UTILIZATION OF *Bacillus* spp. AS PLANT PROBIOTICS

by

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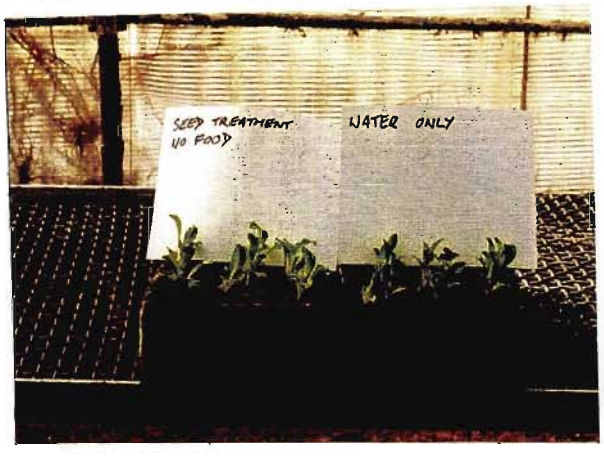
FRONTISPIECE

Comparison of *Bacillus licheniformis* on lettuce seedlings applied by seed treatment plus weekly bacterial drench with or without NutriStart-AC (Nutrient supplement)

Comparison of lettuce seedlings inoculated with *Bacillus licheniformis* and NutriStart-AC with seedlings that received NutriStart-AC



Comparison of *Bacillus licheniformis* seed treated lettuce seedlings without NutriStart-AC with seedlings that received water only

Comparison of lettuce seedlings inoculated with *Bacillus licheniformis* and NutriStart-AC with seed inoculated lettuce seedlings without NutriStart-AC

Comparison of *Bacillus licheniformis* on lettuce seedlings applied by seed treatment plus a weekly bacterial drench and seed treatment alone with NutriStart-AC

Comparison of *Bacillus licheniformis* seed treated plus a weekly bacterial drench lettuce seedlings with NutriStart-AC compared with seedlings that received water only

"A truly extraordinary variety of alternatives to the chemical control of insects is available. Some are already in use and have achieved brilliant success. Others are in the stage of laboratory testing. Still others are little more than ideas in the minds of imaginative scientists, waiting for the opportunity to put them to test. All have this in common: they are *biological* solutions".

Rachel Carson

***Silent Spring*, 1994** (Reprint)

ABSTRACT

Numerous microorganisms produce beneficial effects on plant development when applied to crop seeds or incorporated into soil. Research efforts worldwide over the past two decades have renewed commercial interest in plant growth promoting rhizobacteria (PGPR).With successes being recorded in PGPR research, it is expected that within the next few years, more commercial PGPR products will be available on the market. In particular, commercial PGPR could be advantageous to plant nurseries if they enabled earlier sale of plants, more rapid turnover of seedlings and further crop production cycles.

Trials were carried out to evaluate the growth stimulation and biological control abilities of Biostart™, a *Bacillus*-based plant probiotic comprising seven *Bacillus* spp. and Biostart® 2000 (a combination of three of the *Bacillus* spp.). The first priority was to determine the survival pattern of six Biostart™ *Bacillus* spp., namely *B. chitinosporus*, *B. uniflagellatus*, *B. laterosporus*, *B. pumilus*, *B. subtilis* and *B. licheniformis* in potting soil in the presence or absence of a crop plant, i.e., cucumber, with or without NutriStart-AC. Bacterial numbers in pots in the absence of cucumber seedlings, with or without NutriStart-AC, declined slowly but steadily. Population sizes in pots without NutriStart-AC decreased steadily from Day 1 to Day 14 for all six *Bacillus* spp. and thereafter remained constant between 6.19 and 6.15 log cfu g^{-1} of wet soil for all six *Bacillus* spp. up to termination of the experiment on Day 35. A similar effect was observed in pots supplemented with one gram of NutriStart-AC. In the presence of cucumber seedlings, population sizes in pots without NutriStart-AC supplement declined faster until Day 14 than those in the NutriStart-AC supplemented pots. Populations remained stable after Day 14 for all six *Bacillus* spp. in the NutriStart-AC unsupplemented pots, while there was a variation in population sizes among *Bacillus* spp. in pots supplemented with NutriStart-AC.

Growth stimulation trials in tunnels were carried out using four crops, i.e., lettuce, tomato, sorghum and beans. Seed treatment and seed treatment plus drenching with or without NutriStart-AC were evaluated. All *Bacillus* spp. used stimulated plant growth. Growth stimulation was more pronounced with a 4% NutriStart-AC supplement.

Growth stimulation was best in lettuce, with Biostart® 2000. There was an increase of 466% compared to the dry biomass of the water control lettuce seedlings. The lowest responses were recorded in sorghum and beans.

Three tomato cultivars, i.e., Roma, Floradade and Rodade and a pepper cultivar Thai were evaluated for growth stimulation by applying Biostart™ as seed treatment and seedling drench. The highest growth stimulation, 96%, was obtained using *B. licheniformis* on Roma as a seedling drench. Growth response was better in Roma and Floradade cultivars than in the Rodade cultivar. Pepper plants drenched with Biostart™ *Bacillus* spp., and supplemented weekly with a 4% NutriStart-AC suspension, showed increased fruit yield. Using *B. subtilis*, a 533% increase in fruit yield was recorded when seedlings were supplemented weekly with a 4% NutriStart-AC suspension. Similar results were recorded using an unidentified *Bacillus* strain CM-33 (433%) and *B. licheniformis* (333%).

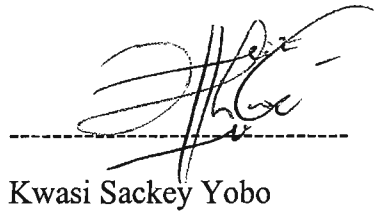
In a nematode control trial, no galls were found on the roots of treated and untreated control seedlings inoculated with *Meloidogyne* spp. Early inoculation of seedlings might have failed because there were no roots for the nematodes to attack at the time of inoculation.

In a biological control trial, Biostart™ *Bacillus* spp. were applied by seed treatment and seedling drench to control *Rhizoctonia* causing damping-off of marigold, cabbage and eucalyptus seedlings. Biostart™ was ineffective under the conditions of this trial. Most seedlings died seven days after pathogen inoculation and by Day 21 about 90% of the seedlings were dead.

The results presented in this thesis have some practical applications to seedling growers in South Africa, especially in growth promotion. Applying Biostart™ probiotic *Bacillus* spp. may increase the turnover of seedlings in nurseries. More trials are needed if the growth promotion and biological control potentials of Biostart™ probiotic *Bacillus* spp. are to be fully exploited.

DECLARATION

I, Kwasi Sackey Yobo, declare that the research reported 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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The Yobo family, for all their sacrifices that have enabled me to pursue my studies.

DEDICATION

To the Yobo family for the support, understanding
and spiritual encouragement during
my studies

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CHAPTER 1

GENERAL INTRODUCTION

More food is needed to feed the world population. There is therefore the need for better agricultural technology to improve crop yields, especially as there is little or no new land available for agricultural practices. This has created a challenge for scientists to search for methods that will result in an increase in crop production/yields in modern agriculture. These methods include the use of environmentally friendly microorganisms to stimulate plant growth, and to control pest and diseases affecting plants used as sources of food.

Chemical control has provided a means of reducing plant diseases. Over time this has proved to have negative side effects such as development of resistance by pathogens, high costs and negative effects on beneficial microorganisms (Utkhede, 1992) as well as environmental, soil and water pollution (Akhtar, 1998). Therefore the use of biological control systems to improve plant growth or control plant diseases or both have been investigated. Biological control of soil-borne pathogens by introduced microorganisms has been studied for over 60 years (Weller, 1988).

The word 'probiotic' is derived from the Greek, meaning 'for life' and has had several different meanings (Fuller, 1992). It was first used by Lilley and Stillwell in 1965 to describe substances secreted by one microorganism which stimulated the growth of another. This therefore meant the exact opposite of an antibiotic (Fuller, 1992). According to Tannock (1999), a 'probiotic' by the generally accepted definition is a "live microbial feed supplement which beneficially affects the host of an animal by improving its intestinal microbial balance". Although referring to the supplementation of farm animals, this definition is easily applied to the human situation. In the context of this study therefore, the term 'plant probiotics' will be referred to as free living rhizosphere microorganisms which benefit plants through provision of plant growth promoters, mobilise soil nutrients and/or control plant diseases.

There is generally a poor link between the ability of a bacterium to inhibit a pathogen *in vitro* and to suppress disease caused by that pathogen *in vivo*. The implication of this is that strains that

produce the largest inhibition zones on agar media are not necessarily the best biological control agents. Therefore, selection of field-effective strains should primarily involve screening for rhizosphere competence. Successful establishment of such strains in the rhizosplane will allow them to exert their biological control activity (Weller, 1988).

Successful biological control of soil-borne plant diseases can thus be effectively achieved through a fundamental understanding of the ecological relationships of the diverse microbial populations (including plant pathogens) and biological control agents in the soil and specifically the rhizosphere (Huang, 1992).

In addition to the generally Gram-negative rhizosphere bacteria that have been considered and used as plant growth stimulants and disease control agents, there are several Gram-positive *Bacillus* spp. Their ability to form heat and desiccation tolerant endospores, has led to investigations for their growth stimulating and biological control ability, despite documentation suggesting they are less effective root colonizers than Gram-negative *Pseudomonas* spp. Spore-forming *Bacillus* spp. are of interest as inoculants because spores are easy to prepare in large quantities (Petras & Casida, 1985), and will retain viability in storage for extended periods, that is, have an extended "shelf life" (Aronson *et al.*, 1986; Young *et al.*, 1995). They also survive in a dormant form until conditions are appropriate for germination and activity (van Elsas *et al.*, 1986). Thus the period of inoculation for a *Bacillus* spp. with, for example a biological control activity, will not be restricted by the need to accurately forecast when conditions would become favourable for the development of the disease (Young *et al.*, 1995).

Growth promotion results in increased seedling emergence, vigour, plant weight, root system development and yield. Although there is a likelihood that many crops may benefit from the application of probiotics, more field trials need to be conducted to further determine their effectiveness on a commercial scale. It is therefore essential to quantify and assess the benefits and costs of using probiotics in commercial crop production.

Since the competency of rhizosphere bacteria, including strains of the same *Bacillus* spp. differ, the present study was aimed at evaluating seven commercially available *Bacillus* spp. and

Biostart®2000 as plant probiotics for growth stimulation and disease control. These are: *Bacillus chitinosporus*; *Bacillus uniflagellatus*; *Bacillus laterosporus*; *Bacillus pumilus*; *Bacillus subtilis*; *Bacillus licheniformis*; an unidentified *Bacillus* strain, CM-33 and Biostart® 2000 (a combination of *B. chitinosporus, B. laterosporus* and *B. licheniformis*).

The objectives of this study were: (i) a general review of:

the genus *Bacillus* paying particular attention to situations where *Bacillus* has been used as a growth stimulant and for disease control; plant growth promotion and mechanisms of growth promotion; biosafety and management of microorganisms intended for use as pest and plant disease control agents

(ii) population dynamic studies of Biostart™ *Bacillus* spp. in soil with/without plant and with/without NutriStart-AC supplement (NutriStart-AC is a commercially prepared nutrient supplement provided by Microbial Solutions[1])

(iii) to ascertain the growth promotion effects of Biostart™ *Bacillus* spp. on common nursery crops with/without NutriStart-AC

(iv) to evaluate the potential of Biostart™ *Bacillus* spp. for the control of *Rhizoctonia* damping-off of seedlings and root-knot nematodes.

1.1 THE GENUS BACILLUS

The genus *Bacillus* belongs to the family Bacillaceae. *Bacillus* spp. are rod-shaped and generally motile bacteria. The motility is an advantage since it enables the bacteria to scavenge more efficiently for limited nutrients excreted from root hairs (Brock & Madigan, 1991).

Many *Bacillus* strains can suppress growth of plant pathogenic organisms by the production of peptide antibiotics (Leifert *et al.*, 1995). These peptide antibiotics are effective against other Gram negative bacteria and some Gram positive bacteria, moulds and yeast (Brock & Madigan, 1991). The antibiotics produced *in vitro* were generally assumed to be compounds responsible for biocontrol *in vivo* (Leifert *et al.*, 1995). In addition to the antibiotics, *Bacillus*, however,

[1]Microbial Solutions (Pty)Ltd., P.O. Box 1180, Strubens Valley 1735, South Africa

produce a range of other metabolites including biosurfactants (Edwards & Seddon, 1992), chitinase and other fungal cell wall-degrading enzymes (Pelletier & Sygusch, 1990; Frändberg & Schnürer, 1994), volatiles (Friddaman & Rossall, 1993, 1994) and compounds which elicit plant resistance mechanisms (Kehlenbeck *et al.*, 1994).

The ability of bacteria to survive and proliferate in soil is an important factor in their success as inoculants for promoting biological control, nutrient solubilisation and bioremediation (Young & Burns, 1993). However, many soil inoculants, shown to be beneficial in the laboratory experiments fail when used in the field (Lethbridge, 1989). This is probably due to a combination of physical (Rattray *et al.*, 1992), chemical (Acea *et al.*, 1988) and biological (Recorbert *et al.*, 1992) stress encountered by the introduced species. It may therefore prove more successful to isolate bacteria from the target soil and screen for beneficial species which can then be reintroduced in much larger numbers. Such bacteria may be more likely to survive and express their properties because they are adapted to the recipient soil environment and should compete effectively with the indigenous microorganisms (Young *et al.*, 1995).

Bacillus spp. have been used for many years in attempts to control plant pathogens and increase plant growth (Turner & Backman, 1991; Holl & Chanway, 1992; Mañero *et al.*, 1996; Kim *et al.*, 1997). *Bacillus* spp. strain L324-92 has been found to show a growth promoting benefit on turf grass when applied to the foliage as a cell suspension (Mathre *et al.*, 1999). This strain was also shown to possess an *in vitro* antibiotic activity against all isolates of *Gaeumannomyces graminis* (Sacci) Arx and Oliver var. *tritici*, as well as species and anastomosis groups of *Rhizoctonia* and all species of *Pythium* tested (Kim *et al.*, 1997). Due to the high growth stimulation response on turf grass, *Bacillus* spp. strain L324-92 was awarded a license in 1998 for further development and commercialisation for use on turf grass (Mather *et al.*, 1999).

Other *Bacillus* spp. have also been reported as potential plant growth stimulants. Two strains of *B. pumilus* and one strain of *B. licheniformis* were found to significantly ($P < 0.05$) promote growth of European alder (*Alnus glutinosa* (L.) Gaertn. (Probanza *et al.*, 1996). These strains increase the aerial surface and aerial length of European alder by 163% and 182% respectively as compared with the controls. Further studies revealed that these three *Bacillus* strains produce

auxin-like (IAA-1) compounds at levels of 1.736 and 1.790 mg IAA-1 L^{-1} culture growth medium. The filtered bacterial growth medium was found to increase plant growth compared to the control (Mañero *et al.*, 1996).

Inoculation of spring wheat (*Triticum aestivum* L.) seeds with *B. subtilis* or *B. pumilus* resulted in rhizosphere populations of 10^5 cfu g^{-1} of root tissue of inoculum bacteria one month after treatment (Juhnke *et al.*, 1987). These findings were contrary to the earlier suggestion that *Bacillus* is a relatively poor rhizosphere colonizer (Lockhead, 1940) and therefore demonstrated that *Bacillus* inoculants can effectively colonize the rhizosphere. Various reports have also shown that *Bacillus* can effectively colonize the rhizosphere (Turner *et al.*, 1991; Asaka & Shoda, 1996; Pandey, 1997).

The most documented mode of action of biological control action of *Bacillus* spp. to suppress plant pathogen growth under laboratory conditions has been antagonism through antibiosis. *Bacillus* antibiotics vary in their mode of action. Their target site or mode of action can be the permeability of the plasma membrane, interference with protein and cell wall synthesis and other membrane functions (Pelczar *et al.*, 1992). Antifungal antibiotics production by two *Bacillus* strains, *B. subtilis* CL27 and *B. pumilus* CL45 were found to show activity against *Alternaria brassicicola* and *Botrytis cinerea* (Leifert *et al.*, 1995). Both free-cell fermentation broth filtrates and washed cells of *B. subtilis* CL27 prevented grey mould disease development on *Astilbe*. Additionally, the concentrations of antibiotics in the culture medium were sufficient to control disease even in the absence of *Bacillus* cells (Leifert *et al.*, 1995).

Bacillus spp. also produce peptide antibiotics. These peptide antibiotics were shown to be produced *in vitro* by *B. subtilis* and *B. brevis*. (Edward & Seddon, 1992). These two *Bacillus* strains were shown to have *in vivo* activity against fungal plant disease. The value of *in vitro* studies into the mode of action was questioned because antibiotic activity produced by different *Bacillus* strains *in vitro* plate assays correlated very little with *in vivo* biocontrol activity (Fravel 1988; Leifert *et al.*, 1993).

The antagonists involved in biological control employ a wide range of mechanisms to reduce/eliminate plant pathogens. Two *B. subtilis* strains, GB03 and GB07, have been marketed as Kodiak and Epic respectively by Gustafon Inc. in the USA for use with several crops as plant growth-promoting rhizobacteria. Field trials have shown that cotton yields were increased by seed treatment with these two strains (Zhang *et al.*, 1996). However, strains GB03 and GB07 have also been reported to be colonizers of cotton roots (Brannen & Backman, 1993). Further studies with *B. subtilis* GB03 and GB07 showed a strong inhibition of *Fusarium oxysporum* f.sp. *vasinfectum* and other *Fusarium* spp. *in vitro* (Zhang *et al.*, 1996). Mechanisms for reducing these *Fusarium* spp. on cotton roots may include antibiosis (Zhang *et al.*, 1996).

Much research on biological control has been focussed on *Pseudomonas* spp. since they are better root colonists than *Bacillus* spp.(Kim *et al.*, 1997). However, the easier to handle *Bacillus* spores in commercially scale, longer 'shelf life' and the production of broad spectrum antibiotics which is necessary for biological control, make *Bacillus* the preferable choice.

1.2 GROWTH PROMOTION

Microbial populations respond to plant growth through the influence of root exudates. In relation to this, microbes in soil can influence plants in a positive or negative way (Curl & Truelove, 1986).

The term rhizobacteria is used to describe the total rhizosphere bacterial population. The rhizosphere is a narrow zone of soil subject to the influence of living roots, as manifested by the leakage or exudation of substances that affect microbial activity (Curl & Truelove, 1986) and comprises the habitat of bacteria that are able to colonize roots (Kloepper *et al.*, 1989). Root colonization reflects the capacity of bacteria to multiply and keep pace with the growing root in field soil (Kloepper *et al.*, 1989). Practically, it is essential that rhizosphere colonization follows as a result of bacterial inoculation. The impact of rhizobacteria on plant growth and health may be classified as neutral, deleterious or beneficial (Kloepper *et al.*, 1989).

1.2.1 Mineral availability and uptake

Deficiencies in soil nitrogen, phosphorus or potassium are most often involved in the limitation of plant growth and frequently must be supplemented by application of commercial fertilizers. The rhizosphere microflora, nourished by root exudates and root debris, indirectly affects plant growth by influencing the availability and uptake of nutrients, resulting in either a beneficial or detrimental effect on the plant.

1.2.1.1 *Mineralization of organic substances*

The most important contribution of microorganisms to plant nutrition involves the decomposition of organic matter, resulting in the subsequent release/formation of ammonia, nitrates, sulfates, phosphates, CO_2, and water. The intensity of these activities is enhanced in the rhizosphere of crop plants where the metabolic activities of organisms, as shown by measured respiration, may be as much as four times higher than in non-rhizosphere soil (Curl & Truelove, 1986).

Soil conditions, such as good aeration, neutral pH and adequate nitrogen supply favour both nitrifying bacteria and plant growth (Curl & Truelove, 1986). The amount of mineral nitrogen in the form of nitrate ions in the soil depends basically on the rate of mineralization from organic matter by microbial action, and the rate of removal by leaching or utilization by crop plants and microbial populations. The rhizosphere has a definite effect on mineralization and ammonification. These processes can be accelerated by the addition of organic matter to soil. Similarly, root exudates and sloughed off root tissues also provide fresh organic substances that stimulate the activities of the rhizosphere flora, resulting in accelerated turnover of nitrogen.

The rhizosphere effect on the soil nitrification process varies with different plant species. This is to be expected as nitrifying bacteria are very sensitive to microbial toxins and to pH changes that occur with qualitative differences in root exudates and the responding microbial activity. In some cases, numbers of *Nitrosomonas* and *Nitrobacter* have been found to increase in response to root exudates. However, in other instances, microbial populations, and nitrification, have been inhibited or nitrogen has been immobilized (Curl & Truelove, 1986). Inorganic nitrogen

compounds, in addition to being taken up by growing plants and microorganisms, or lost by leaching, can also be reduced through denitrification processes.

Nitrates, in the presence of the required reductases and associated electron transport compounds, are converted to gaseous nitrogen and nitrous oxide which then escape into the atmosphere. The denitrification process is carried out mainly by facultative anaerobic bacteria (of which the most common include members of the genera *Pseudomonas*, *Micrococcus*, and *Bacillus*) under poor aeration conditions, such as in waterlogged soils. These organisms grow well in the presence of oxygen, but utilize nitrate as a hydrogen acceptor under limited oxygen supply. According to Russell (1973), a low level of microbiological activity is required for denitrification under low oxygen tension. Nevertheless, the process occurs readily in aerated soils when large amounts of decomposable organic matter are applied. In such cases, soil oxygen is being used up by the highly intensified microbial activity to a greater extent and at a faster rate than it is replaced by diffusion from the atmosphere (Russell, 1973).

1.2.1.2 *Availability of phosphate and absorption by plants*

Microorganisms, through the decomposition of organic compounds and the oxidation or reduction of inorganic compounds, make elements such as phosphorus, potassium, sulphur, calcium and iron in the soil available to plants. Different species of plants grown in similar environments may differ in chemical composition. This is due in part to differences in nutrient availability at the root-soil interface and the varying capacity of different species for nutrient absorption and utilization (Curl & Truelove, 1986). The role of microbial mineralization in the release of phosphates, sulphates and other important elements from organic sources is well documented in the literature. The qualitative and quantitative nature of the microbial population present, and the experimental techniques employed, may either restrict or enhance the availability of nutrients and their uptake in non sterile systems (Curl & Truelove, 1986). Phosphorus is an important element that performs an essential role in plant growth and soil biology. It occurs as a constituent of both organic and inorganic compounds in soil, plants and microorganisms.

According to Alexander (1977), microbial communities in both the rhizosphere and bulk soil regulate the phosphorus cycle in three distinct processes:

ii) heterotrophic mineralization of organic phosphorus compounds and the regeneration of orthophosphates;

iii) immobilization of inorganic phosphorus by autotrophic and heterotrophic microorganisms, resulting in a lower available phosphorus supply and

iv) solubilization of aluminium, iron and calcium phosphates.

As extracellular mineralization by soil phosphatase of microbial origin proceeds, the regenerated phosphate is rapidly immobilized under conditions that are favourable for microbial activity, such as that occurring in the presence of root exudates. Microflora also play a major role in nutrient cycling (Cole *et al.*, 1978). Bacteria assimilate and retain labile inorganic phosphorus as carbon substrates in the rhizosphere. These are metabolized and the bacterial phosphorus is mineralized and returned to the inorganic phosphorus pool by bacteriophagous amoebae. Cole *et al.*, (1978) suggested this process from the results of an experiment designed to stimulate biological activities in the rhizosphere by using glucose amendments to represent supply of root exudates to microorganisms in the presence or absence of amoebae. Nematodes also participate in this process in a similar manner, but less effectively. The contributing role of microorganisms in determining the availability of phosphate through mineralization or immobilization is therefore evident.

Since the rate of diffusion of ions through soil to the roots is extremely slow, phosphorus and certain other essential elements must be in solution in the immediate vicinity of roots before they can be adequately absorbed.

According to Gardner *et al.* (1983), phosphate solubilization is largely a function of soil pH, cation exchange capacity of roots, adsorption and absorption of calcium from calcium phosphate, and the complexing of aluminium and iron by organic anions to solubilize Al and Fe phosphates. These processes are usually induced by, or related to, the action of root exudates and the activities of microorganisms at the root-mineral interface. Under natural conditions, the phosphate dissolving power of plants depends on the presence of both root exudates and associated

microbial products, which together are referred to as 'rhizosphere products' (Moghimi *et al.*, 1978).

Microorganisms also contribute positively to the process of nutrient absorption. The rate of nutrient uptake is closely linked to the rate of diffusion of ions through soil and the rate of their arrival at the root-soil interface (Russell, 1977). This suggests that microbes in the root environment do not effect nutrient availability through mineralization processes only but also through the dissolution of relatively insoluble materials. The rate of nutrient diffusion towards the root also depends, in part, on the uptake rate and consequent lowering of the concentration at the root surface. Microbial activity may be involved in this process if competition with the plant for nutrients in the rhizosphere is sufficient to accelerate the formation of a nutrient void.

Microorganisms are often not considered when experimental results on nutrient uptake by plants are interpreted, even though the plants may be cultured in non-sterile environments (Curl & Truelove, 1986). However, specific evidence of a microbial role in nutrient absorption has been obtained with plants grown in highly artificial systems. Barber and Frankenburg (1971) established that roots growing under non-sterile conditions have a greater capacity for ion uptake than roots growing in the absence of microorganisms. In addition, greater incorporation of phosphate into plant nucleic acids occurs in the presence of microorganisms. This was verified by culturing excised roots of barley in sterile and non-sterile solutions of KH_2PO_4 and measuring the absorption of phosphate ions.

In effect, whether microorganisms significantly affect phosphate uptake and distribution depends to a large extent on the existing concentration of phosphate in the soil or in the experimental growth medium (Benians & Barber, 1974). When the phosphate supply is adequate to meet the metabolic requirements of both the plant and microorganisms, any effect of microbial activity becomes masked and probably negligible. However, in low concentrations of soil phosphate, competition occurs between plants and microorganisms with a resultant restriction of phosphate uptake by the plant.

1.2.1.3 *Availability and uptake of other elements*

Microbes on the root surface and on root hairs can affect the availability and uptake of other ions beside phosphate. Chelating compounds in the root exudates together with the action of microorganisms might increase the availability and uptake of minor elements such as zinc. Some differences have been observed between plant species regarding the solubilization and absorption of calcium in the root zone. This has been attributed to the effects of root exudates which probably mediate a change in pH (Curl & Truelove, 1986).

The absorption of rubidium, as well as phosphorus, has been found to be greater in roots infested with microorganisms than in plants grown under sterile conditions (Barber & Frankenburg, 1971). At concentrations above 0.2mM, Thalium, despite being phytotoxic, may also be absorbed readily by plant roots in non sterile-soil (Barber, 1974).

However, the effect of microorganisms has, more often, been one of reduced nutrient availability or uptake by plants, thus reflecting the capacity of microbes to concentrate and tie up elements on the root surface, particularly at sites of increased exudation where microbial activity is intensified (Curl & Truelove, 1986).

1.2.1.4 *Effect of microorganisms on root morphology*

According to Curl & Truelove (1986), the absorptive capacity of roots is related to:

i) density of the root system
ii) total root surface area
iii) volume of soil occupied by roots and root hairs

These features are governed by the genus/species and age of the plant, soil type, moisture and level of fertilization. Microorganisms on root surfaces directly or indirectly affect root morphology and ultimately enhance or reduce nutrient absorption. Root stunting and retarded root hair development have been observed in several crops following exposure of the root system to soil-water suspensions. However, these effects were absent when diluted suspensions, which

contained reduced numbers of microorganisms, were applied as inocula (Curl & Truelove, 1986).

1.2.1.5 *Activities of fauna on nutrient uptake*

Small fauna in the rhizosphere can influence nutrient availability and uptake by plants indirectly through their predatory action upon the microflora. Bacteriophagous protozoa and nematodes are thought to consume sufficiently high numbers of bacteria to interfere with the normal mineralization of nutrients. However, it is more likely that such feeding will liberate nutrients immobilized in bacterial cells and thus accelerate the mineralization process. Elliot *et al.*, (1979) demonstrated the latter in gnotobiotic microcosms where soils containing both amoebae and bacteria, or nematodes and bacteria, mineralized significantly more NH_4-N and inorganic phosphorus than soils with bacteria alone.

Populations of the microphagous small arthropods (*Acari* and *Collembola*) are especially abundant in habitats of dense, fibrous root systems, suggesting a close relationship with roots for feeding and reproduction (Curl & Truelove, 1986). The common occurrence of bacteria, fungal spores, and mycelial fragments among their gut contents is evidence that these arthropods consume a portion of the soil microflora.

Collembola are attracted to living roots, and can transport bacteria and fungal spores on their bristled bodies into the rhizosphere (Wiggins & Curl, 1979). These activities suggest there is a potential for altering the quantitative and qualitative nature of the microflora around roots. In controlled experiments, certain seedlings, initiated from surface-disinfected seed and grown in sterilized soil, grew 3 cm taller when field-collected *Collembola* were added than they did in sterile soil, lacking these arthropods (Wiggins & Curl, 1979).

One or more of the following activities explains the stimulated plant growth:

i) insect-transported bacteria proliferating at the root-soil surface release additional nitrogen or phosphorus for plant absorption
ii) bacteria synthesize plant-growth stimulating factors, or
iii) microbial degradation of toxins formed during heat sterilization of the soil removed the

inhibitory effects promoting plant growth. In either case the insects probably served only as vehicles for the microflora.

1.2.2 Plant responses to microbial metabolites

Plants respond to specific microorganisms applied to seeds or roots. Usually the response leads to either growth stimulation or growth inhibition. Growth inhibition may also occur in the presence of non-parasitic bacteria or fungi. The mechanisms leading to growth stimulation and growth inhibition may be related to a combination of factors such as increased availability and absorption of nutrients, biological activity against pathogens and production of growth-promoting or growth-inhibiting metabolites by rhizosphere microorganisms (Curl & Truelove, 1986).

1.2.2.1 *Growth promoting factors*

The responses of plants to bacterial inoculation usually occur in the form of:

i) increased vegetative growth (Dashti *et al.*, 1997)

ii) early flowering (Curl & Truelove, 1986)

iii) change in root-to-shoot weight ratio (Probanza *et al.*, 1996), and

iv) increased yields (Suslow & Schroth, 1982; Turner & Backman, 1991; Dashti *et al.*, 1997).

While the effects of some of the above factors on plant growth could be attributed largely to the nitrogen-fixing activities of *Azotobacter*, this organism, along with a wide range of other microorganisms, can also produce growth-regulating substances in the root zone. According to Curl & Truelove (1986), microorganisms in the rhizosphere and rhizoplane of wheat release growth regulating substances with the properties of indole-3-acetic acid (IAA) and the gibberillins, which can be readily absorbed in the region of root-hair development. Microorganisms also synthesize vitamins in the rhizosphere and these vitamins have a definite role in plant growth.

Bacillus subtilis and *Streptomyces griseus* Krainsky, when applied to seeds of barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), wheat (*Triticum aestivum* L.) and carrots (*Daucus carota* L.)

can induce increased marketable yields of these crops. These organisms are antagonistic to *Rhizoctonia solani* Kühn, but since seed bacterization does not necessarily control the pathogen and disease incidence, the observed benefits to plant development are probably due to other factors, including growth substances synthesized by the applied microorganisms. In some instances, growth-stimulating bacteria in the rhizosphere are known to inhibit weakly pathogenic bacteria and fungi (Suslow & Schroth, 1982). Thus, in a natural soil environment, it is most likely that plant growth is affected both by microbially synthesized growth factors and the competitive interactions of growth-promoting versus deleterious microorganisms at the root surface.

Although bacteria are most frequently implicated with the production of substances affecting plant growth, a number of fungi isolated from the rhizosphere of certain plants also synthesize auxins and gibberillins when grown in culture. Practical methods for promoting the multiplication of growth benefiting microorganisms on the root surface, while concomitantly excluding the growth inhibiting organisms, are at a developmental stage. Even among plant growth-promoting microorganisms, some species induce undesirable changes in root morphology.

1.3 MECHANISMS OF GROWTH PROMOTION BY MIXED PGPR

There are several ways in which different PGPR have been reported to directly facilitate the proliferation of their plant host (Glick, 1995). PGPR can synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells (Loper, 1988); they can synthesize several phytohormones that can enhance various stages of plant growth (Lambert & Joos 1989; Mañero *et al.*, 1996). A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. A number of plants are able to use bacteria iron-siderophore complexes as a means of obtaining iron from soil (Wang *et al.*, 1993). Without this mechanism for obtaining iron, the growth of most plants in most soils would be severely limited. However if the effect of a PGPR on plant growth were limited to providing the plant with sufficient iron, one might expect treated plants to vary in their response to the PGPR according to differences in the amount of available iron in the soil (Glick, 1995).

The mechanism most commonly invoked to explain the various effects of PGPR on plants is the production of phytohormones. Most of the attention has focussed on the role of the phytohormone auxin (Mañero *et al.*, 1996). Auxins are a class of plant hormones and the most common and well characterised is IAA which is known to stimulate both rapid and long term response in plants (Cleland, 1990). Plants as well as many PGPR can synthesise auxin. It is absolutely imperative to distinguish the auxin synthesised by the plants in response to PGPR stimulation and the auxin synthesised by the PGPR itself when assessing the effect of PGPR on plants (Gaudin *et al.*, 1994). A relatively straightforward way to directly monitor the effects of bacterially synthesised auxin is to compare plants treated with either wild-type PGPR strains or mutant strains that either do not produce or else overproduce auxin. For example, mutant strains of *Azospirillum brasilence* that synthesise only very low levels of IAA, when compared with the wild type strain, no longer promoted the formation of lateral roots of wheat seedlings (Barbieri & Galli, 1993). On the other hand, a mutant strain of *Pseudomonas fluorescens* BSP53a that overproduce IAA stimulated root development of black currant softwood cuttings and inhibited that of cherry (Dubeikovsky *et al.*, 1993). The result indicated that the growth of plants treated with an IAA- secreting PGPR is affected by the amount of IAA that the bacterium produces. The response observed may also vary from one species of plant to another (Glick, 1995). Hence PGPR facilitate plant growth by altering the hormonal balance within the effected plant.

A hitherto unsuspected mechanism of plant growth promotion involves the plant hormone ethylene. It has been demonstrated that *P. putida* GR12-2 contains the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Jacobson *et al.*, 1994). This enzyme hydrolyses ACC, the immediate biosynthetic precursor of ethylene in plants. When *P. putida* GR12-2 was chemically mutagenised, three independent mutants that lacked ACC deaminase activity were selected. Unlike the wild type, none of these selected mutants were able to promote growth of canola seedling roots under gnotobiotic conditions. This implies that the enzyme ACC deaminase is involved in the mechanism that *P. putida* GR12-2 uses to stimulate canola root elongation (Glick *et al.*, 1994). One model that can be used to explain this observation is that *P. putida* GR12-2 binds to seed coats and during seed imbibition the bacterium sequesters and then hydrolyses ACC, thereby lowering the level of ethylene in the developing plant (Glick, 1995). *P. putida* GR12-2 synthesises IAA and the ACC deaminase activity may prevent IAA which

normally stimulates the enzyme ACC synthase in the plant, from increasing ethylene synthesis. Thus *P. putida* GR12-2 that contains the enzyme ACC deaminase binds to the seed coat of the developing seedling and acts as a mechanism for ensuring that the ethylene level does not become elevated to the point where root growth is impaired (Glick, 1995). This model predicts that any bacterium that contains the enzyme ACC deaminase and can bind to plant seed or roots in the soil should also be able to promote root elongation (Glick, 1995).

