BIOLOGICAL CONTROL AND PLANT GROWTH PROMOTION BY SELECTED *TRICHODERMA* AND *BACILLUS* SPECIES

By

Kwasi Sackey Yobo

BSc (Hons) Nigeria, BSc (Hons) Natal, MSc (Natal)

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Faculty of Science and Agriculture
University of KwaZulu-Natal
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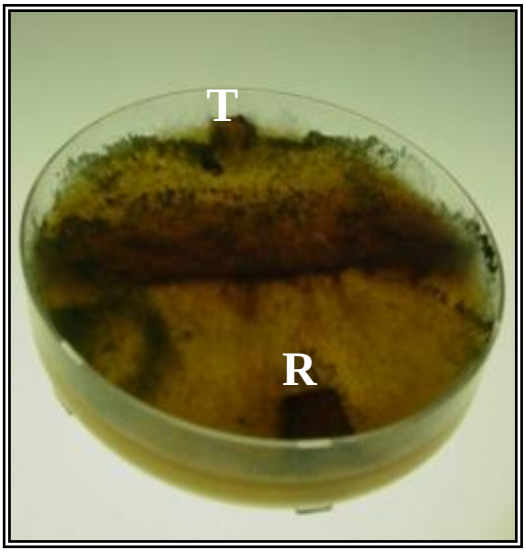
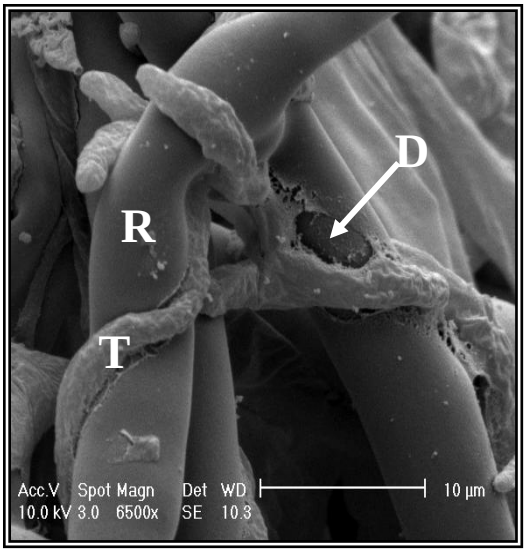
FRONTISPIECE





In vitro interaction between *T. atroviride* SY3A (T) and *R. solani* (R) on a V8 agar medium (left) with brownish discolouration of the *R. solani* mycelium occurring at the point of interaction. Scanning electron micrograph (right) of *Trichoderma* (T) mycoparasitizing *R. solani* hyphae (R) from the interacting regions on V8 agar medium (left). Cell wall disintegration (D) at the point of contact between hyphal strands is evident.

Growth comparison between unfertilized control plant (left) and plant treated with combination of *Trichoderma harzianum* Eco-T® + *Bacillus* B69 (right) 35 days after planting. Treated plant shows healthier, more vigorous growth than the control plant.

ABSTRACT

Various *Trichoderma* and *Bacillus* spp. have been documented as being antagonistic to a wide range of soilborne plant pathogens, as well as being plant growth stimulants. Successes in biological control and plant growth promotion research has led to the development of various *Trichoderma* and *Bacillus* products, which are available commercially. This study was conducted to evaluate the effect of six *Trichoderma* spp. and three *Bacillus* spp. and their respective combinations, for the biological control of *Rhizoctonia solani* damping-off of cucumber and plant growth promotion of dry bean (*Phaseolus vulgaris* L.). *In vivo* biological control and growth promotion studies were carried out under greenhouse and shadehouse conditions with the use of seed treatment as the method of application.

In vitro and *in vivo* screening was undertaken to select the best *Trichoderma* isolates from 20 *Trichoderma* isolated from composted soil. For *in vitro* screening, dual culture bioassays were undertaken and assessed for antagonisms/antibiosis using the Bell test ratings and a proposed Invasive Ability rating based on a scale of 1-4 for possible mycoparasitic/hyperparasitic activity. The isolates were further screened *in vivo* under greenhouse conditions for antagonistic activity against *R. solani* damping-off of cucumber (*Cucumis sativus* L.) cv. Ashley seedlings. The data generated from the *in vivo* greenhouse screening with cucumber plants were analysed and grouped according to performance of isolates using Ward's Cluster Analysis based on a four cluster solution to select the best isolates *in vivo*. Isolates exhibiting marked mycoparasitism of *R. solani* (during ultrastructural studies) viz, *T. atroviride* SY3A and *T. harzianum* SYN, were found to be the best biological control agents *in vivo* with 62.50 and 60.06% control of *R. solani* damping-off of cucumber respectively. The *in vitro* mode of action of the commercial *Trichoderma* product, Eco-T®, and *Bacillus* B69 and B81 suggested the production of antimicrobial substances active against *R. solani*.

In vitro interaction studies on V8 tomato juice medium showed that the *Trichoderma* and *Bacillus* isolates did not antagonise each other, indicating the possibility of using the two organisms together for biological control and plant growth promotion studies. Greenhouse studies indicated that combined inoculation of *T. atroviride* SYN6 and *Bacillus* B69 gave the greatest plant growth promotion (43.0% over the uninoculated control) of bean seedlings in terms of seedling dry biomass. This was confirmed during *in vivo* rhizotron studies.

However, results obtained from two successive bean yield trials in the greenhouse did not correlate with the seedling trials. Moreover, no increase in protein or fat content of bean seed for selected treatments was observed. In the biological control trials with cucumber seedlings, none of the *Trichoderma* and *Bacillus* combinations was better than single inoculations of Eco-T®, *T. atroviride* SY3A and *T. harzianum* SYN.

Under nutrient limiting conditions, dry bean plants treated with single and dual inoculations of *Trichoderma* and *Bacillus* isolates exhibited a greater photosynthetic efficiency that the unfertilized control plants. *Bacillus* B77, under nutrient limiting conditions, caused 126.0% increase in dry biomass of bean seedlings after a 35-day period. Nitrogen concentrations significantly increased in leaves of plants treated with *Trichoderma-Bacillus* isolates. However, no significant differences in potassium and calcium concentrations were found. Integrated control (i.e. combining chemical and biological treatments) of *R. solani* damping-off of cucumber seedlings proved successful. *In vitro* bioassays with three Rizolex® concentrations, viz., 0.01g.l^{-1}, 0.1g.l^{-1} and 0.25g.l^{-1} indicated that the selected *Trichoderma* isolates were partly sensitive to these concentrations whereas the *Bacillus* isolates were not at all affected. In a greenhouse trial, up to 86% control was achieved by integrating 0.1g.l^{-1} Rizolex® with *T. harzianum* SYN, which was comparable to the full strength Rizolex® (1g.l^{-1}) application. Irrespective of either a single or dual inoculations of *Trichoderma* and/or *Bacillus* isolates used, improved percentage seedling survival as achieved with the integrated system, indicating a synergistic effect.

The results presented in this thesis further reinforce the concept of biological control by *Trichoderma* and *Bacillus* spp. as an alternative disease control strategy. Furthermore, this thesis forms a basis for *Trichoderma-Bacillus* interaction studies and proposes that the two organisms could be used together to enhance biological control and plant growth promotion.

PREFACE

The experimental work presented in this thesis was carried out in the School of Applied Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Mark D. Laing and Mr Charles H. Hunter.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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K. S. Yobo (Candidate)

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Professor M. D. Laing (Supervisor)

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Mr C. H. Hunter (Co-supervisor)

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Finally, I would like to thank the Almighty God for seeing me through this study.

DEDICATION

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

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TABLE OF ACRONYMS

ANOVA	=	Analysis of variance
ARC	=	Agricultural Research Council
BCA(s)	=	Biological control agents
CAS	=	Chrome azurol S
CMC	=	Carboxymethylcellulose
CPB	=	Composted pine bark
ESEM	=	Environmental scanning electron microscopy
GLM	=	General linear model
IAA	=	Indole acetic acid
ISR	=	Induced systemic resistance
PCNB	=	Pentachloronitrobenzene
PDA	=	Potato dextrose agar
PEA	=	Plant efficiency analyser
PGPF(s)	=	Plant growth promoting fungi
PGPR(s)	=	Plant growth promoting rhizobacteria
PPRI	=	Plant Protection Research Institute
TSM	=	*Trichoderma* selective medium
YIB	=	Yield increasing bacteria

INTRODUCTION

The fungus *Trichoderma* and the bacterium *Bacillus* spp. are among the most prominent organisms that have been investigated for biological control and plant growth promotion applications. Over the years, research has repeatedly demonstrated that both of these genera contain representatives that can be used as antagonists against various plant pathogens as well as plant growth promoting agents. As a result of extensive research, various *Trichoderma* and *Bacillus* spp. products have been developed and produced commercially (Gardener and Fravel, 2002).

Apart from a report by Jisha and Alagawadi (1996) on the combined effect of *T. harzianum* and *B. subtilis* on nutrient uptake and yield of sorghum (*Sorghum bicolor* L. Moench), there appears to be a notable lack of information with regards to interactions between these two groups of organisms and possible synergistic effects, if any, on biological control and plant growth promotion. The overall aim of this study was to investigate the efficacy of using *Trichoderma* and *Bacillus* isolates singly and in combinations to achieve enhanced levels of biological control and/or plant growth promotion. The objectives of this study were to:

a) Review available literature on the use of bacteria and fungi for biological control and plant growth promotion studies with specific reference to *Trichoderma* and *Bacillus* spp.;
b) Isolate and screen *Trichoderma* spp. for biological control activity against *R. solani in vitro* and *in vivo*;
c) Investigate the possible mechanisms of action of three *Bacillus* spp. isolates, previously shown to demonstrate antagonistic activity against *R. solani* (Kubheka, 2003) and five *Trichoderma* isolates selected from *in vivo* greenhouse screening;
d) Evaluate the effects of single and dual inoculations of *Trichoderma* and *Bacillus* spp. for biological control of *R. solani* damping-off of cucumber (*Cucumis sativus* L.) and growth promotion on dry bean (*Phaseolus vulgaris* L.). A commercial *Trichoderma* product, Eco-T® was used as a standard;
e) Evaluate the effect of single and dual inoculations of *Trichoderma* and *Bacillus* spp. on photosynthetic efficiency, growth promotion and nutrient uptake under nutrient limiting conditions; and

f) Evaluate the combined application of chemical and biological control methods on *R. solani* damping-off in cucumber seedlings.

The following dissertation has been written in the form of seven chapters, each chapter covering a specific aspect of the research conducted on biological control and plant growth promotion by *Trichoderma* and *Bacillus* isolates. With the exception of the literature review and the general overview chapters, each of the chapters were set up independently and prepared in the format of a scientific paper.

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CHAPTER ONE

Literature Review

Application of *Trichoderma* and *Bacillus* species for plant growth promotion and biological control of soil-borne plant pathogens

Development of sustainable agriculture has been earmarked as one of the major strategies to reduce the harmful effects on the environment as a result of indiscriminate use of fungicides and pesticides on agricultural crops (Schippers *et al*., 1995). Possible options include combinations of a gradual reduction in the use of pesticides and fertilizers and an increase in the application of biological control systems. Although the precise definition of sustainable agriculture varies, one key aspect is the reduction in agrochemical inputs with a gradual shift to alternative crop protection strategies, such as cultural practices, organic amendments, resistant or tolerant crop varieties and biological control systems (Kloepper, 1996).

The influence of soil-borne microorganisms on plant development has been a generative and interesting field of research. The effects of these microorganisms on plant growth and development, besides their involvement in mineralization or plant disease activities, have drawn the attention of numerous researchers. In this review, the contribution and role of microbial and fungal species, with specific reference to *Trichoderma* and *Bacillus* species, towards plant growth promotion and biological control strategies are discussed.

1.1 Definition of biological control and plant growth promotion

Firstly, it is useful to define a working definition for biological control and plant growth promotion.

The term "rhizobacterial plant growth promotion" could be defined as the beneficial effects of rhizosphere bacteria on plants, resulting in increased plant growth, faster seed germination and better seedling emergence (Lazarovits and Nowak, 1997). These bacteria are now commonly called plant growth promoting rhizobacteria (PGPR) (Kapulnik, 1991; Lazarovits and Nowak, 1997) and yield increasing bacteria (YIB) in China (Shen, 1997).

Plant growth promoting rhizobacteria (PGPR) can therefore be defined as root inhabiting bacteria that stimulate plant growth (Kloepper and Schroth, 1978; Kloepper *et al*., 1989). Not only have free-living bacteria been shown to enhance plant growth, fungi such as

Trichoderma spp. have also been found to improve seedling emergence and plant establishment (Paulitz *et al*., 1986; Baker, 1988; Ousley *et al*., 1994a). In this review, PGPR will encompass both beneficial bacteria and fungi, specifically *Trichoderma* spp.

The concept of biological control has been in practice long before its definition. By definition, biological control, as defined by Smith (1919) and cited by Waage and Greathead (1988), "is the introduction of exotic insect natural enemies for the permanent suppression of insect pests". A more accurate definition of biological control is that established by Baker and Cook (1974). This definition is specific for the biological control of plant disease and proposes that biological control is "the reduction of inoculum density or disease-producing activity of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally, or through manipulation of the environment, host or antagonist by mass introduction of one or more antagonist". The part of this definition, which will form the focus of this review, in terms of biological control, is the reduction of inoculum density or disease producing activity of a pathogen by mass introduction of one or more antagonists. These antagonists hereafter will be referred to as biological control agents (BCAs).

1.2 Scope and potential application of PGPRs and BCAs

Bacteria and fungi are continuously being studied and exploited for their possible role in plant growth and health. While some of these investigations are in infant or laboratory stages, others are under greenhouse and field studies, some at the point of commercialisation and others are already commercialised and are being sold currently on the market. There are various reports and reviews on the effects of PGPRs on crop plants. Among them is the use of *Rhizobium* spp. to improve growth of legumes through symbiotic nitrogen fixation (Dorosinsky and Kadyrov, 1975; Hernandez and Hill, 1983). Other organisms include *Azospirillum* (Burdman *et al*., 1996; Fallik and Okon, 1996; Jacoud *et al*., 1999), *Trichoderma* spp. (Ousley *et al*., 1993; Inbar *et al*., 1994; Ousley *et al*., 1994b; Bjorkman *et al*., 1998; Bell *et al*., 2000; Rabeendran *et al*., 2000), *Bacillus* and *Pseudomonas* spp. (Seong *et al*., 1992; Ikram, 1994; Schippers *et al*., 1995; Probanza *et al*., 1996; Lazarovits and Nowak, 1997).

Successes in crop yield have been observed in PGPRs application to crops. Evaluation of 20 years worldwide field inoculation by Okon and Lanbandera-Gonzalez (1994) indicates that inoculation with *Azospirilla* increased crop yield as much as 30 percent. Fallik and Okon

(1996) also reported that inoculation of maize (*Zea mays* L.) with *Azospirillum brasilense* increased ear and kernel weight compared to the uninoculated control. Radish (*Raphanus sativus* L.) dry weight was increased after six weeks growth by 150 to over 250 percent by addition of *Trichoderma* spp. (Baker, 1988). Fresh weight of lettuce (*Lactuca sativa* L.) was consistently increased by up to 54 percent when inoculated with *Trichoderma* (Ousley *et al*., 1993). Elsewhere, a 37 percent yield increase has been reported in peas (*Pisum sativum* L.) treated with *Bacillus subtilis* (Turner and Backman, 1991).

In as much as numerous successes have been achieved in plant growth and yield through PGPR applications, some inconsistencies, and to a lesser extent, negative growth promotion have been observed with some PGPR strains. Rabeendran *et al*. (2000) reported that inoculation of cabbage (*Brassica oleracea* L. var. *capitata*) seedlings with *Trichoderma* isolates increased leaf area, shoot and root dry weight compared to the uninoculated control, but similar results were not recorded in the second trial under glasshouse conditions. Probanza *et al*. (1996) reported a negative influence on European alder (*Alnus glutinosa* L.) when inoculated with *Pseudomonas fluorescens* strains. All *P. fluorescens* strains used had negative effects on plant growth and this was expressed by a decrease in values of growth parameters measured, compared to control values.

Most PGPR strains have also been used as biological control agents (BCAs). Several and diverse microbial species have been demonstrated as natural antagonists to several plant pathogens (Cook, 2000). Like growth promotion, a wide range of bacterial and fungal species has been investigated for the control of soil-borne plant pathogens. Among the organisms that have been investigated as BCAs include *Epicoccum purpurascens* Ehrenberg, *Coniothyrium minitans* Campbell, *Talaromyces flavus* (Klocker) Stolk & Samson, *Trichothecium roseum* (Pers.) Link (Huang *et al*., 2000), *Trichoderma* spp. (Chet and Baker, 1981; Kok *et al*., 1996; Huang *et al*., 2000; Mishra *et al*., 2000), *Bacillus* spp. (Kim *et al*., 1997a; Adejumo *et al*., 1999), *Gliocladium* sp.(Lumsden and Locke, 1989; Zhang *et al*., 1996), *Aeromonas* sp. (Inbar and Chet, 1991), *Pseudomonas* spp. (De Boer *et al*., 1999; Borowicz and OmerSaad, 2000), *Chaetomium* (McLean and Stewart, 2000), *Aureobasidium pullulans* (de Bary) Arnaud. and *Cryptococcus albidus* (Saito) Skinner (Dik and Elad, 1999), *Serratia marcescens*, *Streptomyces viridodiaticus* and *Micromonospora carbonacea* (El-Tarabily *et al*., 2000). It is worth noting that not all the organisms that have been investigated as potential BCAs have been mentioned here. Successes with these BCAs in suppressing

plant diseases have been reported in various research articles. *Aeromonas caviae* applied as seed treatment reduced *Rhizoctonia solani* Kühn and *Fusarium oxysporum* f.sp.*vasinfectum* (Atkinson) Snyder et Hansen infections in cotton (*Gossypium hirsutum* L.) by 78 and 57 percent respectively under glasshouse conditions (Inbar and Chet, 1991). The same organism, *Aeromonas caviae*, effectively controlled *Sclerotium rolfsii* Sacc. in beans (*Phaseolus vulgaris* L.) (Inbar and Chet, 1991). *Sclerotium cepivorum* Berk., the causal agent of white rot disease of onions (*Allium cepa* L.) was effectively controlled under glasshouse conditions with applications of *T. harzianum* Rifai, *T. koningii* Oudem., *T. virens* (Miller) von Arx and *Chaetomium globosum* Kunze (McLean and Stewart, 2000). Similar successes have also been reported using various BCAs. These include the use of *Serratia marcescens* and *Streptomyces viridodiasticus* on *Sclerotinia minor* Jagger (El-Tarabily *et al*., 2000), *Talaromyces flavus* and *T. virens* on *Sclerotinia sclerotium* (Lib.) de Bary (Huang *et al*., 2000), *A. pullulans* on *Botrytis cinerea* Pers.: Fr. (Dik and Elad, 1999), and fluorescent *Pseudomonas* spp. on *Fusarium oxysporum* Schlectend.:Fr. (De Boer *et al*., 1999). Below is a table summarising some of the common PGPRs and BCAs that have been investigated for their growth promoting and biological control potentials.

