EVALUATION OF SELECTED FREE-LIVING DIAZOTROPHIC BACTERIA FOR PLANT GROWTH PROMOTION AND BIOLOGICAL CONTROL OF DAMPING-OFF FUNGI

By

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THESIS SUMMARY

Inoculation with free-living diazotrophic bacteria is well documented to enhance vegetative growth and yield increases of various crops coupled with suppression of sublethal pathogens. The use of microbial inoculants has been identified as an alternative or supplement to use of nitrogenous fertilizers and agrochemicals for sustainable agriculture. The search for effective free-living diazotrophic bacterial strains for formulation as biofertilizers has been on going since the 1970's and a number of inoculant biofertilizers have been developed and are commercially available.

In the current study, 250 free-living diazotrophic bacteria were isolated from soils collected from the rhizosphere and leaves of different crops in different areas within KwaZulu-Natal, province, Republic of South Africa. These were evaluated for plant growth-promotion and biological control of damping-off fungi initially by *in vitro* screening. The growth promotion traits tested included, phosphate-solubilization, production of indole-3-acetic acid, production of ammonia and acetylene reduction. Biocontrol traits evaluated included siderophore-production, antibiosis, and production of hydrogen cyanide (HCN). Biochemical and molecular bioassay tests were conducted to identify the twenty most promising isolates selected in the *in vitro* study**.** The twenty isolates were further tested in combination with various levels of nitrogenous fertilizer for growth-promotion of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) under greenhouse conditions. The five most promising isolates identified for growth promotion under greenhouse conditions for each crop were assessed for their effects on the germination of maize *in vitro* and growth and yields of two maize and two wheat cultivars, when combined with a low dose of nitrogenous fertilizer in field trials. The five *Bacillus subtilis* (Ehrenberg) Cohn isolates that suppressed the growth of a wide range of pathogenic fungi *in vitro* were tested for their efficacy against damping-off of wheat caused by *Rhizoctonia solani* Kühn in the greenhouse. These isolates were further studied for their modes of action against *R. solani in vitro*. The modes of action tested included antibiosis, production of siderophores, extracellular enzymes, production of hydrogen cyanide (HCN) and antibiotic resistance.

The twenty most promising bacterial isolates identified from the *in vitro* screening reduced acetylene to ethylene, produced indole-3-acetic acid and siderophores, one isolate solubilized phosphate, and 9 inhibited the growth of *R. solani*. These twenty isolates enhanced growth of maize and wheat above the Uninoculated Control under greenhouse conditions. The growth enhancements varied with bacterial isolate x crop species interactions, which identified five different isolates for each of the two crops. Relative to the Uninoculated Control, the best five isolates significantly ($P = 0.001$) enhanced the growth of maize and wheat at all fertilizer levels for a number of growth parameters: increased chlorophyll levels and heights of maize, shoot dry biomass of maize and wheat, and enhanced root development of maize in the greenhouse. Inoculation of maize and wheat with the two most promising isolates identified from the field trial for each crop, in combination with 65% and 50% of the recommended amount of nitrogenous fertilizer for maize and wheat, respectively, caused the same increases in shoot biomass as the Fully Fertilized Control. Application of a combination of the best bacterial isolates and 35% nitrogenous fertilizer resulted in the same or greater shoot dry biomass and yields of both maize and wheat under field conditions. Shoot dry biomass of wheat increased by 75% above the Uninoculated Control and 30% above the Fully Fertilized Control. The wheat yield increased by 95% above the Uninoculated Control and 43% above the Fully Fertilized Control. Seed inoculation with the best isolates combined with 35% N increased yields of maize by 41% above the Uninoculated Control and 15% above the Fully Fertilized Control. The best isolates significantly ($P < 0.001$) increased plant height, chlorophyll levels and shoot biomass of maize relative to the Uninoculated Control. There was a positive correlation between chlorophyll level and yield, chlorophyll level and shoot dry biomass, height and shoot dry biomass and height and yield of maize at $P = 0.01$ with r values of 0.87, 0.77, 0.92 and 0.81, respectively**.** The isolates that exhibited multiple plant-growth promoting traits *in vitro*, increased shoot biomass of both maize and wheat in the greenhouse and field, and caused yield increases in the two crops under field conditions. Five *B. subtilis* isolates inhibited the growth of some of the pathogenic fungi tested *in vitro* up to 95%. Seed inoculation with the same isolates significantly ($P = 0.001$) suppressed *R. solani* damping-off of wheat under greenhouse conditions and exhibited multiple mechanisms of disease control *in vitro*.

The use of microbial inoculants in combination with low doses of nitrogenous fertilizers can enhance crop production without compromising the yields. The *B. subtilis* isolates obtained in this study can effectively control *R. solani* damping-off of wheat, fix nitrogen and enhance plant growth. The use of microbial inoculants can contribute to the integrated production of cereal crops with reduced nitrogenous fertilizer inputs, as a key component of sustainable agriculture.

Key words: Free-living bacteria; plant growth-promotion; diazotrophs; biological nitrogen fixation; phosphate-solubilization; siderophores; indole-3-acetic acid; biocontrol; damping-off

DECLARATION

The research reported in this thesis was carried out in the School of Agricultural, Earth and Environmental Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, Republic of South Africa, under the supervision of Dr Kwasi S. Yobo and Professor Mark D. Laing.

This thesis represents my original work and has not been submitted in any form for any other degree or diploma to any University. Where use has been made of the work of other authors, it has been duly acknowledged in the text.

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DEDICATION

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THESIS INTRODUCTION

Increases in crop production are essential to meet the food requirements of the increasing world population. Poor soil fertility, pests and diseases are major constraints to crop production. Increased crop production currently depends on the use of chemical nitrogen (N) fertilizers for nutrients, and agrochemicals for management of pests and diseases. However, crop uptake of chemical forms of N fertilizers is low (Garabet *et al.*, 1998; Dobbelaere *et al*., 2002; Halvorson *et al*., 2002). This inefficiency is usually attributed to ammonia volatilization, dinitrification and leaching of nitrates into ground water (Bijay-Singh *et al*., 1995). Continuous use of nitrogen fertilizers therefore may impact negatively on the environment (Shrestha and Ladha, 1998**;** Wairiu and Lal, 2003)**.** Ammonia volatilization and nitrification result in production of greenhouse gases like ammonia and nitrous oxide which lead to pollution of the environment (Reeves *et al*., 2002), while leaching of nitrates lead to contamination of ground water (Shrestha and Ladha, 1998). The high cost of fertilizers and agrochemicals also inflates the cost of crop production. The use of microorganisms in agriculture has therefore been identified as a cheaper and more environmentally sound alternative or supplementary mechanism to improve crop production and minimize production costs (Wu *et al*., 2005). Several free-living bacteria genera have been reported to enhance plant growth and to reduce the deleterious effects of pathogenic microorganisms, subsequently increasing yields of these crops (Glick, 1995; Shen, 1997; Kennedy *et al*., 2004; Kloepper *et al*., 2004; Idris *et al*., 2007). Improvements in growth parameters resulting from the use of microbial inoculants, combined with reduced doses of chemical fertilizers, have been reported in previous research (Okon and Labandera-Gonzalez, 1994; Biswas *et al*., 2000). Research on the use of microbial inoculants to enhance growth and increase yields of crops and control plant diseases has been the focus of many researchers (Dobbelaere *et al*. 2001; Riggs *et al*., 2001; Mehnaz *et al.,* 2010). Typically these beneficial microorganisms have been isolated from the rhizosphere of plants and formulated into microbial inoculants.

The objectives of the current study were to isolate nitrogen-fixing bacteria from the rhizosphere and leaves of different cereal crops, and evaluate their plant growth promotion and biocontrol effects.

The specific objectives were as follows;

- To review the literature on the use of microbial biofertilizers
- To isolate diazotrophic bacteria from the rhizosphere and leaves of wheat and maize and to characterize and identify them.
- To screen these bacteria *in vitro* for plant growth-promotion and biological control traits.
- To evaluate the effect of bacteria as inoculant biofertilizers on the growth of maize and wheat in both the greenhouse and field.
- To test for compatibility of the various novel *Bacillus* isolates, and Eco-T® (a commercial biocontrol agent), aiming for the formulation of a multi-strain inoculant biofertilizer/biocontrol agent.
- To evaluate the effects of selected *Bacillus subtilis* isolates on some pathogenic fungi *in vitro,* and against damping-off of wheat caused by *Rhizoctonia solani* Kühn in the greenhouse.
- To determine the optimum dose of nitrogenous fertilizer to be used in combination with biofertilizer inoculation aiming to integrate the application of chemical fertilizers with biofertilizers with an optimal yield to cost ratio.

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CHAPTER ONE

Literature Review

1.1 Biofertilizers

A heterogeneous array of bacteria inhabits the rhizosphere and phyllosphere of plants. These bacteria can have beneficial, deleterious or neutral effects on plants (Antoun *et al*., 1998). Beneficial bacteria are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1989). These bacteria are isolated and formulated into microbial inoculants that are used to enhance plant growth and suppress pathogens by various mechanisms. Saharan and Nehra, (2011) classified these bacteria into three major groups based on their mechanisms of growth promotion. Those that promote plant growth directly by providing or facilitating the uptake of certain nutrients by the plant from the environment are referred to as biofertilizers, while those that enhance growth by production of growth regulators are biostimulants. Bioprotectants promote plant growth indirectly by lessening or preventing the deleterious effects of phytopathogens through production of antimicrobial compounds or by biopriming of systemic resistance. Application of beneficial microorganisms in agriculture started more than 60 years ago.

Biofertilizers are products containing living cells of microorganisms with the ability to promote plant growth by enhancing availability and uptake of mineral nutrients by the plants (Vessey, 2003). In recent years biofertilizers have emerged as an important component of the integrated nutrient supply system (Wu, *et al*., 2005). At a global level many microbial products have been developed and commercialized for use in agriculture (Shen, 1997). Several PGPR-based products became available in the United States (US) in the 1990's and more were under development, most of which were *Bacillus-based* (Kloepper *et al*., 2004). The first US product consisting of a PGPR strain of *Bacillus subtilis* (Ehrenberg) *Cohn* sold as Quantum® is commercially available (Turner and Bockman, 1991). Two *B. subtilis* strains, GBO3 and GBO7 have been marketed commercially as Kodiak® and Epic® respectively by Gustafson Inc. Plano TX US for use on several crops (Gardener and Fravel 2002).

Azo-Green™ a product of *Azospirillum brasilense* Corrig sold by the Company Genesis Turfs and Forages is used for grasses in Utah (Okon and Labandera-Gonzalez, 1994). Yield-increasing

bacteria (YIB), which have been in commercial development for over 20 years in China, have been used to increase yields of many crops (Shen, 1997; Glick, 1995). BioGrow® which is made up of *Pseudomonas fluorescens* (Flügge) Migula, yeast, *B. amyloliquefaciens* Priest *et al.* and *B. subtilis* has been utilized in Vietnam for rice production (Nguyen *et al*., 2003) and BioPower® whose composition is not known is in use in Pakistan (Malik *et al*., 2002).

1.2 Mechanisms of plant growth promotion

Microbial inoculants improve plant health and productivity through enhancement of host nutrition and growth, antagonism of pests and pathogens and stimulation of host plant defense mechanism (Jacobsen *et al.,* 2004; Kloepper *et al*., 2004). Dobbelaere *et al*. (2003) and Vessey (2003) reviewed the mechanisms by which PGPR promote plant growth. A number of free-living bacteria have the ability to fix nitrogen and increase its availability for plants (Husen, 2003). Indole-3-acetic acid (IAA) produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots which enhance absorption of water and nutrients from the soil (Van Loon *et al*., 1998; Vessey, 2003). Phosphate solubilization increases available phosphorus for the plants in soils with large amounts of clay-bound phosphates (Alagawadi, 2006). Bacteria release phosphates by secretion of organic acids and phosphatases that solubilise and mineralize phosphates and make them available to plants (Kim *et al*, 1997; Alagawadi, 2006). Under iron limiting conditions**, s**iderophore-producing bacteria sequester the limited amount of iron in the soil and transfer it to the microbial cells, thereby reducing its availability for growth and proliferation of the pathogens (Alexander and Zuberer 1991; Jagadeesh, 2006). Bacteria also inhibit proliferation of pathogens through production of antimicrobial compounds such as antibiotics, hydrolytic enzymes and hydrogen cyanide (Glick and Bashan, 1998). Productivity of cereal crops can be enhanced by application of selected novel *Bacillus* strains that combine nitrogen fixation with the production of plant growth promoting substances (Beneduzi *et al*., 2008a). Numerous *Bacillus* and *Paenibacillus* strains express multiple plant growth promoting activities. A number of *Bacillus* strains have already been commercially developed as biological fungicides, insecticides, nematicides and plant growth promoters (Beneduzi *et al*., 2008b). Gardener (2004) reviewed the use of these strains in agriculture. Although many species of

Bacillus and *Paenibacillus* have been found to contribute greatly to plant growth and health there are relatively few studies on these spore formers in the Republic of South Africa.

1.2.1 Biological nitrogen fixation

Biological nitrogen fixation (BNF) is the enzymatic reduction of the atmospheric dinitrogen (N_2) to ammonia catalyzed by nitrogenase (Kennedy and Tchan, 1992; Pedraza, 2008). Global contribution of BNF from both terrestrial and marine sources is estimated to be between 200-300 metric tones of fixed nitrogen per year (Galloway *et., al*, 1995; Karl *et al*, 2002). Plants require nitrogen for formation of chlorophyll and synthesis of proteins and other organic compounds for their growth and sustainability (Kumar, 2006). Plant-associated nitrogen fixing bacteria (diazotrophs) have been considered as one of the possible alternatives to inorganic nitrogen fertilizers for promoting plant growth and increasing yields of agricultural crops (Ahmad *et al.*, 2006; Pedraza, 2008). The first associative diazotroph was reported by Beijerinck in 1925 after which several genera have been reported to have diazotrophs (Dobbelaere *et al*., 2003; Kennedy *et al.,* 2004). These genera include: *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella* and *Pseudomonas* (Dobbelaere *et al*., 2003; Ahmad *et al,* 2006). The ability of free-living bacteria to fix nitrogen was demonstrated by the capability of certain bacteria to grow in what were considered to be N_2-free media and by Kjeldahl determination of increases in total nitrogen contents of incubated soils or culture media (Willis *et al*., 1996). Nitrogen fixation by these bacteria and cyanobacteria has been observed in the laboratory, field and greenhouse (Borsht*et al*., 1993; Boddey *et al*., 2001; Lucy *et al*., 2004; Cakmakci *et al*., 2006; Ahmad *et al*., 2008). Increases in crop yields have been observed in many plants after inoculation with these free-living diazotrophs. However, the amount of nitrogen fixed is believed to be much lower than in symbiotic systems and estimation of the amount of nitrogen fixed in the field has been problematic (James, 2000; Dobbelaere *et al*., 2003; Kennedy *et al*., 2004; Unkovich and Baldock, 2008). Nitrogen fixation in associative systems is determined by temperature, low oxygen, soil water, combined fertilizer nitrogen and availability of carbon substrates (Unkovich and Baldock, 2008).

Unkovich and Baldock (2008) reviewed the various methods used in field measurements of the quantity of nitrogen fixed in associative systems and their limitations. Plant growth promotion by

microorganisms has been attributed to a number of microbial mechanisms in addition to BNF. However, there are a number of cases where evidence exists that their stimulation of plant growth was due to their ability to fix nitrogen (Vessey, 2003) (Table 2.1).

Table 2.1 Plant growth promoting bacteria for which evidence exists that their stimulation of plant growth was due to their ability to fix nitrogen (Adapted from Vessey, 2003)

Plant Growth Promoting bacteria	**Relationship to host**	**Host crop**	**Country**	**References**
Azospirillum	Rhizospheric	Maize	Argentina	de Salamone *et al.*, 1996
		Wheat	Brazil	Boddey *et al.,* 1986
		Rice	Pakistan	Malik *et al.,* 1997
Azoarcus	Endophytic	Kallar grass	Germany	Hurek *et al.,* 2002
		Sorghum	Germany	Stein *et al*., 1997
		Rice	Germany	Egener *et al*., 1999
Azotobacter spp.	Rhizospheric	Maize	India	Pandey *et al.,* 1998
		Wheat	Serbia	Mrkovacki and Milic, 2001
Bacillus polymyxa	Endophytic	Rice	Egypt	Omar *et al.,* 1996
*Cyanobacteria**	Rhizospheric	Rice	Bangladesh	Hashem, 2001
		Wheat	Serbia.	Obreht *et al*.., 1993
Burkholderia spp.	Endophytic	Rice	Brazil	Baldani *et al*., 2001
Gluconacetobacter. diasotrophicus	Endophytic	Sorghum	Italy	Isopi *et al*., 1995
		Sugarcane	Brazil	Boddey *et al*., 2001
		Sugarcane	USA	Sevilla *et al*., 2001
Herbaspirillum spp	Endophytic	Rice	Philippines	James *et al* 2002
		Sorghum	Brazil	James *et al.,* 1997
		Sugarcane	Brazil	Pimentel *et al*., 1991

1.2.2. Phosphate-solubilization

Phosphorus is second to nitrogen as an essential mineral nutrient for crop production comprising about 0.2% of plant dry matter (Alagawadi, 2006; Mittal *et al.,* 2008). Root growth is regulated by phosphorus availability and in early stages of plant growth its availability leads to formation of abundant roots that grow deeper into the soil (Alagawadi, 2006; Lynch, 2007). Soil phosphorus is in the form of poorly soluble mineral phosphates such as tricalcium phosphate, dicalcium phosphate, rock phosphate and hydroxyapatite (Inorganic phosphates) which are not available for nutritional transport and assimilation (Dobbelaere *et al*., 2003; Alagawadi, 2006). An alternative form of phosphorous is found in organic matter (Organic phosphates) which is a high molecular weight material. The two forms of phosphorus must be transformed into soluble ionic and low molecular weight organic phosphate and made available to plants (Tao *et al*., 2008). Several heterotrophic and chemotrophic bacteria, fungi, actinomycetes and algae have the capacity to solubilise phosphates and make them available to plants. They have been reported to solubilise varying quantities depending on strains (Rodriquez and Fraga, 1999; Alagawadi, 2006; Ahmad *et al.,* 2006; Ahmad *et al*., 2008; Mittal *et al*., 2008; Tao *et al*., 2008). They produce organic acids which chelate the cationic portion of the insoluble phosphate compounds (Illmer *et al*., 1995; Jones, 1998). This results in acidification of the surrounding soil, releasing soluble orthophosphate ions (H_2PO_4 and HPO_4) that can be readily taken up by plants (Dobbelaere, 2003). Strains of *B. licheniformis* Chesterand *B. Amyloliquefaciens* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other acids identified among phosphate-solubilizing bacteria include glycolic, malonic, oxalic and succinic acids (Illmer and Skinner, 1992, 1995). The contribution of phosphate solubilizing bacteria to plant growth has been demonstrated in a number of experiments and species of *Bacillus* are among the rhizobacteria that have been implicated (Chabot *et a*l., 1996a**;** 1996b; de Freitas *et al*., 1997; 1998; Pal, 1998; Kumar and Narula, 1999; Bent *et al*., 2002; Cakmakci *et al.*, 2006)**.** Experiments performed with phosphate solubilizing diazotrophs are few and results obtained are diverse, varying according to plant or bacterial species (Dobbelaere *et al.,* 2003).

1.2.3 Production of siderophores

Siderophores are low-molecular weight extra-cellular compounds with very high affinity for ferric iron (Glick and Bashan, 1997; Jagadeesh, 2006). Under iron-limiting conditions, siderophores sequester iron with a high specific activity and transport it into the microbial cells making it unavailable to pathogens (Briat, 1992; O'Sullivan and O'Gara, 1992; Glick and Bashan, 1997; Dwivedi and Joshi, 2003). They transfer ions from where concentrations are low in soils in a form that the ions cannot be used by pathogens thereby reducing their number or activity (Thomashow and Weller, 1990; Loper and Henkels, 1997; Jagadeesh, 2006). Siderophores produced by pathogenic fungi have a lower affinity for iron than those produced by PGPR (Schippers *et al*., 1987). *Bacillus* strains produce siderophores which have been implicated in the control of a number of pathogenic fungi (Husen, 2003; Ahmad *et al.,* 2006; Ahmad *et al*., 2008; Yobo, *et al*., 2011). Biocontrol of pathogens by production of siderophores by *Pseudomonas* species against *Pythium* and *Fusarium* species has been demonstrated by some researchers (Loper and Buyer, 1991; Duijff *et al,*. 1994; Whipps, 2001).

1.2.4 Production of phytohormones

The production of phytohormones by PGPR has been proposed as one of the mechanisms, besides nitrogen fixation, to explain plant growth-promotion (Glick, 1995; Dobbelaere *et al*., 2003; Husen, 2003; Vessey, 2003; Ahmad *et al.,* 2006; Ahmad *et al.*, 2008). Phytohormones are plant growth regulators which are organic substances that influence physiological processes of plants at extremely low concentrations (Dobbelaere *et al*., 2003). Production of auxins, cytokinin-like substances and gibberellin-like substances were proposed for *A. brazilense*, since the increased number of root hairs and lateral roots observed after inoculation could be mimicked by the application of a mixture of indole-3-acetic acid (IAA), kinetin and gibberellic acid (GA_3) (Tien *et al*., 1979). Other studies have also proposed that increased plant growth observed after inoculation with *Azospirillum* was due to bacterial phytohormone production (Fuentes-Ramirez *et al*., 1993; Dobbelaere *et al*., 1999; Ahmad *et al*., 2006). Indole acetic acid stimulates cell expansion, division and differentiation in plants (Ryu and Patten, 2008).

The most common well-characterized and widely studied growth regulator is IAA (Brick *et al.,* 1991; Husen, 2003; Ahmad *et al.*, 2006). Indole-3-acetic acid is a naturally occurring auxin with broad physiological effects (Pedraza, 2008). It has been implicated in plant pathogenesis and plant growth promotion (Patten and Glick, 1996). It has been estimated that 80% of bacteria isolated from the rhizosphere can produce IAA (Patten and Glick 1996). The first evidence for the role of IAA in plant growth promotion was demonstrated by Harari *et al* (1988). They showed that inoculation of wheat (*Triticum aestivum* L.) with *A. brazilense Cd* produced the same effects (increased root length, increased number of root hairs and lateral roots) as application of pure IAA to the roots. Isolation and quantification of IAA from diazotrophic rhizosphere bacteria has been studied by many researchers (Brick *et al.,* 1991; Patten and Glick, 1996; Husen, 2003; Ahmad *et al*., 2006; Ahmad *et a*l., 2008). A number of *Bacillus* species were found to also influence plant growth through production of IAA (Pal *et al*., 2001, Myongsu *et al*, 2005; Rajkumar and Freitas, 2008).

1.2.5 Biological control of pathogens

Phytopathogens can reduce crop yields by 25-100% (Glick and Bashan, 1997; Kulkarni, 2006). Pesticides are used to reduce these losses. However, these pesticides are hazardous to animals and humans and may persist and accumulate in natural ecosystems (Glick and Bashan, 1997; Niranjan *et al.,* 2003). The use of microbial strains has been suggested as an alternative or supplementary mechanism to chemical control (Handlesman *et al.,* 1990; Berger *et al*., 1996; Sherga and Lyon, 1998). In previous years, research has demonstrated that microorganisms can act as natural antagonists to plant pathogens (Chet and Inbar, 1994; Bacon *et al*., 2001). They play an important role in plant disease control (Mathre *et al*., 1999). Glick and Bashan (1997) reviewed the mechanisms by which PGPR control plant pathogens. These include out-competing the pathogen for niche and nutrients, physical displacement, secretion of siderophores to prevent their proliferation in the rhizosphere, synthesis of antibiotics (low-molecular metabolites that inhibit growth and activity of the pathogen), enzymes that degrade their cell walls and biopriming of systemic resistance of plants. Biocontrol agents may be applied by, seedling root dip, seed coating, and incorporation into the growth rooting medium or as a foliar spray (Kim *et al.*, 1997; Schisler *et al*., 2004).

The most popular bacterial genera studied and exploited as biocontrol agents include: *Agrobacterium*, *Bacillus*, *Pseudomonas and Streptomyces*, (Cook, 1993; Larkin and Fravel, 1998; Ahmad *et al.,* 2008). *Bacillus* and *Pseudomonas* antagonize plant pathogens by antibiosis, site competition, and production of siderophores, HCN, NH_3, hydrolytic enzymes, fluorescent pigments, antifungal volatiles and biopriming of host plant resistance (Glick and Bashan 1997; Ryder *et al*., 1999; Gardener *et al*., 2000; Pal *et al*., 2001; Dobbelaere *et al.,* 2003; Ahmad *et al*., 2008). A number of *Bacillus*-based biocontrol products are commercially available in the United States (Gardener and Fravel, 2002; Schisler *et al*., 2004) (Table 2).

1.2.6 Multi-strain microbial inoculants

Non-symbiotic diazotrophs are genetically diverse, occupy a wide range of habitats and exhibit a variety of plant growth promoting mechanisms (Kennedy *et al*., 2004). A multi-strain inoculant may be required to obtain maximum effects on plant growth. Multi-strain inoculation has been found to produce better results than single strains in some cases (Naseby *et al*., 2000; Kumar *et al*, 2001; Chandanie *et al.*, 2006). Research reports indicate that combinations of BCAs and PGPR increase disease suppression (Guetsky *et al*., 2002), enhance plant nutrient uptake and subsequently improve crop yields (Glick, 1995,; Glick and Bashan, 1997; Dobbelaere *et al*., 2003). For example application of *Azospirillum* with *Rhizobium* resulted in increased nitrogen fixation, greater number of nodules and yield increases in legumes (Iruthayathas *et al*., 1983; Rai, 1983; Sarig *et al*., 1986). A 10-20% increase in crop yield was reported in field trials using a combination of *B. megaterium* deBary and *A. chroococcum* Beijerinck (Brown, 1974). Co-inoculation of *Bacillus* sp. and fluorescent *Pseudomonas sp.* controlled collar rots, root rots and wilting of maize (Pal *et al*., 2001). Co-inoculation of *Vigna radiata* (L.) R. Wilczek T44 with *Bradyrhizobium* Jordan along with other rhizosphere bacteria gave better results than those inoculated with *Bradyrhizobium* alone (Ahmad *et al.,* 2006). Co-inoculation of *Bacillus* Isolate B69 with *Trichoderma atroviride* SYN6 gave a plant growth promotion of 43% and increased nitrogen concentration in leaves of bean seedlings over non-inoculated control plants in the greenhouse (Yobo *et al*., 2011).A multi-strain inoculant biofertilizer BioPower is in use in Pakistan (Malik *et al*., 2002).

Another multi-strain inoculant biofertilizer, BioGro, made up of *Pseudomonas florescens*, yeast and *B. amyloliquefasciens* increased rice yields and straw in Vietnam by 21% and 3.5%, respectively, over non-inoculated control (Kennedy *et al*, 2004).

1.3 The genus *Bacillus*

The genus *Bacillus* belongs to the family Bacillaceae and contains the rod-shaped aerobic or facultatively anaerobic spore formers (Priest, 1993). *Bacillus* species have a ubiquitous distribution in soil, water and air with the majority inhabiting the soil (De Freitas *et al*., 1997; Gardener, 2004). Variation in nutrient utilization, motility and physiochemical growth optima allow these bacteria to inhabit diverse niches in agro-ecosystems (Brock and Madigan, 1991; Gardener, 2004). Most species can survive as saprophytes in soils, where most viable cells occur as inactive spores (Nicholson, 2002). Multiple species can be recovered as epiphytes and endophytes of plants and animals as well as from foodstuffs and compost (Slepecky and Hemphill, 1992; Stahly *et al.*, 1992; Priest, 1993). *Bacillus* species are isolated by pasteurizing the samples at 80°C for 10 minutes to remove vegetative cells (Foldes *et al*., 2000). The majority of strains grow well on commercial nutrient agar and tryptone soy agar (Ahmad *et al.,* 2006; Beneduzi *et al*., 2008). *Bacillus* species are identified using their morphology as described in Bergy's Manual of Determinative Bacteriology (Holt, 1993, Holt, *et al*., 1994) and by physiological and biochemical tests (Kim *et al*., 1997). They are aerobic or facultatively anaerobic, endospore-forming gram-positive flagellated rods which appear singly, in pairs or in chains (Priest, 1993). *Bacillus* species have several advantages over other bacteria due to their long shelf life resulting from their ability to form endospores and the broad spectrum activity of their antibiotics (Kim *et al*., 1997; Cavaglier *et al*., 2005). They have been used extensively in agriculture both for plant growth promotion and as biocontrol agents (Shen, 1997, Ryder *et al*, 1999; Niranjan *et al*., 2003; Dobbelaere *et al*., 2003; Vessey, 2003). Increases in crop yields have been observed in many plants after inoculation with these diazotrophs. *Bacillus* isolates fixed nitrogen and increased the growth of sugar beet under greenhouse conditions (Cakmakci *et al*., 2006). *Bacillus* species increased shoot and root growth of rice (Beneduzi *et al*., 2008) and increased plant biomass, root length, plant nitrogen and phosphorus content of wheat (Hafeez *et al*., 2006), increased yields of rice (Khan *et al*., 2003), wheat (de Freitas, 2000), sugar beet and barley (Cakmakci *et al*., 2001) and maize (Pal, 1998).

A number of *Bacillus* strains referred to as yield increasing bacteria (YIB) have been used in agricultural production in China for over 20 years (Shen, 1997; Niranjan *et al*., 2003). *Bacillus* strain L324-9 with ability to control many pathogenic fungi was registered as a commercial biofertilizer in 1998 for use on turf grass (Kim *et al*., 1997; Mathre *et al.,* 1999). A number of *Bacillus* species can control plant pathogens (Turner and Bockman, 1991; Kim *et a*l., 1997). Several members of *Bacillus* produce antibiotics and secondary metabolites against pathogenic microorganisms (Leifert *et al*., 1995; Foldes *et al*., 2000). *Bacillus subtilis* has been used for many years to control pathogens and increase crop yields. It was produced commercially by Gustafson Inc. TX as Quantum-4000 for use on peanuts and has been available since 1983 (Turner and Bockman, 1991). *Bacillus* strain L324-9 controlled take-all disease caused by *G. graminis* var. *tritici*, *Rhizoctonia* root rot caused by *R. solani* and *Pythium* root rot of wheat caused by *P. irregulare* Buisman and *P. ultimum* Trow (Mathre *et al.,* 1999). *Bacillus subtilis* Strain RB14 suppressed damping-off of tomato (*Lycopersicon esculentum* L.) caused by *Rhizoctonia solani Kühn* (Asaka and Shoda, 1996). *Bacillus* isolates B69 and B81 inhibited the growth of *R. solani in vitro* (Yobo *et al*., 2005). A number of *Bacillus* strains reduced take-all diseases, *Rhizoctonia* root rot of wheat and stimulated wheat seedling growth in Australia (Ryder *et al.,* 1999). Several studies have shown the ability of *Bacillus* strains to control damping-off diseases of diverse crops (Handlesman, *et al*., 1990; Mahaffee and Backman, 1993). Five *Bacillus* isolates inhibited the growth of *Fusarium oxysporum* f. sp. *conglutinans* (Wollenw) Snyder and Hansen up to 90% *in vitro* and reduced disease incidence and severity by 10-49% in cabbage seedlings (Kidane, 2004). (Cawoy *et al*., 2011) reviewed *Bacillus*-based commercial biocontrol products in several countries. A number of microbial products are commercially available in South Africa under the names Eco-Bt, Eco-T Eco-77 Bb plus, Bb weevil and Yieldplus but, information on commercial *Bacillus*-based microbial products is scarce.

Table 2.2 Examples of selected *Bacillus*-based plant disease biocontrol commercial products in USA (Gardener and Fravel, 2002; Schisler *et al*., 2004)

Product name	**Company**	***Bacillus* component**	**Formulation type**	**Primary target**
Serenade®	AquaQuest, Davis, CA	*B. subtilis* QST 713	WP, Aqueous suspension	Fungi Bacteria Multiple vegetables, Fruits
Ecoguard®	Novozymes Salem VA	*B. lichenformis* SB3086	Flowable	*Sclerotinia homoecarpa* on turf
Kodiak®	Gustafson, Plano TX	*B. subtilis* GBO3	WP (Conc.) flowable	Fungi on cotton, large-seeded legumes, soybeans.
Yield Shield®	Gustafson Inc.	*B. pumilus* GB34	WP (Conc.)	Fungi on soybean
Bio Yield®	Gustafson Inc.	B. *amyloliquefaciens* GB99+*B. subtilis* GB122	Dry flake	Fungi on bedding plants in potting mixes
Subtilex®	Bekerunderwood Ames, IA	*B. subtilis* MB1600	WP(Conc.)	Fungi on cotton, large-seeded legumes, soybeans
HistickL+Subtilex®	Bekerunderwood Ames IA	*B. subtilis* MB1600+*Rhizobium*	Flowable	Fungi on soybean, peanut
Epic®	Gustafson Inc	*B. subtilis*	WP (Conc.) flowable	Root pathogens, damping-off fungi, *Fusarium* spp., *Rhizoctonia* spp.
Companion®	Growth Products	*B. subtilis GBO3 other B. subtilis* *B. lichenformis* *B. megaterium*	Liquid	Damping-off fungi in greenhouse and nursery

WP = Wettable powder; (Conc.) = Concentrate

1.4 Benefits and limitations of biofertilizers in agriculture

The use of biofertilizers in agriculture has several advantages. They are farmer friendly, environmentally sound and cost-effective (Shen, 1997; Kennedy *et al*., 2004). Use of biofertilizers minimizes the use of chemical fertilizers. In previous research, results obtained with biofertilizers in combination with reduced chemical fertilizers were not significantly different from those with the full amount of fertilizer recommended for various crops (Adenesemoye *et al*., 2009; Akbar *et al*., 2011). Ten microbe-based biofertilizers in the Philippines which are used for the production of rice, maize and other crops have reduced the use of chemical fertilizer by 30% to 50% (Mansalud, 2008). Inoculation of wheat with *Azotobacter* reduced the crop's nitrogen requirement by 50% under greenhouse conditions (Soliman *et al*., 1995; Hegazi *et al.,* 1998). Use of biofertilizers therefore, can reduce the cost of crop production without compromising the crop yields. Biofertilizers improve and maintain soil fertility. Many biofertilizers have been found to promote plant growth and suppress soil borne pathogens at the same time (Kloepper *et al*., 1997; Ryder *et al*., 1999). Biofertilizers degrade soil contaminants, synthesize compounds that decrease plant stress hormone levels and chelate and deliver key plant nutrients (Gehard *et al*., 2009). Self-replication of the microorganisms circumvents the need for repeated application as is the case with chemical fertilizers. Target organisms do not develop resistance (Gloud, 1990; Shen. 1997).

Limitations of biofertilizers include inconsistencies in the results obtained especially in field trials (Dobbelaere *et al*., 2001; 2003). Very little nitrogen is contributed to the plants in the case of free-living bacteria (Chalk, 1991; Rao *et al*., 1998; Kumar, 2006) so that they have to be used in combination with reduced amounts of chemical fertilizers for better results. Some of the rhizobacteria are host specific (Boddey *et al*., 1991). Some are rhizosphere-incompetent due to environmental effects such as non-optimal temperature and moisture level (Tyler *et al*., 2008) such that after their introduction into the rhizosphere their populations decrease to insignificant levels.

1.5 Scope and potential for the application of biofertilizers

The effects of plant growth promoting diazotrophic bacteria on plant performance and yield increase under nutrient limiting conditions have been discussed for many years (Jha *et al*., 2008, 2009). The interest in these bacteria intensified from the 1970s and 1980s with the discovery of endophytic diazotrophs in graminaceous plants such as sugarcane, maize, wheat and rice (Baldani *et al*., 1997; Dobbelaere *et al*., 2003). Okon and Labandera-Gonzalez (1994) reviewed the results of field experiments with *Azospirillum* inoculants in many countries over a period of twenty years. These results showed that the inoculants were capable of increasing yields of agriculturally important crops from 5% to 30%. A number of rhizosphere bacteria have been developed as biofertilizers and biofungicides to minimize excessive use of inorganic fertilizers and protect the environment and plant health (Kennedy *et al*., 2004; Ahmad *et al.,* 2006). Microbial products have been developed for agricultural purposes and are commercially available worldwide (Shen, 1997). The first US product consisting of a PGPR strain Quantum (*B. subtilis)* is available commercially (Gardener and Fravel, 2002; Niranjan *et al*., 2003; Schisler *et al*., 2004). Gardener and Fravel, (2002) and Schisler *et al*. (2004) reviewed *Bacillus*-based products available in the USA (Table 2.2). Yield increasing bacteria, a commercial product of a multi-strain microbial agent made up of *Bacillus brevi Gordon et al., B. cereus Frankland*, *B. coagulans Hammer*, *B. firmus*, *B. lichenformis*, *B. sphericus and B. subtilis*, has been used for crop production for more than a decade in China (Mei *et al*., 1990; Tang, 1994; Shen, 1997). Ten microbe-based biofertilizers available on the market in the Philippines which are used for the production of rice, maize and other crops have reduced the use of chemical fertilizer by 30% to 50% (Mansalud, 2008). In Indonesia there are 41 commercial biofertilizers in use (Husen, *et al*., 2007). There are many reports worldwide on continuous research on the effects of PGPR which include laboratory, greenhouse and field experiments (Okon and Labandera-Gonzalez, 1994). The role played by other bacteria and Cyanobacteria genera on plant growth and health is in continuous investigation. Laboratory and greenhouse experiments have been very successful whereas field results have been inconsistent (Dobbelaere *et al*., 2001)).The variability in the performance of these PGPR has been associated with various environmental factors that may affect their growth and effects on plants (Ahmad *et al*., 2008).

Plant growth promoting rhizobacteria are constantly being screened worldwide to identify strains that are rhizosphere-competent for commercialization both as biofertilizers and biopesticides. *Bacillus* has been extensively investigated both for plant growth promotion and as a biological control agent over the years in many countries**.** *Bacillus* species have been tested and developed into commercial biofertilizers in a number of countries including China, India, Indonesia and USA (Cawoy *et al*., 2011). Its importance stems from the fact that it is the most abundant of the soil bacteria, is easy to isolate, rhizosphere competent, spore-bearing which makes it stress resistant thereby prolonging its shelf life and the spores are easy to produce in large quantities (de Freitas *et al.*, 1997; Kim *et al.*, 1997; Cavagalier *et al*., 2004). Microbial inoculants may be applied as seed treatments, foliar sprays or soil amendments (Creus *et al*., 1996; Islam and Bora, 1998; Singh *et al*., 1999; Niranjan *et al*., 2003). Soil amendment with microbial inoculants has been found to produce better results (Kennedy *et al*., 2004; Kidane, 2004; Niranjan *et al*., 2004).