1.4 INOCULUM POTENTIAL

Generally, the major influence of the rhizosphere on both the saprophytic and parasitic activities of root-infecting organisms is mediated through the action of root exudates (Curl & Truelove, 1986). Direct effects of exudates in the rhizosphere are reflected in pathogen population changes, effects on growth and survival, and the germination of infective propagules. The direct influence of exudates in the rhizosphere are schematically represented in Figure 1.1

Indirect effects are imposed by the general microbial population responding to root exudates, this activity contributing to nutrient availability and uptake by plants, synthesis of growth factors that affect both host plant and pathogens, and the initiation of antagonistic phenomena. All these activities influence the inoculum potential of a pathogen, defined as the energy of growth of a fungal parasite available for infection of a host at the surface of the host organ to be infected, per unit area of the host surface (Garrett, 1970). The measure of the maximum capacity of a pathogen population to infect fully susceptible plant tissue under optimum conditions is termed the absolute inoculum potential (Mitchell, 1979). This attribute is controlled by the gene complement of the pathogen, which determines how the pathogen population will respond to environmental factors in the microecosystem. Therefore, inoculum potential will vary with the inherent nature of different pathogens to produce propagules, to survive in the soil, and to infect host tissues. In general, for a root disease to occur, the following requirements must be met:

i) a susceptible host must be present
ii) a sufficient pathogen population or inoculum density at the root surface
iii) a nutrient energy source for rapid propagule germination and host infection
iv) a biotic and physicochemical environment favourable for pathogen activity

Hence, disease potential, which is the susceptibility of the host as influenced by disease proneness can be considered separately to inoculum potential. Disease can therefore be equated to inoculum potential x disease potential (Baker, 1978).

1.5 PATHOGEN POPULATIONS

Pathogen population is defined as the number of propagative units of bacteria, fungi or nematodes per unit of soil contributing to the inoculum potential, or the chance that disease will occur (Curl & Truelove, 1986). Since the inoculum density of a pathogen contributes to inoculum potential, assessment of viable populations in field soils is often used to predict disease incidence and severity. Usually such assessments have no immediate relation to rhizosphere populations since the estimates are usually made prior to planting a crop. However, the rhizosphere effects of various crop plants used in a rotation system may determine the concentration and nature of inoculum available for infection from season to season. Broad field assessments of seed-borne pathogen populations may hold little relevance as sufficient inoculum result in an epidemic, can develop from the initial colonization of the rhizosphere of the germinating seed.

1.5.1 Bacteria and fungi

Under certain environmental conditions, common rhizosphere bacteria can become minor pathogens. In particular, species of *Pseudomonas* and some species of the Enterobacteriaceae and the Corynebacteriaceae, produce substances that either inhibit plant growth or stimulate fungal pathogens such as *Pythium* spp. to colonize roots, thus predisposing plants to disease. These organisms, sometimes called "deleterious rhizobacteria" are opposed to "plant-growth-promoting rhizobacteria" which are predominantly *Pseudomonas* spp. (Suslow & Schroth, 1982). The inoculum density required by fungal pathogens to produce disease varies widely among different pathogens, and is dependent on the type of inoculum. According to Baker and Cook (1982), the inoculum density required for induction of disease ranges from less than one unit g^{-1} of soil for

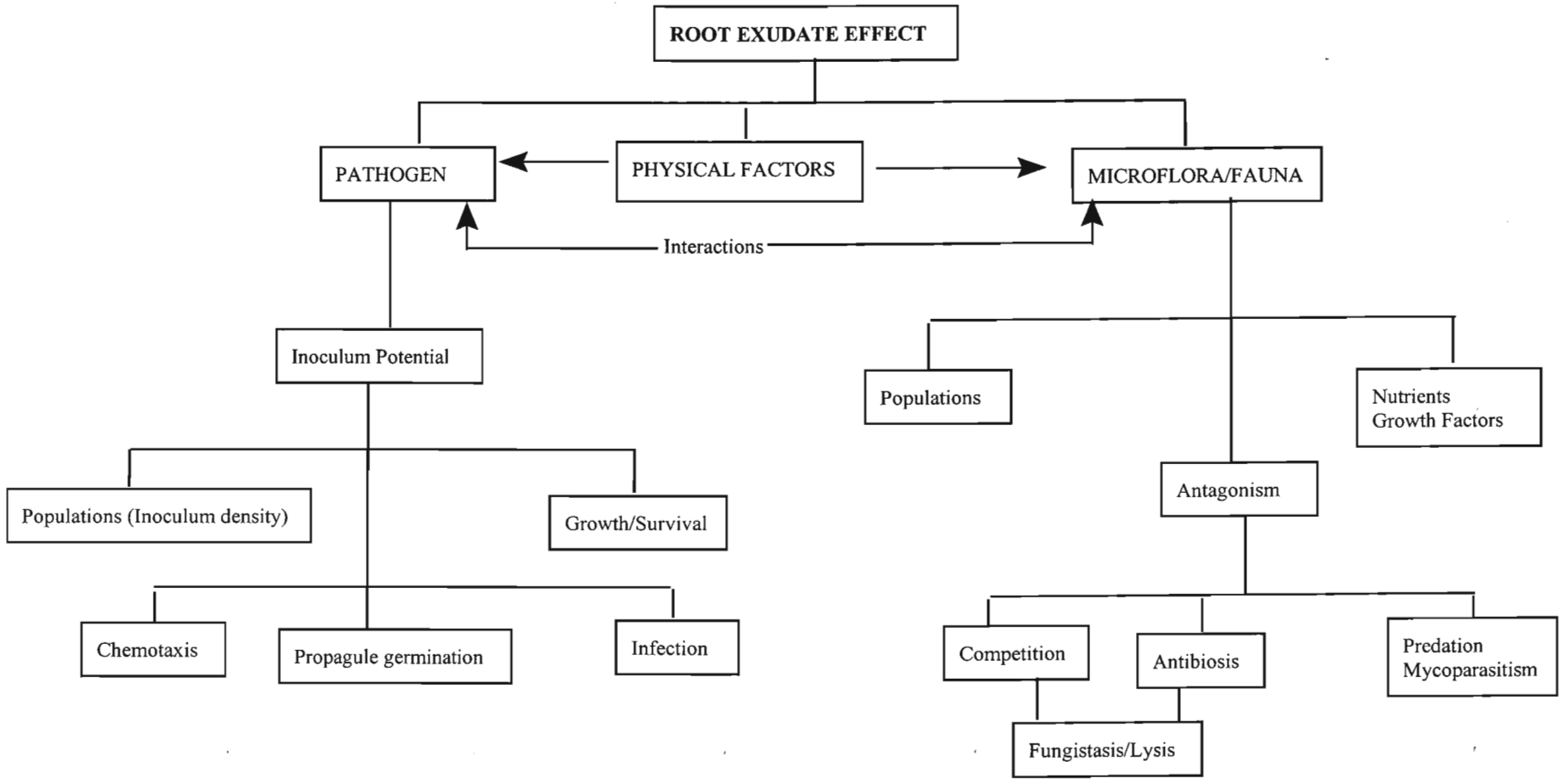

Fig.1.1 Schematic representation of root exudate effects on pathogen activities imposed either directly, or mediated by interactions with, saprophytic microbial populations (Curl & Truelove, 1986).

pathogens such as *Sclerotium rolfsii* (Sacc.), *Phymatotrichum omnivorum* (Shear) Duggar and *R. solani* that form multicellular structures (sclerotia) to more than 1000 units g^{-1} for *Fusarium solani* (Mart.) Appel. and Wr. f. sp. *phaseoli* (Burk.) Synd. and Hans. and *Thielaviopsis basicola* (Berk. and Br.) that persist as thick-walled resting spores. In the rhizosphere, root exudates provide the energy source for vegetative growth and the production of new propagules, which may vary in size according to the quantity of nutrient and the quality of exudates.

Generally, the size as well as numbers of inoculum units contributes to the inoculum density and the potential for infection and disease to occur. A high frequency of cropping with susceptible plants in a rotation system is frequently accompanied by increased populations of a pathogen and severity of disease. Usually, populations of a pathogen are not reduced by rotations with non-susceptible crops, even though general recommendations for disease control may include such practices. According to Davis and McDole (1979), *Verticillium dahliae* (Kleb.) and *R. solani* populations were not reduced in potato fields when the potato crop was rotated with barley. A higher infestation of *R. solani* occurred during a potato-grain rotation sequence than in continuous potato culture. Both these fungi produce sclerotia in dead tissue of diseased plants. Thus, a rhizosphere effect from living roots is of primary significance only when sclerotia are induced to germinate by root exudates, followed by production of secondary sclerotia. Volatile chemicals emanating from root exudates and the volatile metabolites from microbial activity in the rhizosphere can also affect growth of a fungal pathogen, either by inhibition or stimulation, thereby influencing the potential for reproduction of spores or sclerotia.

The extreme complexity of the soil ecosystem poses great difficulties in pinpointing specific factors that stimulate or suppress the reproduction of pathogens under field conditions. The rhizosphere effect also plays a prominent role. The very nature of modern agriculture, i.e., growing plants in pure stands, offers the pathogen a favourable and abundant substrate for growth and reproduction in the rhizoplane, and for further reproduction as the host declines to a state of dead refuse.

1.5.2 Nematode populations

The majority of nematodes in field soils are free-living, feeding superficially on fungal hyphae, algae, and bacteria that occur on underground stems, roots and organic debris. Since the availability of food for these animals may be influenced significantly by root exudates and related factors, their reproductive capacity is obviously subject to rhizosphere effects. Plant parasitic nematodes, though having a soil phase in their life cycle, feed directly upon living plant tissue and in this respect, their populations are influenced by susceptible host roots (Curl & Truelove, 1986).

Galls on host plant roots may also reflect nematode population size since they may contain these animals. Nematode populations may increase suddenly, as when eggs hatch, or decrease suddenly due to drastic environmental changes. Soil environmental factors also determine the distribution and numbers of nematodes in the soil. On the other hand, because nematodes tend to congregate around roots of growing plants, populations are usually greater than the average per unit weight or volume of the bulk soil. The amount of food available to a nematode is affected by the number feeding at the same site; this could be construed as competition in the rhizoplane, resulting in increased or decreased populations of species. However, the actual rhizosphere effect is limited to the stimulation of egg-hatching by root exudates or the inhibition of nematode activity by toxic substances released by roots of some plants. According to Baker and Cook (1982), lower numbers of *Pratylenchus penetrans* (Cobb) Sher. and Allen. were observed near marigold (*Tagetes* spp. Willd) roots than near other plants, whereas numbers of cysts of *Heterodera rostochiensis* (Wollenweber) were unaffected. The stubby-root nematode, *Trichodorus christiei* (Allen) multiplies rapidly on tomato roots, but does not feed on asparagus roots, which produce a toxic glucoside. Cyst-hatching of *H. rostochiensis*, a golden nematode of potato, can be prevented by growing mustard with the potatoes. The effective chemical is phenyl isothiocyanate released by the mustard plants.

1.6 SAFETY OF MICROORGANISMS INTENDED FOR USE AS PEST AND PLANT DISEASE CONTROL AGENTS

Microorganisms are an enormous, but largely untapped, natural resource for use in biological, control of pests and plant diseases. Microbial biocontrol agents include natural enemies and antagonists of pests. According to Cook *et al.* (1996), two primary reasons why microbes are underemployed for pest and disease control, are:

i) technical difficulties of using microorganisms for biological control, owing to a lack of fundamental information on them and their ecology, and
ii) cost to laboratories, agencies or companies of product development and obtaining regulatory approval, which commonly cannot be justified because the pest-and/or environmental-specific nature of these agents limit their use to niche markets.

Agriculture and forestry benefit greatly from the autochthonous communities of microorganisms responsible for naturally occurring biological control of pest species. However, additional benefits are achieved by introducing or applying microorganisms when or where needed.

Regardless of the approach, the risk factor is the combination of hazards and exposure. Thus, the risks associated with using an agent with some known hazardous properties can be reduced by limiting the exposure. The use of an agent with known hazards and high exposure presents little or no risk. Unfortunately, the hazards associated with microorganisms are often not properly identified and evaluated and the resulting risk or benefit analysis is therefore inaccurate (Cook *et al.*, 1996).

1.6.1 Biosafety Issues

Cook *et al.* (1996) identified four unintended but potentially adverse effects of microbial biocontrol agents on nontarget organisms against which safety measures are required. Humans, domesticated animals, and wildlife were included as examples of nontarget organisms.

These potential safety issues include:

i) competitive displacement

ii) allerginicity

iii) toxigenicity of antibiotics and other biologically active metabolites and

iv) pathogenicity.

Only competitive displacement, allerginicity, and toxigenicity will be considered in this review. These four safety issues represent the unintended adverse effect on target organisms whether the microorganism is allochthonous or autochthonous, naturally occurring or modified by classical genetic or recombinant DNA techniques. Gene transfer offers a means to introduce a specific trait for new or more precise intended effects or to eliminate traits for potential adverse effects.

Through gene transfer, potentially desirable or undesirable traits of microbial biocontrol agents can also be transferred naturally to other microorganisms in the environment. Gene transfer of this kind could result in a new genotype of naturally occurring microorganism less able, more able, or of the same ability as the source microorganism, to establish and maintain its population in competition with other microorganisms. Usually a safety issue would arise if the gene transfer result in a microorganism with the potential to display one of the four unintended adverse effects listed above. Any risks involved would basically depend on factors such as the biology of the recipient organism, nature of the trait transferred, and the environment (Cook *et al.*, 1996).

1.6.1.1 *Competitive displacement (target effect)*

According to Cook *et al.* (1996), the term competitive displacement describes an array of effects resulting from microbe-microbe interactions. These include exclusion, and other outcomes with potential overtime to allow a microorganism introduced or applied for biological control to assume the habitat of nontarget native organisms. A practical example is the application of the saprophytic fungus *Peniophora gigantea* (Fr.:Fr.) Donk as a spore suspension to a freshly cut surface of a pine stump. The application allows the fungus to become established in advance of the arrival of airborne spores of *Heterobasidion annosum* (Fr.:Fr). Bref. which is the main cause of the disease annosus root rot of pine. Without the prior colonization of the stump surface by

P. gigantea, *H. annosum* is capable of colonizing the entire stump whereupon pine trees with roots naturally grafted to those of the colonized stump are attacked. Biological control therefore results from preemption of the foodbase needed by the pathogen to infect pine roots. Application of *P. gigantea* spores can be effected by suspending the spores in a bucket of water and brushing them on freshly cut pine stumps. Alternatively the spores can be suspended in oil used to lubricate the chain saws (Artman, 1972).

Some microorganisms such as yeasts and bacteria have the potential to protect wounds and other infection sites on fruit by prior-establishment and competition with pathogens for infection sites and nutrients.

Nonpathogenic bacteria that produce siderophores (natural iron-chelating compounds), if established in adequate populations in the rhizosphere, proved to be biological control agents of certain root pathogens by depleting iron resources (Kloepper, *et al.*, 1980; Schippers *et al.*, 1987). Biological control can also result from rhizosphere-inhabiting non-pathogens out-competing the pathogen for carbon and energy or nitrogenous compounds.

Biological control, through competitive displacement using strains closely related and ecologically similar to pathogens, has great potential for plant disease management. This includes either naturally occurring non-pathogenic relatives of the pathogen or a pathogen rendered non-pathogenic by deletion or modification of critical genes (Freeman & Rodríguèz, 1993).

1.6.1.2 *Competitive displacement (non-target effect)*

Microorganisms introduced for biological control purposes can potentially preempt or displace non-target microorganisms as one of many microbe-microbe interactions mediated through competitors for infection sites or nutrients. For example, the early and deliberate establishment of the saprophytic *P. gigantea* on freshly cut stumps of pine to preempt establishment of the annosus root rot fungus, could theoretically, also preempt the establishment of some other wood-colonizing saprophyte. Usually this kind of effect is no different from the effects of many other kinds of temporal and spatial displacements of non-target microorganisms in the rhizosphere,

within crop residue, on plants, or elsewhere in the environment associated with many common agricultural practices. Moreover, if the preempted or displaced non-target saprophyte is widespread in nature, and has the ability to colonize other substrates, its unintentional preemption, along with a target pathogen as a colonist of stumps would seem inconsequential to the ecology of the non-target microorganism (Cook *et al.*, 1996). According to Cook *et al.* (1996), there is no reliable way to monitor and document the effects of competitive displacement on the ecology of non-target microorganisms. It might therefore be instructive to determine the extent to which preemption of *H. annosum* as a colonist of freshly cut stumps has impacted on the ecology of this fungus in forest ecosystems.

1.6.1.3 *Allergenicity (target effect)*

According to Cook *et al.* (1996), there are no intended target effects for allergenicity as a mechanism of microbial biocontrol.

1.6.1.4 *Allergenicity (non-target effect)*

Certain kinds of pollen and airborne fungal spores are inevitably present in the air we breath and cause allergies in sensitive people, domestic animals, and wildlife. However, it is only a very small proportion of fungal species that produce spores that cause allergies or allergic reaction in humans. Potentially, a biocontrol microorganism released into the air could cause allergies or elicit allergic reactions in humans (Cook *et al.*, 1996). It has been reported that workers in production facilities exposed repeatedly to high concentrations of spores of fungi such as *Beauveria* or *Metarhizium* spp. may develop hypersensitive reactions, although such reactions are not known for people living in application areas. Allergenicity is therefore a potential safety concern as a result of direct exposure of workers at the production centre, or the application site, but is not likely to be a public health issue. Exposure to allergenic particles of all types is common in agricultural settings. Therefore, allergies resulting from the use of microbial biocontrol agents, although not a new problem, should nevertheless be addressed as a safety issue during development and application.

1.6.1.5 *Toxigenicity (target effect)*

Antibiosis as defined by Cook and Baker (1983), is the inhibition or disruption of the behaviour of one organism by the metabolites of another organism. Endophytes live within leaves, or other plant parts, where they derive a benefit from their host while also producing chemicals disruptive to feeding by insects.

The antibiotic, gliotoxin, has been implicated in the biological control of *Pythium* and *Rhizoctonia* damping-off diseases by the soil-inhabiting fungus *Gliocladium virens* Miller, Giddens and Foster. A product based on this fungus (Gliogard) has been registered for use against *Pythium* and *Rhizoctonia*. According to Lumsden *et al.* (1992), use of root-associated microorganisms that protect roots by producing antibiotics, presents major opportunities for greater use of microbial biocontrol. Typical of antibiotic-producing microorganisms generally, *G. virens* produces its antibiotic only after inoculum has been introduced into the soil.

1.6.1.6 *Toxigenicity (non-target effect)*

Substances such as alkaloids, produced by endophytes in leaves of ryegrass and fescue, that offer protection from insect pests of these grasses also cause ryegrass staggers and fescue toxicosis in livestock allowed to graze on these infected plants (Siegel *et al.*, 1987). Presumably deer and other wildlife that feed on grasses could possibly be affected by endophytes established in grasses used for golf courses, lawns and landscapes.

Antibiotics produced by microorganisms introduced into soil, or other habitats, or with the planting material, for biological control purposes could potentially be toxic to non-target microorganisms naturally present in these habitats. While the potential exists, there are no known or documented examples of such non-target effects, possibly because of the minute quantities of these compounds required for biocontrol activity and /or because of the small-scale use of such biocontrol practices (Cook *et al.*, 1996). *Pseudomonas fluorescens* strain 2-79 shows biocontrol activity against wheat take-all (*Gaeumannomyces graminis* (Sacci) Arx and Oliver var. *tritici* Walker). Since it is able to inhibit the pathogen through the production of phenazine-1-

carbosylate. Paulitz & Linderman (1989) reported that this compound produced in the rhizosphere has no effect on the establishment of mycorrhizal fungi.

Antibiosis is a universal phenomenon in habitats occupied by microorganisms. Furthermore, certain antibiotic-producing traits are highly conserved in bacteria (Cook *et al.*, 1995). As an example, the ability to produce the antibiotic 2,4-diacetylphloroglucinol is a trait of bacteria associated with the natural protection of roots of wheat against take-all in Washington, sugar beet (*Beta vulgaris* L.) against *Pythium* infections in Ireland (Shanahan *et al.*, 1992), and tobacco (*Nicotina tabacum* L.) against black root rot in Switzerland (Défago *et al.*, 1991). Mazzola *et al.* (1992) reported that antibiotic-producing abilities is a natural mechanism of bacteria in the rhizosphere.

1.7 MANAGEMENT OF MICROORGANISMS INTENDED FOR USE AS PEST AND DISEASE CONTROL AGENTS

As defined by Cook *et al.* (1996), "safe use" of biocontrol agents includes not only assessment but also management of any risks or potential risks that may be identified. Usually microorganisms known or suspected to cause unacceptable adverse effects on plants, man and animals are eliminated in the initial stages of the research projects. Some may however undergo further tests either before or after being used commercially. This depends on the benefits and on whether the organism, or its unintended adverse effects, can be reasonably managed. Basically, there are many steps in the research and development process and subsequent commercial use whereby knowledge of, and experience with, the microorganism are accumulated to aid in management of its adverse effects. Safety to workers should be assured at all stages of the research and development process by good agricultural practices. The following management principles or practices are described by Cook *et al.* (1996).

1.7.1 Management based on knowledge of the organism

Predictive value is considered to be one of the useful functions of taxonomy. If a microorganism is known to have certain properties, then a taxonomically related organism will frequently have

similar properties. Although this does not preclude the need to study each organism, it does mean that general predictions can be made about an organism and further studies can be focussed on testing these predictions. As more information is gathered about a genus, each species does not have to be treated as if it was a completely unknown organism, except possibly to gain a better understanding of its real or potential hosts and geographic ranges. Knowledge relevant to the organism may be derived from information provided for purposes of registration of related microbial biocontrol agents.

1.7.2 Management based on knowledge of the environment

A great number of applications of microbial biocontrol agents are made into managed environments. These possibly include, managed non-agricultural environments, such as urban areas, parks, lakes and waterways, and forests; agricultural environments for perennial and annual crops, including ranges, pastures, orchards, open fields, and woodlands, and contained environments such as commercial green houses, households, and processing and storage facilities. Each of these environments, in turn, offers some unique, as well as some common options and challenges for management of microbial biocontrol agents.

1.7.3 Management based on experience with other microorganisms

Much information, based on wide experience, relating to the management of microorganisms or their adverse effects in the environment, is available. This includes experience with the management of economically important plant pathogens and beneficial or economically important microorganisms such as *Rhizobium* spp. and mycorrhizal fungi. Usually the same principles and methods for management of these microorganisms applies to the management of microbial biocontrol agents that produce unintended adverse effects in the environment.

1.7.4 Management during basic research in the field

It is necessary to carry out field experiments during the course of conducting research with microbial biocontrol agents (Cook *et al.*, 1996). In order to obtain pertinent information about

safety and performance of any microbial biocontrol agent, small-scale preliminary field trials are usually required. Such trials may include experiments to obtain more information on survival /persistence as well as dispersal/dissemination of the specific biocontrol agent and its interactions with other microorganisms. Genetically marked microorganisms have been used as a means to study the population dynamics of microbial biocontrol agents (Kluepfel,1993). This allows for more information about their ability to spread and survive in nature. Studies of this nature have confirmed that plant-associated microorganisms introduced into soil remain virtually at the site where introduced and decline to undetectable populations soon after, and sometimes before, the supporting plant completes its life cycle (Cook *et al.*, 1996).

During field research, the main safety issue with biocontrol microorganisms will most likely be their pathogenicity to non-target organisms. According to Cook *et al.* (1996), the potential for such an outcome is remote, since such experiments with non-indigenous microorganisms are carried out only when judgements based on results from studies in the greenhouse or growth chamber, experience in other countries, or reports in the scientific literature indicate with reasonable certainty that the microorganism is safe. Microorganisms with known potential to spread and to multiply as pathogens might require special management during the course of basic field studies.

Several approaches exist for managing microbial biocontrol agents intended for use on plants and for which there is insufficient preliminary safety information (Cook *et al.*, 1996). As an example of this, field trials can be conducted in a remote area, or the experimental site can be protected with buffer strips of the same or different plants. Microorganisms introduced into soil and for which there are safety concerns can be eliminated at the end of the trials by soil fumigation. In several cases, plant associated microorganisms can be effectively managed by no longer growing the supporting plant species. Use of bush, fallowing or crop rotation can be practised if deemed necessary.

1.7.5 Management during production and formulation

Enclosed facilities are typically used in the production and formulation phases of research and development of microbial biocontrol agents. This virtually eliminates the chances for adverse pathogenic effects on non-target plants and animals but increases the chances for worker exposure to microorganisms with known or suspected toxigenic or allergenic effects. With good agricultural practices, these safety issues can be managed effectively with the use of appropriate filters on the equipment and facilities and the use of appropriate dust masks and protective clothing by the workers.

1.7.6 Management during application or release

During application or release of microbial bicontrol agents, workers can be protected by wearing appropriate clothing and gloves to prevent exposure of the skin, or dust masks if airborne spores are involved. Timing of the applications could further minimise the potential for undesirable non-target effects. Potential problems such as drift and other unwanted dissemination can be managed by site-directed methods of application and by timing of applications (Cook *et al.*, 1996).

1.7.7 Post-application management

In most cases, potential unintended adverse effects of microbial biocontrol agents will have been eliminated or prevented by interventions based on experimental data or scientific literature before the microorganism is introduced or applied in the environment. Risks however, may exist after the application is made (Cook *et al.*, 1996). Most of the principles of disease and pest management, including integrated pest management, are relevant to management of unwanted or unintended adverse effects of microbial biocontrol agents after field application or introduction (Cook *et al.*, 1996). Examples of such practices include the use of crop rotation and tillage. Chemical pesticides may be needed in extreme cases or emergencies.

1.7.8 Management with public oversight

It is axiomatic that no responsible scientist involved in the development and implementation of microbial biocontrol would deliberately introduce, or apply as inoculum, a microorganism with known potential for an unmanageable adverse effect on humans or the environment (Cook *et al.*, 1996). Professional standards of scientific conduct are established and continually improved through the informal but highly effective procedures of peer review. Most countries also depend on formal oversight by way of a statutory requirement for permits and approvals. Unfortunately, requirements for microorganisms intended for pest or disease control have been based on requirements developed for chemical pesticides and have not been particularly applicable or appropriate for microorganisms (Cook *et al.*, 1996). For example, regulation of microbial biocontrol agents in the USA is further complicated by a lack of consistently applied, clear definitions for the terms "indigenous" and "non indigenous".

1.8 USE OF MICROBIAL BIOCONTROL: STRATEGIES

Virtually all pest species and plant diseases are subject to some level of natural biological control imposed by pathogenic and other antagonistic effects of microorganisms already present in the environment and are interactive with pest agents (Cook *et al.*, 1996). Usually crop rotation and organic amendments are typical examples of farming practices designed to take advantage of, or enhance the activities of, resident populations of microbial biocontrol agents without having to introduce them. Agriculture and forestry benefit greatly from the resident communities of microorganisms, pathogenic or inhibitory to pest species. Morever, this type of natural biological control is always adequate enough by itself. This can be greatly enhanced by introducing or applying additional microorganisms when and where need arises.

1.8.1 Strategies

There are basically three strategies for use of microorganisms introduced/applied for biological control. These comprise:

i) inoculative release

ii) augmentative application, and

iii) inundative application

While the aim in choosing a strategy could be to reduce cost, limit exposure of non-target organisms or optimize efficiency, the strategy is usually dictated by the biology of the microbial biocontrol agent, the target pest or both (Cook *et al.*, 1996).

Inoculative release usually seeks to introduce the agent once or only occasionally into the environment, with the intention that it will establish itself as a sustained population and impose some degree of biological control. This strategy, followed for biological control of an established allochthonous pest species with an autochthonous natural enemy (pathogen) of that pest species, is defined as "classical biological control".

Augmentation applications seek to supplement the resident population of a microbial biocontrol agent by applying a microorganism already present, either naturally or because of a previous introduction/application (Cook *et al.*, 1996). Usually biological control results from the subsequent increase of the microbial population to an effective population density prior to economic damage caused by the target pest.

Inundative applications seek to elevate the population of a microbial biocontrol agent to an instantly very high and timely population density to ensure maximum and rapid suppression or elimination of the target pest species. According to Cook *et al.* (1996), there is nothing inherent in the three strategies, viz; inoculative, augmentative, or inundative, that raise a safety issue. Considerations should therefore be given to how the microbial biocontrol agent is applied. For instance, a microbial biocontrol agent with potential to cause an allergy would more likely raise a question of risk if applied aerially, as an aerosol or dusting than if applied directly to soil, seeds or water. An agent with known or suspected toxigenic properties would more likely raise a question of risk if it was used to treat plant parts consumed by people, livestock, or wildlife than if introduced into soil or applied as a seed treatment or root-dip for transplants.

The search for rhizosphere microorganisms for plant growth stimulation and biological control of soil-borne plant pathogens has come to stay. More research must be carried out on soil physical and chemical factors influencing root colonization and the expression of traits important to bacterial antagonism in the rhizosphere. Identification of these factors will be advantageous because they will make it possible to manipulate these factors in the field to enhance root colonization. Formulation and delivery of bacterial preparations and also on development of inexpensive and easily applied bacterial preparations that will remain active even under less than optimal conditions, must still be evaluated.

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CHAPTER 2

2[1] Survival of Biostart™ *Bacillus* spp. introduced into soil

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Biostart™ comprising six *Bacillus* spp.: *Bacillus chitinosporus*, *B. uniflagellatus*, *B. laterosporus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, was assessed for their ability to survive in soil in pots. Initially, the *Bacillus* spp. populations declined rapidly in soils planted with cucumber seedlings and supplemented with or without NutriStart-AC. A similar situation was observed in soils without cucumber seedlings and supplemented with or without NutriStart-AC, but populations stabilized at Day 21 through Day 35 for all six *Bacillus* spp. Few background indigenous *Bacillus* colonies were counted on *Bacillus* medium plates from the control experiments.

2.1 INTRODUCTION

Bacteria have frequently been introduced into soils for the promotion of plant growth (Broadbent *et al.*,1977) and suppression of soilborne plant pathogens (Alderich & Baker, 1970). Although some noteworthy successes have been obtained in agricultural trials, a major problem has been the poor reproducibility, and variability in results obtained. Reasons could be varying degrees of establishment and survival of the introduced bacteria. In general, population sizes of bacteria decline rapidly once introduced into natural soils. Furthermore growth of introduced microbial populations in undisturbed soils is a rare phenomenon and is referred to as microbiostasis (Ho & Ko, 1985). The growth/survival inhibitory effect has been attributed to a paucity of available nutrient sources to such introduced microbes in soil and also to the hostility of the soil environment to incoming microbes, due to a myriad of adverse abiotic and biotic factors (van Veen *et al.*, 1997).

[1]Chapter format according to Biocontrol Science and Technology

Abiotic factors such as soil moisture, temperature, pH, texture, oxygen and nutrient availability have been suggested as major factors governing the survival of introduced bacteria in soil (van Veen *et al.*, 1997). On the other hand, predation by protozoa, microbial antagonism and competition are considered the main biological factors affecting non-indigenous microorganisms. Despite this knowledge, the comprehensive understanding necessary to predict bacterial survival and population dynamics under field conditions is still lacking.

Quantitative studies of the dynamics of bacterial populations in the rhizosphere are essential in elucidating bacteria - root interactions (Suslow, 1982). If the deliberate introduction of microbial antagonists to improve crop yields is to be optimized, then the population dynamics of the introduced strains has to be monitored.

An organism(s) introduced into soil must have a selective characteristic which does not interfere with its inherent ability to survive or colonize the environment (Schippers *et al.*, 1987). Population decline has been observed for a wide variety of newly introduced bacteria, irrespective of their origin.

This study provides an opportunity to assess and study the survival of useful and available commercial strains of probiotic *Bacillus* spp. in soil. Various spp. of *Bacillus* have been reported as biological control agents (Weller, 1988; Oedjijono *et al.*, 1993), with plant growth promoting abilities (Shishido *et al.*, 1995).

The purpose of this work was two fold: Firstly, to study the population trends of Biostart™ in soil in the absence and presence of a crop plant (cucumber seedlings) *Cucumis sativus* L. Secondly, NutriStart-AC was evaluated as a supplement in both trials.

2.2 MATERIALS AND METHODS

Microorganisms

Six *Bacillus* spp. were used. The *Bacillus* spp. were provided commercially by Microbial Solutions[2] as concentrated spore suspensions at a concentration of 10^9 cells ml^{-1}.

[2] Microbial Solutions (Pty)Ltd., P.O. Box 1180, Strubens Valley 1735, South Africa

Indigenous *Bacillus* population estimation

A cultivated Hutton soil containing fertilizer and organic matter collected from Pietermaritzburg was used for this study. Soil samples were weighed in duplicate and serially diluted in 9 ml quarter-strength Ringer's solution to make up a 10^{-1} single dilution. The two samples were heat treated at 80 °C for 15 minutes in a water bath shaker at 80 rpm. Serial 10-fold dilutions were made from the heated samples and 1 *ml* of appropriate dilutions (10^3-10^4) were plated on *Bacillus* medium (Atlas, 1993). Plates were incubated at 30 °C and colonies counted after 24 h. This was done to estimate the number of indigenous *Bacillus* populations in the original soil.

Bacterial population in soil with or without additional NutriStart-AC

Twenty-four 12.5 cm (about 550 *ml*) pots were filled with soil. The soil was analysed by the Cedara Fertilizer Advisory Services[3] according to Farina & Channon, (1988). An analysis of the soil used is presented in Table 2.1. Prior to inoculation of the pots, each of the six *Bacillus* spp. were cultured separately overnight in 250 *ml* conical flasks. For culturing, 1.2 g of NutriStart-AC were weighed into six separate 250 *ml* conical flasks. Fifty *ml* quantities of distilled water were added to each flask and swirled gently to form a homogeneous mixture. This was sterilised for 15 min at 121 °C and cooled. Two *ml* quantities of concentrated spore suspension (2 x 10^9 cells) of each *Bacillus* spp. were added separately to each of the six conical flasks, labelled and incubated in a water bath shaker at 30 °C for 18 h at 150 rpm. Prior to inoculation into pots, colony forming units (cfu's) were determined for each of the cultures by dilution plating on *Bacillus* medium (Parkinson *et al.*, 1971). Two and a half *ml* aliquots of each culture were inoculated separately into four pots. Two of the pots were supplemented with 1 g of NutriStart-AC dissolved in 5 *ml* of distilled water while the other two pots were unsupplemented. Four uninoculated pots, two containing 1 g of NutriStart-AC dissolved in 5 *ml* of distilled water and two unsupplemented, served as controls. All treatments were in duplicate. The pots were randomly arranged in a polycarbonate seedling tunnel where temperatures were controlled at approximately 26 °C by an evaporative cooling system. Pots were watered three times daily by microjet irrigation. The water used contained soluble fertilizer [3.1.3 (38)] Ocean Agriculture[4])

[3]Cedara Agricultural Development Institute, Private Bag X9059, Pietermaritzburg 3200 South Africa

[4]Ocean Agriculture, P.O.Box 741, Mulders Drift 1747, Republic of South Africa

applied at a rate of 1 g l^{-1} to give 100 mg l^{-1} N, 33 P and 100 K.

