In vitro and *in vivo* screening of *Bacillus* spp. for biological control of *Rhizoctonia solani*

By

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DEDICATION

To my wife, Bongi for all understanding and emotional support when needed during my studies

DECLARATION

I, Bongani Petros Kubheka, declare that the research report in this thesis, except where otherwise indicated, is my own original research. This thesis has not been submitted for any degree or examination at any other university.

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ABSTRACT

The increasing concerns about chemical pesticides that are environmentally hazardous and the continuous development of resistance by pathogens to chemical pesticides have led to this study. Many studies have shown that some Gram-negative bacteria, such as *Pseudomonas flouresens*, control plant diseases and promote plant growth. In this study Gram positive bacteria, *Bacillus* sp., were chosen because of their ability to produce endospores. Endospores can be used in stable, dry formulations. The advantage of using endospores is their ability to survive harsh conditions such as droughts and high temperatures, which give a long shelf life to the biological control agent.

Bacillus isolates were recovered from the rhizosphere of 12 different crops, and were subsequently screened *in vitro* for their antimicrobial activity. Of 130 isolates, 87 exhibited antimicrobial activity against the test organisms: *Rhizoctonia solani, Pythium* sp., *Phytophthora cinnamoni, Fusarium* sp., and single representatives of Gram negative and Gram positive bacteria, namely, *Erwinia carotovora* and *Staphylococcus aureus* respectively. The *Bacillus* isolates B77, B81 and B69 inhibited all the test organisms investigated, which suggests that they produced broad spectrum antimicrobial compounds or more than one antimicrobial compound. Of the isolates that showed antimicrobial activity, 78 of them did not inhibit *Trichoderma harzianum* K D, which is a registered biological control agent; indicating their potential for combined application.

Selected *Bacillus* isolates were tested for the biological control of *R. solani* under greenhouse conditions in wheat, cabbage, tomato, maize, and cucumber seedlings. *Bacillus* isolates were applied as seed treatments, and the inoculated seeds were planted in *R. solani* infested speedling trays. Shoot dry weight measurement of seedlings indicated that 12 out of 19 *Bacillus* isolates showed significantly different shoot dry weight in wheat whereas all the isolates tested in tomato and cucumber gave significantly different shoot dry weight. No significantly different shoot dry weight was obtained for maize or cabbage. Seed emergence findings indicated that none of the *Bacillus* isolates

gave significantly different emergence percentage on wheat, cabbage, tomato, and maize but all of them showed significantly different emergence percentage on cucumber. The results indicate that both the pathogen and the biological control agents exhibited varying levels of specificity on each crop tested.

The biological control potential of the best *Bacillus* isolates was tested on bean and maize crops in the field. Green bean and maize seeds were coated with the selected *Bacillus* isolates and then sown under field conditions. For each isolate, four replicate treatment plots were established, with and without a *R. solani* inoculum. Percentage emergence, plant survival levels to harvesting and yield of maize cobs and green beans pods were measured. For all parameters measured the positive and negative controls were not significantly different thereby rendering the results for the entire field study inconclusive. However, *Bacillus* isolates B77, B11, R5 and R7 improved green bean pod yield and *Bacillus* Isolate B81 increased maize yield, indicating their potentials as plant growth promoting rhizobacteria (PGPR).

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CHAPTER 1

LITERATURE REVIEW

1.1 BIOLOGICAL CONTROL AS A DISEASE CONTROL OPTION

The overuse of chemical pesticides has caused global concern with regards to soil pollution and their potentially harmful effects to human beings and animals (Hayes, 1994; Nemec *et al.*, 1996; Jensen *et al.*, 2000). The Montreal Protocol, 1994, earmarked the soil fumigant methyl bromide as an ozone-depleting substance, and its use has been gradually phased out (Hayes, 1994). The loss of methyl bromide as a soil fumigant has stimulated a search for alternatives. In the United States, the impact of the loss of methyl bromide has been greatest on the California strawberry and the Florida vegetable industries (Nemec *et al.*, 1996). This is because several important diseases were controlled by methyl bromide. One of the most important is tomato root and crown rot caused by *Fusarium oxysporum f.* sp. *radicis lycopersici.* This disease is prevalent in both greenhouse and field operations worldwide. Its control with fungicides and most fumigants is often ineffective, whereas its control using selected biological control agents may be feasible (Nemec *et al.*, 1996).

Pepper production has also been affected by the loss of methyl bromide, which was used to control fungal pathogens such as *Pythium aphanidermatum*, the causal organism of root and crown rot (Orie and Shoda, 1996). Accordingly, biological control of soil-borne diseases has received increased attention as an attractive alternative (Jensen *et al.*, 2000). Research on biological control of plant pathogens received major impetus after the 1963 international symposium "Ecology of Soil-borne Plant Pathogens-Prelude to Biological Control", held at the University of California in Berkerly (Orie and Shoda, 1996). In addition, plant growth-promoting rhizobacteria (PGPR's) hold great promise as potential agricultural and forestry inoculants and, if effective, could reduce or eliminate the use of toxic or environmentally damaging chemical fertilizers and pesticides (Bent and Chanway, 1998). PGPR's have been shown to enhance tree seedling growth in the nursery and at

reforestation sites and, in some cases, to improve the survival of out planted seedlings (Bent and Chanway, 1998).

Microorganisms play a crucial role in plant disease control. As naturally occurring residents of the infection court, they play a role as competitive antagonists of the invading pathogen (Larkin and Fravel, 1998). This feature can be harnessed, managed and exploited to achieve effective biological control. For instance some root-associated microorganisms have broad-spectrum antibiotic activity and are able to suppress more than one pathogen (Emmert and Handelsman, 1999). However, these microorganisms often exhibit other specificities such as host preference or adaptation to certain soil types (Mathre *et al.*, 1999).

One of the most important characteristics necessary for the acceptance and effectiveness of a biological control agent is its ability to survive in environments foreign to its origin (Nemec *et al.*, 1996). In addition to this an organism must be able to successfully colonize plant roots during the period that protection against pathogens is required (Chao *et al.*, 1986)

1.2 RHIZOSPHERE ENVIRONMENT

The rhizosphere is a narrow zone of soil around the root. This zone has been much studied to track populations of introduced bacteria associated with roots (Parmar and Dadarwal, 1999). Many bacterial cells in close proximity to plant roots or adhering to root surfaces are dispersed or redistributed by water filtration in soil. Such bacterial redistribution through the soil can enhance root colonization (Chao *et al.*, 1986). The microorganisms that colonize root surfaces utilize particular components of the root exudates and produce metabolites of their own so that the net biochemical profile of a root results from the interaction of the host plant and its rhizosphere microflora. Early colonizers modify the nature of the rhizosphere for the later colonizers (Gilbert *et al.*, 1996).

Rhizosphere microflora may be either deleterious to plants, or plant growth promoting (Gutierrez Manero *et al.*, 1996). Plant Growth Promoting Rhizobacteria (PGPR) promote

plant growth in two ways; some by solubilizing insoluble compounds such as phosphates, making them available to plants in a usable form (Kumar and Narula, 1999) or they produce phytohormones such as indol-3-acetic acid (IAA) (Mahaffee and Kloepper, 1994; Monier *et al.*, 1998). Other PGPR's promote plant growth by defending the plant from attack or infection by plant pathogens or deleterious microorganisms, by producing antimicrobial compounds such as antibiotics, siderophores and many other different compounds against plant pathogens (Schippers *et al.*, 1995).

The ability to colonize the rhizosphere is essential for selected bacteria to function as biological control agents of soil-borne plant pathogens (Bent and Chanway, 1998). Failure to adequately colonize roots may account for the unreliability of many biological control agents (Knudsen *et al.*, 1997). Root colonizing ability appears to be strain-specific rather than a genus- or species-specific trait (Weller, 1988). For example, *Bacillus subtilis* Strain D-39Sr exhibited greater relative colonization of crown roots of wheat than did Strain D-56Sr (Millus and Rothrock, 1993). The ability of microorganisms to colonize roots, otherwise known as rhizosphere competence, is therefore an essential criterion in screening and selecting suitable biological control agents (Millus and Rothrock, 1993).

1.3 NATURAL OCCURRENCE OF ENDOSPORE FORMING, *BACILLUS* SP.

Members of the genus *Bacillus* are common residents of the soil and rhizosphere environment (Holl and Chanway, 1992; Mazzola, 1999). Kloepper *et al.* (1992) observed that *Bacillus*, *Pseudomonas*, and *Flavobacterium* were the dominant bacterial genera in root-free soil, rhizosphere and the geocarposphere of peanut plants. In another study, the most frequently isolated bacterial endophytes from roots of sweet corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) were found to belong to the genera *Bacillus*, *Burkholderia* and *Pseudomonas* (McInroy and Kloepper, 1995).

Cattelan *et al.* (1998) found that 86.2% of isolates cultured from root-free soil and rhizosphere of nodulating and non-nodulating soybean growing in sandy loam and silt loam soils were from the genus *Bacillus*, with *Bacillus megaterium* the most commonly

identified species in both soils. Overall, *Bacillus* numbers were higher in the rhizosphere of the nodulating and non-nodulating soybean than in the root-free soil. *Bacillus* sp. was offset by higher numbers of *Pseudomonas* in the soybean rhizosphere (Cattelan *et al.*, 1998). Mahaffee and Kloepper (1997) also reported that *Bacillus* was the dominant genus in soil. According to their study, *Bacillus* comprised 67% of the genera isolated. In the rhizosphere of cucumber, they found that *Bacillus* and *Pseudomonas* both dominated. *Bacillus* sp. were also found to dominate the endorrhiza (Mahaffee and Kloepper, 1997). These studies reveal that bacilli occur naturally in the root zone of many plants and hence their application as plant growth promoters or biological control agents is valid and warranted.

1.4 BIOLOGICAL CONTROL ACTION

Members of the genus *Bacillus* have been considered less effective as rhizosphere colonists than fluorescent pseudomonads (Millus and Rothrock, 1993). However, there is a growing list of examples that suggests that selected *Bacillus* species can successfully colonize roots after being introduced as seed inoculants (Adejumo *et al.*, 1999). Endospore formers offer several advantages over well-known biological control agents such as *Pseudomonas fluorescens* and other Gram-negative bacteria. These advantages include their ability to form endospores, which inherently improves shelf life, and the broad-spectrum activity of their antibiotics (Mavingui and Heulin, 1994). The key advantage of Gram-positive bacteria over the Gram-negative bacteria is that they have a natural formulation, the spore, which is stable and stability of the formulation is critical to successful biocontrol (Emmert and Handelsman, 1999). Several strains of *Bacillus subtilis* show antifungal action by producing antifungal volatiles (Fiddaman and Rossal, 1994). Table 1.1 shows some examples of *Bacillus* sp. used as biological control agents. Manipulating media composition or the culture environment can enhance production levels of antifungal compounds.

Table1.1: Examples of *Bacillus* isolates that show biological control action

Organism	Disease/pathogen controlled	Reference
Bacillus sp. Strain L324-92	*Rhizoctonia* root rot caused by *R. solani* AG8 in wheat	Kim *et al.*, 1997
	Take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*	Kim *et al.*, 1997
	Pythium root rot caused by *P. irregulare* and *P. ultimum* in wheat	Kim *et al.*, 1997
Bacillus pumilus	Fusiform rust in Loblolly pine.	Enebak and Carey, 2000
	Penicillium digitatum	Huang *et al.*, 1992
Bacillus subtilis Strains MB1600 and MB1205	*Sclerotinia sclerotiorum*, *Phytophthora sojea*, *P. infestans*, *P. cactorum*, *P. cambivora*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium oxysporum*.	Knox *et al.*, 2000
Bacillus cereus Strain A47 and *B. subtilis* Strain B908	Take-all of wheat	Ryder *et al.*, 1999
	Rhizoctonia root rot in wheat	Ryder *et al.*, 1999
	Sclerotium rolfsii	Ryder *et al.*, 1999
	Pythium ultimum	Ryder *et al.*, 1999

1.5 USE OF *BACILLUS* SP. AS BIOLOGICAL CONTROL AGENTS

Gram-negative bacteria, especially *Pseudomonas* strains, have been intensively investigated as biological control agents with regard to the production of anti-microbial metabolites (Thomashow, 1996). Gram-positive bacteria, especially *Bacillus* sp. have not received as much attention (Orie and Shoda, 1996). This trend is slowly changing as the benefits of *Bacillus* sp. are recognized. Advantages of *Bacillus* sp. include: ubiquity, ability to form

resistant endospores, and their ability to produce a multitude of broad-spectrum antibiotic compounds (Rytter, *et al.*, 1989).

Examples of *Bacillus* species that have been used as biological control agents are presented as follows:

1.5.1 *Bacillus* sp. Strain L324-92 used to control Take-all of wheat, *Rhizoctonia* root rot, and *Pythium* root rot of wheat

Bacillus sp. Strain L324-92 exhibits broad-spectrum inhibitory activity and grows at temperatures ranging from 4 to 40°C (Kim *et al.*, 1997). Being able to grow at 4°C is thought to contribute to its biological control activity on direct-drilled winter and spring wheat. Other well-known biological control agents such as *Pseudomonas fluorescens* cannot grow at temperatures of 4°C. *Bacillus* sp. Stain L324-92 can control three root diseases of wheat: Take-all caused by *Gaeumannomyces graminis* var. *tritici*; *Rhizoctonia* root rot caused by *R. solani* AG8; and *Pythium* root rot caused mainly by *P. irregulare* and *P. ultimum.* These three root diseases are major yield limiting factors in the US Northwest, especially where wheat is direct-drilled into the residue of a previous cereal crop (Kim *et al.*, 1997). Antimicrobial activity of this organism against the above-mentioned pathogens was shown in both *in vitro* and *in vivo* assays. The results from the *in vitro* assay showed that *Bacillus* sp. Strain L324-92 inhibited all isolates of *G.graminis* var. *tritici*, *Rhizoctonia* species, and *Pythium* species tested.

In vivo, field trials are done to verify the results from *in vitro* assays. The results from the field trials showed that yields following inoculation with *Bacillus* sp. Strain L324-92 were comparable to those obtained following soil fumigation or difenoconazole seed treatment. It was also found that in plots where spring barley was the previous crop, *Bacillus* sp. Strain L324-92 treatment was significantly better than treatment with the chemical pesticide, difenoconazole (Kim *et al.*, 1997).

1.5.2 *Bacillus subtilis* as a biological control agent against soil-borne plant pathogens

1.5.2.1 Bacillus subtilis Strain RB14 control of Rhizoctonia solani in Tomato

Bacillus subtilis RB14 produces two antibiotic substances, Iturin A and Surfactin. These antibiotics suppressed *R. solani* the causal agent of damping-off of tomato seedlings. To confirm that it was Iturin A and Surfactin that suppressed the damping-off of tomato seedlings grown in the soil, experiments aimed at recovering the antibiotics from the soil were conducted. The outcome showed that the Iturin A and Surfactin produced by *B. subtilis* RB14 were present in the inoculated soils but absent in the uninoculated control soils. These two antibiotics were further tested in *in vitro* studies to confirm that they inhibit *R. solani* (Orie and Shoda, 1996).

1.5.2.2 Bacillus subtilis Strain 3 control of geranium rust

Rytter *et al.* (1989) isolated strains of *B. subtilis* that consistently inhibited *Puccinia pelargonii-zonalis*, the causal agent of geranium rust, under greenhouse conditions. It was found that *B. subtilis* Strain 3 significantly reduced disease severity when compared with the water and nutrient broth controls. Since it was suspected that *B. subtilis* produces anti-microbial substances, washed bacterial cells and the corresponding cell-free culture filtrate were tested. It was found that the culture filtrate reduced the incidence of rust by 84% whereas the washed cell treatment only controlled the rust by 71%, compared to the water control (Rytter *et al.*, 1989).

1.5.2.3 Bacillus subtilis Isolate FZB24®, a registered plant probiotic bioproduct.

Bacillus subtilis Strain FZB24® has been used widely in treating ornamental and vegetable seedling diseases caused by *R. solani, Fusarium* sp., and *Alternaria radicina*; soil-borne fungal root diseases of maize; *Fusarium*-wilt disease of tomatoes; and *Rhizoctonia*-scurf and common scab in potatoes (Bochow, 1995). *B. subtilis* Strain FZB24® produces a metabolite spectrum that contains different physiologically active substances, which promote plant growth on one hand and increase resistance and tolerance against pathogen

attack on the other hand. These metabolites may act as signals to initiate phytohormone production, especially auxin synthesis by the plant (Srinivasan *et al.*, 1996). The phytohormone balance can also regulate the production of tannin, which acts as a defense substance against pathogens of tomato seedlings. The effectiveness of *B. subtilis* Strain FZB24® is based on the stimulation of plant growth by excretion of physiologically active metabolites following root colonization and, secondly, by direct antibiotic effects (antibiosis) against microbial phytopathogens (Bochow and Dolej, 1999).

1.5.2.4 Control of Sclerotinia sclerotiorum, R.solani, and Fusarium oxysporum by B. subtilis Strains MB1205 and MB1600

Bacillus subtilis can use nitrate as a terminal electron acceptor in the event of oxygen depletion in the environment. This enables *B. subtilis* to maintain its metabolic activity and hence, retain its anti-fungal action even in anaerobic environments (Fiddaman and Rossal, 1994).

Knox *et al.* (2000) confirmed the importance of nitrate to biological control agents under anoxic conditions. They showed that when fungal isolates and selected *Bacillus* isolates were co-cultured on aerobic agar plates, there was a significant reduction in the rate of fungal growth. The addition of nitrate to the growth medium caused no significant difference in the radial growth of the fungi. However, studies conducted under anoxic conditions in sealed plates showed a significant reduction in fungal radial growth. When nitrate was added to the anoxic medium, at concentrations of 10 and 100 mM, *B. subtilis* Strains MB1205 and MB1600 antagonized *S. sclerotiorum, P. infestans, P. cactorum, P. cambivora, P. sojae, M. phaseolina, R. solani* and *F. oxysporum. Bacillus subtilis* Strain MB1205 showed some antagonism towards *Pythium ultimum* as well, whereas *B. subtilis* Strain MB1600 showed no antagonism towards this pathogen (Knox *et al.*, 2000).