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CHAPTER TWO

In vitro screening of free-living diazotrophic bacteria for multiple plant growth-promotion and biological control traits

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Abstract

Two hundred and fifty bacterial isolates were screened *in vitro* for plant growth-promotion and biocontrol traits which included the production of ammonia, siderophores, phytohormones, hydrogen cyanide, phosphate solubilization and antifungal activity. Quantitative analysis for the same traits was carried out with the twenty most promising isolates. Biological nitrogen fixation (BNF) was quantified indirectly using the acetylene reduction assay (ARA), while indole-3-acetic acid (IAA) production was estimated using Salkowiski's reagent. Siderophore production and tricalcium phosphate solubilization were tested using Chrome Azurol S (CAS) agar and Pikovskaya's Medium, respectively. The presence of hydrogen cyanide (HCN) was detected using picric acid and sodium carbonate solutions. Antimicrobial activity was tested using *Rhizoctonia solani* Kühn. The results obtained showed that all twenty isolates produced ethylene ranging from 28.4-62.2 nmol l^{-1} h^{-1} although there was no significant ($P > 0.05$) difference between the amounts produced by the different isolates. The amount of IAA produced was significantly different ($P = 0.001$) among the isolates and ranged from 1.6 mg ml^{-1} to14.1 mg ml^{-1}. All isolates produced siderophores, indicated by halos with diameters that differed significantly ($P = 0.001$), ranging from 7 mm to 36 mm. One isolate was positive for phosphate solubilization, and none produced HCN. The isolates tested displayed multiple growth-promoting traits. These results suggest that free-living bacteria enhance plant growth through multiple plant growth-promoting mechanisms.

Key words: Plant growth promotion; phosphate solubilization; siderophore; IAA; BNF; biocontrol

2.1 Introduction

Significant increases in vegetative growth and grain yield following inoculation with non-symbiotic bacteria has been observed in a number of non-leguminous crops (Kennedy *et al*., 2004) Yield increases were observed in rice (*Oryzae sativa* L.) (Yanni and El-Fattah, 1999), wheat (*Triticum aestivum* L.) (Hegazi *et al*., 1998) and cotton *Gossypium hirsutum* L. (Anjum *et al*., 2007) following inoculation with *Azotobacter* species. Okon and Labandera-Gonzalez (1994) reviewed the effects of *Azospirillum* Corrig inoculation on growth and yield of a wide range of crops under field and greenhouse conditions over a period of twenty years in various countries. Other non-symbiotic bacteria found to influence plant performance include the genera *Acetobacter Beijerinck* on sugarcane *(Saccharum officinarum* L.) (Boddey *et al*., 1991; Lee *et al*., 2002), *Azoarcus* Anders *et al.* on Kallar grass (*Leptochloa fusca* (L.) Kunth) (Reinhold-Hurek *et al*., 1993), and rice (Hurek, 2002), *Burkholderia* Palleron and Holmes on sugarcane (Reis *et al*., 2000), *Herbaspirillum* Baldani *et al*. on rice, sugarcane, sorghum (*Sorghum bicolor* L.) and wheat (Baldani *et al*., 2000; James *et al*., 2000; Kennedy and Islam 2001). The genera *Bacillus* Cohn, *Beijerinckia* Beijerinck, *Klebsiella* Trevisan, *Pseudomonas* Migula *and Serratia* Bizio have also been found to exert beneficial effects on various crops (Glick, 1995; Dobbelaere *et al*., 2003; Kennedy *et al* 2004; Joseph *et al*., 2007; Ahmad *et al*., 2008). These bacteria inhabit the rhizosphere, rhizoplane and phyllosphere of plants and some live as endophytes inside the plants. The exact mechanisms by which they affect the performance of plants are not clearly understood (Dey *et al*., 2004). Initially growth responses observed in plants were attributed to the ability of these microbes to fix nitrogen and supply it to the plants (Christiansen-Weneger, 1992; Boddey and Dobereiner, 1995). However, subsequent research revealed that besides BNF, other mechanisms also contributed to the growth responses observed in non-leguminous plants (Glick 1995; Dobbelaere *et al*., 2003; Vessey, 2003). These mechanism include: phosphate solubilization (Gaur, 1990; De Freitas *et al*., 1997; Richardson and Hadobas, 1997; Rodriquez and Fraga, 1999; Richardson, 2001; Chen *et al*., 2006; Rodriquez *et al*., 2006), siderophore production (Glick and Bashan, 1997; Husen, 2003, Ahmad *et al*. 2008; Yobo *et al*., 2011), and the production of phytohormones (Glick, 1995; Patten and Glick 2002; Zahir *et al*., 2004).

Indirectly they were found to inhibit the deleterious effects of other pathogenic microorganisms by production of siderophores and hydrolytic enzymes (Loper and Buyer, 1991; O´Sullivan and O´Gara, 1992; Elad, 1996; Glick *et al*., 1998; Yobo *et al*., 2011), antibiotics (Shanahan *et al.,* 1992; Leifert *et al*., 1995; Asaka and Shoda, 1996 ; Kim *et al*., 1997), HCN (Flaishman *et al*., 1996; Bloemberg and Lugtenberg, 2001; Ahmad *et al*., 2008) and by priming for systemic resistance in plants (Gaur, 1990; De Freitas *et al.,* 1992; Boddey and Dobereiner, 1995; Glick, 1995; Dobbelaere *et al.,* 2003; Vessey, 2003). Several bacteria have been found to display multiple plant growth promoting activities through which they influenced plant growth directly, indirectly, or by synergistic effects (Khalid *et al*., 2003; 2004; Dey *et al*., 2004; Ahmad *et al*., 2006; 2008; Cakmakçi, 2007; Egamberdieva, 2008).

In vitro screening techniques have been employed to select effective strains of bacteria with multiple plant growth-promoting and biocontrol traits (Husen, 2003; Khalid *et al*., 2003; Ahmad *et al*., 2006; 2008; Cakmakci, 2007; Engamberdieva, 2008). These techniques are simple and efficient for screening large numbers of isolates (Campbell, 1989). Although a lack of consistency in correlation between the results obtained *in vitro* and *in vivo* has been reported, (Chanway and Holl, 1993; Williams and Asher, 1996; Yobo *et al*., 2011), some authors have reported a positive correlation (Askew and Laing, 1994; Glick, 1995; Noel *et al*., 1996; De Boer *et al*., 1999; Zhang *et al.,* 1999; Khalid *et al*., 2004; Yobo *et al.,* 2011). The variability observed in field results has been attributed to environmental conditions or competition from the indigenous soil microbial flora (Khalid *et al*., 2004; Ahmad *et al.,* 2008). However, a combination of *in vitro* and *in vivo* screening can lead to identification of effective strains for sustainable agriculture. The current study was therefore aimed at identifying bacterial strains with multiple plant growth-promoting traits for use in crop production.

2.2 Materials and methods

2.2.1 Sample collection and isolation of bacteria.

Soil samples were collected from the rhizosphere of maize (*Zea mays* L.) and wheat from different sites by uprooting the root system and placing them in plastic bags for transport to the laboratory. They were stored at 4 C for subsequent analysis. Excess soil was shaken off and the soil adhering to the plant roots was collected from each soil sample. Ten grams of each soil sample were transferred to a 250 *ml*-Erlenmeyer flask containing 90 *ml* sterile distilled water and shaken at 150 rpm in an orbital shaker incubator[1] for 30 minutes. Serial dilutions were made from 10^1-10^5 cfu ml^{-1}. These dilutions were heated in a water bath at 100°C for 15 minutes. Aliquots of 0.1 ml were taken from dilutions of 10^3–10^5 cfu ml^{-1} and spread on plates containing Burke's N-free medium in triplicate. The medium contained in a liter of distilled water: glucose, 10.0g, KH_2PO_4, 0.14g, K_2HPO_4, 0.52g, Na_2SO_4, 0.05g, $CaCl_2$, 0.2g, $MgSO_4.7H_2O$, 0.1g, $FeSO_4.7H_2O$**,** 0.0025g $Na_2MoO_4.2H_2O$ and agar, 15.0g, (pH, 7 ± 0.1) (Myongsu *et al*., 2005). The plates were incubated for 7 days at 28°C. Leaf samples were cut into small pieces and disinfected in 0.2% sodium hypochlorite followed by 70% ethanol and ground using a sterile mortar and pestle. Serial dilutions were made in the same way as the soil samples and plated on the same medium. Morphologically different colonies were sub-cultured, purified and stored in 30% glycerol at -80°C for subsequent use. Bacteria colonies on each plate were counted for an estimation of the bacteria population in each soil and leaf sample. Morphological and biochemical studies were carried out for basic characterization of the isolates. The various isolates were screened for plant growth-promoting activities *in vitro*. Quantitative analysis was carried out with twenty most promising isolates. Gram's reaction and KOH tests were done to distinguish between gram-positive and gram-negative bacteria. Endospore staining was done to identify *Bacillus* species. Bacterial cultures were sent to the DNA sequencing facility at Stellenbosch University[2] for

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[1]Shalom Laboratory Supplies c.c.,132 Commercial Road, International Plaza Durban 4001, P. O. Box 57030 Musgrave Road , Durban 4062.

[2] Central analytical facilities DNA Sequencing Unit, Stellenbosch University, Private Bag X1 Matieland, 7602, Republic of South Africa.

sequencing. Species identity was also determined using a MALDI Biotyper Real-time Classification (RTC) using MALDI Biotyper 3.0 from Bruker Daltonics.[3]

2.2.2 Biochemical characterization of bacterial isolates

Bacterial isolates were biochemically characterized using Gram's reaction, a KOH test, endospore-staining and a lactose fermentation test.

2.2.3 Ammonia production

Bacterial isolates were tested for ammonia production in peptone water, as described by Cappuccino and Sherman (1992) and Ahmad *et al*. (2008). Freshly grown cultures were inoculated into 10 ml of peptone water prepared per liter of distilled water10.0g, peptone and 5.0g sodium chloride, adjusted to pH 7.0 in a 100 *m*l conical flask and incubated for 48 hours at 28ºC in an orbital shaker incubator at 150 rpm. Nessler's Reagent (0.5*ml*) was then added to each flask after incubation. The cultures were observed for development of a brown to yellow color as a positive test for the presence of ammonia. The amount of nitrogen fixed was determined indirectly by the Acetylene Reduction Assay (ARA) (Rennie, 1981). In this procedure a pure culture of each isolate was inoculated into a semi-solid nitrogen free medium in a vial, closed with rubber septum and aluminum cap and incubated for 72 hours at 28ºC in an orbital shaker incubator. An airtight syringe was used to draw out one milliliter (*ml*) of gas from the vial. One *ml* of acetylene was injected into each of the vials. The vials were then incubated for 24 hours at 28ºC. One *ml* gas samples were taken from each vial and analyzed for amount of ethylene formed using a Gas Chromatography.

2.2.4 Hydrogen cyanide production

The method described by Miller and Higgins (1970); Wu *et al*. (2005) and Ahmad *et al.* (2006) was used to detect the presence of HCN produced by bacterial isolates. Tryptone soy agar was amended with glycine at 4.4 g l^{-1}. Bacteria were inoculated onto the modified agar plates.

[3] Bruker Daltonics Inc., 40 Manning, Road Billerica, MA 01821.,USA

A Whatman filter paper No.1 was soaked in a mixture of picric acid and sodium carbonate solution (12.5g Na_2CO_3 + 2.5g Picric acid in 1000 *ml* of distilled water) and placed onto the agar. The plates were sealed with parafilm and incubated for 2-4 days at 28ºC.

The plates were observed for development of orange to red color which is a positive test for the presence of HCN. The reactions were scored as: Weak-yellow to brown; Moderate-brown; Strong-reddish brown.

2.2.5 Indole-3-acetic acid production

The modified method of Brick *et al*. (1991) was followed, as described by Sarwar and Kremer (1995); Husen (2003) and Ahamad *et al.* (2006); (2008). A culture of the test isolate was inoculated in 100 *ml* flask with 10 *ml* tryptone soy broth amended with 500 mg of L-tryptophan and incubated for 48 hours at 28ºC. The cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2 *ml*) was mixed with 4 *ml* Salkowiski's reagent (50 *ml* of 35% perchloric acid and 1 *ml* of 0.5M ferric chloride), and 2-3 drops of orthophosphoric acid and incubated at room temperature for 25-30 minutes. The tubes were observed for development of a pink color, which is a positive test for IAA. For quantitative analysis of IAA, the optical density of the mixture was taken at 530 nm using a Milton Roy Spectronic 301 spectrophotometer after color density reached maximum (after 25-30 minutes). The concentration of IAA produced was estimated using standard graph of IAA obtained in the range 10-100mg ml^{-1}.

2.2.6 Phosphate solubilization

Bacterial isolates were tested for phosphate solubilization using Pikovskaya's medium (Husen, 2003; Ahmad *et al*., 2008), in a liter of distilled water 10.0g glucose, 5.0g tribasic phosphate, ($Ca_3(PO_4)_2$) 0.2g KCl, 0.1g $MgSO_4.7H_2O$, 0.002 $MnSO_4.H_2O$, 0.002, $FeSO_4.7H_2O$, 0.5g yeast extract and 15.0 agar, adjusted to a pH of 7.0. A culture of the test isolate was inoculated onto agar plates and incubated for 48 hours at 28ºC. The plates were observed for a clearing zone around the bacterial colonies, which is a positive test for phosphate solubilization. The diameter of the halos formed was measured.

2.2.7 Siderophore-production

Bacteria isolates were tested for siderophore production on CAS Medium (Schwyn and Neilands, 1987; Alexander and Zuberer, 1991; Ahmad *et al*., 2008). Chrome Azurol S agar plates were prepared and divided into equal sectors. The sectors were spot-inoculated with the test isolates in triplicate and incubated for 48-72 hours at 28ºC. The plates were observed for development of a yellow to orange halo around the colonies, which is a positive test for siderophore production. The diameter of the halos formed was recorded.

2.2.8 Antifungal bioassay

The dual culture technique was followed as described by Paulitz *et al*. (1992), Landa *et al*. (1997; Idris *et al.* (2007). Three paper discs dipped in bacterial suspension were placed at the margin of potato dextrose agar (PDA) plates and incubated for 48 hours at 28ºC. Agar plugs (4 mm) of *Rhizoctonia solani* Kühn culture were placed at the center of the plate for each bacterial isolate and incubated for seven days. These were replicated three times. Radii of the fungal colonies towards and away from the bacterial colonies were measured. Growth inhibition was calculated using the following formula,

% inhibition = $(R-r)/R\times100$, where,

r is the radius of the fungal colony opposite the bacterial colony.

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

Isolates with > 30% mycelia growth inhibition against the selected pathogen were considered effective.

2.2.9 Statistical analysis

The data was subjected to analysis of variance (ANOVA) using GenStat 12.1 statistical package. (VSN International, 2011). Treatment mean separation was done using Duncan's Multiple Range Test at 5% level of significance.

2.3 Results

2.3.1 Qualitative analysis

All isolates were rod-shaped. The rods appeared as single cells, in pairs or in chains. Some were motile while others were non-motile. Colony morphology varied with isolates. The qualitative tests indicated that 100% of the isolates were positive for production of ammonia, 75% for indole-3-actic acid, 98% for siderophore production, 0.4% for phosphate-solubilization and 60% inhibited fungal growth *in vitro*. The twenty most promising isolates were selected for biochemical characterization and quantitative analysis for multiple plant growth-promoting traits. Results for the morphological and biochemical characterization are presented in Table 2.1 and Figures 2.1 to 2.5.

2.3.2 Quantitative analysis.

2.3.2.1 Acetylene reduction assay

All twenty isolates produced varying amounts of ethylene. However, there was no statistical significant difference ($P > 0.05$) between the amounts of ethylene produced by the different isolates. The greatest volume (62.2 nmol l^{-1} h^{-1}) was produced by Isolate BS612 and the least (28.4 nmol l^{-1} h^{-1}) by Isolate BL1 (Table 2.2).

2.3.2.2 Indole acetic acid production

All the twenty isolates produced IAA. There was a significant difference (P = 0.001) between the amounts produced by different isolates but Isolate BS10 produced the greatest amount (14.5mg ml^{-1}) and Isolate BS612 the least (1.6 mg ml^{-1}) (Table 2.2 and Figure 2.1).

2.3.2.3 Siderophores Production

All isolates formed yellow to orange halos around the bacteria growth, indicating the presence of a siderophore. There was a significant difference (P = 0.001) between the diameters of siderophore halos formed by different isolates. The widest diameter was created by Isolate BS36 (30.7 mm) while the smallest halo was created by Isolate BL1 (7 mm) (Table 2.2 and Figure 2.3)

2.3.2.4 Phosphate solubilization

Phosphate solubilization was only detected in the plates inoculated with Isolate BS431. A clearing zone was observed around the bacterial growth with a diameter of 30 mm (Figure 2.2)

2.3.2.5 Antifungal bioassay

The twenty isolates were tested for antimicrobial activity against *R. solani*. The level of inhibition varied with each bacterial isolate, while some of the isolates (Isolate BS7) had no biocontrol activity. The greatest inhibition (88%) was observed with Isolate BS10 and the least (40%) with Isolate BL5 (Figure 5).

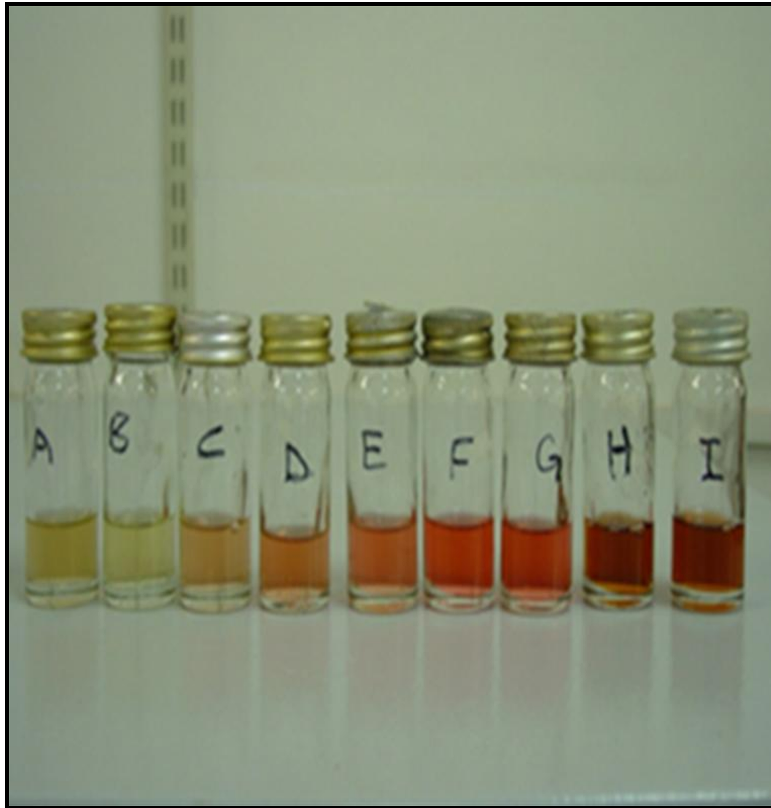

Figure 2.1: Indole-3-acetic acid production.
A,C,D,E,F,G,H and I: Range of colours resulting from different amounts of indole-3-acetic acid produced by different bacterial isolates; B: Control.

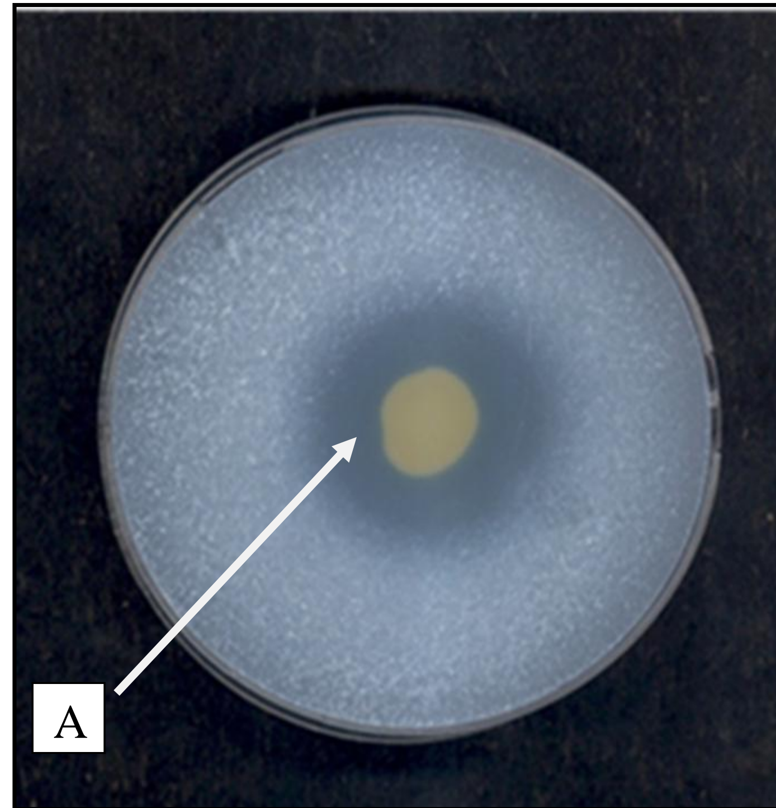

Figure 2.2: Phosphate solubilization
A: A clear zone produced by Isolate BS431 indicative of phosphate solubilization

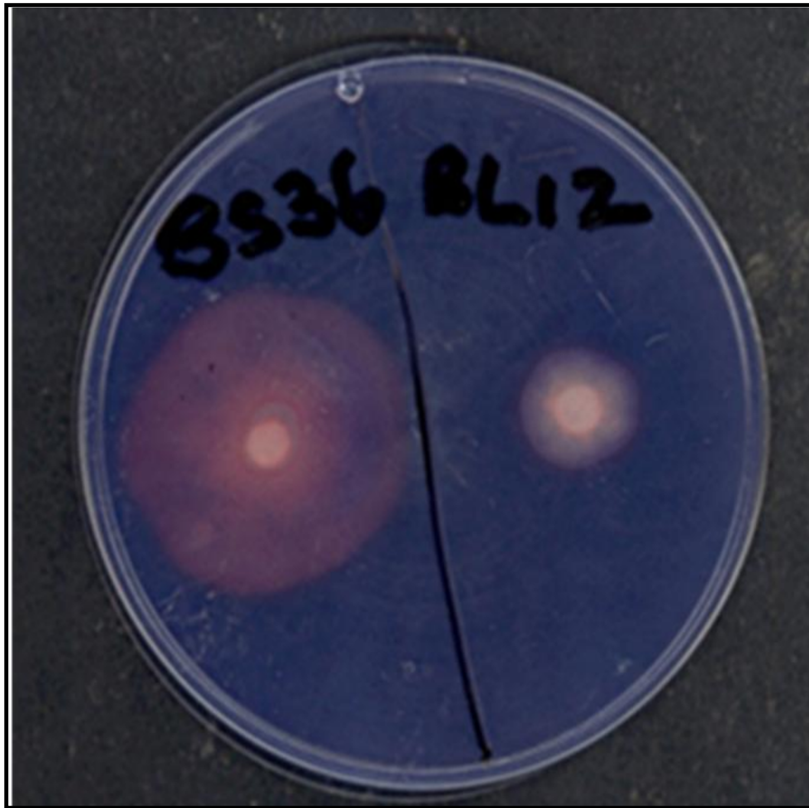

Figure 2.3: Siderophore production by Isolates BL12 and BS36

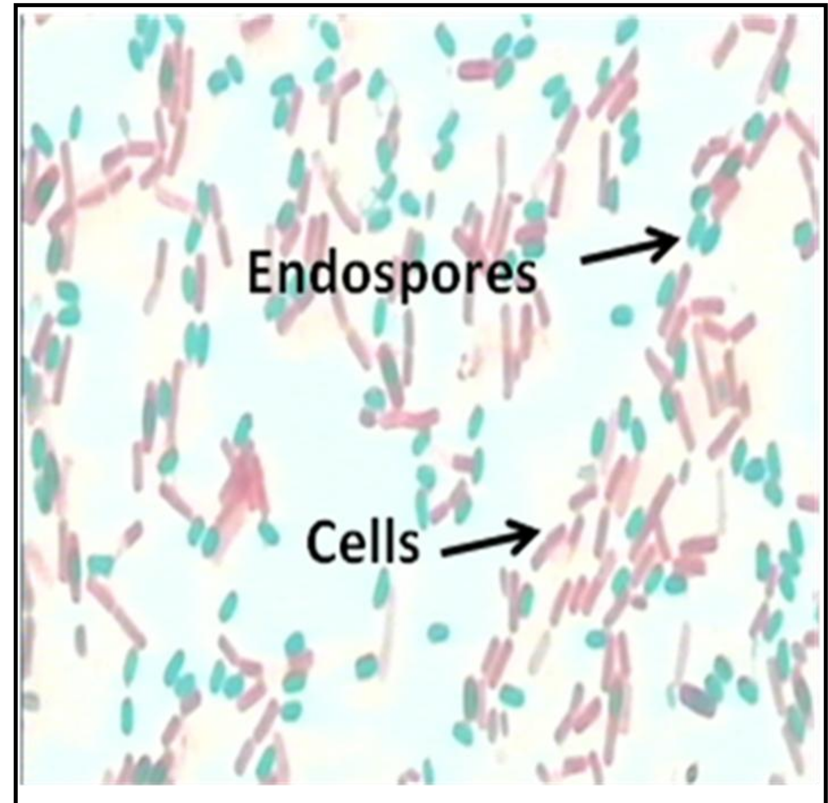

Figure 2.4: Bacterial endospores formed by Isolate BL5

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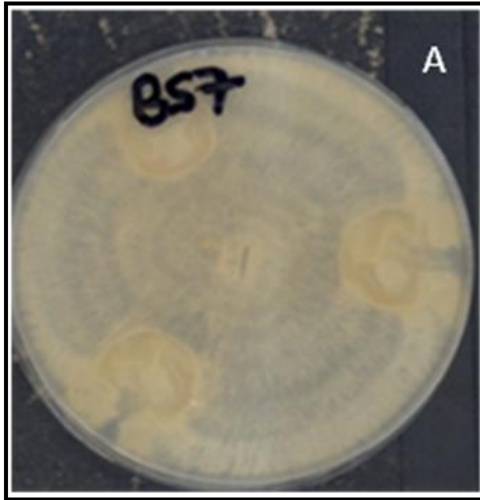

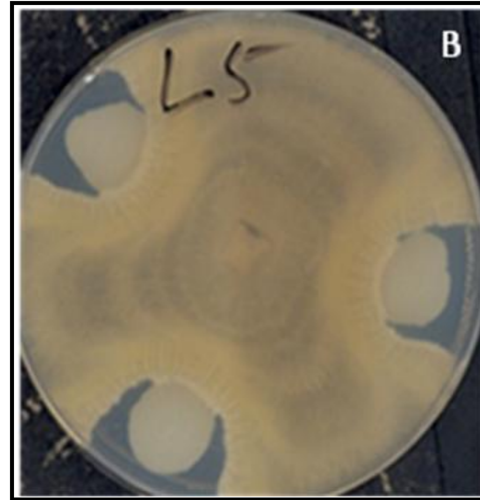

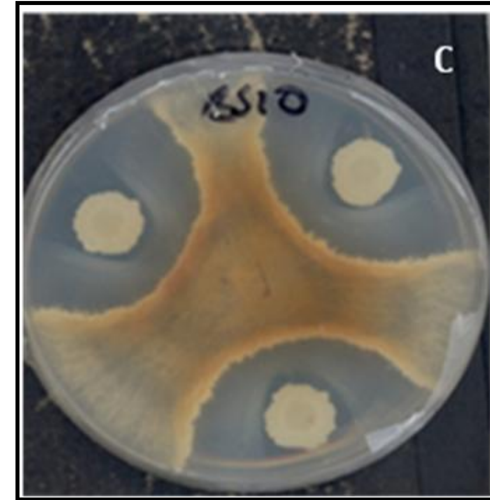

Figure 2.5: Antifungal activity of bacterial isolates against *Rhizoctonia solani.*

A: Isolate BS7: No zone of inhibition indicative of lack of antifungal activity

B: Isolate BL5: Zone of inhibition indicative of antifungal activity

C: IsolateBS10: Zone of inhibition indicative of antifungal activity

Table 2.1: Morphological and biochemical characteristics of selected bacterial isolates

Isolate	Colony morphology	Gram's reaction	KOH reaction	Endospore formation	Motility	Lactose fermentation	Genus	Species
BS1	Round, flat with smooth margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*cereus*
BS16	Round, raised with smooth margin	Negative	Negative	Absent	Motile	Negative	*Proteus*	*vulgaris*
BS36	Round, smooth, grows into medium	Negative	Negative	Absent	Motile	Negative	*Proteus*	*vulgaris*
BS37	Round, raised smooth margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*thuringiensis*
BS43	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS44	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS49	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS431	Round, flat undulate margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS69	Round umbonate with serrated margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*subtilis*
BS612	Round, smooth , grows into medium	Negative	Negative	Absent	Motile	Negative	*Proteus*	*vulgaris*
BS7	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS713	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS817	Round umbonate with serrated margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*subtilis*
BS820	Round umbonate with serrated margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*subtilis*
BS914	Round, raised smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*
BS10	Round umbonate with serrated margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*subtilis*
BL1	Round, raised with smooth margin	Negative	Negative	Absent	Motile	Negative	*Serratia*	*liquefasciens*
BL3	Round flat with serrated margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*subtilis*
BL5	Round, flat with smooth margin	Positive	Positive	Present	Motile	No reaction	*Bacillus*	*cereus*
BL12	Round, raised with smooth margin	Negative	Negative	Absent	Non-motile	Positive	*Klebsiella*	*pneumoniae*

Table 2.2: Production of siderophore, ethylene and IAA by selected bacterial isolates

Siderophore			Ethylene			IAA Production		
Bacterial isolate	**Halo diameter (mm)**		**Isolate**	**C2H2 (nmol l^{-1} h^{-1})**		**Isolate**	**IAA (mg ml^{-1})**	
BS1	7.00	a	BL1	28.4	a	BS612	1.6	a
BL1	9.00	ab	BS36	35.6	a	BL5	2.1	b
BL5	9.7	abc	BS817	35.7	a	BS817	2.1	b
BS820	9.7	abc	BS820	37.6	ab	BS1	2.4	b
BS914	10.7	bcd	BL5	39.0	ab	BL1	2.5	bc
BS44	11.3	bcde	BL3	39.0	ab	BS820	2.7	c
BS37	11.3	bcde	BS431	39.3	ab	BS7	3.1	d
BL3	12.0	bcdef	S12	43.1	ab	BS36	7.8	e
BS612	12.3	bcdefg	BS16	45.4	ab	BS713	9.2	f
BS431	12.7	cdefg	BS43	46.3	ab	BS44	11.5	g
BS49	12.7	cdefg	BS1	46.7	ab	BS16	11.7	g
BS713	13.3	defg	BS914	46.7	ab	BS12	11.7	g
BS7	13.7	defg	BS37	46.7	ab	BS49	12.4	h
BS43	14.3	efg	B69	47.4	ab	BS69	12.7	hi
BS10	15.3	fg	BS49	48.4	ab	BS43	12.9	i
BS12	15.3	fg	BS7	48.8	ab	BS914	13.0	i
BS817	15.7	g	BS713	50.9	ab	BL3	13.0	i
BS16	20.7	h	BS44	51.1	ab	BS37	13.7	j
BS69	24.0	i	BS10	51.3	ab	BS431	14.1	k
BS36	30.7	j	BS612	62.2	b	BS10	14.6	l
F-Value	**28.6**			**1.0**			**2169.9**	
P-Value	**0.001**			**0.5**			**0.001**	
S.E.D.	**1.5**			**10.4**			**0.2**	
CV%	**12.8**			**28.6**			**2.1**	

Means in a column followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test

2.4 Discussion

Nitrogen fertilizers are a major input in crop production globally. Plants utilize nitrogen in the formation of chlorophyll, proteins and amino acids for their growth and sustainability (Kumar, 2006). All isolates in the current study grew well on nitrogen-free media, formed ammonia in peptone water and reduced acetylene to ethylene. Similar observations were made in previous studies (Cappuccino and Sherman, 1992; Ahmad *et al.,* 2006; 2008). This confirmed the ability of the best 20 isolates to produce nitrogenase, which is a characteristic of diazotrophic bacteria. The acetylene reduction assay (ARA) used to estimate the amount of ethylene formed in this study has been identified as the simplest and cheapest indirect method for estimating the amount of nitrogen fixed *in vitro* (Brock *et al*., 1994; Unkovich and Baldock, 2008). The 20 isolates belonged to the genera *Bacillus, Klebsiella, Proteus* Hauser and *Serratia*, all of which have been listed as diazotrophic bacteria in the literature (Okon and Labandera-Gonzalez 1994; Glick, 1995; Dobbelaere *et al*., 2003; Ahmad *et al.,* 2006; Joseph *et al*., 2007). Isolates of the two species, *Proteus vulgaris* Hauser and *Bacillus subtilis* (Ehrenberg) Cohn that generated the largest volume of ethylene subsequently increased the biomass and yield of maize, and wheat respectively under field conditions (Chapters 4 and 5). Biological nitrogen fixation is well documented for contributing to growth and yields of plants (Malik *et al*., 1997; Boddey *et al*., 2001; Hurek *et al*., 2002; Cakmakci *et al.,* 2007). It has been estimated that 20-40% of the plant nitrogen requirement of several non-leguminous crops can be supplied through BNF by non-symbiotic microorganisms (Dobereiner, 1997). The isolates in this study significantly increased shoot biomass and yields of maize and wheat under field and greenhouse conditions (Chapters 3, 4 and 5).

Phytohormone-production is one of the mechanisms proposed for growth promotion by bacteria, and IAA is the most common and widely studied phytohormone (Patten and Glick, 1996; Husen 2003; Ahmad *et al*., 2008). Indole acetic acid stimulates cell expansion, division and differentiation (Ryu and Patten, 2008). Indole-3-acetic acid production was detected from all 20 isolates studied. Results exist in the literature on isolation, identification and quantification of IAA from diazotrophic bacteria (Brick *et al*., 1991; De Freitas, 1997; Ahmad *et al*., 2006).

The number of rhizosphere bacteria with ability to produce IAA has been estimated to be 80% (Patten and Glick, 1996) and 60% (Ahmad *et al.,* 2006). In the current study all isolates (100%) produced IAA. A number of bacterial species have been recorded for influencing plant growth by production of IAA (Pal *et al*., 2001; Myongsu *et al.,* 2005; Cakmakci *et al*., 2007; Beneduzi *et al*.,; 2008; Ragjkumar and Freitas, 2008). Khalid *et al*. (2004) demonstrated that there was a positive correlation between the amount of IAA produced and the yield increases observed in wheat, which might apply to these 20 isolates too.

Phosphorus is an important mineral nutrient required by crops (Mital *et al*., 2008; Kundu *et al.*, 2009; Khan *et al.,* 2010). It is required for a wide range of biochemical activities. It also promotes the development of abundant and long roots in plants (Alagawadi, 2006; Lynch 2007). One common problem affecting soil fertility of crops is that in heavy soils with high clay content, phosphates may bind to the clay so strongly that it is not available for uptake by plants. Several microorganisms have the ability to solubilize phosphates that bind to acid clay particles making the phosphate available to plants. The level of phosphate solubilization varies with bacterial strains (Rodriquezi and Fraga 1999; Mittal *et al.,* 2008; Tao *et al.,* 2008). Contributions by phosphate-solubilizing bacteria to plant growth have been demonstrated by a number of researchers (Chabot *et al*., 1996; De Freitas *et al*., 1997; Kumar and Narula, 1999; Cakmakci *et al.,* 2006). In the current study Isolate BS431 (*K. pneumoniae)* had the ability to solubilize phosphates. *Klebsiella* is among the genera identified for acid phosphatase production and the ability to solubilize phosphates (Thaller *et al*., 1995; Rodriquezi and Fraga, 1999; Jha and Kumar, 2007; Kundu *et al.*, 2009). This isolate significantly increased both the dry biomass and the yield of maize relative to the Uninoculated Control under greenhouse and field conditions (Chapters 3 and 4). This suggests that bacterial phosphate solubilization is one of the mechanisms through which bacteria enhance plant growth.

Siderophore-production was exhibited by all isolates under study. Siderophores have been implicated in the control of some pathogenic fungi (Ahmad *et al.,* 2006, 2008; Yobo *et al*, 2011). Plant growth promoting bacteria produce siderophores with a high affinity for ferric ions (Fe^{3+}).

Siderophores bind ferric ions, making them unavailable to pathogenic microorganisms, thus reducing their deleterious effects to plants (Briat, 1992; Glick and Bashan, 1997; Jagadeesh, 2006). Siderophore-production by PGPR *Pseudomonas* strains has been implicated in the promotion of nodulation and yield increases in groundnuts (*Arachis hypogea* L.) (Dey *et al.,* 2004).

Some of the isolates under study inhibited growth of *R. solani in vitro* while others did not show any antifungal activity. The most effective biocontrol isolates were *Bacillus* species. Plant growth-promoting bacteria can enhance plant growth by inhibiting the deleterious activities of pathogenic microorganisms. *Bacillus* isolates have demonstrated their ability to control plant pathogens (Turner and Bockman, 1991; Mahaffee and Bockman, 1993; Kim *et al*., 1997). *Bacillus subtilis* controlled *R. solani* in wheat, brown spot in wheat and damping-off in tomato (*Lycopersicon esculentum* L.). *Bacillus* Strain L324-9 controlled take-all disease caused by *G. graminis var, tritici*, *Rhizoctonia* root rot caused by *R. solani* and *Pythium* root rot caused *by P. irregulare* Buisman and *P. ultimum* Trow (Mathre *et al*., 1999). Several *Bacillus* biocontrol products are available commercially (Schisler *et al*., 2004; Cawoy *et al*., 2011). The most promising biocontrol isolates in this study inhibited the growth of a wide range of fungal pathogens, exhibited a number of biocontrol mechanisms *in vitro* and suppressed the effects of *R. solani* in wheat under greenhouse conditions (Chapters 6 and 7). These isolates were identified as *B. subtilis* species.

The majority of the isolates under study displayed three or more plant growth-promoting activities. These results suggest that non-symbiotic bacteria can influence plant growth and yield through a variety of mechanisms. *In vitro* screening can be a useful tool for selecting bacterial strains with growth-promoting traits.

