboratory and greenhouse studies, this study warrants further screening and selection of the best isolates based on field tests and additional modes of action studies to ascertain their full potential as biocontrol and growth promoting agents.

6. 1. Introduction

In order for plant growth promoting rhizobacteria (PGPR) to be developed for commercial applications, an effective selection and screening procedure is necessary so that the most promising organisms are selected (Nelson, 2004). In selecting the isolation conditions and screening assays, it is important to consider host plant specificity or adaptation to a particular soil, ecosystem, climatic conditions or pathogens to be targeted (Nelson, 2004). Some of the most important approaches for

selection of microorganisms with the potential to control soilborne phytopathogens include isolating from soils that are suppressive to the pathogens, selection based on traits such as root colonization, ACC deaminase activity production of antibiotics and siderophores (Cattelan *et al.,* 1999; Glick and Bashan, 1997; Weller et al., 2002) On the other hand, selection of organisms with the potential to enhance plant growth in pathogen free systems must include isolation based on traits which are involved in the direct promotion of plant growth by PGPR such as nitrogen fixation, solubilization of phosphorus and iron, production of phytohormones such as auxins and cytokinins (Glick and Bashan, 1997).

Nakkeeran *et al.,* (2005), reviewed that the selection of best antagonistic bacterial isolates plays a major role in commercialization of the isolates for disease management. Apart from the above criteria, selection of the best biocontrol and growth promoting isolates should also take into consideration of the genetic stability, shelf life, growth rate and consistency of the isolates in order to develop potent commercial products (Personal comm. with L. Korsten). Non target effect of the inoculated PGPR on other organisms is also worth considering for a biocontrol agent to be developed into a commercial product (Nelson, 2004).

In this chapter an attempt was made to select only the best performing isolates taking into consideration the *in-vitro* and *in-vivo* results of the experiments done in chapters 2, 3, and 5 and the ability of the bacteria to show some of the major traits involved in the biological control of phytopathogens and growth promotion in plants.

6. 2. Materials and Methods

6. 2. 1. Selection of effective biocontrol isolates

To make a selection of the best biocontrol isolates, results of the *in-vitro* and *in-vivo* antagonistic activities were compared. Isolates showing *in-vitro* and *in-vivo* inhibition of both *Fusarium oxysporum* and *Pythium ultimum* were selected in preference to those with inhibition ability against only one pathogen. An isolate with inhibition potential against only one pathogen has been selected if it rendered a disease suppression of more than 80 % in the *in-vivo* experiment. Isolates which met these criteria were further compared in terms of additional criteria based primarily on the ability of the bacterial isolates to show the major traits commonly expressed during antagonism in the rhizosphere, at least those tested in this particular study. These included the ability to colonize the rhizoplane/rhizosphere at or beyond the threshold level of concentration needed by a rhizobacteria to

achieve a high rhizosphere competence. As most of the effective isolates maintained their initial inoculum concentration (10^8 cfu/ml) in the rhizosphere and/or on the roots, the selection favoured those isolates with concentration between 7-8 $\log_{10}$ cfu/g. Isolates rendering a root colonization level below 7 $\log_{10}$ cfu/g were not selected. Production of antibiotic substances in culture was the other criteria we used. To simplify the selection process, criteria were chosen in such a way that an isolate that produced antibiotic substances against only one of the two pathogens i. e. *F. oxysporum* or *P. ultimum* or not at all should also test positive for at least one trait among chitinolytic activity, siderophore production and induction of systemic resistance in sorghum. On the other hand an isolate positive for antibiosis against both pathogens but negative for siderophore production, chitinolytic activity and induction of systemic resistance was selected based on its ability to colonize the roots/rhizosphere at a higher level and its ability to result in significant *in-vitro* and *in-vivo* inhibition against either of the pathogens.

6. 2. 2. Selection of effective plant growth promoting isolates

For selection of the best growth promoting isolates in pathogen free soil, the selection was based on criteria such as the ability of the rhizobacterial isolates to colonize the rhizoplane/rhizosphere sufficiently and result in significant growth promotion. For isolates from the rhizosphere of grasses in South Africa, due to the many isolates which fulfilled these criteria, additional criteria were included viz. the ability of the isolates to produce the phytohormone IAA, to solubilize phosphate and produce siderophores which are thought to be the direct modes of action in the enhancement of plant growth by rhizosphere bacteria.

As most of the effective isolates from South Africa fulfil at least one of the above criteria, we also took into consideration the growth pattern of the bacterial isolates over a 48 hour period in a broth culture medium, comparing the mean generation time and the growth rate constants. The mean generation time or doubling time (g) is the average time required for all components of the culture to double and is calculated from the following equation (Hardy, 2002).

$$\log_{10} N_t = \log_{10} N_0 + g \log 10\ 2$$

$$g = (\log N_t - \log N_0) / \log_{10} 2$$

Where N_0 is the number of cells at time zero and N_t is the number of cells at time t.

