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.

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the predominant root rot, leaf tip and leaf blade necrosis symptoms, more than 90 % of the isolates were *P. ultimum* (unpublished data).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

3. 5. References

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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.

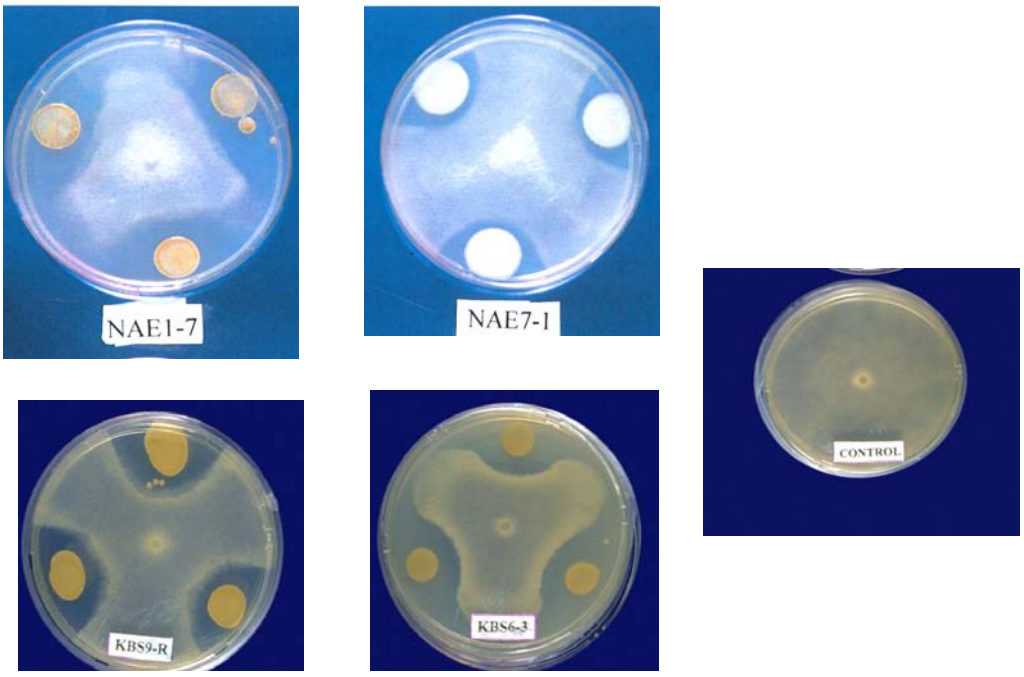


Figure 3. 1. *In-vitro* inhibition of *Pythium ultimum* by selected rhizobacterial isolates from Ethiopia (top) and South Africa (bottom). Note that control plates inoculated with sterile nutrient broth were entirely overgrown by the fungal pathogen with no inhibition zones (right).

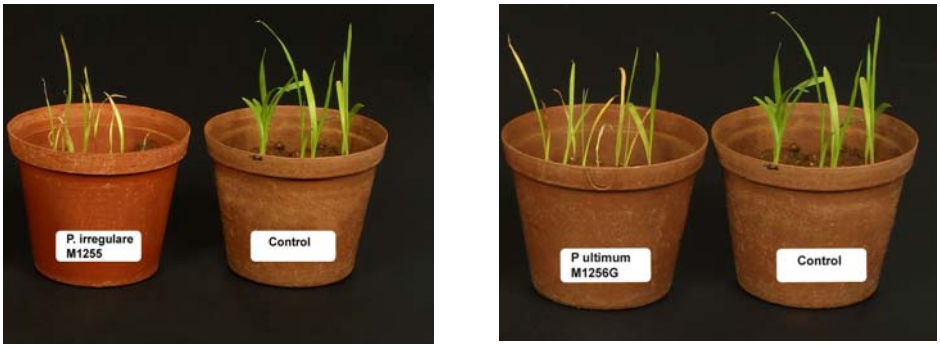


Figure 3. 2. Effect of *Pythium irregulare* M1225 and *Pythium ultimum* M1256G on sorghum seedlings two weeks after inoculation. Yellowing (necrosis of the leaf tip and blade) in the seedlings treated with the two pathogens was evident in comparison with the un-inoculated controls.

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).