1.5.2.5 *Control of Rhizoctonia solani and Gaeumannomyces graminis var. tritici by Bacillus subtilis Strain B908 and B. cereus*

B. subtilis Strain B908 and *B. cereus* are biological control agents originally isolated in China (Ryder *et al.*, 1999). These bacteria were found to be plant growth promoting as well as control agents of root diseases. These strains have been used commercially to increase plant yield in China and are termed "yield increasing bacteria" (YIB) (Ryder *et al.*, 1999).

B. subtilis Strain B908 and *B. cereus* inhibit plant pathogens such as *R. solani* and *Gaeumannomyces graminis* var. *tritici*. The possible mechanisms involved in biological control by these *Bacillus* strains include the production of water soluble and volatile anti-fungal substances. *B. subtilis* Strain B 908 produces large inhibition zones on potato dextrose agar (PDA), indicating that they synthesize low molecular mass compounds that are active against these pathogens. *B. cereus* was found to produce a moderate amount of siderophore when grown on CAS medium (Ryder *et al.*, 1999).

1.5.3 The use of *Bacillus pumilus* to control *Penicillium digitatum*

Penicillium digitatum, a major citrus post-harvest pathogen, was previously controlled by strategic use of fungicides, particularly benzimidizoles (Huang *et al.*, 1992). However, this pathogen has increasingly developed resistance against benzimidizoles, causing a serious problem. Another chemical fungicide, imazalil, has been used to control benzimidizole-resistant strains. However, some strains of *P. digitatum* have also developed resistance against imazalil. Biological control has been proposed as a possible solution to this problem. *Bacillus pumilus* has shown great promise in controlling *P. digitatum* both *in vitro* and *in vivo* testing. Large and distinct inhibition zones (34.6mm) in the *P. digitatum* growth were found surrounding the *B. pumilus* colonies, which was significantly larger than the inhibition zones produced by benomyl (0mm), imazalil (15.3mm) and guazatine (22.7mm) (Huang *et al.*, 1992).

1.5.4 The use of *Bacillus pumilus* to induce systemic protection against Fusiform rust

Strains of *Bacillus pumilus* have also been found to induce systemic protection against Fusiform rust in loblolly pine (Enebak and Carey, 2000). Pine seeds were treated separately with prepatations namely, *B. pumilus* Strain SE34; *B. pumilus* Strain INR7; *B. pumilus* Strain SE52; and *Serratia marcescens* (90-166) at sowing time. After a month the systemic fungicide Bayleton DF was used to treat uninoculated seedlings of the same age which served as a positive control. Untreated seeds inoculated with the pathogen served as a negative control. After four weeks pathogen basidiospores were sprayed onto all the seedlings. The results showed that *B. pumilus* strains SE52, INR7, and SE34 significantly reduced the number of galls, compared to those seedlings, which were not treated with bacteria. Averaged over both years of the study, 31% of the control seedlings (seedlings from untreated seeds) were infected with Fusiform rust, while those seedlings treated with *B. pumilus* Strains SE34, INR7, SE52, and *S. marcescens* (90-166) had only 13; 15; 16 and 14% infection respectively (Enebak and Carey, 2000).

1.6 SOIL-BORNE PLANT PATHOGENS

Soil-borne plant pathogens are deleterious microorganisms that can be found in the plant rhizosphere. These pathogens include *Rhizoctonia* sp., *Fusarium* sp., *Pythium* sp. and *Phytophthora* sp. and they cause diseases such as root rots, damping-off or wilt in various crops (Singh *et al.,* 1999, Cook *et al.*, 2002, Howel, 2002).

Rhizoctonia solani is a plant pathogen that causes losses in a wide range of vegetables and flowers, several field crops, turf grasses, as well as perennial ornamentals, shrubs, and trees (Gross *et al.*, 1998). Symptoms may vary on different crops, with the stage of growth at which the plant becomes infected, and with the prevailing environmental conditions. The most common symptoms are damping-off of seedlings, root rot, stem rot, or stem canker of growing plants (Agrios, 1997). *Rhizoctonia* damage can occur at any time during the growing season, but is more severe on young seedlings (Mathre *et al.*, 1999).

Damage caused by *Rhizoctonia* is frequently confused with diseases caused by other seedling pathogens such as *Pythium, Phytophthora* and *Fusarium* sp. In the case of pre-emergence damping-off, it is difficult to identify the responsible pathogen since the symptoms are similar for *Pythium, Phytophthora, Fusarium* and *Rhizoctonia* sp. (Bauske *et al.*, 1997). *Rhizoctonia*-infected plants typically have reddish-brown, sunken cankers on the lower stem or hypocotyls (Agrios, 1997). Damping-off is probably the most common symptom caused by *Rhizoctonia* on most plants that are vulnerable to the pathogen (Krause *et al.*, 2001). Very young seedlings may be affected before or soon after they emerge from the soil.

Rhizoctonia can also attack the stems of newly emerged seedlings making them water soaked, soft, and incapable of supporting the seedling, which then falls over and dies. In older seedlings, the fungus forms tan to reddish brown lesions, which may increase in length and width until they finally girdle the stem. Shortly before the plant dies, the stem turns brownish black and may bend or twist without breaking, giving the disease the name wire stem (Krupa and Dommergues, 1979).

Rhizoctonia can subsequently attack seedlings that escape initial damping-off resulting in a seedling stem canker, which is also known as sore-shin. The sore-shin lesions appear as reddish brown, sunken cankers that range from narrow to those that completely girdle the stem near the soil line (Klein-Gebbinck and Woods, 2002).

Rhizoctonia sp. such as *R. solani* AG-8 and *R. oryzae* are causal organisms of *Rhizoctonia* root rot. This disease is among the most destructive and widespread of plant diseases. It is also difficult to control because resistant crop varieties are not readily available and few effective chemicals are registered (Duffy, 2000).

As soil temperatures rise later in the growing season, affected plants may show partial recovery due to new root growth. Stem canker is common and destructive in cotton, tobacco, and bean seedlings that escape damping-off. *Rhizoctonia* species causing root lesions, which first appear just below the soil line, can attack other seedlings and partly

grown or mature plants (Agrios, 1997). In cool, wet weather the lesions may increase in size and number to include the whole base of the plant and most of the roots. This results in weakening, yellowing, and sometimes death of the plant (Mazzola *et al.*, 1996).

On plants such as cabbage and lettuce the lower leaves, which are touching the ground, are attacked at the petioles and midribs. Sunken lesions which are reddish-brown and slimy develop and the entire leaf becomes dark brown and slimy. The infection spreads from the lower leaves up to the next leaves until most or all leaves are infected (Agrios, 1997).

Rhizoctonia solani may live in the soil as mycelium and sometimes as small sclerotia that show no internal tissue differentiation (Agrios, 1997). Mycelial cells of *R. solani* are multinucleate whereas mycelial cells of several other *Rhizoctonia* species are binucleate ((Tsror *et al.*, 1997). The mycelium is colourless when young but later turns yellowish or light brown with age. Hyphae consist of long septate cells and produce branches that grow at right angles to the main hypha. The branching characteristics are the primary means of identifying this fungus (Agrios, 1997).

The pathogen can over-winter as mycelium or sclerotia in the soil or in/on infected perennial plants, or in propagative material such as potato tubers (Tsror *et al.*, 1997). In some hosts the fungus may even be carried in the seeds. The fungus is present in most soils and, once established in a field, remains there indefinitely. The fungus is spread by rain, irrigation, and floodwater, with tools carrying contaminated soil and by infected propagative material. The disease is more severe in soils that are moderately wet rather than in soil that is waterlogged or dry. Infection of young plants is most severe when plant growth is slow because of adverse environmental conditions (Agrios, 1997).

Control of *Rhizoctonia* diseases is difficult since for most vegetables, no effective fungicides are available. However, chlorothalonil, thiophanate-methyl and iprodione are sometimes recommended as sprays on the soil before planting, and once or twice on the seedlings soon after emergence (Abawi and Widmer, 2000; Duffy, 2000). Over the years, much effort have gone into developing alternative, more effective means of controlling

Rhizoctonia diseases. Such methods include mulching of field with certain plant materials or with photodegradable plastic, avoiding application of some herbicides that seem to increase *Rhizoctonia* diseases in certain crops and, especially, biological control (Abawi and Widmer, 2000). Several microorganisms such as fungi, soil myxobacteria, and mycophagous nematodes have been found to parasitize *Rhizoctonia* (Lucas *et al.*, 1993). So far, however, many biological control agents for use against *Rhizoctonia* are still at an experimental stage and are not available for use by farmers.

Research conducted in recent years has shown biological control of soil-borne plant pathogens can be enhanced through incorporating farming practices such as crop rotation and adding organic amendments to soil (Mathre *et al.*, 1999). Crop rotations allow time for resident antagonists to sanitize the soil or for propagules of specialized pathogens to die, whereas the addition of organic amendments to soil stimulates growth of resident antagonist populations (Abawi and Widmer, 2000).

Other sanitation practices include soil fumigation, which has been used as an alternative for crop rotations (Abawi and Widmer, 2000). Tillage is also important in biological control in some agri-ecosystems as a means of accelerating the displacement of certain pathogens by resident antagonists (non-pathogens) in crop residues through exposure of new sites for colonization on the residue fragments and through intensification of microbial activity that accompanies soil disturbance (Abawi and Widmer, 2000). Much of what has been said is contrary to agricultural practices, which have moved towards less use of organic amendments and crop rotations (Agrios, 1997). With greater understanding and application of microbiological principles it is hoped that these trends will be reversed. This approach to crop protection involves the incorporation of microorganisms, as introduced antagonists. Bacterial antagonism appears to be an important factor in disease suppression (Zheng and Sinclair, 2000).

It has been published widely that autochthonous soil microorganisms can antagonize numerous plant pathogens. It is widely held that modern biological control agents, if effective, will emerge as products sold by industry. Much of this focus can be attributed to

the emphasis within governmental and academic research institutions on patents and products that can be licensed to industry.

According to Zheng and Sinclair (2000), good biological control agents must be able to colonize roots. This conclusion is based on intensive studies where it was found that the bacterial strains that colonized the roots best were the ones that provided the best suppression of *Rhizoctonia* root rot. It was also found that root colonization by a biological control agent is subject to a dynamic equilibrium within a complex microbial community. Success in colonization may vary depending on timing and numerous factors in the soil environment. Therefore, results with biological control agents may vary from application to application.

The assessment of the efficacy of a biological control agent is difficult since biological control agents often reduce the level of disease rather than completely preventing it (Lindow and Wilson, 1999). Quantitative assays, therefore, are required to properly assess biological control efficacy. For those diseases that are characterized by discrete lesions, the disease severity can be assessed by counting the lesions on a given plant or plant part, but this also is not accurate since visual estimates are often unreliable, being greatly influenced by the observer and by the size, shape, and number of lesions on a leaf (Lindow and Wilson, 1999). For these reasons, it is important that pictorial reference guides are used for comparative purposes. Because of these shortcomings, in this study assessment of disease severity in treated plants as an indication of the efficacy of potential biological control agent, was based on percentage emergence and shoot dry weight rather than on the unreliable, purely visual methods often employed.

The aim of this study was to isolate and screen rhizosphere competent endospore formers, as plant growth promoters and / or biological control agents of selected soil-borne plant pathogens. Screening was done in *in vitro*, greenhouse and field trials. The aim of the *in vitro* trial was to isolate antibiotic producing *Bacillus* sp. Selected isolates were used in greenhouse trials to evaluate their ability to control *R. solani* in different rhizospheres. The

best performing isolates were then tested in field trials to check their rhizosphere competency.

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CHAPTER 2

ISOLATION AND *IN VITRO* SCREENING OF *BACILLUS* ISOLATES AS BIOLOGICAL CONTROL AGENTS OF SELECTED SOIL BORNE PLANT PATHOGENS

Of 130 *Bacillus* isolates recovered from the rhizosphere of different crops and screened *in vitro* for antimicrobial activity, 87 inhibited growth of at least one of the following test organisms: *Rhizoctonia solani*, *Pythium* sp., *Phytophthora cinnamoni*, *Fusarium* sp., and single representatives of Gram negative and Gram positive bacteria, namely, *Erwinia carotovora* and *Staphylococcus aureus* respectively. Isolates B77, B81 and B69 inhibited all test organisms, indicating that they are capable of producing broad-spectrum antimicrobial compounds or more than one active compound. Of the isolates that showed antimicrobial activity 78 of them did not inhibit *Trichoderma harzianum* K D, a registered biological control agent, which creates the potential for applying mixtures of *Trichoderma harzianum* K D and *Bacillus* isolate for improved biological control.

2.1 INTRODUCTION

Biological control involves the control of plant pathogens using biomolecules or microorganisms (Curl, 1986). Microorganisms that can be used as biological control agents against soil-borne plant pathogens are ideally those that are found in the rhizosphere (Skinner and Carr, 1976). The rhizosphere is a narrow zone of soil adjacent to living roots, which is subject to the influence of root exudates that have a direct effect on microbial activity. Rhizosphere-associated microorganisms in turn play a significant role in plant growth and development (Curl, 1986).

Plant growth promoting rhizobacteria (PGPR's) have been shown to promote plant growth by mechanisms such as phytohormone production (e.g. indole-3-acetic acid) (Srinivasan *et al.*, 1996), mineral solubilization (Kumar and Narula, 1999) and disease suppression (Kim *et al.*, 1997; Duffy, 2000). It has been hypothesized that rhizosphere microorganisms that

produce antimicrobial substances act as a first line of defense to roots against attack by soil borne pathogens (De Freitas *et al.*, 1997).

De Freitas *et al.* (1997) found that the most common bacteria associated with roots of field crops are *Bacillus* species, which made up 34% of the total microbial population in the rhizosphere whereas pseudomonads, contributed only 17%. Representatives of the genus *Bacillus* are appealing candidates for biological control of plant pathogens because they produce endospores, which enable them to survive under environmentally stressful conditions such as droughts, pH changes, and high temperatures (Prescott *et al.*, 1993). In addition, endospore-forming bacteria inherently have a longer shelf life than non-endospore forming bacteria, which is favorable for commercialization of a biocontrol product (Adejumo *et al.*, 1999). For example, *Bacillus subtilis* strain GB03 is sold as a treatment to protect crops from *Fusarium* sp. and *Rhizoctonia* sp. and also as a plant growth stimulant under the brand name Kodiak (Emmert and Handelsman, 1999).

Many *Bacillus* sp. are known to produce antimicrobial substances *in vitro* (Dijksterhuis *et al.*, 1999). *Bacillus cereus* UW85 produces two fungistatic antibiotics, Zwittermicin A and Antibiotic B, which have been shown to inhibit the growth of fungal pathogens such as *Phytophthora medicaginis*, the causal organism of damping–off in alfalfa (Silo-suh *et al.*, 1994). Zwittermicin A and Antibiotic B interfere with germination and/or germ tube elongation of fungal spores (Silo-suh *et al.*, 1994). Another example is *Paenibacillus polymyxa,* which has shown antimicrobial activity both *in vitro* and *in vivo*. It produces compounds such as polymyxin and fusaricidin A as well as cell wall degrading enzymes such as chitinases and glucanases. These compounds damage the hyphae and microconidia of the pathogen *Fusarium oxysporium* (Dijksterhuis *et al.*, 1999).

This study was undertaken to isolate pure culture of rhizosphere competent *Bacillus* species and screen them *in vitro* for antimicrobial activity against selected fungal plant pathogens. *Bacillus* isolates were obtained from the roots and rhizosphere soil of a range of crops and vegetables and subjected to *in vitro* bioassays against *Rhizoctonia solani, Pythium* sp.,

Phytophthora cinnamoni, Fusarium sp., and representatives of Gram negative and Gram positive bacteria, namely, *Erwinia carotovora* and *Staphylococcus aureus* respectively.

2.2 MATERIALS AND METHODS

Root material and associated rhizosphere soil were collected from various sites in KwaZulu-Natal, RSA. The crops and vegetables that were sampled included Swiss chard, green pepper, potato, cabbage, carrot, tomato, beetroot, pumpkin, sweet potato, which are vegetables and maize, papaya, and beans, which are crops.

2.2.1 Method for isolation of aerobic endospore formers

Root samples were harvested by carefully pulling the plants from the soil and shaking off the excess soil. The root material was then placed in plastic bags and stored at 4°C until further processing. Approximately 1g of rhizosphere soil / root sample was suspended in 99ml of sterile 1/4 strength Ringer's solution and shaken vigorously for 2 minutes. The suspension was then heat treated at 80°C for 15 minutes to destroy vegetative cells. A 10^{-6} dilution of the suspension was made and 0.1 ml plated out on duplicate plates of Tryptic Soy Agar (TSA) using the spread plate technique. The agar plates were incubated for three days at 25°C, after which representative colonies were arbitrarily selected and streaked onto fresh TSA plates to obtain single colonies. Sub-cultures were made from the resulting colonies on 10% (w/v) TSA agar slants and after incubation at 25°C for 48 hrs, they were stored at 4°C for future use. Isolates were stored in this manner for a maximum of four months, after which they were subcultured onto the same medium. For long-term storage and preservation isolate suspensions were mixed with 15% (v/v) glycerol in a ratio 1:1 and stored in a MDF-U71V Sanyo ultra-low temperature freezer at –80°C. This method allowed for storage periods greater than two years. Gram staining procedures were employed to verify that the isolates were Gram-positive endospore formers.