During the exponential growth phase, a bacterial culture mimics that of a first order chemical reaction, i.e. the rate of increase of cells is proportional to the number of bacteria present at that time. This

constant of proportionality (μ) is an index of growth rate and is called the growth rate constant which can also be determined from the following equation (Hardy, 2002).

$$\ln N_t - \ln N_0 = \mu(t - t_0)$$

This can be simplified into,

$$\log_{10} N_t - \log_{10} N_0 = (\mu / 2.303)(t - t_0)$$

$$\mu = (\log_{10} N_t - \log_{10} N_0)\, 2.303 / t - t_0$$

Whenever isolates performed the same for all the other criteria, comparison of the mean generation time and the growth rate constant was used to exclude bacterial isolates with higher generation time and a lower growth rate constant.

6. 3. Results

6. 3. 1. Selection of effective biocontrol isolates

Ten isolates from sorghum rhizosphere in Ethiopia have been initially selected based on their *in-vitro* and *in-vivo* suppression of both *F. oxysporum* and *P. ultimum* (Table 6. 1). An additional four isolates which were effective against only one of the two pathogens but which resulted in an *in-vivo* disease suppression of ≥ 80% were also selected. Selection out of the 14 best performing isolates was further conducted based on their ability to colonize the roots at a concentration of 7-8 log cfu g^{-1}. The final selection which was based on the ability of the isolates to produce antibiotics, siderophores and show chitinolytic activity resulted in five Ethiopian isolates viz. *B. stearothermophilus* (KBE5-7), *B. cereus* (KBE5-1), *Chromobacterium violaceum* (KBE9-1), *B. subtilis* (NAE1-7), and *B. circulans* (NAE5-7) being selected as the best performing biocontrol isolates (Table 6. 1).

Similarly, seven isolates initially isolated from the rhizosphere of grasses in South Africa have been selected based on their *in-vitro* and *in-vivo* suppression of both *F. oxysporum* and *P. ultimum*. Six other isolates which were effective against only one of the two pathogens but which resulted in an *in-vivo* disease suppression of ≥ 80% were also selected. Of the 13 isolates, ten isolates which colonized the roots at a concentration between 7-8 log cfu g^{-1} were selected. As with selection of the Ethiopian isolates, the final selection, which considered the ability of the isolates to produce antibiotic substances, siderophores or show chitinolytic activity, resulted in selection of the final six isolates as the best performing biocontrol isolates. These isolates were *Pseudomonas fluorescens* (KBS6-17),

Chryseomonas luteola (KBS5-F), *B. cereus* (KBS5-H), *S. marcescens* (KBS9-R, KBS6-H) and *B. cereus* (KFP9-K) (Table 6. 2).

6. 3. 2. Selection of effective plant-growth-promoting isolates

Amongst the isolates from Ethiopia, only three isolates viz. *B. cereus* (KBE7-8), *B. cereus* (KBE5-1) and *C. violaceum* (KBE9-1) resulted in significant growth promotion and two of these i.e. KBE7-8 and KBE9-1 were selected as the best performing plant growth promoters based on additional criteria including root colonization, phosphate solubilization, indoleacetic acid and siderophores production. Nine isolates from South Africa which resulted in significant growth promotion in sorghum and showed abilities to successfully colonize the roots produce indole acetic acid and/or siderophores, and/or to solubilize phosphate were further compared by monitoring their growth pattern in broth media (Table 6.3; Fig. 6. 1). The nine best isolates from the Nylsvlei Nature Reserve in South Africa displayed some variation in their growth rate and mean generation time in liquid culture over a 48 hour period. Compared to the rest of the isolates, cultures of KBS5-F, KBS5-H, KBS6-H and NAS4-3 reach a stationary phase at a cell density of about 9 log cfu ml $^{-1}$ after 24, 16, 16, 16 hours respectively. All four isolates rendered a relatively lower generation time ranging between 23 – 46 min. and higher growth rate constants between 0.8 – 1.4. Based on their growth rate and mean generation time, the final selection of the following five isolates was made as the best performing plant-growth promoting South African isolates: *C. luteola* (KBS5-F), *B. cereus* (KBS5-H), *S. marcescens* (KBS6-H), *S. maltophilia* (KBS9-B) and *B. cereus* (NAS4-3).

6. 4. Discussion

The aim of the present study was the selection of a few best performing bacterial isolates as biological control and plant growth promoting agents that can further be used in future for field trials, registration and commercialization applications.