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CHAPTER THREE

Effects of selected free-living diazotrophic bacteria on growth of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) under greenhouse conditions

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Abstract

Twenty bacterial isolates selected for their multiple plant growth promoting activities *in vitro* were screened for their potential to enhance growth of maize and wheat under greenhouse conditions. Five most promising isolates identified for each crop were tested for their effect on seedling growth of the two crops at reduced nitrogen (N) fertilizer levels under greenhouse conditions. Two isolates identified in a field trial as the most promising for each of the two crops were tested for their potential to enhance plant growth when combined with reduced levels of N fertilizer or co-inoculated with Eco-T® (*Trichoderma harzianum* Rifai) a commercial biocontrol agent, Their effect on root growth of maize was investigated. Parameters studied included chlorophyll level, plant height, shoot dry biomass, and root development. Preliminary screening revealed that all isolates significantly ($P = 0.001$) increased shoot dry biomass relative to the Uninoculated Control in both crops. The five most promising isolates identified for each crop were as follows, Maize: BS431, BS612, BS16, BS36 and BL5, and wheat: BS10, BS69, BS7, BL1 and BL5. Shoot dry biomass of plants inoculated with isolates plus different fertilizer levels increased with increased fertilizer levels, although there was no significant difference between biomass obtained with 35% N and 45% N in both crops. Isolates BS431 and BS10 were the most promising isolates on maize and wheat, respectively. Co-inoculation of these crops with each isolate plus Eco-T® resulted in higher biomass than when either Eco-T® or the bacterial isolates were applied in isolation. Bacterial inoculation increased root length by 6.4%-100.5% and root biomass by 16.4%-27.4% relative to an Uninoculated Control. Shoot dry biomass resulting from bacterial inoculation at levels of 65% N and 50% N for maize and wheat, respectively, was not significantly ($P > 0.05$) different from that of the Fully Fertilized Control, although the fully fertilized plants had the highest biomass. Selected diazotrophic free-living bacteria have the potential to enhance plant growth. When combined with reduced N fertilizer levels, they may reduce N fertilizer requirements without compromising the crop yields.

Key words: Plant growth promotion; dual inoculation; chlorophyll level; biomass; phytohormones; siderophores; phosphate solubilization

3.1 Introduction

Increases in crop production are essential to meet the food requirements of the increasing world population. Poor soil fertility, pests and diseases are major constraints to crop production.
Increased crop production depends on the use of chemical fertilizers for nutrients, and agrochemicals for management of pests and diseases. Besides the high cost of production of these fertilizers and agrochemicals, their continuous use may impact negatively on the environment (Malakoff, 1998; Shrestha and Ladha, 1998**;** Wairiu and Lal, 2003)**.** Reports from previous studies have shown that chemical fertilizer-N use efficiency is low (Garabet *et al.*, 1998; Choudhury and Khanif, 2001; Gyaneshwar; 2002; Halvorson *et al*., 2002). This inefficiency is usually attributed to ammonia volatilization, dinitrification and leaching of nitrates into ground water (Bijay-Singh *et al*., 1995). Use of microorganisms in agriculture has been identified as a cheaper and more environmentally friendly alternative to improve crop production. These beneficial microorganisms maybe isolated from the rhizosphere of plants and formulated into microbial inoculants. These inoculants may be applied as seed treatments, foliar sprays or soil amendments (Creus *et al*., 1996; Islam and Bora, 1998; Singh *et al*., 1999**).** Free-living bacteria are well-documented for significantly increasing plant growth and grain yield of agronomic crops (Fallik*et al*., 1994; Dobbelaere *et al.*, 2002; Kennedy *et al*., 2004; Cakmakci *et al,* .2006; Narula *et al.,* 2006**;** Anjum *et al*., 2007; Rhokzadi *et al*., 2008; Mehnaz *et al.,* 2010). These microorganisms are believed to influence plant growth through improved nutrient availability and acquisition, production of plant growth regulators and suppression or inhibition of plant diseases caused by pathogenic microorganisms (Pal *et al*., 2001; Patten and Glick, 2002; Dobbelaere *et al*., 2001, 2003; Canbolat *et al*., 2006). Enhanced seed germination, seedling vigor, stand health, plant height, root and shoot length, and biomass, increased nitrogen and chlorophyll content and yields, early bloom and increased nodulation in legumes have been observed in plants following bacterial seed inoculation (Bashan, 1998; Muthukumarasamy *et al*., 1999; Balandreau, 2002; Saharan and Nehra, 2011).

Plant growth promoting rhizobacteria are thought to be more efficient under nutrient limiting conditions (De Freitas and Germida, 1992; Shaharoona *et al*., 2008). Improvements in growth parameters resulting from the use of microbial inoculants, combined with reduced doses of chemical fertilizers, have been reported in previous research (Okon and Labandera-Gonzalez, 1994; Biswas *et al*., 2000; Dobbelaere *et al*., 2001; Riggs *et al*., 2001). Use of multiple strains was suggested for optimum results (Kennedy *et al*., 2004). Dual or multi-inoculation with bacterial strains or bacteria in combination with fungi or arbuscular mycorrhiza fungi can yield better results than single inoculations (Belimov *et al*., 1995; Egamberdieva and Höflich, 2004; Lucy *et al*., 2004; Han and Lee, 2005; Ryu *et al*., 2007). The objectives of this study were to investigate the effects of combining bacterial inoculation with reduced levels of N fertilizer, co-inoculation of different bacterial isolates with Eco-T®, and bacterial inoculation on root growth.

3.2 Materials and methods

3.2.1 Source of bacterial cultures

Bacteria isolates used in this study were selected through *in vitro* screening of 250 diazotrophic bacteria for multiple plant growth promoting activities (Chapter 2). Twenty isolates that displayed the most growth promoting activities were selected for secondary screening for enhancement of seedling growth under greenhouse conditions.

3.2.2 Source of seed

The maize seed (*Zea mays* L.) used in these studies was kindly provided by Linkseed Company.[4] The wheat seed *Triticum aestivum L.* (PAN 3434) was supplied by ARC-Small Grain Institute[5]

[4] Link Seed (Pty) Ltd, P.O. Box 755, Greytown, Republic of South Africa,3250

[5]ARC-Small Grain Institute, Private Bag X29, Bethlehem, Republic of South Africa, 9700

3.2.3 Inoculum preparation.

Bacterial cultures were inoculated into tryptic soy broth and incubated for 48 hours at 28ºC in an orbital shaker incubator[6] at 150 rpm. Cells were harvested by centrifuging at 10,000 rpm for 15 minutes at 4ºC. Cell numbers were then adjusted to 10^8 cfu ml^{-1} by dilution method using sterile distilled water. Cell counts were done using a counting chamber and viability confirmed by plate count method. This procedure was repeated for each subsequent experiment.

3.2.4 Seed inoculation

Seeds were disinfected by soaking in 0.02% sodium hypochlorite for 2 minutes, then rinsing them several times in sterile distilled water. Seed inoculation was done by soaking the seed in a bacterial suspension in 2% carboxymethylcellulose (CMC) for two hours to enhance adhesion of the cells onto the seed. For the control the seeds were soaked in a suspension of 2% CMC in sterile distilled water. The seed was then dried under the lamina flow overnight. This procedure was followed for seed inoculation in all other experiments.

3.2.5 Preliminary screening of bacterial isolates for their effects on seedling growth under greenhouse conditions

A randomized complete block design was used. Twenty two treatments consisting of twenty bacterial isolates, and two controls were used. Each treatment consisted of three pots with a top diameter of 200 mm that held 2kg of commercially prepared composted pine bark (CPB) with a pH of 5.5, 75%, water holding capacity 60% air-filled porosity, 650 kg/ton bulk density, 0.57% Nitrogen , 0.18% Phosphorus, 0.38% Potassium, 0.82% Calcium, 16.53% Carbon 0.14% Magnesium, 73mg/kg Zinc and 12293mg/kg Iron. Each pot was seeded with five seeds.

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[6] Shalom Laboratory Supplies c.c., 132 Commercial Road, International Plaza Durban 4001, P. O. Box 57030, Musgrave Road Durban 4062

The pots were kept in the greenhouse with a temperature range of 25-30°C and a relative humidity of 50%-70%. Each treatment and experiment was replicated three times and CPB was used for all greenhouse experiments.

Pots with each of the five isolates were watered daily with an equal amount of a nutrient solution of hydroponics soluble fertilizer containing in g l^{-1} of water NPK, [3:1:3 (38) Complete™], 0.25, micronutrients (Microplex), 0.02 (Ocean Agriculture, Mulder's Drift, South Africa)[7], with phosphorus and potassium levels adjusted to the full amounts recommended for each crop. The Uninoculated Control was watered with tap water and the Fully Fertilized Control with a solution of NPK, [3:1:3 (38) Complete™] at a rate of 1g l^{-1} w/v). The seedlings were thinned to three and five plants per pot for maize and wheat, respectively, after germination. Plants from each pot were harvested at the shoot base after six weeks and kept in brown paper bags. They were then dried at 70°C in an oven for 72 hours and weighed to obtain shoot dry biomass.

3.2.6 Effect of bacterial inoculation on maize and wheat seedlings growth at different N fertilizer levels under greenhouse conditions

The five best isolates for each crop selected from preliminary screening were used in this study. The experimental design was followed as in Section 3.2.5, with seed treated as in Section 3.2.4 above and consisted of 25 treatments. These treatments included three fertilizer doses: 25% N, 35% N and 45% N (as percentage of the full dose recommended by the local Fertilizer Advisory Service)[8], five isolates alone, five isolates plus each of the three fertilizer levels, 100% NPK and a zero Control without isolate or fertilizer. Each pot was seeded with five plants, which were thinned to three for maize, and eight thinned to five for wheat, after germination. The pots were kept in the greenhouse with a temperature range of 25-30°C and watered daily with equal amounts of nutrient solutions as in Section 3.2.5 above.

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[7] Ocean Agriculture (Pty) Ltd. P. O. Box 741, Mulders Drift, Republic of South Africa, 1747.

[8] Fertilizer Advisory Services, KwaZulu-Natal, Department of Agriculture and Environmental Affairs; Pietermaritzburg, Republic of South Africa.

The chlorophyll levels in maize leaves were measured using a chlorophyll meter[9] at the fifth to eighth leaf stage to give a chlorophyll content index (CCI). The plants were harvested at the root base after six weeks, placed in brown paper bags and dried at 70°C in the oven for 72 hours. The dry samples were weighed for shoot dry biomass determination. The experiment was replicated three times and means pooled for statistical analysis.

3.2.7 Effects of single and dual inoculation of Eco-T® and bacterial isolates on seedling growth of maize and wheat under greenhouse conditions.

A dual inoculation method was used to test for *in vitro* compatibility between two most promising isolates, BS612 and BS431 for maize, and BS10 and BL5 for wheat, and Eco-T®. For the seedling trial, maize and wheat seeds were inoculated as in Section 3.2.4 above, with suspensions of Eco-T® in 2% CMC. For each crop five treated seeds maize and eight for wheat were planted in a pot with a top diameter of 200 mm that held 2 kg of composted pine bark. The pots were kept in the greenhouse with a temperature range of 25-30 ºC. The seedlings were thinned to three and five plants per pot for maize and wheat, respectively, after germination. A suspension of each bacterial isolate prepared as in Section 3.2.3 was applied as a drench after germination at a rate of 5 ml per seedling weekly. The plants were watered with a nutrient solution prepared as in section 3.2.5. One control was treated with a solution of 100% NPK and the Uninoculated Control with tap water. After six weeks the plants were harvested at the shoot base, put into brown paper bags and dried in an oven at 70ºC for 72 hours. The samples were weighed to obtain shoot dry biomass. Each trial was replicated three times and the means pooled for statistical analysis.

3.2.8 Rhizotron studies on the effect of bacterial seed inoculation on root development in maize under greenhouse conditions

Maize seeds were treated with bacterial suspension, as in Section 3.2.4 above. Rhizotrons were filled with composted pine bark.

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[9] CCM-200 Plus, Opti-Science Inc., 8 Winn Avenue, Hudson, NH, USA, 03051.

Three rhizotrons were planted, each with three seeds, which were thinned to one plant per rhizotron after germination. The rhizotrons were covered with aluminum foil to protect the roots from direct sunlight. The rhizotrons were kept in a greenhouse with a temperature range of 25-30ºC and watered daily with a nutrient solution prepared as in Section 3.2.5 with NPK at a rate of 0.35g l^{-1} of water. After six weeks plants were harvested and separated into roots and shoots. The roots lengths were measured using a steel-blade retractable tape measure[10]. The roots and shoots were then dried in the oven at 70ºC for 48 hours and weighed to obtain dry biomass. The experiment was replicated three times and means pooled for statistical analysis.

3.2.9 Statistical analysis

The data was subjected to analysis of variance (ANOVA) using GenStat 12.1 statistical package (VSN International, 2011). Treatment mean separation was done using Duncan's Multiple Range Test at 5% level of significance.

3.3 Results

3.3.1 Effect of bacterial seed inoculation on maize and wheat seedling growth under greenhouse conditions.

In preliminary screening, bacterial inoculations significantly (P = 0.001) increased shoot dry biomass relative to the Uninoculated Control in both maize and wheat. The biomass varied with bacterial isolate x crop species interaction and did identify the five different most promising isolates for each crop. The five most promising isolates for maize and wheat were BS36, BS431, BS612, BS16 and BL5, and BL1, BL5, BS7, BS69 and BS10, respectively. Maximum biomass was obtained with BS16 in maize and BS10 in wheat.

[10] Steelbank Merchants (Pty) LTD., 19 Trotter Road, Pinetown, 3610 Pinetown, KwaZulu-Natal, Republic of South Africa.

3.3.2 Response of maize and wheat to bacterial inoculation at different levels of N fertilizer under greenhouse conditions.

Inoculation with bacterial isolates significantly ($P < 0.05$) increased biomass compared to the Uninoculated Control in both crops. Individual isolates plus different levels of N fertilizer gave better results compared to application of isolates alone or the various fertilizer doses applied alone. The biomass obtained with bacterial isolates at 35% N and 45% N levels in wheat were not significantly different. Shoot biomass increased with fertilizer concentration and in both crops the highest biomass was at 45% N. Best results were obtained with Isolate BS431 in maize and Isolate BL5 in wheat (Figures 3.1 and 3.2 and Appendix 3.1).

3.3.3 Effect of single and dual inoculation with Eco-T® and bacterial isolates on maize and wheat seedling growth under greenhouse conditions.

All bacterial isolates were compatible with Eco-T®, except BS10. The combined inoculation of Eco-T® and different bacterial isolates resulted in higher dry biomass, in both maize and wheat, compared to single inoculations with either bacteria or Eco-T®. The greatest dry biomass in maize was obtained with dual inoculation of Eco-T® and Isolate BS612, while single inoculations with Eco-T® had the least biomass. In wheat maximum shoot dry biomass was obtained from co-inoculation of Eco-T® and Isolate BS10. However, maximum biomass was obtained with 100% NPK (Figures 3.3 and 3.4).

Table 3.1 Preliminary screening of bacterial isolates for seedling growth enhancement in maize and wheat under greenhouse conditions.

Maize			Wheat		
Bacterial Isolates	**Shoot dry biomass (g)**		**Bacterial Isolates**	**Shoot dry biomass (g)**	
Uninoculated Control	6.96	a	Uninoculated Control	1.043	a
BS1	12.66	b	BS713	1.240	ab
BS713	13.54	bc	BS43	1.567	b
BS49	13.64	bc	BS49	1.637	b
BL1	13.67	bc	BS44	2.190	c
BL3	13.70	bc	BS37	2.327	c
BS43	13.98	bcd	BS817	2.393	c
25%N	14.58	bcde	BS1	2.447	c
BS44	14.87	cde	25%N	2.447	c
BS10	14.93	cde	BL12	2.543	cd
BS914	15.49	cde	BS36	2.490	cd
BS69	15.53	cde	BS16	2.567	cd
BS7	15.57	cde	BS612	2.580	cd
BL12	15.95	de	BS820	2.583	cd
BS37	16.00	de	BL3	2.590	cd
BS820	16.09	de	BS914	2.597	cd
BS817	16.23	e	BS431	2.617	cd
BS36	16.34	e	BL1	2.648	cd
BS612	16.34	e	BS69	2.687	cd
BS431	16.50	e	BS7	2.710	cd
BL5	16.54	e	BL5	2.720	cd
BS16	16.61	e	BS10	3.073	d
100%NPK	46.76	f	100%NPK	6.657	e
F-Value	**116.1**			**27.3**	
P-Value	**0.001**			**0.001**	
S.E.D.	**0.94**			**0.2**	
CV%	**7.1**			**11.0**	

Means in the same column followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

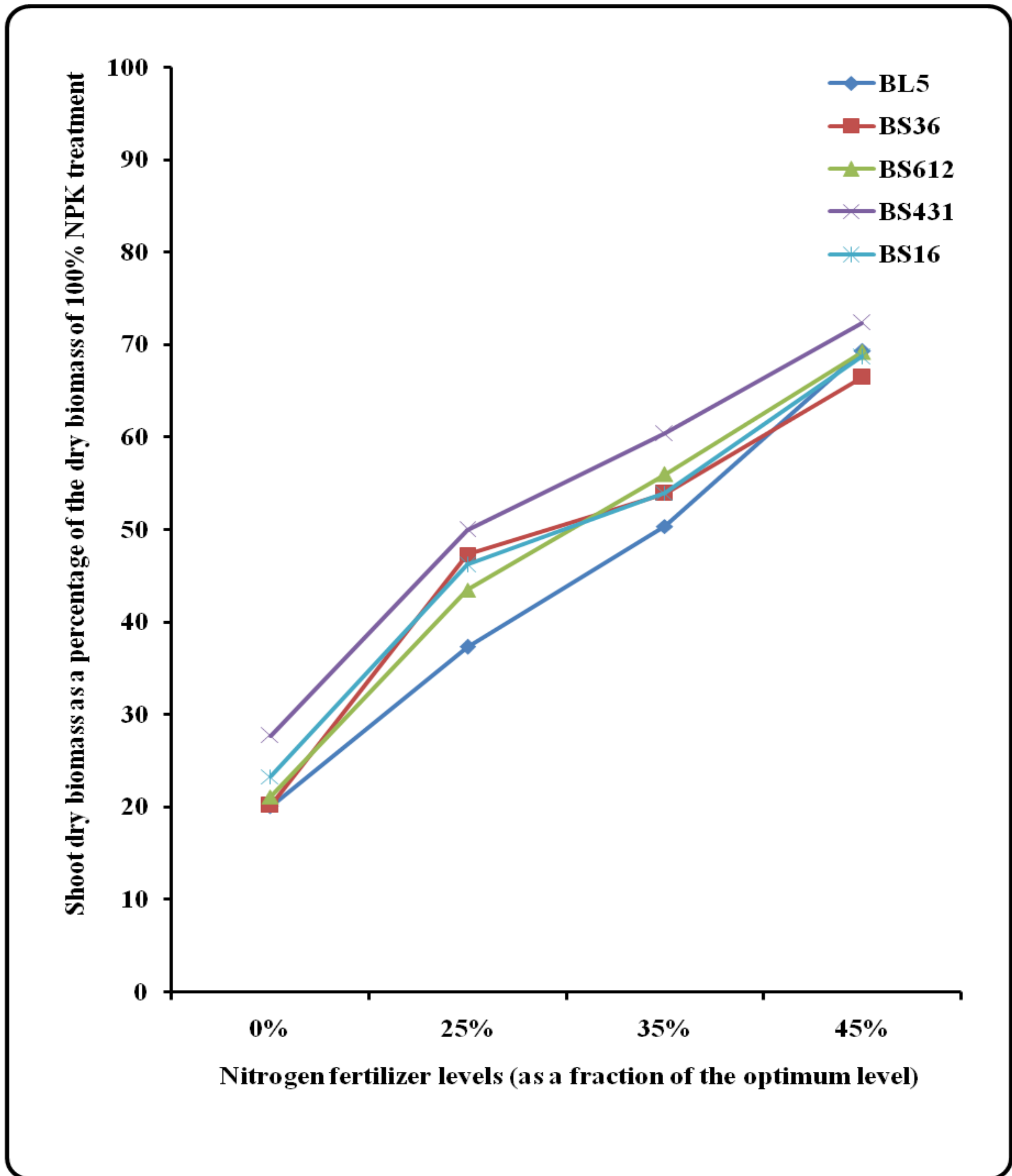

Figure 3.1: Effect of bacterial inoculation at different N fertilizer levels on shoot dry biomass of maize under greenhouse conditions

Nitrogen fertilizer levels as a percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa

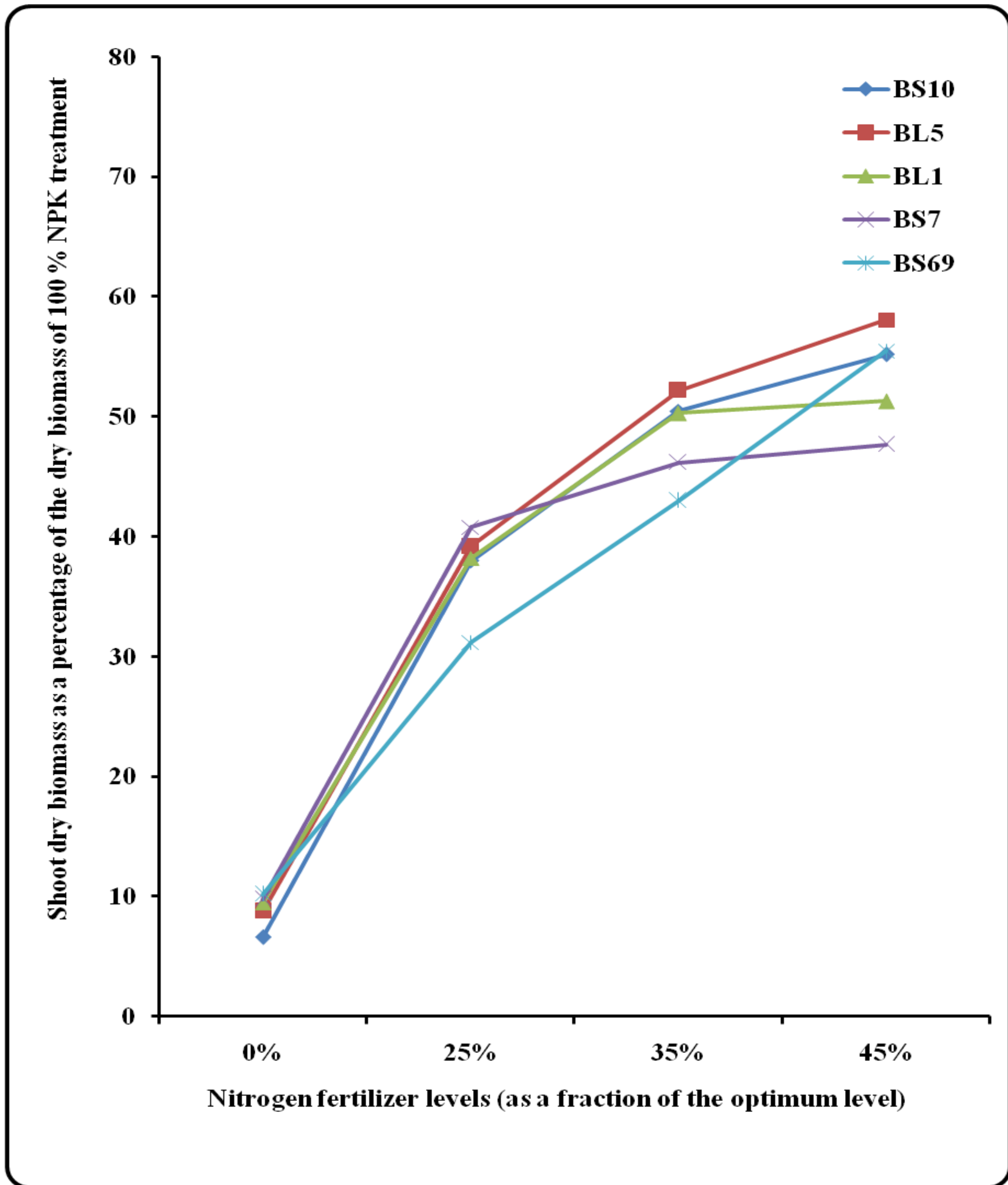

Figure 3.2: Effect of bacterial inoculation at different N fertilizer levels on shoot dry biomass of wheat under greenhouse conditions

Nitrogen fertilizer levels as a percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa

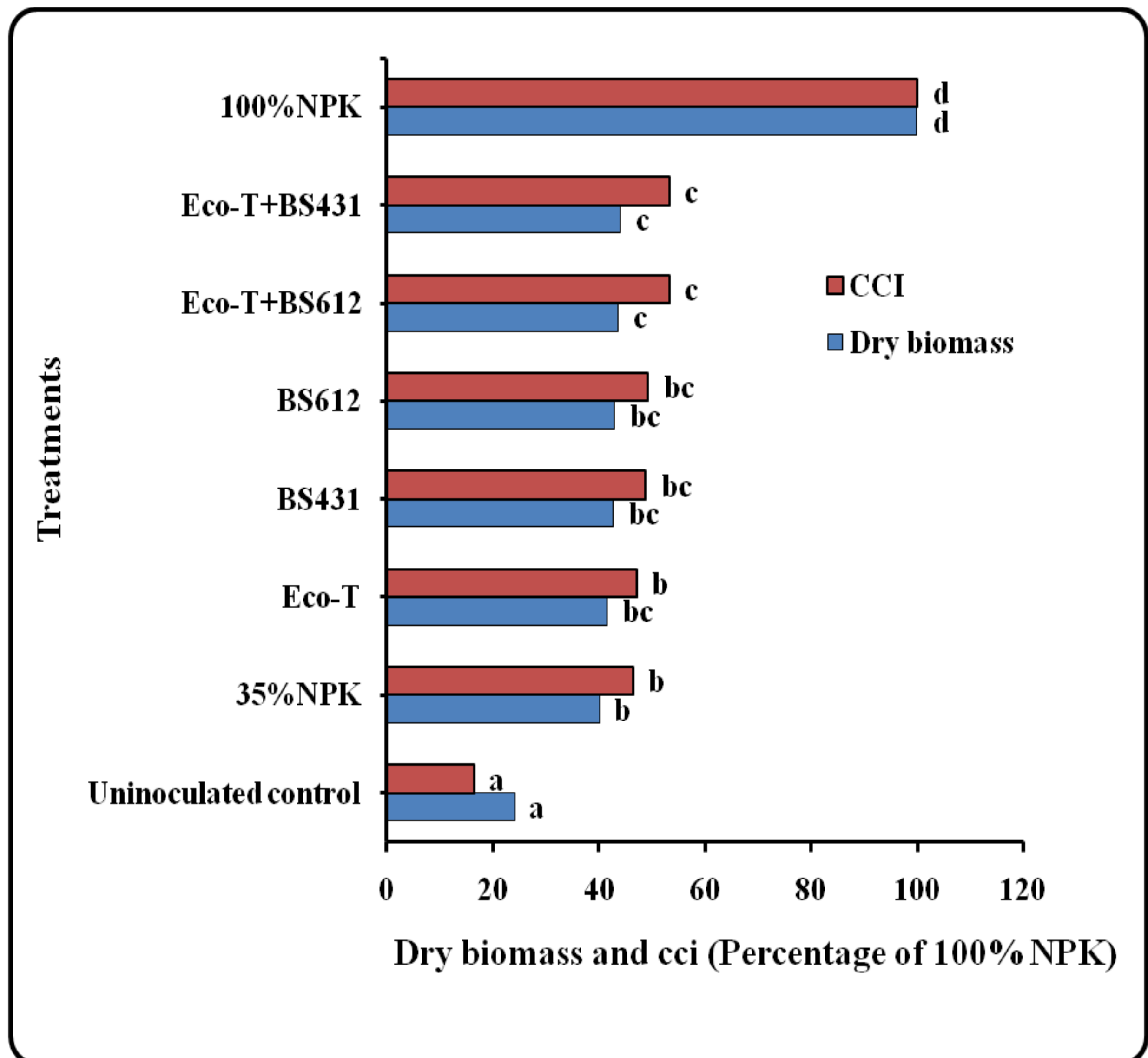

Figure 3.3 Effect of single and dual inoculation of Eco-T® *(Trichoderma harzianum* Rifai) and bacteria (Isolate BS431, (*Klebsiella pneumoniae* (Shcroeter) Trevisan) and Isolate BS612, (*Proteus vulgaris Hauser*) on shoot dry biomass and chlorophyll level (CCI) of maize under greenhouse conditions.

Treatments: Bacterial isolates plus Eco-T®, a commercial BCA; Uninoculated Control: No bacterial isolate or fertilizer applied

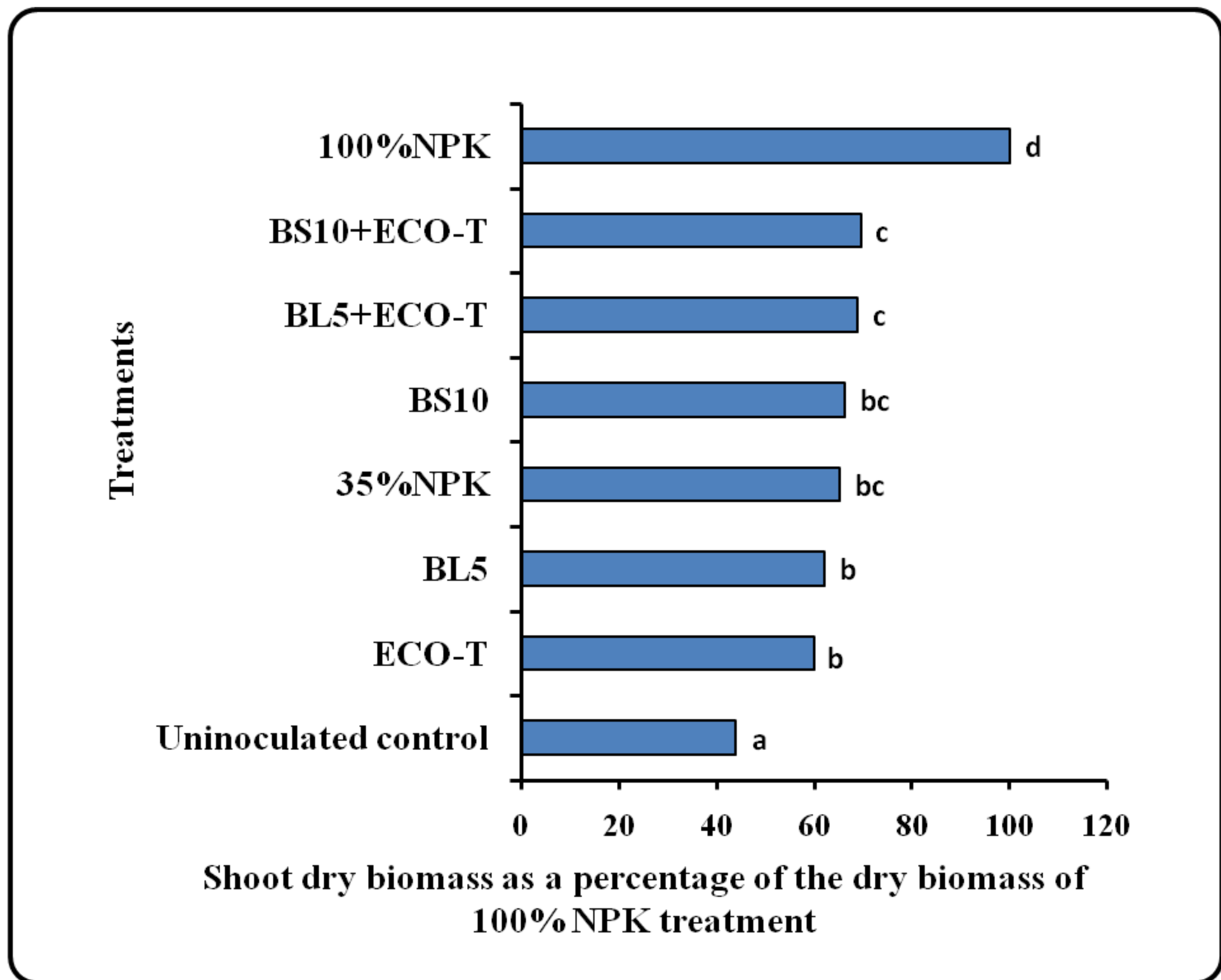

Figure 3.4 Effects of single and dual inoculation of Eco-T® (*Trichoderma harzianum*) a commercial BCA and *Bacillus* isolates (Isolate BL5, (*B. cereus)* and Isolate BS10, (*B. subtilis*) on wheat shoot dry biomass under greenhouse conditions.

3.3.4 Effects of bacterial inoculation on root growth of maize under greenhouse conditions

Root lengths resulting from seed inoculation with different isolates were significantly (P = 0.001) different from each other, from the Uninoculated and the Fully Fertilized Controls. Seed inoculation resulted in longer roots than the Fully Fertilized Control. Maximum length was obtained with Isolate BS612 and minimum length with Isolate BL5.

Root dry biomass of the inoculated plants was significantly (P = 0.001) different from both the Uninoculated and the Fully Fertilized Controls and was highest in the Fully Fertilized Control but lowest in the Uninoculated Control (Table 3.2). There was no significant (P > 0.05) difference in shoot biomass obtained with the various isolates but Isolate BS36 caused the highest and Isolate BL5 the lowest biomass (Table 3.3). Bacterial inoculation resulted in formation of longer roots, numerous lateral roots and root hairs and increased root length by 6.4%-110.3% and root biomass by 16.4%-27.4 above the Uninoculated Control. The Fully Fertilized Control plants developed the maximum root biomass. Isolate BS612 treatment resulted in the greatest length and Isolate BS36 caused the greatest dry biomass (Table 3.2).

Table 3.2 Effects of bacterial inoculation on root growth under greenhouse conditions

Bacterial Isolates+35% N fertilizer	**Root Length (mm)**		**Root Length (%)**	**Root dry biomass (g)**		**Root dry biomass (%)**
35%N	203.300	a	78.20	1.535	a	19.100
Uninoculated control	203.300	a	78.20	0.603	a	8.400
BL5	220.000	a	84.60	1.763	bc	24.800
100%NPK	260.000	b	100.00	7.103	e	100.000
BS431	306.700	c	118.00	2.393	cd	33.700
BS16	410.000	d	157.70	2.340	cd	32.900
BS36	436.700	d	168.00	2.543	d	35.800
BS612	490.000	e	188.50	1.927	bcd	27.100
F-value	**83.920**			**94.28**		
P-Value	**0.001**			**0.001**		
L.S.D	**37.850**			**0.614**		
S.E.D.	**17.650**			**0.286**		
CV%	**6.800**			**14.000**		

Means in a column followed by the same letter are not significantly different from each other at 5% level of significance according to Fisher's L.S.D. test.

3.3.5 Response of maize and wheat to bacterial inoculation at various N fertilizer levels under greenhouse conditions

Plant heights increased with increases in fertilizer level but there was no significant (P >0.05) difference between the inoculated plants at different fertilizer levels and the Fully Fertilized Control.

The heights obtained at the different fertilizer levels alone were lower than those with the combination of bacteria and reduced fertilizer. Isolate BS612 gave greater heights than BS431 at all fertilizer levels (Figure 3.5). The chlorophyll level of the Fully Fertilized Control was significantly ($P = 0.001$) higher than the inoculated plants at reduced fertilizer levels. The chlorophyll levels obtained with different isolates did not differ significantly but the maximum level was obtained with Isolate BS612 at 75% N fertilizer (Figure 3.6). Shoot dry biomass of maize obtained with bacterial inoculation plus 65% N or 75% N was not significantly different from the Fully Fertilized Control. Biomass obtained from plants inoculated with bacteria plus different fertilizer levels was higher than plants treated with the different fertilizer level alone. Isolate BS612 gave greater biomass at all fertilizer levels. In wheat, inoculated plants at different fertilizer levels gave more shoot biomass compared to those with different fertilizer levels alone. Maximum biomass was obtained by application of Isolate BS10 plus 65% N. The biomass obtained with inoculation at 50% N, 65% N or 75% N in wheat were not significantly ($P > 0.05$) different from the Fully Fertilized Control plants although the Fully Fertilized Control plants had the highest biomass (Table 3.3).