2.2.2 Fungal bioassay

Fungal pathogens used as test organisms in the bioassays included *Rhizoctonia solani*, *Pythium* sp., *Fusarium* sp., and *Phytophthora cinnamoni*. These fungal isolates were sourced from virulent pathogenic strains isolated, cultured and maintained within the Discipline of Plant Pathology, University of Natal, Pietermaritzburg. The pathogens were first grown on TSA plates at 25°C for three days to check their ability to grow well on this medium. The dual-culture bioassay technique described by Adejumo *et al.* (1999) was employed. Square agar blocks (1x1 cm) were cut from colonized TSA plates and placed at the center of fresh TSA plates. Bacterial isolates were inoculated at three equidistant points on the peripheral region of the plate (Fig 2.1). Each bacterial isolate was tested in triplicate. The plates were then incubated at 25°C for seven days and viewed on a daily basis for signs of fungal inhibition.

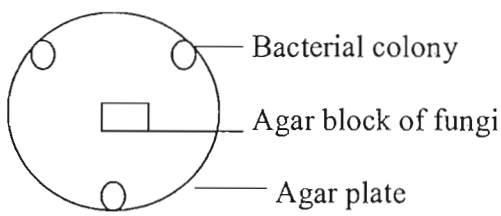

Figure 2.1 Line diagram of the fungal bioassay

Isolates that showed antimicrobial activity were then tested for inhibition against *Trichoderma harzianum* K D, a registered biological control agent, using the dual culture bioassay technique (Adejumo *et al.*, 1999) as outlined above.

2.2.3 Bacterial bioassays

A single representative of Gram positive and negative bacteria was used: viz. *Staphylococcus aureus* and *Erwinia carotovora* respectively. They were obtained from the culture collection maintained within the Discipline of Microbiology, University of Natal, RSA. Although *S. aureus* is not a plant pathogen, it was chosen as a representative Gram-positive bacterium for the bioassay. Both test organisms were streaked on TSA plates in

two well spacer parallel lines, covering three quarter of the plate. On the remaining quarters a *Bacillus* isolate was streaked perpendicular to the two test organisms (Figure 2.2) (It is important that the isolate does not touch the test bacteria). The plates were incubated at 25°C for seven days and viewed on a daily basis for signs of inhibition.

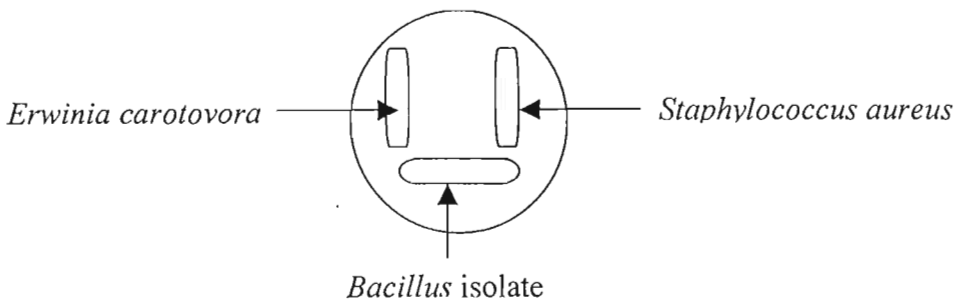

Figure 2.2 Line diagram of bacteria bioassay

2.3 RESULTS

A total of 130 *Bacillus* isolates were selected for further testing after heat treatment of the suspension. Table 2.1 details the numbers of isolates collected as well as their sources. Isolations were made from rhizosphere soil of a range of different plants taken from different locations so as to minimize the possibility of isolating the same strain of bacterium repeatedly.

Gram staining, confirmed that all the isolates were Gram-positive endospore formers. The size, shape and location of endospores within the cells differed between isolates. In some instance, endospores were positioned at the center of the cell and others were situated at the terminal end. The size and shape of the endospore were different. Some cells were distended and others were not.

Table 2.1 Number of *Bacillus* isolates obtained from the rhizosphere of various plants, their locations and designated code numbers.

Plant type	Number of isolates	Source	Isolate designation
Swiss chard	4	Lincoln Meade	B1-B4
Green pepper	5	Lincoln Meade	B5-9
Carrot	11	Lincoln Meade	B23-33
Maize	21	Edendale	B10-22andB123-130
Beet-root	14	Pinetown	B34-47
Papaya	23	Pinetown	B48-B70
Pumpkin	11	Scottsville	B71-B81
Beans	13	Edendale	B82-B94
Sweet-potato	4	Edendale	B95-98
Tomato	13	Edendale	B110-122
Cabbage	4	Lincoln Meade	B99-B102
Potato	7	Lincoln Meade	B103-B109

Examples of fungal inhibition are shown in Plate 2.1. Isolate B44 and B126 both show positive inhibition of *R. solani* whereas B65 showed no inhibition. Table 2.2 shows the percentage of isolates that inhibited each test organism. The number of isolates that inhibited more then one test organism is shown in Table 2.3.

Table 2.2 Percentage of isolates that inhibited each test microorganism

Test microorganisms	Number of isolates showing antimicrobial activity	Percentage of isolates showing antimicrobial activity
R. solani	44	33.8
Pythium sp.	43	33.1
Fusarium sp.	41	31.5
P. cinnamoni	31	23.9
S. aureus	19	14.6
E. carotovora	16	12.3

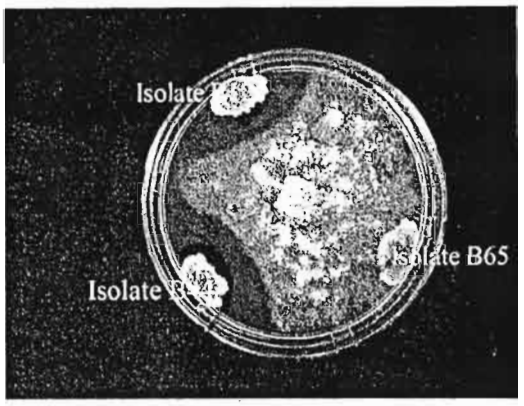

Plate1: Inhibition of *Rhizoctonia solani* by *Bacillus* isolates

Table 2.3 Number of isolates that inhibited one or more test organisms

Number of pathogens inhibited	Number of isolates
1	27
2	29
3	13
4	8
5	5
6	3

Eighty-seven out of the total of 130 isolates showed antimicrobial activity. Three isolates B77, B81, B69 inhibited all test organisms.

After employing the streak method to screen for bacterial inhibition 14.6 and 12.3% of the isolates inhibited *S. aureus* and *E. carotovora* respectively (Table 2.2). Examples of bacterial inhibition are shown in Plate 2.2 (a and b). Isolate B125 inhibited *E. carotovora*, but failed to inhibit *S. aureus,* whereas Isolate B126 inhibited both bacterial test organisms.

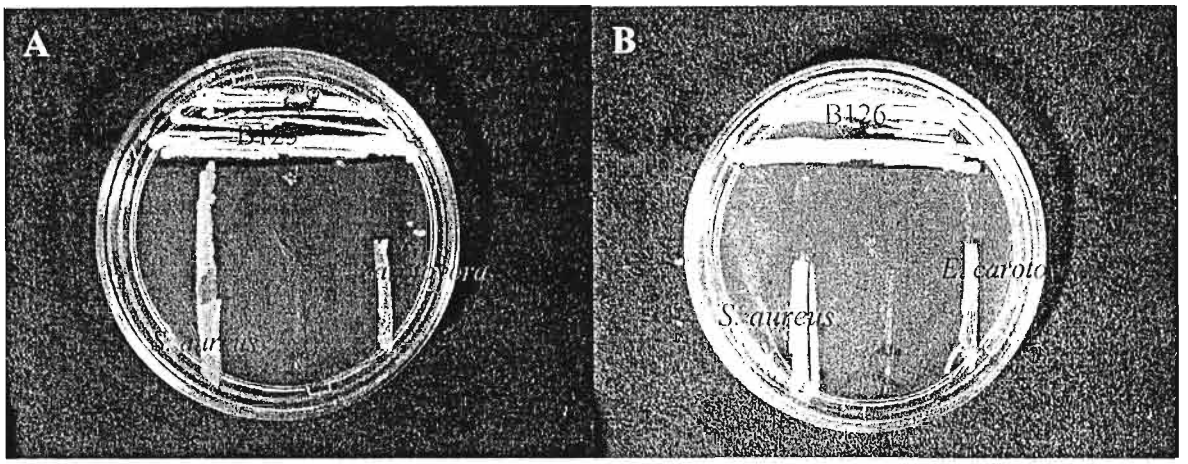

Plate 2.2a and b. Inhibition of both *S. aureus* and *E. carotovora* by Isolate B126.

Of the *Bacillus* isolates that showed antimicrobial activity 89.5% did not inhibit *Trichoderma harzianum* K D. (Plate2.3)

Plate 2.3 Inhibition of *T. harzianum* KD by Isolate B40, but not by Isolate B126.

Table 2.5 *In vitro* bioassay results

Isolate	*P. cinnamoni*	Fusarium sp.	*R. solani*	*Pythium* sp.	*E. carotovora* (Gram -)	*S. aureus* (Gram+)	*T. harzianum* KD
1	+	+	-	-	-	-	-
2	+	-	-	-	-	-	-
3	+	+	-	-	-	-	-
4	-	+	-	+	-	+	-
5	+	-	-	+	-	-	-
6	+	-	-	+	-	+	-
7	-	-	-	+	-	+	-
8	+	-	-	+	+	+	-
9	-	+	-	+	-	-	-
10	-	+	-	-	-	-	-
11	-	+	+	-	-	-	-
12	-	+	+	-	-	-	-
13	-	+	+	-	-	-	-
14	-	-	-	-	-	-	
15	-	+	+	-	+	+	-
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-
18	-	-	-	-	-	-	
19	-	-	-	-	-	-	
20	-	+	+	-	-	+	+
21	-	-	+	-	-	-	-
22	-	-	-	-	-	-	
23	-	-	-	+	-	-	+
24	-	-	-	+	-	-	+
25	+	-	-	+	-	-	-
26	-	-	-	+	-	-	+
27	-	+	-	-	-	-	-
28	+	-	-	+	-	-	-
29	-	-	-	+	-	-	-
30	-	-	-	+	-	-	-
31	+	-	-	+	-	-	-
32	-	-	-	+	-	-	
33	+	-	-	+	-	-	
34	-	-	-	-	-	-	
35	-	-	-	+	-	-	
36	-	-	-	-	-	-	
37	-	-	-	-	-	-	

Table 2.5 (continued) In vitro bioassay results

Isolate	*P. cinnamoni*	*Fusarium* sp.	*R. solani*	*Pythium* sp.	*E. carotovora* (Gram -)	*S. aureus* (Gram+)	*T. harzianum* K D
38	-	+	+	-	-	-	-
39	-	+	+	-	-	-	-
40	-	+	+	-	-	-	+
41	-	+	+	-	-	-	+
42	-	+	+	-	-	-	-
43	-	-	+	-	+	-	-
44	-	-	+	-	-	-	-
45	-	-	-	-	-	-	
46	-	+	+	+	-	+	-
47	-	+	+	-	-	-	-
48	-	+	+	+	-	-	-
49	-	-	-	-	-	-	
50	-	+	-	-	-	-	-
51	-	+	+	+	-	-	-
52	-	-	+	+	-	-	-
53	-	-	-	+	-	-	-
54	-	-	-	+	-	-	-
55	-	-	-	+	-	-	-
56	-	+	+	+	-	-	-
57	-	+	+	+	-	-	-
58	-	+	+	+	-	-	-
59	-	-	-	-	-	-	
60	-	+	-	+	-	-	-
61	+	+	+	-	-	-	-
62	-	+	-	+	-	-	-
63	-	+	-	+	-	-	-
64	-	-	-	-	-	-	
65	-	+	+	-	+	-	-
66	+	-	+	+	-	+	+
67	+	-	-	+	-	-	-
68	+	-	-	-	-	-	-
69	+	+	+	+	+	+	-
70	-	-	-	-	-	-	
71	-	-	-	-	-	-	
72	-	+	+	-	-	-	+
73	-	+	+	-	-	-	-
74	-	-	-	-	-	-	

Table 2.5 (continued) *In vitro* bioassay results

Isolate	*P. cinnamoni*	*Fusarium* sp.	*R. solani*	*Pythium* sp.	*E. carotovora* (Gram -)	*S. aureus* (Gram+)	*T. harzia* K
75	-	-	-	-	-	-	
76	-	-	-	-	-	-	
77	+	+	+	+	+	+	-
78	+	-	+	+	+	+	-
79	-	-	-	-	-	-	
80	+	+	+	-	+	-	-
81	+	+	+	+	+	+	-
82	+	-	-	-	+	+	-
83	-	-	-	-	-	-	
84	-	-	-	-	-	-	
85	-	-	-	-	-	-	
86	-	-	-	-	-	-	
87	+	-	+	+	+	+	-
88	+	-	+	+	-	-	-
89	+	-	-	-	-	-	-
90	-	-	+	+	-	-	-
91	-	-	+	-	-	-	-
92	-	-	-	-	-	-	
93	-	-	-	-	-	-	
94	-	-	-	-	-	-	
95	-	+	-	-	-	-	-
96	-	-	-	-	-	-	
97	-	-	-	-	-	-	
98	+	-	-	-	-	-	-
99	+	-	-	-	-	-	-
100	+	-	-	-	-	-	-
101	-	-	-	-	-	-	
102	-	-	-	-	-	-	
103	-	-	-	-	-	-	
104	-	-	+	-	-	-	-
105	-	-	+	-	-	-	-
106	-	-	-	-	-	-	
107	-	-	-	-	-	-	
108	-	-	-	-	-	-	
109	-	-	+	-	+	-	-
110	+	-	-	-	-	-	-
111	-	-	-	-	-	-	

Table 2.5 (Continued) ***In vitro*** **bioassay results**

Isolate	*P. cinnamoni*	*Fusarium* sp.	*R. solani*	*Pythium* sp.	*E. carotovora* (Gram -)	S. aureus (Gram+)	*T. harzianum* K D
112	+	-	+	+	+	+	-
113	-	-	-	-	-	-	
114	+	-	-	-	-	-	-
115	+	-	+	+	+	+	+
116	-	-	-	-	-	-	
117	-	-	-	-	-	-	
118	+	-	-	-	-	-	-
119	-	-	-	-	-	-	
120	-	-	+	+	-	+	+
121	-	-	-	-	-	-	
122	-	-	-	+	+	+	-
123	-	-	-	-	-	-	
124	-	-	-	-	-	-	
125	+	-	+	+	+	+	-
126	+	-	+	+	-	+	-
127	-	+	-	-	+	-	-
128	+	+	-	-	-	-	-
129	-	-	+	+	+	+	-
130	-	-	-	-	-	-	

2.4 DISCUSSION

Up to 66.9% of the isolates exhibited an ability to produce antimicrobial substances (Table2.5). This shows that *Bacillus* species have great potential for use as biological control agents. The ability of *Bacillus* species to produce spores also gives an advantage over other microorganisms in terms of formulations as biopesticides.

The results showed that *Bacillus* sp. were present in all the rhizosphere environments sampled in this study (Table 2.1). This confirms previous findings that *Bacillus* is a common resident of the rhizosphere of a wide range of plants (Priest, 1997). The difference in spore arrangement, colony morphology and colour indicate that a range of *Bacillus* sp./ Strains were isolated.

The results indicated that selected *Bacillus* isolates produced antimicrobial compounds active against a range of test organisms (Table 2.5). It is hypothesized that *Bacillus* evolved the ability to produce antibiotics as a mechanism to inhibit other microorganisms, which compete with them for nutrients (Silo-suh *et al.*, 1994). Antibiotics are produced as secondary metabolites during the stationary phase of growth but it also appears that in some *Bacillus* species the antibiotics are produced during the growth phase (Madigan *et al.*, 1997). Antibiotics can inhibit a wide range of organisms, including fungi and bacteria. Isolate B77, B81 and B69 confirmed this by inhibiting all test organisms. These isolates either produce a broad-spectrum antibiotic or more than one antimicrobial compound (Emmert and Handelsman, 1999). For instance, *Bacillus cereus* UW85 has been shown to produce two antibiotic compounds namely Zwittermicin A and an unidentified Antibiotic B (Silo-Suh *et al.*, 1994).

The *Bacillus* isolates tested appeared to produce different
antibiotics or variations within a single type or class of antibiotics. This was indicated by their ability to inhibit different test organisms i.e. fungi from different families, Gram-negative and Gram-positive bacteria (Table 2.5). Some antibiotics were antifungal whereas others were antibacterial. Some of the antifungal antibiotics were also different since the test fungi belong to different families, which mean they have different properties (Carlile and Watkinson, 1994). Some *Bacillus* species, such as *B. polymyxa*, produce both antifungal and antimicrobial antibiotics (Kleinkauf and von Dohren, 1997). *Bacillus* Isolates B77, B81 and B69 are classical examples of this since they inhibited all the test organisms, i.e., both bacteria and fungi.

The antibacterial activities were also different; selective inhibition of Gram-negative and Gram-positive genera was observed and in some cases both Gram positive and Gram negative organisms were inhibited. Kleinkauf and von Dohren (1997) showed that some *Bacillus* species produce antibiotics that are specific to Gram-negative bacteria only whereas others produce antibiotics that inhibit the growth of both Gram-negative and Gram-positive bacteria.

The possible cause of the difference in test organisms response might be the mode of action of the antibiotics being different or due to the inherent resistance of the test organism to certain types of antimicrobial compounds. For example, some antimicrobial compounds such as chitinases and glycanases attack only the cell wall of the organism. Such antimicrobial compounds cannot inhibit organisms, such as bacteria that do not have chitins or glycans in their cell wall (Dijksterhuis *et al.*, 1999). Other antimicrobial compounds such as Zwittermicin A and Antibiotic B interfere with germination and/or germ tube elongation of fungal spores (Silo-suh *et al.*, 1994).