In this selection process, the initial strategy was to identify the best performing biocontrol isolate based on the consistency of inhibition results between the *in-vitro* and greenhouse experiments. Isolates which fulfilled this criterion were further selected according to their ability to show important modes of action directly involved in biocontrol activity. Although an attempt was not made to conduct a qualitative study for antibiotic production by the rhizobacterial isolates, the antibiotic substances produced in the culture filtrates of two Ethiopian isolates viz. *B. subtilis* (NAE1-7) and *C. violaceum* (KBE9-1) and one South African isolate *S. marscesens* (KBS9-R) were effective against both *F.*

oxysporum and *P. ultimum.* Several studies have demonstrated that many of the antibiotics produced by biocontrol agents have broad spectrum activity. For example the antibiotic diacetylphloroglucinol (DAPG) produced by several strains of *Pseudomonas fluorescens* have been shown to have activity against a wide range of plant pathogenic fungi (Keel *et al.,* 1992; Thomashow and Weller, 1996). Zwittermycin A is another antibiotic produced by a strain of *Bacillus cereus* which adversely affects the growth and activity of a wide range of phytopathogenic fungi particularly *Phytophthora* and *Pythium* spp. (Silo-Suh *et al.,* 1998). The three isolates which produced antibiotic substances in culture filtrates against *F. oxysporum* and *P. ultimum* in the current study also resulted in significant inhibition of the two pathogens in the dual culture assay. Of the three isolates that tested positive for antibiotic production, *B. subtilis* (NAE1-7) tested negative for the production of siderophores and chitinolytic activity unlike *S. marcescens* (KBS9-R) and *C. violaceum* (KBE9-1). The selection of *B. subtilis* (NAE1-7) as one of the best performing isolates was favoured as this isolate resulted in significant *in-vitro* and *in-vivo* suppression of the two pathogens and was also found to colonize the sorghum roots successfully.

Three isolates obtained from sorghum rhizosphere in Ethiopia viz. *B. stearothermophilus* (KBE5-7), *B. cereus* (KBE5-1) and *B. circulans* (NAE5-7) and seven isolates from the rhizosphere of grasses in South Africa viz. *B. cereus* (KFP9-A), *B. cereus* (NAS4-3), *S. maltophilia* (KBS9-B), *B. cereus* (KBS9-H) and *B. cereus* (KBS5-H), *P. fluorescens* (KBS6-17) and *C. luteola* (KBS5-F) produced antibiotic substances active against only *F. oxysporum*. These isolates were selected as the best performing biocontrol isolates as each of the isolates tested positive for production of siderophores and chitinolytic activity or resulted in significant *in-vitro* and *in-vivo* suppression of the target pathogens. Strains KBS5-F and KBS6-17 which inhibited *F. oxysporum* and *P. ultimum* respectively both *in-vitro* and under greenhouse condition were capable of producing siderophores, while strain KBS5-H produced chitinase on chitin agar medium.

Siderophore production by rhizobacteria has been reported to be responsible for biocontrol activity of various PGPR strains. This biocontrol activity of siderophore producing rhizobacteria involves the suppression of deleterious rhizosphere microorganisms in some cases and suppression of known soil borne pathogens (Scher and Baker, 1982). The selection of siderophore producing isolates KBS5-F and KBS6-17 is therefore based on the fact that siderophore production is an important mechanism by which some strains of bacteria protect plants against root pathogens (Becker and Cook, 1988).

Lysis is a very efficient antifungal mode of action by many strains of rhizobacteria (Chet *et al.,* 1990). Selection of effective biocontrol isolates having strong chitinolytic activity is therefore another

important strategy because chitinases inhibit fungal spore germination, germ tube elongation and lyse hyphal tips (Ordentlich *et al.,* 1988). Chitinolytic activity by isolate KBS5-H in our study was probably the mode of action responsible for the 100 % suppression of *P. ultimum* as this isolate was negative for siderophore production and antibiotic substances against this pathogen.

Whether a bacterial isolate is producing antibiotics, siderophores, or has a strong chitinolytic activity, the ability of the rhizobacteria to colonize the plant rhizosphere is more important. Inconsistent performance of PGPR in the field emanates from poor rhizosphere competence which comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable period of time in the presence of indigenous microflora (Weller, 1988; Lugtenberg and Dekker, 1999). Landa *et al.* (2004), also indicated that inconsistency in disease suppression by introduced bacteria is due to limited rhizosphere colonization irrespective of other modes of action.

In the selection process in the current study, we also considered the root colonization ability of the rhizobacterial isolates. The ability to colonize roots is a highly variable phenomenon among different groups of rhizobacteria, a characteristic reflecting their ability to compete for ecological niches in the rhizosphere. In this study, we have observed that most of the isolates effective in the biocontrol activity against *F. oxysporum* and *P. ultimum* colonized the root at a higher concentration (between 7-9 log cfu/g). This is an interesting result because Bull *et al.* (1991) and Parke (1990), reported that root colonization by rhizobacteria is correlated with disease suppression in only a few instances.

