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.

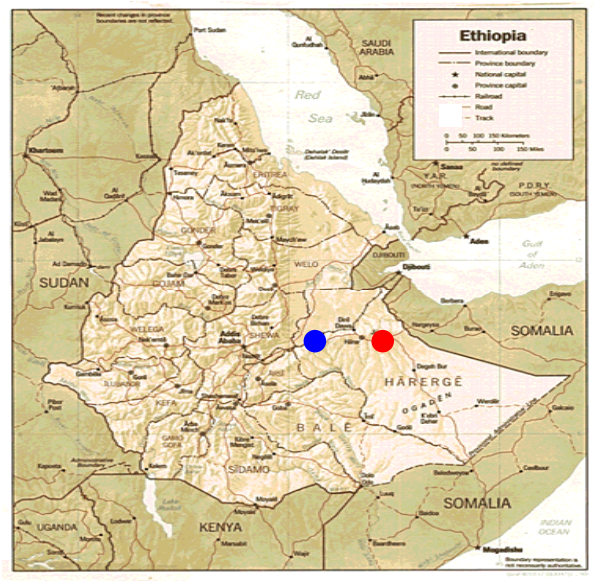
2. 2. MATERIALS AND METHODS

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

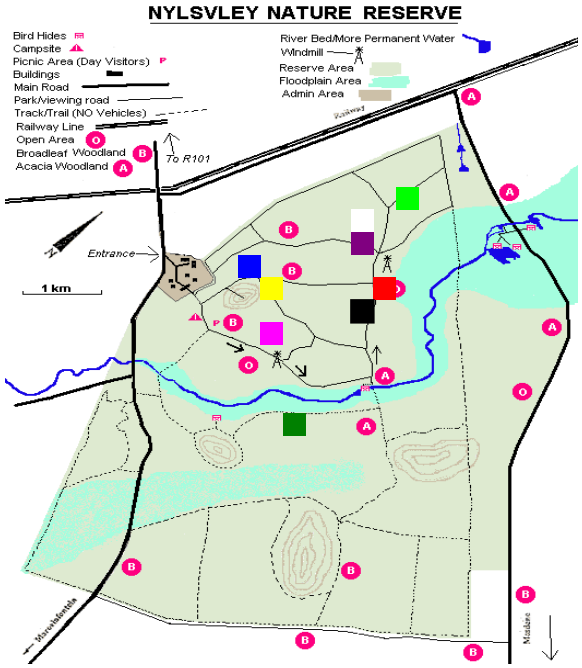


Plate 1. Maps illustrating sampling points in Ethiopia (A) and Nylsvlei Nature Reserve in South Africa (B). ● = Meeson & ● = Jijiga sampling points in Ethiopia. The nine sampling sites at the Nylsvlei are represented on the map using coloured squares: sample no.1= red, 2= black, 3= violet, 4 = white,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