A number of isolates failed to produce zones of inhibition. Possible explanations for this include:

1. The isolate might have produced antimicrobial agents that could not diffuse through the TSA agar. The isolates that exhibit this property possibly stopped the test organism from growing over it. On some plates the fungal pathogen did not grow over the colonies of the isolates but in other instances, colonies were overgrown.
2. The isolates might not have produced antimicrobial compounds under the prevailing conditions. This was evident on some plates where the fungal test organism grew over the colonies of the isolates.
3. The isolates might have produced antimicrobial agents, which can inhibit some fungi but fail to inhibit others (Table 2.5). This suggests that a range of antimicrobial agents were produced with varying activity spectra.

The results indicated that of the isolates that displayed antimicrobial activity, 89.7% did not inhibit *Trichoderma harzianum* K D. This is good from a biological control perspective, because it means that these isolates can be used together with *T. harziarium* K D, thus, enhancing biological control of the plant pathogens used in this study. A mixture of biological control agents may give rise to greater antagonism compared to a single biological control agent (Raupach and Kloepper, 1998). Raupach and Kloepper (1998) tested plant growth promoting rhizobacteria (PGPR) strains INR7 (*B. pumilus*), GB03 (*B. subtilis*), and ME1 (*Curtobacterium flaccumfaciens*) singly and in combinations for biological control against multiple cucumber pathogens. They found that a three-way

mixture of PGPR strains (INR7 + ME1 + GB03) as a seed treatment resulted in intensive plant growth promotion and disease reduction to a level statistically equivalent to the synthetic elicitor Actigard applied as a spray.

The positive *in vitro* tests on a number of *Bacillus* isolates gave clear direction for further testing in greenhouse trials and field trials to evaluate the selected *Bacillus* isolates as biological control agents.

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CHAPTER 3

BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* IN SELECTED CROPS BY SELECTED *BACILLUS* ISOLATES UNDER GREENHOUSE CONDITIONS

Nineteen selected *Bacillus* isolates that exhibited antifungal activity *in vitro* were tested under greenhouse conditions for the control of *Rhizoctonia solani* on wheat (*Triticum aestivum* L.), cabbage (*Brassica oleracea var. capitata* L.), tomato (*Lycopersicon esculentum* L.), maize (*Zea mays* L.) and cucumber (*Cucumis sativus* L.) seedlings. The bacteria were applied as seed treatments and the inoculated seeds were planted in *R. solani*-inoculated speedling trays. Shoot dry weights of the seedlings, measured six weeks after planting, indicated that 12 of 19 isolates caused significantly different shoot dry weight ($p \leq 0.05$) on wheat whereas all isolates tested in tomato and cucumber gave significantly different shoot dry weight compared to the negative control. No significantly different shoot dry weight results were recorded for *Bacillus* applied to maize and cabbage. Analysis of seedling emergence indicated that none of the *Bacillus* isolates gave significantly different emergence percentage on wheat, cabbage, tomato, and maize but all of them showed significantly different emergence percentage in cucumber. The results indicated that both the pathogen and the biological control agents exhibited specificity on each crop tested.

3.1 INTRODUCTION

Rhizoctonia solani causes root rots in many crops such as tomato, cucumber, and wheat (Ryder *et al.*, 1999; Duffy, 2000) and is also associated with pre- and post-emergence damping-off in cucumber, cabbage, tomato, and maize (Asaka and Shoda, 1996; Chuang *et al.*, 2001; Georgakopoulos *et al.*, 2002). Consequently there is a need for methods to control this pathogen.

Management of *R. solani* and other root diseases is largely dependant on cultural practices such as soil structure disturbance where the seed will be planted, use of wide row spacing

to allow rapid warming and drying of soil beneath the crop residue, and placing fertiliser under the seed at planting time (Cook *et al.*, 2002).

Root rots caused by *R. solani* have a major impact on plant growth; with yield reductions of up to 65% reported in cabbage (Klein-Gebbinck and Woods, 2002). This highlights the importance of its control by using reliable and environmentally safe control methods. Some *Bacillus* sp., e.g. *B. subtilis*, have shown suppressive responses to root rot disease complexes, and are known to enhance plant growth (Ali Siddiqui and Ehteshamul-Haque, 2001).

The aim of this study was to screen for *Bacillus* isolates that produce antimicrobial compounds to control *R. solani* in the rhizosphere environments of cabbage, maize, tomato, cucumber and wheat by investigating their effects on emergence percentage and shoot dry weight.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms

Based on their antimicrobial activity *in vitro* screening tests (Chapter 2), nineteen *Bacillus* isolates were selected for further testing in green house trials. A commercially available plant growth promoting rhizobacterium (PGPR) *Bacillus licheniformis*, obtained from K.S. Yobo[1], was used as a benchmark. All bacterial cultures were grown and maintained on Tryptic Soy Agar.

Tryptic Soy Broth (TSB) pure cultures of each isolate were established by tranfering one full loop of cells from a single colony into 4ml of sterile TSB and incubating at 30°C in a shaker (200rpm) overnight. The culture suspension was transferred into 100ml sterile TSB in a 500ml Erlenmeyer flask and cultured at 30°C for 72 hours in a water bath (GFL®) set

[1] K. S Yobo, Discipline of Plant Pathology, University of Natal, Pietermaritzburg, RSA

at 200rpm. Each culture was then centrifuged for 20 minutes in a Beckman J2-HS centrifuge at 10 000 rpm (17 700xg) using a JA10 rotor. The supernatant was discarded and the pellet re-suspended and washed in 100 ml autoclaved distilled water. The suspension was again centrifuged at 10 000 rpm (17 700 xg) for 20 minutes and the supernatant discarded. The resultant pellet was re-suspended in 100 ml sterile distilled water. A Carboxy-Methyl Cellulose (CMC) (2%w/v) suspension was added to each pellet to act as a sticker to attach the bacterial cells to the seeds.

3.2.2 Pathogen

Barley seeds were soaked in distilled water for 20 minutes and then autoclaved at 121°C for 15 minutes. They were then inoculated with plugs of *Rhizoctonia solani* (obtained from E. Ugoji)[2] that had been grown on V8 agar. The pathogen was allowed to colonize the grain for seven days at 23°C in an incubator prior to use in greenhouse trials.

3.2.3 Crops evaluated

Wheat (*Triticum aestivum* L.)

Cabbage (*Brassica oleracea var. capitata* L.) cv. Glory of Enkhuizen

Tomato (*Lycopersicon esculentum* L.) cv. Floradade

Maize (*Zea mays* L.)

Cucumber (*Cucumis sativus* L.) cv. Ashley

Since all seeds used had been chemically treated they were washed with distilled water prior to use. All seeds were obtained from McDonald Seeds[3].

3.2.4 Seed treatment and planting procedure

Before planting, seeds were dipped in a suspension of the *Bacillus* isolate under investigation in a 2%(w/v) CMC solution and then dried overnight at room temperature. Sixty-three Speedling24® trays per crop were prepared to allow for the 19 treatments and

[2] E. Ugoji, Discipline of Plant Pathology, University Of Natal, PMB, RSA

[3] McDonald Seeds, 61 Boshoff Street, Box 238, Pietermaritzburg, 3201, RSA

two controls to be repeated three times. Sixty trays were three quarters filled with pine-bark and a barley grain colonized by *R. solani* placed in each well. The inoculum was then covered with a thin layer of pine-bark. This procedure was used for all treatments except the three trays comprising the positive control where no infected barley seed was added. Seeds were placed on top of this layer and the wells filled to capacity by adding more pine-bark. The speedling trays were left for 24 hrs in the germination room before being placed in a greenhouse at 21-28°C for six weeks. Uncoated seeds grown without *R. solani* were used as positive controls (C). Uncoated seeds grown with *R. solani* were used as negative controls (CP).

Trays were placed in a randomised complete block design and were irrigated three times a day by microjet irrigation. The water used contained soluble fertilizer [3.1.3(38) complete] Ocean Agriculture[4] applied at a rate of $1g\,l^{-1}$ to give approximately 33 mg l^{-1} P, 100 mg l^{-1} K and 100 mg l^{-1} N.

3.2.5 Evaluation and statistical analysis

Emergence percentages were monitored by directly counting seedlings every two days for one week. After six weeks, seedlings were harvested; the root systems removed and shoot dry weight per tray measured after drying the shoots at 75°C for 48hrs. Results were analyzed using Statistical Analysis System (SAS) and Genstat, 5th edition (SAS, 1987).

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[4] Ocean Agriculture, P.O.Box 741 Mulders Drift 1747, RSA

3.3 RESULTS

The results for seedling emergence and shoot dry weight of wheat, cabbage, tomato, maize and cucumber are shown in Figures 3.1A, 3.1B, 3.2A and B, 3.3A and B, 3.4A and B, and 3.5A and B respectively.

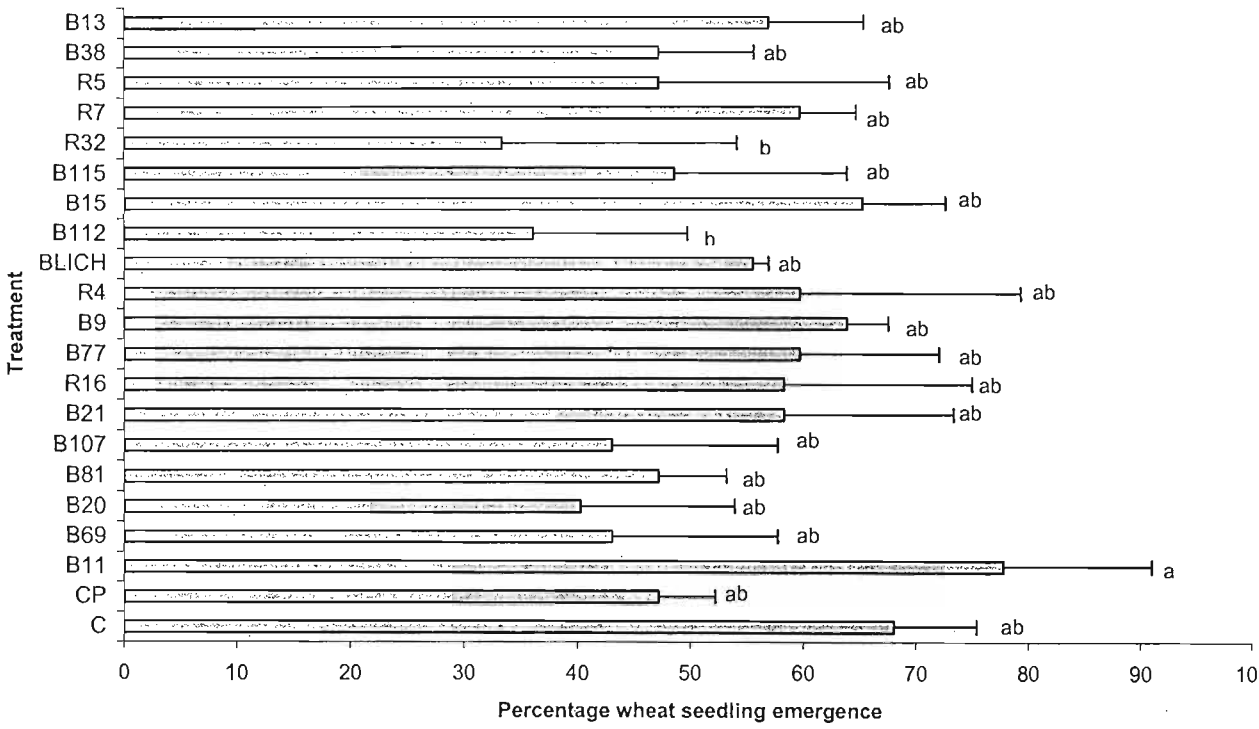

Figure 3.1A Percentage emergence of wheat seeds seven days after planting

C = positive control, where the pathogen was not applied

CP = Negative control, where the pathogen was applied

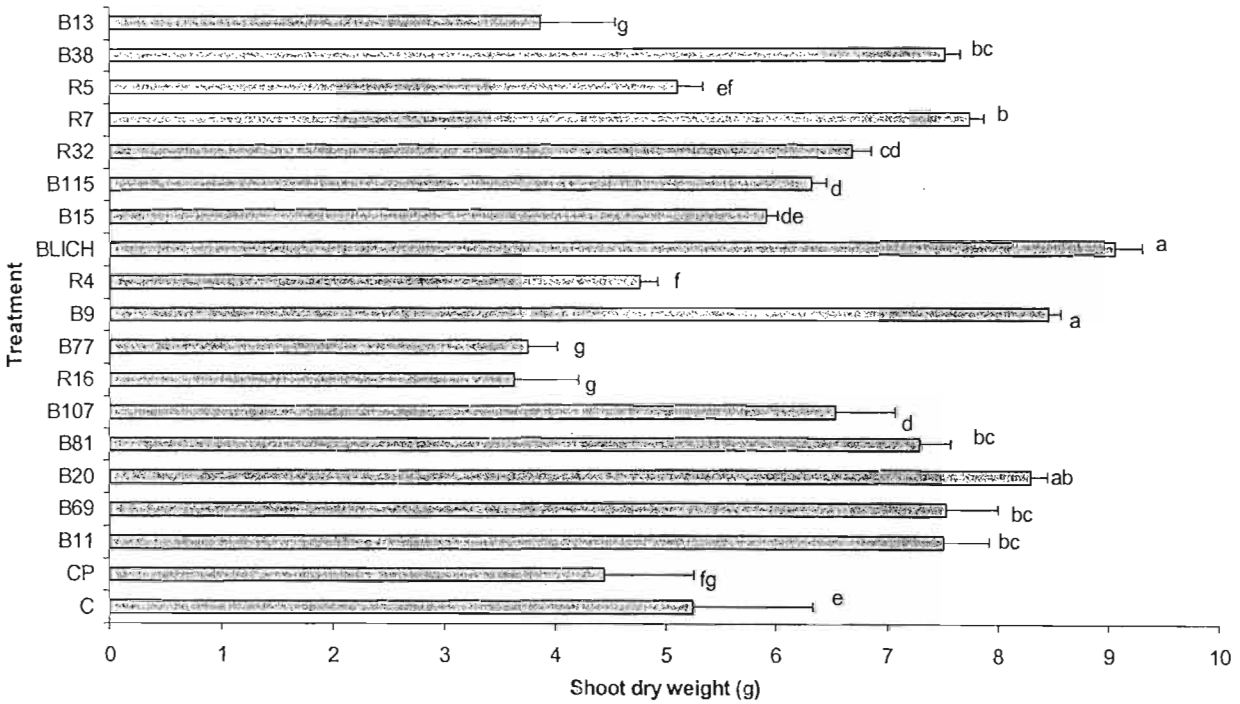

Figure 3.1B Shoot dry weight of wheat harvested six weeks after planting

CP = Negative control, where the pathogen was applied

C = Positive control, where the pathogen was not applied

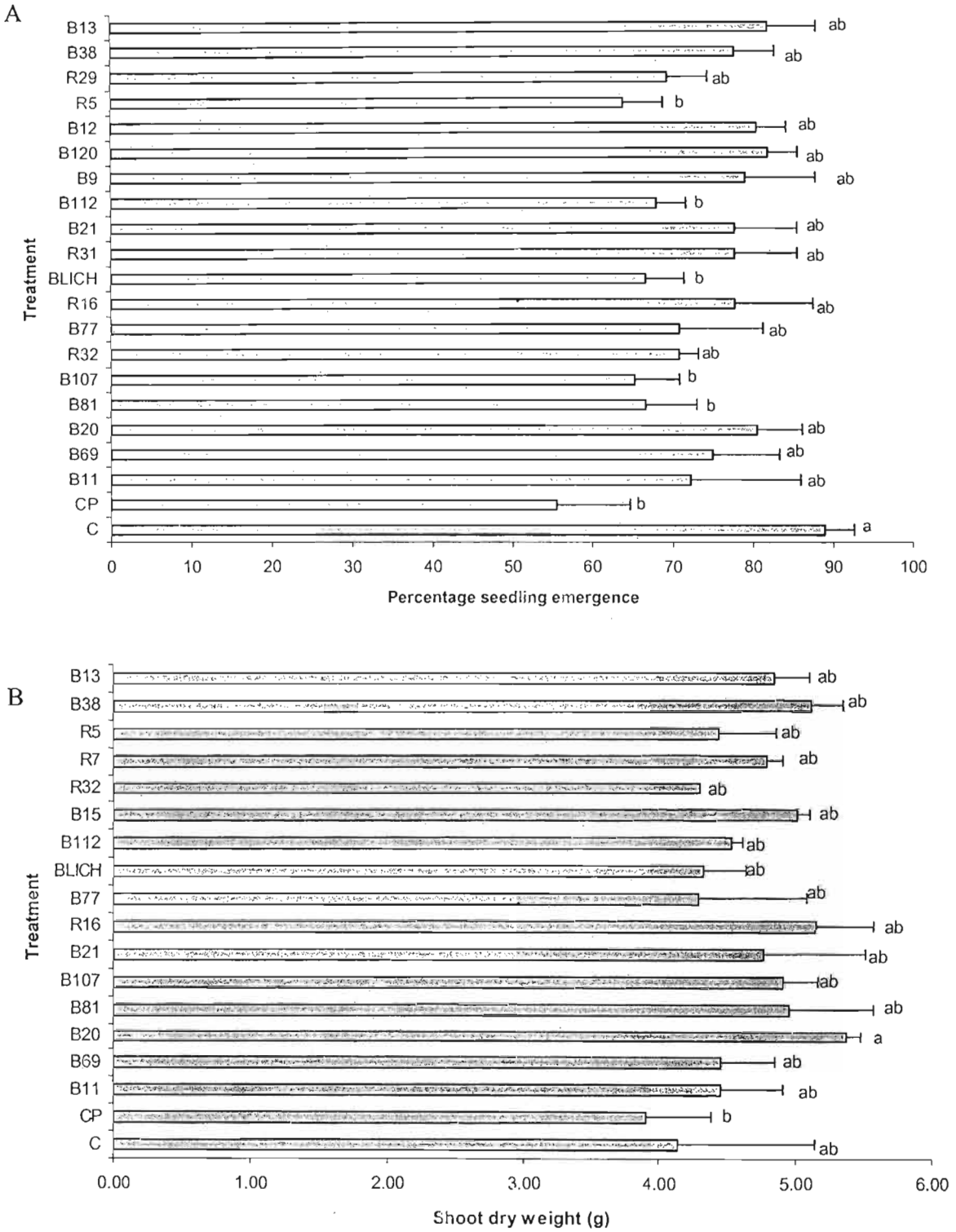

Figure 3.2A Percentage emergence and B. shoot dry weight of cabbage after seven days and six weeks respectively