In the current selection study for the best biocontrol and growth promoting isolates, 13 isolates (more than 60 %) selected based on the various criteria (Tables 6. 1, 6. 2 & 6. 3) belong to members of the endospore forming *Bacillus* spp. The spore forming ability of these isolates is important as one of the commercialization criteria. Because of their ability to survive for extended periods of time ensuring superior shelf life characteristics at the end, they can be readily adaptable to commercial formulation and field applications (Bai *et al.,* 2003). All 13 isolates have been shown to be genetically stable as evidenced by their unchanging cultural and microscopic morphology and consistency of the *in-vitro* and *in-vivo* test results over an extended period of time.

Most of the PGPR based products that became commercially available in the past contain strains of *Bacillus* spp. as other non-spore forming species failed due to lack of long term viability (Kloepper *et al.,* 2004). According to the report by Mathre *et al.* (1999), *Bacillus* spp. may be the only genus of bacteria that meets the shelf life standard required by a commercial microbial product. In addition to

long term viability, strains of *Bacillus* spp. have become commercially successful due to their ability to effectively colonize plant roots, produce antifungal compounds, and secrete volatile substances that can directly stimulate plant growth (McSpadden and Fravel, 2002).

Commercialization of PGPR is mainly focused on *Bacillus* species rather than other efficient biocontrol strains such as pseudomonads (Kloepper et al., 2004). However, control of soilborne pathogens and enhancement of plant growth has been achieved by Bacillus, *Pseudomonas, Serratia* and *Azosprillum* species (Montesinos, 2000) but not to the extent as *Bacillus* spp. Such Gram-negative strains as *P. fluorescens*, *P. syringae* and *B. cepacia* are also being commercialized (Montesinos, 2002). The non-spore forming Gram-negative isolates *P. fluorescens* KBS6-17, *S. marcescens* (KBS9-R, KBS6-H), *C. luteola* KBS5-F and *C. violaceum* KBE9-1 selected in this study all showed the fastest growth rate and unchanging cultural and microscopic morphology. Therefore these species were also included in this selection.

In terms of selecting the best plant growth promoting isolates in pathogen free soils in the current study, the major selection criteria used were significant root colonization and growth promotion. Further selection was based on the modes of action involved in direct plant growth promotion as well as comparison of their growth pattern in liquid culture. Substantial production of IAA, siderophores and phosphate solubilizing enzymes suggests a potential growth promoting ability of PGPR (Ayyadurai *et al.,* 2006). Similar approaches to the selection of plant growth promoting rhizobacteria have previously been developed (Berg *et al.,* 1990). These approaches involved selection of bacteria that are able to achieve large *in-situ* population size, the ability to colonize roots *in-vitro* profusely and a high level of plant growth promoting activity. The approaches have been used to select potential inoculants for rice and resulted in positive effects following seed inoculation of the bacteria (Omar *et al.,* 1989).

6. 5. References

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Table 6. 1. The main criteria for selection of the best biocontrol isolates against *Fusarium oxysporum* and *Pythium ultimum* with regard to rhizobacteria obtained from sorghum rhizosphere in Ethiopia

Bacterial isolate	*In-vitro* inhibition %		Root rot suppression (%)		Root colonization (Log cfu/g)	Antibiotic substances		Siderophore production	Chitinolytic activity	Induced Resistance
	F. oxysporum	*P. ultimum*	*F. oxysporum*	*P. ultimum*		*F.oxysporum*	*P. ultimum*			
KBE4-3	37.43	30.33	95.53	54.21	6.56	-	+	-	+	NT
KBE4-4	35.66	-	-	-	4.68	-	-	-	-	NT
KBE5-1*	33.83	-	100	-	8.09	+	-	-	+	+
KBE5-2	56.53	33.40	77.57	35.26	5.90	-	-	-	-	NT
KBE5-4	48.86	-	86.22	-	4.98	-	-	-	-	NT
KBE5-7 *	40.7	21.63	100	42.98	8.19	+	-	-	+	-
KBE5-8	-	35.86	-	52.89	8.06	-	-	-	-	NT
KBE6-1	-	35.3	-	77.56	5.99	-	-	-	-	NT
KBE6-2	-	31.63	-	51.71	5.14	-	-	-	-	NT
KBE6-3	42.73	18.13	68.00	55.96	5.97	-	-	-	-	NT
KBE6-5	42.3	-	-	-	4.82	-	-	-	-	NT
KBE7-6	56.36	-	57.25	-	7.03	-	-	-	-	NT
KBE7-8	38.56	-	71.71	-	8.19	+	-	-	+	NT
KBE8-2	46.70	-	71.08	-	5.84	+	-	-	+	NT
KBE8-3	46.80	27.50	76.34	41.36	7.93	+	-	-	+	NT
KBE9-1*	66.33	47.36	84.45	77.54	8.08	+	+	+	-	+
KBE9-8	41.00	45.73	60.43	53.51	4.69	-	-	-	-	NT
NAE1-7*	33.20	30.20	59.93	86.23	7.56	+	+	-	-	NT
NAE2-4	39.06	-	61.15	-	4.52	-	-	-	-	NT
NAE5-5	37.46	-	100	-	6.98	+	-	-	-	NT
NAE5-7*	26.80	-	95.83	-	7.94	+	-	+	-	NT