Table 1.1 List of some common PGPRs and BCAs that have been investigated for plant growth promotion and biological control on different crops and on different plant pathogens

Organism(s)	**Intended use**	**Target crop(s)**	**Mode of application**	**Reference(s)**
Nonfluorescent *Pseudomonas* spp.	Growth promotion	Potato (*Solanum tuberosum* L. ssp. *tuberosum*)	Applied to tissue explants	Frommel *et al*. (1991)
Pseudomonas fluorescens	Growth promotion	Tomato (*Lycopersicon esculentum* L.)	Added to peat based substrate	Gagné *et al*. (1993)
P. aeruginosa; *P. putida*	Growth promotion	Tropical Kudzu [*Peuraria phaseoloides* (Roxb). Benth.]	Seedling drench	Ikram (1994)
P. aeruginosa strain 7NSK2	Growth promotion	Spinach (*Spinacea oleracea* L.), maize.	Mixed with growth medium	Seong *et al*. (1992)
T. harzianum	Growth promotion	Cucumber (*Cucumis sativus* L.), Sweet corn, marigold (*Tagetes* spp.), petunia	Root spray, seed coating, potting mix	Chang *et al*. (1986); Ousley *et al*. (1994a); Björkman *et al*. (1998); Bell *et al*. (2000)
T. viride	Growth promotion	Marigold, petunia, Verbena (*Verbena* spp.)	Mixed with growth medium	Ousley *et al*. (1994b)

Organism(s)	Intended use	Target crop(s)	Mode of application	Reference(s)
Trichoderma spp.	Growth promotion	Tomato, tobacco (*Nicotiana tabacum* L.), lettuce	Mixed with growth medium	Windham *et al*. (1986); Ousley *et al*. (1994a)
T. harzianum	Growth promotion	Cucumber, pepper (*Capsicum* spp.)	Incorporated into wheat-bran peat and mix with growth medium	Inbar *et al*. (1994)
T. longipile, T. tomentosum	Growth promotion	Cabbage	Transplant dip	Rabeendran *et al*. (2000)
Bacillus polymyxa	Growth promotion	Lodgepole pine (*Pinus contorta* L.)	Seed treatment	Shishido *et al*. (1995)
B. subtilis	Growth promotion	Peanut (*Arachis hypogaea* L.)	Seed treatment	Turner and Backman (1991)
Frankia	Growth promotion	*Alnus spp.*	Root dip	Berry and Torrey (1985)
Azospirillus brasilense	Growth promotion	Maize, Foxtail bristle grass [*Setaria italica* (L.) Beauv.]	Incorporated into granular peat	Fallik and Okon (1996)
A. lipoferum CRT1	Growth promotion	Maize	Peat carrier	Jacoud *et al*. (1999)
Aeromonas caviae	Biocontrol of *R. solani*, *S. rolfsii*, *Fusarium oxysporum* f.sp. *vasinfectum*	Bean, cotton	Seed treatment	Inbar and Chet (1991)
Pseudomonas spp.	Biocontrol of *Fusarium oxysporum*	Radish	Mixed into potting sand:soil	De boer *et al*. (1999)
Pseudomonas fluorescens	Biocontrol of *Fusarium oxysporum* f.sp. *ciceris*	Chickpea (*Cicer arietinum* L.)	Broadcast and seed treatment	Vidhysekaran and Muthamilan (1995)
Gliocladium virens, *T. hamatum*	Biocontrol of *R. solani* and *Pythium ultimum*	Zinnia (*Zinnia* spp.), cotton	Incorporated into growth medium	Lewis *et al*. (1996)
T. hamatum, *Chaetomium globosum*	Biocontrol of *R. solani* and *Pythium* spp.	Pea, radish	Seed treatment	Harman *et al*. (1980)
T. viride, *G. virens*, *T. hamatum*, *T. harzianum*	Biocontrol of *R. solani*	Eggplant (*Solanum melongena* L.), zinnia, cucumber, cabbage	Incorporated into growth medium	Lewis and Lumsden (2001)
T. harzianum	Biocontrol of *Phytophthora capsici*	Pepper	Potting mix	Ahmed *et al*. (1999)
T. hamatum, *Pseudomonas fluorescens*, *G. virens*	Biocontrol of *Fusarium* spp.	Tomato	Drenching/mixed into growth medium	Larkin and Fravel (1998)
B. subtilis, *G. virens*	Biocontrol of *Fusarium*, root-knot nematode	Cotton	Seed treatment	Zhang *et al*. (1996)
B. subtilis, *B. pumilus*, *B. cereus*, *Pseudomonas fluorescens*	Biocontrol of *R. solani*, *S. rolfsii*	Cotton, bean	Root dip	Pleban *et al*. (1995)

Organism(s)	Intended use	Target crop(s)	Mode of application	Reference(s)
Bacillus spp. L324 - 92	Biocontrol of *Gaeumannomyces graminis* var. *tritici*, *R. solani*	Wheat (*Triticum aestivum* L.)	Seed treatment	Kim *et al*. (1997a)
B. pumilus	Postharvest biocontrol of *Penicillium digitatum*	Citrus fruit	Spray/injection into fruit wounds	Huang *et al*. (1992)
T. virens, *T. longibrachiatum*	Biocontrol of *Rhizopus oryzae*, *Pythium* spp.	Cotton	Seed treatment	Howell (2002)
T. koningii, *T. harzianum*, *Bacillus* spp.	Biocontrol of *Protomycopsis phaseoli*	Cowpea (*Vigna unguiculata* (L.) Walp.	Foliar spray	Adejumo *et al*. (1999)
T. koningii, *T. harzianum*, *Trichoderma* spp.	Biocontrol of *Macrophomina phaseolina*	Cowpea	Seed treatment	Adekunle *et al*. (2001)
C. globosum, *Coniothyrium minitans*, *T. harzianum*, *T. virens*, *T. koningii*	Biocontrol of *Sclerotium cepivorum*	Onion (*Allium cepa* L.)	Soil additive	McLean and Stewart (2000)
Epicoccum purpurscens, *Talaromyces flavus*, *Trichothecium reseum*	Biocontrol of *Sclerotinia sclerotiorum*	Dry bean	Spray	Huang *et al*. (2000)

It should be noted that the above table summarises only some of the research done on PGPRs and BCAs. There are many more which have not been listed here. Through research, some of the successful PGPR and BCA has been commercialised and are currently marketed. There is no doubt that many of these beneficial bacteria and fungi are currently under intense research. The present status of some commercial PGPR and BCA products throughout the world are summarised in the table below, on the next page.

These are by no means the only commercial PGPR/BCA products available in the world market. The table serves to represent the general overview of the available products, their specificities and the degree of success of PGPR/BCA as commercial biological control and plant growth promotion products. So far, only one local commercial *Trichoderma* biological control product, under the name Eco-T®, is currently available and being sold in South Africa. Although other commercial products exist, these are all foreign products and not registered as a local commercial South African product (Dr Mike Morris, 2003 Personal Communication).

Table 1.2 Summary of some common commercial PGPR and BCA products available to growers on the world market with specific reference to target pathogen, crop and mode of application (adapted and modified from Gardener and Fravel, 2002)

Product name	**Biocontrol agent**	**Target pathogen/disease**	**Target crop**	**Formulation type**	**Application method**	**Manufacturer/distributor**
Actinovate	*Streptomyces lydicus*	Soil-borne disease	Greenhouse and nursery crops, turf	Water dispersable granule	Drench	Natural Industries Inc.
AQ Biofungicide	*Ampelomyces quisqualis* isolate M-10	Powdery mildew	Apples (*Malus sylvestris* Mill.), cucurbit, grapes (*Vitis vinifera* L.), ornamentals, strawberries (*Fragaria* spp), tomatoes	Water-dispersable granule	Spray	Ecogen, Inc
Aspire	*Candida oleophila* I-82	*Botrytis* spp., *Penicillium* spp.	Citrus, pome fruit	Wettable powder	Post harvest application as drench, drip or spray	Ecogen, Inc.
BioJect Spot-Less	*Pseudomonas aureofaciens*	Dollar spot, Anthracnose, *Pythium aphanidermatum*, pink snow mold	Turf and others	Liquid	Overhead irrigation; can only be used with the BIOJECT automatic fermentation system	Eco soil system, Inc.
Binab-T	*T. harzianum* (ATCC 20476) and *T. polysporum* (ATCC20475)	Pathogenic fungi causing wilt, take-all, root rot and internal decay of wood products and decay in tree wounds	Not applicable	Wettable powder and pellets	Spay, mixing with potting substrate, mixing with water and painting on tree wounds, inserting pellets in holes drilled on woods	Bio-Innovation AB
Biofox C	*Fusarium oxysporum* (non pathogenic)	*Fusarium oxysporum* and *Fusarium moniliforme*	Not applicable	Dust and alginate granules	Seed treatment or soil incorporation	S.I.A.P.A
Bio-Fungus	*Trichoderma* spp.	*Sclerotinia*, *Phytophthora*, *R. solani*, *Pythium* spp., *Fusarium*	Not applicable	Granular wettable powder, sticks and crumbles	After fumigation; incorporate into soil, sprayed or injection	Grondortsmettingen Decuestern .V.

Product name	**Biocontrol agent**	**Target pathogen/disease**	**Target crop**	**Formulation type**	**Application method**	**Manufacturer/distributor**
Bio-save 10LP, 110	*Pseudomonas syringae*	*Botrytis cinerea*, *Penicillium* spp., *Mucor pyroformis*, *Geotrichum candidum*	Pome fruits, citrus, cherries (*Prunus* spp.), potatoes	Lyophilized products, frozen cell concentrated pellets	Pellets added to water to produce liquid suspension, postharvest application to fruits as drench, dip or spray	Village Farms LLC
BlightBan A506	*Pseudomonas fluorescens*	Frost damage, *Erwinia amylovora*, and russet-reducing bacteria	Almond (*Amygdalus communis* L.), apple, apricot (*Prunus armeniaca* L.), blueberry (*Vaccinium* spp.), cherry, peach (*Prunus persica* L. Batsch.), pear (*Pyrus* spp.), potato, strawberry, tomato	Wettable powder	Bloom time spray of the flower and fruit	NuFarm Inc.
Blue Circle	*Burkholderia cepacia* (*Pseudomonas*) *cepacia* type Wisconsin	*Fusarium*, *Pythium* and many nematodes	Not applicable	Peat carrier or liquid formulation	Seed treatment or drip irrigation	CTT Corp.
Cedomon	*Pseudomonas chlororaphis*	Leaf stripe, net blotch, *Fusarium* spp., spot blotch, leaf spot, and others	Barley (*Hordeum vulgare* L.) and oats (*Avena sativa* L.) potential for other cereals	Seed treatment	Seed dressing	BioAgric AB
Companion	*Bacillus subtilis* GB03, other *B. subtilis*, *B. licheniformis*, *B. megaterium*	*Rhizoctonia*, *Pythium*, *Fusarium* and *Phytophthora*	Greenhouse and nursery crops	Liquid	Drench at time of seedling and transplanting or as a spray for turf	Growth products
Contans WG, Intercept WG	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	All agricultural soils	Water dispersable granule	Spray	PROPHYTA Biologischer Pflanzenschutz GmbH

Product name	Biocontrol agent	Target pathogen/disease	Target crop	Formulation type	Application method	Manufacturer/distributor
Deny	*Burkholderia cepacia* type Wisconsin	*Rhizoctonia*, *Pythium*, *Fusarium* and disease caused by lesion, spiral, lance and sting nematodes	Alfalfa (*Medicago sativa* L.), barley, beans, clover (*Trifolium* spp.), cotton, grain, sorghum, vegetable crops and wheat	Peat-based dried biomass from solid fermentation; aqueous suspension	Applied to seeds with a sticking agent in planter box (aqueous suspension formulation is for use in drip irrigation or as a seedling drench)	Stine Microbial Products
Eco-T®	*T. harzianum*	Root diseases	Vegetable, ornamentals, eucalyptus (*Eucalyptus* spp.)	Wettable powder	Drench and seed treatment	Plant Health Products Pty Ltd.
Epic	*Bacillus subtilis*	*Fusarium*, *Alternaria* and *Aspergillus* spp., which attack roots. Also *R. solani*	Not applicable	Dry powder	Added to slurry, mix with chemical fungicide for commercial seed treatment	Gustafson, Inc.
Galltrol	*Agrobactrium radiobacter* strain 84	Crown gall disease caused by *Agrobactrium tumifaciens*	Fruit, nut and ornamental nursery stock	Petri plates with pure culture grown on agar	Bacterial mass from one plate transferred to one gallon of non-chlorinated water; suspensions applied to seeds, seedlings, cuttings, roots, stems and as soil drench	AgBioChem, Inc.
HiStick N/T	*Bacillus subtilis* MB1600	*Fusarium*, *Rhizoctonia*, *Aspergillus*	Soyabean [*Glycine max* (L.) Merr.], alfalfa, dry/snap beans, peanuts	Not applicable	Slurry, damp and dry inoculation of seeds	Becker Underwood Inc. MicroBio Groups Ltd.
Intercept	*Burkholderia cepacia*	*R. solani*, *Pythium* spp., *Fusarium* spp.	Maize, vegetables, cotton	Not applicable	Not applicable	Soil Technologies Corp.
Kodiak (several formulations)	*Bacillus subtilis* GB03	*R. solani*, *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp. that attack roots	Cotton, legumes	Dry powder; usually applied with chemical fungicides	Added to a slurry mix for seed treatment; hopper box treatment	Gustafson, Inc.
Messenger	*Erwinia amylovora* HrpN harpin protein	Many	Field, ornamental and vegetable crops	Powder	Drench or spray	EDEN Bioscience Corporation

Product name	**Biocontrol agent**	**Target pathogen/disease**	**Target crop**	**Formulation type**	**Application method**	**Manufacturer/distributor**
Mycostop	*Streptomyces griseoviridis* strain K61	*Fusarium* spp., *Alternaria basicola*, *Phomopsis* spp., *Botrytis* spp., and *Phytophthora* spp.	Field, ornamental and vegetable crops	Powder	Drench, spray or through irrigation system	Kemira Agro Oy.
Promote	*T. harzianum* and *T. viride*	*R. solani*, *Pythium* and *Fusarium* spp.	Not applicable	Liquid conidial suspension	Seed treatment or soil/potting medium drench	JH Biotech.
RootShield, PLANT shield, T-22 Planter box	*T. harzianum* strain KRL-AG2 (T-22)	*R. solani*, *Pythium* and *Fusarium* spp.	Trees, shrubs, transplant, all ornamentals, cabbage, tomato, cucumber	Granules or wettable powder	Granules mixed with soil or potting medium; powder mixed with water and added as soil drench	Bioworks, Inc.
Serenade	*Bacillus subtilis* strain QST716	Powdery and downy mildew, *Cecospora* leaf spot, early and late blight, brown rot, fire blight and others	Curcubits, grapes, hops (*Humulus lupulus* L.), vegetables, peanuts, pome fruits, stone fruits and others	Wettable powder	Spray	AgraQuest, Inc.
SoilGard	*Gliocladium virens* (a.k.a *T. virens* GL-21)	Damping-off and root rot pathogens, especially *Rhizoctonia* and *Pythium* spp.	Ornamental and food crop plants grown in greenhouses, nurseries, homes and interiorscapes	Granules	Granules are incorporated in soil or soilless growing media prior to seeding	Certis, Inc.
YieldShield	*Bacillus pumilus* GB34	Soil-borne fungal pathogens causing root diseases	Soyabean	Dry powder formulation	Dry powder added to a slurry mix for seed treatment; hopper box treatment	Gustafson, Inc.

1.3 *Bacillus* and *Trichoderma* as BCAs and PGPRs

The genus *Bacillus* belongs to the family Bacillaceae. Species belonging to this genus are rod-shaped and are generally motile. One important advantage of this genus is their motility since it allows the bacteria to scavenge more efficiently for limited nutrients from root exudates (Brock and Madigan, 1991). *Bacillus* spp. have widely been used for many years in extensive research in an attempt to increase plant growth and suppress the activities of soil-borne plant pathogens (Turner and Backman, 1991; Holl and Chanway, 1992; Gutierrez Mañero *et al*., 1996; Kim *et al*., 1997a; Paulitz and Bélanger, 2001). Probanza *et al*. (1996) in an experiment held that two strains of *B. pumilus* and one strain of *B. licheniformis* showed significantly ($P < 0.05$) increased growth of European alder [*Alnus glutinosa* (L.) Gaertn.]. They reported that the *Bacillus* strains used increased the aerial surface and length of European alder by 163 and 182 percent respectively compared to the untreated controls. Enebak *et al*. (1998) also reported that strains of *B. subtilis* and *B. pumilus* were able to increase germination speed and dry biomass of loblolly pine (*Pinus taeda* L.) and slash pine (*Pinus elliottii* L.) seedlings. According to Shishido *et al*. (1995), two strains of *B. polymyxa* inoculated onto lodgepole pine seeds under greenhouse conditions increased seedling length, shoot and dry biomass by 18, 24 and 27 percent respectively, compared to uninoculated control. Turner and Backman (1991) reported an increase of 17 percent in peanut yield after seeds were treated with *B. subtilis* and grown under field conditions.

Other reported benefits of using *Bacillus* spp. include the ability to control soil-borne plant pathogens (Rytter *et al*., 1989; Asaka and Shoda, 1996; Kim *et al*., 1997a,b), enhance plant survival (Pleban *et al*., 1995) and induced systemic resistance to plant pathogens (Wei *et al*., 1996). *Bacillus* spp. have been used, apart from growth promotion, in an attempt to control a wide range of plant pathogens. Most research articles have not specifically focussed on a particular pathogen or crop. Among the pathogens that have been suppressed using *Bacillus* spp. include *Gaeumannomyces graminis* (Sacc.) Arx & D Olivier var. *tritici* J.C. Walker and *R. solani* (Pleban *et al*., 1995; Asaka and Shoda, 1996; Kim *et al*., 1997a), *Sclerotium rolfsii* (Pleban *et al*., 1995), *Fusarium oxysporum* f.sp. *vasinfectum* (Zhang *et al*., 1996), *Pythium* spp. (Utkhede *et al*., 1999), *Phytophthora* (Utkhede, 1984), *Penicillium* (Huang *et al*., 1992) and *Puccinia* (Rytter *et al*., 1989). The predominant *Bacillus* sp. used in most biological control studies is *B. subtilis* (Rytter *et al*., 1989; Kloepper, 1991; Krebs *et al*., 1993; Pleban *et al*., 1995; Asaka and Shoda, 1996; Zhang *et al*., 1996; Utkhede *et al*., 1999).

A number of successes have been achieved using *B. subtilis* to control plant pathogens on crops. Utkhede *et al*. (1999) demonstrated that *B. subtilis* strain BACT-0 increased fruit yield and fruit number of cucumber plants inoculated with *Pythium aphanidermatum* (Edson) Fitz. in soilless culture under greenhouse conditions. Most of these successes have been achieved under greenhouse conditions (Pleban *et al*., 1995; Utkhede *et al*., 1999), some under growth chamber conditions (Zhang *et al*., 1996) and field conditions (Kim *et al*., 1997a). In most cases, bacterial seed treatment with either spores or vegetative cells has been employed (Zhang *et al*., 1996; Kim *et al*., 1997a). Spore suspensions (Utkhede *et al*., 1999) and root dip (Pleban *et al*., 1995) has also been used.

Use of *Bacillus* spp. to increase plant growth and suppress plant pathogens has yielded some successes in the field of commercialisation. Due to the high growth stimulation on turf grass, *Bacillus* spp. Strain L324-92 was awarded a license in 1998 for further development and commercialisation for use on turf grass (Mathre *et al*., 1999). Also a strain of *B. subtilis* has been marketed in USA, under the name KODIAK®, for seed and furrow applications on cotton and peanuts (Paulitz and Bélanger, 2001). Other commercialised *Bacillus* spp. products include Companion® (*B. subtilis* GB03, *B. licheniformis*), HiStick N/T® (*B. subtilis* MBI600), Serenade® (*B. subtilis* QST716) and YieldShield® (*B. pumilus* GB34) (Anonymous, 2003). From the list above, one can deduce that strains of *B. subtilis* dominate the *Bacillus* spp. commercial products in the market as is the case of research done on *Bacillus* spp. as plant growth promoters and biological control.