Table 2.1. General analysis of experimental potting soil

Soil density	g ml^{-1}	1.16
Phosphorus (P)		85
Potassium (K)		270
Calcium (Ca)	mg l^{-1}	1128
Magnesium (Mg)		213
Zinc (Zn)		17.5
Manganese (Mn)		3
Exchange acidity	cmol l^{-1}	0.11
Total cations		8.18
Acid sat.		1
NIRS organic carbon	%	2.7
NIRS clay		1.8
pH (KCl)	-	5.58

NutriStart-AC (Nutrient supplement) analysis

A laboratory analysis on NutriStart-AC was done to determine the C:N ratio and also the macro- and micro-nutrients present. The analysis was done by Cedara Fertilizer Advisory Services using the methods of Farina (1981); Perstorp (1993) and Matejovis (1996). Table 2.2 below presents the results of the NutriStart-AC analysis. The difference between the combustion and the Kjeldahl nitrogen is due to greater efficiency of the combustion method. The combustion method determines nitrogen regardless of the complexity of the nitrogen-containing compounds present in the sample. Nitrogen values using the Kjeldahl method are commonly lower when compared to a combustion value. This analysis was used as a basis of research in Chapters 2,3,4,5 and 6.

Table 2.2. General analysis of NutriStart-AC (Nutrient supplement). Data is on a 100% dry matter basis.

CNS		
Nitrogen (N)		5.19
Sulphur (S)	%	0.7
Carbon (C)		43.6
Kjeldahl		
Nitrogen (N)	%	4.01
Protein		25.04
Calcium (Ca)		2.8
Magnesium		0.55
Potassium (K)	%	4.32
Sodium (Na)		0.1
Phosphorus (P)		0.73
Zinc (Zn)		31
Copper (Cu)	ppm	11
Manganese (Mn)		316
Boron (B)		7

Calculated C:N = 8:1 (based on the values obtained from the combustion method)

Soil samples were taken from each duplicate pot on Days 1, 2, 4, 8, 14, 21, 28 and 35. After mixing, two grams were weighed out from each soil sample, mixed together and one gram was weighed out and suspended in 9 ml quarter-strength Ringer's solution. This was heated at 80 °C for 15 minutes in a water bath shaker at 80 rpm. This treatment was performed to eliminate all non-spore formers in order to quantitate only spore-forming bacilli. For enumeration, appropriate dilutions of the treated samples were plated on *Bacillus* medium in duplicates and incubated at 30 °C. Colonies were counted after 24 h of incubation and the mean numbers of colonies calculated from the duplicate plates. Soil samples were taken from the same region in each pot at each sampling time.

Bacterial population in soil in the presence of cucumber seedlings with or without NutriStart-AC

Twenty-four cucumber seeds were planted in each of 24 12.5 cm diameter soil-filled pots. The pots were transferred to a polycarbonate seedling tunnel after three days in a germination room (20-24 °C). A week after germination, the pots were drenched separately with cultures of the six *Bacillus* spp. prepared as described for the soil test. Twelve of the pots were supplemented with a drench containing 1 g of NutriStart-AC dissolved in 5 *ml* distilled water. All treatments were duplicated. The pots were watered and fertilized daily as described previously. Soil samples (1 g wet soil) were taken from the root zone from each pot on Days 1, 2, 4, 8, 14, 21, 28 and 35. Samples were taken from the same region in each pot at each sampling time and treated as described previously. Enumeration of cfu's per gram wet soil was performed as described before. Results were presented as Tables and Figures. Tables give the details of individual *Bacillus* spp., while Figures show the survival trend of the introduced *Bacillus* spp. Population sizes were presented as log cfu g^{-1} of wet soil as described by Liu & Sinclair, (1992); Podile, (1994); Kim *et al.*, (1997).

Statistical Analysis

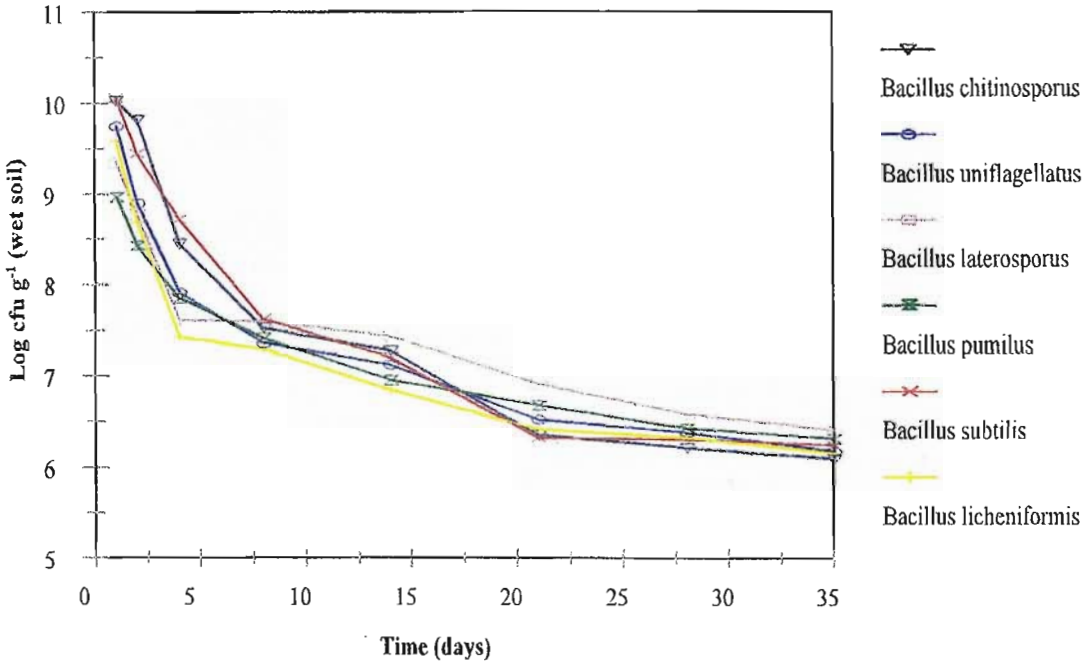
A general linear model (GLM) was used to run an Analysis of Variance and a Linear Regression on the results using the computer statistical package, Statistical Analysis System (SAS, 1987).

2.3 RESULTS

Table 2.3. Populations of Biostart™ cultures used to inoculate potting soil with or without cucumber seedling

Biostart™ cultures	Pots without cucumber seedlings (log cfu)	Pots with cucumber seedlings (log cfu)
Bacillus chitinosporus	11.25	10.35
Bacillus uniflagellatus	11.38	11.56
Bacillus laterosporus	11.40	11.54
Bacillus pumilus	11.32	10.24
Bacillus subtilis	11.28	11.47
Bacillus licheniformis	11.30	10.51

a)



b)

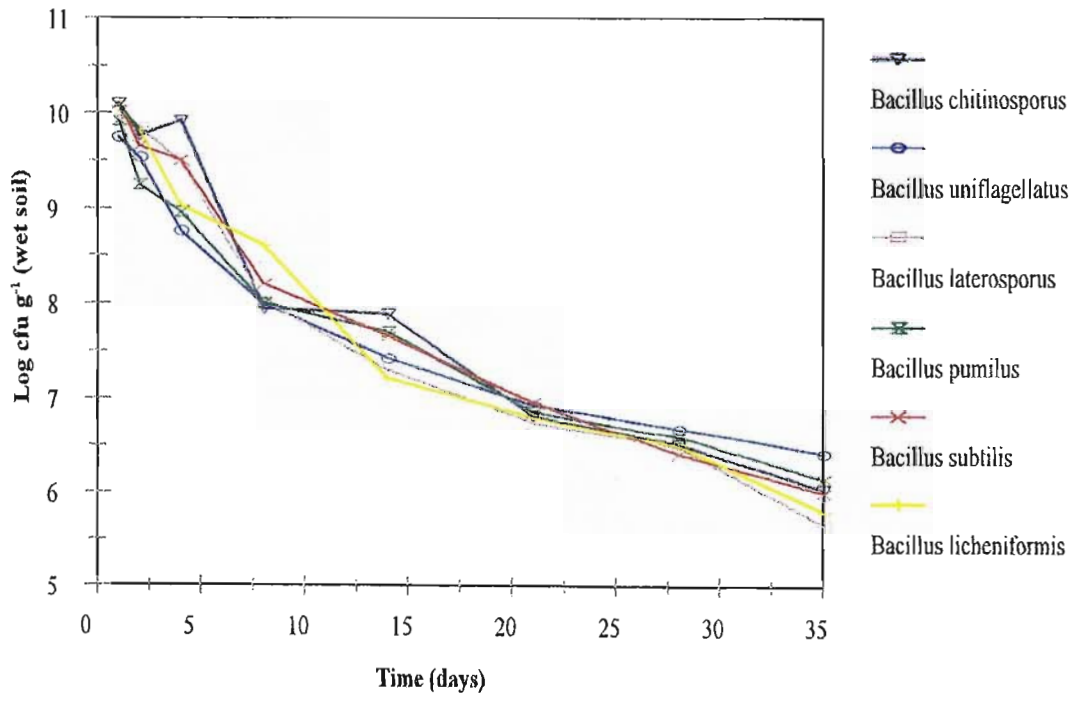
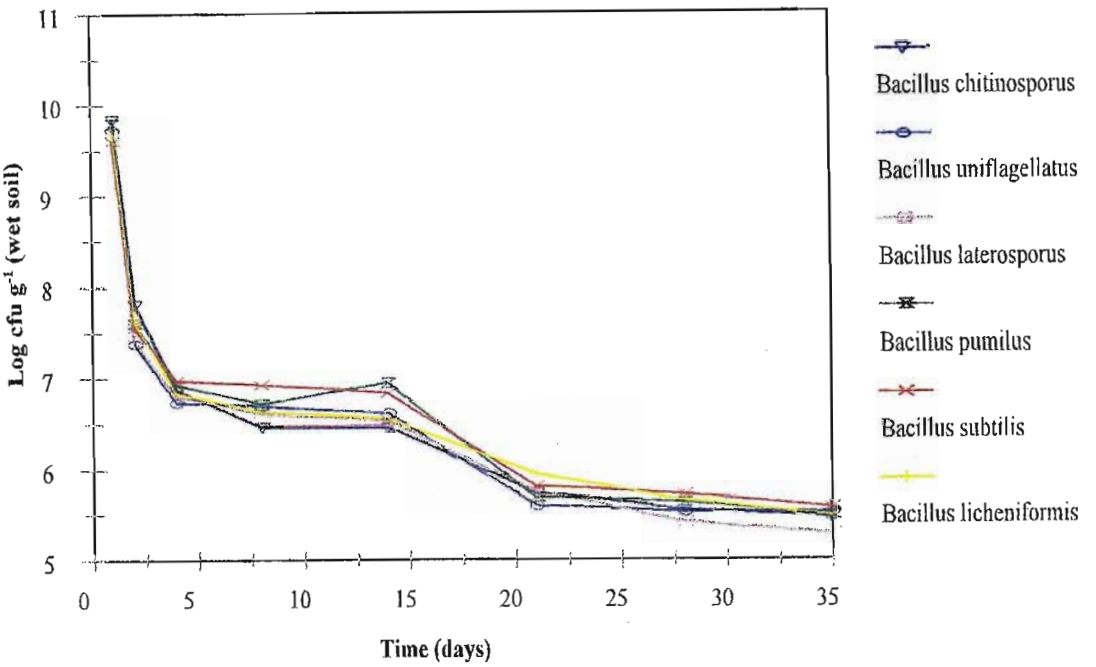


FIGURE 2.1. Population trends of the six Biostart™ *Bacillus* species introduced into the potting soil: a) in the absence of cucumber seedlings without NutriStart-AC, and b) in the absence of cucumber seedlings with NutriStart-AC over a period of 35 days.

a)



b)

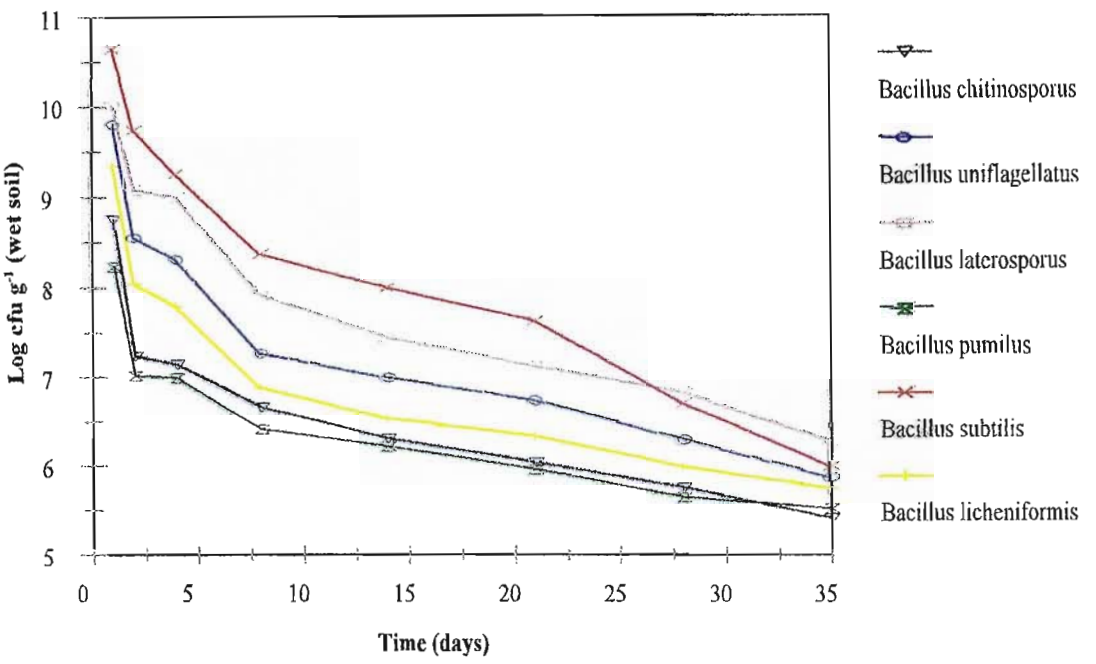


FIGURE 2.2. Population trends of the six Biostart™ *Bacillus* species introduced into potting soil: a) in the presence of cucumber seedlings without NutriStart-AC, and b) in the presence of cucumber seedlings with NutriStart-AC over a period of 35 days.

Bacterial population trends in soils with or without NutriStart-AC

In pots without NutriStart-AC supplement, all six introduced *Bacillus* spp. decreased in population numbers from Day 1 to Day 14 and then stabilized between 6.32 and 6.09 log cfu g^{-1} of wet soil from Day 21 till the termination of the experiment on Day 35 (Figure 2.1; Appendix 2.1). No significant difference (P = 0.76) was observed between survival rates/trends in numbers among all six *Bacillus* spp. (Appendix 2.1). On the other hand, a highly significant difference (P = 0.0001) was observed between the numbers of bacteria on the various sampling days (Appendix 2.1).

In pots with NutriStart-AC supplement, no significant difference (P = 0.63) was observed between survival rates/trends of all six *Bacillus* spp. introduced into soil (Appendix 2.1). However, significant differences (P = 0.001) was observed between bacteria numbers on the various sampling days (Appendix 2.1).

Population sizes differed in magnitude for all six *Bacillus* spp. introduced into soil. Population sizes of:

- *B. chitinosporus* decreased from 10.03 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 3 log cfu. The population size then declined by 1 log cfu and stabilized between 6.35 and 6.09 log cfu g^{-1} of wet soil from Day 21 till the termination of the experiment on Day 35. With NutriStart-AC supplement, population sizes declined from 10.11 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 2 log cfu. A further 2 log cfu decline was observed on Day 14. Populations then stabilized between 6.28-6.04 log cfu g^{-1} of wet soil from Day 21 through to Day 35. Population numbers on Day 4 was 1 log cfu more than what was observed in pots without NutriStart-AC supplement.
- *B. uniflagellatus* declined from 9.75 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 2 log cfu. Population then stabilized between 6.52-6.17 log cfu g^{-1} of wet soil from Day 21 to Day 35. With NutriStart-AC supplement, population decreased from 9.75 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 1 log cfu as compared to 2 log cfu decrease in potting soil without NutriStart-AC supplement. A further 1 log cfu decrease was observed on Day 14 and populations remained constant between 6.93-6.42 log cfu g^{-1} of wet soil until termination of the experiment on Day 35.

- *B. laterosporus* decreased from 9.35 log cfu g^{-1} of wet soil from Day 1 to Day 4 through Day 8 to Day 14 by 2 log cfu. Populations then stabilized from between 6.91-6.60 log cfu g^{-1} of wet soil from Day 21 till Day 35. With NutriStart-AC supplement, population sizes decreased from 10.02 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 1 log cfu and a further 1 log cfu less on Day 8 as compared to a 2 log cfu decrease in unsupplemented NutriStart-AC potting soil. Populations then stabilized between 6.75-6.55 log cfu g^{-1} of wet soil from Day 21through Day 35 (Appendix 2.1).
- *B. pumilus* declined from 8.97 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 3 log cfu. Population remained stable between 6.68-6.31 log cfu g^{-1} of wet soil from Day 21 through Day 35. With NutriStart-AC supplement, population numbers decreased from 9.92 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 2 log cfu as compared to a decrease of 3 log cfu in soil unsupplemented with NutriStart-AC. A further decline of 1 log cfu was observed on Day 21 and then populations stabilized between 6.86-6.15 log cfu g^{-1} of wet soil from Day 21 through Day 35.
- *B. subtilis* decreased from 10.06 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 2 log cfu. A further decrease of 1 log cfu was observed by Day 14. Population then stabilized between 6.32-6.24 log cfu g^{-1} of wet soil from Day 21 till termination of the experiment on Day 35. In pots with NutriStart-AC supplement, population sizes decreased from 10.09 log cfu g^{-1} of wet soil from Day 1 to Day 8 by 2 log cfu as compared to 3 log cfu in soil unsupplemented with NutriStart-AC. Population numbers then stabilized between 6.96-6.00 log cfu g^{-1} of wet soil from Day 21 through Day 35 after a further decline from Day 8 to Day 14 by 1 log cfu.
- *B. licheniformis* decreased from 9.58 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 3 log cfu. Population then stabilized between 6.14-6.15 log cfu g^{-1} of wet soil from Day 21 through Day 35. In pots supplemented with NutriStart-AC, population sizes decrease from10.09 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 1 log cfu as compared to 2 log cfu in the unsupplemented NutriStart-AC soil. A further decline of 2 log cfu was observed on Day 21 and population numbers then stabilized between 6.80-6.02 log cfu g^{-1} of wet soil from Day 21 till the termination of the experiment on Day 35.

Population trends in potting soil in the presence of cucumber seedlings with or without NutriStart-AC supplement

The fate of all six introduced *Bacillus* spp. in the presence of cucumber seedlings with or without NutriStart-AC is shown in Figure 2.2. Detailed population numbers, expressed in log cfu g^{-1} of wet soil is presented in Appendix 2.2. In pots without NutriStart-AC supplement, no significant difference (P = 0.93) was observed between survival rates/trends in numbers among all six *Bacillus* spp. (Appendix 2.2). However, a highly significant difference (P = 0.0001) was observed between the bacterial numbers on the various sampling days (Appendix 2.2).

In pots with NutriStart-AC supplement, a highly significant difference (P = 0.0001) was observed between survival rates/trends in numbers among all six *Bacillus* spp. introduced into potting soil. Likewise, a highly significant difference (P = 0.0001) was observed between the bacterial numbers on the various sampling days.

Population sizes of:

- *B. chitinosporus* decreased from 9.85 log cfu g^{-1} of wet soil from Day 1 to Day 8 by 3 log cfu in pots without NutriStart-AC supplement. A further decrease in numbers, 1 log cfu, was observed from Day 8 to Day 21 and then stabilized between 5.74-5.48 log cfu g^{-1} of wet soil by Day 35. In pots supplemented with NutriStart-AC, population sizes declined by 2 log cfu from Day 1 to Day 8 as compared to 3 log cfu in pots without NutriStart-AC supplement. Population then stabilized between 5.56-5.41 log cfu g^{-1} of wet soil from Day 28 to Day 35.
- *B. uniflagellatus* decreased from 9.73 log cfu g^{-1} of wet soil from Day 1 to Day 8 by 3 log cfu in pots unsupplemented with NutriStart-AC. Population numbers then stabilized between 5.6-5.8 log cfu g^{-1} of wet soil from Day 21 till termination of the experiment on Day 35 after a decrease of 1 log cfu from Day 4 to Day 21. In pots supplemented with NutriStart-AC, population numbers decreased from 9.81 log cfu g^{-1} of wet soil from Day 1 to Day 8 by 2 log cfu as compared to 3 log cfu in pots unsupplemented with NutriStart-AC. A further decline of 3 log cfu was observed till termination of the experiment on Day 35.
- *B. laterosporus* decreased from 9.64 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 3 log

cfu in pots unsupplemented with NutriStart-AC. A further decline of 1 log cfu was observed from Day 4 to Day 21 and stabilized between 5.76-5.29 log cfu g^{-1} of wet soil from Day 21 to Day 35. In pots supplemented with NutriStart-AC, population numbers decreased from 10.01 log cfu g^{-1} of wet soil from Day 1 to Day 4 as compared to 3 log cfu decrease in pots unsupplemented with NutriStart-AC. A further decrease of 3 log cfu was observed from Day 4 to Day 28. Population remained constant between 6.84-6.28 log cfu g^{-1} of wet soil from Day 21 till termination of the experiment on Day 35.

- *B. pumilus* decreased from 9.80 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 3 log cfu in pots without NutriStart-AC supplement. A further decline of 1 log cfu was observed from Day 4 to Day 21 where population stabilized between 5.69-5.50 log cfu g^{-1} of wet soil by Day 35. In pots supplemented with NutriStart-AC, population decreased from 8.24 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 1 log cfu as compared to 3 log cfu in pots unsupplemented with NutriStart-AC. Further decrease of 2 log cfu was observed from Day 4 to Day 21 where population stabilized between 5.96-5.52 log cfu g^{-1} of wet soil from Day 21 to Day 35.
- *B. subtilis* decreased from 9.61 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 3 log cfu in pots unsupplemented with NutriStart-AC . A further decline in numbers of 1 log cfu was observed from Day 4 to Day 21 and then stabilized between 5.82-5.58 log cfu g^{-1} of wet soil from Day 21 to Day 35. In pots supplemented with NutriStart-AC, population decreased from 10.65 log cfu g^{-1} of wet soil from Day 1 to Day 14 by 2 log cfu as
- compared to 3 log cfu in pots unsupplemented with NutriStart-AC. A further decline of 2 log cfu was observed from Day 14 to Day 28 where population remained stable between 6.72-6.00 log cfu g^{-1} of wet soil from Day 28 to Day 35.
- *B. licheniformis* decreased from 9.69 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 3 log cfu in pots without NutriStart-AC supplement. A further decline of 3 log cfu in population numbers was observed from Day 4 to Day 21. Population then stabilized between 5.59-5.49 from Day 21 to Day 35. In pots supplemented with NutriStart-AC, population decreased from 9.35 log cfu g^{-1} of wet soil from Day 1 to Day 4 by 2 log cfu as compared to 3 log cfu in pots without NutriStart-AC supplement. A further decrease of 2 log cfu was observed between Day 4 and Day 35.

Generally all six probiotic *Bacillus* spp. decreased in numbers by 3 log cfu in pots without NutriStart-AC supplement from Day 1 to Day 14. The trend was quite different from the pots supplemented with NutriStart-AC.

Few colonies of indigenous *Bacillus* spp. strains were isolated from uninoculated control soil. Background *Bacillus* spp. that were isolated from the soil were therefore low enough to permit selective enumeration of the introduced bacteria throughout the study. The highest number of background *Bacillus* spp. (15 colonies) were counted at 10^{-3} dilution on Day 35 and were therefore not listed in the results.

2.4 DISCUSSION

Information regarding the fate of bacteria introduced into soil is essential before the organism can be used in the rhizosphere to manage or control plant disease (Liu & Sinclair, 1992). This information will assist in the understanding of the relationship between the bacteria and the indigenous microflora.

Population dynamics of six commercially available *Bacillus* spp. were studied in an attempt evaluate their survival in soil under tunnel conditions. These *Bacillus* spp. were selected because they are widely being sold in South Africa as plant probiotics. These microbial systems were originally formulated in USA and it is essential that their survival is studied in a South African soil if it is to be continually used as plant probiotics in the country.

The most obvious evidence was that all six probiotic *Bacillus* spp. survived after 35 days of introduction into potting soil. Generally all six probiotic *Bacillus* spp. populations stabilized by the time the experiment was terminated. Although there is a general decrease in population for all six *Bacillus* spp., decrease in population numbers were different for all *Bacillus* spp. The net results were that the population sizes of the six *Bacillus* spp. were roughly equal in pots without cucumber seedlings by the termination of the experiment. A similar situation was observed in seedling trials without NutriStart-AC supplement, but the situation was quite different with the seedling trial supplemented with NutriStart-AC. Population sizes of *B. laterosporus* and

B. subtilis in seedling trials with NutriStart-AC supplement were higher than the remaining four *Bacillus* spp.

None of the *Bacillus* spp. increased in population size except *B. chitinosporus* which increased in population size in pots without cucumber seedlings but supplemented with NutriStart-AC. Population of *B. chitinosporus* increased slightly from Day 2 to Day 4 but decreased afterwards. The reason for this increase is unknown. According to Van Elsas *et al.*, (1986), population sizes of a *B. subtilis* strain was also found to decrease in field soil and maintained at a stable level over a period of 120 days. Similar results were recorded by Kim *et al.*, (1997) who reported that populations of *Bacillus* spp. L324-92R_{12} remained constant or increased slightly over the period of 150 days. *Bacillus subtilis* strain AF1 was recovered after 28 days upon introduction into non-sterile soils (Podile, 1994). These observations could confirm our results that although the introduced *Bacillus* spp. population declined soon after introduction into potting soil, they could be recovered after long periods of time.

NutriStart-AC did not have any major detectable effect on the bacteria population sizes. Apart from the slow decline in numbers where NutriStart-AC was added as a nutrient additive, the net population sizes for all six *Bacillus* spp. were roughly equal in pots without cucumber seedlings. A slightly detectable difference was observed in pots with cucumber seedlings where the population sizes of the various *Bacillus* spp. differed by the termination of the experiment. The absence of a lasting effect of NutriStart-AC supplement in potting soil suggests either a lack of additional nutrients or rapid exhaustion of NutriStart-AC.

The ability of these six *Bacillus* spp. to survive in soil through a period of 35 days could be important in preventing plant disease and enhance growth promotion. If the antifungal /antibacterial implicated in biological control by *Bacillus* spp. is exploited (Leifert *et al.*, (1995), then the application of these probiotic *Bacillus* spp. to either seeds or directly to plant roots as a drench could protect the seed or plant from soil-borne pathogens. Also *Bacillus* spp. has been implicated for the production of plant growth promoting metabolites (Manero *et al.*, 1996). This implies that persistence or survival in soil could enhance plant growth by the production of plant growth metabolites if these six *Bacillus* spp. are applied to seed or as a soil drench to seedlings.

Alternatively, large populations of these *Bacillus* spp. on either germinating seeds or plant roots could consume nutrients that are consumed by microbial flora that usually colonise plant roots or seeds. Competition of these nutrients could alter the composition of rhizosphere microbial communities, affect the density of heterotrophic bacteria in the rhizosphere, and contribute to disease prevention (Halverson & Handelson, 1991).

The heat treatment technique employed in this study to select introduced *Bacillus* spp. could have negative effects on the *Bacillus* cells as some may be killed or destroyed. The 80 ^{0}C heat treatment applied could cause damage to the *Bacillus* cells, especially if vegetative cells are present. In future trials, a more precise method such as use of immuno detection methods with specific antibodies for labelling could be employed. This will allow easy identification of introduced *Bacillus* spp. under greenhouse and field conditions. It will also facilitate easier follow up procedures to determine what happens to the introduced *Bacillus* spp. in soil.

More work, however, needs to be done in this area before selected bacteria could be routinely applied for plant growth promotion and disease control purposes.

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2.6 APPENDIX

Appendix 2.1. Population trends of the six *Bacillus* spp. in the absence of cucumber seedlings: a) without NutriStart-AC, and b) with NutriStart-AC. Figures are presented as log cfu's g^{-1} of wet soil.

a)

Organisms	SAMPLING DAYS							
	1	2	4	8	14	21	28	35
	LOG CFU							
B. chitinosporus	10.03	9.82	8.45	7.52	7.29	6.35	6.21	6.09
B.uniflagellatus	9.75	8.9	7.91	7.36	7.12	6.52	6.38	6.17
B. laterosporus	9.35	8.78	7.61	7.59	7.43	6.91	6.58	6.40
B. pumilus	8.97	8.43	7.85	7.41	6.96	6.68	6.43	6.31
B. subtilis	10.06	9.45	8.72	7.62	7.20	6.32	6.30	6.24
B. licheniformis	9.58	8.69	7.42	7.29	6.85	6.41	6.32	6.15

Analysis of Variance table of results

Source	Degree of freedom	Sum of Square	Mean Square	F value	P Value
Isolates	5	2.26	0.45	0.51	0.76^{ns}
Days	7	130.07	18.58	20.99	0.0001***
Isolates*Days	35	7.13	0.20	0.23	1.00^{ns}

ns = not significant at $P > 0.05$

*** = highly significant at $P \leq 0.0001$

C.V. = 12.49

Linear Regression Analysis

Error Mean Square = 0.63

Intercept = 9.74

Slope = - 0.49

R- square = 0.67

Fitted Equation Y = 9.74 - 0.49 * X

b)

Organisms	SAMPLING DAYS							
	1	2	4	8	14	21	28	35
	LOG CFU							
B. chitinosporus	10.11	9.76	9.92	7.94	7.89	6.82	6.52	6.04
B.uniflagellatus	9.75	9.53	8.76	7.99	7.42	6.93	6.69	6.42
B. laterosporus	10.02	9.85	9.48	8.02	7.30	6.75	6.48	6.55
B. pumilus	9.92	9.25	8.96	8.00	7.70	6.86	6.60	6.15
B. subtilis	10.09	9.65	9.50	8.20	7.65	6.96	6.41	6.00
B. licheniformis	10.07	9.82	9.02	8.61	7.21	6.80	6.50	6.02

Analysis of Variance table of results

Source	Degree of freedom	Sum of Square	Mean Square	F value	P Value
Isolates	5	1.52	0.30	0.69	0.63^{ns}
Days	7	191.6	27.37	62.29	0.001**
Isolates*Days	35	6.35	0.18	0.41	0.99^{ns}

ns = not significant at $P > 0.05$

** = significant at $P \leq 0.001$

C.V. = 8.30

Linear Regression Analysis

Error Mean Square = 0.26

Intercept = 10.69

Slope = - 0.59

R- square = 0.87

Fitted Equation Y = 10.69 - 0.59 * X

Appendix 2.2. Population trends of the six *Bacillus* spp. in the presence of cucumber seedlings: a) without NutriStart-AC, and b) with NutriStart-AC. Figures are presented as log cfu's g^{-1} of wet soil.

a)

Organisms	SAMPLING DAYS							
	1	2	4	8	14	21	28	35
	LOG CFU							
B. chitinosporus	9.85	7.81	6.88	6.46	6.46	5.74	5.56	5.48
B.uniflagellatus	9.73	7.38	6.74	6.7	6.62	5.6	5.53	5.53
B. laterosporus	9.64	7.41	6.81	6.61	6.54	5.76	5.42	5.29
B. pumilus	9.8	7.61	6.93	6.72	6.59	5.69	5.64	5.50
B. subtilis	9.61	7.56	6.98	6.93	6.84	5.82	5.73	5.58
B. licheniformis	9.69	7.62	6.84	6.64	6.56	5.59	5.66	5.49

Analysis of Variance table of results

Source	Degree of freedom	Sum of Square	Mean Square	F value	P Value
Isolates	5	0.4	0.08	0.26	0.93^{ns}
Days	7	162.94	23.3	74.78	0.0001***
Isolates*Days	35	0.78	0.02	0.07	1.00^{ns}

ns = not significant at $P > 0.05$

*** = highly significant at $P \leq 0.0001$

C.V. = 8.25

Linear Regression Analysis

Error Mean Square = 0.49

Intercept = 9.08

Slope = - 0.51

R- square = 0.74

Fitted Equation Y = 9.08 - 0.51 * X

b)

Organisms	SAMPLING DAYS							
	1	2	4	8	14	21	28	35
	LOG CFU							
B. chitinosporus	8.75	7.24	7.15	6.67	6.04	6.04	5.76	5.41
B.uniflagellatus	9.81	8.56	8.32	7.28	7.00	6.85	6.30	5.88
B. laterosporus	10.01	9.08	9.00	7.92	7.45	7.13	6.84	6.28
B. pumilus	8.24	7.02	7.00	6.42	6.22	5.96	5.65	5.52
B. subtilis	10.65	9.75	9.26	8.38	8.00	7.63	6.72	6.00
B. licheniformis	9.35	8.05	7.80	6.90	6.55	6.34	6.00	5.76

Analysis of Variance table of results

Source	Degree of freedom	Sum of Square	Mean Square	F value	P Value
Isolates	5	41.44	8.28	30.24	0.0001***
Days	7	123.13	17.59	64.17	0.0001***
Isolates*Days	35	5.44	0.15	0.57	0.95^{ns}

ns = not significant at $P > 0.05$

*** = highly significant at $P \leq 0.0001$

C.V. = 7.14

Linear Regression Analysis

Error Mean Square = 0.69

Intercept = 9.50

Slope = - 0.48

R- square = 0.64

Fitted Equation Y = 9.50 - 0.48 * X

CHAPTER 3

3[1] Evaluation of Biostart™, a *Bacillus*-based plant probiotic as a plant growth stimulant on containerised seedlings

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The effect of plant growth of seven probiotic *Bacillus* spp. and Biostart® 2000 (a combination of three of the seven species) were studied on four crops. All species were found to stimulate plant growth of all four crops tested especially when supplemented with 4% NutriStart-AC. Growth stimulation as high as 466% was recorded on lettuce treated with Biostart® 2000. Similar results were recorded for *B. laterosporus*, *B. chitinosporus*, *B. licheniformis* and *B. subtilis*.

3.1 INTRODUCTION

The beneficial effects of plant growth-promoting rhizobacteria (PGPR) have been studied for several field and nursery crops. The success of plant growth promotion by the introduction of PGPR depends largely on their timely establishment and persistence throughout the growing season (Schippers *et al.*, 1987). There are several mechanisms by which PGPR enhances plant growth. These include production of extracellular growth-promoting chemical substances and iron-chelating siderosphores (Schippers *et al.*, 1987) and antibiotics (Weller, 1988). Plant growth promoting rhizobacteria products tend to reduce the population of major root pathogens and compete for energy-yielding nutrients (Elad & Chet, 1987). They also induce plant resistance and mineralize soil nutrients which results in enhancement of nutrient uptake by the plant (Lifshitz *et al.*, 1987). Most studies of bacterial colonization and growth in the rhizosphere have focused on fluorescent pseudomonads or on rhizobia under various field, greenhouse, and growth chamber conditions (Kloepper *et al.*, 1980; Weller, 1983; Mowad *et al.*, 1984; Loper *et al.*, 1985;

[1]Chapter format according to Biocontrol Science and Technology

Bahme & Schroth, 1987; Howie *et al.*, 1987; Scher *et al.*, 1988; Liddell & Parke, 1989; Osburn *et al.*, 1989; Abaido *et al.*, 1990; Gupta *et al.*, 1995; Dashti *et al.*, 1997; Kim *et al.*, 1997).

