CHAPTER 6

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

Abstract

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

6. 1. Introduction

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

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

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

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

6. 2. Materials and Methods

6. 2. 1. Selection of effective biocontrol isolates

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

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

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

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

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

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

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

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

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

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

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

This can be simplified into,

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

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

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

6. 3. Results

6. 3. 1. Selection of effective biocontrol isolates

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

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

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

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

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

6. 4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

6. 5. References

Ayyadurai, N., Ravindra Naik, P., Srihari Rao, M., Sunish Kumar, R., Samrat, S. K., Manohar, M., and Sakthivel, N. 2006. Isolation and characterization of novel banaba rhizosphere bacterium as fungal antagonist and microbial adjuvant in micro propagation of banana. *Journal of Applied Microbiology*. 100, 926-937.

Bai, Y., Zhou, X. and Smith, D. L. 2003. Enhanced soybean plant growth resulting from coinoculation of *Bacillus* strains with *Bradirhizobium japonicum*. *Crop Science*, 1774-1778.

Becker, O. and Cook, R. J. 1988. Role of siderophores in suppression of *Pythium* species and production of increased growth response of wheat by fluorescent pseudomonads. *Phytopathology*. 78, 778-782.

Berg, O., Heulin, T., Balandeau, J. 1990. Diversity of diazotroph populations in the rhizosphere of maize (Zea mays L.) growing on different French soils. *Biology and Fertility of soils*. 11, 210 - 215.

Bull, C. T., Weller, D. M., Thomashaw, L. S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. tritici by *Pseudomonas fluorescens* strain 2-79. *Phytopathology*. 81, 954-959.

Cattelan, A. J., Hartel, P. G., Fuhrman, J. J. 1999. Screening for plant growth promoting rhizobacteria to promote early soybean growth. *Soil Science Society of the American Journal*. 63, 1670-1680.

Chet, I., Ordentlich, A., Shapira, R., Oppenheim, A. 1990. Mechanisms of biocontrol of soilborne plant pathogen by rhizobacteria. *Plant and Soil*. 129, 85-92.

Glick, B. R. and Bashan, Y. 1997. Genetic manipulation of plant-growth promoting bacteria to enhance biocontrol of phytopathogens. *Biotechnology Advances*. 15, 353-378.

Hardy, S. P. 2002. Human Microbiology . Tyler and Francis, CRS Press. Pp. 37-48.

Keel, C., Schnider, U., Maurhofer, M.., Voisard, C., Laville, J., Burger, U., Wirthner, P., Hass, D. and Defago, G.1992. Suppression of root diseases by *Pseudomonas fluorescens* CHAO: Importance of the bacterial secondary metabolite 2, 4-diacetylphloroglucinol. *Mol. Plant-Microbe Interact*. 5, 4-13.

Kloepper, J. W., Ryu, C-M. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94, 1259-1266.

Landa, B. B., Navas-Cortes, J. A. and Jimenez-Diaz, R. M. 2004. Influence of temperature on plant rhizobacteria interactions related to biocontrol potential for suppression of Fusarium wilt of chickpea. *Plant Pathology*. 53, 341-352.

McSpadden, G. B. and Fravel, D. 2002. Biological control of plant pathogens: Research, commercialization and application in the USA. Online. Plant Health Progress. doi: 10. 1094/PHP-2002-05-10-RV.

Montesinos, E. 2000. Pathogenic plant-microbe interactions. What we know and how we benefit. *International Microbiology*. 3, 69-70.

Montesinos, E., Bonaterra, A., Badosa, E., Frances, J., Alemany, J., Llorente, I. and Moragrega, C. 2002. Plant-microbe interactions and the new biotechnological methods of plant disease control. International Microbiology. 5, 169-175.

Lugtenberg, B. J. J. and Decker, L. C. 1999. What makes *Pseudomonas* bacteria rhizosphere competent ? *Environmental Microbiology*. 1, 9-13.

Mathre, D. E., Cook, R. J. and Callan, N. W. 1999. From Discovery to use: Traversing the world of commercializing biocontrol agents for plant disease control. Plant Disease. 83, 972-983.

Nakkeeran, S., Fernando Dilantha, W. G. and Siddiqui, Z. A. 2005. Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. *In*: Z. A. Siddiqui (ed.). PGPR: Biocontrol and Bio-fertilization. pp 257-296. Springer, Dordrecht, The Netherlands.