There are some reports on the mechanisms by which these *Bacillus* spp. is thought to promote plant growth and control plant diseases (Glick, 1995). Several mechanisms have been suggested to explain the phenomenon by which *Bacillus* spp. promote plant growth. Among these mechanisms are the production of phytohormones such as Indole Acetic Acid (IAA) (Selvadurai *et al*., 1991; Lebuhn *et al*., 1997) and cytokinins (Brown, 1974). Other auxin-like compounds such as IAA-1, which is different from IAA, have been reported to have a plant growth promoting effect (Selvadurai *et al*., 1991). Gutierrez-Mañero *et al*. (1996) reported that *B. licheniformis* and *B. pumilus* produced an IAA-like compound which promoted growth of European alder. Further investigations revealed that these two *Bacillus* strains produce IAA-1 compounds at levels of 1.736 and 1.790 mg.l^{-1} in culture growth medium. Selvadurai *et al*. (1991) also reported a similar effect of indole-3-acetic acid analogues produced by strains of *B. cereus*. These strains were found to increase the dry weight of tomato and wheat

seedlings through the action of indole-3-acetic acid production. Solubilization of phosphates has also been shown as a mechanism of growth promotion by *Bacillus* spp. (Alagawadi and Gaur, 1992; Rojas *et al*., 2001). The combination of *B. polymyxa*, a phosphate solubilizing bacteria with *Azospirillum brasilense* increased yield of sorghum in the field under rainfed conditions (Alagawadi and Gaur, 1992).

Antibiotics has widely been reported as one of the main mode of action by which *Bacillus* spp. control plant pathogens (Swineburne *et al*., 1975; Utkhede, 1984; Leifert *et al*., 1995; Asaka and Shoda, 1996; Kim *et al*., 1997b; Mathre *et al*., 1999). Apart from antibiotics, other inhibitory metabolites have been reported as mechanisms involved in biological control by *Bacillus* spp. (Fränberg and Schnürer, 1994; Pleban *et al*., 1995; Podile and Prakash, 1996), production of volatile compounds (Wright and Thompson, 1985; Fiddaman and Rossall, 1993) and production of biosurfactants (Edwards and Seddon, 1992). Whether these range of antibiotics and volatile compounds produced *in vitro* are solely responsible for biological control activity *in vivo* are not clear since it is difficult to establish whether the antibiotics produced *in vitro* is the same antibiotics produced *in vivo* in plant rhizospheres (Leifert *et al*., 1995) or whether a totally different compound has been elicited *in vivo* on plants that does not correlate to the compound produced *in vitro* (Leifert *et al*., 1995). However, Asaka and Shoda (1996) reported that Iturin A and Surfactin produced by *B. subtilis* RB14 were responsible for the biological control of *R. solani* damping-off of tomato. Iturin A and Surfactin were recovered from autoclaved soils with *B. subtilis* RB14. They therefore concluded that Iturin A and Surfactin played a significant role in suppressing damping-off caused by *R. solani*.

Pleban *et al*. (1995) reported the involvement of a chitinase enzyme produced by *B. cereus* in controlling *R. solani*. Podile and Prakash (1996) also reported the involvement of a chitinase enzyme produced by *B. subtilis* AF1 in controlling crown rot caused by *Aspergillus niger* van Tieghem on groundnut. In the same experiment, they held that an extracellular protein precipitate from *B. subtilis* AF1 significantly retarded the growth of *A. niger*. This goes to support the statements made by Chet (1987) and Weller (1988) that an efficient biological control agent may exhibit one or more mechanisms or a combination of different mechanisms of action by which it suppresses a pathogen. Weller (1988) further maintained that the importance of any of these mechanisms might differ with the physical and chemical conditions of the rhizosphere.

In most studies, the use of *Bacillus* spp. as plant growth promoters and biological control has mainly been at seedling stages in the greenhouses, and some extended to yield trials. Although seedling trials are important as they provide quick assessments of *Bacillus* strains, the ultimate goal is to increase crop yield by minimising the activities of clinical and sub-clinical plant pathogens, which stresses the importance of adult plant field experimental trials.

The genus *Trichoderma* is among the most prominent and commonly used organisms for plant growth promotion and biological control of plant pathogens (Papavizas, 1985; Tronsmo and Hjeljord, 1998). They are filamentous deuteromycetes and are commonly found in all soils (Samuels, 1996). Most species of the genus are photosensitive and sporulate easily on a range of natural and artificial media (Papavizas, 1985).

Application of *Trichoderma* spp. to crop seeds, seedlings and to pathogen-free soils has been reported to stimulate plant growth (Inbar *et* al., 1994; Rabeendran *et al*., 2000). Several authors have reported increase in plant growth as a result of *Trichoderma* application on several crops and plant species. These include marigold, petunia and verbena (Ousley *et al*., 1994b), sweet corn (Björkman *et al*., 1998), cabbage and lettuce (Rabeendran *et al*., 2000) and cucumber (Chang *et al*., 1986; Bell *et al*., 2000). Furthermore, several reports have also reported the use of *Trichoderma* spp. to control plant pathogens on a wide range of economically important crops (Lewis *et al*., 1996; Ahmed *et al*., 1999; Mathre *et al*., 1999; Harman, 2000). Table 1.3 summarises how extensively *Trichoderma* spp. have been used to increase plant growth and control plant diseases.

Various formulations and applications have been used in various reports for applying *Trichoderma* spp. for plant growth promotion and biological control studies. Seed coating/treatment, root-dip in conidial suspension, soil additives either in peat-bran, wheat-bran, peat-sand formulations are some of the methods that have been used in plant growth promotion and biological control studies (Chang *et al*., 1986; Windham *et al*., 1986; Ousley *et al*., 1993; Inbar *et al*., 1994; Koch, 1999; McLean and Stewart, 2000).

Table 1.3 List and effects of some common *Trichoderma* spp. investigated for plant growth promotion and biological control of plant pathogens on different crops

***Trichoderma* strain**	**Intended use**	**Target crop**	**Mode of application**	**Reference(s)**
T. harzianum	Growth promotion	Petunia, pepper, cucumber	Soil additive; sprayed on roots as conidial suspension	Chang *et al*. (1986)
T. harzianum, *T. koningii*	Growth promotion	Tomato, tobacco, radish	Soil additive	Windham *et al*. (1986)
T. harzianum, *T. viride*	Growth promotion	Lettuce	Potting mix (50:50 peat:sand compost mixture)	Ousley *et al*. (1993)
T. harzianum	Growth promotion	Cucumber, pepper	Incorporation into peat-bran mixture	Inbar *et al*. (1994)
T. harzianum	Growth promotion	Lettuce	Soil additive (potting mix)	Ousley *et al*. (1994a)
T. harzianum	Growth promotion	Sweet corn	Seed treatment with polyox sticker	Björkman *et al*. (1998)
T. harzianum	Growth promotion	Cucumber	Seed coating; spore-coated organic pellets; dry biomass powder	Bell *et al*. (2000)
T. longipile, *T. tomentosum*; *T. virens*	Growth promotion	Cabbage, lettuce	Root dip (conidia suspension); incorporation into potting mix	Rabeendran *et al*. (2000)
T. harzianum	Biocontrol of *S. rolfsii*	Tomato, blue lupin (*Lupinus angustifolius* L.), peanut	Liquid formulation sprinkled on soil (growth medium) surface	Wells *et al*. (1972)
T. harzianum	Biocontrol of *Phytophthora cinnamomi*	Pine (*Pinus* spp.)	Incorporated into soil as impregnated clay granule formulation	Kelly (1976)
T. harzianum	Biocontrol of *R. solani*	Eggplant, tomato, bean	Incorporated into soil as wheat-bran formulation	Hadar *et al*. (1979)
T. hamatum	Biocontrol of *R. solani* and *Pythium* spp.	Radish, pea	Seed treatment	Harman *et al*. (1980)
T. harzianum	Biocontrol of *S. rolfsii* and *R. solani*	Beans, cotton	Applied to soil as wheat-bran formulation	Elad *et al*. (1980)
T. hamatum	Biocontrol of *Pythium aphanidermatum*, *P. ultimum*, *P. oligandrum*	Radish, pea, bean	Addition of conidia into growth medium	Chet and Baker (1981)
Trichoderma spp., *T. hamatum*	Biocontrol of *R. solani*	Radish	Applied as wheat-bran formulation to soil (growth medium)	Mihuta-Grimm and Rowe (1986)
T. harzianum, *T. koningii*, *Trichoderma* spp.	Biocontrol of *Phytophthora* root and crown rots	Apple	Applied to growth medium as peat-bran formulation	Smith *et al*. (1990); Roiger and Jeffers (1991)

***Trichoderma* strain**	**Intended use**	**Target crop**	**Mode of application**	**Reference(s)**
T. harzianum, *T. viride*	Biocontrol of *R. solani*	Lettuce	Applied in the form of peat-bran formulation into growth medium	Maplestone *et al*. (1991)
T. harzianum, *T. hamatum*, *T. viride*	Biocontrol of *R. solani*	Cotton	Applied as alginate pellet, powder or bran/germling formulation to soil	Lewis and Papavizas (1991)
T. harzianum, *T. koningii*, *Trichoderma* spp.	Biocontrol of *S. cepivorum*	Onion	Soil additive as sand:bran:fungal homogenate formulation	Kay and Stewart (1994)
T. harzianum	Biocontrol of *R. solani*	Sugar beet (*Beta vulgaris* L.)	Application to soil as processed manure pellets formulation	Kok *et al*. (1996)
T. harzianum, *T. hamatum*, *T. viride*	Biocontrol of *R. solani*	Eggplant	Applied to growth medium as extruded granule formulation	Lewis and Larkin (1997)
T. harzianum, *T. hamatum*, *T. viride*	Biocontrol of *R. solani* and *P. ultimum*	Radish, cucumber	Seed coating	Cliquet and Scheffer (1997)
T. harzianum, *T. hamatum*, *T. viride*, *T. virens*	Biocontrol of *R. solani*	Eggplant, pepper	Applied to soilless mix as commercial Biodac formulation	Lewis *et al*. (1998)
T. harzianum	Biocontrol of *Phytophthora capsici*	Pepper	Added to potting mix in peat and wheat bran	Ahmed *et al*. (1999)
T. harzianum, *T. viride*	Biocontrol of *R. solani* and *P. ultimum*	Cucumber, peas	Potting mix in wheat-bran:vermiculite formulation; also mix in water for irrigating plants	Koch (1999)
T. koningii	Biocontrol of *R. solani* and *P. ultimum* var. *sporangiiferum*	Pepper	Potting mix on grown rice hulls	Harris (1999)
T. virens	Biocontrol of *Sclerotinia sclerotium*	Beans	Foliar spray	Huang *et al*. (2000)
T. harzianum, *T. koningii*, *T. virens*	Biocontrol of *S. cepivorum*	Onion	Applied to growth medium as sand:bran:fungal mix	McLean and Stewart (2000)
T. harzianum, *T. koningii*, *Trichoderma* spp.	Biocontrol of *Macrophomina phaseolina*	Cowpea	Seed treatment	Adekunle *et al*. (2001)
T. hamatum, *T. virens*	Biocontrol of *R. solani*	Eggplant, zinnia, cucumber, cabbage, pepper	Incorporated into growth medium in a formulated form	Lewis and Lumsden (2001)

Other formulations such as processed manure pellets (Kok *et al*., 1996), impregnated clay granules (Kelly, 1976) and foliar sprays (Huang *et al*., 2000) have also been investigated. However inconsistencies in growth promotion studies reported by Rabeendran *et al*. (2000)

with *Trichoderma* strains may not have been due to the mode of application of the *Trichoderma* strains but rather the set of growth conditions under which the experiment was conducted. After observing differences in controls in two separate experiments, Rabeendran *et al*. (2000) held that expression of growth promotion is more apparent under sub-optimal growth conditions than under optimal growth conditions. However, they suggested that the differences between the two controls could possibly be due to sub-optimal growth conditions, as water and nutrients availability were not limiting.

Trichoderma harzianum and *T. viride* Pers.:Fr. applied to peat-based media as dried fermented biomass increased fresh shoot and dry weights of marigold seedlings by 40 and 52 percent respectively (Ousley *et al*., 1994b). However, increase in flowers, fresh shoot and dry weights were dependent on *Trichoderma* concentrations added to the growth medium.

Growth promotion experiments by Kleifeld and Chet (1992) suggest that increase in plant growth does not only depend on the effectiveness of the *Trichoderma* strain used, but also on the mode of inoculum application. They reported that growth response with *T. harzianum* applied as wheat-bran preparation was much more pronounced than when *T. harzianum* inoculum was applied as seed coating or conidial suspension. Bell *et al*. (2000) also reported the effect of inoculum delivery methods on plant growth promotion and biological control. They stressed need for isolates to be tested under different environmental conditions using different inoculum delivery methods before being tagged as plant growth promoters or biological control agents.

Trichoderma strains have been reported to control a wide range of soil-borne plant pathogens (Wells *et* al., 1972; Harman *et al*., 1980; Papavizas, 1985; Ahmed *et al*., 1999; Huang *et al*., 2000; McLean and Stewart, 2000; Adekunle *et al*., 2001). So far, a number of *Trichoderma* products have successfully been commercialised and available on the world market. These include SUPRESIVIT® (*T. harzianum*), TRI002 (*T. harzianum*), ECOFIT® (*T. viride*) (Koch, 1999) and TRICHODEX® (*T. harzianum*) (Paulitz and Bélanger, 2001).

Several mechanisms have been suggested to be responsible for the increased plant growth promotion and biological control observed with *Trichoderma* spp. The mechanisms suggested to be involved in plant growth promotion include control of sub-clinical pathogens (Windham *et* al., 1986; Ousley *et al*., 1993; Inbar *et al*., 1994), production of plant growth hormones (phytohormones) (Chang *et al*., 1986; Baker, 1988), release of soil nutrients and

minerals by increasing saprophytic activity in soil (Ousley *et al*., 1994b), production of vitamins, conversion of non-utilisable materials into utilisable form for uptake by plants and increase in uptake and translocation of minerals (Kleifeld and Chet, 1992). However, it would be more beneficial if a particular growth promoting strain could exhibit two or more of these mechanisms. Since environmental conditions have been suggested to have effect on *Trichoderma* activity (Bell *et al*., 2000), a particular mechanism may be hampered by environmental factors.

Trichoderma spp. possesses multiple mechanisms of action by which they control plant pathogens (Papavizas, 1985; Chet, 1987; Tronsmo and Hjeljord, 1998; Paulitz and Bélanger, 2001). Among these mechanisms include mycoparasitism, which involves parasitism of a pathogenic fungus (Chet *et al*., 1981; Benhamou and Chet, 1993; Gupta *et al*., 1999; Zhang *et al*., 1999; Ortiz and Orduz, 2000). The destructive stage of the mycoparasitic process, which involves the degradation of the host cell wall, is mediated by the production of lytic enzymes such as glucosidases and chitinases (Elad *et al*., 1983; Chérif and Benhamou, 1990). The production of these enzymes, which is very important in the mycoparasitic process, has been been elucidated (Elad *et al*., 1983; Lima *et al*., 1997; Thrane *et al*., 1997; Menendez and Godeas, 1998). Lectin has also been suggested to play a role in the mycoparasitic process (Elad *et al*., 1983). Elad and Kapat (1999) implicated the involvement of protease enzymes produced by *T. harzianum* in the biological control of *B. cinerea*.

Competition for nutrients and space is one of the likely mechanisms involved in the biological control of plant pathogens (Tronsmo and Hjeljord, 1998). Usually the biological control agent grows and out competes the pathogen for nutrients and space. The pathogen is suppressed in the process leading to a population reduction, which no longer becomes a problem (Anonymous, 2001). Sivan and Chet (1989) suggested competitive displacement and free nutrient competition as the mechanism involved in the biological control of *F. oxysporum* on cotton by *T. harzianum* T35.

Antibiotic production has been well documented as one of the mechanisms employed by *Trichoderma* spp. in the suppression of plant pathogens (Lifshitz *et al*., 1986; Ghisalberti and Sivasithamparam, 1991; Graeme-Cook and Faull, 1991; Calistru *et al*., 1997; Mischke, 1997). Other mechanisms reported by other workers include production of volatile organic compounds (Wheatley *et al*., 1997), induced systemic resistance (De Meyer *et al*., 1998) and siderophore production (Weller, 1988).

Many *Trichoderma* spp. possess one or more of the above mentioned mechanisms. However, the relative importance of these mechanisms may depend on the specific *Trichoderma* isolate and the pathogen involved. Environmental conditions also play a major role in the expression of these mechanisms (Tronsmo and Hjeljord, 1998).

Bacillus and *Trichoderma* spp. are commonly found and readily isolated from soil. Reports proposing *Bacillus* and *Trichoderma* spp. being rhizosphere competent have made these two organisms an attractive "commodity" for biological control and plant growth promotion (Ahmad and Baker, 1987; Ahmad and Baker, 1988; Maplestone and Campbell, 1989; Holl and Chanway, 1992; Kim *et al*., 1997a). *Bacillus* spp. form endospores and these endospores are tolerant to heat and desiccation (Lazarovits and Nowak, 1997). These properties led to the investigation of their growth promoting and biological control ability (Petras and Casida, 1985). *Bacillus* spp. are of interest as inoculants because spores are relatively easy to prepare in large quantities (Petras and Casida, 1985) and remain viable for extended periods, thereby imparting an extended "shelf life" to commercially formulated PGPR products (Aronson *et al*., 1986; Young *et al*., 1995). The endospores survive in a dormant form until conditions are appropriate for germination (Van Elsas *et al*., 1986). In addition, the timing for inoculation of *Bacillus* spp. is not restricted by the need to accurately predict conditions favourable for the development of the plant pathogen (Young *et al*., 1995).

On the other hand, *Trichoderma* spp. form conidia and chlamydospores (Samuels, 1996). These could easily be produced in liquid and on solid fermentation media (Lewis and Papavizas, 1983). These structures can easily be produced in large quantities on a commercial scale and formulated or incorporated into peat/wheat bran (Mihuta-Grimm and Rowe, 1986; Maplestone *et al*., 1991) or in powder and pellet formulations (Lewis and Papavizas, 1991). These factors coupled with the various mechanisms exhibited by *Bacillus* and *Trichoderma* spp. make these two organisms attractive choices for biological control and plant growth promotion.

1.4 Interactions between introduced BCAs and PGPRs for biological control and plant growth promotion studies

When discussing the activities of BCAs and PGPRs, it is important to consider a key concept of interactions – the concept of antagonism and synergism that may exist between two beneficial microorganisms. BCAs and PGPRs encounter an array of conditions when

introduced into the rhizosphere. These conditions range from interactions with the resident microbial community (Handelsman and Stabb, 1996), and biotic and abiotic conditions (Guetsky *et al*., 2001; Guetsky *et al*., 2002). There are claims and counter-claims that introduction of a single BCA often results in inconsistent positive effects (Weller, 1988; Schippers, 1992; Mathre *et* al., 1999; Harman, 2000) while other reports maintain that application of mixtures of BCAs and PGPRs could be more beneficial than single strain applications (De Boer *et al*., 1999; Guetsky *et al*., 2002).