Plant growth promoting rhizobacteria are thought to improve plant growth by colonizing the root system and preempting the establishment or suppression of deleterious rhizosphere microorganisms (DRMO) (Weller, 1988). Studies in the Netherlands suggest that PGPR promote potato growth primarily by suppressing cyanide-producing DRMO (Schippers *et al.*, 1987).

Probanza *et al.*, (1996) reported that *Bacillus pumilus* and *Bacillus licheniformis* stimulated growth of the forest tree, European alder [*Alnus glutinosa* (L.) Gaertn.]. These two *Bacillus* species are among the seven species used for this study.

Although significant increases in yields by seed and tuber inoculations with PGPR have been demonstrated in the field, results vary from field to field and from year to year in the same field (Kloepper *et al.*, 1980). Variations seem to be due to unfavourable environmental factors, resulting in inadequate distribution and establishment of introduced rhizobacterial strains or failure of their antagonistic activity towards DRMO.

In this study, the enhancement of plant growth was evaluated using seven commercially available *Bacillus* spp. as plant probiotics used on containerised seedlings.

3.2 MATERIALS AND METHODS

Microorganisms

Seven *Bacillus* species viz: *B. chitinosporus*, *B. uniflagellatus*, *B. laterosporus*, *B. pumilus*, *B. subtilis*, *B. licheniformis*, an unidentified strain CM-33 and Biostart® 2000 (a combination of *B. chitinosporus*, *B. laterosporus*, and *B. licheniformis*) were used in this experiment. The species were provided commercially as concentrated spore suspensions by Microbial Solutions[2].

[2]Microbial Solutions (Pty)Ltd., P.O. Box 1180, Strubens Valley 1735, Republic of South Africa

NutriStart-AC (Nutrient supplement) Analysis

A laboratory analysis on NutriStart-AC was done to determine the C:N ratio and also the macro and micro nutrients present. The analysis was done by Cedara Fertilizer Advisory Services[3] using the methods of Farina (1981); Perstorp (1993) and Matejovis (1996).

Results were as presented in Table 3.1 below.

Table 3.1. General analysis of NutriStart-AC. Data is on a 100% dry matter basis

CNS		
Nitrogen (N)		5.19
Sulphur (S)	%	0.7
Carbon (C)		43.6
Kjeldahl		
Nitrogen (N)	%	4.01
Protein		25.04
Calcium (Ca)		2.8
Magnesium		0.55
Potassium (K)	%	4.32
Sodium (Na)		0.1
Phosphorus (P)		0.73
Zinc (Zn)		31
Copper (Cu)	ppm	11
Manganese (Mn)		316
Boron (B)		7

Calculated C:N = 8:1 (based on the values obtained from the combustion method)

[3]Cedara Agricultural Development Institute, Private Bag X9059, Pietermaritzburg 3200 South Africa

The difference between the combustion and the Kjeldahl nitrogen is due to greater efficiency of the combustion method. The combustion method determines nitrogen regardless of the complexity of the nitrogen-containing compounds present in the sample. Nitrogen values using the Kjeldahl method are commonly lower when compared to a combustion value.

Crops Evaluated

Crops evaluated were:

tomato (*Lycopersicum esculentum* Mill.) cv. Floradade, Seed Lot no. AY 068 RV,

lettuce (*Lactuca sativa* L.) cv. Frosty, Seed Lot no. YD 069 RV,

red sorghum (*Sorghum bicolor* (L) Moench) Reg. No. V2428 (ACT 36/1947), and

beans (*Phaseolus vulgaris* L.) cv. Sodwana, Seed Lot no. AD 021 RE.

Seeds were obtained from McDonald Seeds[4].

Tunnel trials were conducted to evaluate two different application techniques and growth stimulation methods for all seven *Bacillus* spp. and Biostart® 2000 as previously listed. These include seed treatment and seed treatment plus drenching with or without NutriStart-AC, obtained from Microbial Solutions.

Seed Treatment

For culturing, 0.6 g of NutriStart-AC were weighed into eight 250 *ml* conical flasks. One hundred and twenty ml quantities of tap water were added to each flask and swirled gently by hand to form a homogeneous suspension. Two *ml* quantities of concentrated spore suspension (2 x 10^9) cells of each *Bacillus* spp. were added separately to each of the eight conical flasks, labelled and incubated in a water bath shaker at 30 °C for 18 h at 150 rpm. The optical density of each culture was noted at 540 nm with a MILTON ROY Spectronic 301 spectrophotometer. The colony forming units (c.f.u's) were determined for each of the cultures by dilution plating (Parkinson *et al.*, 1971).

[4]McDonald Seeds (Pty)Ltd, 61 Boshoff Street, P.O.Box 238, Pietermaritzburg, Republic of South Africa

Two grams of a sticker, Pelgel® nutrient adhesive[5] were dissolved in 100 *ml* of tap water, stirred and allowed to stand for 1 h. This was to allow the substance to dissolve and form a homogeneous suspension. The suspension was further divided into eight 250 *ml* beakers, each containing 10 *ml* aliquots of the sticker.

To each of the beakers containing the sticker, 10 *ml* of the 18 h cultures was added separately, labelled and stirred. This resulted in a total volume of 20 *ml* of bacterial suspension in each of the eight beakers, giving a ratio of 1:1 sticker-bacterial suspension.

An appropriate number of tomato seeds was placed separately into each of the eight bacterial suspensions and stirred. The seeds were left for two hours to allow bacterial adhesion to the seed coat. The treated seeds were then placed on paper towels and air-dried overnight. The seeds from the combination of adhesive and each *Bacillus* spp. were planted into six Speedling® 24 trays filled with composted pine bark, giving a total of 48 Speedling® 24 trays.

The trays were watered with tap water and left in a germination room (20-24 °C) for three days. The trays were then moved to a plastic covered tunnel (20-30 °C) for seven weeks.

Seed Treatment plus Drenching

Seeds were treated with probiotics as described above. The treated seeds were planted into six Speedling® 24 trays filled with composted pine bark. Trays were watered and left with the seed treated trays.

For drenching, 1.2 g of NutriStart-AC were weighed into eight 500 *ml* conical flasks. To each flask 240 *ml* of tap water were added and the contents swirled gently to facilitate mixing. Two ml quantities of concentrated spore suspensions (2 x 10^9 cells) of each *Bacillus* spp. was added separately to each of the eight conical flasks, labelled and incubated at 30 °C for 18 h at 150 rpm in a water bath shaker. The optical density of each culture was noted at 540 nm. The concentration of cells was determined by using a dilution series. This process was repeated each week for seven weeks in order to ensure fresh inoculum for weekly inoculations.

[5]LiphaTech, Inc., Milwaukee, Wisconsin, U.S.A

One week after seedling emergence, each of the eight broth cultures was separately dispensed in 1 *ml* aliquots directly onto the composted pine bark growing medium. Thus six trays, each containing 24 seedlings, were inoculated per drench volume, and per each *Bacillus* spp. This procedure was repeated each week for seven weeks.

Application of NutriStart-AC onto seedlings

Forty grams of NutriStart-AC were weighed into a 2 *l* conical flask. Tap water (1 *l*) was added to the flask and swirled gently to mix. This resulted in a 4% NutriStart-AC suspension. This process was repeated weekly for seven weeks for weekly applications of NutriStart-AC to the trays.

One week after seedling emergence, 1 *ml* aliquots of the dissolved NutriStart-AC were applied as a drench separately onto the composted pine bark growing medium. Applications were performed in triplicate in the tunnels. This resulted in six out of the total of 12 trays (six for seed treatment and six for seed treatment plus drenching) being supplemented with NutriStart-AC. The remaining trays were not supplemented with NutriStart-AC and served as separate treatments.

Controls

Two controls were set up in this study. Untreated tomato seeds were planted in six Speedling® 24 trays filled with composted pine bark. Three of the trays were labelled as Control One and received water only. The other three served as Control Two and were supplemented weekly with 1 *ml* of 4% NutriStart-AC solution.

Thus for each of the seven *Bacillus* spp. and Biostart® 2000:

1. three trays were seed treated with no NutriStart-AC supplement;
2. three trays were seed treated and supplemented weekly with 1 *ml* of 4% NutriStart-AC suspension;
3. three trays were seed treated, drenched weekly but with no NutriStart-AC supplement;
4. three trays were seed treated, drenched weekly and supplemented weekly with 1 *ml* of 4% NutriStart-AC solution;
5. three trays were treated weekly with NutriStart-AC and

6. three trays were not treated with bacteria or NutriStart-AC.

This gave a total of 102 Speedling® 24 trays.

Growth of seedlings was monitored for seven weeks. Seedlings were irrigated three times a day by microjet irrigation. Irrigation water contained soluble fertilizer [3.1.3 (38) Complete] (from Ocean Agriculture[6]), applied at a rate of 1g l^{-1} to give 100 mg l^{-1} N , 33 P and 100 K.

For seedling dry weight, the number of plants in each tray was noted so that the mean weight per seedling could be determined. Seedlings from each tray were harvested at maturity at their base and placed in a brown paper bag. The plant material was subsequently dried in an oven at 55 °C. Once dried, the content of each bag was weighed and the mean weight per seedling shoot calculated.

Statistical Analysis

A general linear model (GLM) was used to run a factorial analysis on the results using the computer statistical package, Statistical Analysis System (SAS, 1987).

The above procedures were repeated for lettuce, beans and sorghum.

It should be noted that no sterile techniques were used. We were replicating field situations and activity thereby avoiding sterile NutriStart-AC or sterile media or water. By having controls of NutriStart-AC alone, the effect of contamination by opportunistic bacteria was picked up. The overall approach was to determine the efficacy of the treatments recommended to growers by Microbial Solutions. Using a sterile NutriStart-AC solution and media will therefore be completely artificial and will not reflect growers experiences. However a comparative trial between sterile and unsterile NutriStart-AC might be useful at some stage. This applies to all trials in Chapters 4, 5 and 6.

[6]Ocean Agriculture, P.O.Box 741, Mulders Drift 1747, Republic of South Africa

It is also worth noting that no fertilizer ran through the watering system during the first three to four weeks of the lettuce trial because the Dosatron fertilizer injector pump failed to function for this period.

3.3 RESULTS

Table 3.2. Biostart™ numbers in each batch of 18 h broth inoculum prepared for weekly seedling drench inoculations

Organism	Optical Density (OD)	Log c.f.u's ml^{-1}
B. chitinosporus	0.544	6.12
B. uniflagellatus	0.433	5.48
B. laterosporus	0.481	6.05
B. pumilus	0.44	5.68
B. subtilis	0.477	6.01
B. licheniformis	0.464	5.98
CM-33	0.404	5.46
Biostart® 2000	0.48	6.14

Table 3.3. Biostart™ numbers in each batch of 18 h broth inoculum prepared for seed treatment

Organism	Optical Density (OD)	Log c.f.u's ml^{-1}
B. chitinosporus	0.598	7.02
B. uniflagellatus	0.54	6.98
B. laterosporus	0.577	6.74
B. pumilus	0.633	7.88
B. subtilis	0.544	6.52
B. licheniformis	0.52	6.32
CM-33	0.574	6.79
Biostart® 2000	0.528	6.42

Table 3.4. Comparisons of seed treatment and seed treatment plus drench methods with or without NutriStart-AC on tomato (Floradade) seedlings after seven weeks

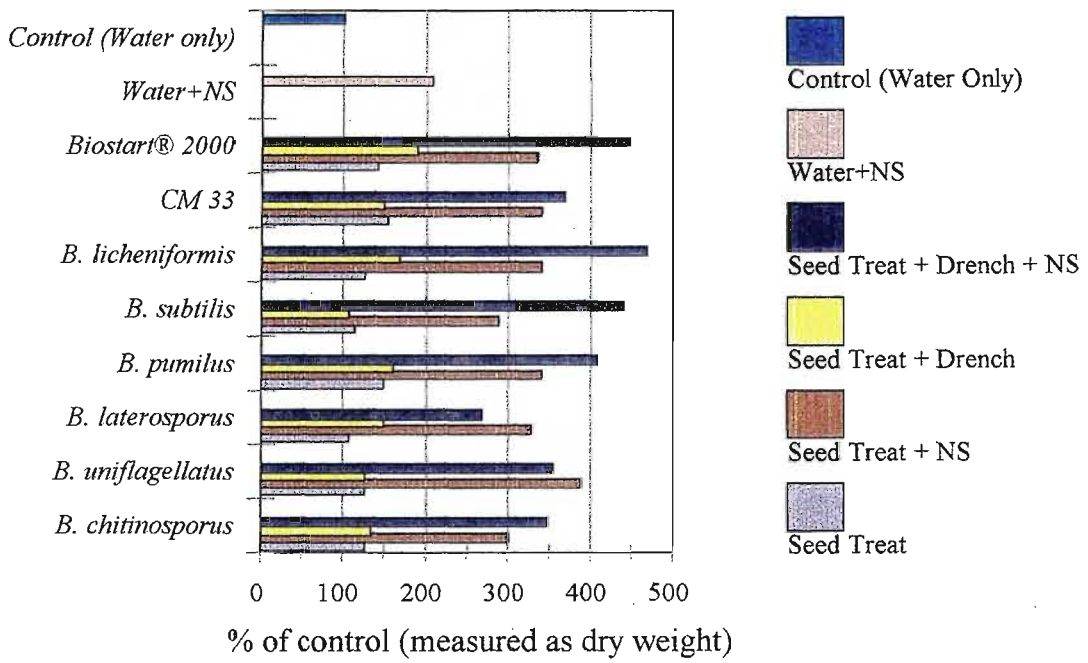
Bacteria	Treatment Type	Nutrient Supplement (NS)	Mean Dry Weight (g)	% Control 1 (Water only)	% Control 2 (Water + NS)
B. chitinosporus	Seed Treat	No	0.19 fg	126	61
B. chitinosporus	Seed Treat	Yes	0.45 abcde	300	145
B. chitinosporus	Seed Treat + Drench	No	0.2 fg	133	64
B. chitinosporus	Seed Treat + Drench	Yes	0.52 abc	346	167
B. uniflagellatus	Seed Treat	No	0.19 fg	126	61
B. uniflagellatus	Seed Treat	Yes	0.58 ab	386	187
B. uniflagellatus	Seed Treat + Drench	No	0.19 fg	126	61
B. uniflagellatus	Seed Treat + Drench	Yes	0.53 abc	353	170
B. laterosporus	Seed Treat	No	0.16 g	106	51
B. laterosporus	Seed Treat	Yes	0.49 abcd	326	158
B. laterosporus	Seed Treat + Drench	No	0.22 fg	146	70
B. laterosporus	Seed Treat + Drench	Yes	0.4b cdefg	266	129
B. pumilus	Seed Treat	No	0.22 fg	146	70
B. pumilus	Seed Treat	Yes	0.51 abcd	340	164
B. pumilus	Seed Treat + Drench	No	0.24 efg	160	77
B. pumilus	Seed Treat + Drench	Yes	0.61 ab	406	196
B. subtilis	Seed Treat	No	0.17 g	113	54
B. subtilis	Seed Treat	Yes	0.43 bcdef	286	138
B. subtilis	Seed Treat + Drench	No	0.16 g	106	51
B. subtilis	Seed Treat + Drench	Yes	0.66 abc	440	212
B. licheniformis	Seed Treat	No	0.19 fg	126	61
B. licheniformis	Seed Treat	Yes	0.51 abcd	340	164
B. licheniformis	Seed Treat + Drench	No	0.25 efg	166	80
B. licheniformis	Seed Treat + Drench	Yes	0.7 a	466	225
CM 33 (unknown)	Seed Treat	No	0.23 efg	153	74
CM 33 (unknown)	Seed Treat	Yes	0.51 abcd	340	164
CM 33 (unknown)	Seed Treat + Drench	No	0.22 fg	146	70
CM 33 (unknown)	Seed Treat + Drench	Yes	0.55 abc	366	177
Biostart® 2000	Seed Treat	No	0.21 fg	140	67
Biostart® 2000	Seed Treat	Yes	0.5 abcd	333	161
Biostart® 2000	Seed Treat + Drench	No	0.28 defg	186	90
Biostart® 2000	Seed Treat + Drench	Yes	0.61 ab	446	196
Control 1 (Water only)	None	No	0.15 g	100	48
Control 2 (Watert +NS)	None	Yes	0.31 cdefg	206	100

Effects	**F- value**	**P- level**	**Significance**	**Description**
Control s	13.39	< 0.001	***	Highly Significant
Bacteria	2.11	0.046	*	Significant
Treat	5.49	0.022	**	Significant
Nutrient supplement	157.4	< 0.001	***	Highly Significant
Bacteria.Treat	1.04	0.412	ns	Not Significant
Bacteria.Nutrient supplement	0.7	0.65	ns	Not Significant
Treat.Nutrient supplement	0.94	0.335	ns	Not Significant
Bacteria.Treat.Nutrient supplement	0.83	0.566	ns	Not Significant
	%CV = 24.6		**S.E = 0.088**	

Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant.

Seed Treat= Application of bacteria to seed with Pelgel® Sticker.

Means with the same letter are not significantly different (P = 0.05) according to Students, Newman and Keuls comparison test.



Nutrient supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Seed treat = Application of bacteria to seed with Pelgel® sticker

Figure 3.1. Response of tomato seedlings (Floradade) to treatment with *Bacillus* probiotics, applied by seed treatment, or seed treatment plus weekly drenches, with or without addition of NutriStart-AC at 40 g l^{-1}

Table 3.5. Comparisons of seed treatment and seed treatment plus drench methods with or without NutriStart-AC on Lettuce (Frosty) seedlings after seven weeks

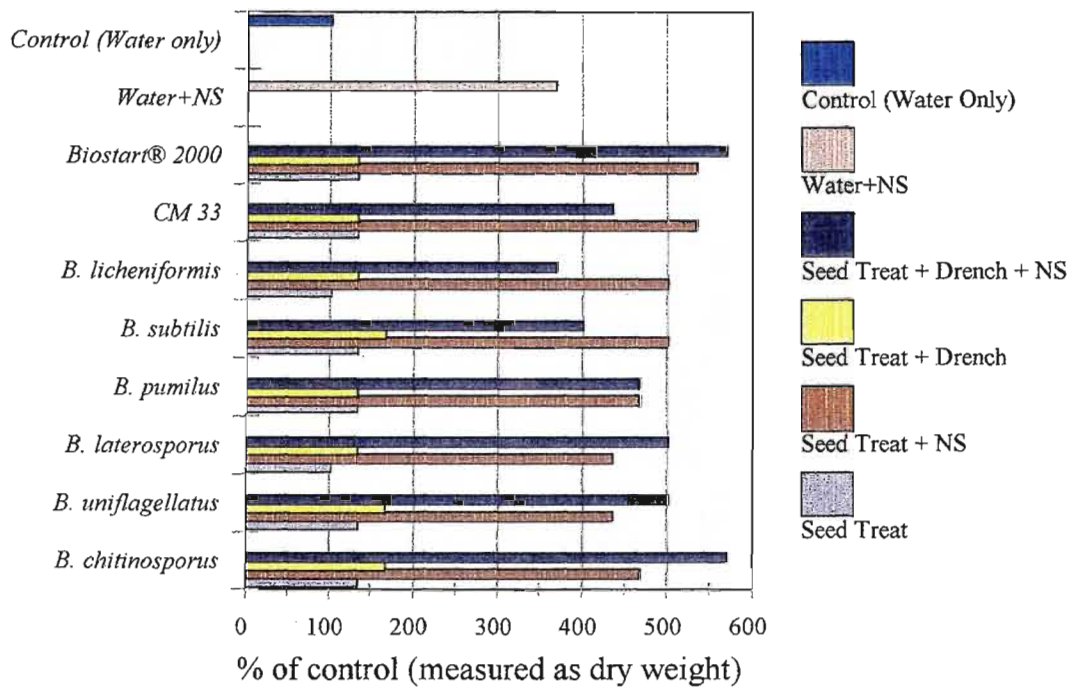
Bacteria	Treatment Type	Nutrient Supplement (NS)	Mean Dry Weight (g)	% Control 1 (Water only)	% Control 2 (Water + NS)
B. chitinosporus	Seed Treat	No	0.04 d	133	36
B. chitinosporus	Seed Treat	Yes	0.14 abc	466	127
B. chitinosporus	Seed Treat + Drench	No	0.05 d	166	45
B. chitinosporus	Seed Treat + Drench	Yes	0.17 a	566	154
B. uniflagellatus	Seed Treat	No	0.04 d	133	36
B. uniflagellatus	Seed Treat	Yes	0.13 abc	433	118
B. uniflagellatus	Seed Treat + Drench	No	0.05 d	166	45
B. uniflagellatus	Seed Treat + Drench	Yes	0.15 abc	500	136
B. laterosporus	Seed Treat	No	0.03 d	100	27
B. laterosporus	Seed Treat	Yes	0.13 abc	433	118
B. laterosporus	Seed Treat + Drench	No	0.04 d	133	36
B. laterosporus	Seed Treat + Drench	Yes	0.15 abc	500	136
B. pumilus	Seed Treat	No	0.04 d	133	36
B. pumilus	Seed Treat	Yes	0.14 abc	466	127
B. pumilus	Seed Treat + Drench	No	0.04 d	133	36
B. pumilus	Seed Treat + Drench	Yes	0.14 abc	466	127
B. subtilis	Seed Treat	No	0.04 d	133	36
B. subtilis	Seed Treat	Yes	0.15 abc	500	136
B. subtilis	Seed Treat + Drench	No	0.05 d	166	45
B. subtilis	Seed Treat + Drench	Yes	0.12 bc	400	109
B. licheniformis	Seed Treat	No	0.03 d	100	27
B. licheniformis	Seed Treat	Yes	0.15 abc	500	136
B. licheniformis	Seed Treat + Drench	No	0.04 d	133	36
B. licheniformis	Seed Treat + Drench	Yes	0.11 bc	366	100
CM 33 (unknown)	Seed Treat	No	0.04 d	133	36
CM 33 (unknown)	Seed Treat	Yes	0.16 ab	533	145
CM 33 (unknown)	Seed Treat + Drench	No	0.04 d	133	36
CM 33 (unknown)	Seed Treat + Drench	Yes	0.13 abc	433	118
Biostart® 2000	Seed Treat	No	0.04 d	133	36
Biostart® 2000	Seed Treat	Yes	0.16 ab	533	145
Biostart® 2000	Seed Treat + Drench	No	0.04 d	133	36
Biostart® 2000	Seed Treat + Drench	Yes	0.17 a	566	154
Control 1 (Water only)	None	No	0.03 d	100	27
Control 2 (Water + NS)	None	Yes	0.11 c	366	100

Effects	F- value	P- level	Significance	Description
Controls	10.99	0.001	***	Highly Significant
Bacteria	4.69	< 0.001	***	Highly Significant
Treat	0.46	0.5	ns	Not Significant
Nutrient Supplement	384.39	< 0.001	***	Highly Significant
Bacteria.Treat	1.37	0.232	ns	Not Significant
Bacteria.Nutrient supplement	2.12	0.062	~*	Marginally Significant
Treat.Nutrient supplement	1.4	0.242	ns	Not Significant
Bacteria.Treat.Nutrient supplement	2.41	0.051	~*	Marginally Significant
	%CV = 20.0		**S.E = 0.018**	

Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant.

Seed Treat= Application of bacteria to seed with Pelgel® Sticker.

Means with the same letter are not significantly different (P = 0.05) according to Students, Newman and Keuls comparison test.



Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Seed treat = Application of bacteria to seed with Pelgel® sticker

Figure 3.2. Response of lettuce seedlings (Frosty) to treatment with *Bacillus* probiotics, applied by seed treatment, or seed treatment plus weekly drenches, with or without addition of NutriStart-AC at 40 g l^{-1}

Table 3.6. Comparisons of seed treatment and seed treatment plus drench methods with or without NutriStart-AC on Red Sorghum seedlings after seven weeks

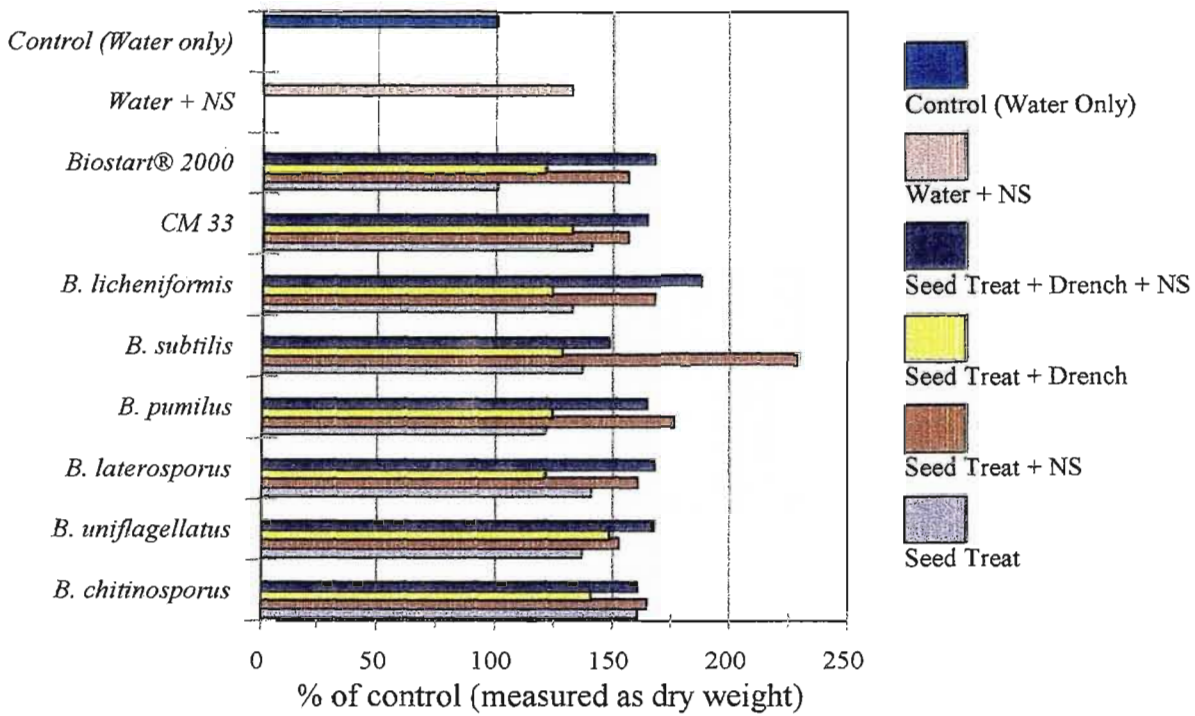
Bacteria	Treatment Type	Nutrient Supplement (NS)	Mean Dry Weight (g)	% Control 1 (Water only)	% Control 2 (Water + NS)
B. chitinosporus	Seed Treat	No	0.4 ab	160	121
B. chitinosporus	Seed Treat	Yes	0.41 ab	164	124
B. chitinosporus	Seed Treat + Drench	No	0.35 ab	140	106
B. chitinosporus	Seed Treat + Drench	Yes	0.4 ab	160	121
B. uniflagellatus	Seed Treat	No	0.34 ab	136	103
B. uniflagellatus	Seed Treat	Yes	0.38 ab	152	115
B. uniflagellatus	Seed Treat + Drench	No	0.37 ab	148	112
B. uniflagellatus	Seed Treat + Drench	Yes	0.42 ab	168	127
B. laterosporus	Seed Treat	No	0.35 ab	140	106
B. laterosporus	Seed Treat	Yes	0.4 ab	160	121
B. laterosporus	Seed Treat + Drench	No	0.3 b	120	90
B. laterosporus	Seed Treat + Drench	Yes	0.42 ab	168	127
B. pumilus	Seed Treat	No	0.3 b	120	90
B. pumilus	Seed Treat	Yes	0.44 ab	176	133
B. pumilus	Seed Treat + Drench	No	0.31 b	124	93
B. pumilus	Seed Treat + Drench	Yes	0.41 ab	164	124
B. subtilis	Seed Treat	No	0.34 ab	136	103
B. subtilis	Seed Treat	Yes	0.57 a	228	172
B. subtilis	Seed Treat + Drench	No	0.32 b	128	96
B. subtilis	Seed Treat + Drench	Yes	0.37 ab	148	112
B. licheniformis	Seed Treat	No	0.33 ab	132	100
B. licheniformis	Seed Treat	Yes	0.42 ab	168	127
B. licheniformis	Seed Treat + Drench	No	0.31 b	124	93
B. licheniformis	Seed Treat + Drench	Yes	0.47 ab	188	142
CM 33 (unknown)	Seed Treat	No	0.35 ab	140	106
CM 33 (unknown)	Seed Treat	Yes	0.39 ab	156	118
CM 33 (unknown)	Seed Treat + Drench	No	0.33 ab	132	100
CM 33 (unknown)	Seed Treat + Drench	Yes	0.41 ab	164	124
Biostart® 2000	Seed Treat	No	0.25 b	100	75
Biostart® 2000	Seed Treat	Yes	0.39 ab	156	118
Biostart® 2000	Seed Treat + Drench	No	0.3 b	120	90
Biostart® 2000	Seed Treat + Drench	Yes	0.42 ab	168	127
Control 1 (Water only)	None	No	0.25 b	100	75
Control 2 (Water + NS)	None	Yes	0.33 ab	132	100

Effects	F- Value	P- Value	Significance	Description
Controls	70.4	0.01	**	Highly Significant
Bacteria	0.96	0.476	ns	Not Significant
Treat	0.07	0.793	ns	Not Significant
Nutrient Supplement	17.92	< 0.001	***	Highly Significant
Bacteria.Treat	1.13	0.356	ns	Not Significant
Bacteria.Nutrient supplement	0.73	0.623	ns	Not Significant
Treat.Nutrient supplement	0.05	0.821	ns	Not Significant
Bacteria.Treat.Nutrient supplent	0.97	0.459	ns	Not Significant
	%CV = 21.4		**S.E = 0.079**	

Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant.

Seed Treat = Application of bacteria to seed with Pelgel® Sticker.

Means with the same letter are not significantly different (P = 0.05) according to Students, Newman and Keuls comparison test.



Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Seed treat = Application of bacteria to seed with Pelgel® sticker

Figure 3.3. Response of red sorghum seedlings to treatment with *Bacillus* probiotics, applied by seed treatment, or seed treatment plus weekly drenches, with or without addition NutriStart-AC at 40 g l^{-1}

Table 3.7. Comparisons of seed treatment and seed treatment plus drench methods with or without NutriStart-AC on Bean (Sodwana) seedlings after seven weeks

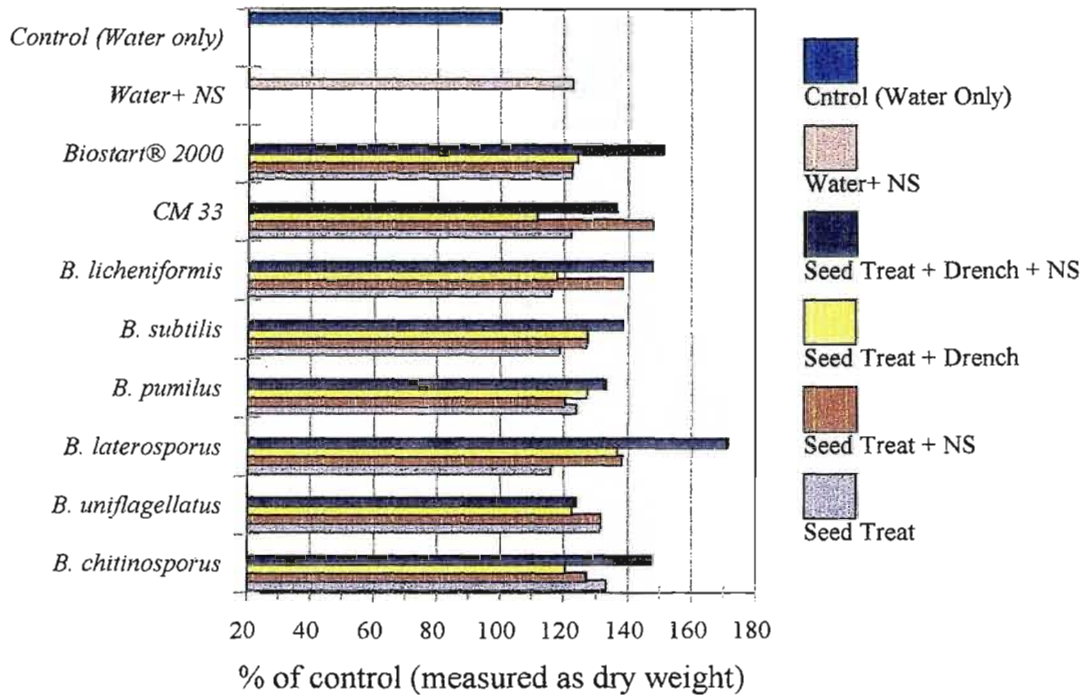
Bacteria	Treatment Type	Nutrient Supplement (NS)	Mean Dry Weight (g)	% Control 1 (Water only)	% Control 2 (Water + NS)
B. chitinosporus	Seed Treat	No	0.6 ab	133	109
B. chitinosporus	Seed Treat	Yes	0.57 ab	126	103
B. chitinosporus	Seed Treat + Drench	No	0.54 b	120	98
B. chitinosporus	Seed Treat + Drench	Yes	0.66 ab	146	120
B. uniflagellatus	Seed Treat	No	0.59 ab	131	107
B. uniflagellatus	Seed Treat	Yes	0.59 ab	131	107
B. uniflagellatus	Seed Treat + Drench	No	0.55 ab	122	100
B. uniflagellatus	Seed Treat + Drench	Yes	0.64 ab	142	116
B. laterosporus	Seed Treat	No	0.52 b	115	94
B. laterosporus	Seed Treat	Yes	0.62 ab	137	112
B. laterosporus	Seed Treat + Drench	No	0.61 ab	135	110
B. laterosporus	Seed Treat + Drench	Yes	0.77 a	171	140
B. pumilus	Seed Treat	No	0.56 ab	124	101
B. pumilus	Seed Treat	Yes	0.54 b	120	98
B. pumilus	Seed Treat + Drench	No	0.57 ab	126	103
B. pumilus	Seed Treat + Drench	Yes	0.6 ab	133	109
B. subtilis	Seed Treat	No	0.53 b	117	96
B. subtilis	Seed Treat	Yes	0.57 ab	126	103
B. subtilis	Seed Treat + Drench	No	0.57 ab	126	103
B. subtilis	Seed Treat + Drench	Yes	0.62 ab	137	112
B. licheniformis	Seed Treat	No	0.52 b	115	94
B. licheniformis	Seed Treat	Yes	0.62 ab	137	112
B. licheniformis	Seed Treat + Drench	No	0.53 b	117	96
B. licheniformis	Seed Treat + Drench	Yes	0.66 ab	146	120
CM 33 (unknown)	Seed Treat	No	0.55 ab	122	100
CM 33 (unknown)	Seed Treat	Yes	0.66 ab	146	120
CM 33 (unknown)	Seed Treat + Drench	No	0.5 b	111	90
CM 33 (unknown)	Seed Treat + Drench	Yes	0.61 ab	135	110
Biostart® 2000	Seed Treat	No	0.55 ab	122	100
Biostart® 2000	Seed Treat	Yes	0.55 ab	122	100
Biostart® 2000	Seed Treat + Drench	No	0.56 ab	124	101
Biostart® 2000	Seed Treat + Drench	Yes	0.68 ab	151	123
Control 1 (Water only)	None	No	0.45 b	100	81
Control 2 (Water + NS)	None	Yes	0.55 ab	122	100

Effects	F- value	P- level	Significance	Description
Controls	9.35	0.003	**	Highly Significant
Bacteria	1.16	0.339	ns	Not Significant
Treat	5.36	0.024	*	Significant
Nutrient Supplement	10.73	< 0.001	***	Highly Significant
Bacteria.Treat	1.35	0.239	ns	Not Significant
Bacteria.Nutrient supplement	1.2	0.316	ns	Not Significant
Treat.Nutrient supplement	4.91	0.03	**	Highly Significant
Bacteria.Treat.Nutrient supplement	0.43	0.878	ns	Not Significant
	%CV = 12.4		**S.E = 0.072**	

Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant.