Nelson, L. M. 2004. Plant growth promoting rhizobacteria (PGPR): Prospects for new inoculants. *Crop Management*, doi:10.1094/CM-2004-0310-05-RV.

Omar, A. M. N., Richard, C., Weinhard, P. and Balandreau, J. 1989. Using the spermosphere model technique to describe the dominant nitrogen fixing microflora associated with wetland rice in two Egyptian soils. *Biology and Fertility of Soils*. 7, 158 – 163.

Ordentlich, A., Elad, Y. and Chet., I. 1988. The role of chitinase of Serratia marcescens in biocontrol of Sclerotium rolfsi. *Phytopathology*. 78, 84-88.

Parke, J. L. 1990. Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of Pythium. *Phytopathology.* 80, 1307-1311.

Scher, F. M. and R. Baker. 1982. Effect of *Pseudomonas putida* and a synthetic non chelator on induction of soil suppressiveness to Fusarium wilt pathogens. *Phytopathology.* 72: 1567-1573.

Silo-Suh, L. A., E. V. Stabb., S. J. Raffel and J. Handelsman. 1998. Target range of Zwittermycin A, an aminopolyol antibiotic from *Bacillus cereus*. *Current Microbiology*. 37, 6-11.

Thomashow, L. S. and Weller, D. M. 1996. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. In: Stacey, G. and N. T. Keen (Eds.), Plant Microbe Interactions, Vol. 1, (pp 187- 236). Chapman and Hall, NY.

Weller, D. M. 1998. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology*. 26, 379-407.