CP = Negative control, where the pathogen was applied

C = Positive control, where the pathogen was not applied

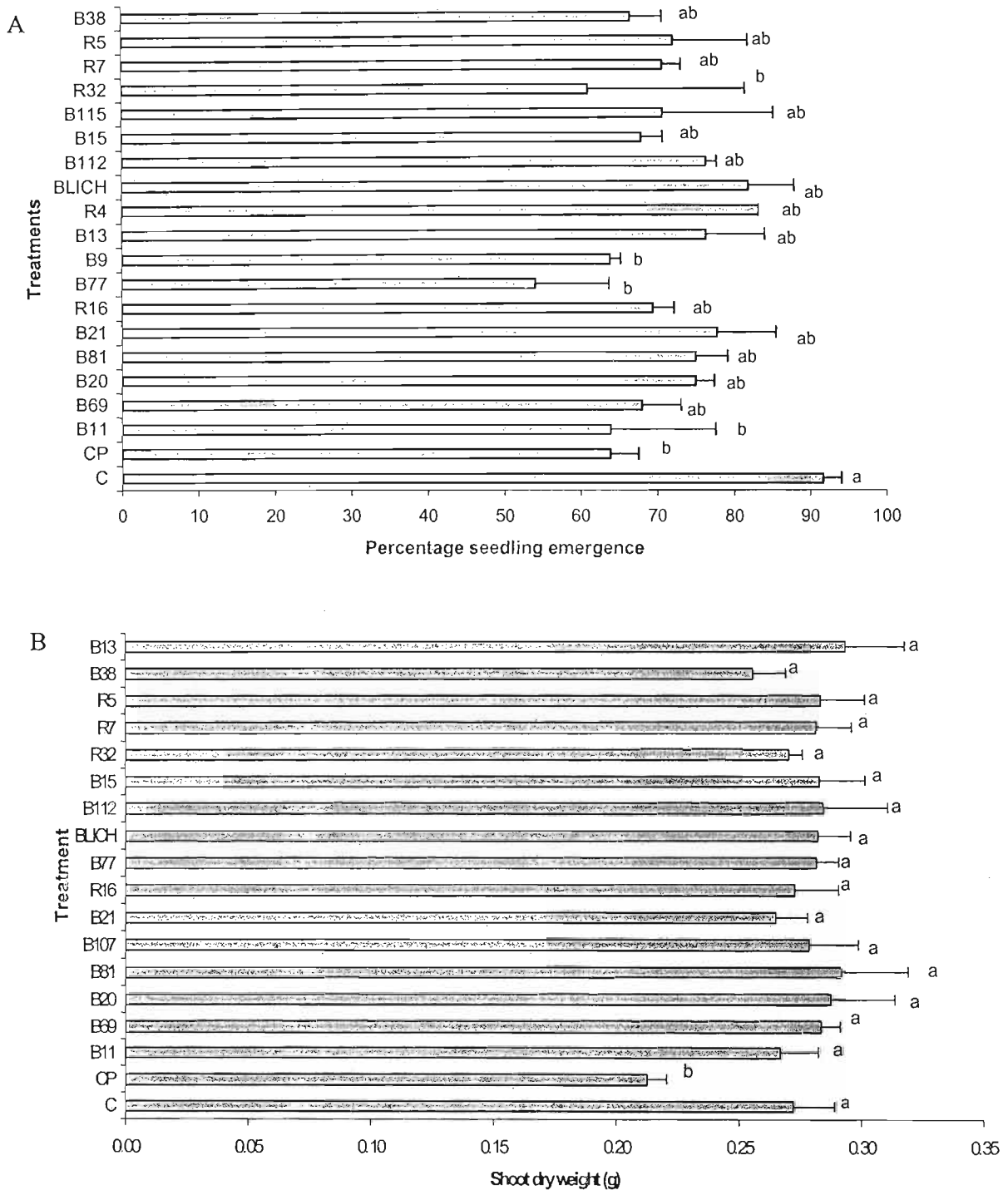

Figure 3.3A Percentage emergence and B. shoot dry weight of tomato after seven days and six weeks respectively

CP = Negative control, where the pathogen was applied

C = Positive control, where the pathogen was not applied

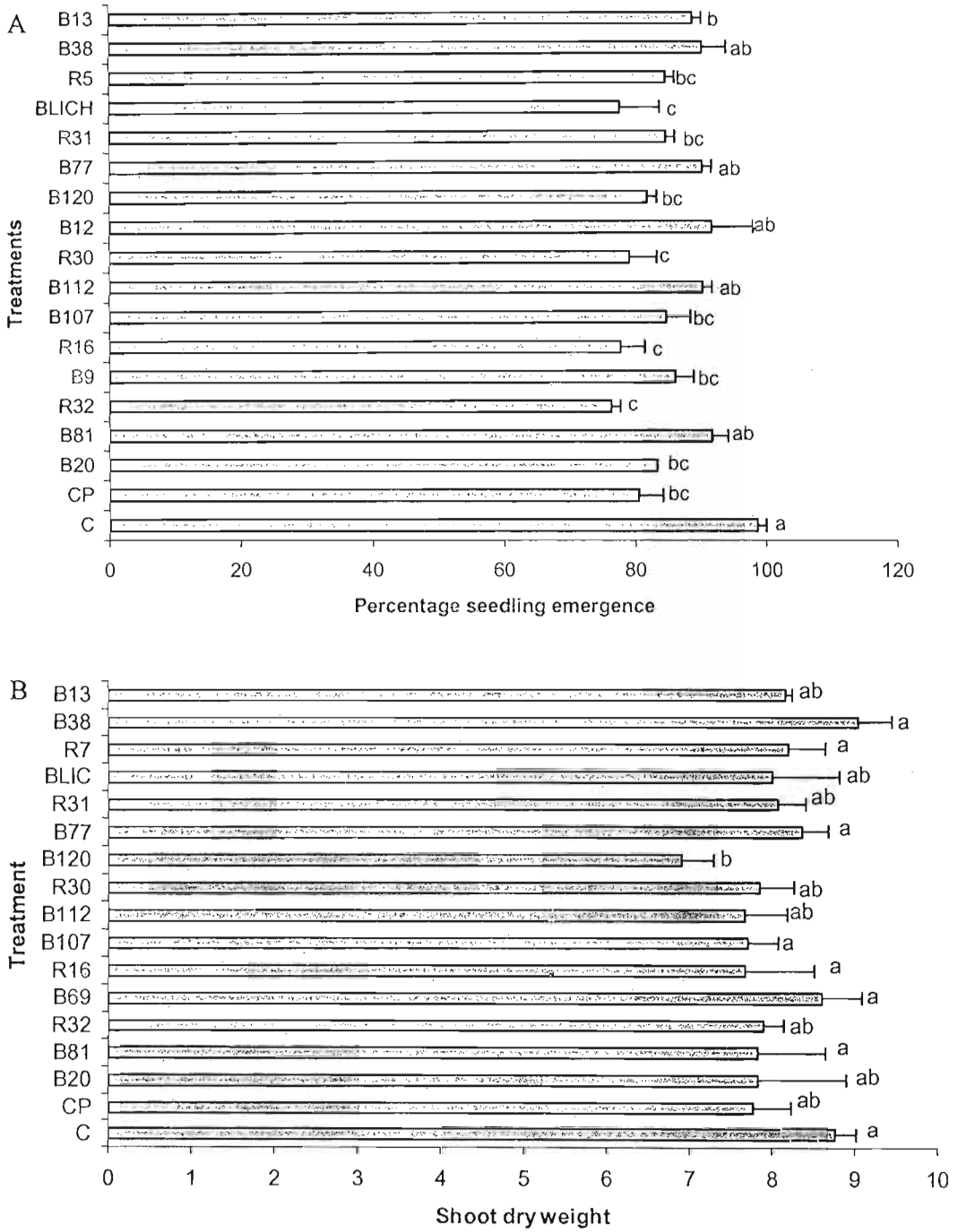

Figure 3.4A Percentage emergence and B. shoot dry weight of maize after seven days and six weeks respectively

CP= Negative control, where the pathogen was applied

C= Positive control, where the pathogen was not applied

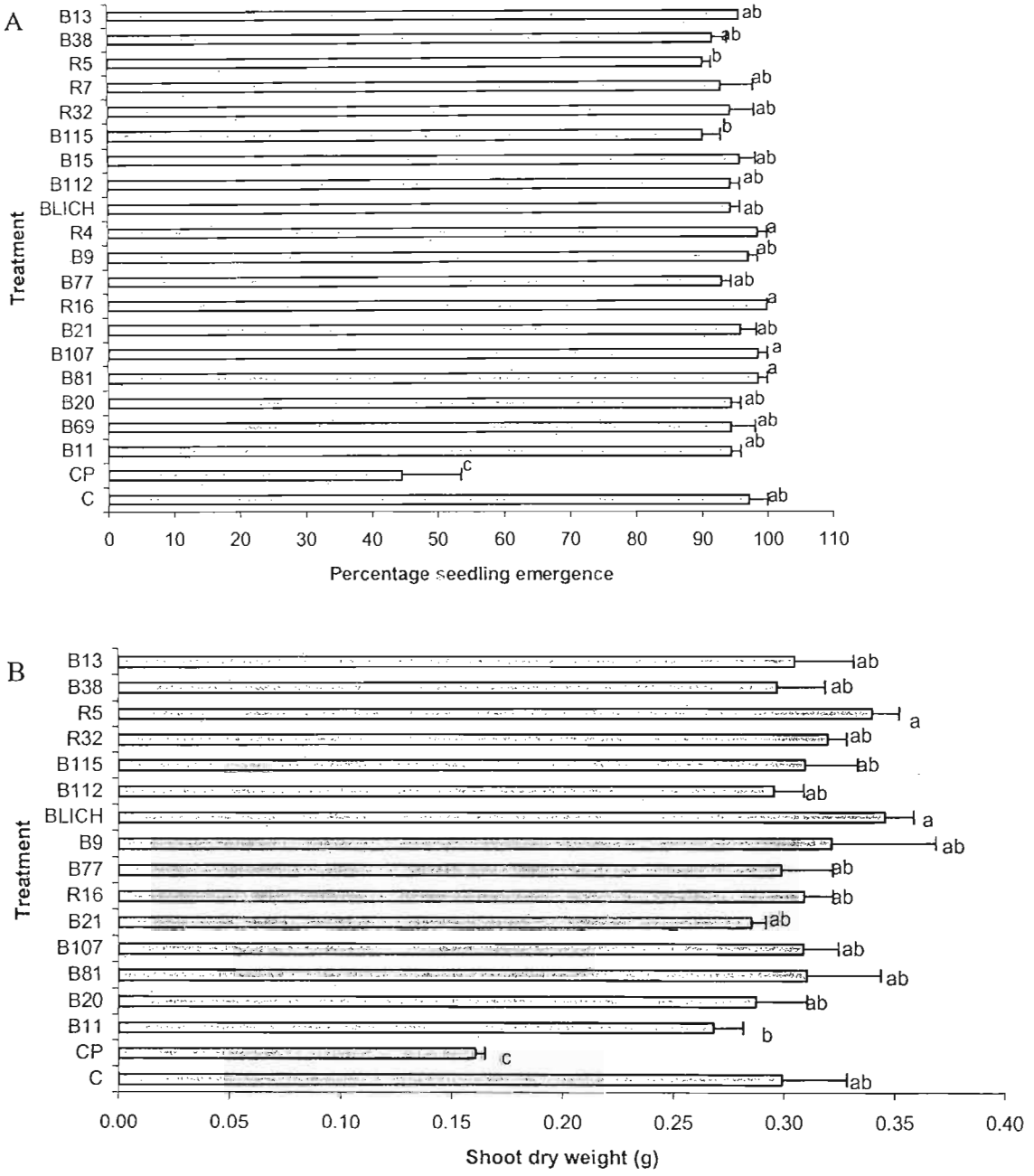

Figure 3.5A Percentage emergence and B. shoot dry weight of cucumber after seven days and six weeks respectively

CP = Negative control, where the pathogen was applied

C = Positive control, where the pathogen was not applied

3.3.1 Emergence

3.3.1.1 Wheat

Statistically there was no significant difference ($p \leq 0.05$) between the percentage emergence of both negative and positive controls (Fig.3.1A). None of the *Bacillus* isolates caused significantly different results compared to the negative control. *Bacillus* Isolate B11 caused significantly higher emergence percentage when compared to *Bacillus* Isolates B112 and R32. *Bacillus* Isolates B13, R7, B15, R4, B9, B77, R16, B21, and B11 caused higher emergence percentage than the negative control, although these findings were not considered significant. The other *Bacillus* Isolates B38, R5, B112, B107, B81, R32, B20, and B69 had lower percentage emergence than both the negative and positive controls, but the effect was also insignificant.

3.3.1.2 Cabbage

Statistically, the positive control was significantly different when compared to the negative control at $p \leq 0.05$ (Fig.3.2A). None of the *Bacillus* isolates caused significantly different percentage emergence when compared to the controls, although all of them caused higher percentage emergence than the negative control.

3.3.1.3 Tomato

According to the emergence percentage of tomato, there was a significant difference between the negative and positive controls (Fig.3.3A). When compared to the negative control, none of the *Bacillus* isolates had significantly different percentage emergence, although all of them caused a higher percentage emergence than the negative control with exception of *Bacillus* isolates B77 and R32.

3.3.1.4 Maize

Statistically, the percentage emergence in the maize positive control (C) was significantly higher than that of the negative control (CP) (Fig.3.4A). Compared to CP, *Bacillus* Isolates B20, B81, B9, B107, B112, B12, B120, B77, R31, R5, B38, and B13 resulted in higher percentage emergence but were not significantly different. The other *Bacillus* Isolates R30,

R16 and R32 as well as the benchmark caused lower emergence percentage when compared to CP, although the differences were also not significant at $p \leq 0.05$.

3.3.1.5 Cucumber

There was a highly significant difference ($P \leq 0.001$) between the negative and positive controls in the case of the percentage emergence of cucumber seeds (Fig. 3.5A). All *Bacillus* treatments caused significantly different results compared to the negative control but none of them was significantly different from the positive control

3.3.2 Shoot dry weight

3.3.2.1 Wheat

There was a significant difference between the negative control (CP) and the positive control (C), with regard to the shoot dry weight in wheat (Fig.3.1B). *Bacillus* Isolates B11, BLICH, B20, R7, B81, B107, B11, B38, R32, B115, B69, and B9 caused highly significant differences in shoot dry weight compared to both the negative and the positive controls. *Bacillus* Isolates R5 and R4 resulted in higher shoot dry weight than CP but these were not significant at $p \leq 0.05$. The other *Bacillus* Isolates B13, B77 and R16 caused lower shoot dry weight than CP but these were also not significant statistically.

3.3.2.2 Cabbage

Between the positive control (C) and the negative control (CP) there was no significant difference, although C had higher shoot dry weight (Fig.3.2B). The presence of all *Bacillus* isolates resulted in higher shoot dry weight than CP, although the shoot dry weight differences were not significant at $p \leq 0.05$ except for *Bacillus* Isolate B20.

3.3.2.3 Tomato

All trays inoculated with *Bacillus* isolates and the positive control (C) showed significantly higher shoot dry weight than the negative control (CP) (Fig.3.3B). Among all the *Bacillus* isolates and C, there were no significant differences.

3.3.2.4 Maize

The positive control (C) had higher shoot dry weight than the negative control (CP) but the difference was not significant at $p \leq 0.05$ (Fig3.4B). Although inoculation with some *Bacillus* isolates caused higher shoot dry weight than CP, the differences were not significant

3.3.2.5 Cucumber

There was a significant difference ($p \leq 0.05$) in shoot dry weight between the positive control (C) and the negative control (CP) (Fig.3.5B). Compared to CP, all *Bacillus* isolates caused significantly different shoot dry weight but they showed insignificant difference to the positive control (C). *Bacillus* isolates R5 and BLICH caused highly significant different shoot dry weight.

Table 3.1 *Bacillus* isolates that showed significantly different percentage emergence and / or shoot dry weight in one or more crops selected when compared to the negative control.