Seed Treat = Application of bacteria to seed with Pelgel® Sticker.

Means with the same letter are not significantly different (P = 0.05) according to Students, Newman and Keuls comparison test.



Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Seed treat = Application of bacteria to seed with Pelgel® sticker

Figure 3.4. Response of bean seedlings (Sodwana) to treatment with *Bacillus* probiotics, applied by seed treatment, or seed treatment plus weekly drenches, with or without addition of NutriStart-AC at 40 g l^{-1}

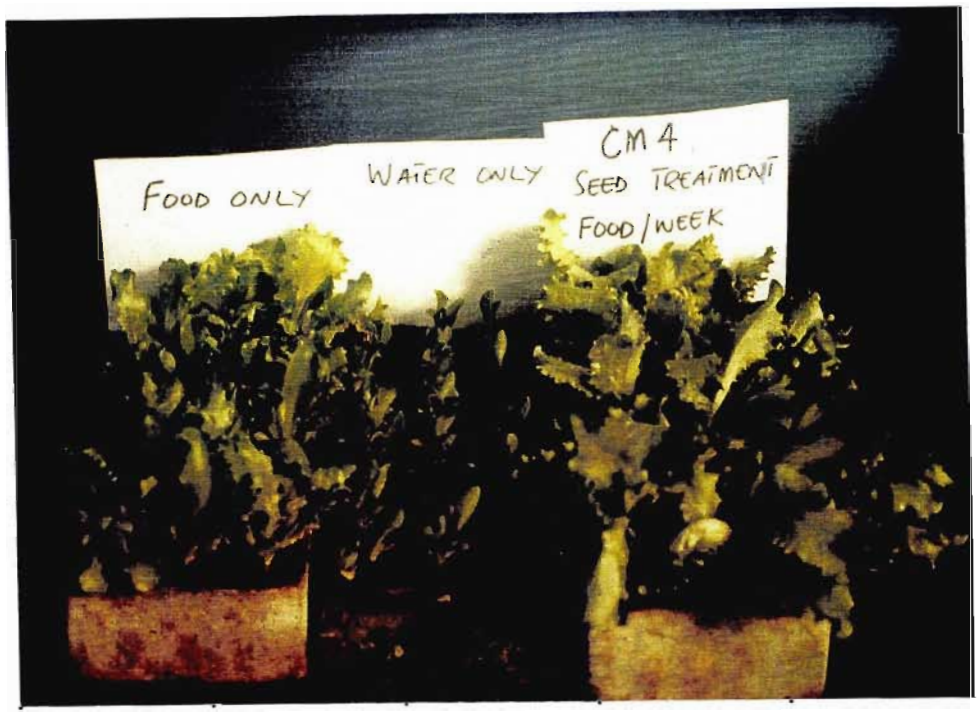


Figure 3.5. Comparison of *Bacillus pumilus* seed treated and NutriStart-AC supplemented lettuce seedlings (far right) with seedlings that received NutriStart-AC only (far left) and water only (middle)

Figure 3.6. Comparison of *Bacillus licheniformis* seed treated and NutriStart-AC supplemented tomato (Floradade) seedlings (middle) with seedlings that received NutriStart-AC only (far right) and water only (far left)

Figure 3.7. Comparison of *Bacillus licheniformis* seed treated plus a weekly bacterial drench of sorghum seedlings with NutriStart-AC supplement (far right) with seedlings that received NutriStart-AC only (middle) and water only (far left)

Biostart™ concentrations used for weekly seedling drench ranged from 5.46-6.14 log cfu ml^{-1} (Table 3.2). Bacterial concentrations used for seed treatment ranged from 6.32-7.88 log cfu ml^{-1} (Table 3.3).

Growth stimulation trials in the tunnel showed that substantial growth increase was obtained when Biostart™ probiotic *Bacillus* spp. were inoculated onto tomato, lettuce, sorghum and beans with or without NutriStart-AC supplement (Tables 3.4-3.7).

For all four crops tested, significant increase in growth was observed when seedlings were further supplemented weekly with a 4% NutriStart-AC solution (Figures 3.1-3.4).

NutriStart-AC played a significant role in growth increase of all four crops. Highly significant differences were observed between the two controls, water only, and the water plus food (NutriStart-AC) for all four crops ($P = 0.001$).

The differences in shoot biomass between seed treated and seed treated plus weekly drenched seedlings without NutriStart-AC supplement was marginal for all four crops (Tables3. 4-3.7). On the other hand, large differences were recorded when seedlings were supplemented weekly with 4% NutriStart-AC as a drench for both the seed treated and seed plus drench treated seedlings. The differences in growth were more pronounced on tomato and lettuce than on sorghum and bean.

For all four crops, growth stimulation differed according to the probiotic *Bacillus* species applied. Results obtained therefore differed for each species from crop to crop; e.g. Biostart® 2000 was more effective on tomato than on sorghum (Tables 3.4 and 3.6).
On tomato; (Table 3.4)

- Only *B. uniflagellatus* and *B. laterosporus* were more effective when applied by seed treatment with NutriStart-AC supplement.
- All other probiotic *Bacillus* spp. and Biostart® 2000 were most effective when applied by seed treatment plus weekly drench with 4% NutriStart-AC supplement.

On lettuce; (Table 3.5)

- *B. chitinosporus*, *B. uniflagellatus*, *B. laterosporus* and Biostart® 2000 were more effective when applied by seed treatment plus weekly drench with NutriStart-AC supplement.
- Only *B. pumilus* had the same effect (366% growth promotion) for both application treatments with NutriStart-AC supplement.
- *B. subtilis*, *B. licheniformis* and CM-33 were most effective when applied by seed treatment with NutriStart-AC supplement rather than as seed treatment plus weekly drench with NutriStart-AC supplement.

On sorghum; (Table 3.6)

- *B. chitinosporus*, *B. pumilus*, and *B. subtilis* were more effective when applied by seed treatment with NutriStart-AC supplement.
- *B. uniflagellatus*, *B. laterosporus*, *B. licheniformis*, CM-33 and Biostart® 2000 were most effective when applied by seed treatment plus weekly drench with NutriStart-AC supplement.

On bean; (Table 3.7)

- Only CM-33 was most effective when applied by seed treatment with NutriStart-AC supplement.
- All other *Bacillus* spp. and Biostart® 2000 were most effective when applied by seed treatment plus weekly drench with NutriStart-AC supplement.

Individual species responded differently to the various treatments and therefore gave varied results in each treatment administered; e.g., lettuce, Biostart® 2000 gave 466% growth increase as seed treatment plus weekly drench supplemented weekly with 4% NutriStart-AC solution, 33% as seed treatment alone and 433% as seed treatment with 4% weekly NutriStart-AC supplement. All species were much less effective with seed treatment and seed treatment plus weekly drench methods when NutriStart-AC supplement was not applied weekly. In this case, none of the probiotic *Bacillus* spp. gave up to 100% growth stimulation on any of the four crops when compared to the water control. The best recorded results of these treatments as compared to the water control was Biostart® 2000, with 86% growth increase on tomato.

No significant difference was recorded between the bacteria, treatment and food for tomato, sorghum and bean. Only lettuce showed a marginal significant difference at P = 0.05 level.

3.4 DISCUSSION

The results of the trials are reported in Tables 3.4-3.7. Below is a summary table of four tables of results, Tables 3.4-3.7. This table makes a coarse summary of the results presented in Tables 3.4-3.7, reflecting the significant or non-significant results from the ANOVAs conducted. Analysis of results, reflected in Table 3.8 are as follows:

Table 3.8. A review of results in Tables 3.4-3.7

Treatments effects and interactions	Treatment Comparisons	Significant	Not Significant
1. Controls	Water control vrs NutriSatrt-AC control	all Four crops	-
2. Bacteria	Differences between seven isolates and Biostart® 2000	tomato, lettuce (highly)	sorghum, bean
3. Treat	Differences between Seed Treatment, Drenching and Seed Treatment plus Drenching	tomato, bean	lettuce, sorghum
4. Nutrient Supplement	Differences between supplemented and unsupplemented application of NutriStart-AC to treatments	all four crops (highly)	-
5. Bacteria* Treat	Interaction between seven isolates, Biostart® 2000 and treatments	-	all four crops
6. Bacteria* Nutrient Supplement	Interaction between seven isolates, Biostart® 2000 and NutriStart-AC application	lettuce (marginal)	tomato, sorghum, bean
7. Treat* Nutrient Supplement	Interaction between all treatments and NutriStart-AC application	bean (highly)	tomato, lettuce, sorghum
8. Bacteria* Treat* Nutrient Supplement	Interaction between seven isolates, Biostart® 2000, all treatments and NutriStart-AC application	lettuce (marginal)	tomato, sorghum, bean

A highly significant difference was observed between the water control and NutriStart-AC control. This suggests that the two controls were different as a result of NutriStart-AC application.

Comparing performances of the individual probiotics, significant differences were found in tomato and lettuce. This shows that the probiotic *Bacillus* spp. responded differently in tomato

and lettuce. Thus the effects of these organisms were different compared to the effects on sorghum and bean. No significant difference was observed between sorghum and bean. This suggests that the probiotic bacteria used responded in the same manner in sorghum and bean. There was no variation in terms of bacteria effect in sorghum and bean. This also suggests that none of the bacteria responded better or worse than another.

Comparing seed treatment and seed treatment plus drench, all probiotic *Bacillus* species responded to seed and seed plus drench treatments. A significant difference was found in tomato and bean. The assumption here is that the added drench might have given an added response after all seeds were treated before sowing. No significant difference was found in lettuce and sorghum. This suggests that all bacteria responded in the same way in all treatments applied. No added response was observed with an added bacterial drench. This implies that there was no difference between the seed treatment and seed treatment plus drench.

Comparing the effect of NutriStart-AC on bacteria growth response on tomato, lettuce, sorghum and bean, a highly significant difference was observed for all four crops tested. This suggests that all four crops tested responded to the addition of NutriStart-AC. The added NutriStart-AC might have acted as a nutrient source or fertilizer for plant growth and development or stimulated rhizobacteria already present in the composted pine bark.

Comparing the responses of the various probiotic *Bacillus* spp. and Biostart® 2000 to the two treatments applied (seed and seed plus drench treatments), no significant difference was found in all four crops. Possibly all the probiotic *Bacillus* species responded in the same manner in all treatments. The added bacterial drench did not add any significant difference or response on all the crops.

Comparing the responses of the various bacteria to the addition NutriStart-AC and the effect on seedling growth response, a marginal significant difference was recorded in lettuce. This suggests that some bacteria responded more to the added food than others. NutriStart-AC might have acted as a boost to some of the bacteria. On the other hand, no significant difference was recorded in tomato, sorghum and bean. There is therefore no difference in response of the bacteria to the

additional NutriStart-AC. All the bacteria responded to the food in the same way, thus NutriStart-AC did not support one bacterium more than the other.

Comparing the responses of seed and seed plus drench treatments towards the addition NutriStart-AC supplement, a highly significant difference was recorded in bean. This suggests that the two treatments responded differently to the added NutriStart-AC. The additional NutriStart-AC therefore had a separately different effect on the seed treatment and the seed treatment plus drench. No significant difference was recorded in tomato, lettuce and bean. The assumption here is that both treatments responded similarly to the NutriStart-AC added. Hence the similar effect seen in all treatments when NutriStart-AC was added as a supplement.

Comparing the responses of the various probiotic bacteria applied by seed and seed plus drench treatments to all four crops towards the addition NutriStart-AC, a marginally significant difference was observed in lettuce. This suggests that the bacteria responded differently to the treatments and NutriStart-AC added. Some bacteria did better than others when NutriStart-AC was supplemented to the various treatments. No significant difference was observed in tomato, sorghum and bean. There were therefore no variations in which all three crops; tomato, sorghum and bean responded to all three factors; bacteria, NutriStart-AC and treatments.

The effects of PGPR on plant growth can be separated into indirect and direct effects (Glick, 1994). The indirect promotion involves lessening or prevention of deleterious effects of one or more phytopathogenic organisms. The most part of the direct promotion entails either providing the plant with a compound that is synthesized by the bacterium, or facilitating the uptake of certain nutrients from the environment.

Appropriate applications of each of seven *Bacillus* spp. and Biostart® 2000 were shown to result in growth stimulation of four crops. Growth increase as high as 466% was recorded for Biostart® 2000 on lettuce as compared to the water control. Similar results were recorded for *B. chitinosporus* (466%), *B. uniflagellatus* (400%), *B. laterosporus*, (400%), *B. subtilis* (400%), *B. licheniformis* (400%) and 433% for an unknown species, CM-33 (Table 3.4). A similar result on growth stimulation was observed when potato seedpieces were treated with two strains of

fluorescent *Pseudomonas* spp. (Kloepper *et al.*, 1980). One strain, *B. licheniformis* and Biostart® 2000 also gave a 466% growth stimulation when applied by seed treatment to lettuce with NutriStart-AC supplement. Three other strains, namely, *B. subtilis, B. licheniformis* and *B. uniflagellatus* also gave a 400% growth stimulation. This suggests that *Bacillus* spp. should not be overlooked as a second choice to fluorescent *Pseudomonas* spp. in terms of growth stimulation and biological control purposes.

Seed treatment (seed bacterization) has been used as a method of applying PGPR for growth stimulation and biological control purposes. Occurrence of *Pythium* damping-off was reduced from 70 to 26% when sugarbeet seeds were treated with *Pseudomonas putida* and sown in soil artificially infested with *Pythium* (Shah-Smith, 1996). Shishido *et al.*, (1995), observed an 18 and 24% increase in height and shoot biomass, respectively compared to uninoculated controls when lodgepole pine seeds were seed inoculated with *Bacillus* spp. strain Pw-2. In this trial, probiotic *Bacillus* spp. gave a considerable increase in shoot biomass when four crops were seed treated and grown in tunnels, especially when seedlings were supplemented with 4% NutriStart-AC suspension. This effect was found to be more pronounced on tomato and lettuce than on sorghum and bean. This might be due to poor bacteria-root colonization on sorghum and bean in an artificial composted pine bark medium.

Seed treatment and seed treatment plus weekly drench without additional nutrients did improve plant growth when compared to the water control. Dramatic growth increase was recorded when seedlings were further supplemented with 1ml of 4% NutriStart-AC solution.

NutriStart-AC alone also stimulated plant growth. Comparing the two controls, it was observed that Control Two, which received 1ml of 4% NutriStart-AC weekly, was comparatively better in terms of growth than Control One which received water and fertilizer only. We therefore suggest that NutriStart-AC not only acted as a nutrient boost for applied probiotics but also as either fertilizer or boosts of the microorganisms already present in the non-sterile composted pine bark used as a growth medium. The authors are not aware of any prior research assessing the effect of a formulated nutrient boost for applied probiotics such as NutriStart-AC on seedling growth and development.

There was not much difference between the dry mass of the seed treated and the seed treated plus weekly drenched seedlings unsupplemented with NutriStart-AC. Neither was there a significant difference between the treatments when seedlings were supplemented weekly with a 4% NutriStart-AC solution. This trend was seen in all four crops. Without food there might have been poor bacterial survival, leading to less bacteria-root colonization, probably due to the poor nutrient status of the composted pine bark growth medium. The presence of NutriStart-AC possibly acted as a bacterial nutrient substitute thereby causing a corresponding increase in bacterial activity and growth increase. With NutriStart-AC supplement, growth increase was more pronounced on tomato and lettuce than on sorghum and beans. A possible reason is that sorghum and beans are field crops and the conditions under which they grow in the field might be quite different from the tunnel conditions used in this trial, and therefore might have affected their growth performance as field crops.

The seed treatment and the seed treatment plus weekly drench methods supplemented weekly with NutriStart-AC was a favourable application method for plant growth stimulation. Biostart™ probiotic *Bacillus* spp. gave some, if not excellent, growth stimulation on all four crops tested. We therefore recommend the seed treatment with weekly NutriStart-AC supplement since there was not much difference between the two. Adopting the seed treatment method will reduce the laborious work involved in weekly seedling drenches.

No significant difference was recorded between the responses of the various bacteria towards the addition of food (NutriStart-AC) and the effect on seedling growth response, except on lettuce where a marginally significant difference was recorded ($P = 0.05$). The implication of this is that some bacteria might possibly have responded more to the added food than others. No significant difference was recorded between bacteria and treatment on all four crops. Possibly, response to treatments were similar for all the *Bacillus* spp. and Biostart® 2000 applied as plant probiotics. Looking at the different treatments visually, differences can be seen between the water plus food control and the two application treatments supplemented weekly with NutriStart-AC. We thus speculate that differences may exist but were not big enough to show up statistically. Further trials with more replicates, are needed to provide decisive results.

No one particular probiotic *Bacillus* spp. could be said to perform best in all four crops. Results

varied from crop to crop as one species performed well on one crop but only performed moderately on another. More trials are therefore needed using a wide range of crops to ascertain which *Bacillus* spp. works best and on which specific crop at a particular time of year in various soils.

Growth stimulation was enhanced when seed treated and drenched seedlings were supplemented weekly with 4% NutriStart-AC. Further research is therefore necessary to prove, beyond doubt, the rhizosphere competence of these probiotic *Bacillus* species.

This work led to the important discovery that legitimate applications of NutriStart-AC alone stimulate plant growth. We suggest that NutriStart-AC must be acting as a direct nutrient source to plants or as a nutrient boost to indigenous and introduced rhizobacteria or both. These effects of NutriStart-AC must be determined if it is continuously used as a nutrient boost to plants and probiotic bacteria. If NutriStart-AC does alone stimulate plant growth, then the chemical analysis must be carried out to find out what plant nutrients are present (N P K, S, Ca, etc) and these must quantified. For further clarification, growth trials in gnotobiotic system must further be carried out with or without NutriStart-AC.

If NutriStart-AC does act as a nutrient boost for indigenous rhizobacteria, then detailed chemical analysis on NutriStart-AC should be carried out to find out what microbial nutrients are present in NutriStart-AC. Also population dynamics trials should be done with different NutriStart-AC dosages as well as counts of indigenous rhizobacteria to further ascertain whether the indigenous rhizobacteria population does increase with NutriStart-AC application or not. Denaturing Gradient Gel Electrophoresis (DGGE) could also used to study the indigenous microbial ecosystem. This works on the principle that the intensity of the bands increases as the microbial population increases.

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CHAPTER 4

4[1] Biostart™ on tomato and pepper seedlings: Effect of seed bacterization and seedling drench on growth and yield

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Biostart™, a *Bacillus*-based plant probiotic applied by seed treatment and seedling drench, was tested for ability to increase the growth of three tomato cultivars and the yield of pepper seedlings. Growth stimulation was more pronounced on the Roma and Floradade cultivars. The best growth stimulation (96%) was obtained using *B. licheniformis* on tomato cultivar Roma. On pepper, as high as 533% increase on fruit yield was achieved when seedlings were drenched with *B. subtilis* and supplemented weekly with 4% NutriStart-AC suspension. Increase in fruit yield was more pronounced in all seven *Bacillus* spp. and Biostart® 2000 when seedlings were supplemented weekly with a 4% NutriStart-AC suspension.

4.1 INTRODUCTION

Seed bacterization has proved to be a successful method for enhancing biological nitrogen fixation in legumes (Stacey *et al.*, 1992; Yobo, 1997). It has also been widely used for growth stimulation/promotion (Brown, 1974; Burr *et al.*, 1978; Suslow & Schroth, 1982; Weller & Cook, 1986; Pierson & Weller, 1994) and biological control (Brown, 1974; Gindrat, 1979; Weller & Cook, 1986; Pierson & Weller, 1994; Hökeberg *et al.*, 1997; Mao *et al.*, 1998; Zaki *et al.*, 1998) purposes.

[1]Chapter format according to Biocontrol Science and Technology

In most cases where seed bacterization is used either for growth stimulation or biological control purposes, the intention is to ensure the bacterial isolates proliferate in the soil, spread to the roots and exert their activity (Hökeberg *et al.*, 1997). This leads to a possible release or production of growth promoting substances such as gibberellin and antibiotics for biological control. However, antibiotics are not necessarily the only disease controlling mechanism (Brown, 1974).

Seedling drench has also been used for growth stimulation and disease control purposes (Berry & Torrey, 1985; Yobo, 1997; Zaki *et al.*, 1998). This method is not widely and frequently used, probably due to the large inoculum volume required and the labour involved in its application.

The purpose of this work was to determine the ability of Biostart™, a *Bacillus*-based plant probiotic, as a seed treatment and seedling drench to increase plant growth.

4.2 MATERIALS AND METHODS

Microorganisms

Seven *Bacillus* spp.: *B. chitinosporus, B. uniflagellatus*, *B. laterosporus*, *B. pumilus, B. subtilis, B. licheniformis,* CM-33 (an unidentified *Bacillus* strain) and Biostart® 2000 (a combination of *B. chitinosporus*, *B. laterosporus*, and *B. licheniformis*) were used in this experiment. The isolates were provided as concentrated spore suspensions by Microbial Solutions[2].

Crops Evaluated

Three tomato cultivars and a pepper cultivar were evaluated. They were:

Tomato (*Lycopersicon esculentum* Mill) cv. Floradade, Seed Lot no. AY 068RV
cv. Rodade, Seed Lot no. YR 030RV and
cv. RomaVF, Seed Lot no. AY 001RO

Seeds were obtained from McDonald Seeds[3].

[2]Microbial Solutions (Pty)Ltd., P.O. Box 1180, Strubens Valley 1735, Republic of South Africa

[3]McDonald Seeds (Pty)Ltd. 61 Boshoff Street, Box 238 Pietermaritzburg, Republic of South Africa

Pepper (*Capsicum frutescens* L.) cv. Thai. seedlings were obtained from Sunshine Seedling Services[4].

Two trials were conducted, one in a plastic covered tunnel and one in a shadehouse, to evaluate the effect of Biostart™ applied by seed treatment and seedling drench on tomato and pepper seeds and seedlings.

Seed Treatment

For culturing, 0.6 g of NutriStart-AC were weighed into eight 250 *ml* conical flasks. One hundred and twenty *ml* quantities of tap water were added to each flask and swirled gently to form a homogeneous suspension. Two *ml* quantities of concentrated spore suspension (2×10^9) cells of each *Bacillus* species were added separately to each of the eight conical flasks, labelled and incubated in a water bath shaker at 30 °C for 18 hrs at 150 rpm. The optical density of each culture was noted at 540 nm and values compared to those established previously (Chapter Three). Each culture was carefully diluted and the optical density noted to produce suspensions of the same optical densities and hence colony forming units (cfu's) for each batch of seed treated.

Two grams of a sticker, Pelgel® nutrient[5] adhesive, were dissolved in 100 *ml* of tap water, stirred and allowed to stand for 1 h. This was to allow the substance to dissolve and form a homogeneous suspension. The suspension was further divided into eight 250 *ml* beakers, each containing 10 *ml* aliquots of the sticker.

To each of the beakers containing the sticker, 10 *ml* of the 18 h cultures were added separately and stirred. This resulted in a total volume of 20 *ml* of bacterial suspension in each of the eight beakers, giving a ratio of 1:1 sticker-bacterial suspension.

[4]Sunshine Seedling Services, Old Wartburg Road P.O.Box 100461, Scottsville, Republic of South Africa

[5]LiphaTech, Inc., Milwaukee, Wisconsin, U.S.A

An appropriate number of tomato seeds were placed separately into each of the eight bacterial suspensions and stirred. The seeds were left for 2 h to allow bacterial adhesion to the seed coat. The treated seeds were then placed on paper towels and air-dried overnight. The seeds from the combination of adhesive and each *Bacillus* spp. were planted into three Speedling® 24 trays filled with composted pine bark, giving a total of 72 Speedling® 24 trays (24 trays per cultivar).

The trays were watered with tap water and left in a germination room (20-24 °C) for three days. The trays were then moved to a plastic covered tunnel (20-30 °C).

Seedling Drench

Prior to growing up inocula of the probiotic organisms, untreated tomato seeds (three cultivars) were planted separately into 24 Speedling® 24 trays filled with composted pine bark, giving a total of 72 trays. The trays were watered and left with the seed treated trays.

For drenching, 1.2 g of NutriStart-AC were weighed into eight 500 *ml* conical flasks. To each flask 240 *ml* of tap water were added and the contents swirled gently to facilitate mixing. Two *ml* quantities of concentrated spore suspension (2 x 10^9 cells) of each *Bacillus* spp. was added separately to each of the eight conical flasks, and incubated at 30°C for 18 h at 150 rpm in a water bath shaker. This process was repeated each week for six weeks in order to ensure fresh inoculum for weekly inoculations. The optical density of each culture was noted at 540 nm and values compared to those established previously (Chapter Three). Each culture was carefully diluted and the optical density noted to produce suspensions of the same optical densities and hence c.f.u's for weekly inoculations.

One week after seedling emergence, each of the eight broth cultures was separately dispensed in 1 *ml* aliquots directly onto the composted pine bark growing medium. Thus three trays, each containing 24 seedlings were inoculated per drench volume per each *Bacillus* species. This procedure was repeated each week for six weeks.

Untreated tomato seeds were used as a control. Each treatment was replicated three times.

All seedlings were irrigated three times a day by microjet irrigation. The water used contained soluble fertilizer (3.1.3[38] complete) from Ocean Agriculture[6] applied at a rate of 1g l^{-1} to give 100 mg l^{-1} N, 33 P and 100 K.

For seedling dry weight, the number of plants in each tray was noted so that the mean weight per seedling could be calculated. Seedlings from each tray were harvested at maturity at their base and placed in a brown paper bag. The plant material was subsequently dried in an oven at 55 °C. Once dried, the contents of each bag was weighed and the mean weight per shoot calculated.

Growth promotion and yield on pepper seedlings

Growth promotion and yield on pepper seedlings was carried in a shadehouse using six-week-old pepper seedlings. Seedlings were planted in 96 18cm diameter pots filled with Perlite. Two seedlings were planted into each pot. The pots were drip irrigated and fertilized using Multicote 5-1-3(43)® a 'slow release fertilizer[7]' as topdressing. Five grams were spread evenly over the surface of each Perlite filled pot. The surface of each pot was further covered with an additional thin layer of Perlite.

For the bacterial seedling drench,1.2 g of NutriStart-AC were weighed into eight 500 *ml* conical flasks. Tap water (240 *ml*) was added to each flask. The flasks were gently swirled to ensure good mixing. Two *ml* of concentrated spore suspension (2 x 10^9 cells) of each *Bacillus* spp. and Biostart® 2000 was added separately to each of the eight conical flasks, and incubated at 30 °C for 18 h at 150 rpm in a water bath shaker. This process was repeated each week for 10 weeks to ensure the weekly inoculum was fresh. The optical density of each culture was noted and values compared to those established previously (Chapter Three). Each culture was carefully diluted and the optical density noted to produce suspensions of the same optical densities and hence c.f.u's for weekly inoculations.

[6]Ocean Agriculture, P.O.Box 741, Mulders Drift 1747, Republic of South Africa

[7]Plaaskem Pty Ltd. P.O.Box/Posbus 87005, Houghton 2041, Gauteng, Republic of South Africa

One week after the seedlings were transplanted, each of the eight cultures was separately dispensed into 2 *ml* aliquots directly onto each seedling in the Perlite growth medium. Thus 12 pots, each containing two seedlings, were inoculated per drench for each culture. Weekly bacterial inoculations were performed for each bacterial culture for a further nine weeks.

Application of NutriStart-AC onto seedlings

Forty grams of NutriStart-AC were weighed into a 2 *l* conical flask. One litre of tap water was added to the flask and swirled gently for good mixing. This resulted in a 4% NutriStart-AC suspension. This process was repeated weekly for 10 weeks in order to have freshly mixed NutriStart-AC for the weekly applications.

The mixed NutriStart-AC suspension (2 *ml*) aliquots were applied as a drench separately onto each seedling in the Perlite growing medium. Six pots were inoculated per each *Bacillus* spp. and Biostart® 2000. This resulted in six out of 12 pots being supplemented with NutriStart-AC. The remaining pots were not supplemented with NutriStart-AC and served as different treatments.

Controls

Two different controls were set up for this trial. Two seedlings per pot were planted in 12, 18 cm diameter pots filled with Perlite growth medium. Six of the pots were used as Control One and received water and fertilizer only. The other six pots served as Control Two and were supplemented weekly with 2 *ml* of 4% NutriStart-AC suspension.

Thus for each of the seven *Bacillus* spp. and Biostart® 2000;

1. six pots received no NutriStart-AC supplement;
2. six pots were supplemented weekly with 4% NutriStart-AC suspension;
3. six control pots receiving water and fertilizer only and
4. six control pots receiving water, fertilizer and 4% NutriStart-AC.

This resulted in a total of 108 pots.

Seedlings were monitored for 10 weeks and the number of flowers, fruits and height of seedlings rated.

Statistical analysis

All results were analysed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS, 1987).

4.3 RESULTS

Table 4.1. Effect of Biostart™ applied by seed treatment and seedling drench on tomato (Roma) after six weeks growth in a tunnel

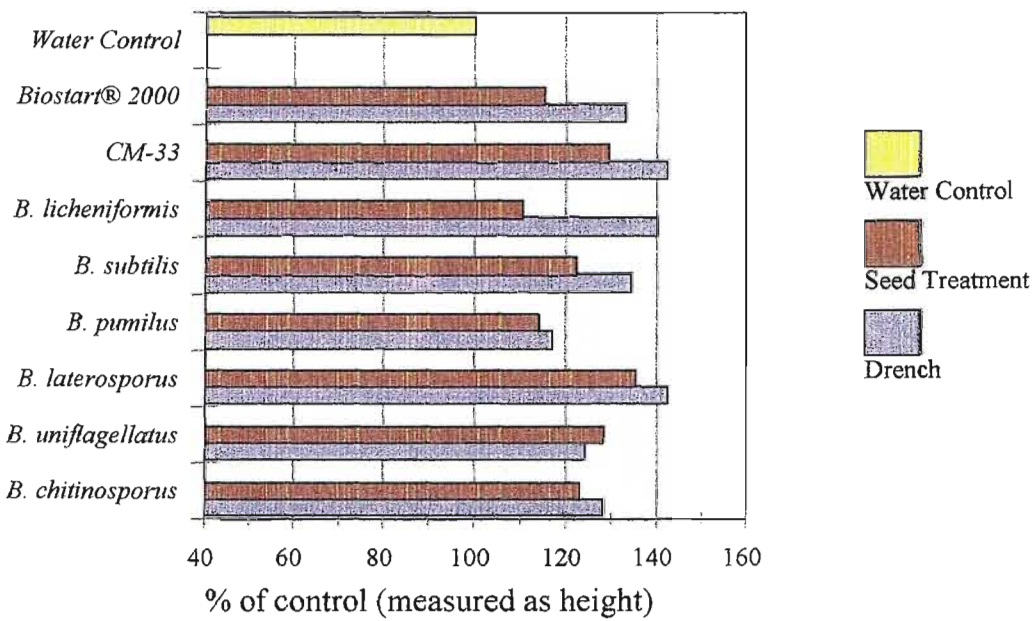
Bacteria	Treatment Type	Mean Height (mm)	% of Control	Mean Wet Weight (g)	% of Control	Mean Dry Weight (g)	% of Control
B. chitinosporus	Drench	201.33 a	128	2.96 ab	138	0.37 ab	132
B. chitinosporus	Seed Treatment	193.29 a	123	3.06 ab	142	0.40 ab	143
B. uniflagellatus	Drench	195.67 a	124	3.26 ab	152	0.43 ab	154
B. uniflagellatus	Seed Treatment	201.42 a	128	2.87 ab	133	0.46 ab	164
B. laterosporus	Drench	223.71 a	142	3.87 a	180	0.53 a	189
B. laterosporus	Seed Treatment	212.17 a	135	3.46 ab	161	0.44 ab	157
B. pumilus	Drench	183.54 a	117	3.11 ab	145	0.4 ab	143
B. pumilus	Seed Treatment	179.67 a	114	2.73 ab	127	0.34 ab	121
B. subtilis	Drench	210.63 a	134	3.43 ab	160	0.48 ab	171
B. subtilis	Seed Treatment	192.25 a	122	2.84 ab	132	0.38 ab	136
B. licheniformis	Drench	220.25 a	140	3.81 a	177	0.55 a	196
B. licheniformis	Seed Treatment	173.21 a	110	2.64 ab	123	0.31 ab	111
CM-33	Drench	222.88 a	142	3.25 ab	151	0.48 ab	171
CM-33	Seed Treatment	202.67 a	129	3.00 ab	140	0.43 ab	154
Biostart® 2000	Drench	208.67 a	133	3.15 ab	147	0.45 ab	161
Biostart® 2000	Seed Treatment	180.84 a	115	3.00 ab	140	0.40 ab	143
Control	Nil	157.17 a	100	2.15 b	100	0.28 b	100
Effects		**P-values**		**P-values**		**P-values**	
Bacteria		0.37^{NS}		0.28^{NS}		0.15^{NS}	
Treat		0.03*		0.006*		0.002*	
Bacteria*Treat		0.75^{NS}		0.55^{NS}		0.07^{NS}	
		% CV = 12.82		% CV = 15.41		% CV = 16.36	
		MSE = 25.66		MSE = 0.49		MSE = 0.07	

1. NS = Not significant; * = significant at P≤ 0.05

2. Means with the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test

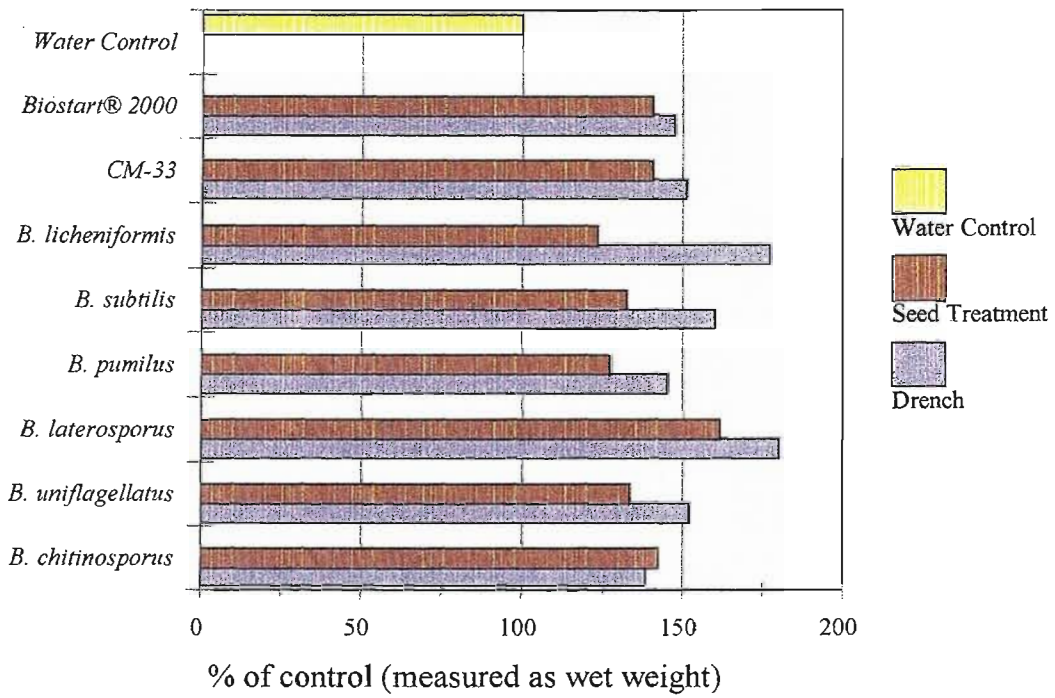
3. Seed treatment = application of bacteria to seed with Pelgel® sticker

4. Drench = Weekly application of bacteria broth cultures, at a rate of 1 ml per plant



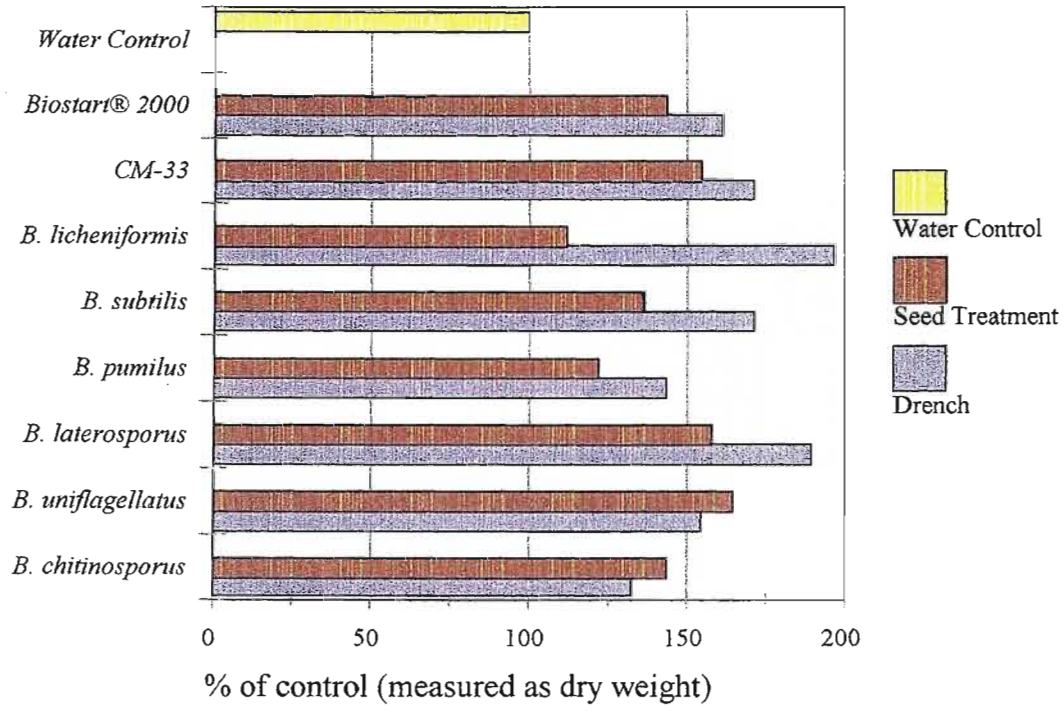
Seed Treatment = Application of bacteria to seed with Pelgel® sticker
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.1. Response of tomato seedlings (Roma) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench.