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

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

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

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

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

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

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

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

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

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

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

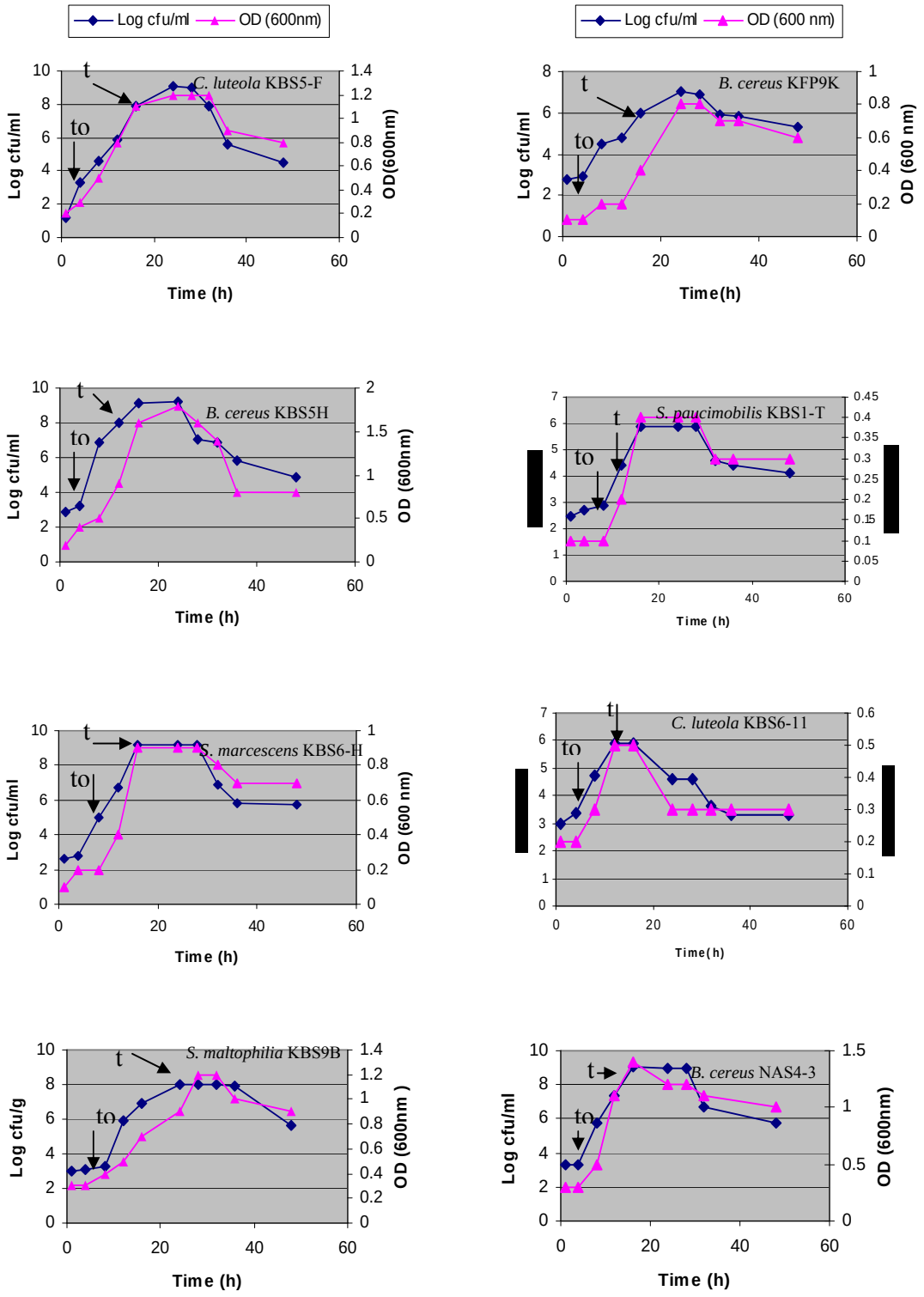

Figure 6. 1. Growth curves of the most effective plant growth promoting bacterial isolates in liquid cultures over 48 h duration in order to select isolates with lower mean generation times and higher growth rates.

CHAPTER 7
GENERAL DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

7. 1. References

Barriuso, J., Pereyra, M. T., Lucas Garcia, J. A., Megias, M., Gutierrez Manero, F. J. and Ramos, B. 2005. Screening for putative PGPR to improve establishment of the symbiosis *Lactarius deliciosus-Pinus* sp. *Microbial Ecology*. 50, 82-89.

Bashan, Y. and de-Bashan, L. E. 2005. Bacteria: Plant growth promoting. In: Encyclopedia of soils in the Environment. (Ed. Hillel, D. Elsevier, Oxford, U. K. Vol. 1, pp. 103-115.

Berg, G., Fritz, A., Roskot, N. and Smalla, K. 2001. Evaluation of potential rhizobacteria from different host plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology*. 91, 963-971.

Donate-Correa, J., Leon-Barrios, M. and Perez-Galdona, R. 2004. Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree shrub legume endemic to the Canary islands. *Plant and Soil*. 266, 261-272.

Idris, H. A., Labuschagne, N. and Korsten, L. 2007. Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. *Biological Control*. 40, 97-106.

Loper, J. E. and Buyer, G. S. 1991. Siderophores in microbial interactions on plant surfaces. Molecular Plant Microbe Interactions. 4, 5-13.

Lugtenberg, B. J. J. and Dekkers, L. C. 1999. What makes Pseudomonas bacteria rhizosphere competent? *Environmental Microbiology*. 1, 9-13.

Lynch, J. M. 1990. The rhizosphere. Wiley-Interscience, Chichester, England.

Martin, F. N. and Loper, J. E. 1999. Soilborne plant diseases caused by *Pythium spp*.: Ecology, Epidemology, and prospects for biological control. *Critical Reviews in Plant Sciences*. 18, 11-181.

Paulitz, T. C. and Loper, J. E. 1991. Lack of a role for fluorescent siderophores production in the biological control of Pythium damping-off of cucumber by a strain of Pseudomonas putida. *Phytopathology*. 81, 930-935.

Raupach, G. S. and Kloepper, J. W. 1998. Mixture of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology*. 88, 1158-1164.

Renwick, A., Campbell, R. and Coe, S. 1991. Assessment of *in-vitro* screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. *Plant Pathology*. 40, 524-532.

Scholte, K. and Jacob, J. 1989. Synergistic interactions between *Rhizoctonia solani* Kühn, *Vertticillium dahliae* Kleb. *Meloidoigyne* spp. and *Pratylenchus neglectus* (Rensch) Chitwood and Oteifa. *Potato Research*. 32, 387-395.

Smit, E., Leeflang, P., Gommans, S., van den Broek, J., van Mil, S. and Wernars, K. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Applied and Environmental Microbiology*. 67, 2284-2291.

Silo-Suh, L. A., Lethubridge, B. J., Raffel, S. J., He, H., Clardy, J. and Handelsman, J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Applied and Environmental Microbiol*ogy. 60, 2023 - 2030.

Walsh, U. F., Morrissey, J. P. and O'Gara, F. 2001. Pseudomonas for the biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Current Opinion in Biotechnology*. 12, 289-295.

Yang, C-H. and Crowley, D. E. 2000. Rhizosphere microbial structure in relation to root location and plant iron nutrition status. *Applied and Environmental Microbiology*. 66, 345- 351.