Sneh *et al*. (1984) reported that no improvement in suppression of *Fusarium* chlamydospores was achieved when lytic and fluorescent bacteria were combined. Dandurand and Knudsen (1993) combined *T. harzianum* and *P. fluorescens* but did not achieve better disease control of *Aphanomyces euteiches* Dresch. f. sp. *pisi* W.F. Pfender & D.J. Hagedon on pea. However, a number of successes have been achieved by others using combinations of BCAs and PGPRs to enhance growth promotion and biological control. Combinations of two fluorescent *Pseudomonas,* strains RE8 and RS111, significantly reduced fusarium wilt, caused by *F. oxysporum* on radish, more than either strain used in isolation (De boer *et al*., 1999). Other successes achieved with BCA and PGPR mixtures are summarised in a table below, on the next page.

A closer look at the above reports suggests that, although some negative interactions and effects have been reported on some crops, other combinations have recorded positive results over single inoculations (El-Tarabily *et al*., 1996; De Boer *et al*., 1999). Successes so far on interaction work by various authors with various BCAs and PGPRs on crop yield and disease control suggest that there is room for improvement on means of increasing plant growth and biological control through bacterial-bacterial, bacterial-fungal or fungal-fungal mixtures. As BCAs and PGPRs may be affected by biotic and abiotic conditions, depending on the environment in which they are used, it is possible that one mechanism may compensate for another where the interacting organisms exhibit an array of mechanisms (Guetsky *et al*., 2002) and may reduce variability in performance with increase in consistency (Larkin and Fravel, 1998).

Negative effects achieved by some researchers could be due to opposing effects between the interacting organisms. These could be due to detrimental interactions, such as the production of antibiotics by one of the organisms, which have a negative effect on the other organism (De boer *et al*., 1999).

Table 1.4. Examples of combinations of BCAs and PGPRs used to improve plant growth promotion and biological control of various plant pathogens on various crops

Interacting Organisms	**Intended Use**	**Target Crop**	**Comments**	**Reference(s)**
Rhizobium and *B. polymyxa* or *P. striata*	Growth promotion	Chickpea	Combined inoculation increased nodulation, nitrogenase activity, available phosphate and grain yield over single inoculation	Alagawadi and Gaur (1988)
Azospirillum lipoferum and *Aspergillus. niger* or *Aspergillus awamori*	Growth promotion	Wheat	Increase in growth when organisms were inoculated in combination. Increased nitrogen uptake and higher grain yield was achieved by combined inoculations	Darmwal and Gaur (1988)
A. brasilense and *P. striata* or *B. polymyxa*	Growth promotion	Sorghum	Significant increase in grain and dry matter yield with increase in nitrogen and phosphorus uptake from combined inoculation compared to single inoculation	Alagawadi and Gaur (1992)
Micromonospora carbonacea, *M. inositola* and *Streptomyces violascens*	Biocontrol of *Phytophthora cinnamomi* root rot	*Banksia grandis*	Combination enhanced disease suppression than each of the isolates used alone. Growth promotion of *Banksia* observed when isolates were used in combination	El-Tarabily *et al*. (1996)
T. koningii and strains of fluorescent *Pseudomonas* spp.	Biocontrol of take-all	Wheat	Mix results obtained. Some combinations better than single inoculations, while some were not different compared to single inoculations	Duffy *et al*. (1996)
B. polymyxa or *P. striata* and *T. harzianum*	Growth promotion	Sorghum	Available phosphorus highest in combined inoculation. Increased in size, ear-head and grain yield with mixtures	Jisha and Alagawadi (1996)

Interacting Organisms	**Intended Use**	**Target Crop**	**Comments**	**Reference(s)**
B. pumilus and *B. subtilis* or *Curtobacterium flaccumfaciens*	Biocontrol of *Colletotrichum orbiculare* and *Erwinia tracheiphila*	Cucumber	Significant and better control with mixtures than single inoculation	Raupach and Kloepper (1998)
Combination of fluorescent *Pseudomonas* spp.	Biocontrol of *Fusarium* wilt	Radish	Disease suppression by compatible strains significantly better than single inoculations. Some combined inoculations were not effective compared to single inoculations	De Boer *et al* (1999)
Rhizobium and *T. harzianum*	Growth promotion	Black gram (*Vigna mungo* (L.) Hepper.	No significant increase in biomass yield was observed in combined inoculation, but marginal increase in seed yield observed in field experiment	Jayaraj and Ramabadran (1999)
B. licheniformis (phosphate solubilizer) and *Phyllobacterium* (nitrogen fixer)	Growth promotion	Mangrove (*Avicennia* spp.) seedlings	Combination enhanced nitrogen fixation and phosphate solubilization. Seedlings developed more leaves in combined inoculations	Rojas *et al*. (2001)
Pichia guilermondii (yeast) and *B. mycoides*	Biocontrol of *B. cinerea*	Strawberry	Combination more effective on conidia destruction than either of the organisms used in isolation	Guetsky *et al*. (2002)

1.4.1 Methods used in interaction studies

Several methods have been employed in interaction studies between BCA and PGPR. Due to variability and inconsistencies observed by some authors in growth experiments (Rabeendran *et al*., 2000), the use of combinations of multiple BCAs and PGPRs has been sought in an attempt to improve disease control and increase in crop yield over the use of a single organism (Guetsky *et al*., 2001).

Methods that have been employed in *in vivo* studies include seed treatment with mixtures of antagonists (equal volumes or amounts) (Raupach and Kloepper, 1998; Jayaraj and Ramabadran, 1999), incorporation of mixtures in wheat-bran carrier into growth medium (El-Tarabily *et al*., 1996) and application of one organism into soil with the interacting organism introduced as seed treatment (Darmwal and Gaur, 1988; Alagawadi and Gaur, 1992; Duffy *et al*., 1996; Jisha and Alagawadi, 1996). Other methods which have been evaluated include soaking of seeds in BCA/PGPR mixtures (Alagawadi and Gaur, 1988), potting soil mix with one organism followed by root dip with the other organism (Sneh *et al*., 1984) and application of mixtures in liquid suspensions on leaf surfaces (Guetsky *et al*., 2002).

In interaction studies where bacterial and fungal BCA/PGPR are involved, the fungus has usually been introduced on carrier materials while the bacterium has been applied as a seed treatment (Duffy *et al*., 1996; Jisha and Alagawadi, 1996). However, interactions involving two bacterial strains have made use of either soaking seeds in a mixture of liquid suspensions (Alagawadi and Gaur, 1988) or as seed treatments with an adhesive (Jayaraj and Ramabadran, 1999). Irrespective of the method employed, a considerable amount of success has been achieved (Jisha and Alagawadi, 1996; De Boer *et al*., 1999; Guetsky *et al*., 2001).

In vitro plating technique (dual culture method) has also been used to study BCA/PGPR interactions (De Boer *et al*., 1999). This gives an insight to whether the interaction is synergistic or antagonistic, especially in a case where one organism produces an inhibitory substance, e.g., an antibiotic. Jisha and Alagawadi (1996) used plate counts to determine the populations of *B. polymyxa*, *P. striata* and *T. harzianum* on selective media after dual inoculation into potting soil. Scanning electron microscopy has also been used in interaction studies. A classical example of this is an experiment by Guetsky *et al*. (2001), where a combination of yeast, *P. guilermondii* and *B. mycoides* was found to cause severe shrinkage of *B. cinerea* conidia. These *in vitro* studies are useful in the sense that they may provide a theoretical explanation to situations *in vivo*.

1.4.2 Benefits and limitations of BCA and PGPR interactions

BCAs and PGPRs have been handicapped by discrepancies in efficacy when applied in different environments. Inconsistencies and variability in plant growth promotion and biological control have been reported (Guetsky *et al*., 2001; Rabeendran *et al*., 2000). Combinations of BCAs and PGPRs have been suggested as a means of reducing variability

and increasing reliability in performance of biological control agents (Guetsky *et al*., 2001). Increase in plant growth by combination of PGPR strains have also been linked to increase in nutrient solubilization and uptake by plants (Jisha and Alagawadi, 1996).

Although mixtures of BCAs and PGPRs are gradually becoming an established approach to combat variations and reduced efficacy of single BCAs and PGPRs under different environmental conditions, there are some limitations to this approach. Although two or more BCAs or PGPRs may seem compatible *in vitro*, competition for the same nutrient source may hinder the synergy between the interacting organisms as one may have a stronger preference for a specific nutrient source. This is usually the case when two or more organisms have the same ecological requirements and will therefore compete for the same nutrient source (Fukui *et al*., 1994; Janisiewicz and Bors, 1995).

1.5 Infection court

The term "infection court" could be defined as a site in or on a host plant where infection can occur. These sites could vary from the roots to the leaves of host plants. Roots of host plants are most ideal as that is where most microbial interactions/activities take place (Deacon, 1991; Bolton *et al*., 1993). Ideally, plant roots are the main target for BCAs and PGPRs applications, hence the activities and conditions prevailing in the rhizosphere are very critical to biological control and plant growth promotion (Deacon, 1991).

The effects of BCAs and PGPRs have been studied on various crops. Some of the target crops include eggplant, cucumber, cabbage, tomato, pepper (Hadar *et al*., 1979; Elad *et al*., 1980; Gagné *et al*., 1993; Inbar *et al*., 1994; Lewis and Larkin, 1997; Lewis *et al*., 1998; Ahmed *et al*., 1999; Dik and Elad, 1999; Harris, 1999; Koch, 1999; Lewis and Lumsden, 2001), onions, lettuce, sugarbeet, celery (*Apium graveolens* L.), radish (Harman *et al*., 1980; Mihuta-Grimm and Rowe, 1986; Budge and Whipps, 1991; Maplestone *et al*., 1991; Kleifeld and Chet, 1992; Kay and Stewart, 1994; De Boer *et al*., 1999; Dik and Elad, 1999; McLean and Stewart, 2000), cowpea, bean, snapbean, chickpea (Papavizas and Lewis, 1989; Vidhyasekaran and Muthamilan, 1995; Adejumo *et al*., 1999; Koch, 1999; Huang *et al*., 2000; Adekunle *et al*., 2001), cotton, sesame (*Sesamum indicum* L.) (Elad *et al*., 1982; Chung and Choi, 1990; Lewis and Papavizas, 1991; Howell, 2002), fruits (Smith *et al*., 1990; Roiger and Jeffers, 1991), cereals (Fallik and Okon, 1996; Hökeberg *et al*., 1997; Mao *et al*., 1997; Vidhyasekaran *et al*., 1997; Björkman *et al*., 1998; Jacoud *et al*., 1999), forest trees such as

European alder and pine, (Kelly, 1976; Probanza *et al*., 1996; Enebak *et al*., 1998) and on flowers such as marigold, petunia and verbena (Ousley *et al*., 1994b). However, this review of published articles show that BCA and PGPR research has focussed more on vegetables and other food crops than on flowers and forest trees. Any positive effect of BCAs and PGPRs on any crop, flowers or forest trees depends largely on rhizosphere competence of these organisms, which is a very important phenomenon in biological control and plant growth promotion (Harman, 2000).

1.5.1 Ecology of BCAs and PGPRs

The success of BCAs and PGPRs largely depends on whether the introduced antagonist/beneficial organism survives, thrives and proliferates in the environment at the infection court (Deacon, 1991). In order for BCAs and PGPRs to effectively fulfil their functions for which they are intended for, they must be introduced into the rhizosphere which is characterised by a rapid change in abiotic and biotic factors, with vigorous, intense and diverse microbial/fungal activities (Handelsman and Stabb, 1996). It is therefore imperative that the expectations of any BCA and PGPR to fulfil its task as plant growth promoter and disease control agent depends on how competitive and how well the organism in question is ecologically adapted to the hostile rhizosphere environment (Deacon, 1991).

The activities of BCAs and PGPRs introduced into soil for the purpose of biological control and plant growth promotion centres around the rhizosphere of the host plant. There is therefore the need to develop an understanding on the types of root exudates (organic compounds) that stimulate microbial growth and activities in the rhizosphere and also the physico-chemical and biological factors that influence the release of these compounds from the plant (Bolton *et al*., 1993). Some of the soluble organic compounds produced by plants get released into the rhizosphere and the nature of these compounds is dependent on the plant species, growth conditions and plant development (Bolton *et al*., 1993). Detailed lists of the various types of root exudates are summarised by Curl and Truelove (1986) and Bolton *et al*. (1993). Types of microbial communities in the rhizosphere of plant roots largely depend on the nature and quantity of root exudates present (Curl and Truelove, 1986). Whether specific BCAs and PGPRs prefer specific root exudates for proper functioning in the rhizosphere where it is introduced is a matter of concern, as different plants species are known to release different types, quantities and composition of root exudates (Curl and Truelove, 1986). Early

works by Rovira (1956; 1959) and Rovira and Harris (1961) demonstrated that different plant species release root exudates that vary in organic composition.

BCAs and PGPRs vary in specificity for plant host, pathogen control, ecological and environmental conditions (Kloepper, 1996). However, some BCAs and PGPRs may be non-specific and therefore may possess a wide range of plant and pathogen hosts and be able to survive and function under variable ecological and environmental conditions (Schroth and Becker, 1990).

One of the conditions, which BCAs and PGPRs must fulfil before being considered true BCAs or PGPRs is the ability to colonize a host plant root system when plant-microbe or microbe-plant association is established (Liu and Sinclair, 1993; Silva *et al*., 2003). For BCAs and PGPRs to be able to colonize the rhizosphere of host plant, they must compete with resident and indigenous microorganisms in an ecological habitat that is new to the introduced BCAs and PGPRs (Schroth and Becker, 1990). Kloepper (1996) maintained that level of colonization by beneficial microorganisms could be observed at various levels varying from crop to crop and from one cultivar to another. Silva *et al*. (2003) reported 28 out of 500 PGPR were able to reduce disease infection caused by *Pseudomonas syringae* pv. *tomato* on tomato. Moreover, they found that these 28 isolates were able to grow and colonize tomato roots *in vitro* in a medium completely devoid of plant nutrients. The conclusion was that these isolates were able to grow *in vitro* due to favourable ecological niches in the rhizoplane and a certain degree of specificity for the root exudates produced. From the concluding remarks, one can deduce that the 28 isolates were able to specifically use the root exudates to support their growth and hence colonized the tomato roots whereas the remaining isolates were unable to utilize the root exudates for growth and colonization, showing non-preference to the root exudates released from the tomato cultivar used.

Yobo (2001) demonstrated that six *Bacillus* spp. were able to significantly increase growth of lettuce and tomato seedlings with a microbial nutrient supplement. These same *Bacillus* isolates were unable to significantly increase growth of red sorghum using the same treatments and under the same growth conditions. Chanway *et al*. (1988) also showed that six out of seven *Bacillus* spp. isolated from the rhizosphere of wheat increased growth of the spring wheat cultivar, Katepwa, but none of the seven isolates increased growth of Neepawa or HY320. The above examples further clarify that some BCA and PGPR strains show

specificity for efficacy in different crops and cultivars as observed with root colonization by Silva *et al*. (2003).

The success or efficacy of BCAs and PGPRs on different ecological and environmental conditions has been debated in some articles and reviews (Kloepper, 1996; Mathre *et al*., 1999; Harman, 2000). Kloepper (1996) reports that *B. subtilis* Strain GBO3 was effective in different regions, under different environmental conditions, on different crops and pathogens in U.S.A. However, Mathre *et al*. (1999) elaborated on the ineffectiveness of a single biological control agent over a wide range of environmental conditions, on different crops and plant pathogens. On the contrary, Harman (2000) stressed that *T. harzianum* Strain T-22 has successfully been used on different crops on different soil types and at different geographical locations with *T. harzianum* Strain T-22 system still maintaining its efficacy. This supports the view of Kloepper (1996). He stated: "*Hence, although a commonly held view of biological control agents is that they are likely to be specific to various regions because of differential responses to the environment, there are exceptions*."

In my view, the concept of BCA/PGPR-plant host specificity raises questions on the choice of plant specific host range by some BCA/PGPR. One tends to doubt whether it is the effect of the different root exudates released by different plant species and possibly cultivars or some BCA/PGPR requiring specific carbon compounds from roots exudates for growth and root colonization. It also raises the question whether non-specificity of BCA/PGPR isolates means that they have no preference for specific root exudates.

1.6 Disease suppression in soil

Plant disease results from an intimate interaction between a pathogen and a susceptible plant host. The extent of disease damage on plants is influenced by the effect of environmental regimes on pathogen and host plant (Alabouvette, 1990). Agricultural soils suppressive to plant pathogens occur worldwide. The nature and biological basis of suppressive soils, and the characteristics such as physical and chemical properties of the soils, has been described elsewhere (Huber and Schneider, 1982; Lyda, 1982; Alabouvette, 1990). Suppressive soils are recognized when an interaction between a susceptible plant host and a pathogen occurs in soil. The suppressiveness is detected as a decrease in pathogen population, activity or minimal disease occurrence as compared to the situation in conducive soils (Baker and Cook, 1974; Hornby, 1983). Several definitions of suppressive soils have been proposed in

literature (Hornby, 1983), but the working definition to be considered is the definition proposed by Baker and Cook (1974).

Baker and Cook (1974) defined suppressive soils as soils which under favourable conditions, a pathogen does not establish; establishes but causes no disease; or establishes and produces disease for a while but then the disease pressure subsequently decreases with time. Hornby (1983) divided suppressive soils into two categories: Long-standing suppression which is a suppressive biological condition naturally associated with the soil from an unknown origin and survives in the absence of plants, while induced suppression sustains its suppressiveness through crop monoculture or by adding the target pathogen.

Weller *et al*. (2002) classified disease suppressiveness in soil into two major categories: General suppression where suppressiveness is based on total microbial biomass in soil and is not transferable between soils, while specific suppression owes its suppressiveness to the effects of individual or selected groups of microorganisms. General suppression is enhanced by the addition of organic matter or build up of soil fertility, which leads to an increase in microbial activity. General suppression is not accredited to any specific microorganisms; hence suppressiveness is due to the activities of diverse communities of microorganisms (Weller *et al*., 2002). While general suppression is non-transferable, transferability is the major characteristic of specific suppression (Scher and Baker, 1980). General and specific suppression lose their activity when soils are autoclaved and gamma radiated. Soil fumigation only reduces the activity of general suppression but does not completely eliminate it while its activity is maintained even at 70^{o}C moist heat (Cook and Rovira, 1976). Specific suppressive soils, however, loses its activity through pasteurisation (Cook and Rovira, 1976; Scher and Baker, 1980; Raaijmakers and Weller, 1998). Suppressive soils to some of the most important diseases caused by soil-borne plant pathogens from various parts of the world have been described elsewhere (Huber and Schneider, 1982; Cook and Baker, 1983; Alabouvette *et al*., 1985; Alabouvette, 1999; Weller *et al*., 2002). These include *R. solani*, (Henis *et al*., 1978; Henis *et al*., 1979; Lucas *et al*., 1993), *P. ultimum* Trow (Martin and Hancock, 1986), *Fusarium* (Alabouvette, 1990), *P. cinnamomi* Rands and *P. infestans* (Mont.) de Bary (Ko and Shiroma, 1989; Adrivon, 1994) and *Thielaviopsis basicola* (Berk. & Br.) Ferraris (Stutz *et al*., 1986).