Isolates	Wheat		Cabbage		Tomato		Maize		Cucumber	
	E %	SDW	E %	SDW	E %	SDW	E %	SDW	E %	SDW
B11	NS	**	NS	NS	NS	*	NS	NS	*	*
B69	NS	**	NS	NS	NS	*	NS	NS		
B20	NS	**	NS	NS	NS	*	NS	NS	*	*
B81	NS	**	NS	NS	NS	**	NS	NS	**	**
B107	NS	*	NS	NS	NS	**	NS	NS	**	**
B21	NS		NS	NS	NS	*	NS	NS	*	*
R16	NS	NS	NS	NS	NS	**	NS	NS	**	**
B77	NS	NS	NS	NS	NS	*	NS	NS	*	*
B9	NS	**	NS	NS	NS		NS	NS	*	*
R4	NS	NS	NS	NS	NS		NS	NS	**	
BLICH	NS	**	NS	NS	NS	*	NS	NS	*	*
B112	NS	NS	NS	NS	NS	*	NS	NS	*	*
B15	NS	*	NS	NS	NS	*	NS	NS	*	
B115	NS	*	NS	NS	NS	*	NS	NS	*	*
R32	NS	**	NS	NS	NS	*	NS	NS	*	*
R7	NS	*	NS	NS	NS	*	NS	NS	*	*
R5	NS	NS	NS	NS	NS	*	NS	NS	*	*
B38	NS	**	NS	NS	NS	*	NS	NS	*	*
B13	NS	NS	NS	NS	NS	*	NS	NS	*	*

Key :

NS = Not significant * = Significant ** = Highly significant

E% = Emergence percentage SDW = Shoot dry weight

None of the *Bacillus* isolates promoted the emergence of wheat, cabbage, tomato and maize (Table 3.1). All *Bacillus* isolates tested promoted the emergence of cucumber seedlings and the shoot dry weight also confirmed that these *Bacillus* isolates inhibited *R. solani*. According to shoot dry weight measurements, only 12 out of 19 of these *Bacillus* isolates promoted growth of wheat seedlings (Table 3.1).

Table 3.2 Mean percentage differences of emergence and shoot dry weight of selected crops with reference to the positive control.

Treatment	Percentage difference of emergence					Percentage difference of dry weight				
	Wh	Ca	To	Ma	Cu	Wh	Ca	To	Ma	Cu
C	100	100	100	100	100	100	100	100	100	100
CP	69.39	62.50	69.69	81.7	46	84.70	94.36	78.8	88.6	53.85
B11	114.29	81.25	69.69	NA	97	142.86	107.49	98.9	NA	89.63
B69	63.27	84.37	74.24	NA	97	143.30	107.41	105.1	98.1	NA
B20	59.18	90.62	81.82	84.5	97	158.03	129.63	106.5	89.3	96.1
B81	69.39	75.00	81.82	93	102	138.79	119.65	107.9	89.3	103.9
B107	63.27	73.44	NA	85.9	102	124.44	118.52	103.2	87.9	103.3
B21	85.71	87.50	84.85	NA	99	NA	115.14	98.15	NA	95.4
R16	85.71	87.50	75.75	78.9	103	69.14	124.07	101.11	87.5	103.5
B77	87.76	79.69	59.09	91.6	96	71.56	103.46	104.3	95.5	100
B9	93.88	89.06	69.69	87.3	100	161.14	NA	NA	NA	107.6
R4	87.76	NA	90.91	NA	102	90.86	NA	NA	NA	NA
BLICH	81.63	75.00	89.39	78.9	97	172.51	104.27	104.6	91.3	115.6
B112	53.06	76.56	83.33	91.6	97	NA	109.34	105.6	87.5	98.89
B15	95.92	NA	74.24	NA	99	112.64	121.1	104.8	NA	NA
B115	71.43	NA	77.27	NA	93	120.38	NA	NA	NA	103.6
R32	48.98	79.69	66.66	77.5	97	127.30	103.70	100.12	90.1	107.0
R7	87.76	NA	77.27	NA	96	147.49	115.62	104.20	93.6	NA
R5	69.39	71.87	78.79	85.95	93	97.40	107.25	104.9	NA	113.7
B38	69.39	87.50	72.72	91.6	95	143.175	123.67	94.7	103.2	99.3
B13	83.67	92.19	83.33	90.1	99	73.7143	117.07	108.6	93.2	102.0

Key: Wh = Wheat; Ca = Cabbage; To = Tomato; Ma = Maize; Cu = Cucumber

C = positive control; CP = Negative control; All other treatments are *Bacillus* isolates.

3.4 DISCUSSION

The *Bacillus* isolates used in the greenhouse trials were selected on the basis of thier antimicrobial activity shown *in vitro*. Some of them did not perform in the greenhouse as expected. It was expected that they would inhibit *Rhizoctonia solani* as they had during the *in vitro* trials. This shows that with some *Bacillus* isolates there is no correlation between their *in vitro* activity and their *in vivo* effects. This confirms the statement made by Knudsen *et al.* (1997) that "Sometimes there is a poor correlation between *in vitro* and *in vivo* performance of biological control agents". These authors related this to the inactivation of antimicrobial compounds such as antibiotics in soil. For example, it was discovered that *Talaromyces flavus* produce four different antibiotics *in vitro* but *in vivo*, in soil, these antibiotics could not be detected. Instead, glucose oxidase was released, which generated hydrogen peroxide from glucose to the detriment of the pathogen.

Based on percentage increase in shoot dry weight, the results indicated that most of the *Bacillus* isolates tested showed antimicrobial activity in the rhizosphere of tomato, wheat and cucumber whereas on cabbage and maize, none of them showed antimicrobial activity (Table 3.1). One of the causes of this might be the effect of root exudates. Root exudates have great influence on the survival and activity of microorganisms in the rhizosphere (Melissa *et al.*, 2001). If the isolates access appropriate exudates, then they grow faster, resulting in the supply of plant growth promoters becoming greater, if the isolate is a plant growth promoting rhizobacterium (PGPR). Different plants produce varying roots exudates, thereby having a significant impact on microbial composition and activity in the rhizosphere. The results indicate that tomato, wheat, and cucumber root exudates were able to support the establishment of certain *Bacillus* isolates enabling them to inhibit *R. solani*.

Highly significant shoot dry weight for *Bacillus* Isolate B81, B107 and R16 were obtained on both tomato and cucumber (Table 3.1). These isolates could be useful for application in hydroponics systems, nursery operations, or greenhouse productions.

In the wheat trial, the results obtained were the most varied, 12 of the 19 *Bacillus* isolates tested caused significantly different shoot dry weight compared to the negative control. Isolates B11, B69, B20, B81, B9, *B. licheniformis*, and B38 had caused highly significant difference shoot dry weights. The results were possibly varied because of the growth conditions: wheat grows best in colder conditions but in this trial it was grown at a temperature range of 21-28°C.

The results also showed that biocontrol activity was specific to certain crops (Table 3.1 and 3.2). A possible explanation for this is that antimicrobial compounds produced *in vitro* have been shown to be inactivated or complexed in soil and/or the rhizosphere by certain plant exudates (Leifert *et al.*, 1995). These authors found that the activities of antimicrobial substances in a soil environment are sensitive to pH, the presence of growth substrates and overall nutrient concentrations. In this study pH, growth substrate, and nutrient concentration were not tested for the inactivation of the antimicrobial substances produced by *Bacillus* isolates but they may have had an impact on the inactivation of the antimicrobial substances.

Plant host specificity might be one of the causes of the different results found with different crops treated with the same treatment (Table 3.2). Different plants produce different exudates with different chemical constituents (Chiarini *et al.*, 1994). These exudates promote different types of microorganisms since different microorganisms have different preferences for nutrients. Because of this, competitive ability in rhizosphere colonization is lower for some microorganisms and greater in others.

Some *Bacillus* isolates did not act as expected. It is possible that they were out-competed by other microorganisms since they were introduced to a new environment. They were isolated from soil but now they had to compete in composted pine bark. Microorganisms colonize different host rhizosphere differently. In one rhizosphere they can be good colonizers and in another they may fail completely to colonize because of competition and factors that influence it (Walker *et al.*, 2002).

Li *et al.* (2002) showed that some biological control agents associate with roots of different crops in different soils for long periods. They also found that some biological control agents produce metabolite(s) with broad-spectrum antibiotic activity. So these two statements suggest that the *Bacillus* isolates that were competent in three different rhizosphere may produce broad-spectrum antimicrobial agents, and that they persist in the rhizosphere.

It is also possible that the growing medium used was not appropriate for the long-term survival of these *Bacillus* isolates and the production of the antimicrobial agents. Different rhizosphere environments have soil particles with different surface charge properties. These charges can have a great effect on the mobility of the antimicrobial substances produce by the biological control agents (Daigle *et al.*, 2001). Some biological control agents can be effective in one rhizosphere and not in another (Foeldes *et al.*, 2000).

The evaluation parameters used in biological control trials must be chosen carefully. For example, in this study percentage emergence and shoot dry weight were chosen but it can be seen from the results that of the two, shoot dry weight was more useful. Emergence depends on the health of the seeds, availability of water, and the distance between the seed and the pathogen. The seed might emerge before the pathogen reaches it. Established seedlings often display increased resistance to pathogen infection (Agrios, 1997). In this case shoot dry weight was the more reliable parameter than the percentage emergence.

This study was done in a greenhouse environment, but it is also important to extend the same study to the field. This will provide better evidence of the competitive ability of the best *Bacillus* isolates and their ability to control *R. solani* in the field environment and on different crops.

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CHAPTER 4

IN VIVO FIELD-TESTING TO DETERMINE THE EFFICACY OF SELECTED *BACILLUS* ISOLATES IN IMPROVING CROP YIELD AND CONTROLLING *RHIZOCTONIA SOLANI* IN MAIZE AND GREEN BEANS

Bacillus isolates that inhibited *Rhizoctonia solani in vitro* and in greenhouse seedling trials were tested for their ability to improve crop yields and for control of the pathogen under field conditions. Green beans and maize seeds were coated with the selected *Bacillus* isolates and sown under field conditions. For each isolate, four replicate treatment plots were established with and without *R. solani* inoculum. Percentage emergence, survival to harvesting and yield of maize cobs and green bean pods were measured. It was observed for all parameters measured that the positive and the negative controls were not significantly different thereby, rendering the results for the study inconclusive. However, *Bacillus* Isolates B77, B11, R5 and R7 promoted increased yields in green bean pods and *Bacillus* Isolate B81 promoted increased maize yields as compared to both controls.

4.1 INTRODUCTION

Root rot is a major disease complex of a large range of crops, causing substantial economic losses (Abawi and Widmer, 2000). *Rhizoctonia solani* is considered one of the most significant root pathogens and has been implicated in reduction of both the yield and the quality of numerous crops (Ryder *et al.*, 1999). *R. solani* is distributed worldwide and has a wide host range including beans, maize, cucumber and many other crops (Agrios, 1997). It can cause different symptoms on the same host depending on the time of infection; these may include pre- and post-emergence damping-off, root rot, foliar blight or fruit rot (Lewis and Lumsden, 2001). *R. solani* causes root rot in bean seedlings by forming severe necrotic lesions on hypocotyls, causing cell disorganization and cell wall degradation (Jabaji *et al.*, 1999).

There is a dire need of effective control strategies for this pathogen since only limited success has been achieved using chemical fungicides which, furthermore, may lose their usefulness due to revised safety regulations, concern on target effects, and / or development of resistance in pathogen populations (Emmert and Handelsman, 1999). Biological control is increasingly gaining popularity as an alternative control measure. *Bacillus* isolates, as biological control agents may be effective in reducing damping-off of bean, tomato, cabbage, cucumber and maize seedlings under greenhouse conditions (Meena and Muthusamy, 1998; Chuang *et al.*, 2001; Lewis and Lumsden, 2001)

Emmert and Handelsman (1999) stated that a successful biological control agent must be able to interact with the pathogen, the plant and the microbial community in the rhizosphere without loosing its effectiveness.

In vitro bioassays (Chapter 2) and greenhouse studies (Chapter 3) have shown that it is possible to isolate, screen and select *Bacillus* isolates which exhibit activity against *R. solani*. In this study *Bacillus* isolates that were effective *in vitro* and showed a positive effect in the greenhouse environment were further tested in the field to evaluate their efficacy under these conditions.

One of the main considerations when undertaking field trials is to take into account the variability in microbial community structure when comparing potting media used in greenhouse trials to the field environment. It is also difficult to ensure that *R. solani* is the sole plant pathogen present during the course of the field trial. This makes the evaluation of the biological control agents in the field difficult since other pathogens can cause the same symptoms as *R. solani*. For example, *Fusarium*, *Pythium*, *Phytophthora* and *Aphanomyces* can also cause damping-off and root rot in many plants (Walker *et al.*, 1998; Mathivanan *et al.*, 2000; Cook *et al.*, 2002; Georgakopoulos *et al.*, 2002).

4. 2 MATERIALS AND METHODS

4.2.1 Maize field trial

4.2.1.1 Trial site

The trial was carried out at Ukulinga Research Farm, University of Natal, Pietermaritzburg, KwaZulu Natal, on well-drained, deep sandy-clay loams. Land preparation prior to planting, involved ploughing and disking, which was carried out in November 2001. Fertilisation with 2x50Kg of 2-3-2 fertilizer (120N: 80P: 60K) obtained from NCD AGRI[5] was done before planting. The overall trial site size was 90x10m, comprising 66 plots. Each plot was 2.5 x 3m with 5 rows 3m long, spaced 0.8m apart. One hundred and fifty plants per plot were used. A complete randomised block Design was employed

4.2.1.2 Culture Preparations

4.2.1.2.1 Microorganisms

Bacillus Isolates B11, B13, B77, B81, R5 and R7 were selected for the trial based on their performance in greenhouse trials. *Trichoderma harzianum KD* (TKD) was used as a benchmark biological control agent. Bacteria were cultured and maintained on Tryptic Soy agar (TSA) whereas the *T. harzianum* K D was grown on V8 agar.

Tryptic Soy Broth (TSB) cultures were established by inoculating one loopful of each *Bacillus* isolate, taken from a single colony on a master plate culture and introduced into 4ml of sterile TSB. The cultures were incubated at 30°C overnight in a shaker water bath (GFL®) set at 200rpm. These cultures were then inoculated into 100ml sterile TSB in a 500ml Erlenmeyer flask and cultured at 30°C for 72hours in a shaker water bath (GFL®) set at 200 rpm. Each culture was then centrifuged at 10 000 rpm (17 700xg) for 20 minutes in a Beckman J2-HS centrifuge, using a JA10 rotor. The supernatant was discarded and the pellet re-suspended and washed in 100 ml sterile distilled water. The suspension was then centrifuged at 10 000 rpm (17 700xg) for 20 minutes and the supernatant discarded. The resultant pellet was resuspended in 100 ml distilled water into which 2%(w/v) Carboxy-

[5] NCD AGRI, 241 Victoria Road, P O Box 378, Pietermaritzburg, 3200, RSA.

Methyl Cellulose (CMC) was dissolved to act as a sticker to coat bean and maize seeds with each test *Bacillus* isolate.

4.2.1.2.2 Pathogen

Barley seeds were soaked for 10 minutes in distilled water and autoclaved for 15 minutes. They were then inoculated with plugs of *R. solani* that had been grown on V8 agar. The pathogen was allowed to colonize the grains for seven days at 23°C in an incubator prior to use in the field trial.

4.2.1.3 Seed treatments and planting procedure

Untreated maize seeds PAN6701 obtained from Pannar Seeds[6] were used for the trial. Batches of seeds were coated with each *Bacillus* isolate (4.2.1.2.1) and planted on the 31st November 2001. Replicate treatment plots were established with and without *R. solani* inoculum. The experimental layout was a randomised complete block design consisting of 66 plots. Each plot consisted of five rows, 3m long and 0.8m apart. Twenty seeds were planted per row resulting in 100 seeds per plot and 5 600 seeds for the whole trial. Pathogen colonized grain was buried between the rows, 7.5cm away from the seeds on both sides of the planted seeds. After planting, $1Lha^{-1}$ of herbicide, Dual®S Gold 915 EC, was sprayed onto the field to control weeds. Emergence of germinated seedlings was monitored daily for 14 days, starting seven days after planting. The positive control (C) comprised of plots that were not inoculated with the pathogen whereas the negative control (CP) plots were inoculated with the pathogen and the seeds were not coated with *Bacillus* isolates. There were eight replicates per treatment, four of them had pathogen inoculum added and the remaining four were not contaminated.

[6] Pannar Seeds Pty (Ltd), P O Box 19, Greytown, 3250, RSA.

4.2.1.4 Harvesting procedure

In each plot, the cobs from only the center three rows were harvested after five months of plant growth. They were oven dried for 24 hours at 75°C to minimize the moisture content. They were then weighed and the results analyzed using Genstat 5.2 statistical analysis of variance (Anonymous, 1987).

4.2.2 Bean Field Trial

4.2.2.1 Trial site

The trial site was adjacent to the one used for the maize trial (4.2.1.1).

4.2.2.2 Culture Preparations

- **Microorganisms**

The cultures were prepared in the same manner as in the maize trial (4.2.1.2.1).

- **Pathogen**

The pathogens were prepared and applied in the same fashion as in the maize trial (4.2.1.2.2).

4.2.2.3 Seed treatments and planting procedure

Green bean seeds (Elangeni cultivar) obtained from Pro-seeds[7], were hand-planted on 16 February 2002. Thirty seeds were planted per row, resulting in 150 seeds per plot. The positive controls consisted of *Bacillus* untreated seeds planted in plots that were not inoculated with the pathogen. The negative controls consisted of *Bacillus* untreated seeds planted in pathogen-inoculated plots. There were eight replicates per treatment, four of them had *R. solani* colonized grain as an inoculum and the other four had no pathogen inoculated. Pathogen colonized grains were buried between the rows, 7.5cm away from the

[7] Pro-seeds cc., 45 Maud Avenue, Pietermaritzburg, 3200, RSA.

seeds. After planting, $1Lha^{-1}$ of herbicide, Dual®S Gold 915 EC was sprayed onto the field to control weeds. After planting Cypermethrin was sprayed at the recommended dose to control cutworms. This was repeated after two weeks. Full cycle irrigation was applied for three hours, three times a week. Emergence of seedlings was monitored daily for 14 days after planting. The results were analyzed using Genstat 5.2, analysis of variance.