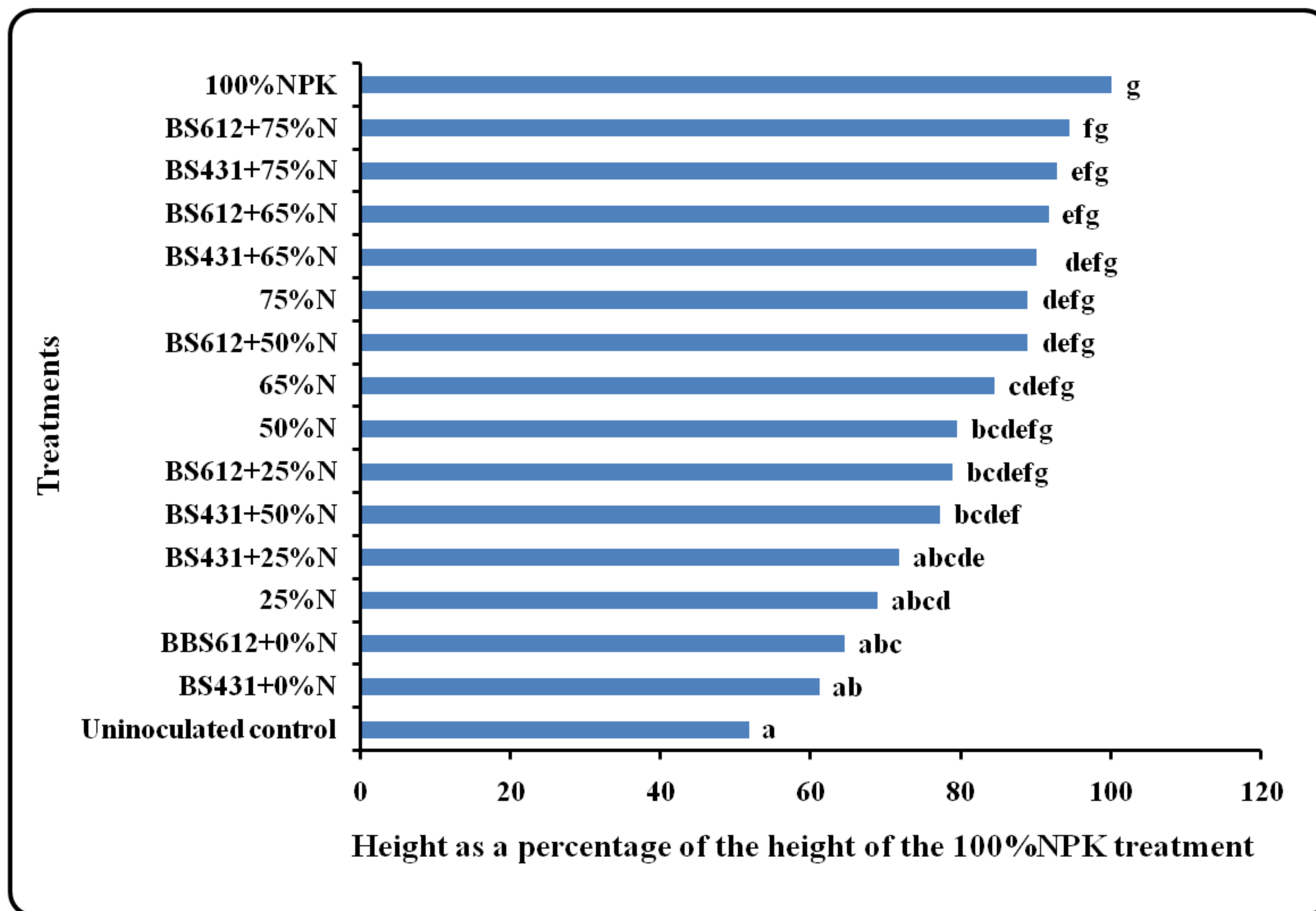

Figure 3.5 Effect of bacterial inoculation at different doses of N fertilizer on plant height in maize under greenhouse conditions

Treatments: Bacterial Isolates plus N fertilizers as a percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa

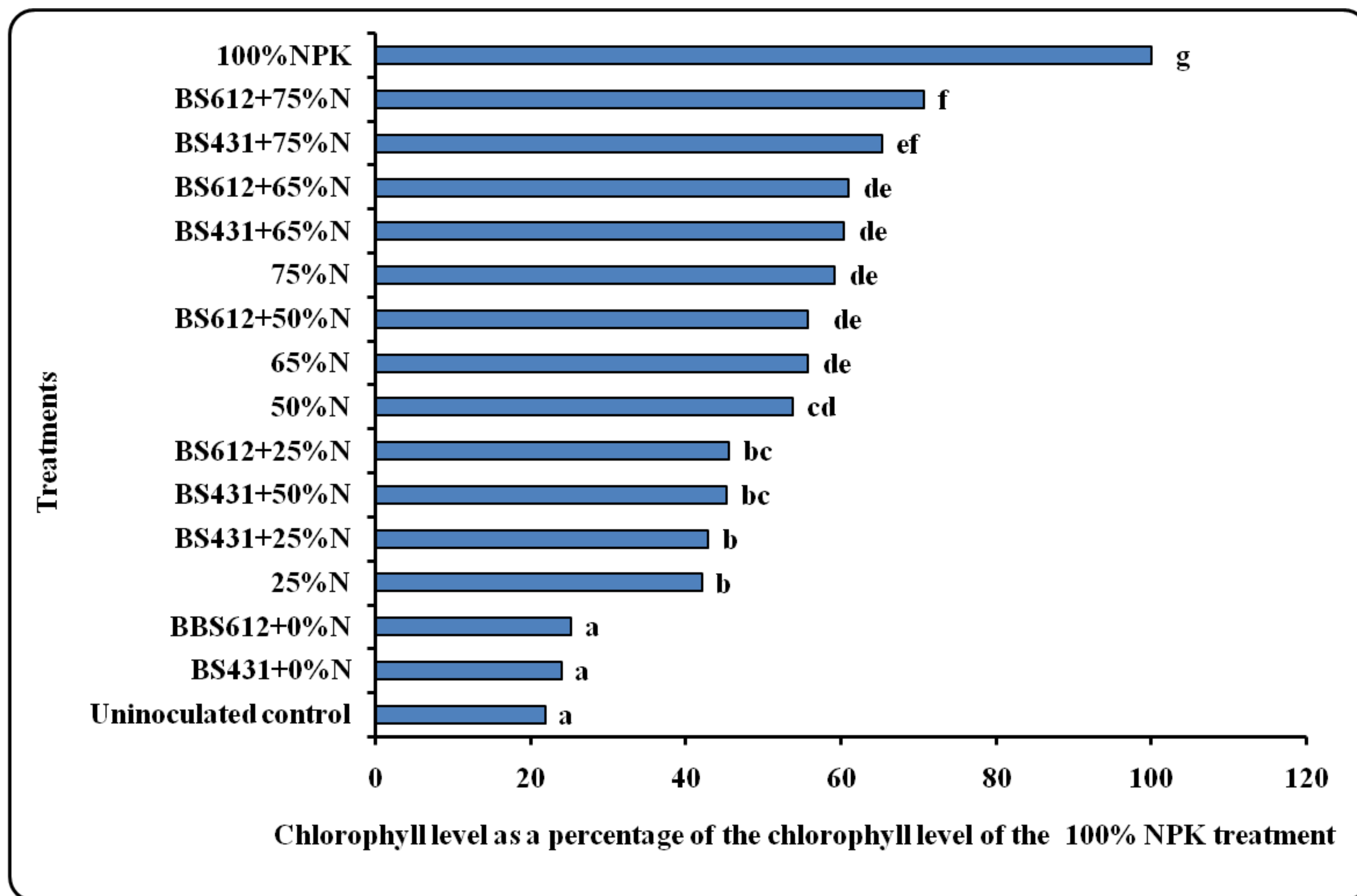

Figure 3 6: Effect of bacterial inoculation at different levels of N on chlorophyll level of maize under greenhouse conditions

Treatments: Bacterial Isolates plus N fertilizers as a percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa

Table 3.3 Response of maize and wheat to bacterial inoculation at varying fertilizer levels under greenhouse conditions

Maize				Wheat			
Treatments	**Shoot dry biomass (g)**		**Shoot dry biomass (%)**	**Treatments**	**Shoot dry biomass (g)**		**Shoot dry biomass (%)**
Uninoculated control	6.4	a	12.5	Uninoculated control	1.9	a	16.2
BS431	6.7	a	13.0	BS10+0%N	2.29	a	19.0
BS612	9.3	ab	18.1	BL5+0%N	2.71	a	22.4
BS431+25%N	19.5	abc	37.9	25%N	7.00	b	57.9
BS612+25%N	21.8	bcd	42.4	BS10+25%N	7.01	b	58.0
25%N	23.2	cd	45.2	BL5+25%N	7.06	b	58.4
50%NP	32.6	cde	63.5	50%N	8.28	bc	65.5
BS612+50%N	34.2	de	66.5	BL5+50%N	8.40	bc	69.5
BS431+50%N	36.9	e	71.5	65%N	8.64	bc	71.5
65%N	39.7	ef	77.2	BL5+65%N	9.06	bc	75.0
BS612+65%N	40.	ef	79.1	BS10+50%N	9.25	bcd	76.5
BS431+65%N	41.3	ef	80.3	75%N	9.860	bcd	81.6
75%N	43.7	ef	85.0	BL5+75%N	9.95	bcd	82.4
BS612+75%N	44.3	ef	86.2	BS10+75%N	9.97	bcd	82.5
BS431+75%N	44.4	ef	86.3	BS10+65%N	10.200	cd	84.2
100%NPK	51.4	f	100.0	100%NPK	12.100	d	100.0
F-Value	**72.9**				**11.5**		
P-Value	**0.001**				**0.001**		
S.E.D.	**2.4**				**1.3**		
CV%	**9.6**				**19.8**		

Means in a column followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test. Treatments: Bacterial Isolates plus fertilizer as a Percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa

3.4 Discussion

Use of microbial inoculants to enhance plant growth and increase yields of agricultural crops has been under investigation for several years. However, a lack of correlation has been reported between results obtained *in vitro* and *in vivo* in many cases. Extensive secondary screening, therefore, is mandatory for identification of effective strains for microbial inoculants.

Preliminary screening indicated that shoot dry biomass of inoculated plants was significantly higher than the Uninoculated Control. This biomass varied with crop species x bacterial isolate interaction. This implies that bacterial inoculation enhanced plant growth and the response to bacterial inoculation may be host specific. Similar results were observed in previous studies (De Salamone and Dobereiner, 1996; Riggs *et al*., 2001; De Oliveira *et al*., 2006; Mehnaz *et al*., 2010). These differences could be due to interaction between bacteria and the host plant caused by production of different types of root exudates by different crops, and the genotype of different cultivars or species of plants (Ladha *et al*., 1986; Frankenberger and Arshad, 1995; Dazzo *et al*., 2000). Khalid *et al*. (2004) suggested that the effect of bacterial inoculation could be influenced by bacterial strain, plant species, cultivar and environmental conditions. Stimulation of plant growth and yield increase due to inoculation with diazotrophs has been observed in wheat (Kloepper *et al*., 1989); maize (Saikia and Bezbaruah, 1995; Niranjan *et al*., 2003, 2004; Kozdroja *et al*., 2004), cotton (*Gossypium hirsutum* L.) (Bashan, 1998); potatoes (*Solanum tuberrosum* L.) (De Freitas and Germida, 1990); sorghum (*Sorghum bicolor (L.)* Moench) (Raju *et al*., 1999), tomatoes (*Solanum lycopersicum L.)* (Gravel *et al*., 2007) and chick pea (*Cicer arietinum L)* (Rokhzadi *et al.,* 2008). The response observed in the current study can be attributed to multiple growth enhancing traits displayed by these isolates *in vitro* (Chapter 2). Multiple plant growth promoting traits were implicated for plant growth and yield increases observed in earlier studies (Gupta *et al*., 1998; Dey *et al*., 2004; Compant *et al*., 2005).Enhancement of growth and yield increases have been attributed to the ability of PGPR to fix nitrogen, solubilize phosphates and produce phytohormones in maize (De Salamone *et al*., 1996: Pandey *et al*., 1998; Egamberdieva, 2007) and wheat (Ozturk *et al*., 2003; Salantur *et al*., 2006).

Direct involvement of phytohormone-production in growth promotion was demonstrated in canola *(Brassica napus* L) and lettuce (*Lactucasativa* L) (Noel *et al*. 1996) and wheat (Khalid *et al*., 2004).

Siderophore-producing fluorescent pseudomonads increased growth and yield of chick pea and soybean (*Glycine max* L (Merr)) (Kumar and Dube, 1992). Vessey (2003) and Kennedy *et al.* (2004) reviewed studies in which plant growth promotion was thought to result from biological nitrogen fixation. Increases in nitrogen levels caused by bacteria inoculation has been recorded in the literature (Boddey *et al.,* 1991; Muthukumarasamy *et al.,* 1999; Baldani *et al*., 2000*;* Islam *et al*., 2002; Malik *et al*., 2002).

Dual inoculation with different bacterial isolates and Eco-T® enhanced chlorophyll level and shoot dry biomass in maize and shoot dry biomass in wheat above the Uninoculated Control, the bacteria or Eco-T® applied singly. In previous experiments multi-strain inoculation was seen to produce better results than single strains (Nasbey *et al*, 2000; Kumar *et al*., 2001). Jisha and Alagawadi (1996) found that combining *Bacillus polymyxa* (Prazmowcoki) Mace and *T. harzianum* enhanced growth of sorghum as compared to either organism applied singly. Combining biological control agents and PGPR increased disease suppression (Guetsky*et al*., 2002) and improved yields and nutrient uptake (Alagawadi and Gaur, 1988). Combining *Bacillus megaterium* deBary and *Azotobacter chroococcum* Beijerinck increased crop yields in field trials by 10-20% (Brown, 1974). Co-inoculation of *Vigna radiata* L. T44 with *Bradyrhizobium* (Kirchner) Jordan along with other rhizosphere bacteria gave better results than those inoculated with *Bradyrhizobium* alone (Ahmad *et al.,* 2006). Dual inoculation of *Bacillus* Isolate B69 with *Trichoderma atroviride* SYN6 increased shoot dry biomass by 43% and nitrogen concentration in leaves of beans (*Phaseolus vulgaris L.*) seedlings over a non-inoculated control in the greenhouse (Yobo *et al*., 2011).

In these trials bacterial inoculation enhanced root growth. This is in agreement with observations made in previous studies (Niranjan *et al*., 2004; Beneduzi *et al*., 2008; Mehnaz *et al*., 2010)**.** These increases can be attributed to IAA production observed in the *in vitro* study (Chapter 2).

Changes observed in root morphology following application of IAA were similar to those obtained following inoculation with *Azospirillum brasilense* Corrig (Tien *et al*., 1979). Dobbelaere *et al*. (1999) demonstrated that plant root development observed following inoculation with *A. brasilense* was due to the effects of IAA produced by this strain. Growth improvements observed following bacteria inoculation were attributed to IAA production in other studies (Biswas *et al.,* 2000; Zahir *et al*., 2000; Khalid *et al*., 2001; 2003; Ahmad *et al*. 2005, 2006, 2008; Egamberdieva, 2005;Muratova *et al.,* 2005; Cakmakci *et al*., 2006). Isolate BS36, which caused the most root dry biomass, produced IAA and produced the most amount of siderophore. This implies that the increases in root biomass caused by this isolate could have resulted from increased nutrient availability through siderophore production, and enhanced water and nutrient uptake by increase in root surface area due to IAA production. Siderophore-production has been implicated in growth improvements observed in other studies (Dey *et al*., 2004; Dell'Amico *et al*., 2008; Dimkpa *et al*., 2008).

Bacterial inoculation at reduced chemical fertilizer levels enhanced chlorophyll level and height of maize and shoot dry biomass of maize and wheat above that of reduced chemical fertilizer or bacterial isolates alone. The shoot dry biomass obtained with the seed inoculation at reduced N doses was not statistically different from the Fully Fertilized Control. Similar results were recorded in tomatoes (Adesemoye *et al*., 2009), maize (Rizwan *et al*., 2008)**,** sunflower (*Helianthus annus* L.) (Akbar *et al*., 2011).Lack of significant difference between results obtained with bacteria inoculation at 65% N and 50% N levels and the Fully Fertilized Control in maize and wheat, respectively, suggest that bacterial inoculation could meet up to 35% and 50% nutrient requirements of the two crops respectively and bacteria inoculation could supplement reduced amounts of fertilizer N without compromising crop yields. Soliman *et al*. (1995) and Hegazi *et al.* (1998) demonstrated that inoculation of wheat with *Azotobacter* Beijerinck could reduce urea N requirements by 50% under greenhouse conditions. The increased plant growth observed in this study can be attributed to increased nutrient availability and enhanced nutrient uptake resulting from biological nitrogen fixation, IAA production, and enhanced fertilizer use efficiency. All strains used in this study produced ammonia, siderophores and IAA (Chapter 2).

Growth improvement in wheat was attributed to enhancement of nutrient use efficiency by Shaharoona *et al*. (2008). Application of bacteria in combination with reduced levels of inorganic fertilizer enhanced nitrogen and phosphorus uptake in tomatoes (Adesemoye *et al*., 2009). Bacterial inoculation of the two crops at different levels of N fertilizer increased shoot dry biomass significantly, although biomass due to inoculation at fertilizer levels above 50% did not differ significantly from that with fertilizer alone. This lack of significant effects at higher fertilizer levels, imply that bacteria may not be effective under conditions of higher nutrient levels. Inoculation of wheat with *Azospirillum brasilense* increased yield and other yield components significantly under low fertilizer rates compared to higher rates under field conditions (Dobbelaere *et al*., 2001). Although there was no significant difference between biomass obtained at 50 % N, 65% N and 75% N and the Fully Fertilized Control in wheat, the best biomass was obtained with bacterial Isolate BS10 inoculation combined with 65% N fertilizer level. This study suggests that use of microbial inoculants may constitute an important component of integrated mineral management for sustainable agriculture.

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Appendices

Appendix 3.1 Response of maize and wheat to bacterial inoculation at different levels of N fertilizer under greenhouse conditions

Treatments	Maize Shoot dry biomass (g)		Treatments	Wheat Shoot dry biomass (g)	
Un-inoculated control	5.57	a	Un-inoculated control	0.710	a
BL5	7.95	b	BS7	0.900	a
BS36	8.01	b	BL1	1.210	a
BS612	8.33	b	BS69	1.300	a
BS16	9.22	b	BS10	1.340	a
BS431	10.96	c	BL5	1.400	a
25%N	14.23	d	25%N	4.157	b
BS612+25%N	17.22	e	BS69+25%N	4.273	bc
BS16+25%N	18.28	ef	BS10+25%N	5.207	bcd
BS36+25%N	18.71	ef	BL1+25%N	5.233	bcd
BL5+35%N	18.73	ef	BL5+25%N	5.373	bcd
35%NPK	19.56	fg	BS7+25%N	5.580	cde
BS431+25%N	19.78	fg	BS69+35%N	5.880	def
BL5+25%N	19.94	fg	BS7+35%N	6.320	defg
BS36+35%N	21.33	gh	35%N	6.440	defg
BS16+35%N	21.38	gh	BS7+45%N	6.530	defg
BS612+35%N	22.14	h	BL1+35%N	6.890	efgh
BS431+35%N	23.89	i	BS10+35%N	6.920	efgh
45%N	25.02	ij	BL1+45%N	7.027	fgh
BS36+45%N	26.31	jk	45%N	7.070	fgh
BSL5+45%N	27.17	kl	BL5+35%N	7.143	fgh
BS16+45%N	27.38	kl	BS69+45%N	7.550	gh
BS612+45%N	27.41	kl	BSS10+45%N	7.593	gh
BS431+45%N	28.65	l	BL5+45%N	7.950	h
100%NPK	39.57	m	100%NPK	13.69	i
F-value	**184.05**			**51.04**	
P-Value	**0.001**			**0.001**	
S.E.D.	**0.85**			**0.59**	
CV%	**5.30**			**13.50**	

Means in a column followed by the same letter are not significantly different from each other at 5% level of significance according to Duncan's Multiple Range Test.

Treatments: Isolates plus fertilizer N as a percentage of the full amount recommended for the crop by the local Fertilizer Advisory Center Cedara, Pietermaritzburg, Republic of South Africa.

CHAPTER FOUR

Effects of free-living diazotrophic bacteria on seed germination, seedling growth and yield of maize (*Zea mays L.*)

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Abstract

Five promising bacterial isolates were selected for enhancing *in vitro* seed germination and seedling vigor and seedling growth of maize (*Zea mays L.*) under greenhouse conditions. These isolates were evaluated for their effects on growth and yield of maize at a reduced nitrogenous (N) fertilizer level (35% N of the total amount of N fertilizer) and full rates of phosphorus (P) and potassium (K) required for the crop as per the local Fertilizer Advisory Services recommendations, on two maize cultivars, LS8527BR (C1) and LS8521B (C2) under field conditions. An Uninoculated Control, where no bacterial isolate or N fertilizer was applied, and a Fully Fertilized Control (100% NPK) were included for comparison. Bacterial inoculation increased seed germination by 5%-17% and vigor index (VI) by 3.9%-34% relative to the Uninoculated Control. There was a significant difference ($P = 0.001$) between germination and VI caused by seed inoculation by some bacterial isolates and the Uninoculated Control. Germination and VI caused by seed inoculation by the best isolates was not significantly ($P > 0.05$) different from that treated with indole-3-acetic acid (IAA). Shoot dry biomass, chlorophyll level (CCI) and height of maize were significantly higher ($P < 0.05$) in the inoculated plants compared to the Uninoculated Control. There was a significant difference ($P < 0.05$) between the yields of the plants inoculated with the best bacterial isolates and the Uninoculated Control in all trials, whereas there was no significant difference $P > 0.05$) between the yields of the plants inoculated with the best isolates and those of the Fully Fertilized Control. Yields increased by 4%-41% in C1 and 6%-29% in C2 relative to the Uninoculated Control. Bacterial inoculation combined with 35% N caused yield increases of 6%-15% above the Fully Fertilized Control. There was a positive correlation between chlorophyll level and yield, chlorophyll level and shoot dry biomass, height and shoot dry biomass and height and yield of maize at $P = 0.01$ with r values of 0.87, 0.77, 0.92 and 0.81, respectively. Both Isolates BS431 and BS612 combined with 35% N caused the best results and there was no significant difference between the results obtained with these treatments and the Fully Fertilized Control. In a combination of *in vitro* and *in vivo* field studies the potential of free-living diazotrophic bacteria to enhance seed germination, seedling growth and increase yields of maize was demonstrated.
Key words: Free-living diazotrophs; maize; seed germination; seedling vigor; chlorophyll level; biomass; height; yield

4.1 Introduction

Maize is a major cereal food crop globally and serves as a stable food crop in developing countries. In South Africa maize is the second largest crop after sugarcane and accounts for 70% of grain production (DoA, 2011). Commercial farmers produce 98% while small scale farmers produce only 2% (Tshilambilu, 2011). Poor soil fertility, drought and diseases are major constraints to crop production in developing countries (Lynch, 2007). Increased food crop production therefore depends on the use of chemical fertilizers to supply nutrients to plants, and agrochemicals to manage pests and diseases. Continuous use of these fertilizers and agrochemicals, however, may result in pollution of water resources, destruction of beneficial microorganisms and insects, and a long term reduction in soil fertility. Resource-poor small-scale farmers usually cannot meet the cost of these agricultural inputs. Microbial inoculants have therefore been identified as an alternative for sustainable agriculture to increase soil fertility and crop production (Wu *et al*., 2005). Research on identification of effective microorganisms for formulation of these inoculants has attracted the interest of many scientists globally. Plant growth-promoting rhizobacteria refers to a heterogeneous group of beneficial bacteria inhabiting the rhizosphere of plants that influence plant growth through a consortium of mechanisms (Kloepper *et al*., 1980; 1989). These bacteria include the genera *Acetobacter*, *Azoarcus*, *Achromobacter*, *Alcaligenes, Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas* and *Serratia* (Okon and Labandera-Gonzalez, 1994; Glick, 1995; Dobbelaere *et al*., 2003; Ahmad *et al.,* 2008). Bacteria can make key minerals available to plants through biological nitrogen fixation (BNF) and phosphate-solubilization. Some bacteria enhance plant growth through the production of phytohormones that influence root development for improved nutrient and water acquisition. Some of them also produce antifungal metabolites that are toxic to plant pathogens and siderophores that bind ferric iron from the rhizosphere, making it unavailable to pathogens (Glick, 1995; Glick and Bashan, 1997; Dobbelaere *et al*., 2003; Vessey, 2003). These bacteria may be isolated from the rhizosphere of plants and formulated into inoculants that are introduced back into the rhizosphere where they may influence plant growth. Use of microbial inoculants may result in productive use of lower doses of chemical fertilizers.

Enhancement of plant growth and increases in crop yields caused by microbial inoculants has been reported by a number of authors (Okon and Labandera-Gonzalez, 1994; Muthukumarasamy *et al.,* 2000; Bashan *et al.,* 2004; Rhokzadi *et al*., 2008; Mehnaz *et al.,* 2010). Use of these products to enhance crop production is practiced in many countries including Australia, Belgium, Brazil, China, Cuba, Egypt, India, New-Zealand, the Netherlands and the United States of America (USA) (Rodriquez and Fraga, 1999).Studies on the positive effects of PGPR on seed germination seedling growth and yield of maize have been reported in the literature (Saikia and Bezbaruah, 1995; Niranjan *et al*., 2003; 2004 and Gholami *et al*., 2009).

The main objective of the current study was to test isolates of diazotrophic bacteria with multiple plant growth promoting traits isolated from the rhizosphere and leaves of maize and wheat for their effects on *in vitro* seed germination and plant growth and yields of two maize cultivars under field conditions

4.2. Materials and methods

4.2.1 Source of bacterial cultures

The bacterial isolates used in these experiments were previously selected through *in vitro* studies for plant growth promoting activities (Chapter 2) and in a greenhouse screening for enhancement of height, shoot dry biomass and chlorophyll levels in maize (Chapter 3).

4.2.2 Source of seed

The seed used in these studies was kindly provided by Link Seed (Pty) Ltd., P.O Box 755, Greytown, 3250, Republic of South Africa.

4.2.3 Inoculum preparation.

Bacterial cultures were inoculated into tryptic soy broth and incubated for 48 hours at 28ºC in an orbital shaker incubator[11] at 150 revolutions per minute (rpm). Cells were harvested by centrifuge action at 10,000 rpm for 15 minutes at 4ºC. Cell numbers were then adjusted to approximately 10^8 cfu using sterile distilled water. Cell counts were done using a counting chamber and viability confirmed by a plate count method.

4.2.4 Effect of seed inoculation on *in vitro* seed germination and seedling vigor of maize

Ten isolates with promising results in growth promotion activities (Chapter 2) were used in the germination test. The test for seed germination was done using the Paper Towel method (ISTA, 1993). The seed was treated with the different bacteria isolate suspensions amended with 2% carboxymethylcellulose (CMC) and left to stand for two hours to allow the bacteria to adhere onto the seed. Excess inoculum was then drained and the seed allowed to dry overnight under in a lamina flow. Seeds treated with each of the isolates and two controls (untreated seed coated with CMC alone and seed treated with 15mg ml^{-1} of IAA), were placed into paper towels soaked in sterile distilled water. For each treatment 25 seeds were placed equidistantly on moist paper and covered with another presoaked paper and rolled up with plastic wrapping to keep the towels moist. The towels were then incubated in a germination chamber at 25°C for seven days. The process was replicated three times and the test done three times. The number of germinated seeds was counted. Seedling vigor index (VI) was determined at the end of seven days using the method described by Abdul Baki and Underson (1973) in which the length of the root and shoot of each seedling was measured and VI calculated using the formula: VI = (mean root length + mean shoot length) x (germination (%)).

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[11]Shalom Laboratory Supplies c.c. 132 Commercial Road, International Plaza Durban 4001, P. O. Box 57030, Musgrave road Durban 4062.

4.2.5 Effect of seed inoculation on growth and yield of maize under field conditions.

Field experiments were conducted at the University of KwaZulu-Natal Ukulinga research farm (30° 24'S, 29° 24'E at an altitude of 700m), from December 2009 to April 2010, and December 2010 to April 2011. The soil pH was 4-5, clay 34%-38%, organic carbon 2.5%-3.2% and organic N 0.36%. Relative humidity varied between 30%-100% throughout the season, temperatures between 20-30°C, and there were 322 mm of rain. A yield experiment (Trial One) was conducted with one maize cultivar (C1) from December 2009 to April 2010. Seeds were treated as in Section 4.2.4 above, with the five isolates selected from the greenhouse test. A split plot design was used in which the whole plot was divided into two equal portions. The treatments were replicated three times and arranged in a randomized complete block design in each of the sub-plots. The whole plot was fertilized with full amount of P and K required by the crop as per the recommendations by the local Fertilizer Advisory Services[12]. The two sub plots were fertilized either with 35% N from lime ammonium nitrate (LAN) or it was Unfertilized. Two thirds of the N fertilizer was applied at sowing and one third five weeks after planting. There were six rows in each plot with a row width of 3 meters and a length of 8.32 meters, with a distance of 29 mm between seeds. Twenty five plants were sampled for the yield. A second field trial (Trial Two) was conducted to study the effect of bacterial inoculation on chlorophyll level, shoot dry biomass, height and yield of two maize cultivars (C1 and C2). A third trial (Trial Three) was done with C2 inoculated with the best two bacteria isolates (BS612 and BS431) identified in the first two trials grown under dry land conditions to assess their performance under these conditions. In this trial the treatments were replicated six times.

4.2.6 Statistical analysis

The data was subjected to analysis of variance (ANOVA) using GenStat 12.1 statistical package (VSN International, 2011). Treatment mean separation was done using Duncan's Multiple Range Test at 5% level of significance.

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[12]Fertilizer Advisory Service, KwaZulu-Natal, KZN, Department of Agriculture and Environmental Affairs; Soil Fertility Analytical Services; Private Bag, X9059, Pietermaritzburg, Republic of South Africa, 3200.

4.3 Results

4.3.1 Seed germination and seedling vigor

Seed inoculation with some bacterial isolates significantly (P = 0.001) enhanced seed germination and seedling vigor of maize above the Uninoculated Control. There was no significant difference in germination and VI as a result of treatment with some isolates, and with IAA. Germination and VI caused by bacteria inoculation increased by 5-17% and 3.9-34%, respectively, above the Uninoculated Control except treatment with Isolate BS44 which was lower than the Uninoculated Control. The best results due to bacterial inoculation were obtained with BS10 (Table 4.1).

Table 4 1: Effects of bacterial seed inoculation on *in vitro* seed germination and seedling vigor of maize after seven days of incubation in a germination chamber at 25°C

Bacterial Isolates	**Germination (%)**		**Vigor Index (VI)**	
BS44	63.3	a	1603	a
Uninoculated Control	63.3	a	1705	a
BS36	68.3	ab	1833	ab
BL5	71.7	abc	1848	abcd
BS431	71.7	abc	1944	ab
BS7	73.3	abc	1976	abcd
BS69	75.0	abcd	2203	cdef
BS16	80.0	bcd	2344	bcde
BS612	80.0	bcd	2380	cdef
BL1	81.7	cd	2433	def
BS10	86.7	de	2526	ef
IAA	96.7	e	2784	f
F-Value	**6.69**		**6.09**	
P-Value	**0.001**		**0.001**	
S.E.D.	**5.29**		**209.80**	
CV%	**8.50**		**12.10**	

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Duncan's Multiple Range Test.

IAA: Indole acetic acid (15 mg ml^{-1}).

4.3.2 Effect of seed inoculation on different growth parameters and yield of maize under field conditions

Bacterial seed inoculation, in combination with 35% N, increased chlorophyll levels significantly ($P < 0.05$) compared to the Uninoculated Control in both trials. Chlorophyll levels caused by bacterial inoculation in combination with 35% N in trials Two and Three were not significantly different ($P > 0.05$) from those resulting from treatment with 100% NPK. Chlorophyll levels were higher in the irrigated trial compared to the dryland trial. Seed inoculation with Isolate BS612 combined with 35%N caused the highest chlorophyll level in irrigated trials (Table 4.2). Plants of C1 were taller at 30 and 60 days after planting, but at 90 days C2 plants were taller. In cultivar C2 there was a significant ($P = 0.001$) difference between the heights of the inoculated plants treated with 35% N compared to the Uninoculated Control. With both cultivars there was no significant difference ($P > 0.05$) between the heights of inoculated plants treated with 35% N and those treated with 100% NPK. Treatment with Isolate BS612 and 35% N resulted in the tallest plants of both cultivars 90 days after planting (Table 4.3). Under dryland conditions, there was a significant ($P = 0.001$) difference between the heights of inoculated plants treated with 35%N and the Uninoculated Control, but they were not significantly different from plants treated with 100% NPK (Table 4.6).

Inoculated plants with 35%N developed significantly ($P < 0.05$)) more shoot dry biomass than the Uninoculated Control but were not significantly different from the 100%NPK treated plants. The greatest biomass was obtained from plants treated with Isolate BS431 and 35% N, in the two varieties (Table 4.4). Shoot biomass obtained following bacterial inoculation were higher than those of the Fully Fertilized Control at 30 and 60 days after planting, but lower after 90 days (Table 4.7).

Trial One yields obtained from plants treated with the best isolates and 35%N were significantly ($P < 0.05$) greater than the Uninoculated Control, but they were not significantly different from yields resulting from treatment with 100% NPK. Seed inoculation of C1 with Isolates BS612 and BS431 in combination with 35% N caused higher yields than seed treated with 100% NPK, with the greatest yields resulting from treatment with Isolate BS612.

In Trial Two there was a significant ($P < 0.05$) difference between the yields caused by bacterial inoculation and 35% N, relative to the Uninoculated Control, but they were not significantly different from those resulting from 100% NPK treatment, in both cultivars. Maximum yields with C1 were caused by treatment with BS612 and 35% N, while the maximum yield with C2 was obtained from treatment with 100% NPK. Seed inoculation in combination with 35% N increased yields of C1 by 4%-41%, and C2 by 6%-29%, above the Uninoculated Control. In C1 seed inoculation and 35% N increased the yields by 15% above the yields caused by treatment with 100% NPK. Seed treatment with Isolates BS612 with 35% N caused the best yields with both cultivars.

The yields in Trial 3 (Dryland) were lower than those of Trials 1 and 2. Seed inoculation with Isolate BS612 and 35% N caused higher yields than those caused by treatment with 100% NPK (Table 4.5). Treatment with Isolate BS612 and 35% N caused the highest yields with both cultivars and in all trials. Seed inoculation of C2 caused higher yields compared to C1 but the difference was not statistically significant. However, seed inoculation with Isolates BS612 and BS431 in combination with 35% N increased yields of C1 above yields caused by treatment with 100% NPK in Trial 2 whereas C2 treated with 100% NPK had the highest yield (Table 4.6).

Table 4.2: Effect of seed inoculation on chlorophyll level (CCI) of two maize Cultivars under field conditions

Trial Two		Trial Two		Trial Three (Dryland)	
Cultivar C1		**Cultivar C2**		**CultivarC2**	
Treatments	**CCI**	**Treatments**	**CCI**	**Treatments**	**CCI**
BS612+0%N	50.60a	BS431+0%N	47.00a	Uninoculated Control	38.45a
Uninoculated Control	51.73ab	BS16+35%N	49.83ab	BS612	44.43ab
BSI6+0%N	55.13bc	Uninoculated Control	51.33ab	BS431	48.12bc
35%N	55.87bc	35%N	51.63ab	35%N	51.07bcd
BS36+0%N	56.03bc	BL5+0%N	53.73abc	BS612+35%N	57.03cde
BL5+0%N	57.10cd	BSI6+0%N	53.90abc	BS431 +35%N	59.28de
BS431+0%N	58.03cd	BS612+0%N	54.90abcd	100%NPK	62.5e
BS16+35%N	61.43de	BL5+35%N	57.13bcd		
BS36+35%N	61.43 de	BS36+35%N	57.20bcd		
BS431+35%N	63.87 ef	BS431+35%N	58.37bcd		
BS612+35%N	66.70fg	BS612+35%N	61.43cd		
100%NPK	68.53g	100%NPK	63.4d		
F-Value	**13.52**		**2.68**		**7.9**
P-Value	**0.001**		**0.019**		**0.001**
S.E.D.	**2.07**		**8.80**		**14.50**
CV%	**4.3**		**3.95**		**4.32**

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Duncan's Multiple Range Test.

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa; 100%NPK: Fully Fertilized Control; Uninoculated Control: No bacterial isolate or fertilizer applied.

Table 4.3 Effect of seed inoculation on plant height (mm) of two maize Cultivars C1 and C2 under field conditions

	Cultivar C1				**Cultivar C2**		
Treatments	**30 Days**	**60 Days**	**90 Days**	**Treatments**	**30 Days**	**60 Days**	**90 Days**
BSI6+0%N	530.0^{a}	1623^{a} a	1840^{ab}	Uninoculated Control	450.0^{a}	1717^{a}	1950^{abc}
Uninoculated Control	531.0^{a}	1852^{bc}	2100^{bc}	BSI6+0%N	503.7^{b}	1779^{abc}	1823^{a}a
BS36+0%N	534.7^{ab}	1741^{ab}	2257^{c}	BL5+0%N	514.3^{bc}	1787^{abc}	1903^{ab}
BL5+0%N	542.3^{abc}	1937^{bc}	2257^{c}	BS431+0%N	517.7^{bc}	1909^{abc}	2349^{bcd}
BS36+35%N	569.0^{abcd}	1747^{ab}	1683^{a}	BS36+0%N	520.0^{bc}	1759^{abc}	200^{abc}
BS16+35%N	576.7^{bcde}	1912^{bc}	2180^{c}	35%N	526.7^{bcd}	1812^{abc}	2015^{abc}
BS431+0%N	582.3^{cdef}	1987^{c}	2270^{c}	BS36+35%N	538.0^{bcde}	1934^{abc}	2388^{bcd}
BL5+35%N	583.7^{cdef}	1954^{c}	2120^{bc}	BS612+0%N	555.3^{cdef}	1657^{a}	2301^{abcd}
BS612+0%N	584.3^{cdef}	1913^{bc}	2322^{c}	BS16+35%N	556.7^{cdef}	1851^{abc}	2319^{bcd}
35%N	590.3^{def}	2054^{c}	2160^{c}	BS431+35%N	571.3^{def}	2008^{bc}	2441^{cd}
BS612+35%N	614.3^{ef}	1936^{bc}	2392^{c}	BL5+35%N	572.0^{def}	1862^{abc}	2370^{bcd}
BS431+35%N	623.3^{f}	2058^{c}	2367^{c}	100%NPK	573.3 ^{ef}	2030^{c}	2389^{bcd}
100%NPK	624.3^{f}	2045^{c}	2398^{c}	BS612+35%N	584.7^{f}	2033^{c}	2540^{d}
F-Value	**6.2**	**4.5**	**5.3**		**7.1**	**1.95**	**2.65**
P-Value	**0.001**	**0.001**	**0.001**		**0.001**	**0.1**	**0.02**
S.E.D	**18.96**	**88.3**	**130.0**		**20.8**	**122.9**	**207.20**
CV%	**4.0**	**5.7**	**7.3**		**4.5**	**8.1**	**11.50**

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Duncan's Multiple Range Test.

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa; 100%NPK: Fully Fertilized Control; Uninoculated Control: No bacterial isolate or fertilizer applied

Table 4.4 Effect of seed inoculation on shoot dry biomass (g) of two maize Cultivars C1 and C2 under field conditions

	Cultivar C1				Cultivar C2		
Treatments	**30 Days**	**60 Days**	**90 Days**	**Treatments**	**30 Days**	**60Days**	**90 Days**
BS36+0%N	31.35a	502.30abcd	703.30bc	BS36+0%N	27.06a	483.3cd	663.3ab
Uninoculated Control	32.04a	427.30a	503.70a	Uninoculated Control	27.33a	320.0a	565.4a
35% N	33.29ab	451.5abc	762.40bcd	BSI6+0% N	29.94ab	356.7ab	696.7ab
BS431+0% N	34.29abc	510.0abcd	882.70cde	BL5+0% N	30.52ab	402.0abc	555.7a
BS36+35% N	35.15abc	527.7abcd	788.30bcd	35% N	31.11ab	383.3abc	657.0ab
BL5+0% N	35.58abc	452.30abc	631.70ab	BS612+0% N	31.79abc	480.0cd	784.3b
BS612+0% N	35.74abc	481.70abc	875.30cde	BS16+35% N	34.85bc	469.7cd	713.0ab
BS16+35% N	37.69abcd	429.30ab	792.00bcd	BL5+35% N	34.86bc	458.7bcd	736.7ab
BL5+35% N	41.25bcd	450.0abc	803.00bcd	100% NPK	35.00bc	486.7cd	1039.3d
BS612+35% N	41.37bcd	547.0bcd	948.30de	BS36+35% N	35.34bc	486cd	652.7ab
100% NPK	41.94cd	598.0d	1040.30e	BS612+35% N	36.30bc	532.7d	972.7cd
BS431+35% N	44.52d	562.7cd	969.00de	BS431+35% N	38.21c	540.0d	820.7bs
F-Value	**3.3**	**2.4**	**5.3**		**3.8**	**3.7**	**5.7**
P-Value	**0.006**	**0.034**	**0.001**		**0.003**	**0.003**	**0.001**
S.E.D.	**3.5**	**49.6**	**89.2**		**2.9**	**49.3**	**84.6**
CV%	**11.8**	**13.3**	**13.6**		**10.7**	**13.3**	**14.1**

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Duncan's Multiple Range Test.

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa; 100%NPK: Fully Fertilized Control; Uninoculated Control: No bacterial isolate or fertilizer applied

Table 4.5 Effect of seed inoculation on yield (kg) of two maize Cultivars C1 and C2 under field conditions.