Henis *et al*. (1978; 1979) reported a decline of *Rhizoctonia* blight of radish after three consecutive plantings in a clay loam soil. They maintained that disease decline was apparent

only when the pathogen, *R. solani*, was present and active. Further investigation into microfloral changes and diversity associated with the radish blight decline revealed an increase in *Trichoderma* spp. propagules in the soil. They concluded that the increase in the number of *Trichoderma* spp. propagules in the soil corresponded to an increase in disease decline. Liu and Baker (1980) later identified *T. harzianum* as the main cause of the radish damping-off decline.

Natural suppression of soils to fusarium wilt has also been studied and reported elsewhere (Hornby, 1983; Alabouvette, 1990; Weller *et al*., 2002). Suppressive soils to fusarium wilts limit the intensity of wilts in crops such as banana, cucumber, cotton and carnation (Alabouvette, 1990). Incidentally, suppressive soils to fusarium wilts are very specific and therefore are not effective against wilts or diseases caused by non-vascular *Fusarium* spp. or other soil-borne plant pathogens (Alabouvette, 1986; Deacon and Berry, 1993; Alabouvette, 1999). Activity in fusarium wilt suppressive soils is eliminated by soil treatment such as methyl bromide, moist heat or gamma radiation (Scher and Baker, 1980). Bacterial and fungal species found to be involved in fusarium wilt suppressive soils include *Trichoderma*, *Bacillus* and *Pseudomonas* spp. (Scher and Baker, 1982; Sivan and Chet, 1989). Suppressiveness in one soil can be transferred into a heat-treated conducive soil through mixing (Alabouvette, 1986).

The fact that the activity of suppressive soils is microbiological in nature does not necessarily mean that there is no relationship between the physical and chemical properties of the soil and an increase or decrease in disease pressure in natural suppressive soils (Alabouvette, 1990). Scher and Baker (1980) maintained that suppression of fusarium wilt is associated with sandy-loam soils and high pH. Lyda (1982) detailed the physical and chemical properties of suppressive soils based on the definition of suppressive soils by Baker and Cook (1974). There is therefore the need to consider the relationship between the soil type and the microbial balance to further elucidate the mechanisms and activities of suppressive soils. It is also important to consider the physical and chemical properties that favour the survival and activities of the dominant microbial and fungal populations in the soil (Alabouvette, 1990).

1.7 Competition in the rhizosphere

BCAs and PGPRs may encounter strong and diverse competition for resources, nutrients, and space or infection sites with resident microbial community when introduced into the rhizosphere of host plants (Deacon, 1991; Bolton *et al*., 1993; Whipps, 2001). Competition therefore is viewed as a likely and important mechanism of biological control (Harman, 2000).

Ahmad and Baker (1987) defined the term "rhizosphere competence" as the ability of a BCA or PGPR to increase its population, and function effectively for the intended purpose in the developing rhizosphere of host plants. Rhizosphere competence of *Trichoderma* and other fungal species used as BCA and PGPR has been reported elsewhere (Ahmad and Baker, 1987; Harman, 2000). Papavizas (1981) studied the populations of *T. harzianum* in the rhizosphere of pea and beans and showed that survival of *T. harzianum* in the rhizosphere of seed treated pea and bean was poor, and neither did the situation improved when a conidial suspension was inoculated directly into the rhizosphere of these two crops. However, survival rate improved when pea seeds were treated with metalaxyl before *T. harzianum* application. Similarly, Ahmad and Baker (1987) reported that rhizosphere colonization by a mutant strain of *T. harzianum* was influenced by benomyl application.

Like fungal species, the rhizosphere competence and population dynamics of *Bacillus* has been reported. Although the genus has been considered as less rhizosphere competent than *Pseudomonas* spp., Maplestone and Campbell (1989) and Kim *et al*. (1997b) demonstrated successful colonization of a wheat rhizosphere by three strains of *Bacillus* spp., namely *Bacillus* spp L324-92R_{12}, *B. pumilus*, and *B. mycoides*. Holl and Chanway (1992) also reported rhizosphere colonization of lodgepole pine seedlings by *B. polymyxa*. All these *Bacillus* strains have either been shown to control plant diseases (Mapplestone and Campbell, 1989) or to increase plant growth (Holl and Chanway, 1992).

In order to reduce competitive interactions in favour of the introduced beneficial BCAs/PGPRs to effectively function and perform their intended task, methods such as fumigation (Harman, 2000) and partial soil sterilization or pasteurisation (Katan, 1980; Jin *et al*., 1990) have been practiced. Such practice reduces the populations of resident microbial communities making the introduced and beneficial microbial/fungal species more competitive and increases their rhizosphere competence (Papavizas, 1981; Ahmad and Baker, 1987).

Harman (2000) observed a yield increase in onions treated with *T. harzianum* T-22, maneb and metalaxyl treatments compared to the *T. harzianum* T-22 treatment and the fungicides in isolation. This result further demonstrated that the maneb and metalaxyl treatment reduced microbial population and competition in the soil prior to *T. harzianum* T-22 application.

1.7.1 Delivery methods of BCA and PGPR

Cook *et al*. (1996) outlined three major modes of BCA/PGPR application delivery. These are:

(i) Inundative application – Application of a microbial or fungal BCA/PGPR with the intention of raising the population to a very high level to ensure maximum plant growth and development as well as rapid disease control. However, this does not imply a permanent population establishment;

(ii) Augmentative application – The BCA/PGPR is applied in large population numbers, expected to multiply and mainly supplement the populations of already introduced strain, related or similar microbial/fungal agent for increased performance;

(iii) Inoculative application – Introduction of a microbial or fungal BCA or PGPR once into an environment with the assumption that it will multiply, establish itself and maintain a permanent population level that would be able to execute its intended task.

Cook *et al*. (1996) discussed examples of these applications/strategies and the safety issues related to their implementation. Two main methods of inoculations of BCAs/PGPRs, which are mostly being used worldwide, are seed treatment (inoculation) and/or soil inoculation (Bashan, 1998). However, seed inoculation is more widely used and preferred since it is relatively easy and requires less inoculum (Bashan, 1998). The merits and demerits of seed and soil inoculations have been described elsewhere (Bashan, 1998).

1.8 Integrated biological control

There has been considerable interest in combining biological control agents with reduced rates of fungicides/pesticides to enhance disease control of plant diseases (Lewis and Papavizas, 1991). This strategy is aimed at reducing the amount of chemical/pesticide residues in food, soil, river and groundwater (Becker and Schwinn, 1993; Lumsden *et al*., 1995).

Integrated control could be defined as a sustainable approach with the aim of curbing the activities of soil-borne plant pathogens and/or control plant diseases by combining biological, cultural and chemical control systems to minimize health and environmental hazards (Andrews, 1983; Lewis and Papavizas, 1991). Integrated biological control with compost-amended substrates is discussed elsewhere (Hoitink and Boehm, 1999). Only integrated biological control with reduced rates of fungicides/pesticides is discussed here.

Becker and Schwinn (1993) compared the strengths and weaknesses of chemical control and biological control agents based on nine major criteria as illustrated in the table below.

Table 1.5. Comparison of the strengths and weaknesses of biological and chemical control systems based on nine criteria as proposed by Becker and Schwinn (1993)

Criteria	Chemical Control	Biological Control
EFFICACY		
- Short term	+ + +	+
- Long term	+	+ + +
- Rapidity of effect	+ + +	+
- Effect in high disease pressure	+ + +	+
- Curative control	+ + +	+
RELIABILITY		
- Environmental influence	+ + +	+
- Influence of plant and soil	+ + +	+
- Shelf life	+ + +	+
EASE OF USE		
- Risk of resistance	+	+ + +
REDUCTION OF HARMFUL SIDE EFFECTS		
- On humans and animals	+	+ + +
- Residues in food	+	+ + +
- Residues in groundwater	+	+ + +
- Persistence in the environment	+	+ + +
PUBLIC ACCEPTANCE	+	+ + +
PROFITABILITY TO PRODUCER	+ + +	+
COST EFFECTIVENESS	+	+ + +
LONG-TERM BENEFIT FOR SOCIETY	+	+ + +

'+ + +' (Most), and '+' (Least) relative advantage of the control method. Table adapted and modified from Becker and Schwinn (1993)

From the table above, one can deduce that chemical control possess some features which are lacking in biological control systems. The question that comes into one's mind is whether a reduced rate of fungicide/pesticide, used in conjunction with biological control systems, will work effectively against soil-borne plant pathogens and/or plant diseases while compensating for the demerits from each control method.

Henis *et al*. (1978) showed that the combined use of *T*. *harzianum* and pentachloronitro-benzene (PCNB) substantially controlled *Rhizoctonia* damping-off on radish. Other notable

examples include control of chickpea wilt (*Fusarium oxysporum* f.sp. *ceceri*) (Kaur and Mukhopadhyay, 1992), cucumber grey mould (*B. cinerea*) (Elad *et al*., 1993), damping-off and root rot of snap bean (*R. solani*) (Keinath *et al*., 2000), cucurbit powdery mildew (*Podosphaera xanthii*) (Shishkoff and McGrath, 2002) and take-all and root rot (*Gaeumannomyces graminis* var. *tritici* and *R. solani*) (Cook *et al*., 2002).

Methods/strategies most commonly used for integrated biological control include seed treatment (Keinath *et al*., 2000; Cook *et al*., 2002), sprays (Elad *et al*., 1993) and soil application/fumigation (Kaur and Mukhopadhyay, 1992; Cook *et al*., 2002). Successes have been achieved and substantial disease control reported irrespective of the integrated method/strategies employed. Elad *et al*. (1993) reported a 96 percent control of cucumber grey mould with a mixture of *T. harzianum* with dicarboximide when used as sprays. Likewise, Cook *et al*. (2002) reported yield increase with soil fumigation and seed treatments with rhizobacteria and various fungicides. However, the success of this method depends the compatibility between the BCA and the fungicide to be applied.

1.9 Induced systemic resistance (ISR)

Induced resistance as defined by Hammerschmidt and Kuć (1995) is the "enhancement of the plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation". Induced systemic resistance (ISR) has been linked to the activation and elevation of a plants defence mechanism by non-pathogenic microorganisms leading to a systemic protection (Kloepper *et al*., 1992; Van Wees *et al*., 1997; Pieterse *et al*., 1998; Van Loon *et al*., 1998). ISR has been demonstrated against bacteria, fungi and viruses in a number of crops and induction is thought to differ in different bacterial strains in different plant species (Leema *et al*., 1995; Van Loon *et al*., 1998).

1.9.1 Methods and examples of ISR mediated by BCA and PGPR

Common methods that have been used to demonstrate ISR include split-root system (Liu *et al*., 1995), seed and soil treatment (De Meyer *et al*., 1998) and cotyledon injection (Liu *et al*., 1995). There are several published reports that demonstrate ISR by BCAs/PGPRs.

De Meyer *et al*. (1998) demonstrated that the biocontrol agent *T. harzianum* T39 reduced grey mould symptoms caused by *B. cinerea* on five crops using the spatial separation system. The authors concluded that the reduction of grey mould symptoms was as a result of ISR by *T.*

harzianum given the spatial separation of the antagonist and the pathogen. They further stated that this mechanism adds to other mechanisms exhibited by *T. harzianum* T39 in controlling *B. cinerea*.

Several authors have demonstrated ISR in cucumber (Liu *et al*., 1995; Yedidia *et al*., 1999). In a split-root system, Liu *et al*. (1995) showed that two PGPR strains, *P. putida* strain 89B-27 and *S. marcescens* strain 90-166 induced ISR against fusarium wilt. The antagonists and the pathogen, *Fusarium oxysporum* Schlectend.:Fr. f.sp. *cucumerinum* J.H. Owen were inoculated on separate halves of two-week old cucumber seedlings. Delayed symptom developments, reduction in disease severity compared to uninoculated controls were used as an index of ISR expression in test plants. Other examples of BCA/PGPR mediated ISR include control of bacterial angular leaf spot in cucumber (Liu *et al*., 1995), cucumber mosaic in cucumber and tomato (Raupach *et al*., 1996) and late blight in tomato (Yan *et al*., 2002).

Reports on ISR have mainly been investigated either in the greenhouse or *in vitro* in the laboratory (Ramamoorthy *et al*., 2001). However, Wei *et al*. (1996) successfully demonstrated ISR under field conditions. Field trials conducted over a two-year period employing seed treatment or seed treatment plus soil drench showed a significant reduction in disease severity of angular leaf spot. Incidence of Anthracnose was significantly reduced coupled with an increase in yield compared to untreated controls (Wei *et al*., 1996).

1.9.2 Mechanisms of ISR

Mechanisms of ISR-mediated by BCA/PGPR include modifications of structural and ultra-structural cell wall (Benhamou *et al*., 1996) and biochemical and physiological changes in host plants (Benhamou *et al*., 1996). These mechanisms and BCA/PGPR determinants of ISR has been fully reviewed by Van Loon *et al*. (1998) and Ramamoorthy *et al*. (2001).

1.10 Opportunities of BCAs/PGPRs

The benefits of using BCAs/PGPRs or their products to control plant diseases and improve crop yield play an important role in sustainable agriculture. There is therefore the need to identify these organisms and understand the mechanisms under which they operate if they are to be exploited. Various opportunities could be drawn from using BCAs/PGPRs in crop productivity. These include:

(i) Minimising the use of chemical pesticides – Essential as many chemicals are being banned from usage on crops due to a recognition of environmental hazards;

(ii) Inability of soil-borne plant pathogens to develop resistance to BCAs/PGPRs – This is crucial due to increasing resistance to fungicides;

(iii) Specific to pathogen control without having any negative effect on other beneficial microorganisms;

(iv) Environmental friendly and pose no danger to humans and animals as opposed to chemical control strategies;

(v) Mineralization of complex organic molecules in soil – This improves soil conditions for plant growth, hence increase in crop yield (Anonymous, 2003).

Becker and Schwinn (1993) discussed in detail a number of opportunities and problems regarding the use of BCAs/PGPRs products.

In South Africa, biological control is fast gaining grounds in the research community. The object of the intensified biological control research is to find alternatives to banned chemical control methods and reduce the risk of environmental hazards. Farmers' conceptions on biological control options are rapidly changing, particularly larger export based farmers (Dr Mike Morris, Personal Communication). A large section of the small-scale farmers largely depend on chemical inputs for disease control. There is the need for a large number of field trials to show the effectiveness and efficacy of biological control systems. Until this is done, these farmers will continuously depend on chemical methods for disease control (Dr Mike Morris, 2003 Personal Communication).

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CHAPTER TWO

Isolation and screening of *Trichoderma* species for biological control of *Rhizoctonia solani*

"*...Screening is target-orientated; we get what we select*…" --Deacon, 1991.

K.S. Yobo[1], M.D. Laing[1] and C.H. Hunter[2]

[1]*Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

[2]*Discipline of Microbiology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

Abstract

Twenty *Trichoderma* strains isolated from composted soil were screened *in vitro* and *in vivo* for antagonistic activity against *Rhizoctonia solani*. Dual culture bioassays were undertaken and assessed for evidence of hyperparasitism, antibiosis and antagonism, rated according to the Bell test scale. Two isolates, SYN and SY3A, which did not show antimicrobial activity *in vitro*, but which were able to rapidly invade and overgrow *R. solani* on V8 agar proved to be the most effective *in vivo*. Compared to the diseased control (23.6% survival) application of isolates SYN and SY3A resulted in the greatest percentage seedling survival, 60.0 and 62.5% respectively. Four isolates, SM1, SYN2, SY2A and SY2E elicited an inhibitory response *in vitro*, indicating the production of antimicrobial substances. However, *in vivo* screening using cucumber plants grown under greenhouse conditions showed that none of the four isolates were able to effectively control damping-off caused by *R. solani*. Poor correlation was found between isolates that performed well in the Bell rating system test and *in vivo* greenhouse study. Cluster analysis, a multivariate analysis technique, was used to group and classify the isolates according to their performance *in vivo*. The implications of these results are discussed here.

Key words: biological control; Bell test; *R. solani*; *Trichoderma*

2.1 Introduction

Rhizoctonia solani Kühn is a soilborne plant pathogen which infects a wide range of crops (Mildenhall and Williams, 1973; Hwang *et al*., 1986; Kloepper, 1991). Post- and pre-emergence damping off (Kloepper, 1991), brown-girdling and seedling blight (Benhamou and Chet, 1993) of crop seedlings are some of the diseases caused by *R. solani*.

Members of the genus *Trichoderma* are saprophytic fungi that are widely distributed in agricultural soils, and which have been cited as potential biological control agents (BCAs) (Papavizas, 1985). Numerous studies have shown that disease caused by *R. solani* can be controlled effectively by the application of selected strains of *Trichoderma* spp. (Elad *et al*., 1980; Elad *et al*., 1981; Maplestone *et al*., 1991; Koch, 1999). As a result of this, extensive research programmes have been established to determine the efficacy of using *Trichoderma* spp. as BCAs (Mishra *et al*., 2000; Lewis and Lumsden, 2001).

A common approach for selecting and screening potential BCAs involves isolating the antagonist from the rhizosphere of target plants followed by *in vitro* and *in vivo* screening of the isolates for biological control activity (Chanway *et al*., 1988; Aziz *et al*., 1997). Dual culture bioassays or Bell tests are typically used *in vitro* to screen for antagonism, antimicrobial activity and/or hyperparasitism (Bell *et al*., 1982; Benhamou and Chet, 1993). *In vitro* testing is usually simple to perform, allowing large numbers of isolates to undergo preliminary screening. Unfortunately, one of the main disadvantages of this approach is that the efficacy of isolates selected *in vitro* does not always correlate with the results observed *in vivo* (Andrews, 1985; Fravel, 1988; Williams and Asher, 1996).

The aims of the research presented in this chapter were to isolate a selection of *Trichoderma* cultures and then subject them to *in vitro* and *in vivo* screening to assess for biological control activity against *R. solani*. Dual culture bioassays were used to screen isolates for *in vitro* antagonism, antimicrobial activity and/or hyperparasitism, whilst inoculated cucumber (*Cucumis sativus* L.) plants grown under greenhouse conditions were used to assess the biological control potential of each isolate *in vivo*. The findings from the *in vivo* and *in vitro* studies were then correlated using SAS-cluster analysis (SAS, 1987).

2.2 Materials and Methods

2.2.1 Source of Trichoderma *strains*

Random samples of a composted soil were collected from a display garden in Scottsville, Pietermaritzburg, KwaZulu-Natal, Republic of South Africa and stored at 4^0C until further processing could be achieved.

Trichoderma spp. strains were isolated from the compost soil and cultured on a *Trichoderma* selective medium (TSM) (Appendix 1) (Askew and Laing, 1993). One gram (1g) of soil was added to 100ml distilled water and serial dilutions were made (10^3). One ml (1ml) aliquots from each dilution were plated in triplicate, on TSM agar plates, using the spread plate method, and then incubated at 26 ± 1^0C for 7 days in the dark. Distinct colonies characteristic of the genus *Trichoderma* were selected and transferred separately onto freshly prepared V8 agar medium (Appendix 1). Each isolate was assigned an isolate code and agar plates were incubated at 26 ± 1^0C for 5 days. A total of 20 isolates were selected for evaluation as BCAs against *R. solani*. Sub-cultures of each isolate were grown on half-strength Potato Dextrose Agar (PDA) (Merck) medium at 26 ± 1^0C for 5 days and then stored in the dark at an ambient temperature ± 20^0C. In addition, colonised agar blocks and spores were stored in double sterilized-distilled water and kept at ± 20^0C.