NAE6-2	30.00	30.00	78.32	45.17	5.23	-		-	-	NT
NAE7-1	40.93	40.93	45.74	78.33	6.06	+	+	-	+	NT

* Selected as the best performing biocontrol isolates, + = tested positive, - = tested negative, NT = not tested.

Table 6. 2. The main criteria for selection of the best biocontrol isolates against *Fusarium oxysporum* and *Pythium ultimum* with regard to rhizobacteria obtained from the rhizosphere and rhizoplane of grasses at Nylsvlei Nature Reserve in South Africa

Bacterial isolate	*In-vitro* inhibition %		Root rot suppression (%)		Root colonization (Log cfu/g)	Antibiotic substances		Siderophore production	Chitinolytic activity	Induced resistance
	F. oxysporum	*P. ultimum*	*F. oxysporum*	*P. ultimum*		*F. oxyporum*	*P. ultimum*			
KBS1-F	-	32.36	-	61.73	-	-	-	-	-	NT
KBS1-T	-	47.50	-	40.83	-	-	-	-	-	NT
KBS2-6	19.28	30	61.1	69.14	5.4	-	+	-	+	NT
KBS2-12	17.62	52.6	51.3	90.7	7.1	-	+	-	-	NT
KBS5-F*	37.6	-	95.83	-	8.4	-	+	+	-	NT
KBS5-H*	16.32	55.8	59.13	100	8.1	+	-	-	+	+
KBS6-H*	37.5	-	60	-	8.2	-	-	+	+	NT
KBS6-3	-	31.66	-	99.12	-	-	+	-	-	NT
KBS6-11	13.85	25.63	37.87	15.46	4.8	-	-	-	-	NT
KBS8-7	15.83	-	14.83	-	4.1	-	-	-	-	NT
KBS9-B	13.5	-	87.5	-	8.3	+	-	-	-	+
KBS9-H	18.5	55.73	85.5	80.4	8.2	-	+	-	-	+
KBS9-N	-	22.5	-	83.95	3.4	+	-	-	-	NT
KBS9-R*	24.8	52.36	64.50	86.25	7.9	+	+	+	-	+
KBS10-9	8.88	-	58.1	-	3.9	-	-	-	-	NT
KFP9-K*	-	38.66	-	96.55	8.2	+	-	-	+	-
KFP9-A	11.48	39.76	94.77	98.7	7.8	+	-	-	-	+
NAS2-B	18.6	-	15.83	-	3.9	-	-	-	-	NT

NAS2-F	15.28	-	49.71	-	4.5	-	-	-	-	NT
NAS4-3	15.46	-	58.5	-	7.9	+	-	-	-	NT
NAS6-2	10.40	-	57.27	-	5.2	-	-	-	-	NT
NAS6-B	18.6	-	91.43	-	6.9	-	-	+	-	NT
NAS6-N	-	30.00	-	4353	-	-	-	-	-	NT

* Selected as the best performing biocontrol isolates, + = tested positive, - = tested negative, NT = not tested

Table 6. 3. Selection of the best performing Ethiopian and South African isolates for growth promotion of sorghum in pathogen free soil

Bacterial isolates	Root [a] colonization (cfu g^{-1})	Siderophore production	Phosphate solubilization	IAA production	*Bacterial growth property*	
					g (min.) [b]	*(μ)c*
KBE5-1	++	-	-	+	ND	ND
KBE7-8	++	-	+	+	ND	ND*
KBE9-1	++	+	+	+	ND	ND*
KBS1-T	+	-	+	-	96	0.48
KBS5-F	++	+	+	-	46	0.8*
KBS5-H	++	-	+	+	30	1.4*
KBS6-H	++	+	+	+	23	1.23*
KBS6-11	+	-	-	+	96	0.72
KBS9-B	++	-	+	+	62	0.67
KBS9-H	++	-	+	+	96	0.56
KFP9-K	++	-	+	-	69	0.59
NAS4-3	++	-	+	+	25	1.1*

[a] + = ≤ 10^5, ++ = ≥ 10^8 [b] g = mean generation time [c] μ = growth rate constant * = selected isolates
ND = not detected.

