CHAPTER 4

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

Abstract

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

4. 1. Introduction

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

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

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

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

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

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

4. 2. Materials and Methods

4. 2. 1. Detection of antibiotic substances

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

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

4. 2. 2. Detection of siderophore production

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

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

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

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

4. 2. 3. Detection of bacterial Chitinolytic activity

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

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

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

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

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

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

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

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

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

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

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

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

4. 3. Results

4. 3. 1. Production of antibiotic substances

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

4. 3. 2. Production of siderophores

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

4. 3. 3. Chitinolytic activity

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

4. 3. 4. Induction of systemic resistance

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

4. 4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. 5. References

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

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

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

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

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

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

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

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

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

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

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

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

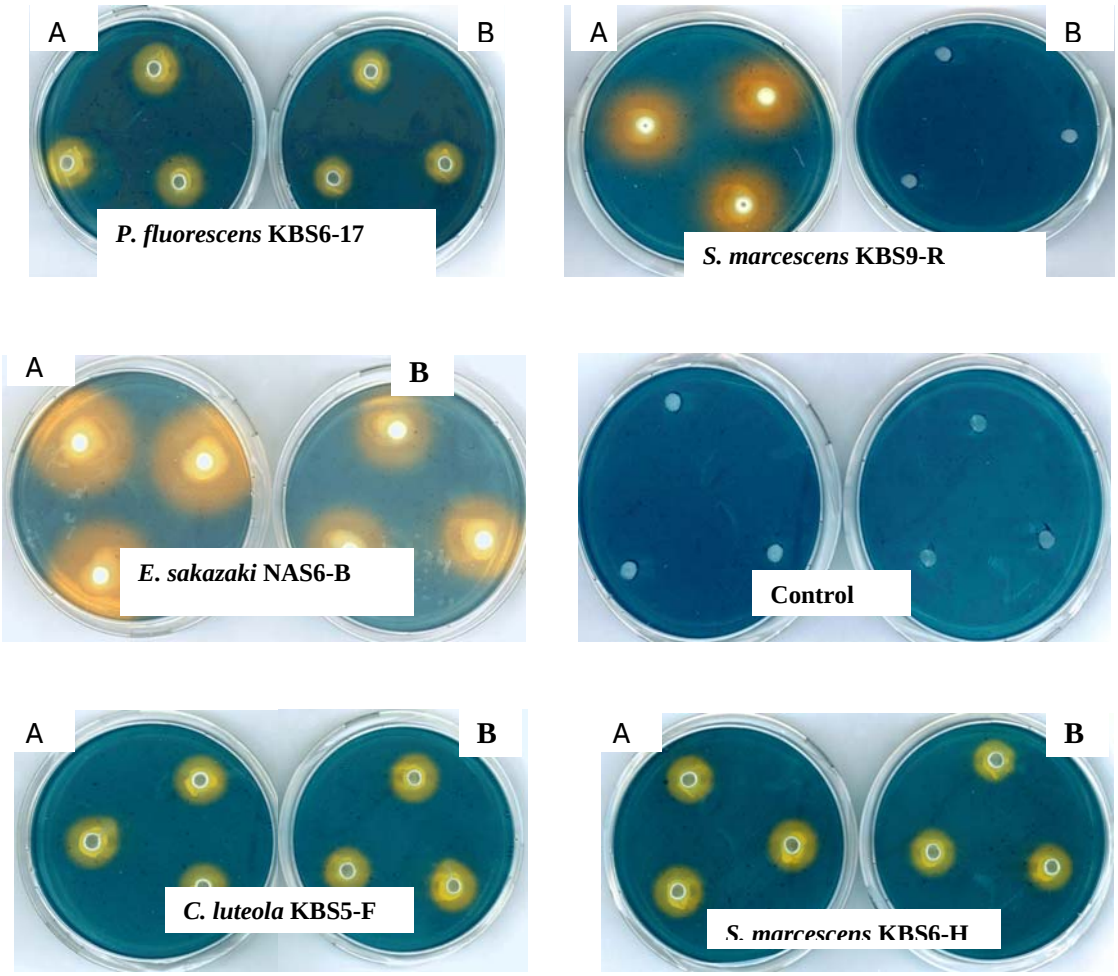

Figure 4. 1. Assay for the production of siderophores in culture supernatants of some of the most effective rhizobacterial isolates under iron restricted (A) and high iron (B) conditions on CAS agar plates. The production of siderophores is confirmed by a change in the colour of the CAS- agar medium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