Cultivar C1	Trial One	Trial Two	Cultivar C2 Trial Two		Cultivar C2 Trial Three (Dryland)		
Treatments	**Yield (kg)**	**Yield (kg)**	**Treatments**	**Yield (kg)**	**Treatments**	**Yield (kg)**	**Yield (kg)**
Uninoculated Control	1.56^{a}	3.83^{a}	ninoculated Control	3.73^{a}	Uninoculated Control	1.97^{a}	(0.28^{a})
BS612+0%N	2.61^{bc}	4.05^{ab}	35%N	3.90^{a}	BS431	2.42^{ab}	(0.37^{ab})
35%N	2.85^{bc}	4.20^{ab}	BL5+35%N	4.16^{ab}	BS612	2.42^{ab}	(0.38^{ab})
BL5+0%N	2.45^{b}	4.41abc	BS36+35%N	4.31 ^{ab}	35%N	2.72^{ab}	(0.43^{b})
BS36+0%N	2.75^{bc}	4.52^{abc}	BS431+0%N	4.39^{abc}	BS431+35%N	2.82^{ab}	(0.41^{ab})
BS431+0%N	2.91^{bcd}	4.54^{abc}	BS36+0%N	4.43^{abc}	100%NPK	2.82^{b}	(0.43^{b})
BS36+35%N	2.99^{bcd}	4.68^{abc}	BL5+0%N	4.45^{abc}	BS612+35%N	3.01^{b}	(0.47^{b})
BS16+35%N	3.27^{cde}	4.88^{abc}	BSI6+0%N	4.54^{abc}			
BL5+35%N	2.99^{bcd}	5.08^{abc}	BS16+35%N	4.59^{abc}			
BSI6+0%N	2.91^{bcd}	5.12^{abc}	BS612+0%N	5.37^{abcd}			
100%NPK	3.19^{cde}	5.17^{abc}	BS431+35%N	5.57^{bcd}			
BS431+35%N	3.78^{e}	5.49^{bc}	BS612+35%N	6.03^{cd}			
BS612+35%N	3.56^{de}	5.95^{c}	100%NPK	6.85^{d}			
F-Value	**7.5**	**1.6**		**3.3**		**1.50**	**(1.9)**
P-Value	**0.001**	**0.2**		**0.006**		**0.21**	**(0.115)**
S.E.D.	**0.3**	**0.7**		**0.7**		**26.80**	**(0.7)**
CV%	**11.9**	**17.1**		**18.2**		**0.40**	**(28.6)**
L.S.D						**0.81**	**(0.1)**

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Duncan's Multiple Range Test; Values in parentheses represent transformed means using a log transformation.

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa; 100%NPK: Fully Fertilized Control; Uninoculated Control: No bacterial isolate or fertilizer applied

Table 4.6Effect of seed inoculation on plant height (mm) of maize Cultivar C2 under dryland conditions

Treatments	30 Days	60 Days	90 Days
Uninoculated Control	487[a]	1652ab	1554[a]
BS612	533[ab]	1554a	1702[a]
BS431	527[ab]	1704ab	1709[a]
35%N	553[bc]	1704ab	1733[ab]
BS612 +35%N	575[bc]	1781b	1923[c]
BS431 +35%N	592[cd]	1746b	1905[c]
100%NPK	633[d]	1794b	1854[bc]
F-Value	**7.9**	**2.1**	**6.3**
P-Value	**0.001**	**0.001**	**0.001**
L.S.D.	**4.9**	**16.5**	**12.5**
S.E.D.	**2.4**	**8.1**	**6.0**
CV%	**7.5**	**8.2**	**6.1**

Means followed by the same letter in a column are not significantly different from each other at 5% level of significance according to Fisher's L.S.D. test

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa. 100%NPK: Fully Fertilized Control; Uninoculated Control:

No bacterial isolate or fertilizer applied

Table 4.7 Effect of bacterial seed inoculation on shoot dry biomass (g) of C2 under dryland conditions

Treatments	**30 Days**	**60 Days**	**90 Days**
Uninoculated Control	34.59[a]	359.8[a]	395.5[a]
BS612	34.99[a]	413.5[ab]	465.9[ab]
BS431	31.35[a]	490.8[abc]	503.6[b]
35%N	39.18[ab]	510.2[bc]	538.6[b]
BS612 +35%N	46.96[b]	516.2[bc]	657.6[c]
BS431 +35%N	46.72[b]	547.5[bc]	661.8[c]
100%NPK	57.54[c]	563.5[c]	677.9[c]
F-Value	**7.7**	**2.7**	**9.9**
P-Value	**0.001**	**0.035**	**0.001**
L.S.D.	**9.6**	**130.4**	**101.5**
S.E.D.	**4.7**	**63.9**	**49.7**
CV%	**19.6**	**22.8**	**15.5**

Means in a column followed by the same letter are not significantly different at 5% level of significance according to Fisher's L.S.D. test

Treatments: Bacterial isolates plus nitrogen (N) fertilizer as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center, Cedara, Pietermaritzburg, Republic of South Africa; 100%NPK: Fully Fertilized Control; Uninoculated Control: No bacterial isolate or fertilizer applied

4.4 Discussion

Free-living bacteria have been documented to enhance seed germination, growth and yield of various crops. Increase in seed germination following inoculation with *Azotobacter* Beijerinck has been reported in chick pea (*Cicer arietinum* L), black gram *(Phaseolus mungo* (L.) Hepper) cowpea *(Vigna cajung* (Burm F) Walp.) and maize (Saikia and Bezbaruah, 1995). *Bacillus* enhanced growth of pepper (*Capsicum anunum* L) and cucumber *(Cucumis sativum* L.) (Han e*t al*., 2006), increased yields of wheat by 43% (Kloepper and Beauchamp, 1992), and increased the yield, growth and nutrition of raspberry (*Rubus occidentalis L.*) (Orhan *et al*., 2006). *Bacillus megaterium* deBary enhanced rooting performance, root length and root dry matter in mint (*Mentha piperita* L.) (Kaymak *et al*., 2008). *Pseudomonas* increased yields of legumes (Johri, 2001) and increased root and shoot length in canola (*Brassica napus* L.) seedlings (Glick *et al*., 1997). *Azospirillum brazilense* Corrig and *Bradyrhizobium japonicum* Jordan strains promoted seed germination and early seedling growth in maize and soybean (*Glycine max* L.) (Casan'na *et. al*., 2009).

In this study it was demonstrated that seed inoculation with selected bacterial strains enhanced seed germination, and seedling vigor *in vitro*, and increased plant height, shoot dry biomass, chlorophyll level and grain yields of two maize cultivars under field conditions. There was a positive correlation between chlorophyll level and yield, chlorophyll level and shoot dry biomass, height and shoot dry biomass and height and yield of maize at $P = 0.01$ with r values of 0.87, 0.77, 0.92 and 0.81, respectively. Similar results have been recorded in previous work with wheat and potatoes (*Solanum tuberosum* L.) (De Freitas and Germida, 1992), potatoes (Frommel, 1993), sorghum (*Sorghum bicolor* (L) Moench) (Raju *et al*., 1999), wheat (*Triticum aestivum* L.) and maize (Dobbelaere *et al.*, 2001), pearl millet (*Pennisetum glaucum* L.) (Niranjan *et al*., 2003, 2004), maize (Kozdroja *et al*., 2004; Gholami *et al*., 2007), wheat (Wu *et al*., 2005; Shaharoona *et al*., 2006); Salantur *et al*., 2006), sunflower (*Helianthus annus* L.) and wheat (Shaukat *et al.,* 2006) and chickpea (Vikram, 2007; Rokhzadi *et al.,* 2008). A number of researchers have measured the ability of bacteria to fix nitrogen, solubilize phosphates and produce phytohormones, resulting in the enhancement of growth and yield increases in various crops (de Salmone *et al*., 1996; Malik *et al*., 1997; Pandey *et al*., 1998; Cattelan *et al*., 1999; Ozturk *et al*., 2003; Cakmakci *et al*., 2006; Egamberdieva, 2007).

Enhancement of seed germination and seedling vigor observed in the current study may have been due to the production of indole-3-acetic acid (IAA). Indole-3-acetic acid enhances cell expansion, division and differentiation (Ryu *et al.,* 2003). Soil bacteria synthesize IAA in pure cultures and in the soil (Arshad and Frankenberger, 1998; Barazani and Friedman, 1999; Biswas *et al*., 2000). Strains of bacteria that produce IAA increased growth and yield of wheat (Khalid *et al*., 2004) and promoted seed germination in orchids (Tsavkelova *et al*., 2007). An IAA producing strain of *Bacillus megaterium* promoted growth of tea (*Camelia sinensis* L.) (Chokrabortry *et al*., 2006). Increases in growth were observed in cucumber, tomato and pepper when inoculated with strains of bacteria that produced IAA (Kidoglu *et al*., 2007). Indole-3-acetic acid producing rhizobacteria are used to enhance growth in rice cultivation (Ashrafuzzaman *et al*., 2009). The five bacterial isolates used in this study produced varying amounts of IAA in broth culture in the presence of 500 mg ml^{-1} of tryptophan, and Isolate BS431 produced the most IAA also caused the best results in some of the parameters measured.

Increased plant heights, chlorophyll levels and shoot biomass may have resulted from the isolates' ability to fix nitrogen. All isolates grew well on nitrogen-free media, produced an orange to red color with Nessler's reagent when inoculated in peptone water, and reduced acetylene to ethylene. Availability of sufficient nitrogen to plants leads to growth of healthy green leaves in crops, with high chlorophyll levels (Varvel *et al*., 2007; Gholizadeh *et al*., 2009). Since nitrogen is a component of chlorophyll and is closely correlated with nitrogen concentration in the leaves (Blackmer *et al*., 1994), increase in chlorophyll levels implies an increase in nitrogen concentration. Gholizadeh *et al*. (2009) demonstrated that there is a strong linear correlation between chlorophyll levels and nitrogen concentration of rice leaves. Management of nitrogen requirements of maize was successfully achieved based on chlorophyll level measurement using a chlorophyll meter (Peterson *et al*., 1993; Rostami *et al*., 2008). Increases in chlorophyll levels observed therefore, may be an indication of an increase in nitrogen levels in the leaves resulting possibly from BNF by the bacterial isolates. Sufficient nitrogen concentration in the leaves leads to an increase in leaf surface area (Varvel *et al*., 2007). Increase in nitrogen concentration may be implicated in the increases in shoot dry biomass observed in this study. However, there is a need to measure the amount of nitrogen in the leaves to determine the changes in nitrogen concentration.

Increased nitrogen levels caused by bacterial inoculation have been recorded in literature (Boddey *et al.*, 1991; Muthukumarasamy *et al.,* 1999; Baldani *et al*., 2000; Malik *et al*., 2002). Biological nitrogen fixation was implicated in growth enhancements observed in previous research as reviewed by Vessey (2003) and Kennedy *et al*. (2004). Isolate BS431 caused phosphate solubilization (Chapter 2) and caused the best results for most of the parameters studied. Contributions of phosphate-solubilizing bacteria in plant growth has been demonstrated by other researchers (Chabot *et a*l., 1996a**;** 1996b; De Freitas *et al*., 1997, 1998; Kumar and Narula, 1999; Peix *et al*., 2001; Cakmakci *et al*., 2006). Growth enhancement and yield increases may have resulted from the suppression of phytopathogens by production of antifungal compounds and siderophores (Dobbelaere *et al.,* 2003; Glick and Pasternak 2003) exhibited by all isolates studied (Chapter 2). Siderophore-producing fluorescent pseudomonads increased growth and yield of chickpea and soybean (Kumar and Dube, 1992), and the growth and yield of groundnuts (Dey *et al*., 2004).

The two maize cultivars differed in their response to bacterial inoculation. Seed bacterial inoculation caused higher yields, with C2 compared to cultivar C1. This suggests that bacterial inoculation may be cultivar specific. Similar responses of crop varieties to bacterial inoculation have been reported in maize (De Salamone and Dobereiner, 1996; Riggs *et al*., 2001; Mehnaz *et al*., 2010). Yobo (2001) reported enhanced growth in lettuce and tomatoes following inoculation with *Bacillus* species, supplemented with a microbial nutrient, while no significant increase was observed with sorghum using the same nutrients under the same conditions. These differences in response of crop cultivars to bacterial inoculation were attributed to interactions between bacteria and the plants, types of root exudates, and gaseous diffusion efficiency (Ladha *et al*., 1986; Frankenberger and Arshad, 1995; Kloepper, 1996; Khalid *et al*. (2005). The low values obtained for the various growth parameters in the dryland trial might have resulted from the effects of the environmental conditions. Khalid *et al*. (2004) demonstrated that the effect of bacterial inoculation on wheat was affected by the interaction between plant genotype, PGPR strain and environmental conditions.

The bacteria tested in this study enhanced seed germination, seedling growth and increased yields of maize when used in combination with a low dose of nitrogen fertilizer and full doses of potassium and phosphorus. Improvements in growth parameters resulting from the use of microbial inoculants combined with reduced levels of chemical fertilizers have been reported in previous research (Okon and Labandera-Gonzalez, 1994; Biswas *et al.*, 2000; Dobbelaere *et al.*, 2001; Riggs *et al.*, 2001). A combination of *in vitro*, greenhouse and field studies were used to select promising bacterial strains for use in maize production.

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CHAPTER FIVE

Response of two wheat (*Triticum aestivum L.*) cultivars to inoculation with five selected free-living diazotrophic bacteria: A field trial

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Abstract

Five bacterial isolates were obtained from the rhizosphere of different plants, and selected for their plant growth promoting activities *in vitro* and for enhancing wheat seedling growth under greenhouse conditions. These isolates were tested for their potential to promote seedling growth and increase yields of two wheat cultivars (C) (PAN 3434 (C1)) and PAN 3490 (C2)) under a low dose of nitrogenous (N) fertilizer in a field trial. Bacterial inoculation in combination with 35% N significantly (P = 0.001) increased shoot dry biomass and yield of wheat and caused the same biomass and yield or greater than the Fully Fertilized Control. Shoot dry biomass increased between 0.2%-75.4% relative to the Uninoculated Control and 4%-29.4% above the Fully Fertilized Control. Inoculation of C1 with Isolate BS10 in combination with 35% N caused the greatest shoot dry biomass, whereas seed inoculation with Isolate BL5 combined with 35% N gave the highest biomass of C2. Bacterial inoculation with some isolates in combination with 35% N significantly increased the yields of both cultivars above the Uninoculated Control and caused the same yields or greater than the Fully Fertilized Control. The best yield was recorded with BS10 plus 35% N applied to C1, and BS7 plus 35% N applied to C2. Yields increased between 1.4 %-96.5% above the Uninoculated Control and 1.4%-43.1% above the Fully Fertilized Control. Increases in yields were consistent with increases in shoot dry biomass and varied with isolate and plant cultivar interactions.

Key words: Free-living bacteria; plant growth-promotion; wheat, biological nitrogen fixation; phosphate-solubilization; siderophores; phytohormones

5.1 Introduction

A diverse group of microorganisms that includes fungi, bacteria, protozoa and Cyanobacteria (Choudhury and Kennedy, 2004; Shridhar, 2012) have been documented for enhancing plant growth. Bacteria inhabiting the rhizosphere of plants that exert beneficial effects to the plants are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1989). Plant growth promoting rhizobacteria promote growth directly by providing nutrients or enhancing nutrient uptake and indirectly by suppressing plant pathogens (Ahmad *et al*., 2006). Research on use of microbial inoculants to enhance growth and increase yields of crops has attracted the interest of many researchers (Okon and Labandera-Gonzalez, 1994; Muthukumarasamy *et al.,* 1999; Riggs *et al*., 2001; Dobbelaere *et al*. 2002; Bashan *et al.,* 2004; Rhokzadi *et al*., 2008; Mehnaz *et al.,* 2010). Several free-living bacteria genera have been reported to enhance growth and increase yields of crops of agronomic importance. Significant increase in growth rates have been reported in sugarcane due to application of *Acetobacter diazotrophicus* Beijerinck (Boddey *et al.,* 1991). Several strains of this species have been isolated from sugarcane (Fuentes-Ramirez *et al*., 1993). Application of *Azospirillum* Beijerinck can increase wheat yields under greenhouse and field conditions (Okon and Labandera-Gonzalez, 1994; Hegazi *et al*., 1998; Ganguly *et al*., 1999). Increases in plant height, tiller number and yields of rice were observed following inoculation with *A*. *lipoferum* (Beijerinck) comb (Mirza *et al*., 2000; Balandreau, 2002). Application of *Azotobacter* Beijerinck can contribute up to 50% of wheat nutrient requirements under greenhouse conditions (Soliman *et al*., 1995), and increase rice yields by 20% in the field (Yanni and El-Fattah, 1999). *Burkholderia vietnamiensis* Gilli*s et al.* increased rice yields by 13%-22% (Tran Van *et al*., 2000) while another species of this genus increased rice biomass by 69% per plant (Baldani *et al*., 2000). *Herbasprillum seropedicae* Baldani. *et al*. increased root and shoot length, grain weight and grain yield of rice in the field (Arangarasan *et al*., 1998), yield in the greenhouse (Mirza *et al*., 2000) has been reported for positive results when applied to maize, sorghum, sugarcane and wheat (James *et al*., 2000). Cyanobacteria and *Azolla* Lam can reduce plant nitrogen requirements by 30%-50% of Urea-N, respectively (Choudhury and Kennedy, 2004)

Wheat serves as a major staple food crop for 35% of the world's population (Joshi and Bhatt, 2011). Increases in food production are needed to meet the demands of the world's increasing population. Nitrogenous chemical fertilizers are essential to enhance food production. However, a large portion of these fertilizers are lost through gaseous emissions, dinitrification and leaching of nitrates into ground water (Bijay-Singh *et al.*, 1995), which impacts negatively on the environment (Rejesus and Hornbaker, 1999). Resource-poor small-scale farmers cannot afford the cost of these agrochemicals. Use of nitrogen fixing (diazotrophic) bacteria has therefore been proposed as an alternative or supplement to reduce excessive use of nitrogenous fertilizers. Use of diazotrophic bacteria in combination with nitrogen fertilizers has been found to reduce the amount of nitrogen fertilizer that needs to be applied to plants (Yanni *et al.*, 1997). In previous investigations the best results were obtained from inoculation in fields with moderate nitrogen fertilization (Okon and Labandera-Gonzalez, 1994; Dobbelaere *et al.*, 2001; Riggs *et al.*, 2001). Fuentes-Ramirez *et al.*(1993) isolated large numbers of *A. diazotrophicus* strains from sugarcane grown under low doses of fertilizer nitrogen as compared to those under high doses.

Bacteria in the genus *Bacillus* are free-living, endospore-forming gram-positive bacteria with a ubiquitous distribution (Gardener, 2004). Joshi and Bhatt (2011) found *Bacillus* to be the most dominant (40%) genus in a wheat rhizosphere. The positive effects of *Bacillus* on plant growth and yields of a number of crops has been reported in the literature (Kloepper *et al.*, 1989; Kim *et al.,* 1997; Shen, 1997; Mathre *et al.,* 1999; Podile 1999; Yobo *et al.*, 2011). Their growing importance in research is attributed to their ability to form resistant endospores which prolongs their shelf life and makes it easy to develop inoculant formulations (Collins and Jacobson, 2003). Strains of *Bacillus* produce substances that inhibit growth of other microorganisms (Lilinares *et al.,* 1994), which ensures their multiplication and survival in the rhizosphere of many plants (Foldes *et al.*, 2000; Shoda, 2000).

The primary objective of the current study was to identify free-living diazotrophic bacterial strains that are capable of enhancing wheat growth and increase yields in the presence of a low dose of N fertilizer, as a potential cheaper option for sustainable agriculture. Their effect on different wheat genotypes was also investigated.

5.2 Materials and methods

5.2.1 Source of bacterial cultures

Bacterial isolates were isolated from the rhizosphere of different plants using standard isolation procedures and selected through in vitro studies for plant growth promoting activities (Chapter 2) and greenhouse screening for enhancement of seedling growth in wheat (Chapter 3).

5.2.2 Source of seed

The seed used in these studies was kindly provided by Willem Boshoff, a wheat breeder at Pannar Seed (Pty) Ltd, P. O. Box 17164, Bainsvlei, 9338, Republic of South Africa.

5.2.3 Inoculum preparation.

Bacteria cultures were inoculated into tryptic soy broth and incubated for 48 hours at 28ºC in an orbital shaker incubator[13] at 150 (rpm). Cells were harvested by centrifuging at 10,000 rpm for 15 minutes at 4ºC (Beckman Coulter Avanti J-26 XPI High Speed Centrifuge)[14]. Cell numbers were then adjusted to 10^8 cfu ml^{-1} by dilution method using sterile distilled water. Cell counts were done using a counting chamber and viability confirmed by plate count method.

5.2.4 Effect of bacterial seed inoculation on growth and yield of wheat under field conditions.

Field experiments were conducted at the University of KwaZulu-Natal Ukulinga research farm (30° 24'S, 29° 24'E at an altitude of 700m), from June–November 2011. The soil pH was 4.2-4.5, clay percentage of 36%, organic carbon of 2.8% and organic N of 0.34%. Two wheat cultivars, C1 (high yield potential) and C2 (low yield potential) were used in this trial. Two trials (T1 and T2) were set up for each cultivar in two plots at the same site. Seeds were treated with the five isolates selected from the greenhouse test. Treatments consisted of five bacterial isolates, and one Uninoculated and one Fully Fertilized Controls.

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[13] Shalom Laboratory Supplies c.c. 132 Commercial Road, International Plaza, Durban 4001, P. O. Box 57030, Musgrave Road Durban 4062.

[14] Beckman Coulter Inc. 4300 N Harbour Boulevard, Box 3100, Fullerton, California, 92834-300. USA.

Each treatment was replicated three times and they were arranged in a split plot design. The whole plot was fertilized with the full amount of P and K recommended according to the soil analysis results and the two sub plots one with 35% of the normal amount of nitrogen (N) from lime ammonium nitrate (LAN) recommended for the crop, and the other was unfertilized. Two thirds of the fertilizer was applied at sowing and one third five weeks after sowing. The plots were 2x1m rectangles. Each plot had six rows spaced at 20 centimeters with a distance of 10 centimeters between plants. Both pre-emergence and post-emergence herbicides were used to control the weeds. Five plants were sampled for shoot dry biomass measurements every 30 days for three months. These plants were harvested at the soil level, dried in an oven at 70ºC for 72 hours and weighed. Yield parameters such as number of spikes, spikelets per spike and grains per spikelet were studied. Forty spikes per treatment were shelled for yield determination.

5.2.5 Statistical analysis

The data was subjected to analysis of variance (ANOVA) using GenStat 12.1 statistical package VAN International (2011). Treatment mean separation was done using Duncan's Multiple Range Test at the 5% level of significance.

5.3 Results

5.3.1 Effect of bacterial seed inoculation on shoot dry biomass under field conditions

Bacterial inoculation in combination with 35% N significantly ($P = 0.001$) increased shoot dry biomass relative to the Uninoculated Control, while there was no significant difference between the biomass of the inoculated plants and those of the Fully Fertilized Control in both cultivars. These increases varied with the interaction between wheat cultivars and bacterial isolates. The highest shoot biomass was obtained with *Bacillus* Isolates BS10 on C1 and BL5 in C2. With C1, shoot dry biomass increased by 0.2%-74.3% above the Uninoculated Control and 5%-28% above the fully fertilized control. Shoot dry biomass increased by 2.2%-75.4% above the Uninoculated Control and 4%-29% above the Fully Fertilized Control with C2. Higher shoot dry biomass was recorded with cultivar C1 in both trials (Figures 5.1 to 5.4 and Appendices 5.1 and 5.2).

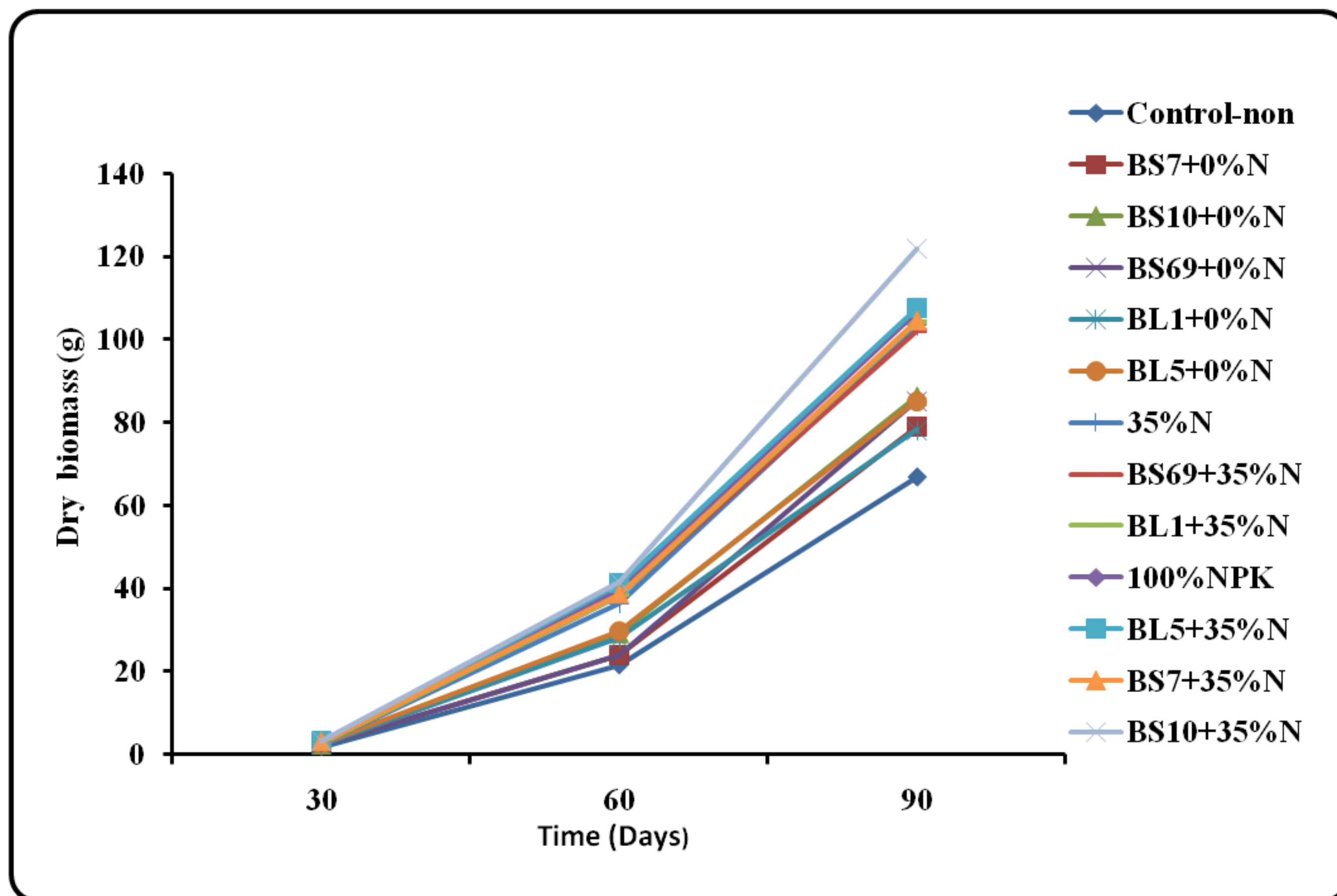

Figure 5.1: Effect of seed inoculation on shoot dry biomass of wheat (T1C1) under field conditions.

Treatments: Bacterial isolates plus Nitrogen (N) as a percentage of amounts recommended for the crop by the local Fertilizer Advisory Center Cedara. T1: Trial One; C1: Cultivar one; Time in days after planting; Control-non: Uninoculated control with no bacterial isolate or N fertilizer applied.

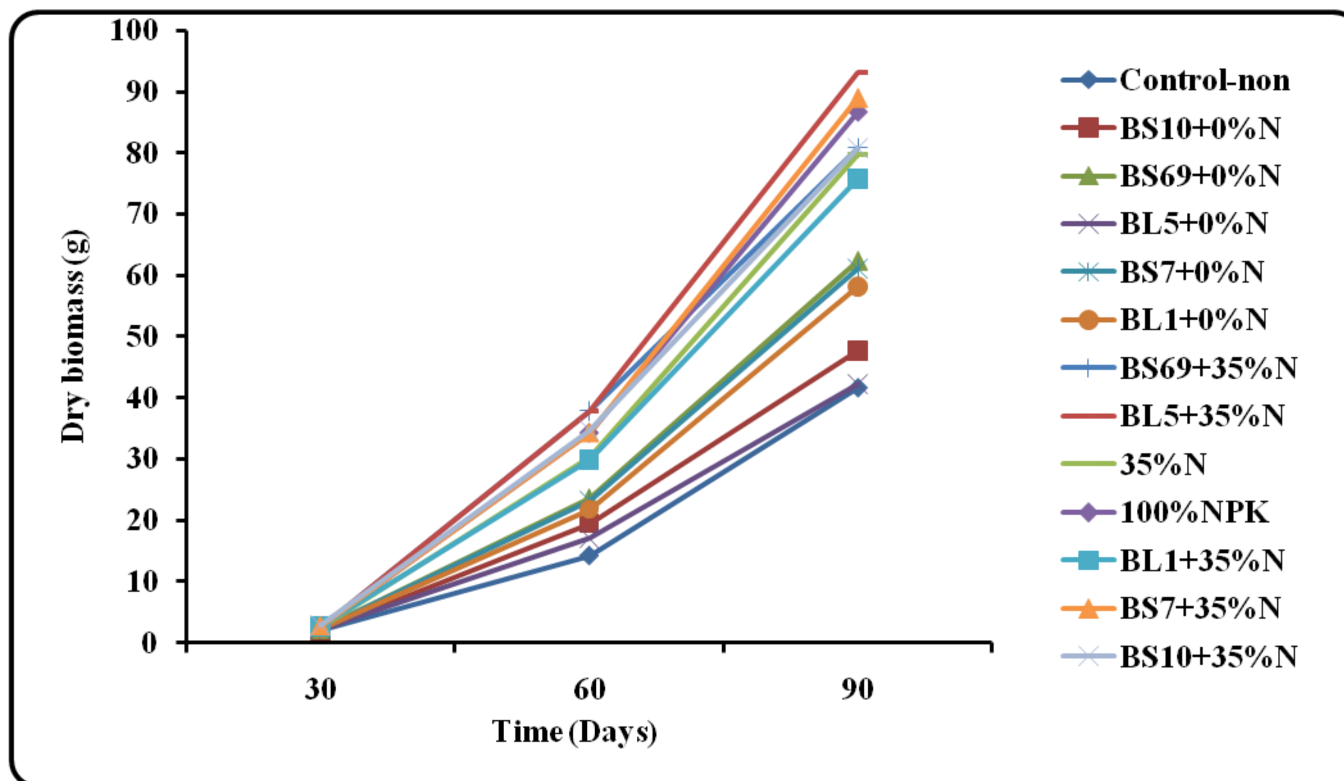

Figure 5.2: Effect of seed inoculation on shoot dry biomass of wheat (T1C2) under field conditions.

Treatments: Bacterial isolates plus Nitrogen (N) as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center Cedara. T1: Trial One; C2: Cultivar Two; Time in days after planting; Control-non: Uninoculated control with no bacterial isolate or N fertilizer applied.

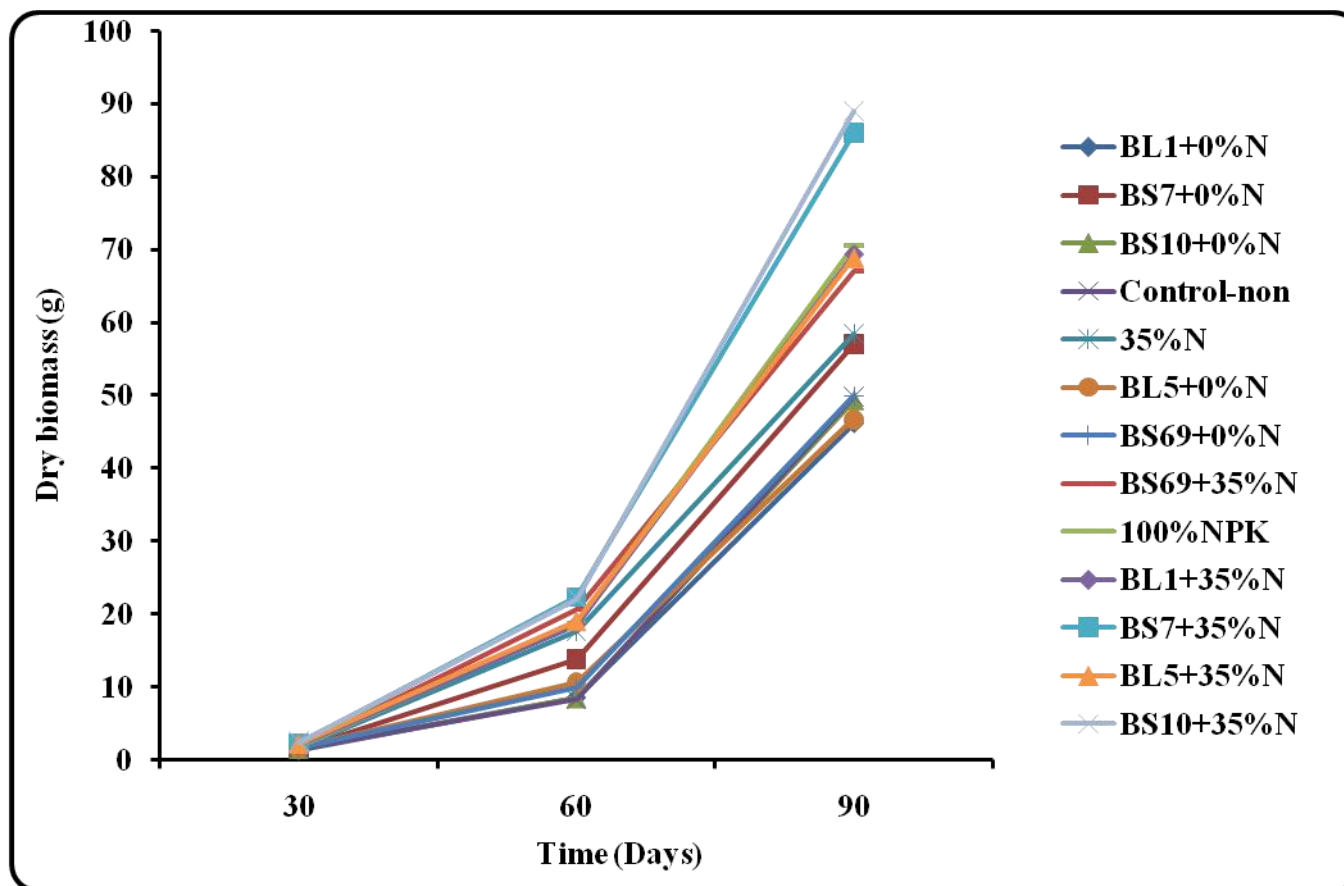

Figure 5.3: Effect of seed inoculation on shoot dry biomass of wheat (T2C1) under field conditions.

Treatments: Bacterial isolates plus Nitrogen (N) as a percentage of the amount recommended for the crop by the local Fertilizer Advisory Center Cedara. T2: Trial Two; C1: Cultivar one; Time in days after planting; Control-non: Uninoculated control with no bacterial isolate or N fertilizer applied.

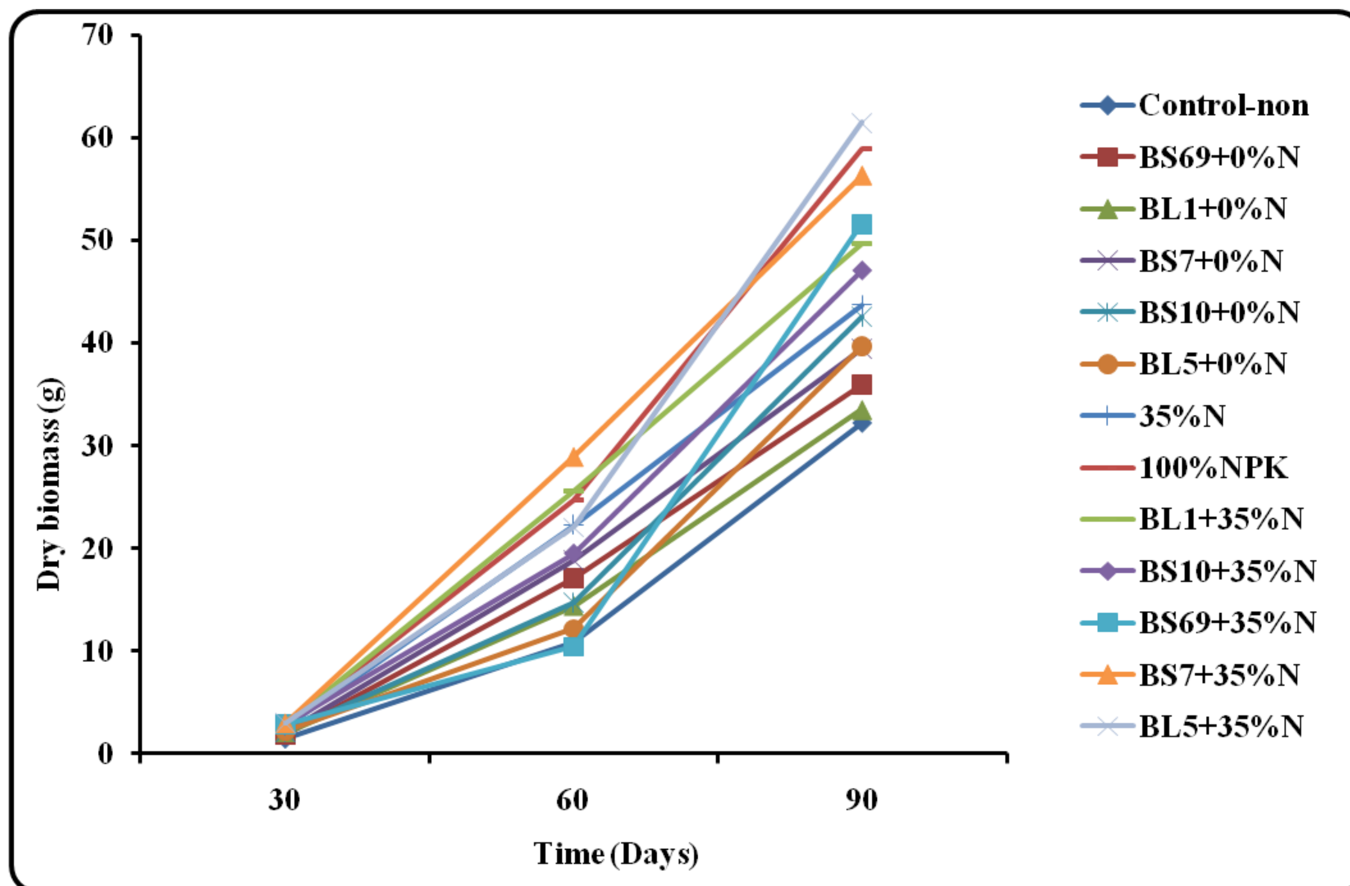

Figure 5.4: Effect of seed inoculation on shoot dry biomass of wheat (T2C2) under field condition.