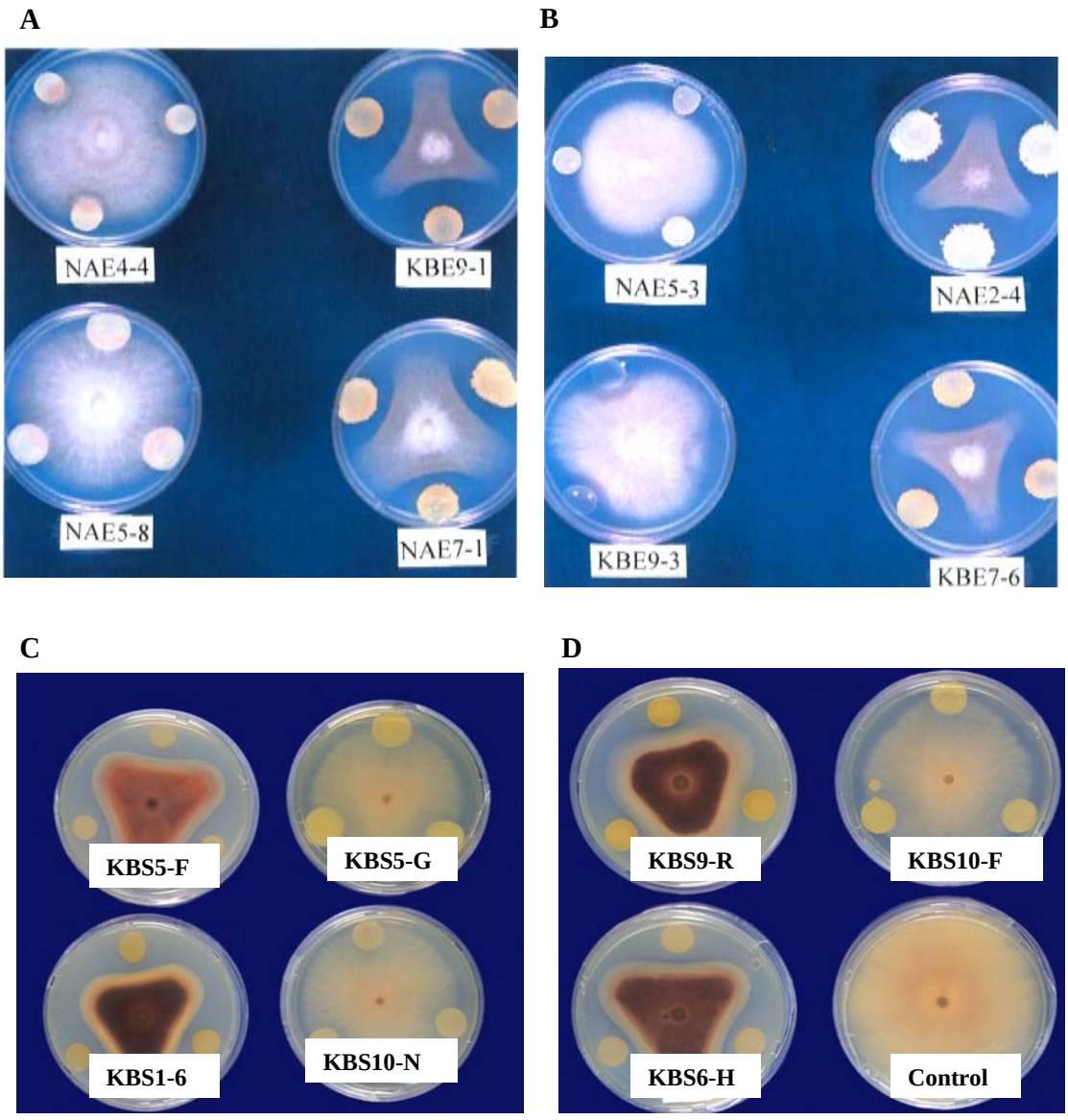

Figure 2. 1. Dual culture assay for *in-vitro* inhibition of mycelial growth of *Fusarium oxysporum* by selected bacterial isolates from sorghum rhizosphere in Ethiopia (**A & B**) and from the rhizosphere and rhizoplane of grasses in South Africa (**C & D**). Note the formation of clearly visible inhibition 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.

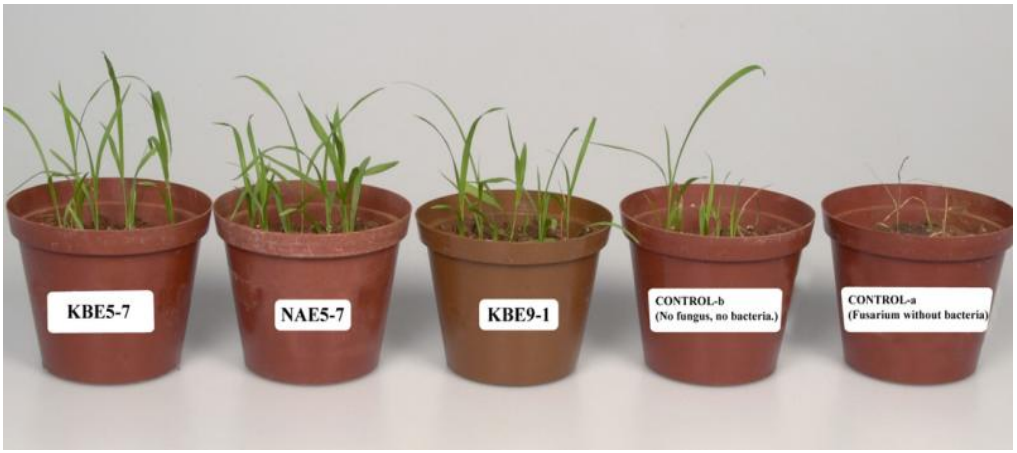

Figure 2. 2. Greenhouse pot experiment illustrating the efficacy of some of the bacterial isolates from Ethiopia in the suppression of root and crown rot diseases by *F. oxysporum* in four weeks old sorghum seedlings. All plants treated with *F. oxysporum* alone died (Control a, far right) whereas 100 % 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