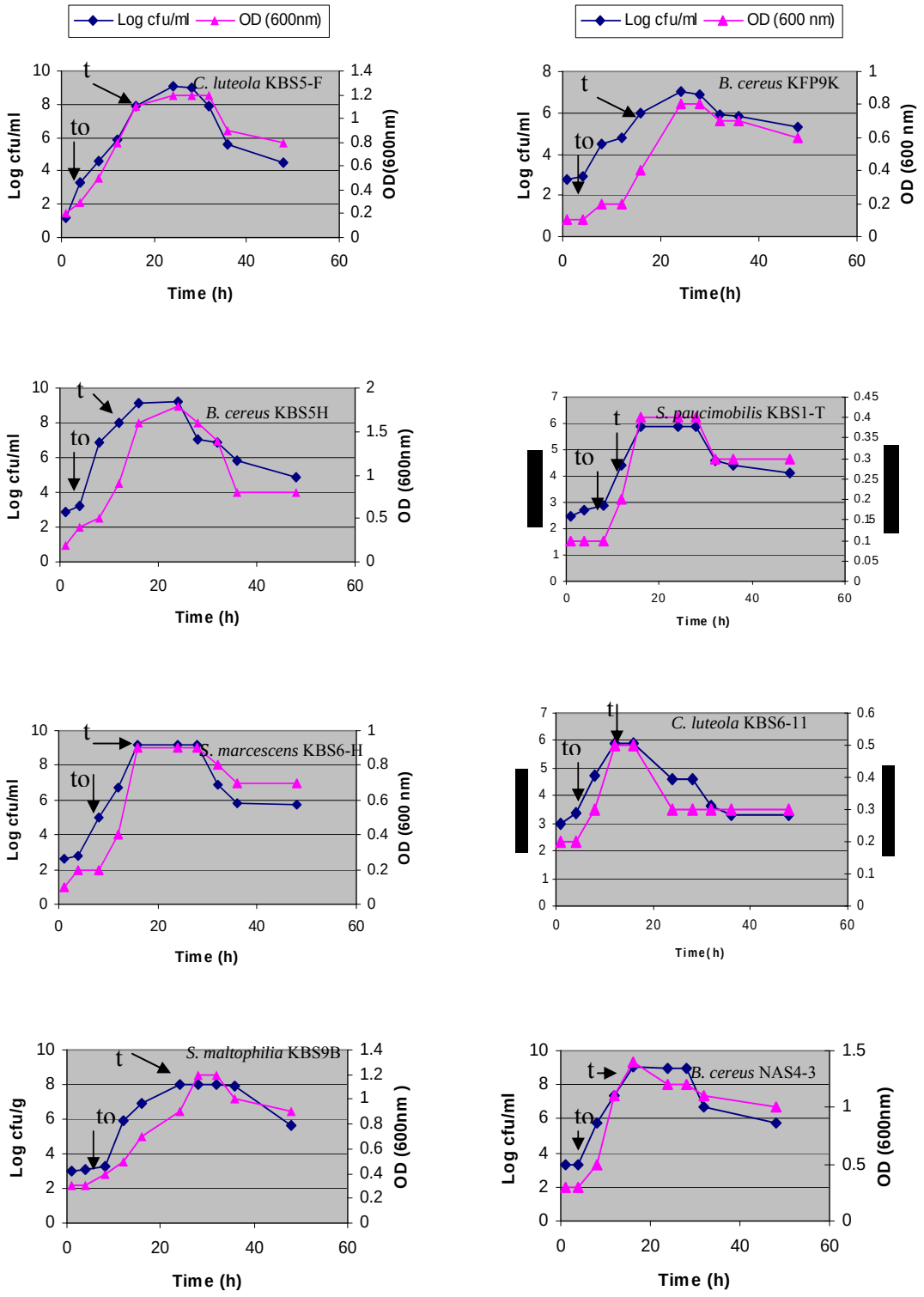

Figure 6. 1. Growth curves of the most effective plant growth promoting bacterial isolates in liquid cultures over 48 h duration in order to select isolates with lower mean generation times and higher growth rates.

CHAPTER 7
GENERAL DISCUSSION

The use of plant growth promoting rhizobacteria (PGPR) as inoculants in agriculture is becoming the method of choice not only for the control of soilborne phytopathogenic fungi, but also for enhancing the growth of several crops in pathogen free soil.