Treatments: Bacterial isolates plus Nitrogen (N) as a percentage of amounts recommended for the crop by the local Fertilizer Advisory Center Cedara. T2: Trial Two; C2: Cultivar Two; Time in days after planting; Control-non: Uninoculated control with no bacterial isolate or N fertilizer applied; 100% NPK: Fully Fertilized Control.

5.3.2 Effect of bacterial inoculation on yield (g) of two wheat cultivars under field conditions

Yields of inoculated plants at 35% N were significantly higher ($P < 0.05$) than the Uninoculated Control but there was no significant difference between the yields of inoculated plants at 35% N and the Fully Fertilized Control. Yields varied with the isolates and wheat cultivar interaction. The highest yields were recorded by C1 treated with Isolates BS10 and C2 treated with Isolate and BS7. Yield increases ranged between 1.4%-96.5% above the Uninoculated Control and 6.6%-43.1% above the Fully Fertilized Control in C1 and 2.5%-37.4% and 4%, respectively, in C2. The highest yield was obtained with inoculation of C1 with Isolate BS10 in combination with 35% N. The three isolates (BS10, BS7 and BL5) which caused the best shoot dry biomass caused the best yield increases. Increases in yields were consistent with increases in shoot dry biomass. There was no significant difference between the yields of the two wheat cultivars (Table 5.3).

Table 5.3: Effect of seed inoculation on yield of two wheat cultivars (C) under field conditions

Cultivar one (C1)					Cultivar two (C2)				
Treatments	T1 Yield (g)	Yield (%)	T2 Yield (g)	Yield (%)	Treatments	T1 Yield (g)	Yield (%)	T2 Yield (g	Yield (%)
Control-non	14.470a	46.6	24.210ab	61.1	Control-non	27.170a	46.6	27.370a	66.8
BL5+0%N	25.530ab	82.3	36.010bcde	91.1	BS69+ 0% N	28.740ab	49.3	31.970ab	78.0
35%N	25.700ab	82.8	33.500bcde	84.5	BL1+0%N	30.620abc	52.5	34.670abc	84.6
BL1+0%N	25.870ab	83.4	18.330a	46.3	BL5+0%N	31.260abc	53.6	32.270abc	78.8
BS10+0%N	27.570abc	88.8	26.730abcd	67.4	BS69+35%N	31.930abc	54.8	33.500abc	81.8
BS7+0%N	28.330bc	91.3	24.730ab	62.4	BL1+35%N	35.720abcd	61.3	35.60abc	86.9
BS69+ 0% N	29.970bcd	96.6	25.170abc	63.5	BS7+0%N	35.920abcd	61.6	34.170abc	83.4
BS69+35%N	30.27bcd	97.6	25.770abcd	65.0	35%N	36.870bcd	63.3	32.400abc	79.1
100%NPK	31.030bcd	100.0	39.630cde	100.0	BS10+0%N	37.550bcd	64.4	33.570abc	81.9
BL1+35%N	38.700bcde	124.7	37.270bcde	94.0	BL5+35%N	39.590cd	67.9	36.770abc	89.7
BL5+35%N	40.530cde	130.6	40.070de	101.1	BS10+35%N	41.730d	71.6	35.870abc	87.6
BS7+35%N	42.170de	135.9	38.370bcde	96.8	BS7+35%N	43.300d	74.3	42.700c	104.2
BS10+35%N	44.400e	143.1	42.230e	106.6	100%NPK	58.290e	100.0	40.970bc	100.0
F-Value	**4.11**		**3.17**			**9.03**		**1.62**	
P- Value	**0.002**		**0.008**			**0.001**		**0.152**	
S.E.D.	**5.81**		**6.18**			**3.82**		**4.37**	
CV%	**22.90**		**23.90**			**12.70**		**15.20**	

Means in a column followed by the same letter are not significantly different ($P < 0.05$), according to Duncan's Multiple Range Test.

Treatments: Bacteria isolates + nitrogen (N) fertilizer as percentage (%) of the amount recommended for the crop by the local Fertilizer Advisory Center Cedara; Control-non: Uninoculated control with no bacterial isolate or fertilizer applied. Cultivar One: PAN 3434; Cultivar Two: PAN 3490, T1 and T2: Trials 1 and 2.

5.4 Discussion

Seed inoculation of wheat with some bacterial strains plus 35% N enhanced seedling growth and increased yields significantly. Reports on increases in wheat dry biomass following inoculation with rhizobacteria are well documented (Ryder *et al*., 1999; Khalid *et al*., 2005). Increases in biomass and yields of crops of agricultural importance after bacterial inoculation in the presence of low doses of N fertilizer have been recorded in previous researches. Kennedy *et al*. (2004) reviewed researches in which significant increases in growth and yield of several crops were reported following inoculation with several free-living bacteria genera in combination with low doses of nitrogen fertilizer. Increase in yields following seed inoculation with *Azospirillum* strains in combination with low doses of nitrogen have been reported by other authors (Favilli *et al*., 1987; Paredes-Cardona *et al*., 1988; Caballero-Mellado *et al*., 1992). Okon and Labandera-Gonzalez (1994) reviewed results obtained with different crops following inoculation with *Azospirillum* strains in several countries over a period of twenty years. Maximum shoot dry biomass and yields in the two cultivars in this study was recorded with Isolates BS10 (*Bacillus subtilis* (Ehrenberg) Cohn). *Bacillus* strains have been used extensively in agriculture both for plant growth promotion and biocontrol of pathogens (Shen 1997; Ryder *et al*., 1999; Niranjan *et al*., 2003). *Bacillus* strains have been used to increase yields of various agricultural crops in China for over 20 years. *Bacillus* Strain L324-9 was registered as a biofertilizer in 1998 in the USA for use on turf grass (Kim *et al.,* 1997; Mathre *et al.,* 1999). *Bacillus* inoculation enhanced *Rhizobium* nodulation in pigeon pea (Podile, 1995). Co-inoculation of *Bacillus* Isolate B69 with *Trichoderma atroviride* Strain SYN6 increased plant growth by 43.4% and nitrogen concentration in leaves of bean seedlings under greenhouse conditions (Yobo *et al*., 2011).Stimulation of plant growth and yield increases in wheat as a result of inoculation with diazotrophs has been documented (Kloepper *et al*., 1989; Boddey and Dobereiner, 1995; Hegazi *et al.*, 1998). The enhanced seedling growth and yield increases observed in this study can be attributed to several causes, such as: biological nitrogen fixation (BNF), production of plant growth hormones, siderophores and biological control of sub-lethal fungal pathogens. All isolates used in the current study reduced acetylene to ethylene, which is a characteristic of diazotrophic bacteria, all produced siderophores and IAA, and some inhibited growth of a wide range of pathogenic fungi *in vitro* (Chapters 2 and 7).

Plant growth promotion and yield increases have been attributed to multiple causes by other researchers (Gupta *et al*., 1998; Dey *et al*., 2004; Compant *et al*., 2005). Biological nitrogen fixation was implicated in plant growth and yield enhancement by other authors (Soliman *et al.,* 1995; Pandey *et al.,* 1998; Boddey *et al*., 2001; Hurek *et al*., 2002; James *et al*., 2002). Malik *et al*. (1997) demonstrated that 26% of nitrogen in Kallar grass (*Leptochloa fusca* (L.) Kunth) was derived from BNF. Nitrogen deficiency symptoms in wheat were relieved after inoculation with *Klebsiella pneumoniae* (Shcroeter) Trevisan Strain Kp342 in the absence of N fertilizer whereas the symptoms persisted in the Uninoculated Control (Iniguez *et al*., 2004). *Klebsiella pneumoniae* has been shown to produce dinitrogen reductase (Chelius and Triplett, 2000). Reviews on growth promotion and yield increases resulting from BNF by bacteria exist in literature (Vessey, 2003; Kennedy *et al*., 2004). Isolates BS10 and BS7 with the best yields in the current study, were among the five isolates, with the largest amount of ethylene produced (Chapter 2). Siderophore-production is a possible contributing factor to plant growth and yield enhancements recorded in this study. All isolates in this study produced varying amounts of siderophores. Siderophores-producing fluorescent pseudomonads increased growth and yield of chickpea (*Cicer arietinum* L.) and soybean (*Glycine max* L (Merr)) (Kumar and Dube, 1992)**.** Phytohormone production has been proposed as a contributing factor to increase in growth promotion observed in inoculated plants by other researchers (Fuentes-Ramirez et *al*., 1993; Glick, 1995; Okon and Vanderleyden 1997; Vessey, 2003; Ahmad *et al*., 2006). Indole-3- acetic acid enhances root development, which allows for improved water and nutrient acquisition by the roots, to which improved plant performance has been attributed (Riggs *et al*., 2001. Direct involvement of phytohormone production in growth promotion has been demonstrated in canola (*Brassica napus* L.) and lettuce (*Lactuca sativa* L.) (Noel *et al*., 1996), and wheat (Khalid *et al.,* 2005). Cakmakci *et al*. (2007) demonstrated that a number IAA-producing *Bacillus* strains enhanced growth in wheat and spinach. Six *K. pneumoniae* IAA producing isolates from the rhizosphere of wheat enhanced germination and root development in moth bean **(***Vigna aconitifolia* (Jacq) Marechal) and significantly increased root length and shoot height of wheat (Sachdev *et al*., 2009). Growth improvement resulting from inoculation with *Azospirillum* species was attributed to the effects of IAA produced by these species.

Dobbelaere *et al*. (1999) and Lambrecht *et al*. (2000) demonstrated that non-IAA producing mutants of *Azospirillum* could not enhance growth in wheat under greenhouse conditions. Treatment with Isolate BS10 resulted in the highest biomass and yield and this isolate produced the largest amount of IAA (Chapter 2). Increases in dry biomass and yields of wheat could have resulted in part from suppression of pathogens in the rhizosphere of this crop. *Bacillus subtilis* (BS10), which stimulated the maximum shoot dry biomass and yield, inhibited the growth of several pathogenic fungi *in vitro* and suppressed the effects of *Rhizoctonia solani* Kühn in wheat under greenhouse conditions (Chapters 2 and 6). Kloepper (1993) reported that most PGPR isolates that have been studied seem to promote plant growth by suppression of deleterious pathogens. Other factors that contribute to enhanced growth include phosphate-solubilization and induced systemic resistance. However the capacity for phosphate-solubilization was not detected in any of the isolates, and induced systemic resistance was not investigated in this study.

Performance of the isolates used in this study varied with the wheat cultivar. Isolate BS10 produced the best results when inoculated onto C1, whereas the best results with C2 were achieved with Isolates BL5 and BS7. Similar responses of crop varieties to bacterial inoculation have been reported in the literature (de Salamone *et al*., 1996; Riggs *et al*., 2001; Dobbelaere *et al*., 2002; Munos-Rojas and Caballero-Mellado, 2003; de Oliveira *et al*., 2006; Mehnaz *et al*., 2010). *Bacillus* species isolated from wheat rhizosphere increased the growth of a wheat cultivar, Katepwa, while no increase was observed in another cultivar, Neepawa HY320 (Chanway *et al*., 1998). Iniguez *et al*. (2004) demonstrated that an isolate of *K. pneumoniae* isolated from wheat rhizosphere relieved nitrogen deficiency symptoms in one cultivar Trenton while the symptoms persisted in two other cultivars Russ and Stoa. The biomass of Trenton and Stoa increased significantly above that of Russ. These differences in response were attributed to interaction between bacteria and plants, resulting from production of different types of root exudates, and by gaseous diffusion efficiency by different crops and genotypes (Frankenberger and Arshad 1995; Kloepper, 1996; Dazzo *et al*., 2000). Khalid *et al*. (2005) concluded that the effect of bacterial inoculation on plants was influenced by interactions between bacterial strain, plant species, cultivar and environmental conditions. Homologous strains gave the highest biomass and yield. Isolates BS10, BS7 were isolated from the rhizosphere of wheat and BL5 from wheat leaves.

This concurs with observations made by other researchers with *Azospirillum* strains (Favilli *et al*., 1987; Sumner, 1990; Wani, 1990). An inoculated low-yielding Cultivar recorded similar biomass and yield increases as the inoculated high-yielding Cultivar. This similarity observed in performance of the two wheat cultivars implies that bacterial seed inoculation might have the capability to improve the performance of low-yielding crop cultivars. Similar observations were recorded with the bacterial inoculation of two maize cultivars (Chapter 4).

There was consistency in the results obtained with these isolates in the laboratory, greenhouse and in the field and the isolates that caused the highest increases in shoot biomass also caused the greatest yield increases. The isolates BS7 and BS10 which caused the best results under field conditions, exhibited a number of growth-promoting traits *in vitro,* and caused the best seedling growth under greenhouse conditions. However, further investigation must be done with more plant genotypes, more fertilizer levels and in different seasons to confirm and expand these results. The lack of significant difference between the shoot biomass and yields obtained after inoculation with some bacterial isolates in combination with 35% N compared with the Fully Fertilized Control implies that inoculation of wheat with these isolates can meet 65% of the crop's nutritional requirements. The use of suitable free-living bacterial strains as inoculant biofertilizers, in combination with a low dose of N fertilizer, could enhance wheat cultivation by both commercial and small scale farmers.

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Appendices

Appendix 5.1 Effect of seed inoculation on shoot dry biomass of two wheat cultivars in Trial One (T1) under field conditions

Cultivar 1				Cultivar 2			
Treatments	**30 Days**	**60 Days**	**90 Days**	**Treatments**	**30 Days**	**60 Days**	**90 Days**
Control-non	1.850a	21.55a	67 00a	Control-non	1.963a	14.22a	41.57a
BS7+0%N	2.230ab	23.81ab	79 00ab	BS10+0%N	2.007ab	19.49ab	47.73ab
BS10+0%N	2.270ab	29.14abc	86.33 ab	BS69+0%N	2.147abc	23.58bc	62.37abc
BS69+0%N	2.283abc	23.83ab	85.33 ab	BL5+0%N	2.170abcd	17.09ab	42.20a
BL1+0%N	2.373abcd	28.1abc	78.33 ab	BS7+0%N	2.187abcd	23.27bc	61.20abcd
BL5+0%N	2.507abcd	29.72abc	85.33 ab	BL1+0%N	2.300abcde	21.79b	58.27abc
35%N	2.537abcd	36.25bc	103.50 bc	BS69+35%N	2.380abcde	37.88e	80.90cde
BS69+35%N	2.687bcde	38.22c	102.33 bc	BL5+35%N	2.437bcde	37.89e	93.24e
BL1+35%N	2.770bcde	38.45c	104.33 bc	35%N	2.443bcde	30.29cde	79.67cde
100%NPK	2.873bcde	39.72c	106.67 bc	100%NPK	2.547cdef	34.26de	86.70cdcde
BL5+35%N	3.043cde	41.11c	107.67 bc	BL1+35%N	2.607def	29.89cd	75.8bcde
BS7+35%N	3.053de	38.69c	104.67 bc	BS7+35%N	2.713ef	34.28de	89.0de
BS10+35%N	3.313e	41.76c	122 00c	BS10+35%N	2.907f	34.69de	80.8cde
F-value	**3.2**	**3.05**	**3.02**		**4.3**	**11.5**	**4.2**
P-Value	**0.007**	**0.01**	**0.01**		**0.001**	**0.001**	**0.001**
S.E.D.	**0.3**	**5.90**	**12.67**		**0.2**	**3.4**	**12.5**
CV%	**15.2**	**21.80**	**16.40**		**9.7**	**15.0**	**22.1**

Means followed by the same letter in a column are not significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.

Treatments: Bacteria Isolates + Nitrogen (N) fertilizer as percentage (%) of the amount recommended for the crop by the local Fertilizer Advisory Center Cedara; Control-non: Uninoculated Control with no bacterial isolate or fertilizer applied.

Appendix 5.2: Effect of seed inoculation on shoot dry biomass of two wheat cultivars in Trial Two (T2) under field conditions

	Cultivar 1					Cultivar 2		
Treatments	**30 Days**	**60 Days**	**60 Days**	**90 Days**	**Treatments**	**30 Days**	**60 Days**	**90 Days**
BL1+0%N	1.330a	8.60a	(0.932a)	46.20a	Control-non	1.500a	10.84a	32.23a
BS7+0%N	1.463ab	13.82abc	(1.135abc)	57.03ab	BS69+0%N	1.863ab	17.15abcde	36.00abc
BS10+0%N	1.487ab	8.44a	(0.913a)	49.27a	BL1+0%N	2.040abc	14.38abc	33.53ab
Control-non	1.530ab	8.34a	(0.193a)	49.80a	BS7+0%N	2.063abcd	18.85bcdef	39.47abcd
35%N	1.620abc	17.66abc	(1.207bc)	58.50ab	BS10+0%N	2.140abcd	14.78abcd	42.57abcd
BL5+0%N	1.633abcd	10.62ab	(1.016ab)	46.80a	BL5+0%N	2.180abcd	12.12ab	39.67abcd
BS69+0%N	1.643abcd	9.90ab	(0.993ab)	49.93a	35%N	2.657bcd	22.29defg	43.73abcde
BS69+35%N	1.847abcd	20.57bc	(1.285c)	67.03abc	100%NPK	2.703bcd	24.75efg	58.93fg
100%NPK	1.903bcd	18.79abc	(1.261c)	70.53abc	BL1+35%N	2.820cd	25.54fg	49.70cdefg
BL1+35%N	1.973bcde	18.57abc	(1.259c)	69.4abc	BS10+35%N	2.840cd	19.5bcdef	47.03bcdef
BS7+35%N	2.127cde	22.30c	(1.334c)	86.07c	BS69+35%N	2.847cd	10.45a	51.63defg
BL5+35%N	2.143de	19.00abc	(1.208bc)	68.93abc	BS7+35%N	2.983d	28.93g	56.33efg
BS10+35%N	2.443e	22.17c	(1.285c)	89.07c	BL5+35%N	3.000d	22.08cdefg	61.50g
F-value	**4.3**	**2.8**	**(4.7)**	**2.9**		**3.1**	**6.3**	**5.0**
P-Value	**0.001**	**0.015**	**(0.001)**	**0.013**		**0.009**	**0.001**	**0.001**
S.E.D.	**0.2**	**4.6**	**(0.1)**	**11.2**		**0.4**	**3.3**	**6.0**
CV%	**15.2**	**36.9**	**(11.2)**	**22.2**		**19.8**	**22.0**	**16.3**

Means in a column followed by the same letter are not significantly (P<0.05) different according to Duncan's multiple range test.

Treatments: Bacteria isolates + nitrogen (N) fertilizer as percentage (%) of the amount recommended for the crop by the local Fertilizer Advisory Center Cedara; Control-non: Uninoculated Control with no bacterial isolate or fertilizer applied.

CHAPTER SIX

Evaluation of selected *Bacillus subtilis* (Ehrenberg) Cohn isolates as potential biological control agents against *Rhizoctonia solani* Kühn damping-off of wheat (*Triticum aestivum* L.) under greenhouse conditions

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Abstract

Five *Bacillus subtilis* isolates (BL3, BS69, BS820, BS817 and BS10) which inhibited the growth of *Rhizoctonia solani in vitro,* were tested for biological control of *R. solani* damping-off of wheat under greenhouse conditions. Two *Klebsiella pneumoniae* isolates (BS7 and BS431) that did not inhibit growth of *R. solani in vitro*, and Eco-T®, a commercial biocontrol agent of *Trichoderma harzianum* Rifai, were included for comparison. The *R. solani* isolate used in this study was isolated from the rhizosphere of common bean. All the biocontrol agents were applied as seed treatments. Tests were conducted both in Speedling®24 trays and rhizotrons under greenhouse conditions. The wheat variety Krokodil was used because it is susceptible to most fungal diseases of wheat. An *in vitro* seedling test was conducted on water agar. Seedlings inoculated with *R. solani* developed brown lesions and died after seven days of incubation while the Uninoculated Control had no disease symptoms. A number of plant species were screened for susceptibility to *R. solani* under greenhouse conditions. All plant hosts tested under greenhouse conditions were susceptible to *R. solani*. Studies in the Speedling® trays showed that bacterial inoculation provided some disease control based on seed germination, seedling survival and shoot dry biomass. Seed germination, seedling survival and shoot dry biomass of seedlings treated with *Bacillus* strains were significantly higher (P = 0.001) than the Diseased Control. There was no significant difference (P =0.001) between germination and seedling survival as a result of treatment caused by *B. subtilis* (Isolate BS10) or Eco-T®, or the Disease-Free Control. Among the isolates, *Bacillus* Isolate BS10 caused the highest seed germination, seedling survival and shoot dry biomass. Seed inoculation increased seed germination by 5%-23%, seedling survival 8%-25% and shoot dry biomass 10%-30% above the Diseased Control. Seed germination, seedling survival and shoot dry biomass were significantly lower than the Diseased Control after inoculation with two *K. pneumoniae* isolates which did not inhibit growth of *R. solani in vitro*. Rhizotron studies with *Bacillus* isolates revealed that seed inoculation enhanced root and shoot growth relative to the diseased control.

Inoculation with Isolate BS10 resulted in the highest root and shoot biomass, which were not significantly different (P = 0.001) from those of the Disease-Free Control. Root biomass increased by 28%-68% and shoot biomass increased by 14%-37% relative to the Diseased Control. These results suggest that, the *Bacillus* isolates used in this study have the potential to control damping-off of wheat caused by *R. solani*.

Key words: *Rhizoctonia solani*; biocontrol; *Bacillus subtilis*; seed germination; seed survival; dry biomass; root development.

6.1 Introduction

Crop losses caused by pathogenic microorganisms range from 25%-100% (Glick and Bashan, 1997; Kulkarmi, 2006). Globally, food loss due to the effects of plant diseases is estimated at 10% (Strange and Scotts, 2005). Agrochemicals are often used to reduce these losses. However, they are hazardous to both animals and humans, and may accumulate in natural ecosystems and eliminate beneficial microorganisms (Glick and Bashan, 1997; Niranjan *et al*., 2003). This calls for an urgent search for eco-friendly biological alternatives for sustainable agriculture. Use of non-pathogenic microorganisms for disease control has been proposed as an option or supplementary mechanism to chemical control (Handelsman *et al*., 1990; Berger *et al.,* 1996; Sharga and Lyon 1998; Raaijmakers *et a*l., 2002; Schisler *et al*., 2004). Several microorganisms can act as natural antagonists to phytopathogens (Chet and Inbar, 1994; Niranjan, *et al*., 2004; Idris *et al*., 2007). Production of antifungal compounds, competition, for nutrients and induced systemic resistance have been proposed as the main mechanisms involved in disease suppression by biological control agents (BCAs) (Chet *et al*. 1990; Glick and Bashan, 1997; Ryder *et al,* 1999; Guetsky *et al*., 2002; Haas and Keel, 2003; Nelson, 2004; Compant *et al.,* 2005; Ryu *et al*., 2005). Bacterial genera commonly studied and exploited as BCAs against plant pathogens include *Pseudomonas* Migula, *Streptomyces* Waksman & Henrici, *Agrobacterium* Conn and *Bacillus* Cohn (Cook, 1993; Larkin and Fravel, 1998; Ahmad *et al*., 2008).

A number of *Bacillus* strains control plant pathogens (Zhang *et al*., 1996; Utkhede *et al*.,1999; Murphy *et al*., 2000; Kloepper *et al*.,2004; Dutta *et al*., 2008). Strains in the genus *Bacillus* are ideal candidates to be BCAs because of their ubiquitous distribution, motility, production of resistant endospores, variation in nutrient utilization and broad spectrum activity (Brock and Madigan, 1991; Priest, 1993; Nicholson, 2002; Gardener, 2004). Several *Bacillus* biocontrol products are commercially available. Gardener and Fravel (2002) and Schisler *et al*. (2004) reviewed *Bacillus*-based biocontrol commercial products in USA, but further information on *Bacillus*-based commercial BCAs is scarce. Species of the genus *Rhizoctonia* are soil-borne fungal pathogens that affect a wide range of important agronomic crops, vegetables, ornamentals, shrubs and trees worldwide (Kloepper, 1991; Agrios, 1997; Ryder *et al*., 1999). Infection may occur at any growth stage but it is most common at the seedling stage (Agrios, 1997; Mathre *et al*., 1999). Reports on root rot in wheat caused by *Rhizoctonia* species are well documented (Ogoshi *et al*., 1990; Paulitz *et al*., 2002; Paulitz and Schroeder, 2005). However in South Africa, information on biological control of *Rhizoctonia* damping-off of wheat by *Bacillus* is scarce. The aim of the current study was to identify promising *Bacillus* strains as potential BCAs for management of *Rhizoctonia* damping-off in wheat.

6.2. Materials and methods

6.2.1 Source of bacteria cultures and inoculum preparation

Five *Bacillus subtilis* isolates, BL3, BS10, BS69, BS817 and BS820, used in this study, were selected through *in vitro* screening for antifungal activity against *R. solani* (Chapter 2). Two *K. pneumoniae* isolates BS7 and BS431, (that did not cause any inhibition i*n vitro*), together with Eco-T® (*Trichoderma harziuanum* Rifai) a commercial BCA, were included for comparison.

Cultures of these isolates were inoculated in tryptic soy broth and incubated in an orbital shaker incubator[15] at 28ºC for 48 hours. Cells were harvested by centrifuging (Beckman Coulter Avanti J-26XPI High Speed Centrifuge) [16]at 10,000 rpm for 15 minutes at 4ºC.
Cell numbers were adjusted to, approximately 10^8 cfu ml^{-1} by the dilution method using sterile distilled water. Cell counts were done using a counting chamber and viability confirmed by a plate count method. This procedure was repeated for each subsequent experiment.

6.2.2 Seed inoculation

Wheat seed of the Cultivar Krokodil was provided by the ARC-Small Grain Institute.[17] Seeds were disinfected by soaking in 0.2% Sodium hypochlorite for 2 minutes and rinsed several times in double sterilized distilled water. Seed inoculation was done by soaking the seed in a bacteria suspension in 2% carboxymethylcellulose (CMC) for two hours with intermittent wrist shaking to enhance adhesion of the cells onto the seed. The seed was then dried under a lamina flow overnight. This procedure was followed for seed inoculation in all experiments.

6.2.3 Isolation of *R. solani* cultures

A number of *R. solani* isolates were isolated using a modification of the toothpick baiting technique used by Kumar *et al*. (1999). Sterile toothpicks were inserted into the rhizosphere of a number of plants species that included soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.), wheat, cabbage (*Brassica oleracea* L.) and tomato (*Solanum lycopersicum* L.). They were then collected after 48 hours and placed one per plate onto basic water agar (pH 8) and incubated at 28°C for 24 hours. The plates were observed under a stereo microscope for fungal growth. To obtain pure cultures, 4-mm mycelia plugs were cut from the edge of young cultures and transferred to fresh agar plates and incubated at 28°C for 3-5 days.

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[15]Shalom laboratory Supplies c.c. 132, Commercial road, International Plaza, Durban, 4001, P. O. Box 51030, Musgrave road, Durban, 4062.
[16] Beckman Coulter Inc. 4300 N Harbour Boulevard, Box 3100, Fullerton, California, 92834-300. USA.
[17]ARC-Small Grain Institute Private Bag, X29, Bethlehem, Republic of South Africa, 9700

They were then transferred onto Potato Dextrose Agar (PDA) and incubated for 3-5 days. Wet mounts were prepared from each plate and observed under a compound microscope. *Rhizoctonia solani* isolates were identified based on morphological characteristics as described by Agrios (1997). Pure cultures were preserved as plugs in double sterilized distilled water or grown on agar slants or barley seed for subsequent use. Stored cultures were revived by plating on PDA and incubating at 28°C for 3-5 days. The culture was sent to Plant Protection Research Institute (PPRI), Agricultural Research Council[18] for identification.

6.2.4 Pathogenicity test

Preserved *R. solani* was plated onto PDA and incubated at 28°C for 3-5 days. For *in vitro* tests, 4 mm mycelia plugs from each isolate were placed at the center of a water agar plate. Wheat seeds were placed at the edge of the plate on opposite sides. A control was set up without the fungus. The plates were incubated at 28°C and observed daily for disease symptoms. For the *in vivo* test, wheat seeds were planted at a depth of 11mm at the center of each cell in the Speedling® 24 tray. Four mm plugs of each isolate were cut from the edge of a young culture and placed on top of the growth medium a few mm from the center of each cell. The trays were placed in the greenhouse and watered three times daily with a solution of NPK soluble fertilizer [3.1.3 (38)] Complete® at a concentration of 1 g l^{-1} using a micro jet overhead irrigation system. The temperatures ranged between 22-26°C and a relative humidity 60%-70%. The plants were observed for typical damping-off symptoms, which included pre- and post-emergence damping-off, wilting, root and shoot rot, and stunting. Damped off seeds and infected roots and shoots were collected and taken to the laboratory for re-isolation of the fungus. To re-isolate the fungus, small pieces of the infected root and shoot, and damped seeds were disinfected in 70% ethanol, rinsed in sterile distilled water, dried on sterile filter paper and plated onto water agar. The plates were incubated at 28°C for 24 hours and observed for growth of the fungus.

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[18]Mycology Diagnostic Services, Biosystematics, ARC-PPRI, Private Bag X134, Queenswood, Republic of South Africa, 0121

The fungus was then sub-cultured on PDA and examined for morphological characteristics of *R. solani*. A test was conducted on a number of crop species to test for susceptibility of different plant hosts.

6.2.5 Screening bacterial isolates as biological control agents of *Rhizoctonia solani* damping-off of wheat under greenhouse conditions

Wheat seeds were treated as in Section 6.2.3 above. Speedling® 24 trays were filled with composted pine bark. The trays were seeded with one seed placed at the center of each cell. R. solani fungal plugs (4 mm) cut from the edge of a young culture were placed a few mm from the center of each cell. Seeds treated with Eco-T®, a commercial strain of *T. harzianum*, were used as a standard control. Seeds treated with CMC alone were used for the diseased and Disease-Free Controls. A Disease-Free Control had uninoculated seed and plain agar plugs. The treatments were replicated three times and the trays were arranged in a randomized complete block design. The trays were kept in the greenhouse and watered three times a day with a solution of NPK soluble fertilizer [3.1.3 (38)] Complete® at a concentration of 1g l^{-1} using micro jet overhead irrigation system. Temperatures were maintained at a range of 22-26°C and relative humidity 60%-70%. Seeds were rated for germination after two weeks and seedling survival after six weeks. The plants were harvested after six weeks at the soil level, dried in the oven and weighed to determine shoot dry biomass of plants per tray. The experiment was replicated three times and means pooled for statistical analysis.

6.2.6 Rhizotron studies on *Bacillus* biocontrol of *Rhizoctonia solani* damping-off in wheat under greenhouse conditions

Wheat seeds were treated as in Section 6.2.3 above with bacteria suspensions of five most promising bacteria isolates identified in the Speedling® 24 trays test. Rhizotrons were filled with composted pine bark.

Three rhizotrons were planted each with three seeds, which were thinned to one per rhizotron after germination. Each rhizotron was covered with aluminum foil to protect the roots from direct sunlight. The rhizotrons were placed in ice cream containers, kept in a greenhouse and watered three times a day with a solution of NPK soluble fertilizer [3.1.3 (38)] Complete® at a concentration of 1g l^{-1} using micro jet overhead irrigation system. Temperatures were maintained at a range of 22-26°C and relative humidity 60%-70%. After six weeks entire plants were harvested and separated into roots and shoots. They were then dried in the oven at 70ºC for 48 hours and weighed to obtain dry biomass.

6.2.7 Data analysis

The data was subjected to analysis of variance (ANOVA) using GenStat 12.1 statistical package. Mean separation was done using Fisher's L.S.D. at a 5% level of significance.

6.3 Results

6.3.1 Isolation of *Rhizoctonia solani*

Hyphae of *Rhizoctonia solani* grew out of the toothpicks after 24 hours of incubation on water agar at 28ºC (Figure 6.1). Pure cultures were white and turned brown when old. The hyphae were large, had septations and branched at 90º with a constriction at the base of the branch. The fungal isolate, accession number 162/745 was identified as *R. solani* AG4 HGI by Dr Mariette Truter.[19] Identification was done by molecular fungal identification based on BLAST results using sequences of the internal transcribed spacer region in GenBank and CBS databases.

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[19]Dr. Mariette Truter, Manager: Identification services, Biosystematics Programme, Mycology Unit PPRI, ARC, Private Bag X134, Queenswood, Pretoria, Republic of South Africa, 0121.

6.3.2 Pathogenicity test

In the *in vitro* test the seedlings had brown lesions on the shoots and roots (Figure 6.2 A and B) while no symptoms were observed on the Control (C). Fungal cultures recovered from the infected seedlings had typical morphological characteristics of *R. solani*.

The *in vivo* test revealed that *R. solani* caused both pre- and post-emergence damping-off. Some seedlings rotted, collapsed and died while others were stunted. *Rhizoctonia solani* was recovered from the infected seeds and seedlings. Typical hyphae emerged from infected seeds and pieces of roots and shoots plated on water agar after 24 hours (Figures 6.3 A and B). A fungal culture re-isolated from these tissues (infected seeds, roots and shoots) had typical morphological characteristics of *R. solani*.

6.3.3 Efficacy of bacterial seed inoculation on biocontrol of *R. solani* damping-off of wheat under greenhouse conditions

The study conducted in Speedling® trays revealed that bacterial inoculation significantly ($P < 0.005$) increased seed germination relative to the Diseased Control (DC). Germination after inoculation with Isolate *B. subtilis* (BS10) was not significantly different ($P > 0.05$) from the Disease-Free Control (DFC) and that inoculated with Eco-T®, the commercial BCA. *Bacillus* inoculation increased seed germination by 5%-23%. There was no significant difference ($P < 0.05$) between seedling survival caused by *Bacillus* inoculation and the DFC. Maximum seedling survival was observed in the DFC and minimum after inoculation with Isolate BS7. Isolate BS10 caused the highest seeding survival relative to the other *Bacillu*s isolates. *Bacillus* inoculation increased seedling survival by 8%-25% above the DC. Shoot dry biomass of inoculated seedlings was significantly different ($P < 0.05$) from the DC. Shoot dry biomass did not differ significantly among the Bacillus isolates but was significantly different ($P < 0.05$) from the DFC which had the highest biomass. *Bacillus* inoculation increased shoot dry biomass by 10%-30% above the DC.

Eco-T® treatment resulted in the highest germination and seedling survival, while BS10 treatment resulted in the highest shoot dry biomass.

The two isolates that did not inhibit growth of *R. solan*i *in vitro* did not enhance seed germination, seedling survival or shoot dry biomass (Table 6.1).

6.3.4 Rhizotron studies on *Bacillus* biocontrol of *R. solani* damping-off of wheat under greenhouse conditions

Bacillus inoculations enhanced root growth. Inoculated plants developed longer and more numerous lateral roots with more root hairs than the DC (Figure 6.4). Seed inoculation with *Bacillus* isolate significantly ($P<0.05$) increased both root and shoot dry biomass above the DC. Seed inoculation increased root biomass by 28%-68% and shoot biomass by 14%-37%. There was no significant difference between the root and shoot biomass obtained from inoculation with Isolate BS10 and the DFC, and seed treatment with this isolate resulted in the highest root and shoot dry biomass among the treated seedlings.

Figure 6.1 Hyphae of *Rhizoctonia solani* growing from the toothpick after 48 hours of incubation at 28°C on water agar

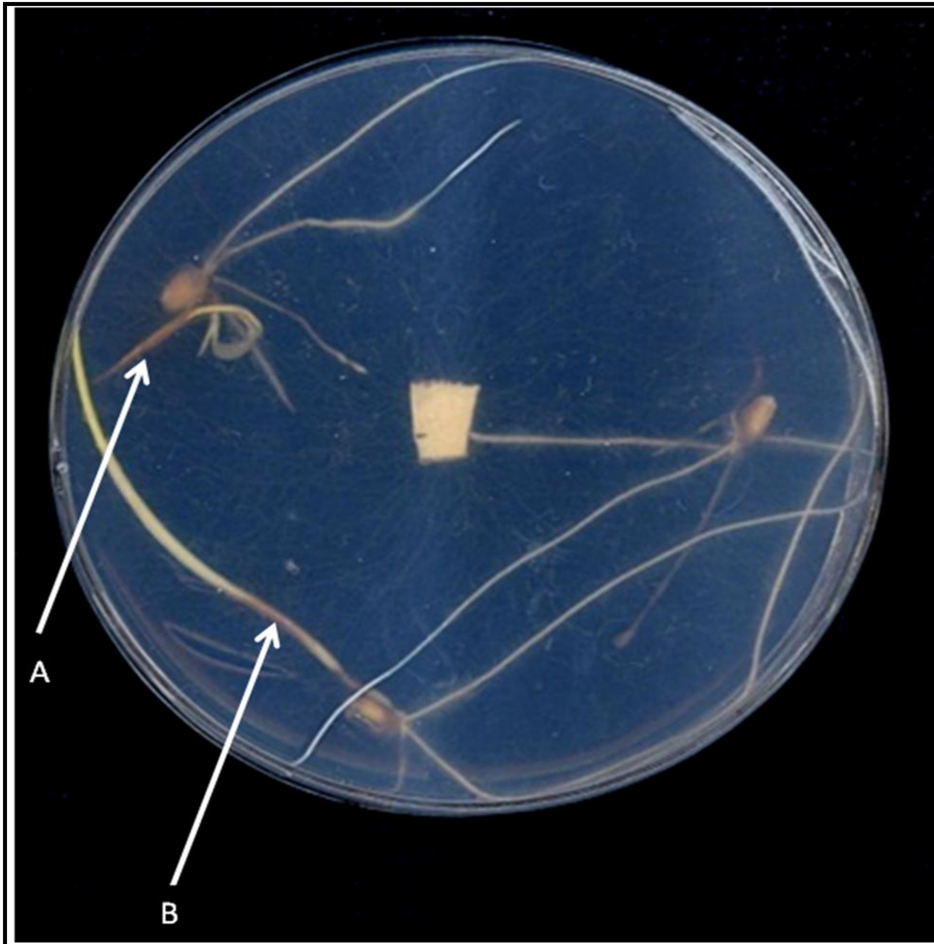

Figure 6.2 A: Seedlings infected by *R. solani* with brown lessions on the root (A) and shoot (B) after 5 days of incubation 28°C.