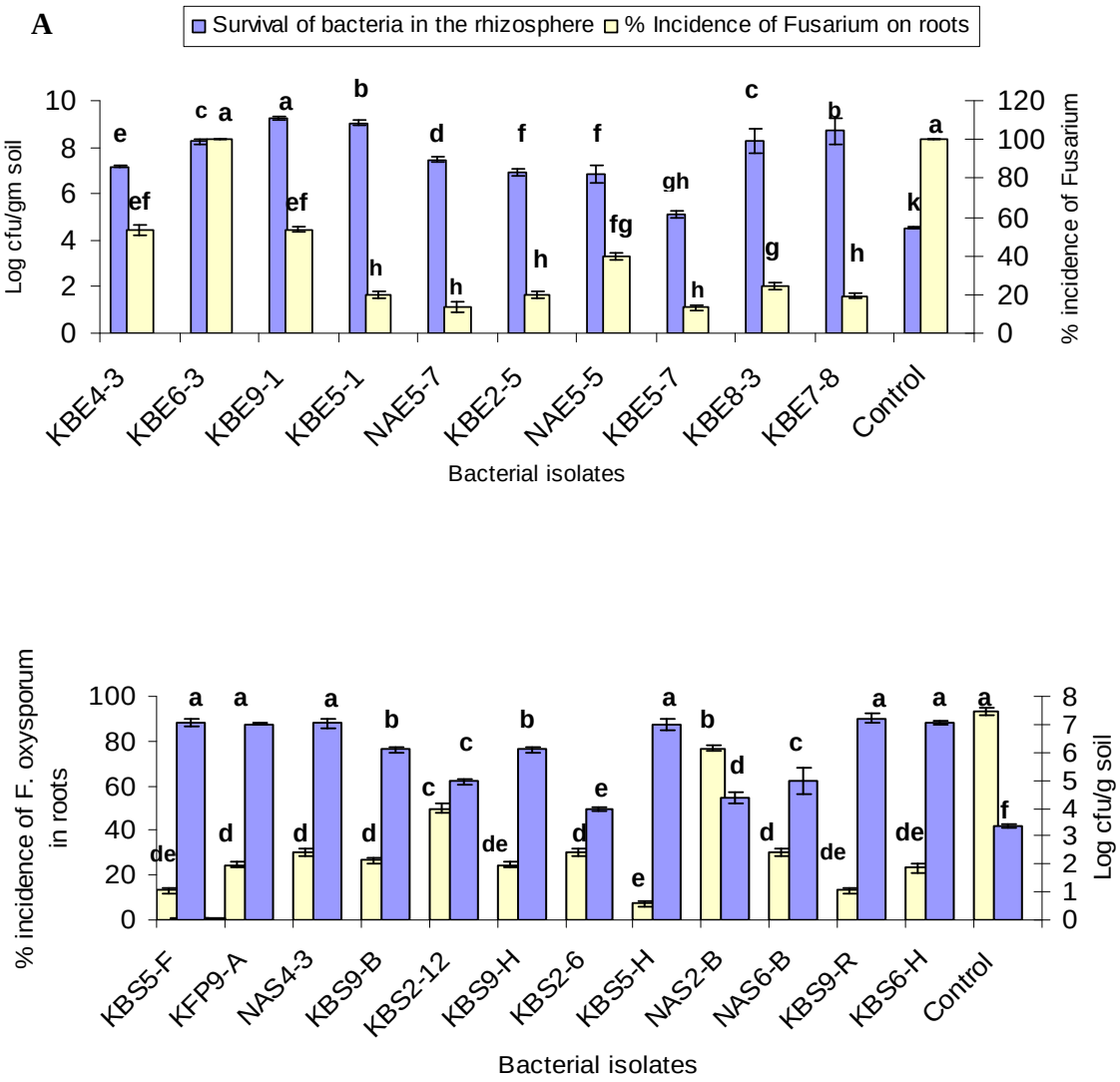

Figure 2. 4. Efficacy of the bacterial isolates obtained from the sorghum rhizosphere in Ethiopia (**A**) and from the rhizosphere of grasses in South Africa (**B**) as biological control agents as illustrated by their high rate of survival in the rhizosphere and low incidence of *F. oxysporum* in roots 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*.