The strategy employed to select a putative biocontrol and growth promoting bacteria was to target the rhizosphere of wild species of grasses at the Nylsvlei Nature Reserve in South Africa, a pristine fluid plain that has no previous history of agricultural farming. In addition the rhizosphere of sorghum from Ethiopia was included. Organic compounds released through exudates by plants in the rhizosphere will contribute to selective growth of certain microbial populations that support and enhance plant growth (Lynch, 1990). This creates a very selective environment where diversity is low (Barriuso *et al.,* 2005). Therefore the rhizosphere of the wild grass species has been selected as the best source for isolating putative plant growth promoting rhizobacteria. The results of identification by means of API as well as partial sequencing of the 16S r DNA of the bacterial isolates from the two sampling sites has shown that *Bacillus* species dominated the microbial flora in the sorghum rhizosphere in Ethiopia. In contrast, the rhizosphere of grasses from the Nylsvlei Nature Reserve in South Africa harboured, in addition to *Bacillus* spp, different strains of Gram-negative isolates effective both in the biocontrol and growth promotion experiments. Such variation in the species composition between the two sites can be contributed to various factors such as differences in soil types, plant species, nutritional status and other environmental factors (Yang and Crowley, 2000).

In total, 302 bacterial isolates were obtained from the two sites, 78 being from Ethiopia and 86 from South Africa. With the initial *in-vitro* antagonistic screening against *Fusarium oxysporum*, 22 Ethiopian and two South African isolates rendered ≥ 30 % inhibition of the mycelial growth. Similarly, 12 isolates from Ethiopia and 14 isolates from South Africa resulted in ≥ 30 % *in- vitro* inhibition of *P. ultimum.* The *in-vitro* screening results obtained in this study were used to facilitate the screening and selection of potential biocontrol agents and subsequently test the ability of the isolates to suppress *F. oxysporum* and *P. ultimum* under greenhouse conditions.

Paulitz and Lopper (1991), and Lopper and Byer (1991), suggested not to associate *in-vitro* inhibition with *in-vivo* activity. However, *in-vitro* methods offer a quicker means of testing the antagonistic potential of selected isolates than greenhouse tests (Renwick *et al.,* 1991). In the *in-vitro* tests in this study, the mycelial growth of *F. oxysporum* was significantly inhibited by many of the isolates tested of which *Chromobacterium violaceum* (KBE9-1) from Ethiopia and *Chryseomonas luteola* (KBS5-F) from South Africa rendered the maximum percent inhibition. A number of *Bacillus* species isolated in this study also produced dramatic inhibition zones in the dual culture assay. In a similar manner, a number of the tested isolates were also able to significantly inhibit the *in-vitro* mycelial growth of *P. ultimum*, the maximum inhibition being rendered by *Bacillus licheniformis* (KBE5-7) from Ethiopia and two *B. cereus* isolates KBS9-H and KBS5-H from South Africa.

The formation of a clear inhibition zone with *F. oxysporum* and *P. ultimum* in the first screening phase is due to the production of antibiotics, toxic metabolites or siderophores responsible for the biocontrol activity of rhizobacteria (Berg *et al.,* 2001). Such clear inhibition zones in the dual culture assay were produced against these pathogens by *B. stearothermophilus* (KBE5-7), *C. violaceum* (KBE9-1), *and B. subtilis* (NAE1-7) from Ethiopia and *B. cereus* isolates (KBS5-H, KBS9-H) and *S. marcescens* (KBS9-R) from South Africa. This probably indicates that the antimicrobial metabolites produced by these isolates might also have antagonistic activity against other soilborne fungal pathogens. Such antagonism of the bacterial isolates towards more than one pathogen is important because of the occurrence of synergistic interactions of pathogens under field conditions (Scholte and Jacob, 1989).

The second stage of the screening procedure namely the selection of potential biocontrol agents under greenhouse condition revealed that isolates highly effective in the dual culture assay have the potential to be used as biocontrol agents. The *in-vivo* selection of isolates as biocontrol agents has an advantage over the *in-vitro* screening as it also selects for root colonization ability and rhizosphere competence of the isolates.

Among the most effective isolates found in this study, *B. stearothermophilus* (KBE5-7), *C. violaceum* (KBE9-1), *B. subtilis* (NAE1-7) and *B. cereus* (KBE8-3) from Ethiopia and *C. luteola* (KBS5-F), *S. marcescens* isolates (KBS9-R, KBS6-H), *B. cereus* isolates (KBS5-H, KBS9-H) and *S. maltophilia* (KBS9-B) from South Africa successfully colonized the roots effectively ($\geq 10^7$cfu/g) with a high level of rhizosphere competence. The same isolates showed consistency in their biocontrol activity both in the *in-vitro* and greenhouse trials against both pathogens confirming the

ability to survive and become established in the rhizosphere which is essential for efficient biocontrol activity (Lugtenberg and Dekkers, 1999).

In this study, rhizobacterial isolates such as *B. stearothermophilus* (KBE5-7), *B. cereus* (KBE5-1), *C. violaceum* (KBE9-1) and *B. subtilis* (NAE1-7) from sorghum rhizosphere and *B. cereus* isolates (KBS9-H, KBS5-H, KFP9-A), *C. luteola* (KBS5-F), *S. marcescens* (KBS9-R) from the rhizosphere of grasses displayed different level of biocontrol efficacy.