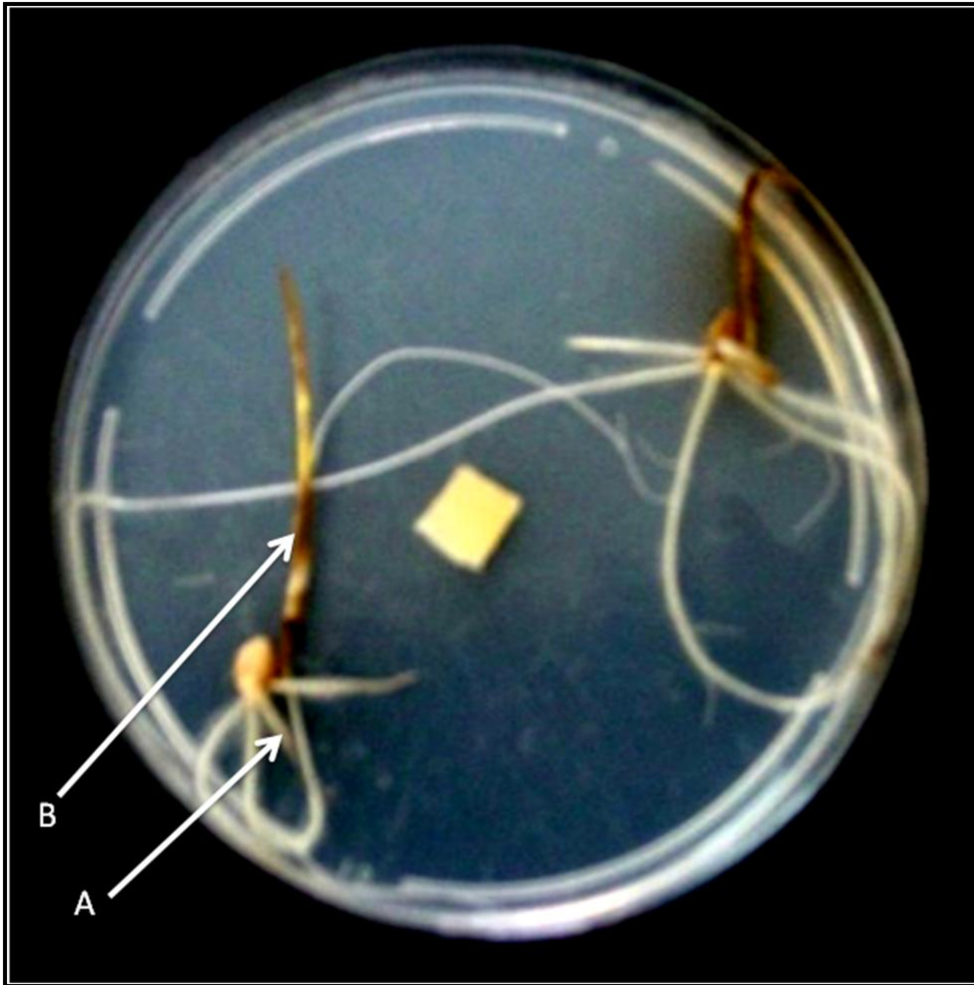

Figure 6.2 B: Seedlings infected by *R. solani* with brown lessions on the root (A) and shoot (B) after 7 days of incubation at 28°C.

Figure 6.2 C: Healthy seedlings (Control without *R. solani*) after 5 days of incubation at 28°C.

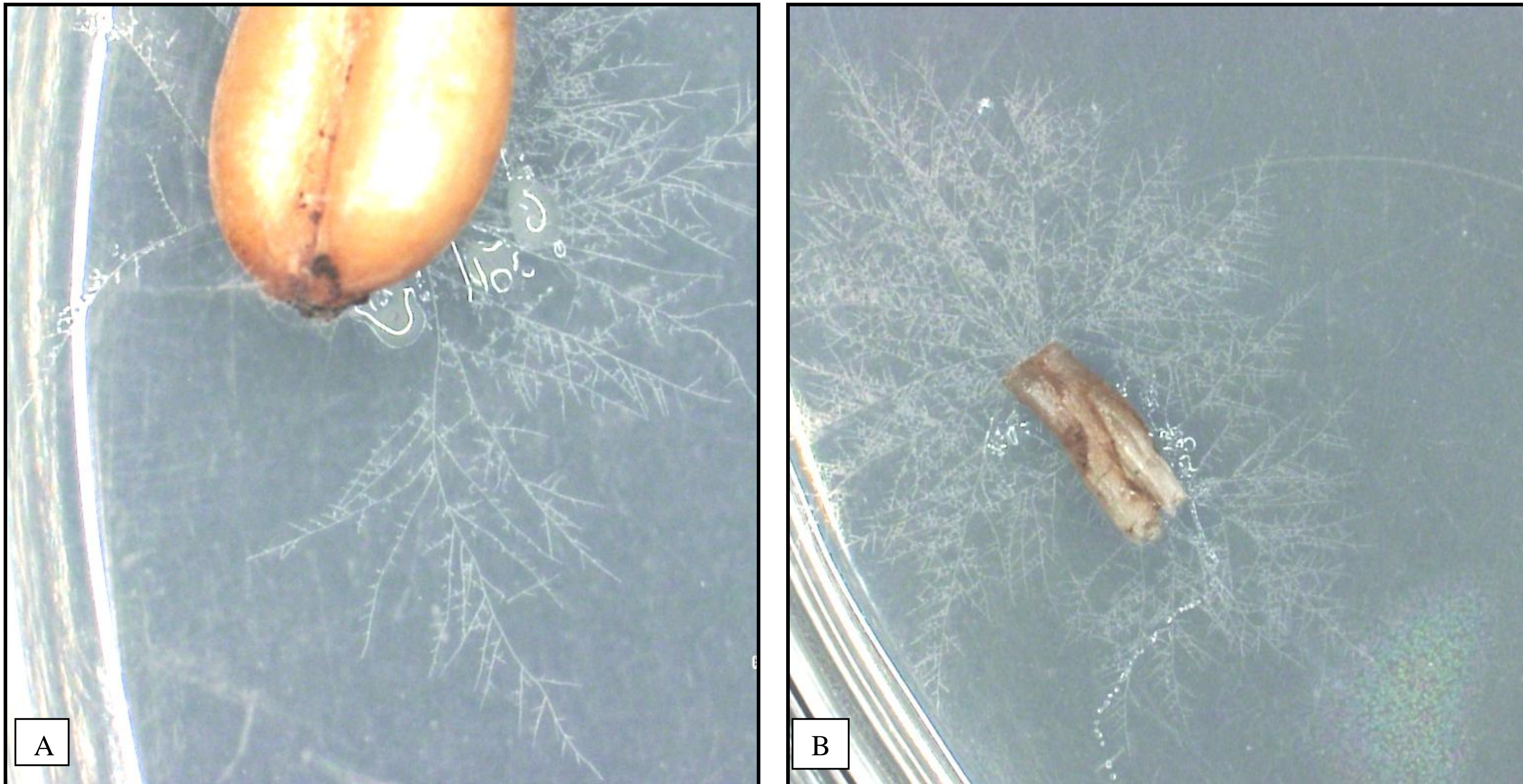

Figure 6.3 Effects of *Rhizoctonia solani* on germinating wheat seed and seedling

Hyphae of *R. solani* emerging from an infected seed (A) and an infected shoot (B) after 24 hours of incubation at 28ºC.

Table 6.1 Effects of bacterial inoculation on *Rhizoctonia solani* damping-off of wheat

Bacterial isolate	**Seed germination (%)**		**Bacterial isolate**	**Seedling survival (%)**		**Bacterial isolate**	**Shoot dry biomass (Percentage of the biomass of DFC)**	
BS7	55	a	BS7	61	a	DC	44	a
DC	57	ab	DC	63	ab	BS431	44	a
BS431	58	ab	BS431	67	ab	BS7	49	ab
BS69	62	abc	BS69	71	abc	BS69	54	abc
BS820	66	bc	BS820	74	abc	BS820	57	abc
BS817	67	bc	BS817	78	abc	BS817	57	abc
BL3	71	cd	BL3	88	abc	BL3	64	abc
BS10	80	de	BS10	88	abc	ECO-T®	68	bc
ECO-T®	80	de	ECO-T®	92	bc	BS10	73	c
DFC	89	e	DFC	98	c	DFC	100	d
F-Value	**11.4**			**8.71**			**7.5**	
P	**0.001**			**0.001**			**0.001**	
L.S.D.	**9.9**			**13.02**			**1.5**	
S.E.D	**4.7**			**6.2**			**0.7**	
CV%	**8.4**			**9.7**			**17.4**	

Means followed by the same letter in a column are not significantly different ($P < 0.05$) from each other according to Fisher's L.S.D.

DC: Diseased Control (With pathogen but without BCA); DFC: Disease-Free Control (Without pathogen or BCA); Eco-T®, (*T. harzianum*), a commercial BCA

Table 6.2 Rhizotron studies on biocontrol of *Rhizoctonia solani* damping-off of wheat using *Bacillus subtilis* under greenhouse conditions

Bacterial isolate	Root dry biomass (g)		Root dry biomass (Percentage of biomass of DFC)	Bacterial isolate	Shoot dry biomass (g)		Shoot dry biomass (Percentage of biomass of the DFC)
DC	0.043	a	9.5	DC	0.197	a	38.1
BL3	0.053	ab	36.4	ECO-T®	0.267	ab	51.6
BS820	0.073	abc	50.0	BL3	0.300	ab	58.1
BS817	0.077	abcd	52.3	BS820	0.350	b	67.3
BS69	0.087	bcd	59.1	BS69	0.370	bc	71.6
ECO-T®	0.093	cd	63.6	BS817	0.387	bcd	74.9
BS10	0.113	de	77.3	BS10	0.503	cd	74.8
DFC	0.147	e	100.0	DFC	0.517	d	100.0
F-Value	**7.4**				**6.08**		
P	**0.001**				**0.002**		
L.S.D.	**0.04**				**0.14**		
S.E.D	**0.02**				**0.06**		
CV%	**24.5**				**21.4**		

Means in a column followed by the same letter are not significantly different ($P < 0.05$) from each other according to Fisher's L.S.D.

DC: Diseased Control (With pathogen but without BCA); DFC: Disease-Free Control (Without pathogen or BCA); Eco-T®, (*T. harzianum*), a commercial BCA

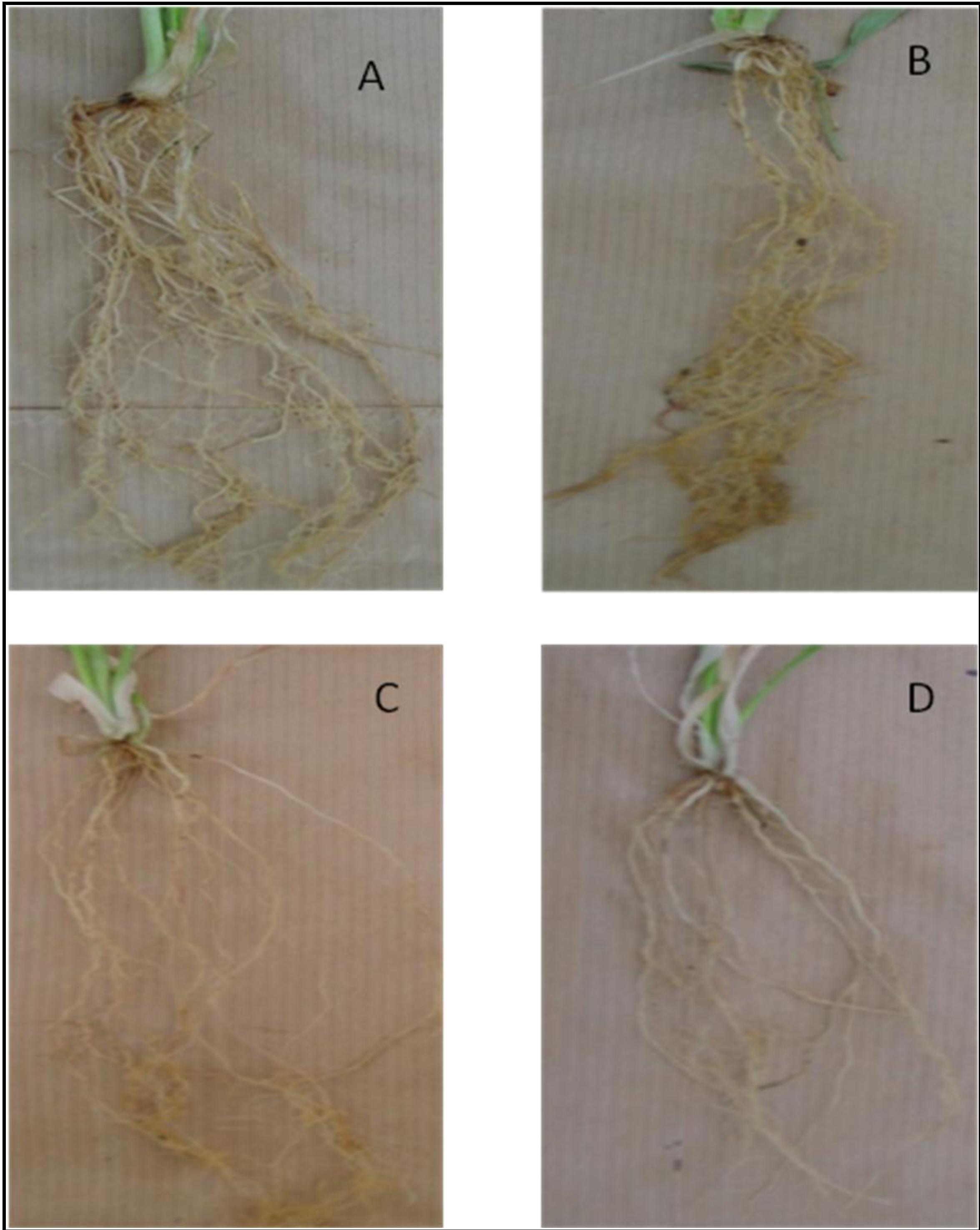

Figure 6.4 Effect of *Rhizoctonia solani* on wheat roots inoculated with *Bacillus* isolates after six weeks under greenhouse conditions.

Key: A: Disease-Free Control (Without pathogen or BCA); B: Isolate BS10 (*Bacillus subtilis*) inoculation plus pathogen; C*:* Eco-T® (*T. harzianum*) a commercial biocontrol agent inoculation plus pathogen; DC: Diseased Control (With pathogen but without BCA)

6.4 Discussion

The current study demonstrated that *Bacillus* strains may control *Rhizoctonia* damping-off of wheat. This was reflected in the higher root and shoot biomass recorded for the inoculated plants compared to the diseased control. *Bacillus* Strain L324-92 controlled take-all disease caused by *Gaeumannomyces graminis* (Sacc.) von Arx and D Olivier var. *tritici* J.C. Walker, *Rhizoctonia* root rot caused by *R. solani* and *Pythium* root rot of wheat caused by *P. irregular* Buisman and *P .ultimum* Trow (Kim *et al.,* 1997). A number of *Bacillus* strains reduced take-all diseases, *Rhizoctonia root* rot of wheat, and stimulated wheat seedling growth in Australia (Ryder *et al.,* 1999). *Bacillus cereus* Frankland Strain UW85 controlled damping-off of alfalfa seedlings (Handelsman, *et al*., 1990). Five *Bacillus* isolates inhibited the growth of *Fusarium oxysporum* f. sp. *conglutinans* Schlecht up to 90% *in vitro*, and reduced disease incidence and severity by 10-49% in cabbage seedlings (Kidane, 2004). *Bacillus subtilis* controlled *Rhizoctonia* damping-off in maize under greenhouse conditions (Ugoji and Laing, 2008). Guetsky *et a*l., (2002) reported that inoculation of biocontrol agents, together with plant growth promoting rhizobacteria, could increase disease suppression. Inoculation of cucumber with a combination of *Bacillus* Isolate B69 and Eco-T® and *Bacillus* Isolate B81 and Eco-T® caused maximum seedling survival and shoot dry biomass *(*Yobo *et al*., 2011). However, the effect of dual inoculation of *Bacillus* and Eco-T® was not investigated in this study. This is because none of the *Bacillus* strains used in this study was compatible with Eco-T® and all strains inhibited its growth *in vitro*. A number of mechanisms may be responsible for biological control of *R. solani* by *Bacillus* isolates observed in the current study. All *Bacillus* isolates used in this study caused up to 88% inhibition to *R. solani* growth *in vitro*. This inhibition was observed without the two organisms coming into contact with each other (Chapter 2 and Chapter 7). This implies that the inhibition could have been due to production of non-volatile antifungal compounds that diffused through the medium that were active against this pathogen, inhibiting its growth *in vitro*. This is because the test for volatile compounds showed that they were not present and the fungal growth was inhibited without the fungal and bacterial growths coming into contact in the plate.

Antibiotics produced by biocontrol agents inhibit fungal cell wall synthesis, which inhibits the growth of pathogens (Subbarao, 1999).

In prior studies antibiotic production has been implicated in biocontrol of pathogenic diseases by *Bacillus* species (Ryder *et al*., 1993; Pierson and Weller, 1994; Leifert *et al*, 1995; Asaka and Shoda, 1996**;** Kim *et al*., 1997**;** Mathre *et al.*, 1999; Ryu *et al.*, 2004). Pure antibiotics isolated from biocontrol bacterial strains inhibited the growth of the same pathogens (Carmi *et al*., 1994). *Bacillus subtilis* Strain RB14 produced antibiotics iturin A and surfactin (Hiraoka *et al.,* 1992**)** that suppressed *Rhizoctonia* damping-off of tomatoes (Asaka and Shoda 1996). *Paenibacillus polymyxa* (Prazmowcoki) Mace Strain E681 inhibited growth of a range of pathogenic fungi through production of antibiotics (Ryu *et al.*, 2006). A non-antibiotic producing mutant of *Pseudomonas fluorescens* Migula could not control *R. solani* damping-off in cotton (Hill *et al*., 1994).

Production of siderophores by *Bacillus* strains in this study may be a further mechanism involved in the biocontrol of *Rhizoctonia* damping-off. All *Bacillus* isolates in this study produced siderophores (Chapter 2). Competition for nutrients is another mechanism by which biocontrol agents inhibit proliferation of pathogens. Siderophores synthesized by BCAs bind ferric ions in the rhizosphere and transport them to the microbial cell wall making them unavailable to the pathogen (Briat, 1992; O'Sullivan and O'Gara, 1992; Glick and Bashan, 1997; Jagadeesh, 2006). Siderophore production has been implicated in suppression of disease in several studies (Thomashow and Weller, 1990; Day *et al.,* 2004; Ahmad *et al*., 2006; Ahmad *et al,*. 2008).
Biological control agents synthesize hydrolytic enzymes such as chitinase cellulase, pectinase, lipase, protease and glucanase, which are believed to attack fungal cell walls, inhibiting the growth of pathogens (Chet and Inbar, 1994; Singh *et al*., 1999; Chernin and Chet, 2002). Two chitinase-producing *Pseudomonas* strains lysed the mycelia and inhibited the growth of four root rot pathogenic fungi (Lim *et al*., 1991; Fridlender *et al.,* 1993). Production of hydrolytic enzymes was implicated in the biocontrol of *R. solani* in this study. All *Bacillus* isolates in this study tested positive for lipase, protease, cellulase and amylase *in vitro* (Chapter 7). Other metabolites such as hydrogen cyanide and biosurfactants have been implicated in some studies but HCN was not detected in any of the isolates tested here (Chapter 2). Induction of systemic resistance, though not investigated in this study, maybe another mechanism responsible for the observed results.

Biological control agents have been reported to suppress pathogenic diseases by inducing systemic resistance in the plant hosts (Ahlstrom, 1991; Liu *et al*., 1995; Van Loon *et al.,* 1998; Zhang *et al*., 2002; Dwivedi and Johri, 2000; Ryu *et al.*, 2004). Some biocontrol agents with multiple plant growth promoting traits may resist disease by improving plant growth and development. Some pathogens can only multiply and establish themselves in stressed plants (Bashan *et al*., 1978; Dab *et al.,* 1982; Hillocks and Chinodya, 1989). The most promising isolate in this study (BS10) was found to posses a number of growth promoting traits in addition to antifungal activity. These growth promoting effects may have contributed to its effectiveness in controlling *R. solani* and produced greater root and shoot biomass than Eco-T®, though Eco-T inoculation resulted in more seed germination and seedling survival. This study has demonstrated that *B. subtilis* strains have the potential to control *R. solani* damping-off of wheat through multiple mechanisms of disease control. Isolate BS10 is a good candidate because it possesses traits for both plant growth promotion and diseases control. However, since the efficacy of BCAs is affected by both abiotic and biotic conditions, the best isolates need to be tested under field conditions to determine their efficacy under natural environmental conditions. There is also a need to establish the exact mechanisms that individual strains employ in disease suppression, for formulation of a multi-strain BCA.

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CHAPTER SEVEN

In vitro studies on selected mechanisms employed by *Bacillus subtilis* (Ehrenberg) Cohn isolates in biological control of *Rhizoctonia solani* Kühn damping-off of wheat (*Triticum aestivum* L.)

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Abstract

Bacillus subtilis isolates that effectively suppressed damping-off caused by *Rhizoctonia solani* in wheat under greenhouse conditions were investigated for putative mechanisms of control. Mechanisms investigated include antibiosis, production of extracellular enzyme, siderophores, hydrogen cyanide (HCN) and resistance against antibiotics. Environmental scanning electron microscope (ESEM) was used for ultrastructural studies on *in vitro* interaction between *B. subtilis* isolates and *R. solani*. All *B. subtilis* isolates inhibited the growth of *R. solani* and a number of other fungal genera tested *in vitro.* Isolate BS10 caused the highest inhibition (88%) of *R. solani*. All isolates tested positive for cellulase, amylase and proteinase production, indicated by clear zones around bacterial growth, and crystal formation due to lipase production. Production of siderophores was detected from all isolates, as indicated by yellow-orange halos of varying diameters around the bacterial growth with the largest from Isolate BS69 (24 mm). None of the isolates produced chitinase, pectinase or HCN. The ESEM micrograph preparations from sections obtained from the edge of the zone of inhibition showed that hyphae of *R. solani* had lost turgidity and shrunk, while hyphal apices were deformed whereas those from the Control looked healthy with branches at 90º. Isolates BL3, BS817, BS820 and BS69 resisted up to 75µg ml^{-1} of ampicillin and BS10 up to 100µg ml^{-1}. All isolates resisted 25µg ml^{-1} of chloramphenicol, BS10 and BS820 resisted tetracycline up to 75µg ml^{-1} and BS10 and BS69 resisted up to 75µg $ml^{-1}$of streptomycin. All isolates were sensitive to all concentrations of kanamycin and gentamycin antibiotics tested. *In vitro* inhibition of different fungal genera, suppression of *R. solani,* resistance to some antibiotics and evidence of different modes of action displayed by these isolates demonstrated that Bi subtilis employs a consortium of mechanisms in biocontrol of damping-off fungi. I*n vitro* screening of *B. subtilis* isolates for putative mechanisms involved in suppression of *R. solani* and other pathogenic fungi could be an effective technique for identification of strains with biocontrol potential.

Key words: Biocontrol; antibiotics; hydrolytic enzymes; siderophores; antifungal activity; *Bacillus subtilis; Rhizoctonia solani*

7.1 Introduction

Various mechanisms have been proposed for the control of fungal pathogens in plants by biological control agents (Chet *et al*., 1990; Glick and Bashan, 1997; Kim *et al*., 1997; Rampach and Kloepper, 1998; Shoda, 2000; Romero *et al*., 2004; Idris *et al.*, 2007). These mechanisms include: antibiosis, (Carmi *et al*., 1994; Leifert *et al*., 1995; Foldes *et al*., 2000; Hass and Keel, 2003), parasitism (Lim *et al*. 1991; Chet and Inbar,1994; Lima *et al*., 1997; .Zhang *et al*., 1999; Whipps, 2001; Manjula & Podile, 2005), production of hydrogen cyanide (Dowling and O'Gara, 1994 ; Flaishman *et al*., 1996; Nelson, 2004), biosurfactants (Stanghellin and Miller, 1997), competition for niche and nutrients (O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999; Compant *et al.,* 2005) and induction of systemic resistance (Liu *et al.,* 1995; Van Loon *et al*., 1998; Bent, 2006). Biocontrol agents may inhibit proliferation of pathogens by the production of an array of antibiotics that inhibit the synthesis of fungal cell walls (Subbarao, 1999). Iron is an essential growth element required by microorganisms. Under iron-limiting conditions BCAs out-compete fungal pathogens by producing siderophores that bind ferric ions in the rhizosphere and transport them to the microbial cell wall, making them unavailable to the pathogens (Briat, 1992; O'Sullivan and O'Gara, 1992; Glick and Bashan, 1997; Dwivedi and Johri, 2003). Biological control agents are also believed to suppress pathogens by production of hydrolytic enzymes that parasitize fungal cell walls and inhibit the growth of these pathogens (Chet and Inbar, 1994; Singh *et al*., 1999; Whipps, 2001; Chernin and Chet, 2002). Induced systemic resistance against pathogenic diseases by plants has been observed following inoculation by BCAs (Kloepper *et al*., 2004). A clear understanding of the various mechanisms employed by BCAs in their fight against pathogenic microorganisms is essential in identification and formulation of effective BCAs for use in agriculture. Combinations of BCAs with different modes of action against pathogenic microorganisms may offer better and efficient protection than a single mechanism. The current study reports on the possible mechanisms employed by *B. subtilis* strains in suppression of *R. solani* damping-off of wheat.

7.2 Materials and methods

7.2.1 Source of *Bacillus subtilis* and *Rhizoctonia solani* isolates

Five *B. subtilis* isolates used in this study were selected for inhibition of *R. solani in vitro* (Chapter 2) and suppression of its effects on wheat under greenhouse conditions (Chapter 6). A pathogenic isolate of *R. solani* was obtained from the rhizosphere of common bean (*Phaseolus vulgaris* L.) and tested for its effects on germination of wheat *in vitro* and for damping-off of wheat under greenhouse conditions (Chapter 6). Other fungal pathogens including *Pythium arhenomonas* Drechsler, *Fusarium oxysporum* Schlecht *F. solani* F. *circinatum* Nirebergan O'Donnell, *Aspergillus flavus* Johann Heinrich Friedrich Link, *Diplodia pinea* (Fr) Dyko & B. Sutton and *Pyricularia oryzae* Cavara tested *in vitro,* were obtained from Plant Protection Research Institute (PPRI)[20].

7.2.2 Antibiosis

A modification of the dual culture technique as described by Paulitz *et al*. (1992), Landa *et al*. (1997) and Idris *et al.* (2007) was used for detection of antifungal activity. Three paper discs dipped in each of the bacterial suspensions were placed at the margin of potato dextrose agar (PDA) plates and incubated for 48 hours at 28ºC. Agar discs (4x4 mm^2) of the fungal culture were placed at the center of each plate and the plates were incubated in the dark for seven days. Radii of the fungal colonies towards and away from the bacterial colonies were measured. Growth inhibition was calculated using the following formula;
% inhibition = (R-r/R) ×100, where, r is the radius of the fungal colony opposite the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony. Isolates with > 30% mycelia growth inhibition against the selected pathogen were considered effective for biological control of the pathogen. The test was replicated three times.

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[20]Mycology Diagnostic Services, Biosystematics, ARC-PPRI, Private Bag X134, Queenswood, Republic of South Africa. 0121

This test was carried out with *Pythium arhenomonas*, *Fusarium oxysporum F. solani* F. *circinatum*, *Aspergillus flavus*, *Diplodia pinea* and *Pyricularia oryzae*. Environmental scanning electron microscope (ESEM), (Zeiss EVO LS 15 VP SEM)[21] was used for ultrastructural studies on interaction between *B. subtilis* and *R. solani in vitro*. In this experiment mycelia growth was obtained from the edge of the inhibition zone for each isolate and the edge of a young growing pure culture of *R. solani* as a control. The mycelia samples were fixed in 3% buffered glutaradehyde for 3 hours. They were rinsed in sodium cacodylate buffer twice for five minutes. They were then fixed in buffered osmium tetroxide for one hour, and then rinsed again two times in buffered sodium cocodylate for five minutes. This was followed by of dehydration in a graded series of ethanol from 10% - 90% for 10 minutes at each concentration and then 3 times in 100% ethanol for 10 minutes each. The samples were then dried in a critical point drier (Hitachi Critical Point Dryer)[22] and then mounted on an ESEM stub. The samples were finally coated with gold in a sputter coater (Eiko IB. 3 ion sputter coater) and viewed under the ESEM.

7.2.3 Siderophores production

The *B. subtilis* isolates were tested for siderophore-production on Chrome Azurol S (CAS) Medium (Schwyn and Neilands, 1987; Ahmad *et al*., 2006, 2008). Chrome Azurol S agar plates were prepared and divided into two equal sectors. The sectors were inoculated with each of the test isolates and incubated for 48-72 hours at 28ºC. The plates were observed for development of a yellow to orange halo around the bacterial growth which is a positive test for siderophore production.

7.2.4 Cellulase production

Cellulase production by *B. subtilis* strains was detected using *Bacillus* medium as described by Atlas and Park (1993), Cattelan *et al*. (1999), Kumar *et al.* (2005) and Yobo *et al.* (2005) supplemented with carboxymethylecellulose (CMC) as a source of cellulose. The medium contained in a liter of distilled water, KNO_3, 1.0g, K_2HPO_4, 1.0g, $MgSO_4.7H_2O$, 0.5g, KCl, 1.0g, glucose, 1.0g, CMC, 5.0g and bacteriological agar, 20.0g.

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[21]Carl Zeiss, (Pty) LTD, 363 Oak Avenue, Ferndale, Randburg South Africa. 2194.

[22]Hitachi Koki Co. Ltd. HCP-2 Tokyo, Japan.

Wells were made at the center of each plate using the wider end of a sterile Pasteur pipette. The wells were filled with 10 µl of each of the bacterial suspensions. A control was set up using sterile distilled water. The plates were incubated in the dark at 28ºC for 3 days. The plates were then flooded with 1% (w/v) Congo Red solution for 15 minutes and then rinsed with 1M sodium hydrochloride for 10 minutes. Clear zones around the wells indicated the presence of cellulase.

7.2.5 Amylase production

The *Bacillus* medium used in Section 7.2.4 supplemented with 2g of starch was used to detect the presence of amylase. Paper discs dipped in each of the bacterial suspensions were placed at the center of each plate and the plates were incubated in the dark at 28ºC for 3-5 days. The plates were then flooded with Lugol's iodine solution. Clear zones formed around the discs indicated the presence of amylase.

7.2.6 Lipase production

The basal medium described by Cattelan *et al.,* (1999); Kumar *et al*., (2005); Yobo *et al.,* (2005) was used to demonstrate lipase production by *B. subtilis* strains. The medium contained in g l^{-1} of distilled water, peptone, 10.0, NaCl 5.0, $CaCl.2H_2O$, 0.1, and bacteriological agar, 20.0. This medium was supplemented with Tween 20 sterilized separately and cooled to 45ºC at a concentration of 10 ml l^{-1}. The plates were inoculated with paper discs dipped in each of the bacterial suspensions and incubated in the dark at 28ºC for 4 days. The plates were observed for formation of crystals under a dissecting microscope fitted with a digital camera (Leica DFC 450 digital camera, Vacutec) [23]as an indication of lipase production.

[23] Vacutec, 63 Main Street, Randburg, Republic of South Africa.

7.2.7 Proteinase production

The medium used in Section 7.2.4 supplemented with gelatin, which was sterilized separately and added to the medium at a concentration of 50ml l^{-1} was used to demonstrate proteinase production. The plates were inoculated with paper discs dipped in suspensions of each of the *B. subtilis* isolates and incubated in the dark at 28ºC for 3 days. The plates were stained with 0.1% (w/v) Amido Black solution in methanol and distilled water at a ratio of 30:10:60 (v/v/v) for 15 minutes and destained with methanol-acetic acid–distilled water, at a ratio of 30:10:60 v/v/v. The presence of proteinase production was indicated by the formation of a clear zone around the bacterial growth (Smibert and Krieg, 1994).

7.2.8 Pectinase production

Production of pectinase by *B. subtilis* isolates was tested using a modified M9 medium described by Atlas and Park (1993); Cattelan *et al*. (1999); Kumar *et al*. (2005); Yobo *et al*. (2005). The medium contained in a liter of distilled water, Na_2HPO_4, 6.0g, KH_2PO_4, 3.0g, NH_4Cl, 1.0g, NaCl, 0.5g, $CaCl_2$, 0.2g, glucose, 1.0g, $MgSO_4.7H_2O$, 0.5g, yeast extract, 1.2g and pectin, 5.0g. Inoculated plates were incubated in the dark at 28ºC for five days. Plates were then flooded with 2M HCl solution. They were observed for formation of clear zones around the discs, indicative of pectinase production.

7.2.9 Antibiotic resistance

The procedure described by Bauer *et al*. (1966) was used to investigate resistance of *B. subtilis isolates* against chloramphenicol, streptomycin, tetracycline, gentamycin, kanamycin and ampicillin at concentrations of 25, 50, 75 and 100 µl ml^{-1} for each antibiotic. For each strain, 10 µl aliquot was spread evenly on tryptone soy agar plates. A paper disc dipped in each of the antibiotic concentrations was placed at the center of the plate and incubated in the dark at 28ºC for three days. This preparation was replicated three times for each concentration for the four antibiotics. The plates were observed for inhibition zones around the discs, which is an indication of sensitivity of the bacterial isolates to the antibiotics. Each test was replicated three times.

7.3 Results

7.3.1 Antibiosis

All isolates inhibited the growth of *R. solani,* indicated by zones of inhibition around the bacterial colony. Hyphae at the edge of the inhibition zone turned brown while the rest of the hyphae only changed color on prolonged incubation. The greatest inhibition (88%) was observed with Isolate BS10 and the least (61%) with Isolate BL3 (Figure 7.1 and Table 7.1). The zones of inhibition persisted after incubation for more than 14 days. Tests with other fungal genera revealed that all isolates inhibited the growth of all genera tested. The highest inhibition with other fungal genera was observed in *Pyricularia oryzae* (85% - 90%) while the least was with *F. solani* (33% - 53%). The ESEM micrographs showed that *R. solani* hyphal cells from the inoculated plates had shrunk, lost turgidity and the hyphal apices were deformed, while those of the control were healthy, with clear branching at 90º (Figures 7.2–7.5).

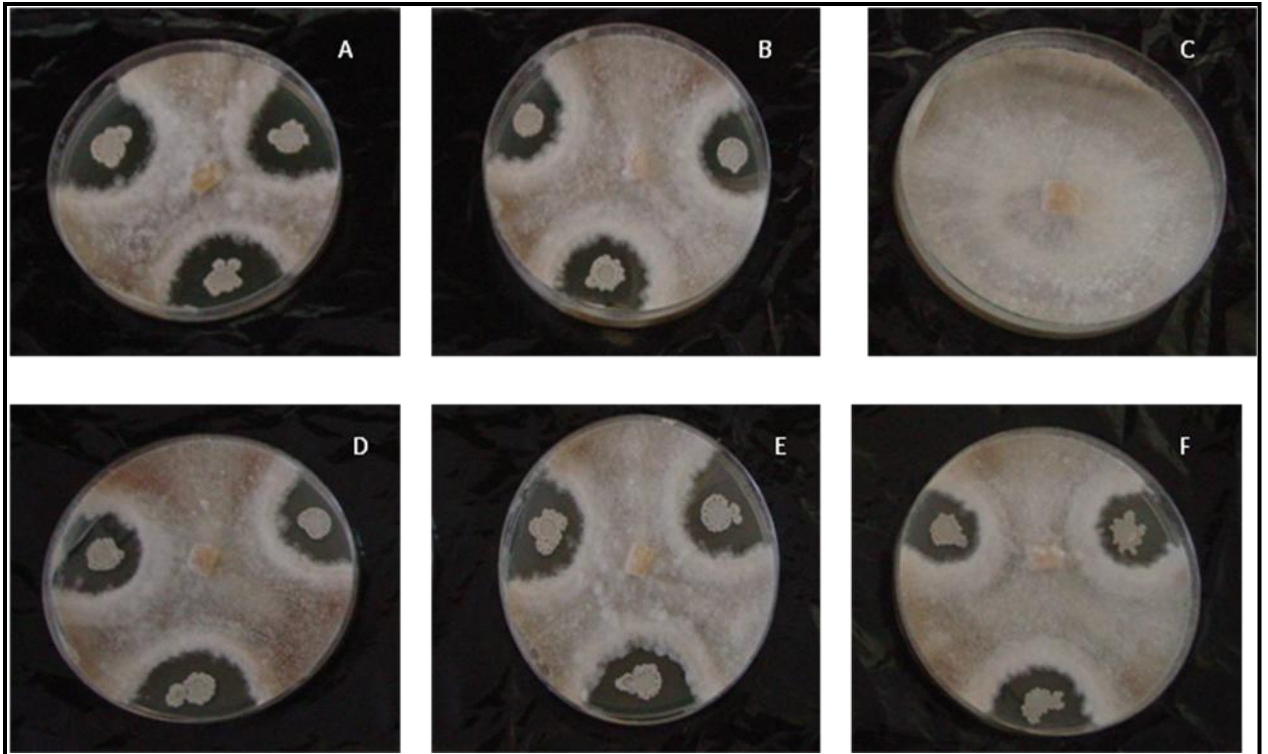

Figure 7.1: *In vitro* growth inhibition of *Rhizoctonia* s*olani* by *Bacillus subtilis* Isolates after 7 days of incubation at 28 ºC. A: Isolate BS10; B: Isolate BL3; C: Control; D: Isolate BS69; E: Isolate BS817; F: Isolate BS820

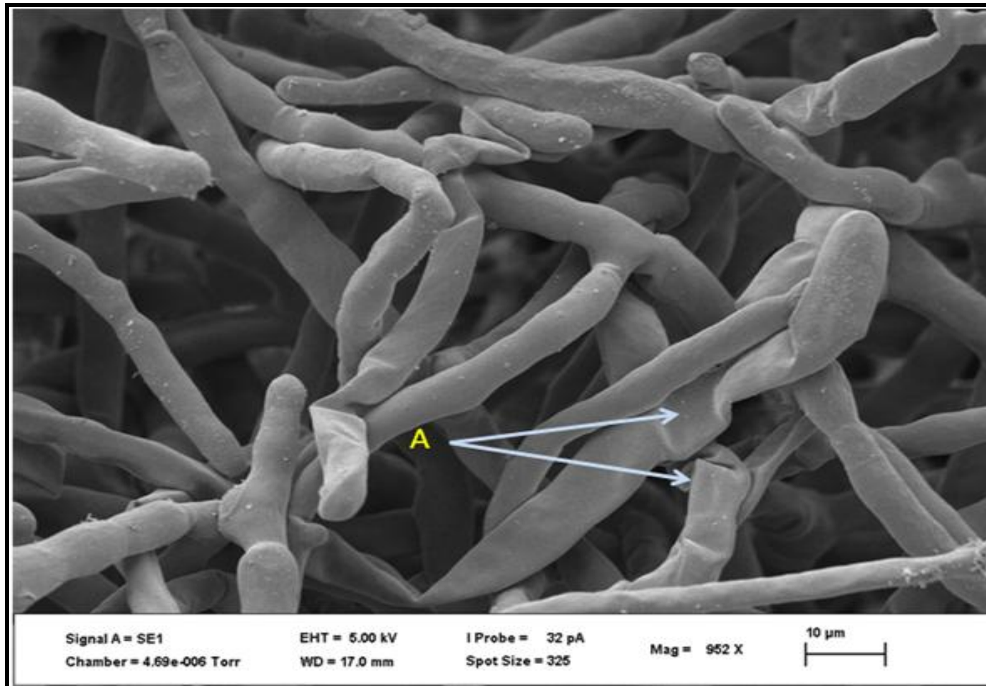

Figure 7.2 Environmental scanning electron micrograph showing deformation of *Rhizoctonia* s*olani* hyphae caused by Isolate BS10. A: Shrunken and deformed hyphae due to loss of turgidity.