2.2.2 Isolation of Rhizoctonia *spp.*

A *Rhizoctonia* sp. culture was isolated from cabbage (*Brassica oleracea* L. var. *capitata*) seedlings, obtained from Sunshine Seedlings Nursery, Pietermaritzburg, South Africa, which exhibited damping-off symptoms. Sections (1cm long) of diseased cabbage seedling roots were washed four times with sterile distilled water and then placed on sterile filter paper in a Petri dish on a laminar flow bench. The sections were then placed on 2% (w/v) water agar plates, incubated at 26 ± 1^0C and examined daily for evidence of mycelial growth. Fungal cultures were transferred onto a freshly prepared water agar medium and incubated as indicated previously. Wet mount slides of each culture were prepared and viewed using bright field microscopy. Samples were confirmed to be *Rhizoctonia* sp. based on characteristic morphological structures as indicated in Barnes (1979). *Rhizoctonia* sp. isolates were stored on water agar blocks immersed in double-sterilized distilled water as well as on half-strength PDA. Stored cultures were revived by transferring fungal matter onto V8 agar medium and incubating at 26 ± 1^0C for 3-7 days. The pathogenicity of the *Rhizoctonia* sp.

was confirmed *in vitro* and *in vivo* using cucumber (*Cucumis sativus* L.) cv. Ashley seedlings. The *Rhizoctonia* isolate used in this study was sent to Dr Isabel Rong (Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, Republic of South Africa) for identification and was subsequently identified to be *R. solani* (PPRI Accession number 03212).

2.2.3 In vitro *antagonism of* Trichoderma *isolates against* R. solani

In vitro dual-culture bioassays were performed by placing colonized agar plugs (4mm diameter) of a selected *Trichoderma* isolate and the test pathogen on opposite sides of a Petri dish (90mm diameter) containing V8 agar medium. The bioassay was replicated three times for each *Trichoderma* isolate and plates were incubated in the dark for 5 days at 26 ± 1^0C. Controls for each *Trichoderma* isolate as well as *R. solani* were also established on V8 agar medium and incubated with the test plates. The experiment was repeated twice.

After five and 7 days of incubation, each dual-culture bioassay plate was assessed for antibiosis, antagonism and/or invasion ability. Antimicrobial activity was interpreted as zones of inhibition on the dual-culture test plates. A distinct zone of inhibition between a *Trichoderma* isolate and *R. solani* was attributed to the production of antimicrobial compounds. The degree of antagonism of each isolate towards *R. solani* was rated according to the rating system of Bell *et al*. (1982), based on a scale of 1-5:

Class 1 = *Trichoderma* completely overgrew *R. solani* and covered the entire medium surface;

Class 2 = *Trichoderma* overgrew at least two thirds of the medium surface;

Class 3 = *Trichoderma* and *R. solani* each colonized approximately one-half of the medium surface and neither organism appear to dominate the other;

Class 4 = *R. solani* colonized at least two-thirds of the medium surface and appear to withstand encroachment by *Trichoderma*;

Class 5 = *R. solani* completely overgrew the *Trichoderma* and occupied the entire medium surface.

According to Bell *et al*. (1982), a *Trichoderma* isolate is considered to be antagonistic towards a fungal pathogen if the mean score is ≤2, but not highly antagonistic if the number is ≥3. Plates were subsequently incubated for a further 2 days and then rated again for invasion ability using the following rating system developed by the authors.

Class 1 = *Trichoderma* completely overgrew *R. solani* and invaded the entire plate and sporulation was apparent on all sections of the plate after 7 days and the *R. solani* mycelium turned brown;
Class 2 = *Trichoderma* completely overgrew *R. solani* and invaded the entire plate and sporulation was evident on all sections of the plate after 7 days. No discolouration of the *R. solani* mycelium occurred;
Class 3 = *Trichoderma* colonized 50% of the plate from the point of contact with *R. solani* and patches of sporulation was evident on sections of plate where *Trichoderma* invaded *R. solani*; and
Class 4 = *Trichoderma* colonized less than 50% of *R. solani* from the point of contact with *R. solani* and little or no sporulation was evident on invaded sections.
Isolates judged as either Classes 1 or 2 were considered to be highly invasive against *R. solani*.

2.2.4 In vivo *screening of* Trichoderma *isolates against* R. solani *damping-off*

(a) Growth and preparation of Trichoderma *inoculum*

Each *Trichoderma* isolate was plated onto six V8 agar plates and incubated at 26 ± 1^0C for seven days until extensive sporulation had occurred. Spores from each isolate were harvested by washing each Petri dish with 150ml of sterile distilled water separately into a sterile 250ml beaker. Mycelial fragments were then filtered off using sterilized cheesecloth. Spore count estimates were made using a counting chamber (Tiefe 0.200mm Fuchs-Rosenthal, Germany) and each spore suspension was adjusted to approximately 10^8 spores.ml^{-1} and were refrigerated at 4^0C until used.

(b) Growth and preparation of R. solani

Rhizoctonia solani was sub-cultured onto V8 agar medium and the agar plates were incubated at 26±1^0C until fully colonized.

(c) Crop

Cucumber seeds cultivar Ashley, were obtained from Starke Ayres Seed Company (Pty) Ltd., Pietermaritzburg, Republic of South Africa.

(d) Seed treatment and greenhouse trials

Cucumber seeds were washed eight times with sterile distilled water to reduce the amount of fungicide residues on the treated seeds and were air-dried under a laminar flow bench. A 2% (w/v) carboxymethyl cellulose (CMC) spore-sticker suspensions was made up for each *Trichoderma* isolate by adding 50ml of spore suspension (± 10^8 spores ml^{-1}) to one gram of CMC in a 200ml sterile beaker. The suspension was placed on a rotary shaker (Model GFL 3005, Labortechnik, Germany) and shaken (150rpm) at ambient room temperature to achieve a homogenous mixture. Approximately 80-90 seeds were added to each batch of spore-sticker suspension and allowed to soak for 1h with intermittent wrist swirling. The treated seeds were removed from the suspension, placed in a sterile Petri dish and air dried on a laminar flow bench overnight (12-18h).

Speedling® 24 trays were half filled with composted pine bark (Potting Mix, Gromed, Crammond, Republic of South Africa). Pathogen inoculation was achieved by placing V8 agar plugs (4mm square) of *R. solani* in the centre of each cell directly on top of the growth medium. The cells were then filled and the treated seeds planted. Controls using seeds coated solely with CMC sticker were also established. Disease free control trays received agar plugs with no *R. solani*, while diseased control trays received plugs with *R. solani* but no *Trichoderma* treatments. There were three replicate trays for each treatment. The trays were watered and left in the germination room at 20-24^{0}C for 2 days. The trays were then moved to a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. Trays were irrigated three times a day by microjet overhead irrigation (Inverted mini wobbler, Sennenger, U.S.A). The water used was maintained at 20^{0}C using a temperature controlled heating system (Pro Heat 2000 Plus, Republic of South Africa) and contained NPK soluble fertilizer [3:1:3 (38)] Complete at a rate of 1g.l^{-1}. Seedling survival was rated after 4 weeks. The plant material was then harvested at their base at soil level and subsequently dried at 70^{0}C for 48h to determine the total dry weight of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated twice.

2.2.5 Statistical analysis

A general linear model (GLM) was used to run an ANOVA on the number of healthy seedlings and dry biomass of healthy seedlings. If the ANOVA was significant, ($P<0.05$), then the means were separated using the Students Neuman Keul's test using SAS (Version 6.12) (SAS, 1987). Furthermore, multivariate cluster analysis was used to group the

performance of all 20 *Trichoderma* isolates based on seedling survival and total seedling dry biomass.

2.3 Results

2.3.1 In vitro *antagonism of* Trichoderma *against* R. solani

The results for the *in vitro* dual culture test are shown in Table 2.1. Four isolates, SM1, SYN2, SY2A and SY2E, produced zones of inhibition against *R. solani*. However, according to the Bell rating scale, these four isolates achieved only a Class 3 rating. Based on the Bell rating scale, none of the isolates screened scored better than a Class 3 rating after 5 days. Based on the Invasion ability rating scale developed in this Chapter, two isolates, SYN and SY3A achieved a Class 1 rating, whilst a further four isolates, (SYN4, SYN6, SY2F and SY3F) achieved a Class 2 rating (Table 2.1). Neither SYN nor SY3A showed antimicrobial activity against *R. solani* after 5 and 7 days. They did, however, give rise to a brownish discolouration when they came into contact with *R. solani*. The discolouration spread over the *R. solani* culture as the two *Trichoderma* isolates subsequently invaded the entire plate. Complete invasion of dual culture plates by Class 1 and 2 isolates occurred within 7 days of inoculation. Sporulation on all sections of the plate was also apparent for these two Classes.

2.3.2 In vivo *screening of* Trichoderma *isolates for activity against* R. solani

In the greenhouse trials pre-emergence damping-off, and to a lesser extent post-emergence damping-off, were the major disease symptoms observed.

Percentage seedling survival for the controls ranged from 23.6% for the diseased control to 83.3% for the disease free control (Table 2.1). The mean dry biomass yield for the diseased control was 24.9% of the yield obtained for the disease free control ($P < 0.0001$).

Isolates SY3A and SYN were the only isolates that produced results statistically significantly better ($P < 0.05$) than the diseased control (Table 2.1) but were still significantly different to the disease free control ($P < 0.05$). The mean dry yields for SY3A and SYN were 70.1 and 70.4% respectively, of the yield obtained for the disease free control. Next in performance *in vivo* were Isolates SYN4, SYN6 and SY2F with percentage survival of 40.2, 44.4 and 45.8% respectively. These were not significantly different from the diseased control ($P > 0.05$).

The four isolates (SM1, SYN2, SY2A and SY2E) that showed *in vitro* antibiosis against *R. solani* were not effective in controlling damping-off *in vivo*. Seedling survival (%) for these isolates (ranging from 16.6 to 34.7%) were not statistically different to the diseased control.

Table 2.1 *In vitro* and *in vivo* screening of *Trichoderma* isolates against *R. solani* using dual culture bioassays and greenhouse based biological control trials with cucumber (*Cucumis sativus* L.) cv Ashley respectively

Isolate/Treatment	Mean number of surviving plants [a] after 4 weeks	% Seedling survival after 4 weeks	Mean dry biomass [a] after 4 weeks	*In vitro* bioassay		
				Bell rating	Invasion ability	Antibiosis
Control (Disease free)	20.00 a	83.33	17.67 a	na	na	na
Control (Disease check)	5.67 def	23.61	4.40 cd	na	na	na
S2	8.00 cdef	33.33	5.88 cd	4	3	+
SM1	7.33 def	30.74	4.22 cd	3	3	+
SYN2	8.33 cdef	34.72	4.98 cd	3	3	–
SYN3	4.33 ef	18.06	2.43 cd	4	4	–
SYN4	9.67 cde	40.28	5.72 cd	3	2	–
SYN5	8.00 cdef	33.33	4.47 cd	4	3	–
SYN6	10.67 bcd	44.44	6.42 cd	3	2	–
T1	7.33 def	30.55	5.41 cd	4	3	–
T2S1	6.67 def	27.78	4.51 cd	4	4	–
SYN	14.41 b	60.06	12.40 b	3	1	–
SY1	7.33 def	30.56	4.26 cd	4	3	–
SY2A	4.00 ef	16.67	3.00 cd	3	4	+
SY2E	6.00 def	25.00	4.03 cd	3	4	+
SY2F	11.00 bcd	45.83	7.02 cd	3	2	–
SY3A	15.00 b	62.50	12.44 b	3	1	–
SY3C	3.00 f	12.50	1.72 d	4	4	–
SY3F	9.00 cde	38.89	4.19 cd	4	2	–
SY5	8.00 cdef	33.33	5.44 cd	4	3	–
SY6	7.67 def	31.95	5.40 cd	4	3	–
SY7	8.33 cdef	34.72	5.55 cd	4	3	–
F-ratio	11.13		14.14			
P-level	0.0001		0.0001			
% CV	23.18		28.26			
Significance	* * *		* * *			

[a] Values followed by different letters within a column are significantly different (Students Newmans Keul's test, P = 0.05); na, not applicable; – +, negative or positive for antibiosis; ***, Significantly different at $P \leq 0.001$.

Three isolates, SYN3, SY2A and SY3C had lower percentage seedling survival compared to the disease control (Table 2.1).

Cluster analysis was used to group the isolates according to their performance *in vivo* on cucumber seedlings against *R. solani* damping-off. Using Ward's clustering method (SAS, 1987), a four-cluster solution was developed based on the numbers of surviving seedlings and seedling dry biomass (Figure 2.1).

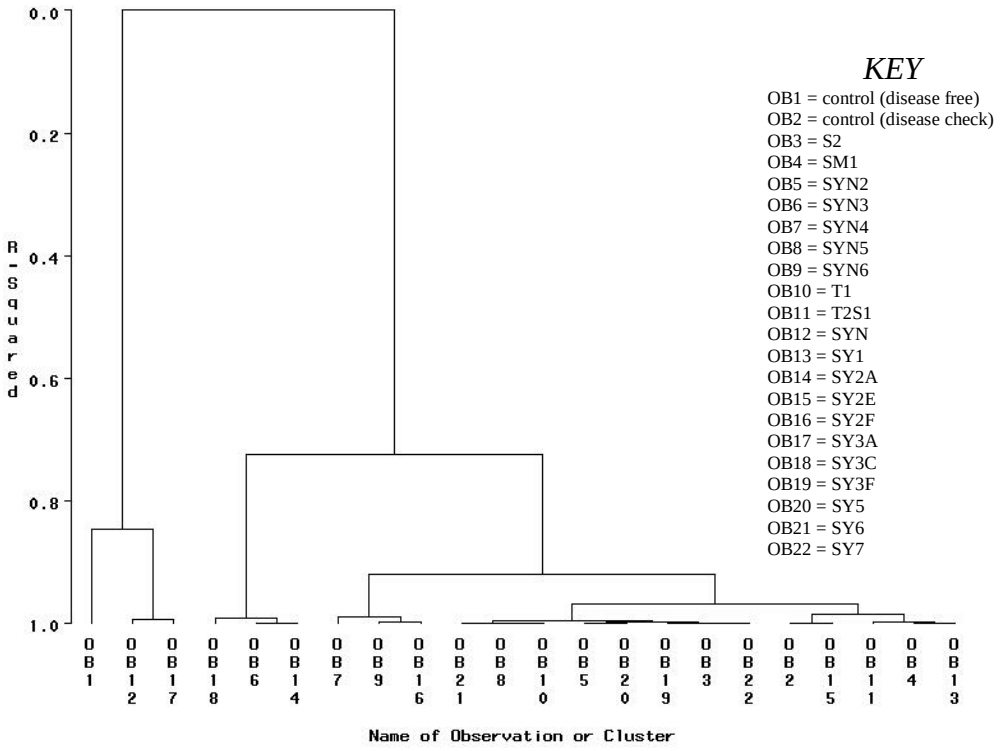

Fig 2.1 Dendogram of isolate groupings according to percentage seedling survival and dry biomass four weeks after planting in the greenhouse

The clusters used were to ensure that isolates/treatments were exclusively classified in one of the defined clusters. Table 2.2 presents the number of cluster groupings, mean values of the variables with the F-ratios and their corresponding significance levels.

Cluster 1 contains only one member, which is the disease free control. It recorded the highest percentage of surviving seedlings and dry biomass. Cluster 2, contains the diseased control with 20% of the isolates, and is characterized by the lowest percentage mean values of surviving seedlings. This Cluster group comprises of isolates/treatments that performed poorly.

Cluster 3 is made up of two members only and accounts for 10% of the isolates. These two were the best performing isolates as characterized by mean percentage values of seedling survival and dry biomass. Finally, Cluster 4, contains the largest number of isolates, accounting for 70% of the isolates considered to have achieved a moderate performance.

Table 2.2 Cluster groupings and members of each group of isolates with the corresponding mean percentage seedling survival and mean dry biomass (g)

Cluster number	Cluster member (s)	Mean seedling survival (%)	Mean dry biomass (g)
1	Control (Disease free check)	83.33	17.67
2	Control (Disease check)	23.61	4.39
2	SYN3	18.06	2.43
2	SY2A	16.67	2.99
2	SY2E	25.00	4.03
2	SY3C	12.50	1.72
3	SYN	60.06	12.40
3	SY3A	62.50	12.44
4	S2	33.33	5.88
4	SM1	30.56	4.22
4	SYN2	34.72	4.98
4	SYN4	40.28	5.71
4	SYN5	33.33	4.47
4	SYN6	44.44	6.42
4	T1	30.56	5.41
4	T2S1	27.78	4.51
4	SY1	30.56	4.26
4	SY2F	45.83	7.02
4	SY3F	38.89	4.19
4	SY5	33.33	5.44
4	SY6	31.94	5.40
4	SY7	34.72	5.55
F-ratio		55.80	110.23
P-level		0.0001	0.0001
%CV		14.85	15.06
Significance		* * *	* * *

***, Significantly different at $P \leq 0.001$

2.4 Discussion

Of the 20 *Trichoderma* originally isolated from composted soil only two, SYN and SY3A, performed significantly better *in vivo* than the diseased control. Neither isolates performed well when ranked against either antimicrobial activity or antagonism as determined by the Bell test (Class 3). Both isolates showed evidence *in vitro* of rapid invasion of *R. solani* after 7 days. This overgrowth was associated with the onset of a brown pigmentation or discolouration of the *R. solani* mycelium from the first point of contact with the *Trichoderma* mycelium. The pigmentation has been attributed to production of toxic metabolites and/or enzymes and is thought to give a good indication of mycoparasitism (Lorito *et al*., 1993; Calistru *et al*., 1997; Menendez and Godeas, 1998). This was later confirmed in an Environmental Scanning Electron Microscopy (ESEM) study (Chapter 3). None of the other isolates screened *in vitro* gave rise to pigmentation effects. Based on the Invasion Ability Scale we developed, there appeared to be good correlation between the *in vitro* and *in vivo* performances. Isolates placed in Classes 1 or 2 showed the best biological control activity *in vivo*.

Findings from the Bell rating system did not correlate well with the *in vivo* plant screening. This was contrary to the findings and recommendations of Askew and Laing (1994) who reported that out of 118 *Trichoderma* isolates, 92% identified as antagonistic to *R. solani in vitro* significantly reduced damping-off in the nursery. Similarly, Williams and Asher (1996) found no correlation between biological control *in vivo* and antifungal activity *in vitro* when bacterial isolates that showed strong antifungal activity against *Pythium ultimum* Trow *in vitro*, were tested *in vivo*. They further concluded that the bacterial isolates that showed strong antifungal action *in vitro* were not necessarily good biological control agents *in vivo*. The Bell rating system makes the assumption that *Trichoderma* isolates rated ≥ 3 are not highly antagonistic (Bell *et al*., 1982). However two isolates, SYN and SY3A which did not show any signs of inhibition *in vitro* were scored Class 3 on a Bell rating and Class 1 on the Invasion Ability Scale significantly reduced damping-off compared to the diseased control. This was confirmed by the four-cluster solution performed in this study to classify isolates/treatments based on their performance *in vivo* on test plants.