Study of the modes of action of PGPR has become an important strategy for improving the efficacy of biocontrol agents (Walsh *et al.,* 2001). In the modes of action studies in chapter 4, the effective biocontrol isolates have been tested for certain specific modes of action. Although no qualitative and quantitative studies have been conducted on the production of antibiotics by the effective isolates in this study, the results from the agar well diffusion assay indicated that antibiosis is one of the mechanisms by which the bacterial isolates suppressed *F. oxysporum* and *P. ultimum*. The production of antibiotics by rhizobacteria is perhaps the most powerful mechanism against phytopathogens (Bashan and deBashan, 2005). The antibiotic substances produced by *C. violaceum* (KBE9-1), *B. subtilis* (NAE1-7) and *S. marcescens* (KBS9-R) inhibited both *F. oxysporum* and *P. ultimum*. This mode of action correlates with the *in-vitro* and *in-vivo* inhibition results suggesting that these isolates have the potential to be developed as biocontrol agents.

It was observed in this study that some isolates which were less effective in the *in-vitro* test showed significant biocontrol efficacy in the *in-vivo* experiments. Among theses isolates were *Stenotrophomonas maltophilia* (KBS9-B) and *B. cereus* (KFP9-A) against *F. oxysporum and B. subtilis* (KBE6-3) and *Bacillus* spp. (KBE5-8) against *P. ultimum*. Such variations are probably due to presence of one or more modes of action other than antibiosis.

It is believed that many of the most effective biological control agents reduce infection by fungal pathogens through more than one mechanism (Silo-Suh, 1994). In the current study, such additional modes of action as production of siderophores, chitinolytic activity and induction of systemic resistance have been detected among the most effective isolates i.e. *S. marcescens* (KBS9-R), *B. cereus* (KBS5-H), *C. luteola* (KBS5-F) and *B. cereus* (KFP9-A).

In chapter 5 of this thesis, the screening for potential plant growth promotion in pathogen free soil has shown dramatic results. Many of the isolates effective in the biocontrol of *F. oxysporum* and / or *P. ultimum* have also been found to have potential plant growth promoting activity in sorghum under

greenhouse conditions. Although several groups of rhizobacteria have been reported to be associated in the growth promotion of various crops, to our knowledge this is the first report of PGPR to be involved in the growth enhancement of sorghum.

In the greenhouse experiments in this study inoculation of single bacterial isolates resulted in promising results both in enhancing the growth of sorghum seedlings as well as controlling root rot caused by *F. oxysporum* and *P. ultimum*. Single biocontrol agents may not be active in all soil environments and most cases of naturally occurring biological control result from mixtures of antagonists. Mixtures of PGPR with compatible microbial antagonists complement the activities of their co-inoculants and improve the efficacy of biological control (Martin and Loper, 1999). The current study provides a foundation for further work to assess mixtures of the PGPR isolates as it was previously shown to be effective by Raupach and Kloepper (1998).

In countries like Ethiopia, primitive agricultural practices most of which are meant for subsistence farming led to the continuous reduction in yields. This situation was worsened by poor soil nutrition and increased infection by phytopathogenic fungi such as *Fusarium oxysporum* and *Pythium ultimum* causing root rot in a wide variety of crops. Efforts to combat these problems using fungicides and chemical fertilizers were not only unsuccessful in these countries, but are also unaffordable (Idris *et al.,* 2007). Current trends in agriculture are therefore focused on the reduction of the use of fungicides and inorganic fertilizers to find alternative ways for more sustainable agriculture (Smit *et al.,* 2001). In this regard, the use of PGPR inoculants as biocontrol agents against phytopathogens and/or biofertilizers is becoming a promising alternative to chemical fertilizers and fungicides (Donate-Corea *et al.,* 2004). The research on the isolation and screening of PGPR and the results obtained in the present study is therefore a step forward towards introducing the application of PGPR in agriculture in these countries.

Plant growth promoting rhizobacteria have existed in the rhizosphere of most agricultural soils despite extensive monoculture systems. However, in most developing countries, their existence and the resulting agricultural significances have barely been described or studied. In developing countries such as Ethiopia, the farming practices are mainly small scale monoculture involving planting of a single crop such as sorghum (*Sorghum biocolor*), teff (*Eragros teff)* or maize (*Zea mays*) over extended period of time. Such extensive monoculture favours the development of several groups of phytopathogenic fungi and bacteria as a result of which the crop fails to give the desired yield. The findings in this study and the subsequent application of PGPR in the mainly monoculture

agricultural soils in Ethiopia may also contribute to the development of soil microbial diversity which are beneficial in agriculture.

7. 1. References

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