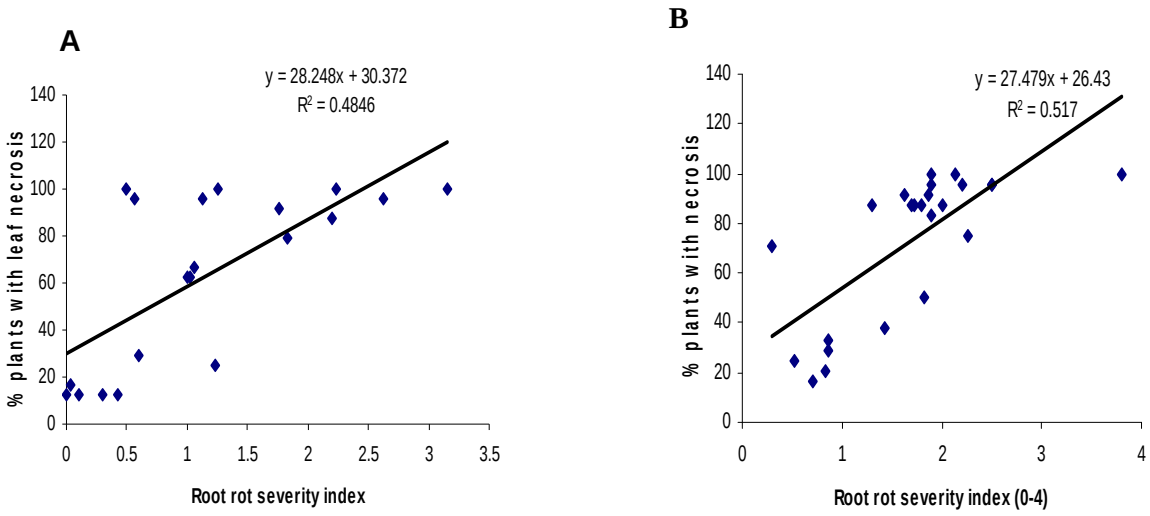
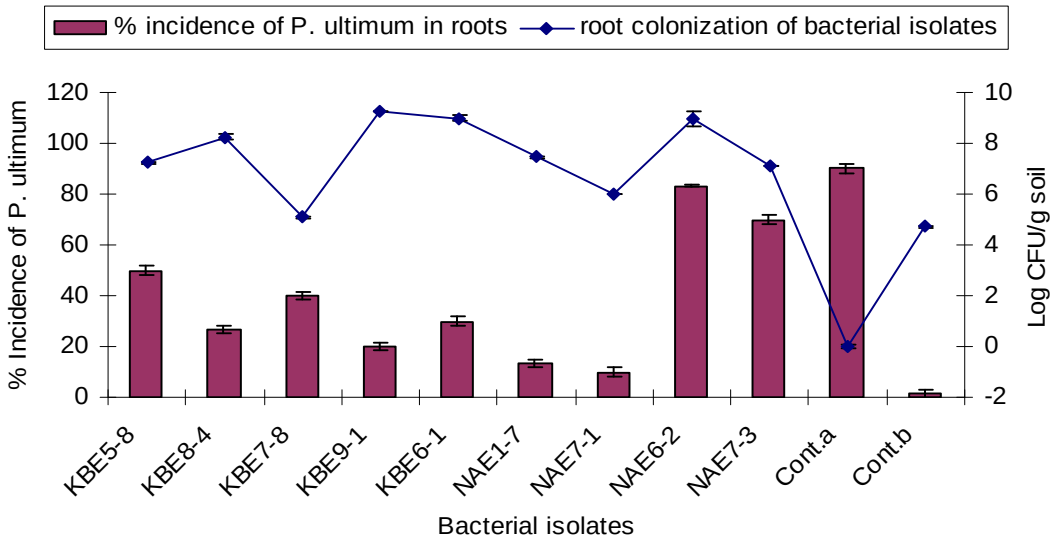


Figure 3. 4. Linear correlation coefficient representing positive relationship (*P= 0.005*) between root rot severity index and the number of plants with leaf tip and blade necrosis in sorghum seedlings after treatment with *Pythium ultimum* and rhizobacterial isolates from Ethiopia (A) and South Africa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

A



B

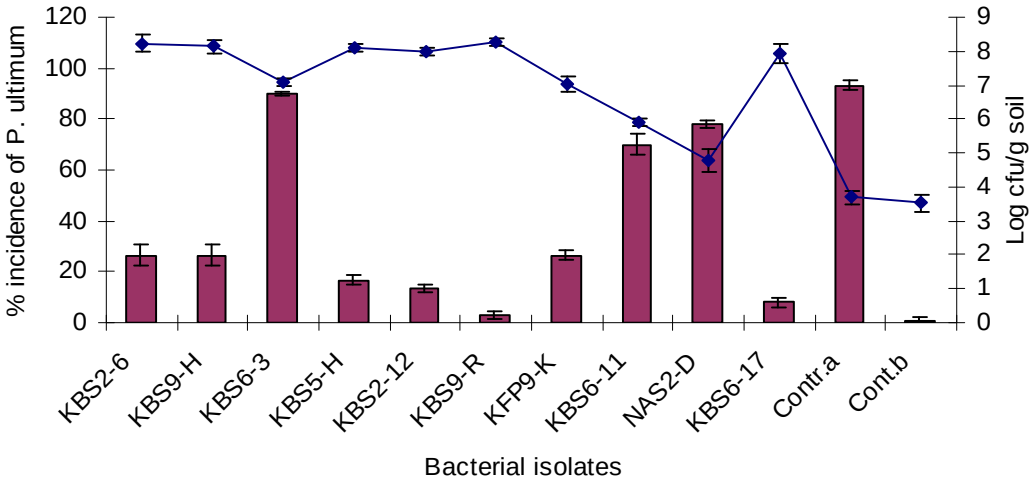


Figure 3. 5. Efficacy of selected rhizobacterial isolates from Ethiopia (A) and South Africa (B) in the biological control of *Pythium ultimum* root rot in sorghum as illustrated by incidence of the pathogen in roots (%) and the survival of bacteria in the rhizosphere (Log cfu/g soil) four weeks after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