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.2. Response of tomato seedlings (Roma) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.3. Response of tomato seedlings (Roma) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench

Table 4.2. Effect of Biostart™ applied by seed treatment and seedling drench on tomato (Rodade) after six weeks growth in a tunnel

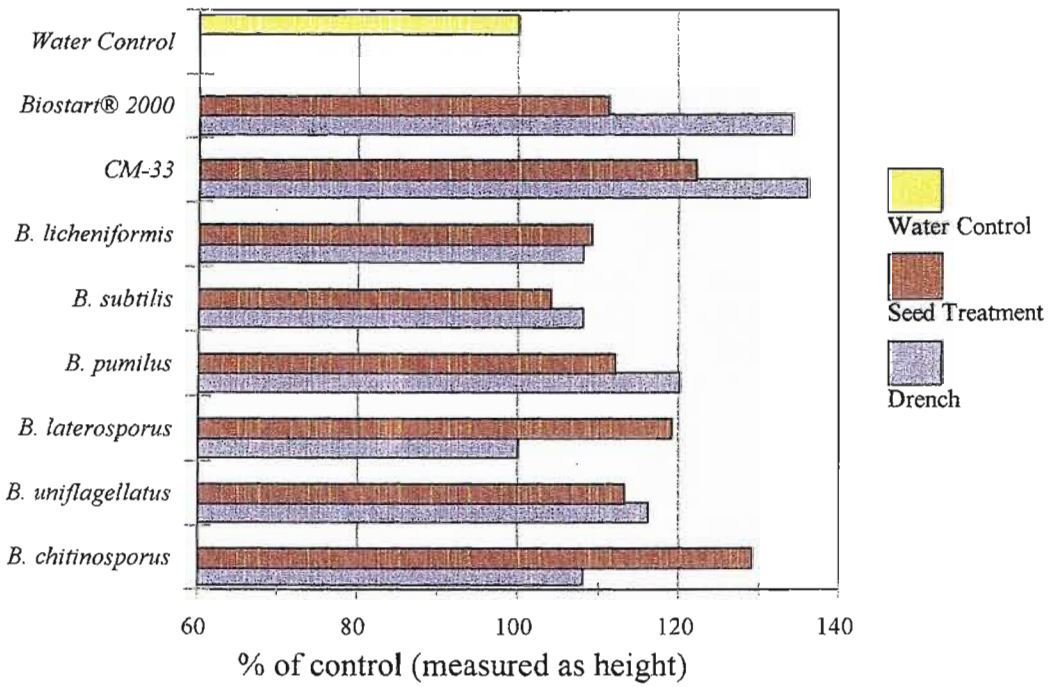
Bacteria	Treatment Type	Mean Height (mm)	% of Control	Mean Wet Weight (g)	% of Control	Mean Dry Weight (g)	% of Control
B. chitinosporus	Drench	169.79 a	108	3.44 a	111	0.51 a	121
B. chitinosporus	Seed Treatment	202.34 a	129	4.04 a	130	0.59 a	140
B. uniflagellatus	Drench	182.34 a	116	3.68 a	119	0.54 a	129
B. uniflagellatus	Seed Treatment	178.21 a	113	3.31 a	107	0.52 a	124
B. laterosporus	Drench	157.55 a	100	3.42 a	110	0.47 a	112
B. laterosporus	Seed Treatment	186.92 a	119	3.37 a	109	0.51 a	121
B. pumilus	Drench	189.25 a	120	3.81 a	123	0.55 a	131
B. pumilus	Seed Treatment	176.71 a	112	3.62 a	117	0.55 a	131
B. subtilis	Drench	170.42 a	108	3.43 a	111	0.48 a	114
B. subtilis	Seed Treatment	162.96 a	104	3.22 a	104	0.47 a	112
B. licheniformis	Drench	170.21 a	108	3.46 a	112	0.49 a	117
B. licheniformis	Seed Treatment	171.46 a	109	3.17 a	102	0.47 a	112
CM-33	Drench	214.21 a	136	3.6 a	116	0.52 a	124
CM-33	Seed Treatment	191.84 a	122	3.42 a	110	0.46 a	110
Biostart® 2000	Drench	210.54 a	134	3.89 a	125	0.61 a	145
Biostart® 2000	Seed Treatment	173.88 a	111	3.43 a	111	0.50 a	119
Control	Nil	157.25 a	100	3.10 a	100	0.42 a	100
Effects		**P-values**		**P-values**		**P-values**	
Bacteria		0.32^{NS}		0.75^{NS}		0.60^{NS}	
Treat		0.74^{NS}		0.36^{NS}		0.58^{NS}	
Bacteria*Treat		0.34^{NS}		0.79^{NS}		0.81^{NS}	
		% CV = 14.71		% CV = 15.26		% CV = 18.71	
		MSE = 26.75		MSE = 0.53		MSE = 0.10	

1. NS = Not significant; * = significant at P≤ 0.05

2. Means with the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test

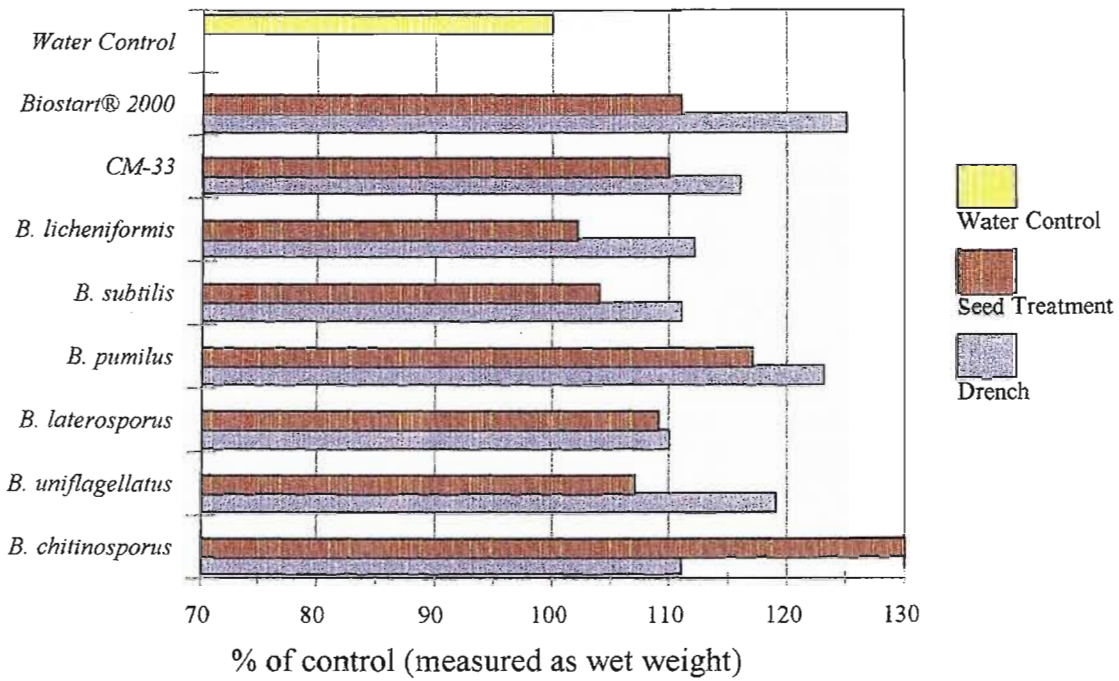
3. Seed treatment = application of bacteria to seed with Pelgel® sticker

4. Drench = Weekly application of bacteria broth cultures, at a rate of 1 ml per plant



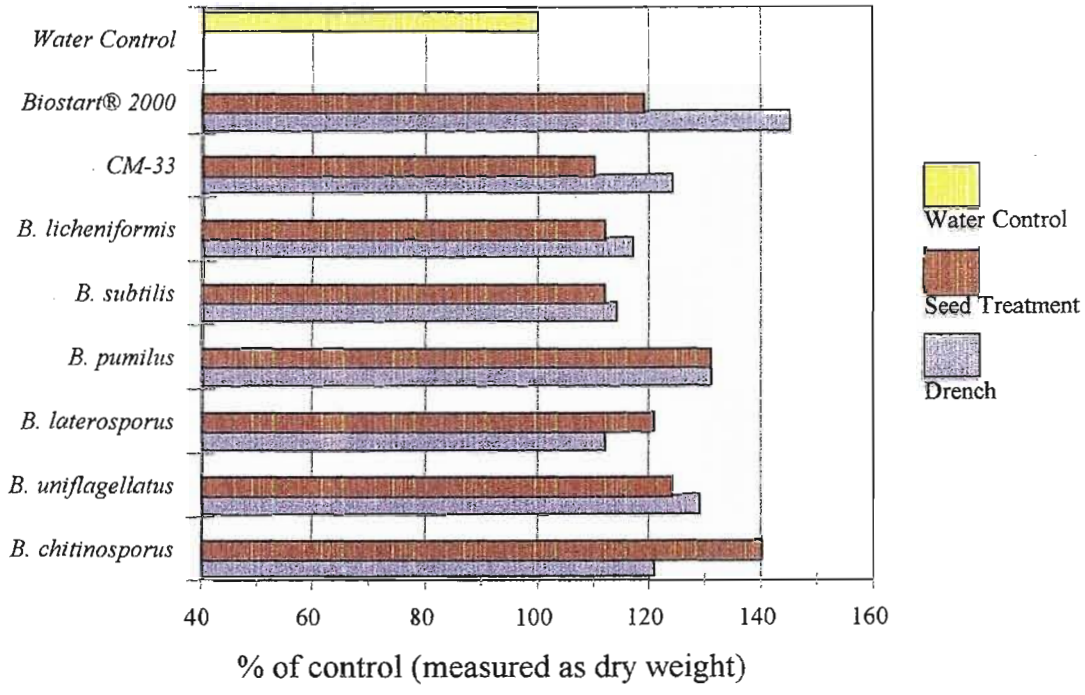
Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.4. Response of tomato seedlings (Rodade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.5. Response of tomato seedlings (Rodade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.6. Response of tomato seedlings (Rodade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench

Table 4.3. Effect of Biostart™ applied by seed treatment and seedling drench on tomato (Floradade) after six weeks growth in a tunnel

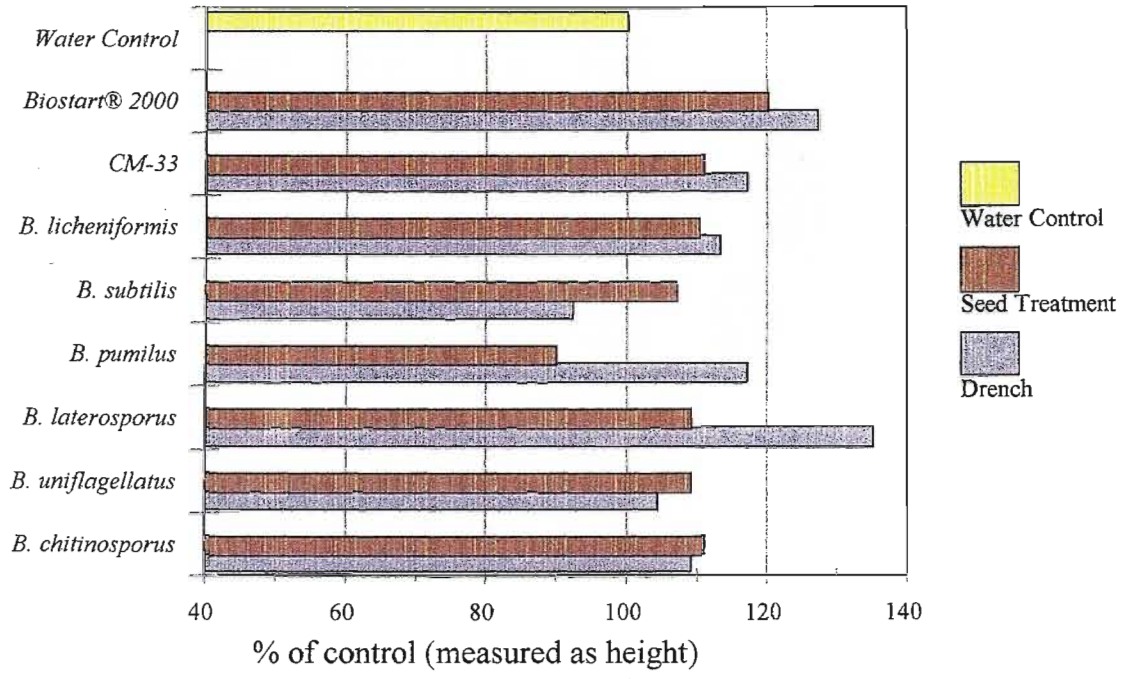
Bacteria	Treatment Type	Mean Height (mm)	% of Control	Mean Wet Weight (g)	% of Control	Mean Dry Weight (g)	% of Control
B. chitinosporus	Drench	170.33 a	109	3.87 ab	116	0.67 ab	126
B. chitinosporus	Seed Treatment	172.46 a	111	3.50 b	104	0.61 ab	115
B. uniflagellatus	Drench	162.34 a	104	3.69 b	110	0.62 ab	117
B. uniflagellatus	Seed Treatment	169.75 a	109	4.18 ab	125	0.70 ab	132
B. laterosporus	Drench	209.71 a	135	5.04 a	150	0.84 a	158
B. laterosporus	Seed Treatment	170.59 a	109	4.08 ab	122	0.65 ab	123
B.pumilus	Drench	182.67 a	117	4.11 ab	123	0.67 ab	126
B.pumilus	Seed Treatment	140.09 a	90	3.51 b	105	0.56 ab	106
B. subtilis	Drench	143.00a	92	3.62 b	108	0.57 ab	108
B. subtilis	Seed Treatment	166.30a	107	3.42 b	102	0.60 ab	113
B. licheniformis	Drench	175.29a	113	3.90 ab	116	0.66 ab	125
B. licheniformis	Seed Treatment	170.92 a	110	3.62 b	108	0.61 ab	115
CM-33	Drench	181.96 a	117	4.71 ab	141	0.75 ab	142
CM-33	Seed Treatment	173.08 a	111	3.56 b	106	0.61 ab	115
Biostart® 2000	Drench	197.88 a	127	4.39 ab	131	0.74 ab	140
Biostart® 2000	Seed Treatment	187.71 a	120	4.07 ab	121	0.68 ab	128
Control	Nil	155.80 a	100	3.35 b	100	0.53 b	100
Effects		**P-values**		**P-values**		**P-values**	
Bacteria		0.22^{NS}		0.005*		0.07^{NS}	
Treat		0.25^{NS}		0.001**		0.02*	
Bacteria*Treat		0.43^{NS}		0.08^{NS}		0.25^{NS}	
		% CV = 15.56		% CV = 10.96		% CV = 13.49	
		MSE = 26.98		MSE = 0.43		MSE = 0.09	

1. NS = Not significant; * = significant at P≤ 0.05

2. Means with the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test

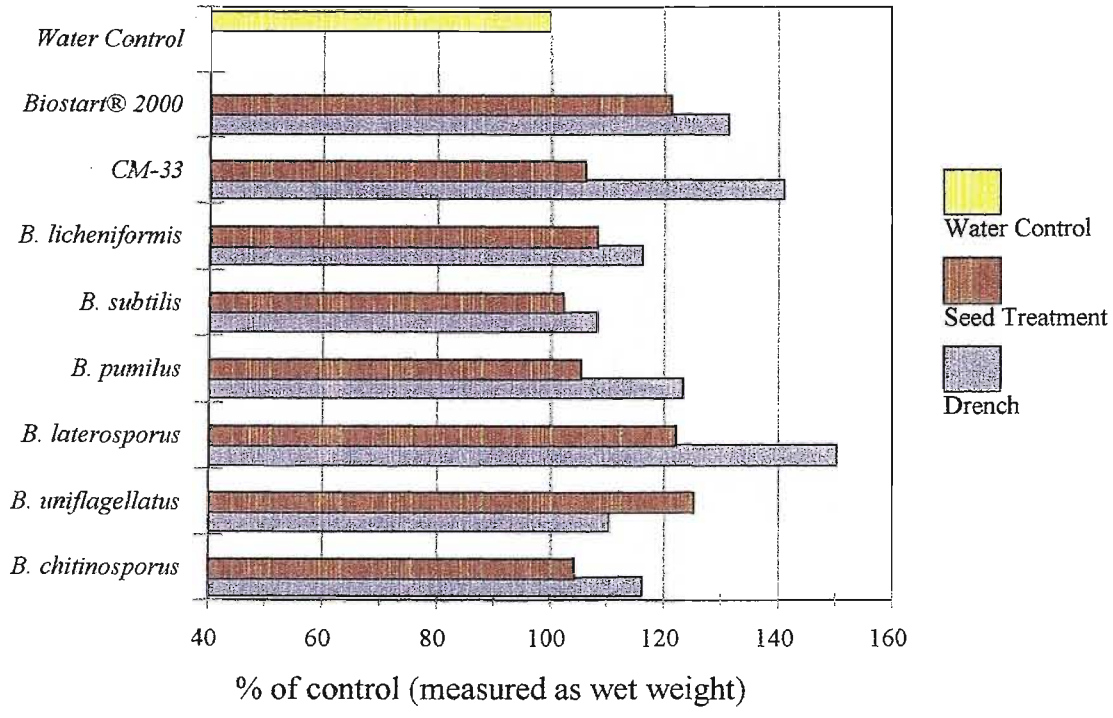
3. Seed treatment = application of bacteria to seed with Pelgel® sticker

4. Drench = Weekly application of bacteria broth cultures, at a rate of 1 ml per plant



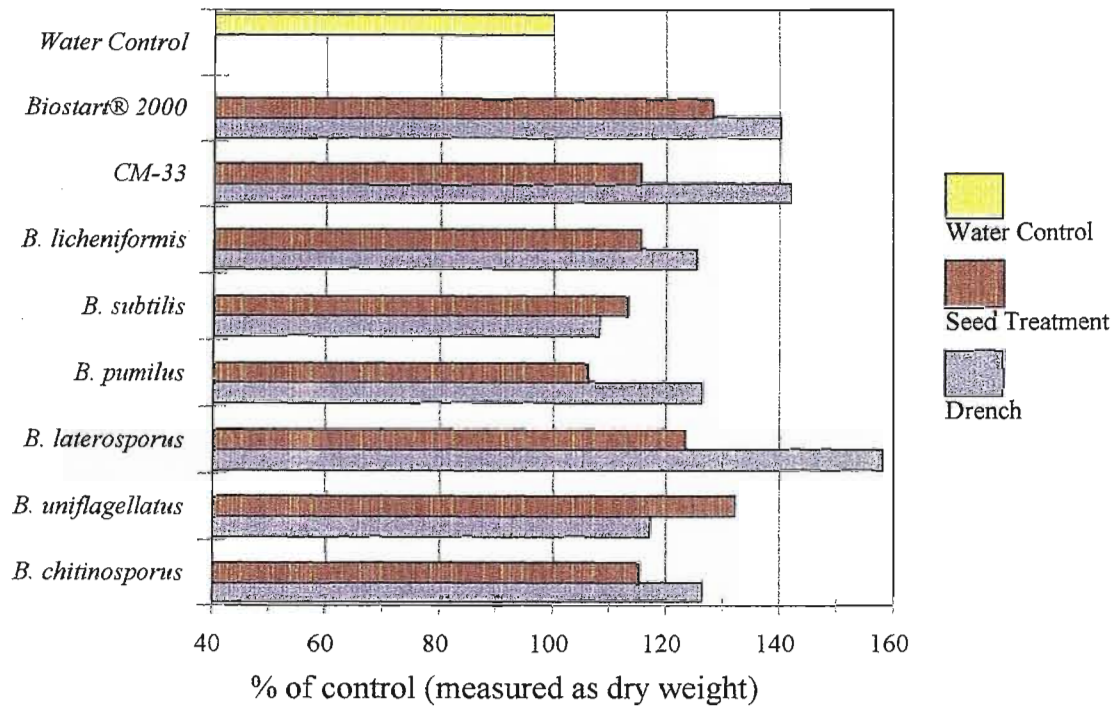
Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.7. Response of tomato seedlings (Floradade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.8. Response of tomato seedlings (Floradade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench



Seed Treatment = Application of bacteria to seed with Pelgel® sticker.
Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

Figure 4.9. Response of tomato seedlings (Floradade) to treatment with *Bacillus* probiotics, applied by seed treatment or weekly seedling drench

Table 4.4. Effect of Biostart™ applied by seedling drench on pepper (Thai) seedlings with or without NutriStart-AC after 10 weeks growth in a tunnel

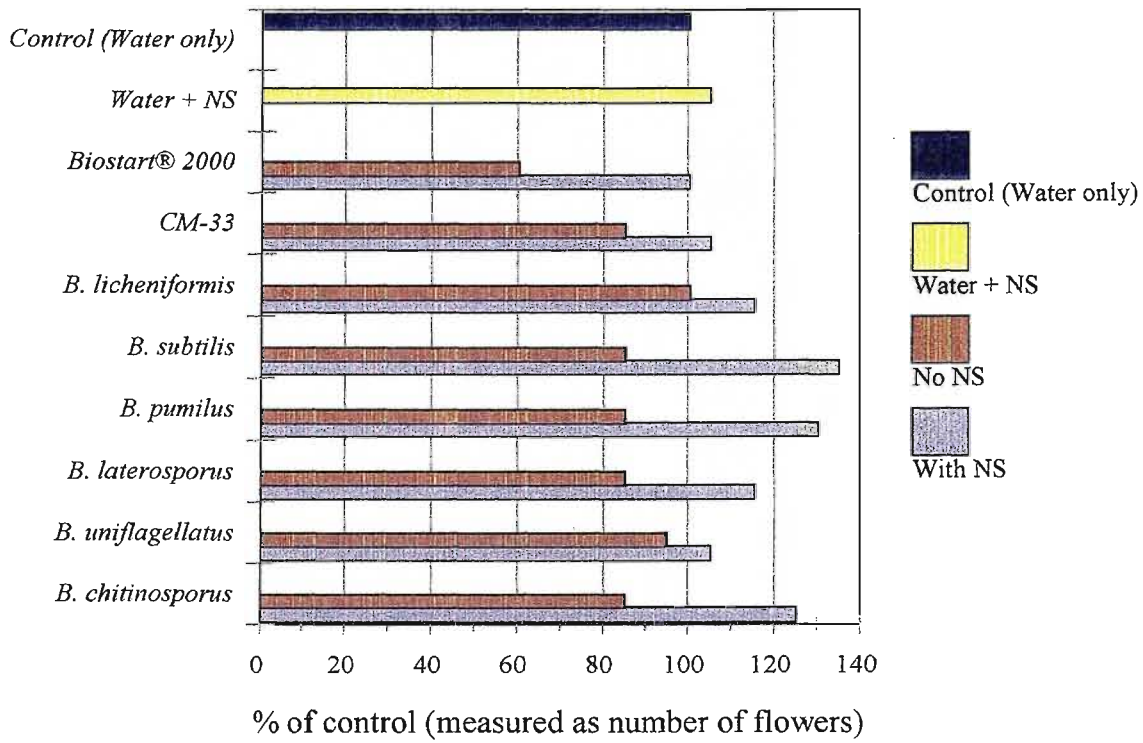
Bacteria	Treatment Type	Nutrient supplement (NS)	Mean no. of Flowers	% Control (Water Only)	Mean no. of Fruits	% of Control (Water Only)	Mean Height	% Control (Water Only)	Mean no. of Fruits + Flowers	% of Control (Water only)
B. chitinosporus	Drench	Yes	25ab	125	15 abc	500	435 a	94	40 ab	174
B. chitinosporus	Drench	No	17 ab	85	7 cde	233	436 a	94	24 bc	104
B. uniflagellatus	Drench	Yes	21 ab	105	12 abcd	400	433 a	93	33 abc	143
B. uniflagellatus	Drench	No	19 ab	95	8 abcde	267	461 a	99	27 abc	117
B. laterosporus	Drench	Yes	23 ab	115	11 abcd	367	459 a	99	34 abc	148
B. laterosporus	Drench	No	17 ab	85	6 de	200	430 a	92	23 bc	100
B. pumilus	Drench	Yes	26 ab	130	14 abc	467	520 a	112	40 ab	174
B. pumilus	Drench	No	17 ab	85	7 cde	233	473 a	102	24 bc	104
B. subtilis	Drench	Yes	27 a	135	19 a	633	495 a	106	46 a	200
B. subtilis	Drench	No	17 ab	85	6 de	200	471 a	101	23 bc	100
B. licheniformis	Drench	Yes	23 ab	115	13 abcd	433	463 a	100	36 ab	157
B. licheniformis	Drench	No	20 ab	100	9 abcde	300	475 a	102	29 abc	126
CM-33	Drench	Yes	21 ab	105	16 ab	533	501 a	108	37 ab	161
CM-33	Drench	No	17 ab	85	6 cde	200	462 a	99	23 bc	100
Biostart® 2000	Drench	Yes	20 ab	100	11 abcd	367	463 a	100	31 abc	135
Biostart® 2000	Drench	No	12 b	60	3 e	100	411 a	88	15 c	65
Water Only	Nil	No	20 ab	100	3 e	100	465 a	100	23 bc	100
Water + Food	Nil	Yes	21 ab	105	12 abcd	400	472 a	102	33 abc	143
Effects			**P-values**		**P-values**		**P-values**		**P-values**	
Bacteria			0.4^{ns}		0.06^{ns}		0.13^{ns}		0.11^{ns}	
Nutrient supplement			0.0001***		0.0001***		0.15^{ns}		0.0001***	
Bacteria*Nutrient supplement			0.64^{ns}		0.14^{ns}		0.72^{ns}		0.42^{ns}	
			% CV = 33.52		% CV = 39.85		% CV = 13.68		% CV = 32.34	
			MSE = 6.74		MSE = 4.02		MSE =63.16		MSE = 9.73	

1. ns = Not significant; *** = significant at P≤ 0.001

2. Means with the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test

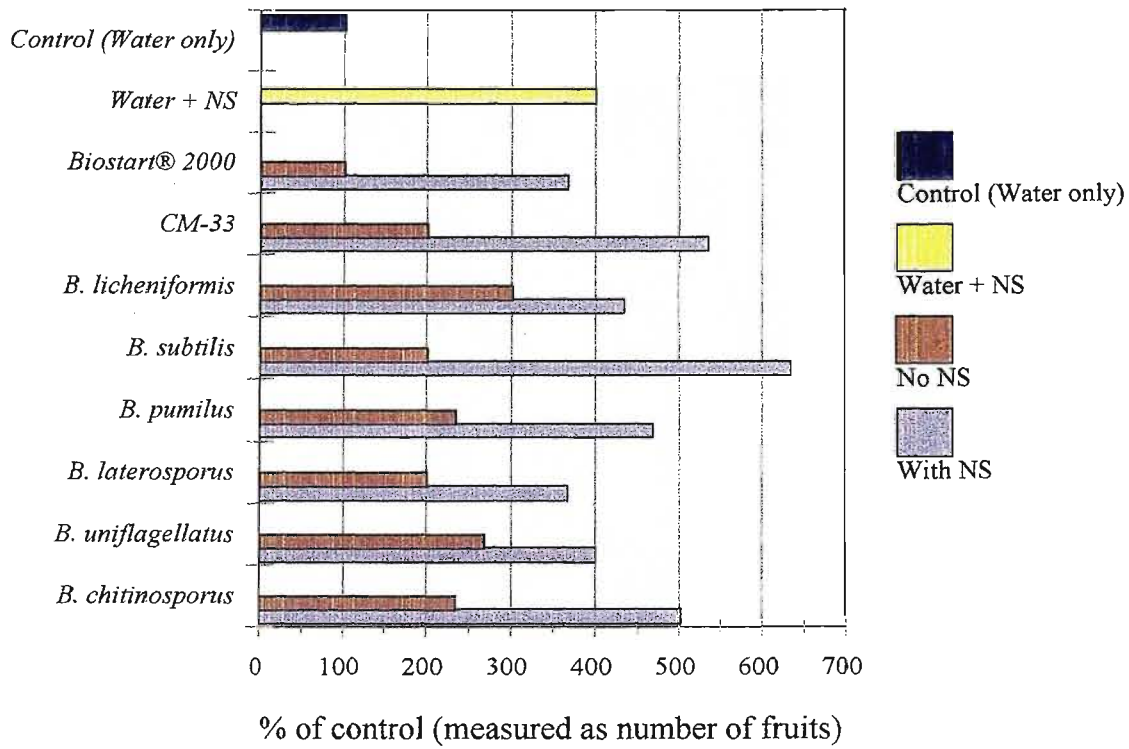
3. Drench = Weekly application of 1 ml of bacteria broth cultures onto seedlings

4. Nutrient Supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant



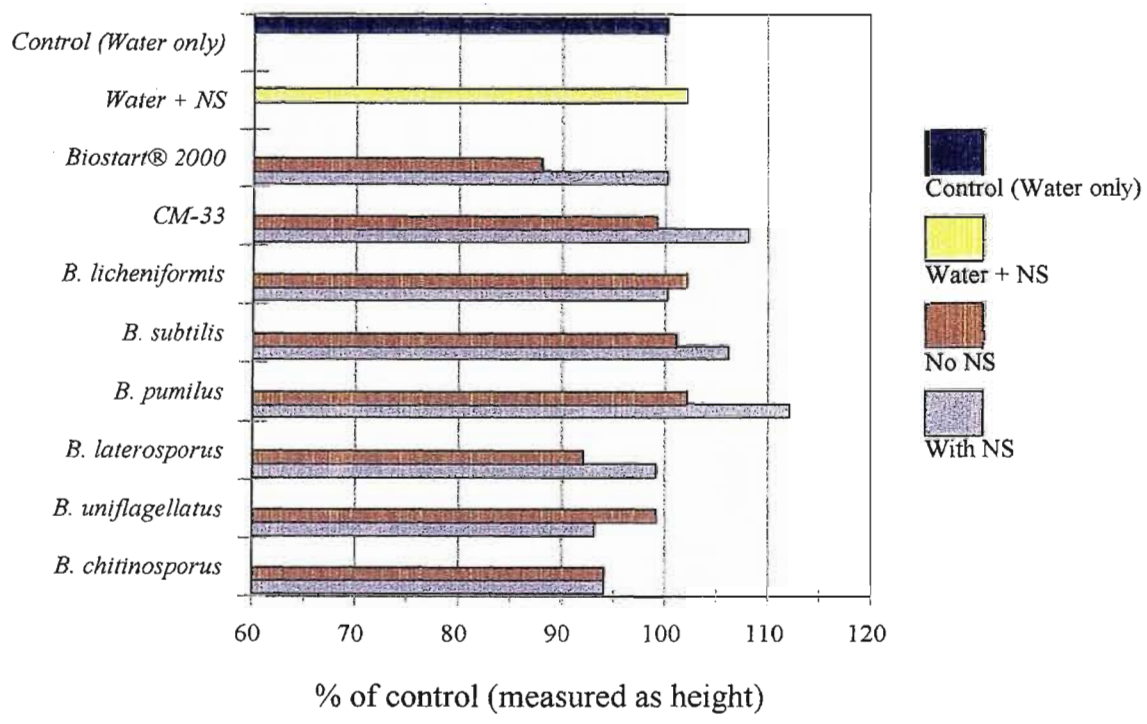
Nutrient supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Figure 4.10. Response of pepper (Thai) seedlings to treatment with *Bacillus* probiotics, applied by weekly seedling drenches, with or without NutriStart-AC at 40 gl^{-1}



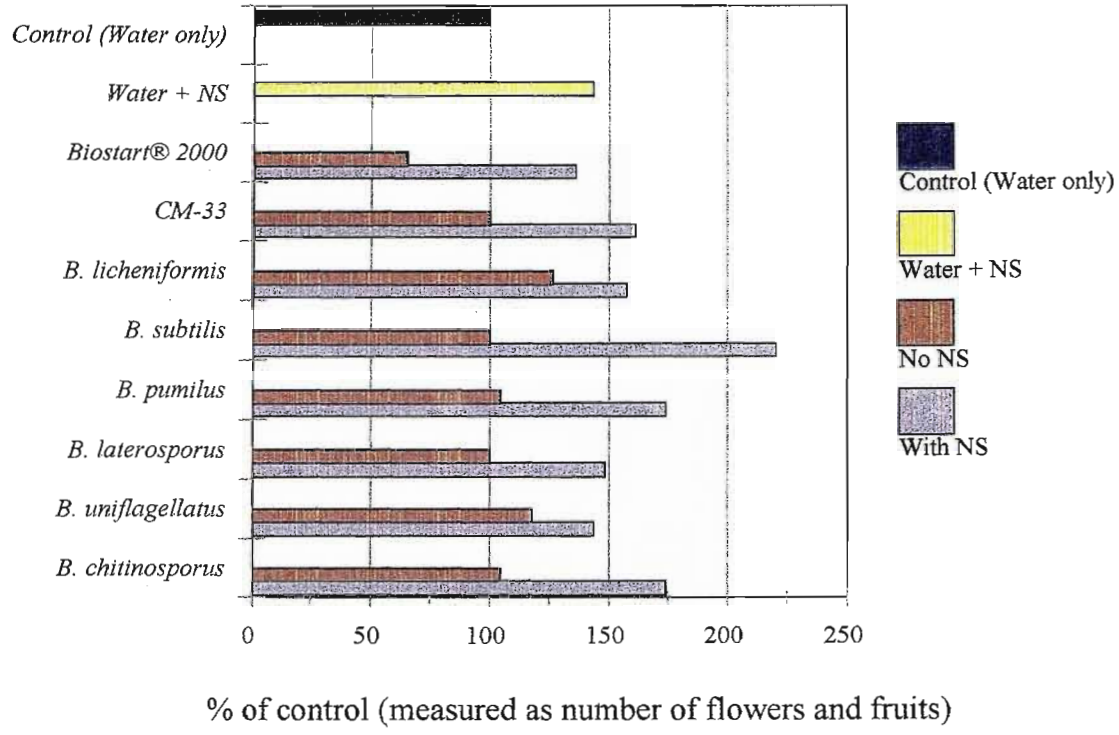
Nutrient supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Figure 4.11. Response of pepper (Thai) seedlings to treatment with *Bacillus* probiotics, applied by weekly seedling drenches, with or without NutriStart-AC at 40 gl^{-1}



Nutrient supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Figure 4.12. Response of pepper (Thai) seedlings to treatment with *Bacillus* probiotics, applied by weekly seedling drenches, with or without NutriStart-AC at 40 $g/^{-1}$



Nutrient supplement (NS) = Weekly drench with NutriStart-AC, at a rate of 1 ml per plant

Figure 4.13. Response of pepper (Thai) seedlings to treatment with *Bacillus* probiotics, applied by weekly seedling drenches, with or without NutriStart-AC at 40 gl^{-1}

Figure 4.14. Comparison of *Bacillus uniflagellatus* seed treated (middle) and seedling drenched (far right) tomato (Roma) seedlings with water control seedlings (far left).