We are not aware of another scale which attempts to specifically rate hyperparasitism against *R. solani in vitro* for biocontrol agents, including *Trichoderma*. An Invasion Ability Scale was developed to assess the *Trichoderma* isolates *in vitro*. Browning discolouration, which

was observed during dual culture studies, was the major characteristic of the Class 1 isolates. Even though the brownish pigmentation or discolouration of the *R. solani* mycelium was used as the major characteristic for the Class 1 isolates on the Invasion Ability Scale, it is not possible to immediately conclude that this reaction or property of the Class 1 isolates was part of a biological control mechanism. The authors are however aware of the yellowish discolouration developed by *Aspergillus flavus* Link. when tested against culture filtrates of *T. harzianum*, instead of a characteristic green colony (Calistru *et* al., 1997) but are not aware of any report on brownish reaction or discolouration of *R. solani* mycelium by *Trichoderma* species *in vitro*. This property, coupled with good *in vivo* activity by the Class 1 isolates, suggests that *R. solani* could be a good indicator organism of hyperparasitism for some *Trichoderma* isolates *in vitro*. This was later confirmed in Chapter Three where *R. solani* mycelial wall destruction was caused by the Class 1 isolates compared to the Class 2 isolates, which did not exhibit the brownish discolouration *in vitro*.

The rapid growth and invasion by the Class 1 and 2 isolates observed during *in vitro* bioassays may indicate that competition for nutrients (Elad, 2000) and space may be possible mechanisms of biological control. These results indicate that the selection criteria for choosing isolates can be very subjective and do not always correlate well with *in vivo* studies.

The inhibition zones associated with Isolates SM1, SYN2, SY2A and SY2E were observed before physical contact with *R. solani* was made suggesting that antimicrobial substances were produced (Askew and Laing, 1994; Calistru *et al*., 1997). Various studies have implicated antibiotic substances, volatile and non-volatile compounds as well as other metabolites in biological control of plant pathogens and other fungal species (Fravel, 1988; Calistru *et al*., 1997; Wheatley *et al*., 1997). Wheatley *et al*. (1997) reported that two *Trichoderma* isolates produced volatile compounds, which inhibited the growth of wood decay fungi. Likewise, Calistru *et* al. (1997) reported the possible role of volatile compounds produced by *Trichoderma* species on colony growth and morphology of *A. flavus* and *Fusarium moniliforme* Sheldon. However, poor correlations have been found between *in vitro* and *in vivo* studies (Williams and Asher, 1996). Very poor biological control however, was achieved with the four antibiosis inducing isolates *in vivo* in the greenhouse. *Trichoderma* spp. exhibits an array of biological control mechanisms. It is recommended that newly selected isolates need to pass through multiple screening techniques as the failure to fulfil the requirements of one mechanism does not indicate failure for the rest. For instance, a

T. harzianum isolate which effectively controlled damping-off on eggplant (*Solanum melongena* L.), bean (*Phaseolus vulgaris* L.) and tomato (*Lycopersicon esculentum* L.) but which failed to produce antibiotics *in vitro*, was found to hyperparasitize the cell walls of *R. solani* (Hadar *et al*., 1979). These results demonstrate and stress the need for more than a single *in vitro* screening strategy for selecting potential biological control agents.

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CHAPTER THREE

Modes of action of selected *Trichoderma* and *Bacillus* isolates exhibiting biological control activity against *Rhizoctonia solani*

"*…The mode of action of an inoculant biological control agent helps to define the circumstances in which it can be used…* --- Deacon and Lorraine, 1993.

K.S. Yobo[1], M.D. Laing[1] and C.H. Hunter[2]

[1]*Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

[2]*Discipline of Microbiology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

Abstract

Possible modes of action of selected *Trichoderma* and *Bacillus* isolates showing biological control of *Rhizoctonia solani in vivo* were investigated. Isolates were screened for antimicrobial activity, extracellular enzyme activity, siderophore production and in the case of *Trichoderma* isolates, mycoparasitism. *In vitro* bioassays indicated that *Trichoderma* isolate Eco-T, and *Bacillus* isolates B81 and B69 inhibited *R. solani* growth, suggesting the production of antimicrobial substances. Ultrastructural studies of each of the *Trichoderma* isolate-*R. solani* interactions demonstrated evidence of mycoparasitism of the *Trichoderma* isolates against *R. solani*. Each of the *Trichoderma* isolates exhibited chitinolytic, cellulolytic, lipolytic and amylolytic activity *in vitro*. Only *T. atroviride* SYN6 showed positive proteolytic activity *in vitro*. All three *Bacillus* isolates produced lipase and protease *in vitro* whereas only B81 exhibited cellulase (on carboxymethyl-cellulose agar) and amylase activity *in vitro*. Chitinase and cellulase (on milled filter paper agar) activity were not detected in any of the *Bacillus* isolates. None of the *Trichoderma* and *Bacillus* isolates showed any pectinolytic activity. However, siderophore production was detected for all *Trichoderma* and *Bacillus* isolates. None of the *Trichoderma* and *Bacillus* isolates antagonised each other *in vitro*. The implications and possible involvement of these mechanisms in biological control of *R. solani* are discussed.

Key words: *Bacillus*; extracellular enzymes; mycoparasitism; *Rhizoctonia solani*; *Trichoderma*

3.1 Introduction

Various modes of action have been demonstrated by which *Trichoderma* and *Bacillus* spp. can suppress or control soilborne fungal plant pathogens. Mechanisms such as the production of extracellular antibiotics (Ghisalberti and Sivasithamparam, 1991; Leifert *et al*., 1995; Calistru *et al*., 1997), enzymes such as chitinase (Lima *et al*., 1997) and β-1, 3- glucanase (Menendez and Godeas, 1998), siderophore production (Scher and Baker, 1982), induction of systemic resistance (Liu *et al*., 1995; Ramamoorthy *et al*., 2001), mycoparasitism (Benhamou and Chet, 1993; Kumar *et al*., 1998; Gupta *et al*., 1999) and competition for key nutrients or elements (Elad, 1996) have all been described. Elad (1996) reported that *T. harzianum* Rifai T39 successfully competes with *Botrytis cinerea* Pers.:Fr. for nutrients or elements responsible for activating germination of the pathogen propagules for infection.

Biological control by *Bacillus* spp. has mainly been implicated with the production of antibiotics (Leifert *et al*., 1995; Asaka and Shoda, 1996; Kim *et al*., 1997a). Other mechanisms such as production of chitinase and other fungal cell wall degrading enzymes (Pelletier and Sygusch, 1990; Frändberg and Schnürer, 1994) and to a lesser extent, the production of antifungal volatiles (Fiddaman and Rossall, 1993; 1994) have been described. Mechanisms involved in the biological control by *Bacillus* spp. have also been implicated as part of the range of mechanisms exhibited by *Trichoderma* spp. Ghisalberti *et al*. (1990) suggested that the array of antibiotic substances secreted by *Trichoderma* spp. are responsible for their antagonistic properties. However, the activities of chitinase and other cell wall degrading enzymes are much more apparent and common in *Trichoderma* spp. (Lorito *et al*., 1993; Lima *et al*., 1997; Menendez and Godeas, 1998). *Trichoderma* spp. also use the mechanism of mycoparasitism (Gupta *et al*., 1999), which complements the other biological control mechanisms of this group of organisms.

Mycoparasitism involves the degradation of a fungal cell wall by an antagonist via the production of lytic enzymes (Lorito *et al*., 1993; Lima *et al*., 1997). Mycoparasitism of plant pathogenic fungi by *Trichoderma* species has often been reported in *in vitro* studies. Enzymes that hydrolyse fungal cell wall components such as chitinase and glucanase have been shown to play a significant role in cell wall lysis (Lorito *et al*., 1993; Lorito *et al*., 1994; Lima *et al*., 1997; Menendez and Godeas, 1998). Hadar *et al*. (1979) provides an early account of mycoparasitism of *Rhizoctonia solani* Kühn by a *T. harzianum* strain *in vitro*. The

study revealed that extracellular β-1, 3-glucanase and chitinase enzymes were involved in the mycoparasitic process.

Bacillus spp. Strain L324-92 exhibited antibiotic activity *in vitro* inhibiting *Gaeumannomyces graminis* (Sacc.) Arx & D Olivier var *tritici* J.C. Walker, the causal organism of take-all in wheat (Kim *et al*., 1997a). L324-92 was found to suppress take-all of wheat *in vivo* as well as root rot caused by *R. solani* and *Pythium ultimum* Trow (Kim *et al*., 1997a). Similarly, *B. subtilis* Strain RB14 known to produce the antibiotics Iturin A and Surfactin suppressed damping-off of tomato (*Lycopersicon esculentum* L.) caused by *R. solani* (Asaka and Shoda, 1996).

Production of hydrolytic enzymes such as cellulase, pectinase, protease and lipase have been suggested to play a role as mechanisms of biological control (Benhamou and Chet, 1996; El-Tarabily *et al*., 1996; Elad and Kapat, 1999; Barbosa *et al*., 2001; De Marco and Felix, 2002). El Tarabily *et al*. (1996) suggested that cellulases produced by *Micromonospora carbonacea in vitro* are involved in antagonism against *Phytophthora cinnamomi* Rands and hence capable of causing cell wall lysis of the pathogen. Elad and Kapat (1999) reported that extracellular protease enzyme from *T. harzianum* T39 partially deactivated hydrolytic enzymes produced by *B. cinerea* with a corresponding inhibitory effect on conidia germination of *B. cinerea*. Barbosa *et al*. (2001) on the other hand suggested that pectinase and cellulase enzymes are needed for pathogenic action and saprophytic activities of BCAs. Elsewhere, amylase has also been suggested to aid in saprophytic survival (De Marco *et al*., 2003).

The mycoparasitic action of *T. harzianum* on phytopathogens has been suggested to be a synergistic action of hydrolytic enzymes such as chitinases, lipases, proteases and glucanases (Benhamou and Chet, 1996).

Effective biological control can be achieved through biological control agent(s) exhibiting multiple mechanisms of action. However, the effectiveness of these mechanisms is often governed by a set of environmental conditions under which they operate (Guetsky *et al*., 2002). The present study was undertaken to investigate the biological control mechanisms of five *Trichoderma* isolates, one commercial product, EcoT® (active ingredient, a strain of *T. harzianum*) and three *Bacillus* isolates which exhibit *in vitro* and *in vivo* activity against *R. solani*. Furthermore, interactions between these two organisms were assessed *in vitro* to

determine possibilities of using combinations of these two organisms *in vivo* to enhance biological control and plant growth promotion.

3.2 Materials and Methods

3.2.1 Fungal and bacterial strains

(a) Fungal strains

Five *Trichoderma* isolates, SY3A, SYN, SYN4, SYN6 and SY2F, selected from preliminary *in vitro* and *in vivo* screening (Chapter 2) for biological control activity against *R. solani*, and one commercial strain, EcoT® (active ingredient, *T. harzianum*), were used in this study. The *Trichoderma* isolates were identified by Dr Isabel Rong, (National Collection of Fungi, Agricultural Research Council (ARC), Plant Protection Research Institute, Pretoria, Republic of South Africa). The isolates were identified as follows: *T. atroviride* P. Karsten (Isolate SY3A); *T. harzianum* Rifai (Isolate SYN); *T. pseudokoningii* Rifai (Isolate SYN4) and *T. atroviride* P. Karsten (Isolate SYN6). Isolate SY2F was unidentified and was referred to as *Trichoderma* spp. SY2F.

(b) Bacterial strains

Three unidentified *Bacillus* isolates, *Bacillus* B81, B69 and B77 were used in this study. The *Bacillus* strains were isolated and screened by Kubheka (2003). Isolates B81 and B69 were reported to have biological control properties against *R. solani* while Isolate B77 was reported to exhibit growth promotion properties. The isolates were grown in tryptone soy broth for 24h, then stored at -80^0C (SANYO VIP series ultra low temperature freezer, Model MDF-U71V, SANYO Electrical Co., Ltd Japan) in 65% glycerol-phosphate buffer solution. Isolates were plated on tryptone soy agar medium (Merck) when needed.

3.2.2 In vitro *dual culture bioassay and ultrastructure studies of* Trichoderma *and* R. solani *interactions*

Mycelial plugs (4mm diameter, cut from the actively growing edge of a 4-day old mycelial mat on V8 agar) of a single antagonist and the pathogen were placed opposite each other on a 90mm diameter petri dish containing V8 agar medium. Each bioassay was replicated four times and was incubated for 5 days at 28^0C in the dark. Mycelial plugs (4mm diameter) from regions of mycoparasitic interaction were collected after 5 days post-inoculation and were fixed in 3% (v/v) glutaraldehyde in cacodylate buffer (0.1M; pH 7.0). After 6h of

refrigeration at 4^0C, the specimens were dehydrated in a graded alcohol-acetone series [10, 20, 50, 70, 80% (v/v)] and twice in 100% (v/v). Dehydrated samples were mounted on copper stubs with double-sided sticky tape and sputter coated with gold-palladium and then kept in a dessicator until examination with Phillips XL30 Environmental Scanning Electron Microscopy (ESEM) on high vacuum at 10kV. Three samples per *Trichoderma-R. solani* interactions were examined.

3.2.3 In vitro *dual culture bioassay of* Bacillus *isolates and* R. solani *interactions*

Cell suspensions of *Bacillus* isolates were obtained by culturing each isolate separately in 250ml Erlenmeyer flasks containing 100ml of tryptone soy broth (Merck). After culturing for 48h in a waterbath shaker (Model GLF® 1083, Labortechnik, Germany) at 30^0C, the cells were centrifuged at 9000 x *g* (Beckman J2 HS) for 20min. Cell pellets were then washed and centrifuged twice (9000 x *g*) with sterile distilled water. Washed cell pellets were then made up to 300ml with sterile distilled water and used for *in vitro* bioassays. For each of the *Bacillus* isolate cell suspension, a single streak was made bisecting a 90mm diameter Petri dish containing potato dextrose agar (PDA) (Merck). A 4x4mm agar disk colonized with *R. solani* was placed at the edge of the plate on either side of the bacterial streak. The dual culture bioassay was replicated four times for each *Bacillus* isolate. Plates inoculated solely with the fungi served as a control. Plates were incubated at 28^0C and were rated on Days 3 and 14. All test plates were compared to the control plates to assess antimicrobial activity.

3.2.4 Enzyme production

The production of extracellular chitinase, cellulase, lipase, protease, amylase and pectinase activity were investigated for all *Trichoderma* and *Bacillus* isolates.

Enzyme bioassays were carried out by inoculating the fungal and bacterial isolates onto agar media containing specific substrates. For the *Trichoderma* isolates, mycelial plugs (4mm diameter), cut from the edge of a 4-day old mycelial mat culture on V8 tomato juice agar were used. Inoculation of the agar plates was achieved by placing a plug from each isolate in the centre of an agar plate. *Bacillus* isolate inoculation was achieved by transferring 10µ*l* of a 48h-old cell suspension onto a 6mm filter paper disc (Whatman no.1) which was already placed in the centre of the agar plates unless otherwise indicated. The cell suspensions for the *Bacillus* isolates were prepared as previously described (Section 3.2.3). Four replicates per isolate were made and all experiments were repeated once.

3.2.4.1 Extracellular chitinase production

The production of chitinase by *Trichoderma* isolates was investigated using a solid agar medium modified from Lima *et al*. (1997). The agar medium contained (g.l^{-1} of distilled H_2O): bacteriological peptone, 1.0; urea, 0.3; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.3; glucose, 1.0; colloidal chitin [prepared from crab shell chitin (Merck), using the method of Hsu and Lockwood (1975)], 15.0 (wet weight); trace elements (Fe^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}), 0.1% (w/v) and agar (Merck), 20.0, adjusted to pH 6.0 (autoclaved for 15min at 121^0C).

Inoculated plates were incubated at 28^0C in the dark and examined daily over a 7-day period for clearing of the opaque agar medium, after which the mycelium on agar plates was washed off with distilled water and zones of clearing scored on a scale of 1-4. Class 1= Complete clearance of agar plate with no traces of colloidal chitin, Class 2= Clearance of agar plate with minor traces of colloidal chitin, Class 3= Partial clearance of agar plate and Class 4= No zones of clearing. Classes 1 and 2 were considered as strong chitinase activity and Class 3 as weak chitinase activity.

The production of chitinase by the three *Bacillus* species was investigated on a modified *Bacillus* agar medium from Atlas and Parks (1993). The medium is made up of (g.l^{-1} of distilled H_2O): colloidal chitin, 15.0 (wet weight); yeast extract, 0.5; $(NH_4)_2SO_4$ 1.0; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 1.0; glucose, 0.5 and agar (Merck), 20.0; adjusted to pH 8.0 (autoclaved for 15min at 121^0C).

Inoculated plates were incubated at 30^0C in the dark and examined daily over a 7-day period. Evidence of extracellular chitinase activity was indicated by clearing of the opaque agar medium.

3.2.4.2 Extracellular cellulase production

The production of extracellular cellulase by the *Trichoderma* isolates and Eco-T® was determined on agar medium supplemented with carboxymethylcellulose (CMC) (El-Tarabily *et al*., 1996) or milled filter paper (Harper and Lynch, 1985). Carboxymethylcellulose was used as a soluble form of a cellulose while milled filter paper (Whatman no.1) was used as insoluble form of cellulose. The modified medium adapted from Lima *et al*. (1997) contained (g.l^{-1} distilled H_2O): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; NH_4NO_3, 1.0; KCl, 0.2; trace elements

(Fe^{2+}, $Mn^{2+}$and Zn^{2+}), 0.2% (w/v); glucose, 1.0; bacteriological peptone, 1.0 and agar (Merck), 20.0, adjusted to pH 6.5. The medium was supplemented with either 5 g.l^{-1} CMC or milled filter paper as sources of cellulose and then autoclaved for 15min at 121^0C.

Carboxymethylcellulose agar plates were incubated at 28^0C in the dark for 4 days. Plates were flooded with 1% (w/v) Congo red solution for 15min, and then de-stained with 1M sodium chloride solution for 10min. Unstained areas (clear zones) of the agar medium provided evidence of extracellular cellulase production.

For milled filter paper agar, plates were incubated at 28^0C in the dark for 28 days. Plates were observed for zones of clearance indicating evidence of extracellular cellulase activity. The mycelium on agar plates were washed off with distilled water and zones of clearing scored on a scale of 1-4 indicated as follows: Class 1= Complete clearance of agar plate with no traces of milled filter paper, Class 2= Clearance of agar plate with minor traces of milled filter paper, Class 3= Partial clearance of agar plate and Class 4= No zones of clearing. Classes 1 and 2 were considered as strong cellulase activity and Class 3 as weak cellulase activity.

Extracellular cellulase activity for the *Bacillus* isolates was determined on a modified *Bacillus* agar medium adapted from Atlas and Parks (1993). The medium contained (g.l^{-1} distilled H_2O): K_2NO_3, 1.0; K_2HPO_4, 1.0; KCl, 1.0; $MgSO_4.7H_2O$, 0.5; glucose, 1.0; and agar (Merck), 20.0. The medium was supplemented with either 5g.l^{-1} CMC or milled filter paper as a source of cellulose and then autoclaved for 15min at 121^0C.

Wells were made in the middle of the agar medium using the bottom of a sterile Pasteur pipette and the agar plugs were removed with a mounted needle. Each well was filled with 0.1ml cell suspension of a single *Bacillus* isolate. Plates supplemented with CMC were incubated for 3 days and were stained as previously described. Plates supplemented with milled filter paper were incubated for 28 days and rated as described above.

3.2.4.3 Extracellular lipase production

Lipolytic activity was determined using a solid agar medium described by Hankin and Anagnostakis (1975). The basal medium contained (g.l^{-1} distilled H_2O): bacteriological

peptone, 10.0; NaCl, 5.0; $CaCl_2.2H_2O$, 0.1; agar, 20.0. The medium was adjusted to pH 6. Tween 20 (Sorbitan monolaurate), was used as the lipid substrate and was sterilised separately at 121^0C after which 10ml.l^{-1} was added to the basal medium after it had been sterilised and cooled to 45^0C. Inoculated plates were incubated at 28^0C for 4 days.