4.2.2.4 Harvesting procedure

For each plot, only the center three rows were harvested. This was done on 20 May 2002. Only the pods were harvested. They were oven dried for 24 hours at 75°C to minimize the moisture content. The pods were then weighed and the results were analyzed, using Genstat 5.2, analysis of variance.

4.3 RESULTS

4.3.1 Maize trial

4.3.1.1 Percentage emergence

After 21 days emergence percentage showed no significant difference between the treatments at $p \leq 0.05$. Only *Bacillus* Isolate R5 and TKD had a mean emergence percentage lower than the negative control. In most plots that were inoculated with *Rhizoctonia solani* the emergence was better than in the uninoculated plots. The positive control was better than the negative control (Fig.4.1).

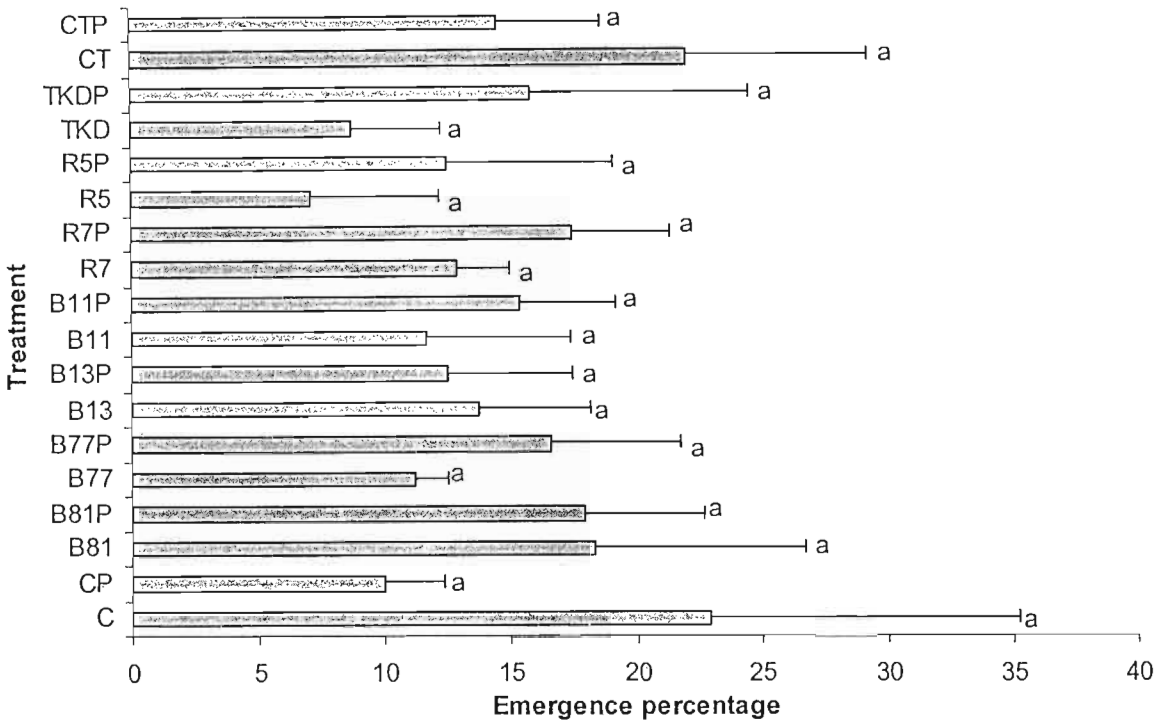

Figure 4. 1 Emergence percentage of maize seedlings 21 days after planting

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots; CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D); C (positive control); CP (negative control)

Table 4.1 Analysis of variance of emergence percentage of maize seedlings after 21 days

Source	Degrees of freedom	Sum of Squares	Mean Square	F pr
Rep stratum	3	362.4	120.8	
Treatments	17	1235.8	72.7	0.925 ns

ns = Not significant at P>0.05

l.s.d = 16.63

4.3.1.2 Percentage of maize plants surviving to harvest stage

There was no significant difference between the positive and the negative controls at $p \leq 0.415$, although the positive control had a higher percentage maize plants survive until the time of harvest than did the negative control. None of the other treatments were significantly better than the controls.

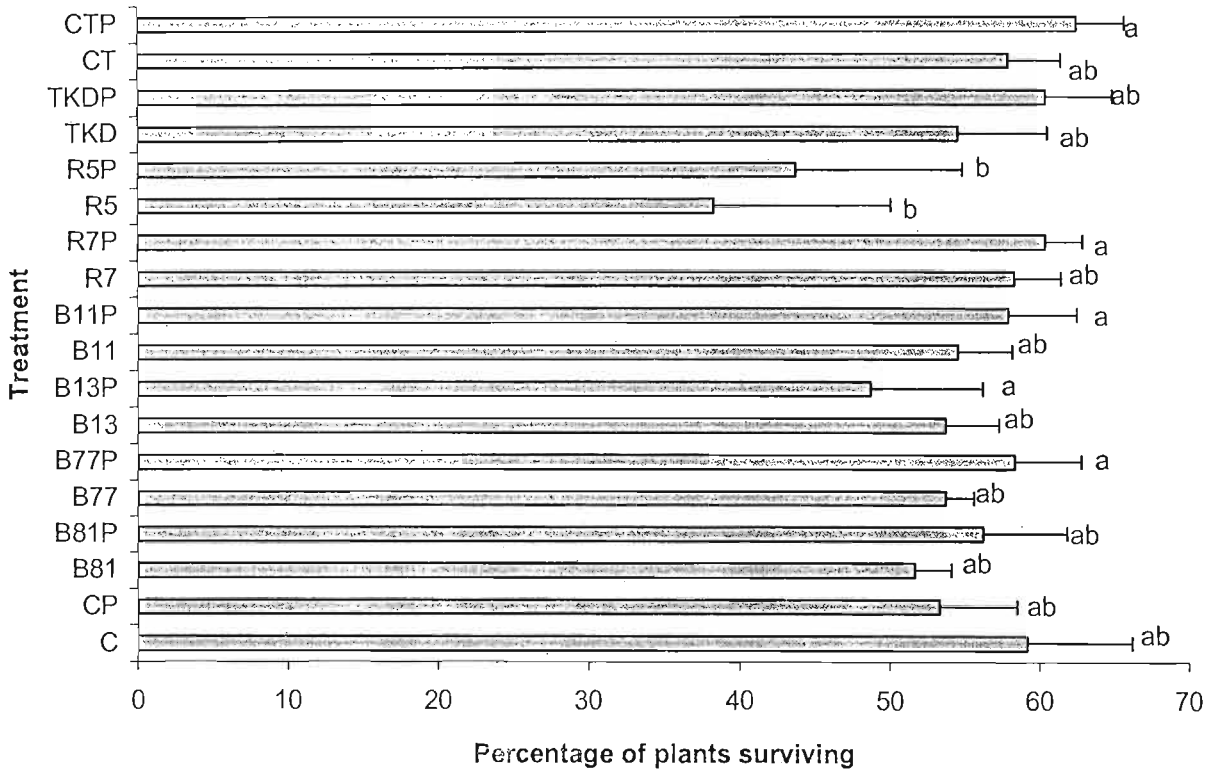

Figure 4. 2 Percentage of maize plants surviving at time of harvest.

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots;
CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D);
C (positive control); CP (negative control)

Table 4.2 Analysis of variance table of maize plant stand survival after 5 months

Source	Degrees of freedom	Sum of Squares	Mean Square	F pr
Rep stratum	3	9.7	3.2	
Treatments	17	2526.4	148.6	0.415ns

ns = Not significant at P>0.05 l.s.d = 16.80

4.3.1.3 Maize weight per plot

There was no significant difference between the negative (CP) and the positive control (C). *Bacillus* Isolate B81 was the only treatment that resulted in a higher weight when compared to the controls, although it was not significant at $p \leq 0.05$. It was significantly different to TKD, R5 and R7 but not to the other treatments.

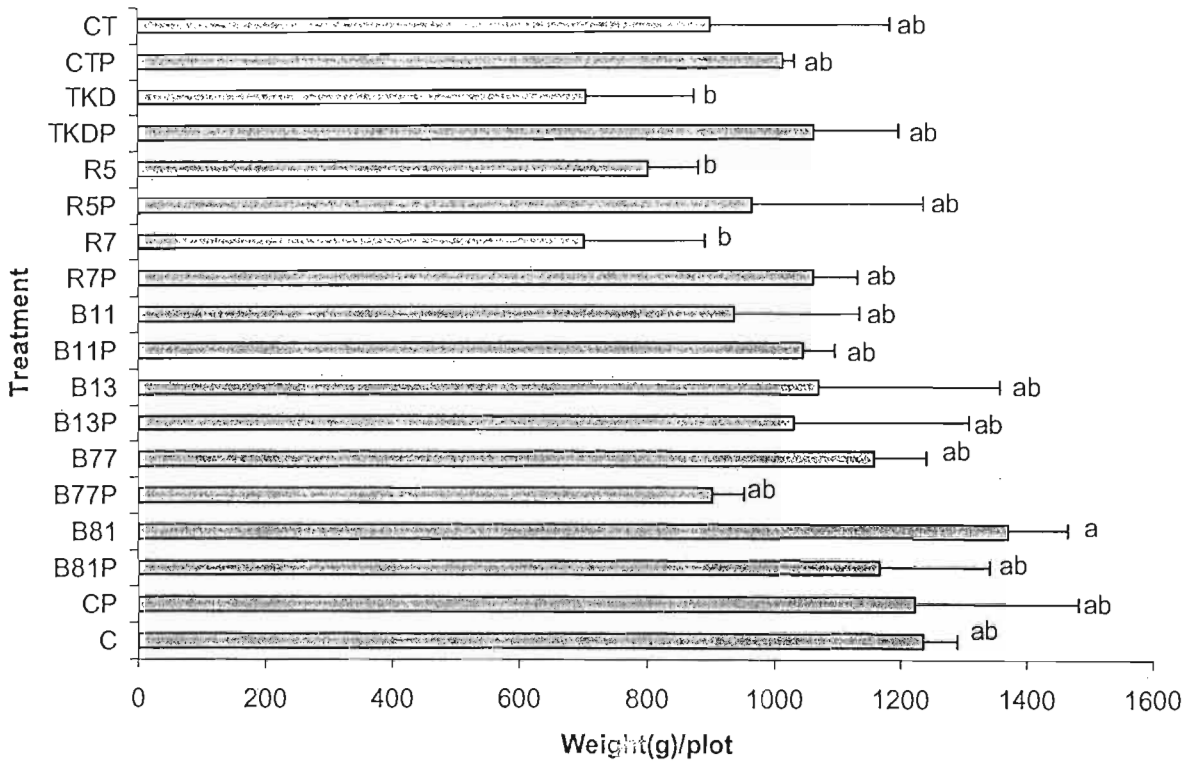

Figure 4. 3 Maize weight per plot harvested after four months

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots;

CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D);

C (positive control); CP (negative control)

Table 4.3 Analysis of variance table of maize weights harvested after five months

Source	Degree of freedom	Sum of Square	Mean Square	F pr
Rep stratum	3	301723	100574	
Treatments	17	2166821	127460	0.474 ns

ns = Not significant at P>0.05

l.s.d = 506.8

4.3.2 Green bean trial

4.3.2.1 Percentage emergence of green bean seedlings

There were no significant differences between the treatments except for *Bacillus* Isolate B77P, which had a significantly lower emergence percentage than the positive control (C). The negative control (CP) mean emergence percentage was lower than that of C and *Bacillus* Isolates R7P, B81P and B13P. Plots that were inoculated with the *Bacillus* isolates only had higher mean emergence percentages than those that were inoculated with both *Bacillus* isolates and the pathogen.

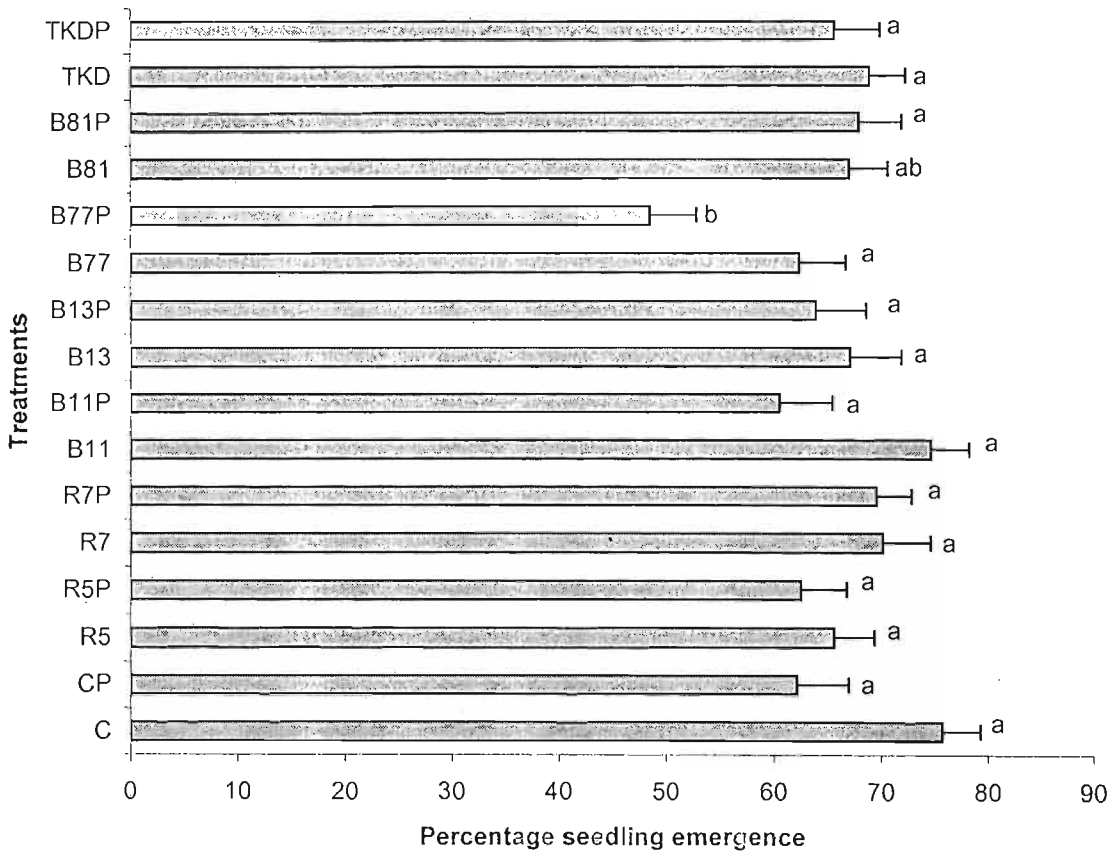

Figure 4. 5 Percentage of green bean seedlings emerged after 14 days.

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots; CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D); C (positive control); CP (negative control)

Table 4.4 Analysis of variance table of percentage of green bean seedlings emerged after 14 days of planting

Source	Degree of freedom	Sum of Square	Mean Square	F pr
Rep stratum	3	5149.4	1716.5	
Treatments	15	10467.0	697.8	0.039 *

* = Significant at P>0.05

l.s.d = 26.68

4.3.2.2 Percentage of bean plants Surviving to harvest stage.

There was no significant difference in number of plants surviving at harvest among the treatments except for *Bacillus* Isolates B77P and B11P which caused significantly lower survival percentage than the positive control (C), and *Bacillus* Isolate B13. The positive control showed a higher percentage of plant surviving at harvest than the negative control, although the difference was not significant at P>0.05.

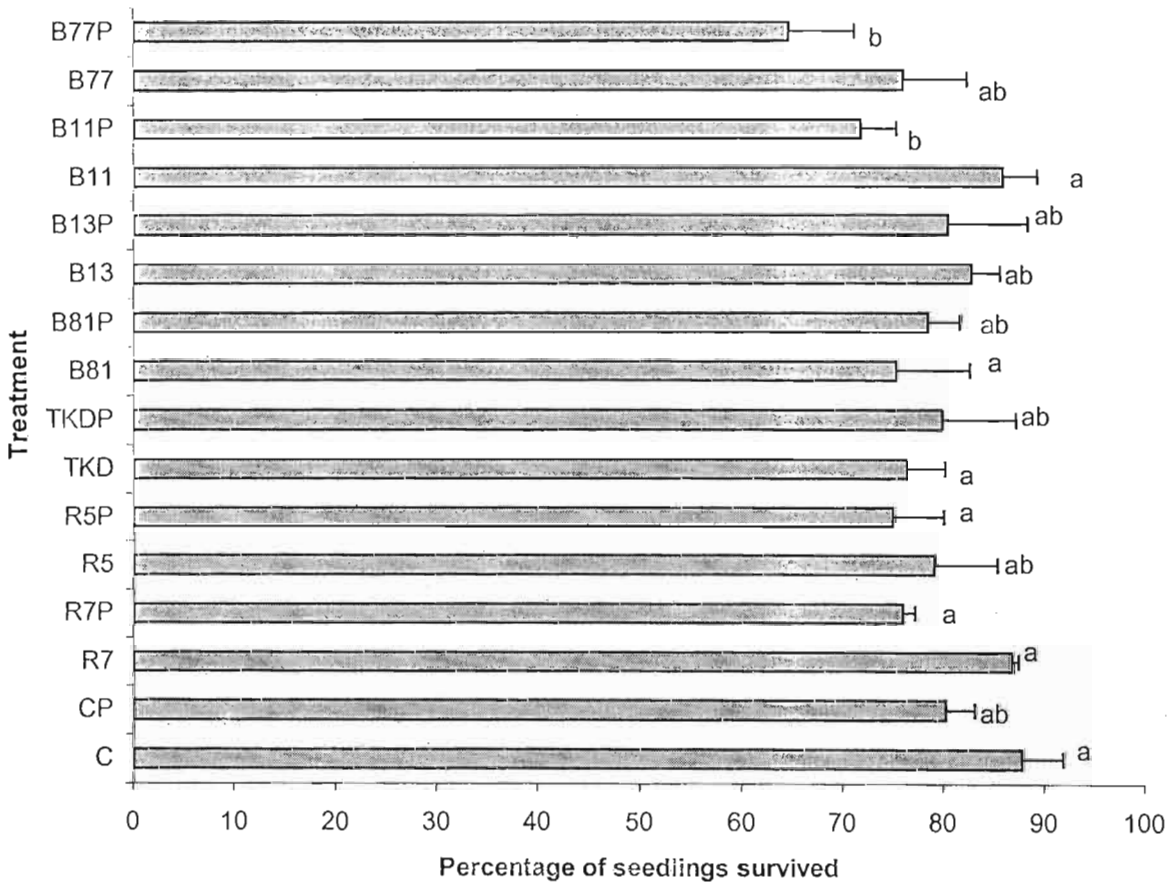

Figure 4. 6 Percentage of green bean seedlings survived until the harvest stage.