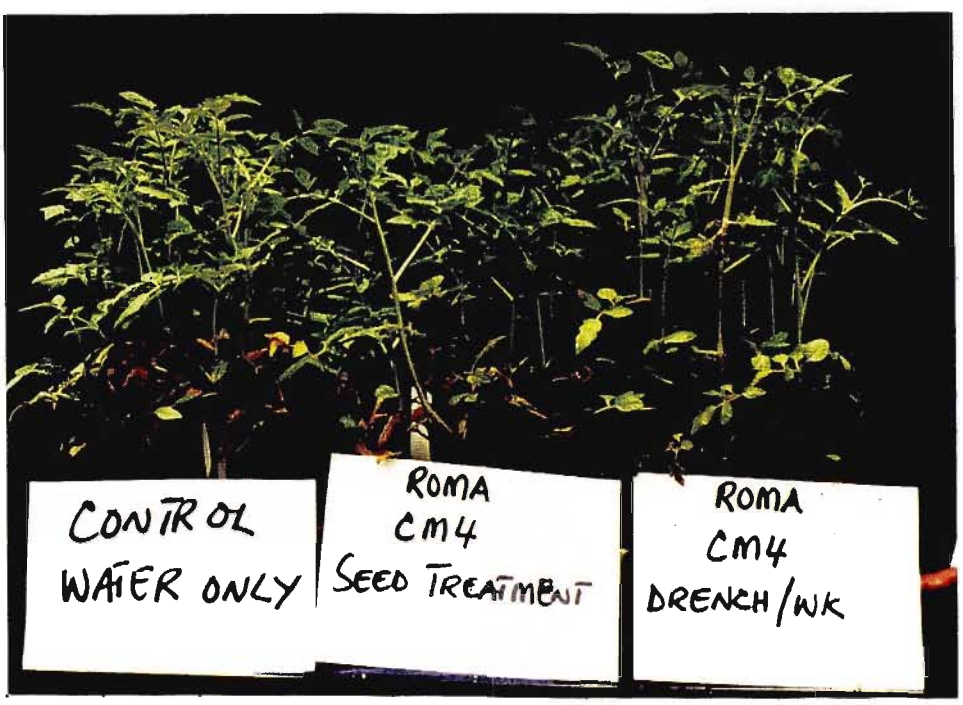


Figure 4.15. Comparison of *Bacillus pumilus* seed treated (middle) and seedling drenched (far right) tomato (Roma) seedlings with water control seedlings (far left)

Note the differential interaction between the treatments, tomato cultivar (Roma) and the *Bacillus* strains.

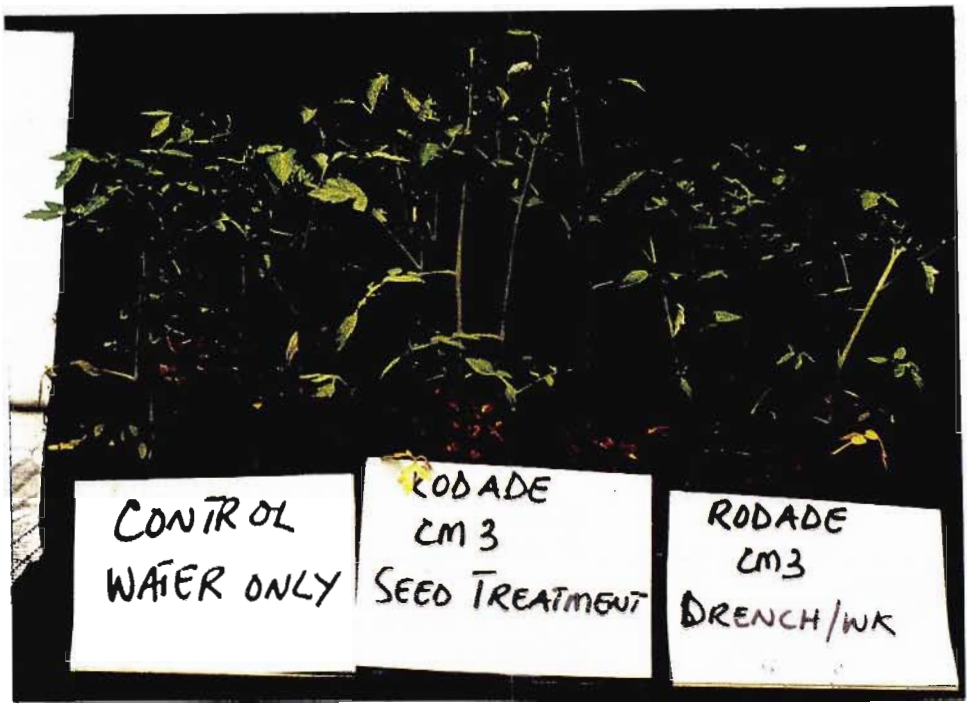


Figure 4.16. Comparison of *Bacillus laterosporus* seed treated (middle) and seedling drenched (far right) tomato (Rodade) seedlings with water control seedlings (far left)

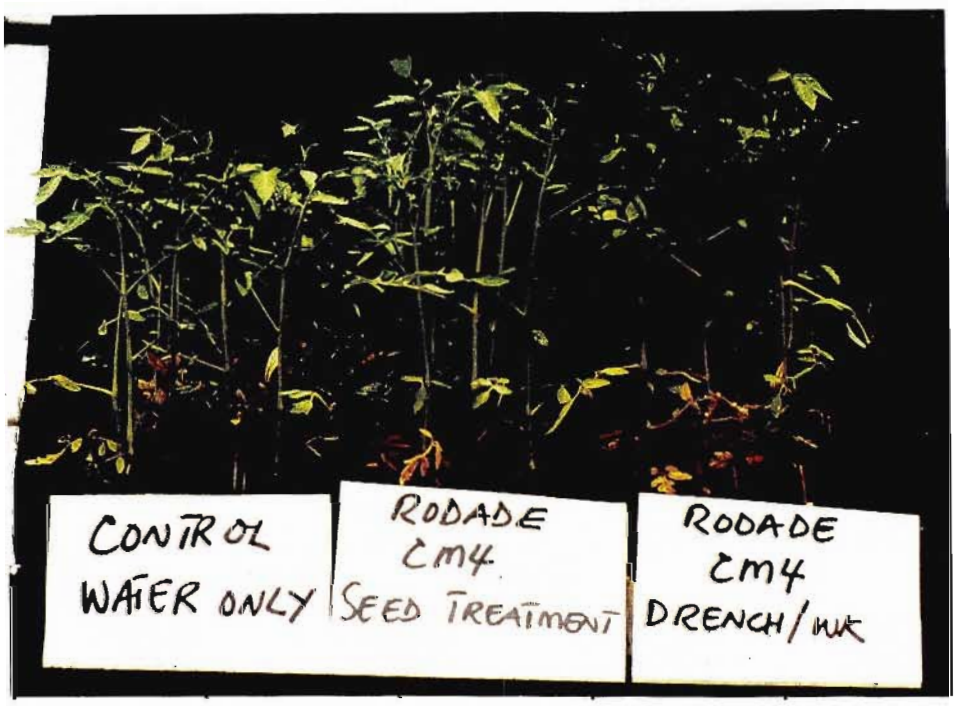


Figure 4.17. Comparison of *Bacillus pumilus* seed treated (middle) and seedling drenched (far right) tomato (Rodade) seedlings with water control seedlings (far left)

Note also the differential interaction between the treatment, tomato cultivar (Rodade) and *Bacillus* strains.

Biostart™, a *Bacillus*-based plant probiotic caused a statistically significant increase of seedling height, wet weight and dry weight of the tomato cultivar, Roma and an increase in seedling wet and dry weights of Rodade and Floradade cultivars. For all three cultivars tested, the least response to the bacterial treatments was recorded on Rodade. No statistically significant increase was recorded on Rodade for all three parameters measured (Tables 4.1-4.3).

The differences in seedling dry weight between seed treated and seedling drenched seedlings were marginal for some bacteria (Tables 4.1-4.3). Major differences were recorded on Roma with *B. licheniformis* with growth increase of 96% (drench) against 11% (seed treatment), *B. laterosporus* recorded growth increase of 89% (drench) against 57% (seed treatment) and *B. subtilis* 71% (drench) against 36% (seed treatment). Similar effects were recorded on wet weights (Table 4.1).

For all three tomato cultivars, growth increase differs according to the Biostart™ *Bacillus* probiotic species applied and the cultivar used. Increase in fresh and dry weight with Biostart™ treatments were as follows:

- Roma recorded an increase ranging from 23-80% (wet weight at P = 0.006) and dry weight increase ranged from 11-96% (at P = 0.002) (Table 4.1, Figures 4.2-4.3)
- Rodade recorded an increase ranging from as low as 2 to 30% on wet weight (P = 0.36) and from 10-45% on dry weight (P = 0.58) (Table 4.2, Figures 4.5-4.6)
- Floradade recorded an increase ranging from 2-50% on wet weight (P = 0.001) while on dry weight increases ranged from 6-58% at P = 0.02 (Table 4.3, Figures 4.8-4.9)

Increase in growth also differed according to the two treatments and bacteria applied. Response differed among the probiotic *Bacillus* species applied. Comparisons based on dry weight are as follows:

On Roma, (Table 4.1):

- Only *B. chitinosporus* and *B. uniflagellatus* were more effective when applied by seed treatment
- All other probiotic *Bacillus* spp. and Biostart® 2000 were most effective when applied by seedling drench method

On Rodade, (Table 4.2):

- Only *B. pumilus* had the same effect (31% growth increase) for both treatments
- *B. chitinosporus* and *B. laterosporus* were more effective when applied by seed treatment than seedling drench
- All other probiotic *Bacillus* species were most effective when applied by seedling drench method

On Floradade, (Table 4.3):

- Only *B. chitinosporus* and *B. subtilis* were more effective when applied by seed treatment than seedling drench
- All other probiotic *Bacillus* spp. were more effective when applied by seedling drench method

Varied results were obtained with regard to individual species in growth response according to the cultivar used and the treatment applied; e.g. on Roma, *B. licheniformis* resulted in the best growth increase (dry weight) when applied by seedling drench (96%) and 11% (dry weight) when applied by seed treatment (Table 4.1). Different results were caused by the same bacteria on Rodade and Floradade (Tables 4.2-4.3).

None of the *Bacillus* spp. recorded a 100% growth increase on any of the tomato cultivars used. The best result of the three cultivars was recorded on Roma by *B. licheniformis* (96% dry weight). Generally, growth response was found to be more pronounced on Roma than on Rodade and Floradade. Rodade responded the least of the three cultivars used in this trial (Tables 4.1-4.3 and Figures 4.1-4.9).

Growth promotion and yield on pepper seedlings

Biostart™ applied by seedling drench to pepper seedlings caused a significant increase in the number of flowers (P = 0.0001), fruits (P = 0.0001) and fruits plus flowers (P = 0.0001) when supplemented weekly with 2 *ml* of 4% NutriStart-AC suspension. Increases as high as 533% (compared to the water control) in the number of fruits was recorded when seedlings were drenched with *B. subtilis* supplemented weekly with 2 *ml* of 4% NutriStart-AC suspension (Table 4.4).

Moreover, a substantial increase in the number of fruits was obtained when seedlings were not supplemented weekly with NutriStart-AC suspension. *Bacillus uniflagellatus* recorded an increase of 167%. The least, 0%, was recorded by Biostart® 2000 as against 267% when supplemented with 4% NutriStart-AC suspension.

A marginal increase was recorded in the number of flowers. The highest increase was recorded after treatment with *B. subtilis* (35%) when supplemented weekly with 4% NutriStart-AC suspension.

No statistically significant results were obtained on height of pepper plants. The largest response was caused by *B. pumilus* resulting in a 12% increase in height.

All seven *Bacillus* spp. and Biostart® 2000 stimulated growth and yield but not at the same level. Varying degrees of results were obtained according to the performance of each species on the pepper cultivar used. The best results among all seven *Bacillus* spp. and Biostart® 2000 was caused by *B. subtilis* on fruits (533%), flowers (35%) and flowers plus fruits (100%).

4.4 DISCUSSION

The results of the trials are reported in Tables 4.1-4.4. Below is a summary of five tables of results, Tables 4.1-4.4. This table makes a coarse summary of the results presented in Tables 4.1-4.4, reflecting the significant or non-significant results from the ANOVAs conducted. Analysis of results, reflected in Table 4.4 is as follows:

Table 4.5. A review of results in Tables 4.1-4.4

Treatment effects and Interactions	Treatment Comparisons	Significant	Not Significant
	Tomato		
1. Bacteria	Differences between isolates and BioStart® 2000	Wet Weight (Floradade)	Height (all three cultivars) Dry weight (all three cultivars) Wet weight (Roma and Rodade)
2. Treat	Differences between Seed Treatment and Drenching	Height (Roma) Wet and Dry weight (Roma and Floradade)	Height (Rodade and Floradade) Wet and d ry weight (Rodade)
3. Bacteria*Treat	Interaction between seven isolates, BioStart® 2000 and treatments applied	-	Height, wet and dry weight (all three cultivars)
	Pepper		
4. Bacteria	Differences between isolates and BioStart® 2000	-	Flowers, Fruits, Height, Flowers plus Fruits
5. Nutrient supplement	Differenced between supplemented and unsupplemented application of NutriStart-AC to treatment (drenching)	Flowers, Fruits, Flowers plus Fruits	Height
6. Bacteria*Nutrient supplement	Interaction between seven isolates, BioStart® 2000 drenching and NutriStart-AC application	-	Flowers, Fruits, Height, Flowers plus Fruits

Comparing performances of the individual *Bacillus* spp. and BioStart®2000 probiotics, a significant difference (P = 0.005) was observed on wet weight on Floradade seedlings. This suggests that the various probiotic *Bacillis* spp. responded differently in terms of wet weight on Floradade seedlings. Thus, the effect of these probiotic species were different compared to the effects on Roma and Rodade seedling wet weights. No statistically significant difference was observed on seedling height (all three cultivars), dry weight (all three cultivars) and wet weight (Roma and Rodade only). This shows that all the *Bacillus* spp. and Biostart® 2000 used as probiotic organisms responded in the same way on seedling height and dry weight of all three tomato cultivars and on wet weight of Roma and Rodade. There was therefore no variation in terms of bacteria effect on seedling height and dry weight of all three tomato cultivars and on wet weight of Roma and Rodade. Bacterial response was neither better nor worse when compared to each other.

Comparing seed treatment and seedling drench, all seven probiotic *Bacillus* spp. Biostart® 2000 responded to the seed treatment and seedling drench methods of application. Significant differences were observed in Roma on seedling height (P = 0.03), wet weight (P = 0.006) and dry

weight (P = 0.0002). In Floradade, significant differences were observed on seedling height (P = 0.001) and dry weight (P = 0.02). A possible explanation is that the bacterial drench might have given an additional response as seedlings were drenched weekly with bacterial suspensions a week after germination. No statistically significant differences were recorded in Rodade on seedling height, wet weight and dry weight and Floradade on seedling height. It is assumed that all the probiotic *Bacillus* spp. and Biostart® 2000 responded in the same manner in the seed and seedling drench treatments applied. No additional response was observed with the weekly bacteria seedling drench. Hence, there was no difference in response between the seed treatment and seedling drench.

Comparing the response of the various probiotic *Bacillus* spp. and Biostart® 2000 to the two treatments applied (seed and seedling drench) treatments, no significant difference was found in all three cultivars. It appears, therefore, that the weekly bacterial drench did not make any significant difference and response on any of the three cultivars.

Comparing performances of the individual probiotics on pepper seedlings, no statistically significant differences were observed on flowers, fruits, seedling height and flowers plus fruits. A possible suggestion is that the probiotic bacteria used responded in the same manner on all four parameters measured. There was, therefore, no variation in terms of bacterial effect on flowers, fruits, seedling height and flowers plus fruits. None of the bacteria therefore responded better or worse than the other.

The effect of NutriStart-AC on bacteria, growth and yield response on pepper seedlings gave a statistically highly significant difference on flowers (P = 0.0001), fruits (P = 0.0001) and flowers plus fruits (P = 0.0001). The added NutriStart-AC might have acted as a nutrient source for flower and fruit production. This suggests that the production of flowers and fruits responded to the addition of NutriStart-AC. Alternatively, NutriStart-AC might have stimulated all rhizosphere bacteria. No significant difference was recorded on height, i.e., the added NutriStart-AC did not increase seedling height.

Comparing the response of the various bacteria towards the addition of NutriStart- AC and the effect on growth and yield, no significant difference was recorded on flowers, fruits, height and flowers plus fruits. There is therefore no difference in response with regards to the various bacteria, thus the addition of food did not support one bacteria more than the other.

Commercially available strains of *Bacillus* spp. applied by seed treatment and seedling drench as plant probiotics induced statistically significant increase in growth of two tomato cultivars (Roma and Floradade) and yield increase in pepper (Thai). A growth increase of 96% was attained by Roma when *B. licheniformis* was applied to seedlings as a drench treatment. Similar results were obtained with *B. laterosporus*, *B. subtilis*, and CM-33 with increases of 89%, 97% and 71% respectively. Weller & Cook (1986) found that a 26% grain yield was recorded following seed treatment with *Pseudomonas fluorescens* biovar I (Q72a-80) on wheat seeds. Results obtained from the tomato cultivar trial show that there is a potential in *Bacillus* spp. to provide growth stimulation if well manipulated by providing the right growth conditions and the best possible application method. It was previously found (Chapter Three) that NutriStart-AC does play an important role in growth stimulation. It is assumed that none of the *Bacillus* spp. was able to achieve a 100% or more growth stimulation due to the absence of NutriStart-AC supplement.

An increase as high as 533% in yield was recorded when pepper seedlings were drenched with *B. subtilis* and supplemented weekly with 2 *ml* of 4% NutriStart-AC suspension. Similar results were obtained using *B. chitinosporus* (400%), *B. uniflagellatus* (300%), *B. licheniformis* (333%), *B. pumilus* (367%) and CM-33 (433%). For most probiotic *Bacillus* spp., an increase in fruit yield with 4% NutriStart-AC supplement was more than two fold compared to fruit yield from plants that received no NutriStart-AC supplement. This confirms the premises established in the previous trial (Chapter Three) that NutriStart-AC does play an important role in growth and stimulation associated with probiotic treatments.

Seed treatment has attracted attention as a method of transferring plant growth promoting rhizobacteria (PGPR) into the rhizosphere for growth stimulation and biological control. A *Pseudomonas* isolate, MA342 was found to suppress disease incidence of *Drechslera teres* Sacc. and *Tilletia caries* (DC.) Tul. in the field when applied by seed treatment (Hökeberg *et al.*, 1997).

Zhang *et al.* (1996) also observed that seed treatment with *Gliocladium virens* Miller, Giddens and Foster strains G-4 and G-6 and with *B. subtilis* strains GB03 and GB07 reduced the colonization of tap roots and secondary roots of cotton seedlings by *Fusarium* spp. In this trial, the probiotic *Bacillus* spp. used gave a considerable increase in wet and dry weight with seed treated seedlings as compared to untreated controls. Growth stimulation was more pronounced on Roma and Floradade than on Rodade. Rodade has a notably small root system, and this may affect the degree of rhizosphere colonization.

The best growth increase in the tomato cultivar trial was recorded using *B. licheniformis* (96%) on Roma applied by seedling drench. Most *Bacillus* spp. performed better when applied as a seedling drench. A possible assumption is that more roots might have been colonised as a result of the weekly bacterial drench. Zaki *et al.* (1998) also found that *Pseudomonas cepacia* used as a soil drench increased a cotton seedling stand significantly ($P \leq 0.05$) relative to the non-treated control in *Rhizoctonia solani* Kühn infested and non-treated blocks.

No one particular probiotic *Bacillus* spp. could be singled out to have performed best in all three tomato cultivars and pepper plants. Results of individual species varied from one cultivar to the other. This further suggests that more trials are needed to assess the efficacy of seed treatment and seedling drench on different plant cultivars. The methods involved in treating seeds and a wide range of seed adhesives (stickers) need to be assessed as some adhesives may contain some chemical components that might have an adverse or deleterious effect on some PGPR while enhancing others. Future trials should also be conducted in the field as conditions in the field are not the same as those in the tunnels and greenhouses and might not favour these probiotic *Bacillus* spp.

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CHAPTER 5

5[1] Evaluation of Biostart™ for control of plant-parasitic nematodes (Root-knot nematodes) on pepper seedlings

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A shadehouse trial was conducted to evaluate the effect of Biostart™, a *Bacillus*-based plant probiotic on root-knot nematodes, (*Meloidogyne* spp.). After 12 weeks growth and monitoring, no galls were found on the pepper (*Capsicum frutescens* L. cv. Thai) seedlings inoculated with *Meloidogyne* spp. We suggest that early inoculation failed because there were no roots for the nematodes to attack at the time of inoculation.

5.1 INTRODUCTION

Plant-parasitic nematodes, especially root-knot nematodes, are important cosmopolitan pathogens affecting the production of tropical and subtropical crops and are one of the major factors limiting crop productivity. For the past two decades, nematode control has been based mainly on the use of chemicals (Duponnois *et al.*, 1998). Chemical nematicides, though effective in providing rapid destruction of nematodes, are now being reappraised due to their environmental hazardousness (such as persistence in soil and their contamination of ground water), human health, their attack on non-target organisms, high cost and limited availability in many developing countries (Akhtar, 1998). An awareness of the problems associated with the use of pesticides, as well as their possible dangers, the time required to develop resistant cultivars, the economic pressures on land use which limit the use of crop rotation and other cultural methods, is the impetus behind the strong movement in determining the potential of biological management of plant parasitic nematodes (Jatala, 1986).

[1]Chapter format according to Biocontrol Science and Technology

Nematologists have now focused their attention on alternative control measures such as cultural and biological methods. Cultural methods include crop rotation, fallow years and cover crops. Biological control strategies include the use of rhizobacteria and fungi to reduce plant-parasitic nematode populations. Several works have been carried out on nematode control using rhizo- and endophytic bacteria (Jatala, 1986; Rodriguez-Kabana *et al.*, 1987; Becker *et al.*, 1988; Sayre, 1991; Spiegel *et al.*, 1991; Duponnois *et al.*, 1998; Hallmann *et al.*, 1998). Moreover, organic soil amendments are also recognized as 'non-conventional' nematode management options (Mankau & Minter, 1962; Rodriguez-Kabana, 1986; Rodriguez-Kabana *et al.*, 1987).

Biological control of nematodes has long been considered an alternative to managing nematodes with pesticides. However, this technology developed slowly because of the effectiveness of chemicals and the limited resources given to the search for alternative control methods (Becker *et al.*, 1988). Overall, more information is needed if biological control, as opposed to chemical control of nematodes is to be fully adopted.

The present study focuses on the potential of Biostart™, a *Bacillus*-based plant probiotic to control root-knot nematodes on pepper seedlings.

5.2 MATERIALS AND METHODS

Microorganisms

Seven *Bacillus* spp.: *B. chitinosporus*; *B. uniflagellatus, B. laterosporus, B. pumilus, B. subtilis, B. licheniformis,* an *Bacillus* strain CM-33 and Biostart® 2000 (a combination of *B. chitinosporus*, B. *laterosporus*, and *B. licheniformis*) were used in this experiment. The species were provided commercially as concentrated spore suspensions by Microbial Solutions[2].

Crop Evaluated

Six-week-old pepper seedlings from Sunshine Seedling Services[3] were used in this trial.

[2]Microbial Solutions (Pty)Ltd., P.O. Box 1180, Strubens Valley 1735, Republic of South Africa.

[3]Sunshine Seedling Services, Old Wartburg Road P.O.Box 100461 Scottsville 3201, Republic of South Africa

Sampling and collection of nematode infested soil

Soil samples were collected from a commercial farm at Tala Valley, near Pietermaritzburg, known to be infested with *Meloidogyne* spp. Soil samples were taken around plant roots with symptoms specific of *Meloidogyne* spp. infection, i.e., stunted plants with galls on the roots from different places on the farm. Soil samples collected around each plant were placed in a separate plastic bag. The samples were stored in a cold room at about 4 ^{0}C.

Nematode extraction from soil

Populations of nematodes were extracted from the soil by a modified Cobb's sieving and decanting technique (Figure 5.1) (Van Beizooijen, 1998). For extraction, about 200-300 *ml* of a well mixed soil sample was placed in a 2 *l* plastic bowl and covered with tap water. The soil and the water were mixed by stirring with a glass rod and allowed to stand for about 30-45 seconds. The water was poured through a sieve with a 0.5 mm pore size into a second 2 *l* plastic bowl. The debris on the sieve was discarded. Materials in the first plastic bowl, consisting of sand and heavy soil particles were discarded. The water in the second plastic bowl was poured back through a 350µ pore size sieve into the first plastic bowl. At this stage, the residue on the 350µ pore size sieve was washed into a third plastic bowl using a washing bottle. The water in the first plastic bowl was poured back through a 175µ pore size sieve into the second plastic bowl. The residue on the 175µ screen was washed into the third plastic bowl and the water in the second plastic bowl poured back through a 100µ pore size sieve into the first plastic bowl. The residue on the 100µ pore size sieve was washed into the third plastic bowl. The water in the first plastic bowl was poured back through a 50µ pore size sieve into the second plastic bowl and the residue on the 50µ pore size sieve washed into the third plastic bowl. This last operation was repeated four times and the debris from the 50µ pore size sieve washed into the third plastic bowl. The suspension obtained from the four sieves was decanted through a 385µ pore size sieve over a 16 cm diameter watch glass onto a double cotton wool filter. The sieve was then placed in a shallow tray filled with water, in order to get an even distribution of the debris onto the filters. Care was taken not to damage the filters. The sieve with the filter was placed in an extraction dish, containing 90 ml of tap water. This was covered and kept until the following day. The final suspensions containing the nematodes were pooled together after removing the filter from the extraction dish.

Counting and standardizing nematode populations

Extractions from different soil samples were pooled to form one suspension. Using a pressure pump, air was blown into the suspension for even nematode distribution. Three different 5 *ml* samples were drawn and placed into three separate counting dishes which had been divided into grids to assist nematode counting. With a dissecting microscope, nematodes in each of the 5 *ml* samples were counted, the numbers noted and the mean number for the five samples calculated. The mean number in 1 *ml* of the suspension was calculated.

Shadehouse Trial

Six-week-old pepper seedlings were planted in 96 18cm diameter pots filled with Perlite. Two seedlings were planted into each pot. The pots were drip irrigated and fertigated using Multicote 5-1-3(43)® a 'slow release fertilizer'[4] as topdressing. Five grams were spread evenly over the surface of each Perlite filled pot. The surface of each pot was further covered with a thin layer of Perlite.

For the bacterial seedling drench,1.2 g of NutriStart-AC were weighed into eight 500 *ml* conical flasks. Tap water (240 *ml*) was added to each flask. The flasks were gently swirled to ensure good mixing. A 2 *ml* concentrated spore suspension (2×10^9 cells) of each *Bacillus* spp. and Biostart® 2000 was added separately to each of the eight conical flasks, labelled and incubated at 30 °C for 18 h at 150 rpm in a water bath shaker. This process was repeated each week for 12 weeks to ensure that weekly inoculum was fresh. The optical density of each culture was noted at 540 nm and the values compared to those established previously (Chapter Three). Each culture was carefully diluted and the optical density noted to produce suspensions of the same optical densities and hence colony forming units (cfu's) for weekly inoculations. Three days after the seedlings were transplanted, each of the eight cultures was separately dispensed into 2.5 *ml* aliquots directly onto each seedling in the Perlite growth medium. Thus 12 pots, each containing two seedlings, were inoculated per drench for each culture. The pots were allowed to stand for a week to allow the bacteria to colonize the roots.

[4]Plaaskem Pty Ltd. P.O.Box/Posbus 87005, Houghton 2041 Gauteng Republic of South Africa

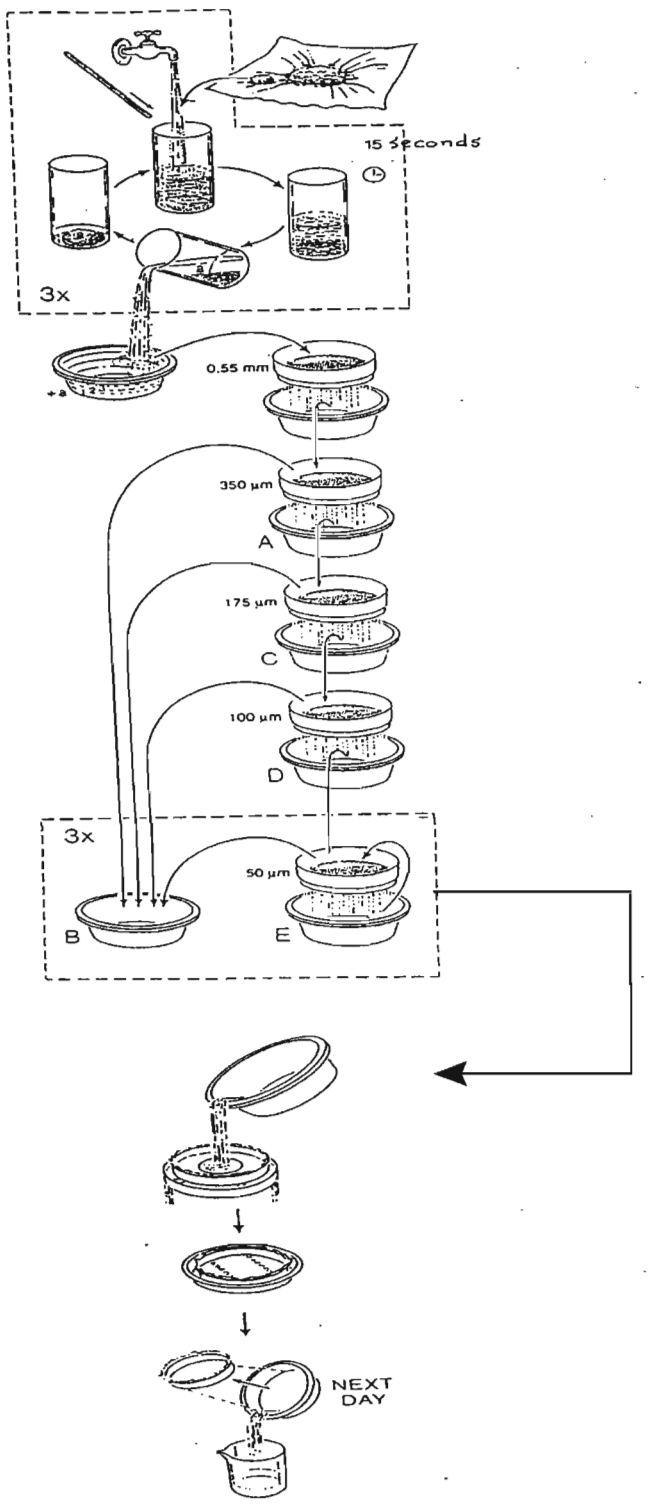

Figure 5.1. Schematic representation of Cobb's modified sieve method for nematode isolation from soil (Van Bezooijen, 1998)

One week after drenching, all 96 pots were inoculated with approximately 500 juvenile *Meloidogyne* spp. per plant. Weekly bacterial inoculations were performed for each bacterial culture for a further 11 weeks.

Application of NutriStart-AC onto seedlings

Forty grams of NutriStart-AC were weighed into a 2 *l* conical flask. A 1 *l* quantity of tap water was added to the flask and swirled gently for good mixing. This resulted in a 4% NutriStart-AC suspension. This process was repeated weekly for 12 weeks in order to have freshly mixed NutriStart-AC for the weekly applications.

Three days after nematode inoculation, 2.5 *ml* aliquots of the mixed NutriStart-AC suspension were applied as a drench separately onto each seedling in the Perlite growing medium. Six pots were inoculated with each *Bacillus* spp. and Biostart® 2000. This resulted in six out of 12 pots being supplemented with NutriStart-AC. The remaining pots were not supplemented with NutriStart-AC and served as different treatments.

Controls

Two different controls were set up for this trial. Two seedlings per pot were planted in 12, 18cm diameter pots filled with Perlite growth medium. Six of the pots were used as Control One and received water and fertilizer only. The other six pots served as Control Two and were supplemented weekly with 2.5 ml of 4% NutriStart-AC suspension.

Thus for each of the seven *Bacillus* spp. and Biostart® 2000;

1. six pots received no NutriStart-AC supplement;
2. six pots were supplemented weekly with 4% NutriStart-AC suspension;
3. six control pots receiving water and fertilizer only and
4. six control pots receiving water, fertilizer and 4% NutriStart-AC.

This resulted in a total of 108 pots.

Gall index rating scale

The following rating scale was used to assess galling on roots (gall formation).

Index	No. of galls per root system	Infection
0	0	No visible infestation
1	1-4	Light infestation
2	5-15	Moderate infestation
3	16-34	Moderately heavy infestation
4	35-50	Heavy infestation
5	> 50	Very heavy infestation

5.3 RESULTS

No galls were found on roots of test or control plants.