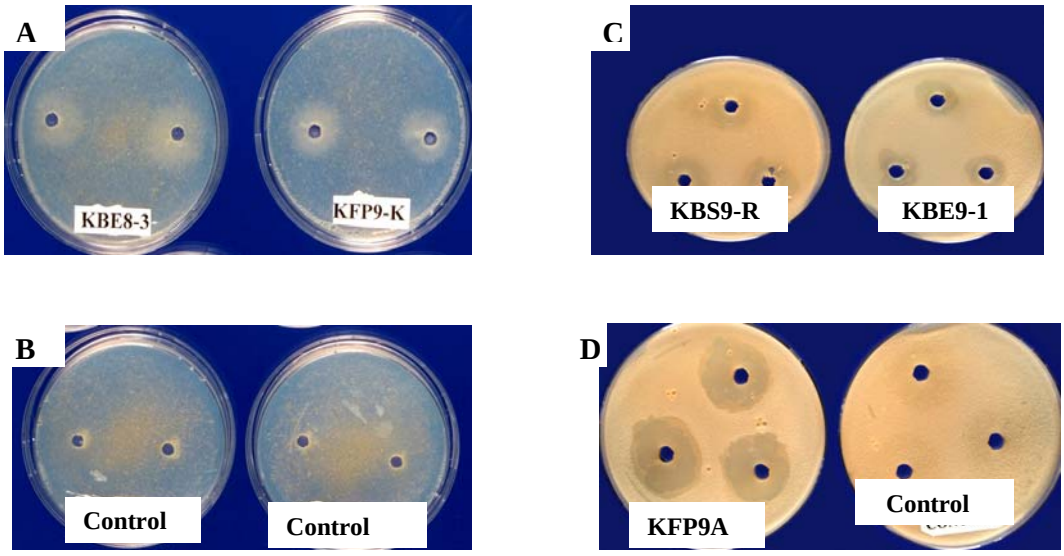

Figure 4. 2. A & B: Chitinolytic activity by bacterial isolates on chitin agar plate as evidenced by the formation of clearing zones. C & D: Inhibition of *F. oxysporum* by antibiotic substances from culture filtrates of bacterial isolates in the agar well diffusion assay.

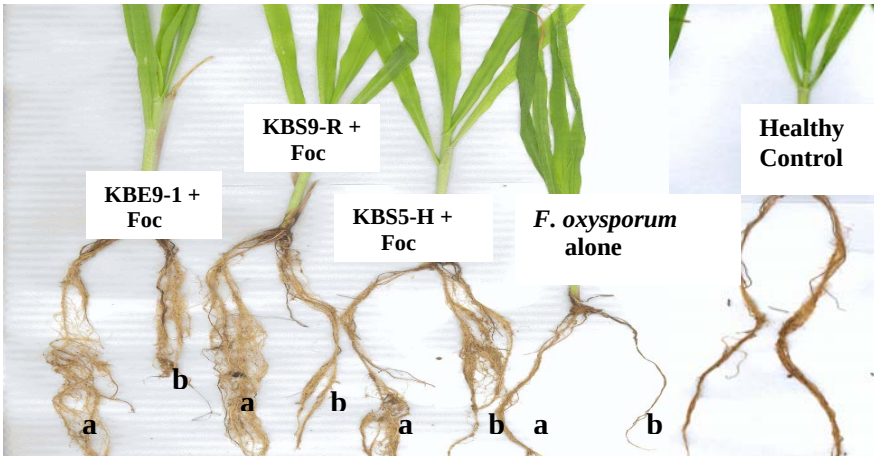

Figure 4. 3. Induction of systemic resistance in sorghum by rhizobacterial isolates KBE9-1, KBS9-R and KBS5-H in the split root experiment in which half of the root was treated with bacterial isolates (a) and the other half with *Fusarium oxysporum* RC331 (b). Half of the root system in the diseased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