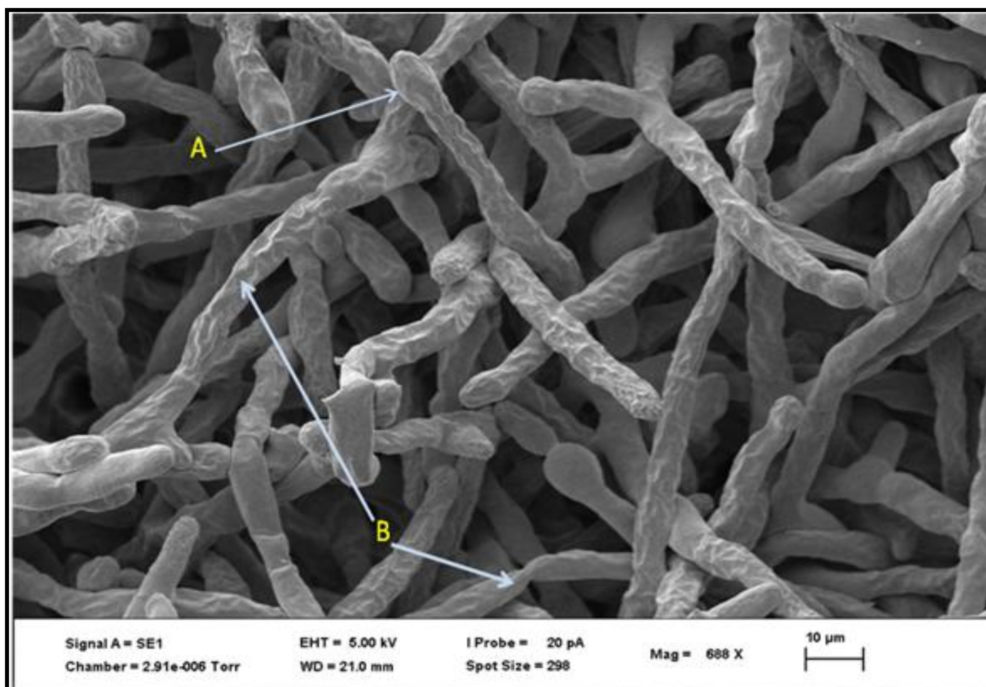

Figure 7.3 Environmental scanning electron micrograph showing deformation of *Rhizoctonia* s*olani* hyphae caused by Isolate BS820. A: A deformed hyphal apex, B: Shrunken and deformed hyphae due to loss of turgidity.

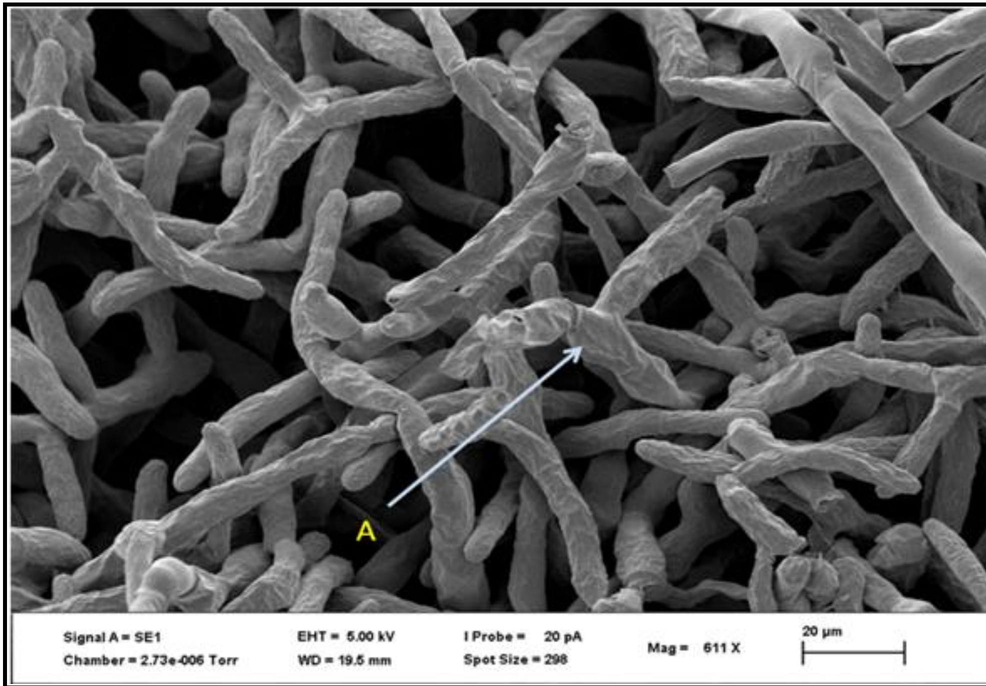

Figure 7.4 Environmental scanning electron micrograph showing deformation of *Rhizoctonia solani* hyphae caused by Isolate BS820. A: Shrunken and deformed hyphae due to loss of turgidity

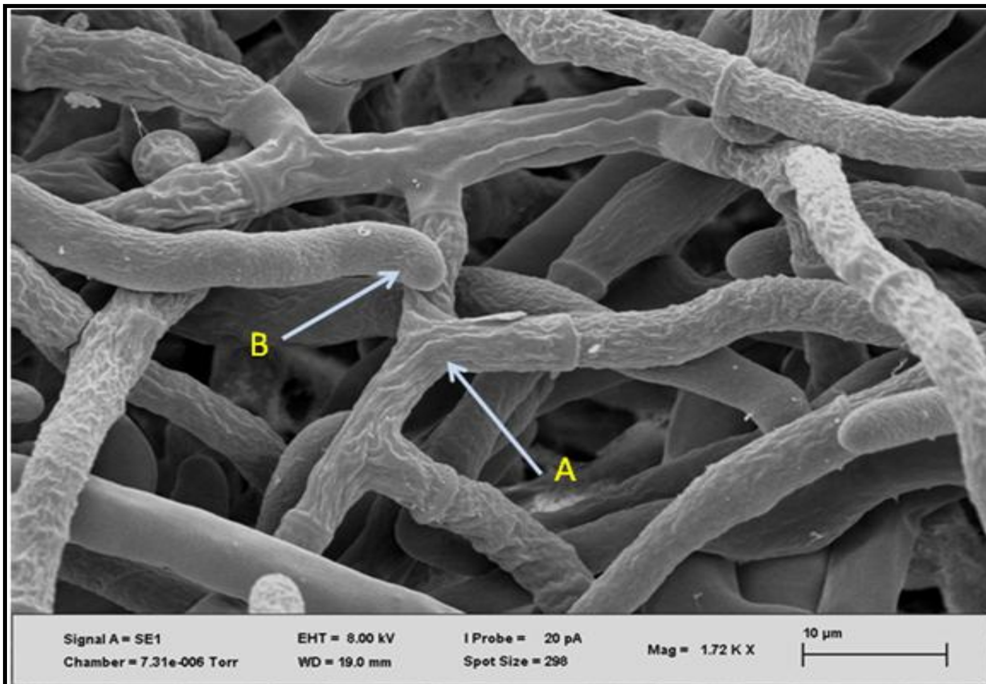

Figure 7.5 Environmental scanning electron micrograph of Healthy normal *R. solani* hyphae (Control without bacterial inoculation). A: A right-angled hyphal branch; B: A healthy hyphal apex.

7.3.2 Siderophores Production

All isolates formed yellow to orange halos around the bacterial growth, indicating the presence of siderophores. The amount of siderophore produced based on the diameter of the halos differed significantly (P = 0.001) between the isolates (Table 7.1). Isolate BS69 formed the largest halo (24 mm) while Isolate BS820 had the smallest (9.7 mm)

7.3.3 Cellulase production

Clear zones were observed around the bacterial growth for all isolates indicating the presence of cellulase while the rest of the plate retained the red color of the stain.

7.3.4 Amylase production

Clear zones were formed around the bacterial growth confirming amylolytic activity while the rest of the medium retained a blue-black color indicative of the presence of starch (Figure 7.6)

7.3.5 Lipase production

White crystals were formed on the medium by all isolates confirming lipase production (Figure 7.7).

7.3.6 Proteinase production

Clear zones were formed around bacterial growth which was indicative of the presence of proteinase.

7.3.7 Pectinase production

The bacteria grew on the medium but no clear zones were observed with any of the isolates indicating the absence of pectinase.

7.3.8 Chitinase production

No clear zones were observed around the bacterial growth, which indicated the absence of chitinase.

Table 7.1 *In vitro* growth inhibition of *Rhizoctonia solani* and siderophore production by *Bacillus subtilis* isolates

Bacterial Isolate	**Siderophore halo diameter (mm)**		**Growth Inhibition zone (%)**	
BS820	9.7	a	81.3	bc
BL3	12.0	a	61.0	a
BS10	15.3	b	88.0	c
BS817	15.7	b	61.3	a
BS69	24.0	c	71.3	ab
F-value	**31.9**		**12.74**	
P-Value	**0.001**		**0.002**	
L.S.D.	**3.1**		**10.97**	
S.E.D.	**1.4**		**4.80**	
CV %	**10.9**		**8.00**	

Means in a column followed by the same letter are not significantly different from each other at 5% level of significance according to Fisher’s L.S.D.

Table 7.2 Mechanisms of biological control exhibited by *Bacillus subtilis* isolates against *Rhizoctonia solani in vitro.*

***In vitro* test**	**Bacterial Isolates**				
	BL3	**BS69**	**BS10**	**BS817**	**BS820**
Antifungal activity	+	+	+	+	+
Amylase production	+	+	+	+	+
Cellulase production	+	+	+	+	+
Chitinase production	-	-	-	-	-
Lipase production	+	+	+	+	+
Pectinase production	-	-	-	-	-
Proteinase production	+	+	+	+	+
Siderophore production	+	+	+	+	+
Hydrogen cyanide production	-	-	-	-	-

+ = Positive for the test; - = Negative for the test

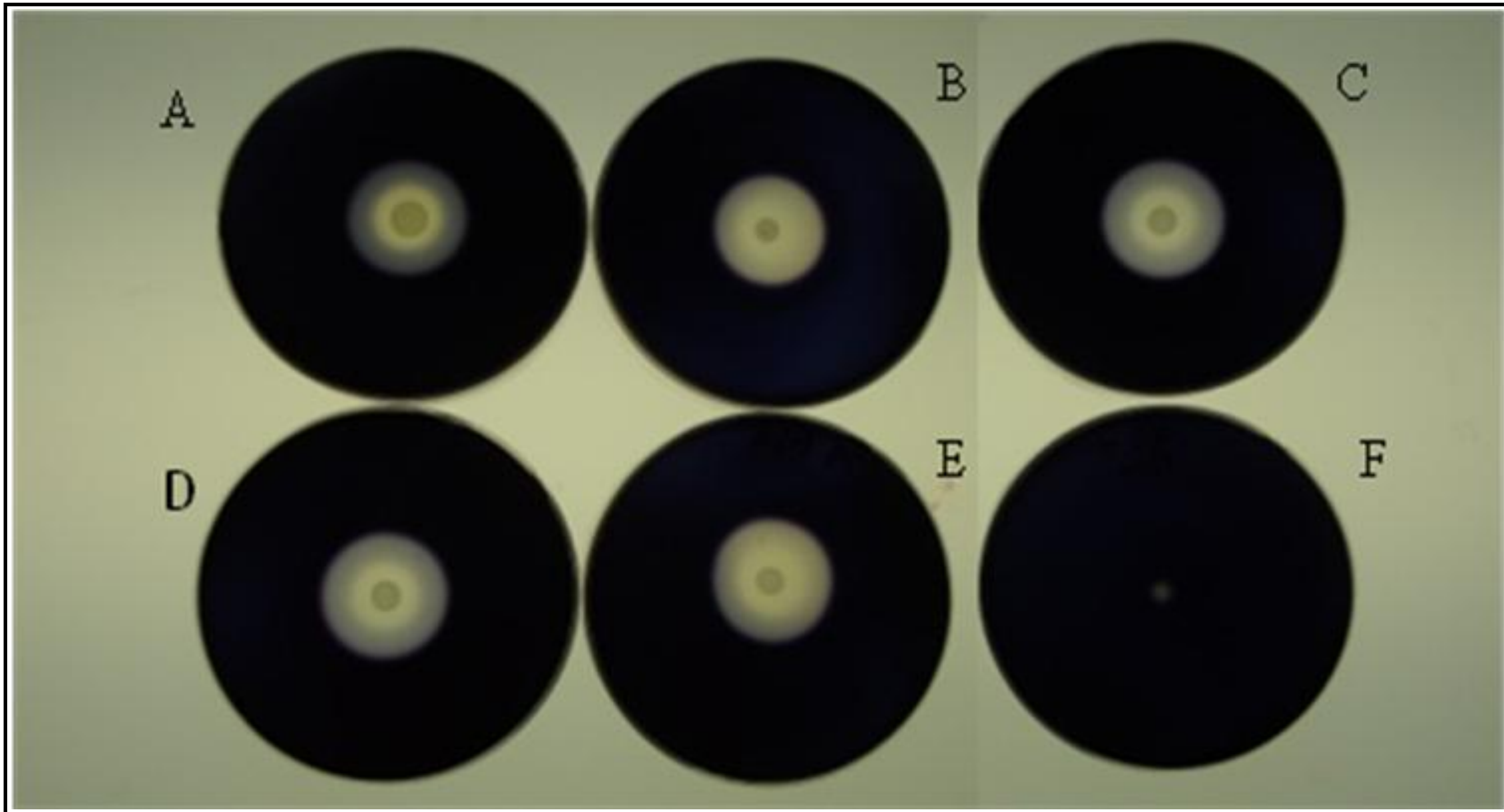

Figure 7.6: Evidence of amylase activity by the five *B. subtilis* isolates on agar medium supplemented with starch at 2g l^{-1} after 3 days of incubation at 28ºC. Clear zones indicate a positive test for amylase production activity. A: Isolate BS817, B: Isolate BS820, C: Isolate BS69, D: Isolate BS10, E: Isolate BL3 F: Control.

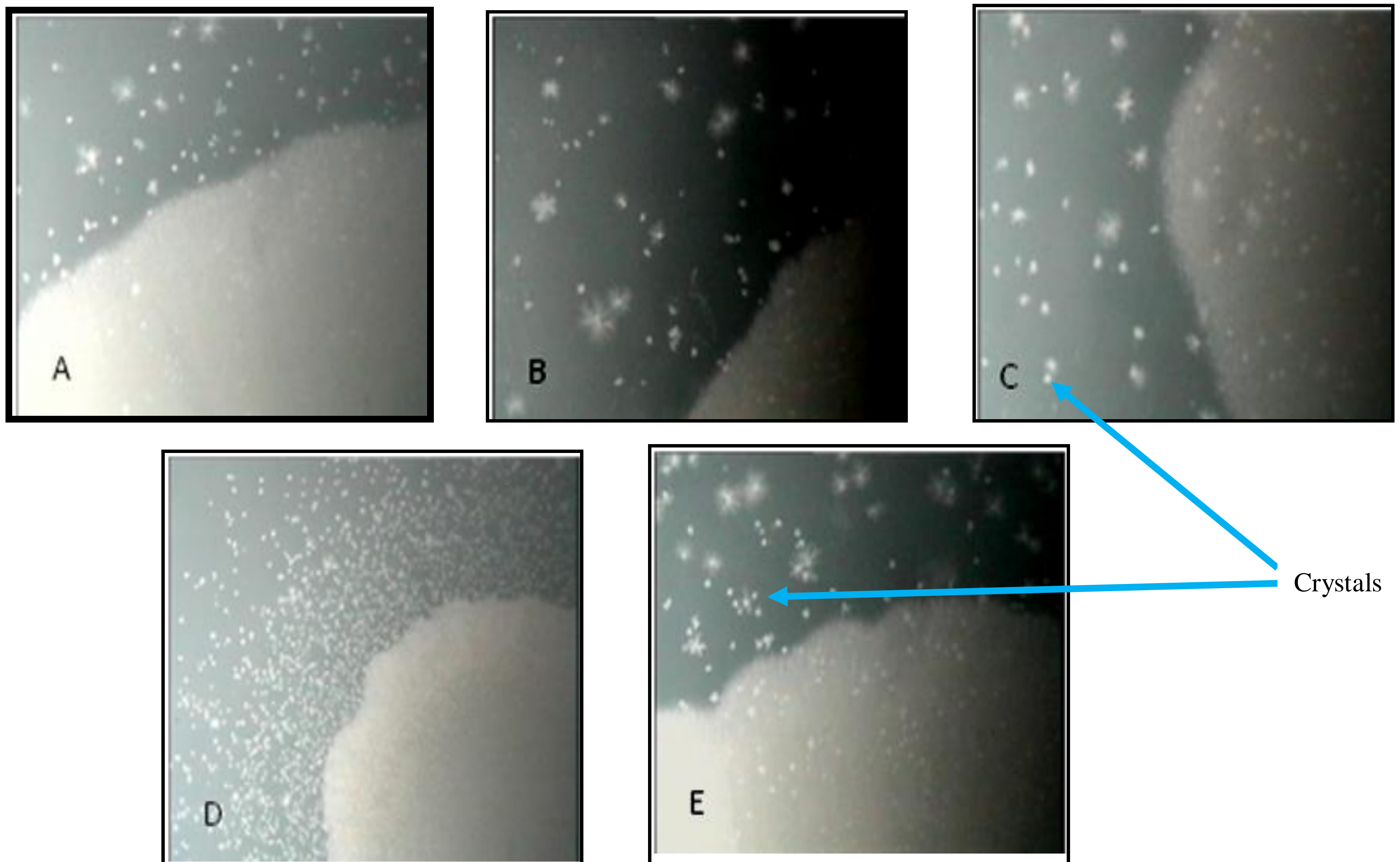

Figure 7.7 Crystal formation by five *B. subtilis* isolates on agar medium supplemented with Tween 20 at a concentration of 10 *ml* l^{-1} after 4 days of incubation at 28°C indicative of lipase production. A: Isolate BS10, B: Isolate BL3, C: Isolate BS69, D: Isolate BS817, E: Isolate BS820.

7.3.9 Antibiotic resistance

Resistance to antibiotics varied with isolate, type of antibiotic and their concentrations. BS10 was the most resistant and BL3 the most sensitive. One isolate (BS10) resisted ampicillin up to a concentration of 100µg ml^{-1}, while the rest resisted up to 75µg ml^{-1}. All isolates resisted tetracycline at a concentration of 25µg ml^{-1} but BS10 and BS820 resisted 50µg ml^{-1}. All isolates resisted 25µg $ml^{-1}$of streoptomycin, all except BS817 resisted 50µg ml^{-1}, BS69 resisted 75µg ml^{-1} and BS10 100µg ml^{-1}. All isolates were resistant to 25µg ml^{-1} of chloramphenicol but sensitive to kanamycin and gentamycin at all concentrations tested (Table 7.2).

Table 7.3 Antibiotic resistance by *Bacillus subtilis* isolates

Antibiotic	**Concentration** (μ**g ml**$^{-1}$)	**Bacterial Isolate**				
		BL3	**BS10**	**BS69**	**BS817**	**BS820**
Ampicillin	25	+	+	+	+	+
	50	+	+	+	+	+
	75	-	+	+	+	+
	100	-	+	-	-	-
Chloramphenicol	25	+	+	+	+	+
	50	-	-	-	-	-
	75	-	-	-	-	-
	100	-	-	-	-	-
Streptomycin	25	+	+	+	+	+
	50		+	+	-	+
	75	-	+	+	-	-
	100	-	+	-	-	-
Tetracycline	25	+	+	+	+	+
	50	-	+	-	-	+
	75	-	-	-	-	-
	100	-	-	-	-	-
Kanamycin	25	-	-	-	-	-
	50	-	-	-	-	-
	75	-	-	-	-	-
	100	-	-	-	-	-
Gentamycin	25	-	-	-	-	-
	50	-	-	-	-	-
	75	-	-	-	-	-
	100	-	-	-	-	-

+ = Isolate resistant to antibiotic, = Isolate sensitive to antibiotic

7.4 Discussion

Inhibition of *R. solani* growth *in vitro* indicated by inhibition zones, browning of the hyphae at the edge of the zone and loss of turgidity by the cells and deformation of the hyphal apices was an indication of production of antifungal compounds by the *Bacillus* isolates. Other authors have reported similar observations (Kim *et al.,* 1997; Shoda, 2000; Kubheka, 2003; Ryu *et al*., 2006; Ahmad *et al*., 2008). Montealegre *et al.* (2003) reported loss of turgidity and hyphal deformation of *R. solani in vitro* following inoculation with *B. subtilis* isolates. This antagonism could be due to the presence of antifungal compounds produced by the bacterial isolates. Persistence of inhibition zones beyond 14 days implies that the antifungal compounds produced by these isolates were fungicidal. Levels of inhibition above 50% caused by all isolates suggest that they can suppress the effects of *R. solani*. Loss of hyphal turgidity and shrinkage, coupled with deformation of hyphal apices, implied interference with the integrity of the fungal cell wall that might have led to leakage of cell contents**.** Antibiotics produced by bacterial strains interfere with cell wall and protein synthesis by fungal pathogens. Inhibition of the growth of a wide range of pathogenic fungal genera *in vitro* by *B. subtilis* isolates suggests that these isolates produce a variety of antifungal compounds that can suppress the growth of several fungal pathogens**.** A number of authors have reported growth inhibition of a number of fungal pathogens *in vitro* by *Bacillus* species (Marten *et al*., 2000; Cazorla *et al*., 2007). *Bacillus subtilis* has been reported to produce a variety of antibiotics (Stein, 2005). Synthesis of antibiotics has been proposed as the most effective mode of action in biocontrol of pathogenic fungi by BCAs (Glick and Bashan, 1997; Raaijmakers and Weller, 1998). Antibiotics produced by BCAs inhibit both cell wall and protein synthesis in pathogenic microorganisms, inhibiting their multiplication (Subbarao, 1999). It has been demonstrated that antibiotics isolated and purified from BCAs can suppress the same pathogen as the BCAs (Carmi *et al*., 1994; Touré *et al.,* 2004), whereas non-antibiotic-producing mutants of BCAs could not suppress diseases controlled by the wild types of the same BCAs (Hill *et al*., 1994; Pierson *et al*., 1994). Production of antibiotics has been implicated in biocontrol of pathogenic fungi by *Bacillus* species by other authors (Leifert *et al.,* 1995; Mathre *et al*., 1999; Guetsky *et al.,* 2000).

A number of researchers have demonstrated production of antibiotics by *Bacillus* species to which they attributed the control of pathogenic diseases by these species (Ryder *et al*., 1993; Pierson and Weller, 1994; Silo-Suh *et al*., 1998; Whipps, 2001; Ryu *et al.*, 2004; *Lee et al*., 2007; Chen *et al.,* 2009; Borisova *et al*., 2010). *Bacillus subtilis* RB14, which produced antibiotics iturin A and surfactin (Hiraoka *et al.,* 1992), suppressed *Rhizoctonia* damping-off of tomatoes (Asaka and Shoda, 1996). Iturin-producing *Bacillus* strains controlled *Fusarium oxysporum* Schlecht and *Rosellinia necatrix* Prill in avocado (*Persea Amaricana* Mill) (Cazorla *et al*., 2012). *Paenibacillus polymyxa* (Prazmowski) Mace Strain E681 inhibited growth of a range of pathogenic fungi through production of antibiotics (Ryu *et al.*, 2006). Iturin was reported to inhibit several fungi (Munimbazi and Bullerman, 1998; Chitara *et al*., 2003). Production of other antimicrobial compounds such as HCN has also been implicated in antagonism against some pathogenic fungi (Dowling and O'Gara, 1994; Ahmad *et al*., 2006, 2008). Production of HCN by pseudomonads was suggested as the mode of control of black root rot of tobacco caused by *Thielavopsis basicola* (Berk. & Br.) Ferraris (Voisard *et al*., 1989). None of the isolates in this study produced HCN (Chapter 2), which implies that this mechanism was not involved in the suppression of *R. solani.*

The yellow-orange halos formed around bacterial growth in the siderophore test were an indication of the production of siderophores by the isolates. Siderophores production could be a possible mode of action employed by these isolates in suppression of *R. solani*, observed both *in vitro* and *in vivo.* Competition for nutrients by production of siderophores has been suggested as one of the modes of action employed by some BCAs against pathogenic microorganisms (Weller and Cook, 1986; Lopper, 1988; Thomashow and Weller, 1990; Loper & Henkels, 1997; Dwivedi and Johri, 2003; Day *et al.,* 2004). Biocontrol of pathogens by production of siderophores by *Pseudomonas* species against *Pythium* and *Fusarium* species has been demonstrated by some researchers (Loper and Buyer, 1991; Duijff *et al,* .1993; Whipps, 2001). However, disease control by production of siderophores has been reported as being host and pathogen specific and also depends on the soil composition, the producing bacterial strain and its affinity for iron (Glick and Bashan1997).

Some BCAs inhibit proliferation of pathogenic microorganisms by production of hydrolytic enzymes such as chitinases, glucanases, and pectinases. Although biocontrol by production of these enzymes has been mostly evident in *Trichoderma* species, some *Bacillus* species have been reported to control pathogenic fungi by production of these enzymes (Chernin *et al.,* 1995; Pleban *et al* 1995; Guetesky *et al*., 2002; Manjula & Podile, 2005). However these enzymes were not detected in any of the *Bacillus* isolates in the current study. Enzymes such as amylase, cellulase, lipase and proteinase were produced by the isolates in this study. Biological control agents may utilize these enzymes in acquisition of nutrients from different substrates in the rhizosphere. These enzymes therefore play an important role in enhancing competition against other microorganisms in the rhizosphere. They may contribute to successful colonization and establishment of BCAs population inhibiting the multiplication of pathogens.

Resistance to antibiotics by BCAs can protect them against antibiotics produced by pathogens and other microorganisms in the rhizosphere and enhance colonization by the BCAs. Resistance against ampicillin by all isolates indicated that these isolates can resist the effects of pathogenic fungi which are known to synthesize ampicillin. Their resistance against streptomycin, chloramphenicol and tetracycline may give them a competitive edge advantage against other bacterial microorganisms such as strains in the genus *Streptomyces.*

Some BCAs inhibit diseases by induction of systemic resistance to the plants. *Bacillus* strains are among BCAs that control diseases by inducing resistance (Bakker *et al.,* 2000; Kloepper *et al.,* 2004; Bent, 2006). Induced resistance offers protection against both bacterial and fungal pathogens (Van Peer *et al*., 1991**).** For example, *Bacillus mycoides* Flugge can suppress *Botrytis cinerea* DeBary in leaves in strawberries by systemic resistance (Guetesky *et al*., 2002). However this mode of action was not investigated in this study.

This study has demonstrated that screening of potential BCAs for the various modes of action used in disease control can be optimized for the selection of effective bacterial strains for use in agriculture. *Bacillus subtilis* isolates produce several antifungal compounds active against a wide range of fungal pathogens. The tested isolates of *B. subtilis* controlled *R. solani* by employing different mechanisms.

Effective isolates identified in this study can provide protection against a wide range of pathogenic fungal genera alone or when combined. *Bacillus subtilis* Isolate BS10 may be a promising strain for formulation as a biocontrol agent for the management of damping-off of wheat caused by *R. solani*.

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THESIS OVERVIEW

The search for replacements or alternatives to fertilizers and agrochemicals has attracted the attention of many researchers globally in the last few decades**.** This interest has been prompted by the need to enhance crop production to meet the demands for food for the increasing world population. Nitrogenous fertilizers are usually used to enhance yields of crops and pesticides are used to control diseases and pests. However, both fertilizers and agrochemicals are costly and may be harmful to the environment and to animal and human health (Bhattacharje *et al*., 2008; Joshi and Bhatt, 2011). Use of microbial inoculants to enhance crop production has therefore been proposed as a cheaper and environmentally sound option for sustainable agriculture (Wu, *et al.*, 2005). Seed inoculation with free-living diazotrophic bacteria has been documented to increase plant growth and yields, and reduce levels of damage caused by plant pathogens (Okon and Labandera-Gonzalez, 1994; Dobbelaere *et al*., 2001; Riggs *et al*., 2001, Mehnaz *et al*., 2010)**.** These bacteria have been shown to influence plant growth and yields through several mechanisms such as biological nitrogen fixation (BNF) phytohormone production and phosphate-solubilization (Dobbelaere *et al*., 2003; Vessey, 2003). They also reduce or inhibit the effects of pathogenic microorganisms by production of siderophores, hydrogen cyanide, antibiotics and extracellular hydrolytic enzymes (Glick and Bashan, 1997). Bacteria widely investigated for growth promotion and biological control of plant pathogens include genera such as *Azospirillum*, *Azotobacter*, *Bacillus, Klebsiella* and *Pseudomonas* (Kim *et al*., 1997; Dobbelaere *et al*., 1999; Iniguez *et al*., 2004; Cakmakci *et al*., 2007; Sachdev *et al*., 2009). A number of microbial-based products have been developed and are commercially available to enhance plant growth (Shen, 1997; Kloepper *et al*., 2004; Schisler *et al.,* 2004; Cawoy *et al.,* 2011).

In this study selected free-living diazotrophic bacterial isolates were investigated for their effects on the growth of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) and for their potential to control damping-off fungi in wheat. *In vitro* studies were conducted to determine the possible mechanisms of plant growth promotion and biological control exhibited by these isolates.

A study was conducted on the *in vitro* interaction of isolates of *Bacillus subtilis* and Eco-T®, a commercial biocontrol agent (BCA), (an isolate of *Trichoderma harzianum* Rifai), for a possibility of applying the two microbes together to enhance plant growth and to provide for the control of *Rhizoctonia solani Kühn*. The effects of application of bacterial isolates in combination with a low dose of nitrogenous fertilizer were investigated under greenhouse and field conditions for their effects on seedling growth and yields of maize and wheat. *Bacillus* isolates were evaluated for their potential to inhibit a wide range of pathogenic fungi *in vitro* and for control of *R. solani* damping-off of wheat under greenhouse conditions. For *in vivo* trials, different bacterial isolates were applied as seed treatments.

In this overview we report the findings of this study and the issues that need to be addressed in future research. The findings from this research were as follows;

- Free-living diazotrophic bacteria exhibited multiple plant growth-promotion activities and a variety of biological control mechanisms *in vitro*.
- A combination of the most promising bacterial isolates from the *in vitro* studies and a low dose of nitrogenous fertilizer enhanced growth of maize and wheat under greenhouse conditions.
- Seed inoculation of maize with some bacterial isolates in combination with a low dose of nitrogenous fertilizer increased shoot dry biomass and yields of maize above the Uninoculated Control and caused the same or greater yield increases than the Fully Fertilized Control under field conditions.
- Seed inoculation of wheat with some bacterial isolates in combination with a low dose of nitrogenous fertilizer caused the same shoot dry biomass and yields or greater than the Fully Fertilized Control, and these were significantly higher than the Uninoculated Control under field conditions.
- Seed inoculation with *B. subtilis* isolates inhibited the growth of a wide range of pathogenic fungi *in vitro* and suppressed *R. solani* damping-off of wheat under greenhouse conditions
- *B. subtilis* isolates exhibited multiple biological control mechanisms against *R. solani in vitro* as indicated by inhibition zones, the browning of mycelia, the loss of hyphal turgidity, and the deformation of hyphal tips, as revealed by the electron microscope ultrastructures of the mycelia.

- *B. subtilis* isolates that exhibited various mechanisms of biological control *in vitro* also suppressed *R. solani* damping-off of wheat under greenhouse conditions.
- All *B. subtilis* isolates were compatible with each other but none was compatible with Eco-T® *in vitro*.

In vitro screening techniques have been utilized in previous studies to select effective strains with multiple plant growth-promotion and biological control traits from a large number of initial isolates (Gupta *et al*., 1998; Ryu *et al*., 2006; Ahmad *et al* 2008).

The main limitation with this technique is that the results obtained *in vitro* do not always correlate with those observed *in vivo* under field conditions (Chanway and Holl, 1993). Apart from this, it is not possible to identify biocontrol agents that do not show antifungal activity *in vitro* through this technique.

In this study bacterial isolates with multiple plant growth promotion and biocontrol traits were selected. Some of these isolates enhanced growth and yields of maize and wheat under greenhouse and field conditions. The selected *B. subtilis* isolates exhibited several modes of action against *R. solani*, effectively inhibited the growth of a wide range of pathogenic fungi *in vitro* and controlled *R. solani* of wheat in the greenhouse. This was revealed by increases in seed germination seedling survival and shoot dry biomass of inoculated plants compared to the Disease-free Control, and those seeds inoculated with Eco-T®, a commercial BCA. *In vitro* screening of diazotrophic bacteria for plant growth–promotion and biocontrol activities provides a quick and viable technique for the selection of effective bacterial strains for use in sustainable agriculture. However, some of the effective isolates selected were subsequently shown to be closely related to bacterial species known to be pathogenic to animals and humans. Therefore, there is still a need to identify simpler techniques that include identification of the isolates that can be used for screening of larger numbers of isolates *in vitro*. Reports on a lack of correlation between results obtained *in vitro* and under field conditions exist in the literature (Schroth and Becker 1990; Williams and Asher, 1996). However, in this study, the most promising isolates identified *in vitro* worked well *in vivo*. They enhanced seedling growth of maize and wheat under greenhouse conditions.

Bacterial Isolate BS431 (*Klebsiella pneumoniae (*Shcroeter) Trevisan) exhibited most of the plant growth-promotion traits tested, enhanced shoot dry biomass in the greenhouse and increased both shoot biomass and yield of maize in the field. Isolate BS10 (*B. subtilis*) caused the best shoot dry biomass and yield in wheat and tested positive for all growth promotion traits studied *in vitro*. This demonstrates that isolates that exhibited the most growth promotion activities *in vitro* enhanced plant growth *in vivo*. Khalid *et al*. (2004) also demonstrated that there was a positive correlation between the *in vitro* indole-3-acetic acid production by rhizobacteria and the increases in host growth parameters.

Plant growth enhancements and yield increases following inoculation of non-legumes with *Azospirillum brasilense* were initially attributed to biological nitrogen fixation by some researchers. However, they were later confirmed to be due to the effects of IAA produced by this species on root morphology (Okon and Labandera-Gonzalez, 1994; Dobbelaere *et al*., 1999; Vessey, 2003). Further research is therefore required to establish the exact mechanism responsible for the observed results, to determine whether these results were due to synergistic effects by the various potential growth enhancement mechanisms.

Seed inoculation in combination with a 65% and 50% nitrogenous fertilizer in maize and wheat, respectively, caused the same increase in shoot biomass as the Fully Fertilized Control, whereas increasing fertilizer doses above these levels did not seem to have any significant effect on the biomass of these two crops. This observation indicates that these bacterial isolates were more effective when combined with low levels of nitrogenous fertilizer. Biswas *et al*. (2000) and Riggs *et al*. (2001) reported improvements in growth parameters of various crops as a result of bacterial inoculations at reduced levels of nitrogenous fertilizers. These findings confirm that the use of suitable microbial inoculants may enhance nitrogen fertilizer efficiency, leading to enhanced crop production at lower doses of these fertilizers. Use of the most promising isolates identified in this study may constitute an important component of integrated mineral management for maize and wheat production. Studies with more crop species and cultivars are required to confirm these results and to expand the potential of the approach to other field crops. There is also a need to test these isolates for the ability to colonize and establish themselves in the rhizosphere of these crops.

Seed inoculation with the most promising bacterial isolates, combined with a low dose of nitrogenous fertilizer, caused the same or greater shoot dry biomass and yield increases of maize and wheat as the Fully Fertilized Control under field conditions. The results varied with cultivar x isolate interactions. Similar observations have been reported by other authors (Dobbelaere *et al*., 2002; Adesemoye *et al*., 2009; Akbar *et al*., 2011). Trials with the most promising isolates and a number of crop cultivars could lead to identification of the best bacterial isolate and cultivar combinations that could serve as an option for maize and wheat production to reduce the cost of production and reduce the adverse effects of agrochemicals to the environment. The most promising *B. subtilis* isolates from this study inhibited the growth of a wide range of pathogenic fungi *in vitro* and controlled damping off by *R. solani* of wheat under greenhouse conditions. The same isolates exhibited various mechanisms of biological control against *R. solani in vitro*.
These isolates should be tested for biological control of *R. solani* under field conditions.
Isolate BS10 (*B. subtilis*) controlled *R. solani* of wheat as effectively as Eco-T®, a commercial BCA, and caused a greater shoot dry biomass than this BCA in the greenhouse. In the field this isolate caused the same or greater shoot dry biomass and yield increases of wheat than the Fully Fertilized Control. Although *Bacillus*-based biocontrol products have been commercialized in other countries (Cawoy *et al*., 2011), there is little information on commercial BCAs in South Africa based on *B. subtilis*. With its ability to promote growth of wheat, and to control *R. solani* damping-off of wheat, this isolate is a promising candidate for formulation of an inoculant biofertilizer for wheat production.

Through this study the following promising bacterial strains were identified:

- Isolate BS431, a strain of *Klebsiella pneumoniae*, has the ability to promote growth and enhance yields of maize.
- Isolate BS10, a strain of *B. subtilis,* has the ability to promote wheat growth and enhance its yields, and to control *R. solani* damping-off of wheat in the greenhouse, to control other pathogenic fungi *in vitro* through multiple biocontrol mechanisms.

The ubiquitous bacterium *K. pneumoniae*, which is sometimes an endophytic bacterium, has been studied widely for growth promotion. In one study, it has been found to fix enough nitrogen to relieve nitrogen deficiency symptoms in wheat (Iniguez *et al*., 2004).

In another study several isolates of this bacterium have been found to synthesize phytohormones (Sachdev *et al*., 2009). The isolate identified in this study therefore could be a promising strain for formulation of inoculant biofertilizers for maize. Although the existence of pathogenic strains of this genus have been reported, there is currently no direct link between rhizosphere isolates and those that are pathogenic to animals and humans. If the necessary precautions are taken to ensure the safety of personnel dealing with the inoculants, these isolate could enhance maize production at a low cost. Alternatively with the current knowledge on genetic engineering, genes responsible for IAA synthesis and BNF can be extracted from these isolate and incorporated into other plant growth promoting bacterial strains and formulated for use in agriculture.

Isolate BS10 of *B. subtilis* promoted growth and enhanced wheat yields through multiple growth promotion mechanisms. This isolate also suppressed the effects of *R. solani* through multiple biocontrol mechanisms. It has the ability to resist a variety of antibiotics and to produce enzymes that can lead to successful colonization and establishment of this strain in the rhizosphere of plants. Therefore this isolate needs to be tested on many wheat cultivars in multiple environments to see if it can be used widely to enhance wheat production. A study on biocontrol of damping-off of *R. solani* in wheat under field conditions is required to confirm the efficacy of this isolate under field conditions as a biological control agent.

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