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots;
CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D);
C (positive control); CP (negative control)

Table 4.5 Analysis of variance of percentage green bean plants surviving at 3 months after planting

Source	Degrees of freedom	Sum of Squares	Mean Square	F pr
Rep stratum	3	227.2	75.7	
Treatments	15	2027.8	135.2	0.233 ns

ns = Not significant at P>0.05 l.s.d = 14.44

4.3.2.3 Pod weight of green beans per plot

Although the mean pod weight of the positive control (C) was greater than the negative control (CP), the difference was not significant. Compared to CP, *Bacillus* Isolates B81, B13, B11, R7 and R5 caused higher pods weight per plot but only R7 produced significantly different pods weight. All plots that were not inoculated with the pathogen had higher pod weights than the corresponding treatments that were inoculated with the pathogen except B81P and B11P.

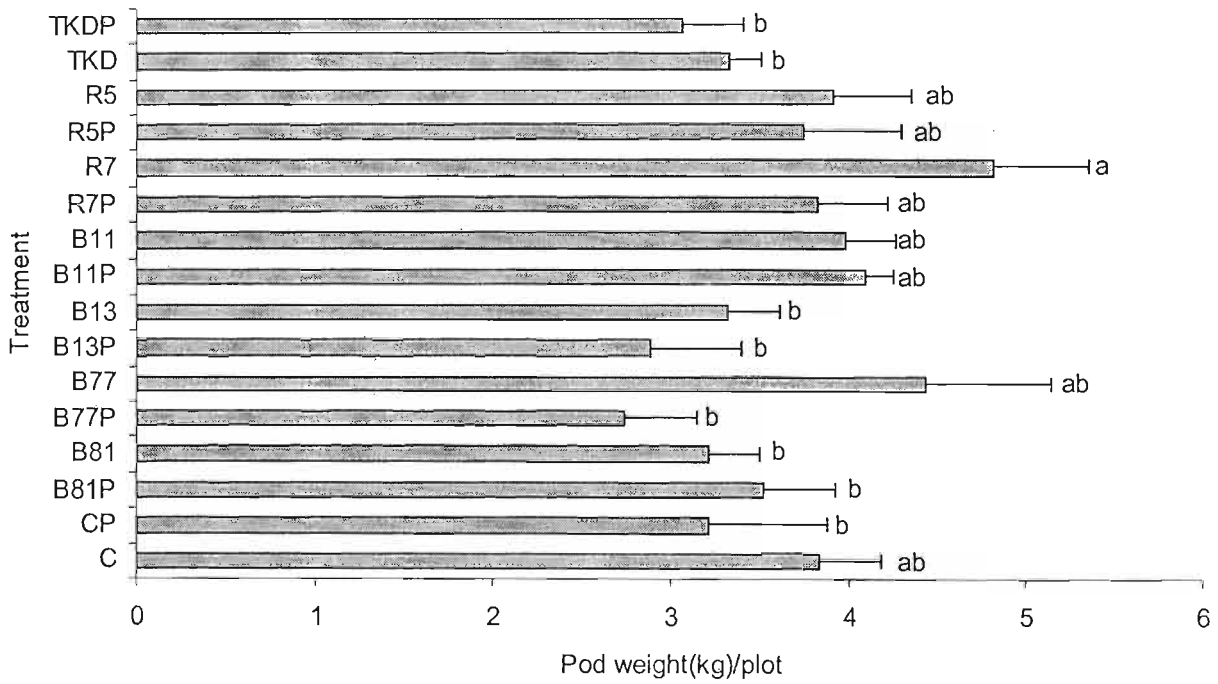

Figure 4. 8 Pod weights of green beans per plot harvested after 3 months

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots; CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D); C (positive control); CP (negative control)

Table 4.6 Analysis of variance of green bean pod weight per plot harvested 3 months after planting

Source	Degrees of freedom	Sum of Squares	Mean Square	F pr
Rep stratum	3	0.6208	0.2069	
Treatments	15	19.0258	1.2684	0.113ns

ns = Not significant at P>0.05

l.s.d = 1.269

Table 4.7 Yield percentages of green bean pods and maize cobs compared to the positive control.

Treatment	Pod yield percentage	Maize cob yield percentage
C	100.00	100
CP	83.61	98.92
B81P	91.74	94.38
B81	83.70	110.83
B77P	71.49	73.16
B77	115.50	93.80
B13P	75.22	83.47
B13	86.30	86.66
B11P	106.80	84.58
B11	103.72	75.88
R7P	99.56	85.99
R7	125.35	56.88
R5P	97.54	78.17
R5	101.96	65.09
TKDP	86.81	86.12
TKD	80.02	57.07

Key: All treatments with suffix 'P' were applied in pathogen-inoculated plots;
CT (chemically treated seeds); TKD (*Trichoderma harzianum* K D);
C (positive control); CP (negative control)

The pathogen did not infect maize and beans significantly so the assessment of biological control agents was inconclusive however, *Bacillus* Isolate B77, B11, R5 and R7 resulted in higher pod yield percentages and B81 resulted in higher maize yield percentage than all other treatments including the controls.

4.4 DISCUSSION

The results obtained for percentage emergence, percentage survival to harvest stage and the yield percentage for both maize and beans showed no significant difference between the positive and the negative controls. This suggests that the pathogen did not have the desired effect on the two crops used in this trial. A general trend observed, however, was that the positive control means were better than the negative control means in all experiments except for maize yield percentage.

A possible explanation for the observed lack of significant difference between the controls may be attributed to insufficient pathogen inoculum. Smith *et al.* (1997) reported that inoculum dosage is very important in the success of biological control agents. In this study the dosage levels of both the pathogen and the biological control isolates were not optimized and this possibly resulted in not obtaining a discernable response to the pathogen.

The inability of *Rhizoctonia solani* to infect crops in the field environment may also be caused by environmental stresses such as competition, change in pH, as well as the presence of compounds that may be deleterious to the pathogen (Paulitz, 2000; Estevez de Jensen *et al.*, 2002). In the greenhouse environment the conditions were well controlled whereas in the field environment the prevailing conditions were subject to uncontrolled fluctuations, e.g. temperature, availability of water, etc. This might have affected the effectiveness of both the pathogen and the biological control agent, resulting from a loss of pathogenicity or viability, respectively. This suggests that greenhouse conditions are more

suited to the growth and proliferations of fungal pathogens than in the natural soil environment.

Another explanation is that the seedlings had passed the susceptable stage before the pathogen, *Rhizoctonia solani*, reached them. The distance between the pathogen inoculum and the seed was greater in the field than in the greenhouse study. It is possible that the distance the pathogen had to travel to infect the seeds allowed the seeds to germinate and develop into healthy seedlings before the pathogen reached them. It has been reported that *R. solani* does not infect established plants or seedlings as aggressively as emerging seedlings (Agrios, 1997). Singer and Munns (1999) state that each soil has, depending on its colloid components, a particular retention capacity for a particular solute or substance. It is thus possible that the pathogen or the virulent factor produced by the pathogen took too long to reach the plant or seed. In the greenhouse trials pine bark was used as the growth medium and since this substance has a very low retention capacity because of its large pore spaces, the pathogen was more mobile than in the field soils.

Some soils are inherently suppressive to soil borne pathogens and inhibit the growth of the pathogen itself through competition and/or inhibition (Cook and Baker, 1996; Hyakumachi, 1999). Soilborne plant pathogens are affected and controlled by the activity of all other surrounding soil microorganisms. The interactions between them determine the survival, reproduction, pathogenic capacity, and fate of the pathogen (Yaacov, 2002). So in the present study *R. solani* might have been suppressed by the soil itself, which would explain why no significant differences between the positive and the negative controls were observed.

There are many strains of *R. solani* and these differ considerably in their virulence. This partially correlates with geographical origin (Pascual *et al.*, 2000). In this present study the lack of a significant difference between the positive and negative controls might have been due to the virulence status of *R. solani* strain used, i.e., it was not virulent enough to infect maize.

Application of *Bacillus* Isolates R7 and B77 resulted in greater pod yields than all the other treatments, including the controls. *Bacillus* Isolate B81 promoted greater maize yields. This indicates that *Bacillus* Isolates R7, B81, and B77 may have plant growth stimulation ability and are rhizosphere competent. The other *Bacillus* isolates tested did not perform as expected.

Another possible explanation for the failure of the *Bacillus* isolates to inhibit *R. solani* in the field is the inactivation of the antibiotics they produced, by other soil microorganisms in the rhizosphere (Cook and Baker, 1996). Even chemical compounds in the soil can react with the antibiotics and inactivate them, depending on the polarity of the antibiotic compound produced (Estevez de Jensen *et al.*, 2002).

For future field trials it is recommended that the dosage of both the pathogen and the biological control agent be optimized to obtain a discernable response as suggested by Smith *et al.*, (1997). Knowledge of the inter relationships between the host plant, pathogen, biological control agent and the environment, must be acquired before applying the biological control agent. This will help in understanding and deriving a procedure for applying the pathogen and the biological control agent properly.

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CHAPTER 5

GENERAL OVERVIEW

The inherent properties of *Bacillus* species led to their selection in this study as potential biological control agents. These properties include their ability to survive harsh conditions (Prescott *et al.*, 1993), rhizosphere competency (Brimecombe *et al.*, 2001), their ability to produce antibiotics (Mangenot and Diem, 1979) and the production of endospores, which can be used in formulations of biological control agents.

Bacillus sp. isolates were isolated in pure culture from different plant rhizospheres and were then tested *in vitro* for antimicrobial activity. The isolates that produced antimicrobial compounds *in vitro* were tested against *Rhizoctonia solani* in greenhouse trials with wheat, cabbage, tomato, maize, and cucumber as the crops invesigated. The isolates that maintained their ability to inhibit *R. solani* and promote plant growth were further evaluated under field conditions.

From *in vitro* bioassays it was found that 83 *Bacillus* isolates out of 130 exhibited antimicrobial activity against the test organisms. A range of antimicrobial activities was observed, suggesting that several different types of antimicrobial compounds were involved. The zones of inhibition varied in size, possibly indicating the production of different compounds at varying concentrations. For further study, characterization of these isolates and their associated antifungal compounds is recommended. This will result in a better understanding of each strain and will enable the optimization of conditions for maximizing production of the antimicrobial agent.

Both antifungal and antibacterial activities were observed. Of the 83 active *Bacillus* isolates, 61 produced antifungal compounds only whereas 22 produced a mixture of antifungal and antibacterial antibiotics. The antifungal compounds produced were possibly different because they exhibited different antifungal activity spectra. Similar findings were

observed for the antibacterial profiles, some isolates inhibited only Gram-negative bacteria whereas others inhibited both Gram-negative and Gram-positive bacteria. This may be the result of the different properties of the cell wall of Gram negative and Gram-positive bacteria. The findings from the *in vitro* study indicate that different strains of *Bacillus* were isolated, thus giving us a large pool of potential biological control agents to select from.

Three isolates, B77, B81 and B69, inhibited all the test organisms used in the *in vitro* bioassays. This indicates that they might produce broad-spectrum antibiotics (Emmert and Handelsman, 1999) or they might produce more than one antibiotic compound (Silo-Suh *et al.*, 1994). Identification and characterization of these antimicrobial compounds are recommended for further study so that this question can be answered.

Of the *Bacillus* isolates that exhibited antifungal activity, 89.7% did not inhibit *Trichoderma harzianum* K D, a registered biological control agent. The potential of using such isolates in conjunction with the registered biological control agent needs to be further investigated since mixtures of biological control agents can potentially give rise to greater plant protection compared to a single biological control agent (Raupach and Kloepper, 1998).

The *Bacillus* isolates that exhibited antimicrobial activity against *R. solani in vitro* bioassays were further tested under greenhouse conditions. *R. solani* is regarded as one of the major plant pathogens causing yield limiting diseases (Kim *et al.*, 1997). All *Bacillus* isolates were tested in wheat, cabbage, tomato, maize, and cucumber rhizospheres for consistency in their antimicrobial activity against *R. solani*.

Percentage emergence and shoot dry weight were used as parameters to evaluate the efficacy of the *Bacillus* isolates as biological control agents under greenhouse conditions. It was found that these parameters did not necessarily give a clear picture of the biological control potential of the isolates. It is recommended that in future studies, other parameters such as root dry weight, regular monitoring of pathogen and *Bacillus* levels in the rhizosphere, leaf size and plant height, be used as well.

The results from the greenhouse trials indicated that *Bacillus* isolates tested were crop specific in respect of plant growth promotion and biological control. According to shoot dry weight, several *Bacillus* isolates promoted growth of wheat, tomato and cucumber whereas none of them promoted growth of cabbage and maize. A possible explanation for this is that the type and concentration of root exudates can vary considerably between different plants, thereby influencing the survival and activity of microorganisms in the rhizosphere (Brown, 1976; Melissa *et al.*, 2001). Another possible explanation is that *R. solani* was avirulent towards some of the crops tested. This is shown in Chapter 3 where no significant difference was observed between the negative and positive controls. It is recommended for future studies that *Bacillus* sp. be isolated directly from the roots of plants or crop types for which protection is being sought. Confirmation of plant host specificity is also recommended. At a later stage, it would be interesting to elucidate the mechanism of attraction; i.e. determine the constituents of the root exudates that promote rhizosphere competency and production of antimicrobial compounds (Foeldes *et al.*, 2000).

Of the 19 *Bacillus* isolates used in the greenhouse studies, 12 gave significant shoot dry weight. The significance of these findings is that they could have commercial application to nursery operations or greenhouse production systems. It is recommended that further investigation on the method and rate of application of these biological control agents be investigated and optimized.

In addition to producing antimicrobial compounds, some biological control agents protect the plant by producing extracellular lytic enzymes, e.g., chitinases, which attack the cell wall of the pathogens (Podile and Prakash, 1996). Others can induce a systemic resistance response in the plant (Enebak and Carey, 2000). It is therefore recommended that isolates also be screened for protection mechanisms other than antibiotic production when selecting potential biological control agents.

The *Rhizoctonia solani* used in this study did not appear to infect and / or produce disease symptoms in maize and cabbage. One of the reasons might be the strong influence of

environmental factors on diseases development. Incidence of disease usually varies substantially from one experiment to the next unless great care is taken to carefully control experimental conditions (Lindow and Wilson, 1999).

The *Bacillus* isolates that gave the best results in greenhouse trials were selected for field-testing using maize and bean crops to evaluate plant growth promotion and their ability to control *R. solani* in the field environment. The positive and negative controls for each trial were not significantly different, indicating that the inoculated *Rhizoctonia* was unable to elicit a disease response. This indicated that the pathogen was either unable to establish itself or the crops were not susceptible to it, or that the fungus lost its pathogenicity. It is recommended that for future studies the pathogenicity and virulence of the pathogen be checked on the target crop prior to establishing extensive field trials.

The results from the field trials showed that *Bacillus* Isolates B77, R7 and B81 possibly produced plant growth stimulating effects. *Bacillus* Isolates B77 and R7 improved green bean pod yield and *Bacillus* isolate B81 improved maize yield. These isolates should be investigated further for verification of their plant growth promoting ability.

A possible explanation for the inability of the pathogen to infect the test plants may be the placement of the *Rhizoctonia* inoculum, which could have influenced the time taken for the pathogen to migrate through the soil and reach the plants. The soil porosity can delay the pathogen. In the greenhouse studies it was easy for the pathogen to spread since the porosity of the pine-bark growing medium was greater than that of the field soil.

Inoculum density is another possible explanation for the inability of the pathogen to infect the plants. In future studies, the inoculum density should be high so that the effect of the physical, chemical and biotic components of the soil is overcome (Mangenot and Diem, 1979).

The following suggestions are proposed as a way forward to improving the screening and selection protocols used in this study:

1. The isolation of the *Bacillus* isolates must be done from the rhizosphere of the plant/crop of interest.
2. A range of media should be used during *in vitro* screening to accommodate the growth of both fungi and bacteria and also to allow for the production of potential antimicrobial compounds, which might not be produced on tryptic soy agar, the medium used in this study.
3. In greenhouse trials, the inoculum densities of *Bacillus* isolates must be evaluated to select the best concentration to facilitate root colonization.
4. Parameters used in evaluating biocontrol performance should include the number of stunted plants present, root dry weight, and plant height / length in addition to emergence percentage and shoot dry weight used in this study.
5. Viability of the pathogen and its pathogenicity must be checked regularly.
6. Crops used in field trials should ideally be the same as those for which positive results were obtained in prior greenhouse trials.
7. The presence of other diseases in the field must be verified.

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