5.4 DISCUSSION

This trial, aimed to control root-knot nematodes on pepper seedlings using Biostart™, a *Bacillus*-based plant probiotic, was unsuccessful. A series of factors can be identified which could explain the failure of the trial.

Greenhouse research on biological control of nematodes has involved using sandy soils or sand amended with organic matter (Stirling, 1984; Becker *et al.*, 1988; Spiegel *et al.*, 1991; Oka *et al.*, 1993; Zuckerman *et al.*, 1993; Duponnois *et al.*, 1995; Bourne *et al.*, 1996., Oka *et al.*, 1997; Tzortzakakis *et al.*, 1997; Duponnois *et al.*, 1998). In all the cases stated above, sand or sandy soil with other potting mixes was used. Galls were found on roots where the biocontrol agent was partially effective. Our trial used Perlite as the medium for plant growth. The absence of sand or soil in the potting Perlite medium might have reduced the chances of the introduced *Meloidogyne* spp. to proliferate and penetrate plants roots to cause infection.

Soil properties are very important to nematodes. According to Dropkin (1989), nematodes build up large populations in sandy soils. Juveniles in sandy soils are able to move horizontally and

vertically over a distance of up to 75 cm in nine days, but migration decreases with increasing clay content of the soil, with no migration in soils with more than 30% of clay (Van Der Wal, 1998).

The number of nematodes inoculated (500 juveniles) onto seedlings seems to be a good number for pot trials and infection to occur. Spiegel *et al.* (1991) inoculated 500-750 *Meloidogyne javanica* (Treub.) Chitwood J2 per 500 ml pot with 2-week old tomato seedlings. Our seedlings were 6 weeks old before they were transplanted and were inoculated with nematodes after 10 days. Nematodes were therefore inoculated when the seedlings were more than 7 weeks old. As the number of nematodes inoculated per seedling was a substantial amount to initiate infection, the already developed seedlings might have acquired some physiological resistance. However, this does not occur that fast (J. van Bezooijen, personal communication)[5].

Flooding or too much watering of the pots might also have caused a drastic reduction in nematode numbers. There is a possibility that the introduced *Meloidogyne* spp. may have washed out or leaked through the holes in the base of the pots as the pots leaked during watering. It must also be noted that the automatic drip irrigation system waters three times a day. This means that the pots were watered twice after the seedlings were inoculated with nematodes as the inoculation was carried out in the morning. The introduced *Meloidogyne* spp. might have been washed out of the pots soon after introduction. *Meloidogyne* spp. densities are known to drop significantly when soils are flooded for prolonged periods of time (Van Der Wal, 1998).

We therefore suggest that for a successful greenhouse biocontrol trial on nematodes, the right conditions must be used. The right growing medium, preferably sandy loam, or sandy soil with an organic matter supplement, favourable pH, and sufficient number of nematodes must be used. It must also be borne in mind that water conditions in the growing medium should be kept at optimum and flooding should be avoided.

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CHAPTER 6

6[1] The use of plant probiotic bacteria (Biostart™) in the biological control of *Rhizoctonia* damping-off of seedlings

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Commercial preparations of seven *Bacillus* spp. and Biostart®2000 (a combination of three of the seven *Bacillus* spp.) were evaluated for their biological control properties on *Rhizoctonia* causing damping-off of marigold, cabbage and eucalyptus seedlings. Results obtained show that the biocontrol agents used were unable to effectively control *Rhizoctonia*. Most of the seedlings died seven days after pathogen inoculation, and by Day 21, about 90% of the seedlings were dead.

6.1 INTRODUCTION

Increased global concern about the environmental impact of the use of pesticides in agriculture has led to an intensive search for alternative plant protection and disease management strategies (Kok *et al.*, 1996). Biological control, which involves the use of microbial antagonists, has become an important component for safe plant disease management. Many genera of bacteria and fungi have shown promise as biological control agents against numerous plant pathogens (Maplestone & Campbell, 1989; Krebs *et al.*, 1993; Vidhyasekaran & Muthamilan, 1995; Cartwright & Benson, 1995; Gupta *et al.*, 1995; Pleban *et al.*, 1995; Kok *et al* ., 1996; Xi *et al.*, 1996; Zhang *et al.*, 1996; Kim *et al.*, 1997; Vidhyasekaran *et al.*, 1997; Walker *et al.*, 1998).

For commercial biocontrol agents to be viable, the antagonist must adapt to diverse soil environments. The importance of abiotic factors on biological control was reviewed by Burpee (1990) and furthermore, the bacterial strain must be rhizosphere competent and be able to adequately colonize roots (Weller, 1988).

[1]Chapter format according to Biocontrol Science and Technology

The purpose of this research was to evaluate the feasibility of biological control for damping-off caused by *Rhizoctonia* using Biostart™, a commercial *Bacillus*-based plant probiotic.

6.2 MATERIALS AND METHODS

Microorganisms

Seven *Bacillus* spp.: *B. chitinosporus*, *B. uniflagellatus, B. laterosporus*, *B. pumilus*, *B. subtilis, B. licheniformis,* an unknown *Bacillus* strain CM-33 and Biostart® 2000 (a combination of *B. chitinosporus*, *B. laterosporus*, and *B. licheniformis*) were used in this experiment. The species were provided commercially as concentrated spore suspensions by Microbial Solutions[2].

Pathogen

The isolate, *Rhizoctonia solani* Kühn used was obtained from C. Clark[3]. The *Rhizoctonia* used was stored on sterised wheat in McCartney bottles.

Crops Evaluated

Cabbage (*Brassica oleracea var. capitata* L.) cv. Glory of Enkhuizen, Seed Lot no. YI 011 RR
Marigold (*Tagetes erecta* L.) cv. Lemon drop, Seed Lot no. 14170
Eucalyptus (*Eucalyptus macarthuri* Deane & Maiden) Seed Lot no. M1697.

Cabbage and marigold seeds were obtained from McDonald Seeds[4] and eucalyptus seeds were obtained from the Institute of Commercial Forestry Research (ICFR)[5].

Trials were done to evaluate the feasibility of a seed treatment and seedling drench application using the different *Bacillus* spp. and the Biostart® 2000.

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[3]C. Clark School of Applied Environmental Sciences, Discipline of Plant Pathology, University of Natal, Pietermaritzburg, Republic of South Africa

[4]McDonald Seeds, 61 Boshoff Street, Box 238 Pietermaritzburg, Republic of South Africa

[5]Institute of Commercial Forestry Research (ICFR), University of Natal, Private Bag X 01, Scottsville 3209, Republic of South Africa

Seed Treatment

For culturing, 0.6 g of each of the NutriStart product were weighed into eight 250 *ml* conical flasks. One hundred and twenty *ml* quantities of tap water were added to each flask before swirling it gently to form a homogeneous suspension. Two *ml* quantities of concentrated spore suspension (2×10^9) cells of each *Bacillus* spp. were added separately to each of the eight conical flasks, labelled and incubated in a shaker water bath at 30 °C for 18 h at 150 rpm. The optical density of each culture was noted at 540 nm and values compared to those established previously (Chapter Three). Each culture was carefully diluted and the optical density noted to produce suspensions of the same optical densities and hence colony forming units (cfu's) for seed treatment.

Two grams of a sticker, Pelgel® nutrient adhesive[6] were dissolved in 100 *ml* of tap water, stirred and allowed to stand for 1 h. This was to allow the substance to dissolve and form a homogeneous suspension. The suspension was further divided into eight 250 *ml* beakers, each containing 10 *ml* aliquots of the sticker.

To each of the beakers containing the sticker, 10 *ml* of the 18 h old cultures was added separately, labelled and stirred. This resulted in a total volume of 20 *ml* of bacterial suspension in each of the eight beakers, giving a ratio of 1:1 sticker-bacterial suspension.

An appropriate number of seeds were placed separately into each of the eight bacterial suspensions and stirred. The seeds were left for 2 h to allow bacterial adhesion to the seed coat. The treated seeds were then placed on paper towels and air-dried overnight.

Seeds of all three crops were treated with a combination of adhesive and each *Bacillus* spp. and Biostart® 2000. Treated seeds were planted into three Speedling® 24 trays filled with composted pine bark, giving a total of 24 Speedling® 24 trays per crop.

The trays were watered and inoculated with *Rhizoctonia*. Pathogen inoculation was achieved by placing a 4 mm square of V8 agar colonized by *Rhizoctonia* upside down directly on top of the

[6]LiphaTech, Inc., Milwaukee, Wisconsin, U.S.A

covered seeds. All trays were left in a germination room at 20-24° C for two days. The trays were then moved to a plastic covered tunnel at 20-30° C.

Seedling drench

For drenching, 1.2 g of NutriStart-AC were weighed into eight 500 *ml* conical flasks. To each flask 240 *ml* of tap water were added and the contents swirled gently to facilitate mixing. Two ml quantities of concentrated spore suspensions (2×10^9 cells) of each *Bacillus* species was added separately to each of the eight conical flasks, labelled and incubated at 30 °C for 18 h at 150 rpm in a water bath shaker. This process was repeated each week for seven weeks in order to ensure fresh inoculum for weekly inoculations. The optical density of each culture was noted at 540 nm and values compared to those established previously (Chapter Three).

Untreated cabbage, marigold and eucalyptus seeds were each planted into 24 Speedling®24 trays filled with composted pine bark. Before seeds were covered with composted pine bark, an 18 hr overnight broth culture of each of the seven *Bacillus* spp. and Biostart®2000 was separately dispensed in 1 ml aliquots directly onto the seeds in the growing medium. Thus three trays, each containing 24 seeds, were inoculated per drench volume, per *Bacillus* spp. and Biostart®2000 per crop. Speedling trays were not watered, but were left overnight to enable bacteria to adhere to the seed coats. They were then watered the next day and inoculated with the pathogen as described above.

Trays were left under the same conditions as stated above. The drenching procedure was repeated a week after seedling emergence for up to six weeks.

Controls

Control treatments were:

1. neither antagonist nor pathogen
2. pathogen only

Controls were also replicated three times with one tray per replicate.

All seedlings were irrigated three times a day by microjet irrigation. The water used contained soluble fertilizer [3.1.3(38) complete] Ocean Agriculture[7] applied at a rate of 1g l^{-1} to give approximately 33 P and 100 K in 100 mg l^{-1}N.

Seedling Ratings

Seedlings for the three crops were rated as follows:

1. percentage germination after 4 days for cabbage and marigold
2. percentage germination after 8 days for eucalyptus only, due to late germination.
3. percentage damping-off after 7 days for cabbage and marigold
4. percentage damping-off after 14 days for eucalyptus only
5. percentage stand (survival) at 4-6 weeks for all three crops

For seedling dry weight, the number of plants in each tray was noted so that the mean weight per seedling could be determined. Seedlings from each tray were harvested at maturity at their base and placed in a brown paper bag. The plant material was subsequently dried in an oven at 55 °C. Once dried, the content of each bag was weighed and the mean weight per shoot calculated.

Statistical analysis

Results were analysed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS, 1987).

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

6.3 RESULTS

Table 6.1. Percentage germination, damping-off and survival of tunnel-grown seed treated and seedling drenched marigold seedlings seven days after inoculation with *Rhizoctonia* spp., using seven *Bacillus* spp. and Biostart® 2000 as biological control agents

Bacteria	Treatment Type	% germination (after 4 days)	% damping-off (after 7 days)	% survival (after 7 days)
B. chitinosporus	Drench	87.5 a	74.6 a	25.4 b
B. chitinosporus	Seed Treatment	83.3 a	50.0 a	50.0 b
B. uniflagellatus	Drench	87.5 a	67.2 a	32.8 b
B. uniflagellatus	Seed Treatment	87.5 a	76.7 a	23.3 b
B. laterosporus	Drench	87.5 a	74.1 a	34.9 b
B. laterosporus	Seed Treatment	83.3 a	69.4 a	31.6 b
B. pumilus	Drench	83.3 a	69.4 a	30.6 b
B. pumilus	Seed Treatment	83.3 a	61.7 a	38.3 b
B. subtilis	Drench	90.3 a	60.0 a	40.0 b
B. subtilis	Seed Treatment	83.3 a	65.7 a	34.3 b
B. licheniformis	Drench	87.5 a	65.1 a	34.9 b
B. licheniformis	Seed Treatment	76.4 a	50.0 a	50.0 b
CM-33	Drench	87.5 a	66.0a	34.0 b
CM-33	Seed Treatment	80.6 a	51.8 a	48.2 b
Biostart® 2000	Drench	87.5 a	72.5 a	27.5 b
Biostart® 2000	Seed Treatment	80.6 a	70.8 a	29.2 b
Water Only	Nil	90.3 a	0.0 b	100 a
Pathogen Only	Nil	83.3 a	81.7 a	18.3 b
Effects		**P-values**	**P-values**	**P-values**
Bacteria		0.71^{NS}	0.62^{NS}	0.68^{NS}
Treatment		0.06^{NS}	0.08^{NS}	0.3^{NS}
Bacteria*Treatment		0.8^{NS}	0.9^{NS}	0.66^{NS}
		% CV = 9.45	% CV = 29.21	% CV = 45.18
		MSE = 1.92	MSE = 3.88	MSE = 3.17

NS = Not significant.

Means followed by the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test.

Seed treatment = application of bacteria to seed with Pelgel®.

Drench = weekly application of bacteria broth cultures, at a rate of 1 ml per plant.

Table 6.2. Percentage germination, damping-off and survival of tunnel-grown seed treated and seedling drenched cabbage seedlings seven days after inoculation with *Rhizoctonia* spp., using seven *Bacillus* spp. and Biostart® 2000 as biological control agents

Bacteria	Treatment Type	% germination (after 4 days)	% damping-off (after 7 days)	% survival (after 7 days)
B. chitinosporus	Drench	33.3 b	66.7 b	33.3 b
B. chitinosporus	Seed Treatment	41.7 b	56.7 b	43.3 b
B. uniflagellatus	Drench	26.4 b	63.5 b	36.5 b
B. uniflagellatus	Seed Treatment	33.3 b	62.5 b	37.5 b
B. laterosporus	Drench	33.3 b	58.3 b	41.7 b
B. laterosporus	Seed Treatment	36.1 b	65.9 b	34.1 b
B. pumilus	Drench	34.7 b	51.8 b	48.2 b
B. pumilus	Seed Treatment	43.1 b	64.7 b	35.3 b
B. subtilis	Drench	38.9 b	57.3 b	42.7 b
B. subtilis	Seed Treatment	33.3 b	54.2 b	45.8 b
B. licheniformis	Drench	40.3 b	69.4 b	30.6 b
B. licheniformis	Seed Treatment	34.7 b	56.2 b	43.8 b
CM-33	Drench	31.7 b	64.9 b	35.1 b
CM-33	Seed Treatment	38.9 b	53.8 b	46.2 b
Biostart® 2000	Drench	33.8 b	54.2 b	45.8 b
Biostart® 2000	Seed Treatment	31.9 b	61.4 b	38.6 b
Water Only	Nil	77.8 a	0.0 a	100 a
Pathogen Only	Nil	20.8 b	53.3	46.7 b
Effects		**P-values**	**P-values**	**P-values**
Bacteria		0.90^{NS}	0.9^{NS}	0.69^{NS}
Treatment		0.74^{NS}	0.53^{NS}	0.56^{NS}
Bacteria*Treatment		0.11^{NS}	0.18^{NS}	0.27^{NS}
		% CV = 34.35 MSE = 2.63	% CV = 50.04 MSE = 2.27	% CV = 31.66 MSE = 0.99

NS = Not significant.

Means followed by the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test.

Seed treatment = application of bacteria to seed with Pelgel®.

Drench = weekly application of bacteria broth cultures, at a rate of 1 ml per plant.

Table 6.3. Percentage germination, damping-off and survival of tunnel-grown seed treated and seedling drenched eucalyptus seedlings seven days after inoculation with *Rhizoctonia* spp., using seven *Bacillus* spp. and Biostart® 2000 as biological control agents

Bacteria	Treatment Type	% germination (after 8 days)	% damping-off (after 14 days)	% survival (after 14 days)
B. chitinosporus	Drench	36.1 b	57.5 a	42.5 b
B. chitinosporus	Seed Treatment	34.7 b	60.2 a	39.8 bc
B. uniflagellatus	Drench	37.5 b	59.3 a	40.7 bc
B. uniflagellatus	Seed Treatment	37.5 b	59.3 a	40.7 bc
B. laterosporus	Drench	34.7 b	56.2 a	43.8 bc
B. laterosporus	Seed Treatment	38.9 b	57.3 a	42.7 bc
B. pumilus	Drench	37.5 b	63.0 a	37.0 bc
B. pumilus	Seed Treatment	36.1 b	58.0 a	42.0 bc
B. subtilis	Drench	40.3 b	62.0 a	38.0 bc
B. subtilis	Seed Treatment	43.1 b	58.3 a	41.7 b
B. licheniformis	Drench	38.9 b	53.8 a	46.2 b
B. licheniformis	Seed Treatment	38.9 b	60.9 a	39.1 bc
CM-33	Drench	37.5 b	63.0 a	37.0 bc
CM-33	Seed Treatment	36.1 b	58.1 a	41.9 bc
Biostart® 2000	Drench	36.1 b	62.0 a	38.0 bc
Biostart® 2000	Seed Treatment	37.5 b	36.0 a	37.0 bc
Water Only		84.6 a	0.0 b	100 a
Pathogen Only		29.2 b	71.4 a	28.6 c
Effects		**P-values**	**P-values**	**P-values**
Bacteria		0.95^{NS}	0.97^{NS}	0.47^{NS}
Treat		0.83^{NS}	0.78^{NS}	0.67^{NS}
Bacteria*Treat		0.99^{NS}	0.95^{NS}	0.18^{NS}
		% CV = 23.29 MSE = 2.10	% CV = 40.36 MSE = 2.14	% CV =18.26 MSE = 0.69

NS = Not significant.

Means with the same letter are not significantly different (P = 0.05) according to Student, Newman and Keuls comparison test.

Seed treatment = application of bacteria to seed with Pelgel®.

Drench = weekly application of bacteria broth cultures, at a rate of 1 ml per plant.

Results obtained were as presented in Tables 1-3. Most of the seedlings died by the Day 7 after pathogen inoculation (marigold and cabbage) and by Day 14 for eucalyptus. Biostart™ failed to be an effective biological control agent, under the conditions of this particular trial.

None of the treatments applied were better than the inoculated control, as reflected by damping-off and survival of seedlings for marigold, eucalyptus and cabbage. Most of the seedlings died by Day 7. By Day 21, about 90% of the seedlings were dead. The highest percentage survival after Day 7 (50%) resulted from seeds treated by *B. chitinosporus* and *B. licherniformis* on marigold applied by seed treatment (Table 6.1). All other survival rates fell below 50% for all probiotic treatments.

6.4 DISCUSSION

The development of biological control agents requires the elucidation of characteristics such as:

(i) Mechanism(s) of action

(ii) Optimum rate(s) and concentration of antagonist applied to target areas

(iii) Carrier or preparation substrate

(iv) Method(s) of application (Hebbar *et al.*, 1992).

Our results demonstrate that none of the seven *Bacillus* spp. and Biostart® 2000 was able to effectively control damping-off on any of the three crops tested: marigold, cabbage and eucalyptus. The results also show poor percentage survival (stand) of seedlings soon after germination. By Day 21, about 90% of the seedlings in some of the trays showed damping-off and death.

We suggest that the following factors may have affected the biological control activity of the bacteria used.

Concentrations of Biostart™ used for seed treatment and seedling drench ranged from log 6.32-log 7.88 ml^{-1} (10^{-6}-10^{-7}) and log 5.46-log 6.14 ml^{-1} (10^{-5}-10^{-6}) respectively as indicated in Chapter Three.

These concentrations may not be high enough to colonize plant roots and overcome the pathogen's activity. It is suggested that correct bacterial antagonist concentrations are essential for efficacy of biological control of plant pathogens. Cartwright & Benson (1995) found that effective disease control using Strain 5.5B of *Pseudomonas cepacia* on *Rhizoctonia* stem rot of Poinsettia (*Euphorbia pulchérrima* Willd.) decreased as bacterial concentration decreased.

No statistically significant difference was observed when the performances of the individual probiotics were compared (Tables 1-3). This suggests that there was no variation in terms of bacterial effect on *Rhizoctonia* damping-off. It also suggests that none of the bacteria was effective in controlling *Rhizoctonia* damping-off.

No significant difference was recorded when comparing the two treatment methods, i.e., seed treatment and seedling drench. Neither of the application treatments worked because Biostart™ probiotic *Bacillus* spp. might have only growth stimulation activity and might therefore have no biological control activity.

The degree of root colonization is one of the factors affecting the efficiency of biological control agents. Weller (1988) suggested that variable root colonization by introduced bacteria, including colonization from plant to plant, and root to root, on a given plant, is probably the main reason for inconsistent control by biological control agents. Percentage seedling stand seven days after pathogen inoculation were higher for marigold than cabbage, though not statistically significant.

The production of metabolites or substances toxic to other microorganisms or plant pathogens may be responsible for the inhibitory action in biological control (Omoifo & Ikotun,1987; Weller, 1988). If antibiosis or toxic substance production is the mode of action in the biological control agents used in this trial, then it is possible that the time period before pathogen attack on seedlings was so short that the biological control agents did not have enough time to manifest and produce the required substances. The medium in which the plants were grown may also not have been an ideal medium for quick bacterial population increase, probably due to low nutrient status. This may have reduced bacterial activities such as growth and multiplication, root colonization and

possibly production of antibiotics and other vital metabolites necessary for biological control.

The quality and amount of nutrients available are important to ensure optimun growth of the antagonist. Growth stimulation trials in Chapters Three and Four revealed that growth of seedlings was enhanced when Biostart™ inoculated seedlings were supplemented with 4% NutriStart-AC suspension. The addition of NutriStart-AC as nutrient supplement could have aided Biostart™ population increase and activities which could possibly enhance antibiotic production and other vital metabolites necessary for biological control. This might have reduced the Biostart™ *Bacillus* spp. activity as an antagonist.

The virulence of the *Rhizoctonia* isolate used may have also contributed to the failure of the trial. The *Rhizoctonia* may have rendered the biological control agents ineffective and may have also contributed to the failure of the trial. Another possibility is that the biological control agents could not inhibit or halt the spread of the pathogen once the pathogen had infected or gained access into the plant. Pathogen inoculation was achieved by placing a 4 mm square of V8 agar colonized by *Rhizoctonia* directly upside down on top of the covered seeds. There is therefore a greater potential for the pathogen to migrate aboveground on the surface of the composted pine bark medium. A possible explanation here is that the pathogen readily attacked the seedlings at the stem on germination. The *Rhizoctonia* had no access to the Biostart™ *Bacillus* spp. in the composted pine bark growth medium found on the roots of the seedlings. The method of pathogen inoculation might not have been the best option. For future studies, the pathogen could be inoculated first and then covered with the growing medium before antagonist inoculation.

In order to develop an effective biological control agent, performance must be consistent (Weller, 1988). To accomplish this will involve research in many diverse areas because the words "biological control" is the culmination of complex interactions between the host, pathogen, antagonist and the environment. Environmental conditions natural for a pathogen namely, extremes in pH, moisture, temperature or low nutrient availability, may be completely unnatural for biological control agents. An antagonist most adapted to the environmental conditions of the pathogen may have the best chance for success in controlling the pathogen (Baker & Cook, 1974). Plant diseases may develop when the abiotic environment is especially favourable for the

pathogen and is unfavourable to either the host or antagonists or both. Plant disease may also occur when the antagonist is in a low population relative to the pathogen population, are inhibited by other microorganisms, lack of nutrients and proper environmental conditions to function as an antagonist (Baker & Cook, 1974). In this trial, the growth medium used was not supplemented with 4% NutriStart-AC suspension. The probable lack of nutrients in the growth medium might have decreased the activities of the introduced Biostart™ *Bacillus* spp. to function as antagonists. The temperature in the growth medium might also have affected the performance and activity of the Biostart™ *Bacillus* spp.

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CHAPTER 7

General Overview

Interest in the application of bacteria to soil or plant roots has increased markedly following numerous publications on the successful application of plant growth promoting rhizobacteria (PGPR) (Lambert & Joos, 1989). Most of these findings have remained at research stages and are yet to be registered and commercialized for large scale applications.

The results presented in this thesis evaluated seven commercial *Bacillus* spp. and Biostart® 2000 as plant growth stimulants and disease control agents. It was established that:

- Growth stimulation was more apparent in some crops than others. This suggests that PGPR could be specific in growth response in some crops while in others it causes a slight increase or none at all.
- Growth response was more pronounced when Biostart™ *Bacillus* spp. were supplemented with a 4% microboost activator, NutriStart-AC. NutriStart-AC was made from local raw materials. This could be used as a replacement of other highly refined media for culturing *Bacillus* spp. in the future.

7.1 Potential for application of bacteria into soil

There are vast possibilities in the application of bacteria to soil for beneficial purposes. This includes the potential for introducing organisms into the soil for specific tasks such as increasing the amount of available nutrients for uptake by plants leading to plant growth stimulation and disease control. Phosphorus status in the soil could be improved by applying bacteria that release fixed phosphorus, such as *Bacillus* spp. and *Pseudomonas* spp. (Brown, 1974). Although phosphorus occurs in the soil, both in organic and inorganic forms of phosphate, only a small fraction of the total soil phosphate is directly available to plants. However, the cycling of phosphorus in the soil could increase its availability.

A wide variety of bacterial genera is applied to soil for their effect on plant development. These bacteria probably promote growth by producing plant growth hormones in the rhizosphere of

plants at the seedling stage (Brown, 1974). In our trials, it was established that Biostart™ *Bacillus* spp. improves plant growth.

Another useful property of rhizosphere bacteria is their capacity to control soil-borne plant pathogens. This is mainly achieved through antagonism or competition (van Elsas & Heijnen, 1990). Fluorescent pseudomonads in particular, are well known for the production and excretion of siderophore iron-chelating agents. These bacteria act by depriving plant pathogens of iron in the rhizosphere, thereby limiting their development.

7.2 Growth Promotion and Disease Control

Bacterial effects on plant growth results from multiple interactions between introduced bacteria, the associated crop and soil microflora (Kloepper *et al.*, 1989). Each of these interactions is determined by multiple environmental variables such as the type, nutrition moisture, and temperature.

Plant growth promoting rhizobacteria (PGPR) could have a great impact on crop yield. Kloepper *et al.* (1989) analyzed the effect of yield of three bacteria inoculants applied to a variety of crops. Compared to controls, increases in yield as high as 160% were observed, but reductions, although smaller in magnitude, were also observed. In our growth stimulation trial, Biostart™ *Bacillus* spp. stimulated plant growth, although growth stimulation differed from one crop to another. Growth increases greater than 400% were observed on lettuce. Maximizing the potentials of these *Bacillus* spp. under both greenhouse and field conditions could aid nurseries and small-scale farmers in South Africa by improving seedling production and increasing crop yield. It could also lessen the amount of fertilizers presently used in nurseries and in the field.

According to Baker (1987), biological control of soil-borne plant pathogens by introduced microorganisms has been studied for over 65 years. Concurrently, there has been a shift to the opinion that biological control can have an important role in agriculture in the near future. It is encouraging that several companies presently have programs to develop biological control agents as commercial products. This increased interest in biological control is, in part, a response to public concern about hazards associated with chemical pesticides.

Bacillus spp. have been tested on a wide variety of plant species for their ability to control disease (Weller, 1988). These bacteria are appealing candidates for biological control because of the production of endospores which are tolerant to heat and desiccation.

7.3 Field trials, yield effects and challenges in product development

Successful PGPR trials in greenhouses would not be of great importance to the agricultural industry if the inoculum was not tested in the field. Field trials are therefore essential if a particular PGPR formulation is intended for large scale production for commercial purposes.

According to Kloepper *et al.* (1989), about 10^7 hectares of land were treated with PGPR and increases of 10-20% were reported in 50-70% of the field trials. Sorghum yield was increased from 15-33% as a result of *Bacillus* inoculation in the field (Broadbent *et al.*, 1977). In our growth trials, Biostart™ *Bacillus* spp. improved seedling growth in tunnels. Therefore the potential for these *Bacillus* spp. to do well in the field should be investigated.

Several challenges need to be resolved if the full potential of a PGPR is to be exploited for commercial purposes. The challenge of developing consistent benefits and product delivery needs to be met. Formulations need not only maintain viability but must possess the ability to sustain growth promotion and biological control potentials of the bacteria. The formulations must be developed with a simple delivery system that allows easy application by small-scale farmers and seed companies with existing equipment and application practices. Bacterial fermentation systems must also be optimized and the quality of their output controlled with respect to inoculum density and biological activity. The product must be tested in the field in different areas, and the environmental limits on the biological activity must be determined. Survival and dispersal of the bacteria in the environment must be also closely monitored.

It is my believe that PGPR research has come to stay and will soon make a major breakthrough in plant pathology in South Africa. The results described in this thesis show that there is great potential in PGPR research. This will ensure food security for the world population, in the form of improving crop production and controlling plant diseases which are presently causing substantial losses to agriculture.

This work has reached a stage that needs continuity to fill in the missing links and solve some of the problems encountered during the course of this study. The soil is a vast reservoir of potential biological control and growth promoting agents. It is my belief that continuation of this work, with either a new bacterial or fungal agent using the results obtained with the Biostart™ system as a standard for comparison, will open the way for new fungal or bacterial agents to be registered in the market.

7.4 Future needs

Early research on free-living bacteria in soil indicated that when certain strains are applied to seeds or roots, they may benefit crops by stimulating plant growth or by reducing damage from soil-borne plant pathogens (Kloepper *et al.*, 1989).

Beneficial effects of rhizosphere bacteria have most often been based on increased plant growth, faster seed germination, and better seedling emergence (Lazarovits & Nowak, 1997). Response of crops to inoculation with PGPR strains vary from year to year and from one field location to another. Interactions of inoculants with soil environmental parameters and microbial communities may hamper PGPR survival and root colonization, thereby limiting their effectiveness as plant growth promoters (Lalande *et al.*, 1989). This might lead to inconsistencies experienced in field trials.

A key factor affecting the success of plant growth promotion and disease control trials using PGPR has been the varying degrees of establishment and survival of the introduced PGPR populations. This area of research, i.e., the assessment of where and in which numbers inoculant cells are localized in soils; how dynamic the situation is in relation to prevailing and local soil conditions, and where inoculant cells are able to grow, is another important area of study. Heat treatment employed as a selective method for *Bacillus* sporeformers in the population dynamics study in this thesis might not be the best approach for following the population trend of *Bacillus* spp. in soil. This raises a question on the fate of the vegetative cells in the soil samples analysed since they could not withstand the heat treatment. Vegetative cells and not spores may be more important in growth stimulation and biological control. In order to determine and quantitatively monitor the number of vegetative cells present, a labelled assay method, such as the immuno

detection method with specific antibodies described by Kluepful (1993), may be needed.

Plant growth promoting rhizobacteria that stimulate growth of one plant species or cultivar may not work or may even retard the growth of another. As established in two of our trials, the effect of Biostart™ on the tomato cultivar Roma was more pronounced than on the Floradade and Rodade cultivars. Response on lettuce was also more pronounced than observed on tomato, sorghum or beans. This requires trials on different plants and cultivars to ascertain which PGPR work best on which plants, although some PGPR may have broad host ranges.

Root colonization is required for consistent beneficial effects of PGPR inocula. Inconsistencies in field performances by PGPR and biological control agents could largely be explained by inferior root colonization (Kloepper *et al.*, 1989). Today, there is no doubt that bacterial inocula can significantly stimulate the yield of various crops, but performance has generally been inconsistent. Evaluation of consistency is necessary not only to characterize the quality of the PGPR inoculum, but also to identify the cause of inconsistency. Knowing more about inconsistency of a particular PGPR inoculum could provide a basis for guiding product development. Successful trials in the greenhouse therefore need to be tested under different field conditions in order to ascertain the conditions under which the strains perform best in the field. In order to efficiently utilize a wider range of potential PGPR, more comprehensive and general information on microbial interaction in the rhizosphere is still needed. Since inconsistency of PGPR performance is still a major problem for the commercial development of bacterial inoculum, fundamental information underpinning PGPR applications and use is therefore vital if PGPR inoculant is to be consistently effective and reliable.

Biological control agents have the potential to fill the gap created by the disappearance of the broad spectrum fungicides (Harman & Taylor, 1990). These biological control agents must be effective, reliable and active against a wide range of potential seed and seedlings attacking pathogens in order to bridge this gap. In our biological control trial on *Rhizoctonia* damping-off of seedlings, Biostart™ was ineffective under the conditions used for the trial. This calls for more effective and reliable methods of application if any of the Biostart™ *Bacillus* spp. are to be exploited in the near future as potential biological control agents.

With legislation of the use of chemicals in agriculture in South Africa becoming increasingly stringent, the need to find alternatives to chemical control or to develop means of applying sublethal doses of chemicals, which provide effective control of soil-borne plant pathogens, is becoming more and more urgent. Biological control provides the ideal solution. The adverse effects on the environment are minimal and synergistic effects become apparent when biological control agents are combined with fungicides and bactericides.

Based on the findings and foundations laid out in this thesis, a forecast of what is needed in future research is as follows:

1. **Promoting *Bacillus*-based plant probiotics**
   - Feasibility studies in the market, especially in South Africa, to know how medium and large scale farmers are willing to use the product if made available.
   - All research on PGPR would be geared towards registration and commercialisation of the product on a large scale.

2. **Population dynamics in soil**
   - The heat treatment technique employed in this thesis to select for introduced *Bacillus* spp. does not give the exact population as the vegetative cells were killed. A more precise and accurate technique is needed to quantify the spores and vegetative cells. Methods such as the use of an antibiotic marker or immuno detection method with specific antibodies for labelling cells will allow easy identification of introduced *Bacillus* spp. under greenhouse and field conditions. This will facilitate easier follow up procedures to determine what happens to the introduced *Bacillus* spp. in soil.

2. **Growth stimulation**
   - Based on the growth promotion findings in this thesis, there is a need for:
     i) more tunnel or greenhouse and field trials with new *Bacillus* and fungal isolates, preferably *Trichoderma* spp.;
     i) assessing the effect of NutriStart-AC on these isolates in growth

stimulation trials;

ii) optimizing the use of NutriStart-AC supplement for growth stimulation related to time and greenhouse and field trials on a specific crop(s) and cultivars to ascertain which crop(s) and cultivars respond best to the bacterial and fungal agents used.

3. **Formulation of an ideal *Bacillus* growth medium**

- We suggest that other formulations, beside NutriStart-AC should be made available and if possible patented.
- Molasses and brewery waste will be tried as a cheap source of raw materials.
- Variation of C:N ratios in different formulations will be identified to find out which combinations work best as a boost.
- an assessment of different packaging methods, storage conditions and optimization in search of the most effective way of storing the formulated NutriStart-AC, or other formulation will be made.

4. **Biological control**

- Little was achieved with Biostart™ *Bacillus* spp. when used as biological control agents.
- We therefore suggest a search for new *Bacillus* and other fungal isolates for this purpose.
- Consideration should be given to mixtures of *Bacillus* and *Trichoderma* isolates for biological control.
- We suggest the use of peat, sand, loam and Perlite mixtures for nematode control trials.
- Consideration should also be giving to integrated control.

5. **Nursery trials**

- Good and top quality growth medium required to support bacterial and fungal growth.

- Treating and sterilizing trays to avoid any source of bacterial or fungal contaminations.
- The need for a highly controlled environment (heating and cooling devices) to facilitate trials in the tunnels or greenhouses.

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