Lipolytic enzyme activity was determined by the observation of a visible precipitate resulting from the formation of crystals of the calcium salt of the lauric acid liberated by lipase enzyme. Crystals were viewed with the aid of a dissecting microscope Stemi SV6 (Zeiss, Germany) and digital images taken (Panasonic Super Dynamic WV-CP450 camera).

3.2.4.4 Extracellular proteinase production

The basal medium used in Section 3.2.4.2 was used to determine proteolytic activity. Gelatine was used as the protein substrate and replaced the CMC or milled filter paper used previously. A 8% (w/v) solution of gelatine was made up and sterilised separately for 15min at 121^0C before being added to the basal medium (50ml.l^{-1}). Inoculated plates were incubated at 28^0C for 3 days.

Proteinase activity was detected by staining the plates with 0.1% (w/v) amido black in methanol-acetic acid-distilled water (30:10:60 v/v/v) for 15min followed by destaining with methanol-acetic acid-distilled water (30:10:60 v/v/v). The appearance of clear zones around *Trichoderma* and *Bacillus* growth on agar plates indicated the presence of extracellular proteinase activity.

3.2.4.5 Extracellular amylase production

The ability of the *Trichoderma* and *Bacillus* isolates to degrade starch was used as the criterion for determining amylase activity. The basal medium used in Section 3.2.4.2 supplemented with 2g.l^{-1} of soluble starch (Merck) was used to determine amylolytic activity. Inoculated plates were incubated at 28^0C for 3-5 days and flooded with Lugols iodine solution. Starch molecules are stained a blue back colour whereas zones of clearing were considered to be indicative of regions of amylolytic activity.

3.2.4.6 Extracellular pectinase activity

Pectolytic activity was determined on a modified M9 agar medium (Atlas and Parks, 1993) amended with 1.2 g.l^{-1} of yeast extract (Cattelan *et al*., 1999). The medium contained (g.l^{-1}

distilled H_2O): Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4Cl, 1.0; NaCl, 0.5; $CaCl_2$, 0.2; glucose, 1.0; $MgSO_4.7H_2O$, 0.5; yeast extract, 1.2; agar, 20.0 and then sterilised for 15min at 121^0C. Apple pectin (Allied Drug Company, Rep. of South Africa) (5g.l^{-1}) was added as a source of pectin. Inoculated plates were incubated at 28^0C. After 3-5 days of incubation, plates were flooded with 2M HCl and isolate colonies surrounded by clear halos were considered positive for pectinase activity (Cattelan *et al*., 1999).

3.2.5 Production of siderophore

To determine siderophore production by the *Trichoderma* and *Bacillus* isolates, a modified chrome azurol S (CAS) agar medium, described by Barghouthi *et al*. (1989) was used. Pantothenic acid was omitted from the composition of the medium.

To prepare the CAS agar medium, 5.3g of NaOH and 30.24g of piperazine-*N*, -*N* '-bis(2-ethanesulfonic acid) (Merck) were dissolved in 750ml of distilled water. To this solution 20g of agar (Merck) was added and 100ml of stock solution containing (g.l^{-1} distilled H_2O): KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 10.0. The solution was then sterilised by autoclaving for 15min at 121^0C. Filter sterilised solutions of the following were added when the CAS medium had cooled to $\pm 50^0$C: Casamino acids (10%) (w/v), 30ml; glucose (20%) (w/v), 10ml; thiamine (200 μg.ml^{-1}), 10 ml; nicotinic acid (200 μg.ml^{-1}), 10 ml; $MgCl_2$ (1M) (2.03g in 10ml), 1ml and $CaCl_2$ (1M) (5.55g in 500ml), 1ml. A sterile solution (100ml) containing a complex of chrome azurol S, iron, and hexadecyltrimethylammonium bromide was added. The medium was mixed gently by wrist swirling and poured into 90mm sterile Petri dishes. The CAS-iron- hexadecyltrimethylammonium bromide solution was prepared by dissolving 60.5mg of CAS (Merck) in 50ml of distilled water and 10ml of 1mM (0.14g in 500ml) $FeCl_3.6H_2O$ (in 10mM HCl) was added. This solution was added slowly to a solution containing 72.9mg of hexadecyltrimethylammonium bromide (Merck) in 40ml of distilled water. The resulting solution was sterilised by autoclaving for 15min at 121^0C.

Inoculated plates were incubated at 28^0C for 6 days. Siderophore production was detected as a yellow-orange halo around the cultured colonies of *Trichoderma* and *Bacillus* in an otherwise a blue agar medium.

3.2.6 In vitro *interaction between* Trichoderma *and* Bacillus *isolates*

In vitro bioassay study to determine the compatibility of the *Trichoderma* and *Bacillus* isolates was done on V8 agar medium (Appendix 1). A Petri dish containing V8 agar medium was inoculated with a cell suspension from a 48h-old cell suspension of each of the three *Bacillus* isolates. The cell suspensions for the three *Bacillus* isolates were prepared as previously described. Each isolate was inoculated in a circular mode at a different corner at the edge of the agar medium and coded beneath the plate. A mycelial plug (4mm diameter, cut from the actively growing edge of a 4 day old mycelial mat on V8 agar) of a single *Trichoderma* isolate was placed in the centre of the three *Bacillus* inoculations. This procedure was repeated for all six *Trichoderma* isolates. Each bioassay was replicated three times and was incubated at 28^0C for 5 days in the dark. Plates were assessed for zones of inhibition or clearance after 5 days of incubation. The experiment was repeated once.

3.3 Results

3.3.1 In vitro *dual culture bioassay and ultrastructure studies of* Trichoderma *and* R. solani *interactions under ESEM*

The dual culture plate tests revealed that contact between the pathogen and each of the *Trichoderma* isolates occurred three days after inoculation. Eco-T® was the only isolate that elicited an inhibitory response towards *R. solani* (Fig 3.1). The remaining *Trichoderma* isolates showed no inhibitory response but overgrew *R. solani*. Complete colonization of the plates by all six fungal antagonists occurred within 6 days of inoculation with the formation of spores on all sections of the plates. Isolates *T. atroviride* SY3A and *T. harzianum* SYN both brought about a brownish discolouration of the *R. solani* mycelium. The brownish discolouration spread over the plates as the two *Trichoderma* isolates grew over *R. solani* (Fig 3.2A and B).

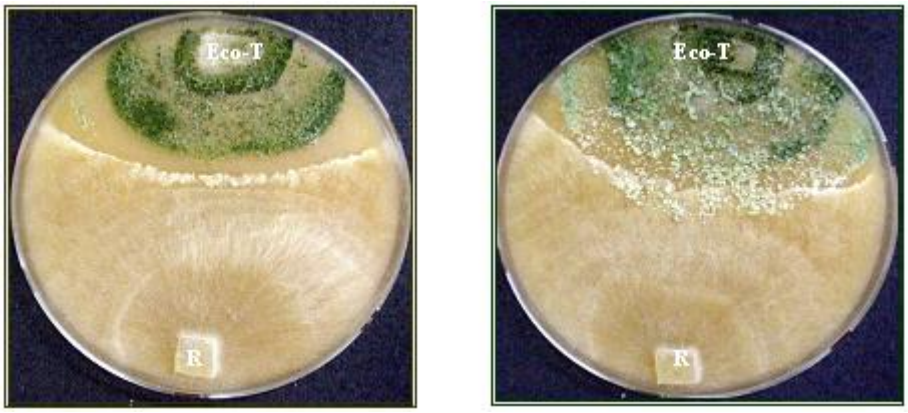

Fig 3.1 *In vitro* interactions between Eco-T and *R. solani* on V8 agar medium showing inhibitory response towards *R. solani* (left) and overgrowth of *R. solani* (right) after 3 and 5 days of inoculation respectively.

ESEM revealed that cell wall lysis of *R. solani* and/or coiling of the *Trichoderma* isolates and Eco-T around *R. solani* occurred where they came into contact. *T. atroviride* SY3A and *T. harzianum* SYN exhibited the most extensive evidence of mycoparasitism. ESEM revealed that when *Trichoderma* came into contact with *R. solani* the *Trichoderma* coiled around the *R. solani* hyphae (Fig 3.3A). Subsequently, evidence of lytic activity, cell wall degradation (Fig 3.3B) and a penetration hole (Fig 3.3D) into the host were apparent. *Trichoderma* mycelium was distinguished from *R. solani* by hyphal diameter (Benhamou and Chet, 1993). Average hyphal diameter of *Trichoderma* was 2μm while that of *R. solani* ranged from 5-6μm.

The integrity of the cell surface of the pathogen, *R. solani*, began to disintegrate where *T. atroviride* SY3A and *T. harzianum* SYN isolates made contact with the *R. solani* cell wall (Fig 3.3B and C). Pronounced collapse and loss of turgor of *R. solani* hyphae were among the typical features of advanced alteration observed (Fig 3.3D). Prolonged contact with the *R. solani* caused cell wall penetration with evidence of penetration holes (Fig 3.3D) and massive cell damage (Fig 3.3D and F). These were seen as features of total cell wall destruction, cell wall breakdown and hyphal disintegration.

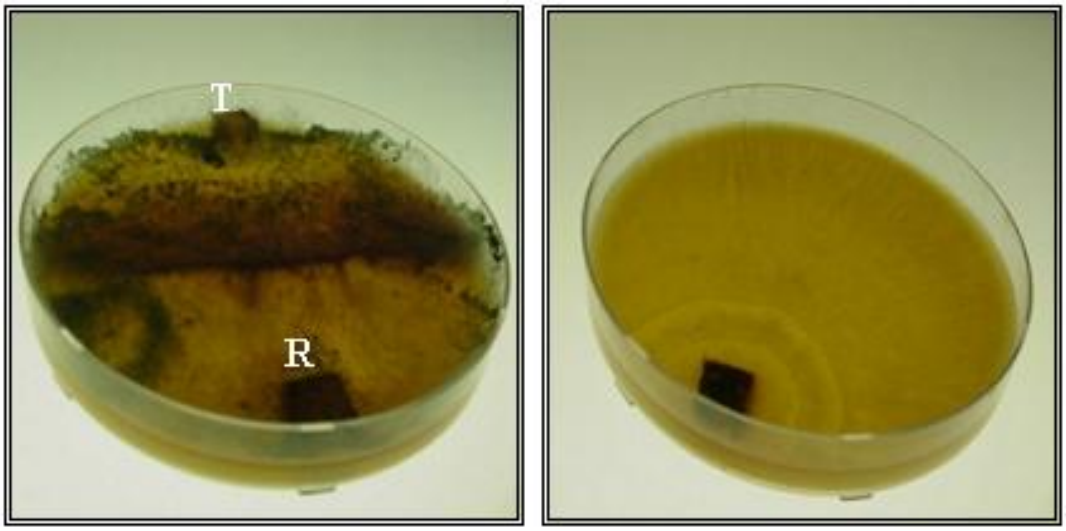

Fig 3.2A. *In vitro* interaction between *T. atroviride* SY3A and *R. solani* on a V8 agar medium (left) compared to *R. solani* control (right). Brownish discolouration of the *R. solani* mycelium occurred at the point of interaction (left) and spread over the plate completely by covering the plate 6 days after inoculation.

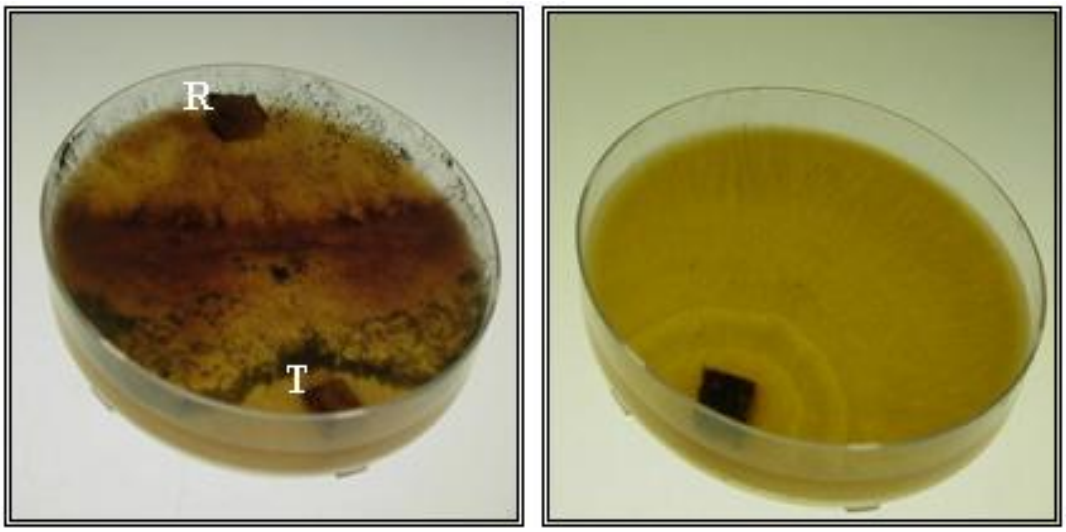

Fig 3.2B. *In vitro* interaction between *T. harzianum* SYN and *R. solani* on a V8 agar medium (left) compared to *R. solani* control (right). Brownish discolouration of the *R. solani* mycelium occurred at the point of interaction (left) and spread over the plate completely by covering the plate 6 days after inoculation.

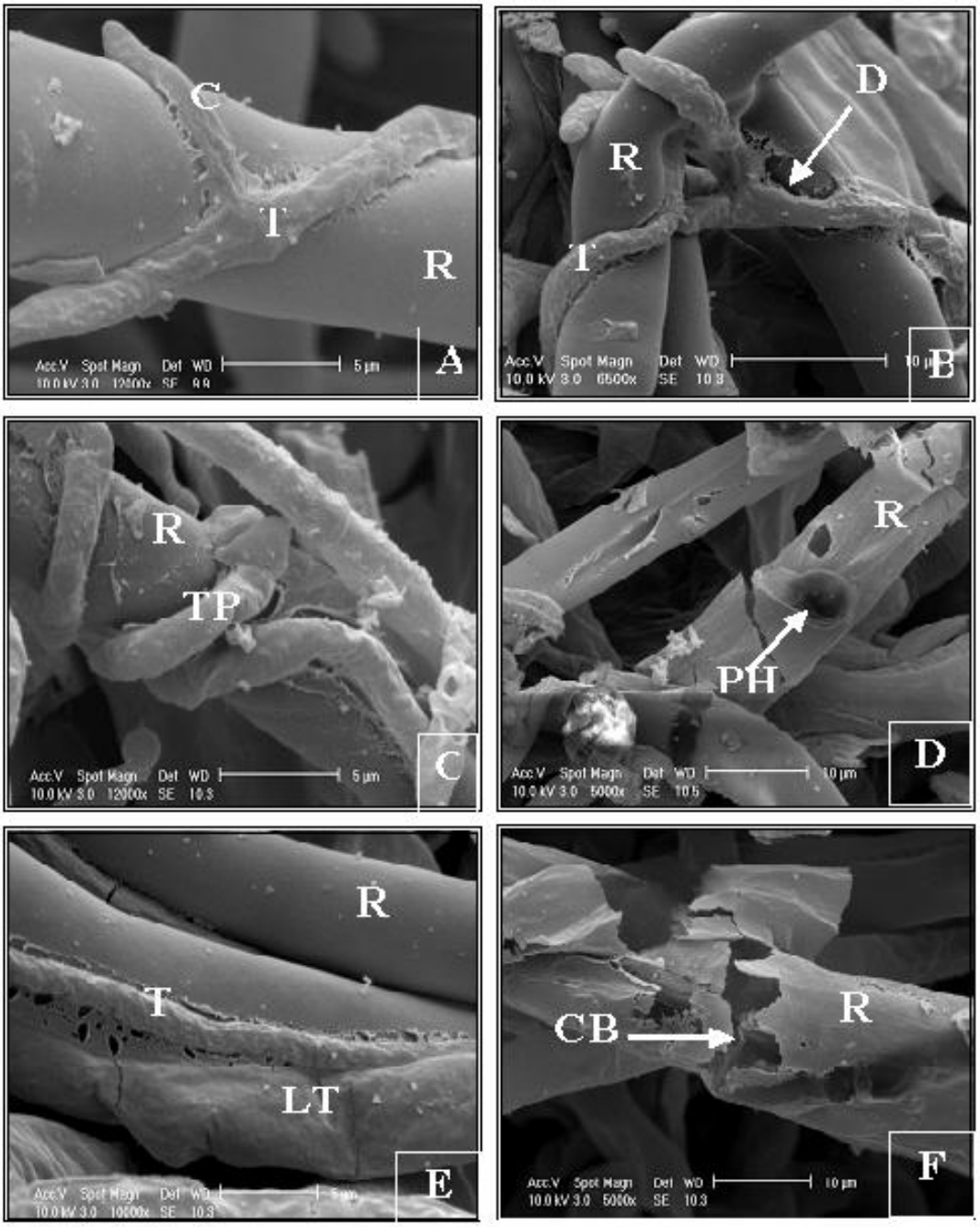

Fig 3.3A-F Scanning electron micrographs of *Trichoderma* (T) mycoparasitising *R. solani* hyphae.
Early stages of mycoparasitism are characterized by branching *Trichoderma* hyphae (T) coiling (C) round *R. solani* (R) (A). Cell wall disintegration (D) at the point of contact between hyphal strands is evident (B). Dense coiling of *Trichoderma* hyphal strands and penetration (TP) of *R. solani* hyphal strands is shown in C. Penetration hole (PH) arising from the formation of an appressorium and penetration peg (not visible) by *Trichoderma* was evident on the surface of a partially degraded *R. solani* hyphal strand (D). Loss of turgor (LT) pressure (E), and cell destruction of (CB) of *R. solani* resulted (F).

3.3.2 In vitro *dual culture bioassay of* Bacillus *species and* R. solani

Of the three *Bacillus* isolates tested *in vitro* for antagonism against *R. solani*, Isolates B81 and B69 were antagonistic to *R. solani* (Fig 3.4A and B) whereas Isolate B77 was not. Zones of inhibition were greater on Day 3 for both isolates. By Day 6 the zones of inhibition arising from Isolate B69 were completely overgrown by whitish powdery *R. solani*. Zones of inhibition on plates inoculated with Isolate B81 remained constant from Day 6 to the last day of observation (Day 14) (Fig 3.4A and B).

3.3.3 Enzyme production

The results of the enzyme bioassays are summarized in Table 3.1.

3.3.3.1 Extracellular chitinase production

All the *Trichoderma* isolates produced zones of clearance on the chitin media after 7 days, indicating evidence of chitinolytic activity. *T. atroviride* SY3A, *T. pseudokoningii* SYN4, *T. atroviride* SYN6 and *T. harzianum* SYN were scored 1 for their zones of clearing. *Trichoderma* sp. SY2F was scored 2 whereas Eco-T® was given a score of 3.

None of the *Bacillus* isolates tested were positive for chitinase production. Although all three isolates grew on the agar medium used, no zones of clearing were seen.

3.3.3.2 Extracellular cellulase production

On CMC agar plates, all *Trichoderma* isolates tested positive for cellulase production. Complete clearance on agar plates was observed for all *Trichoderma* isolates after 4 days of incubation. Agar plates were completely covered with fungal mycelium and spores on all sections of the agar plate for all the *Trichoderma* isolates and Eco-T®.

On agar medium supplemented with milled filter paper all *Trichoderma* isolates produced cellulase, with varying zones of clearing after 4 weeks of incubation. *Trichoderma* sp. SY2F, *T. pseudokoningii* SYN4, and *T. atroviride* SYN6 were all given a score of 1.

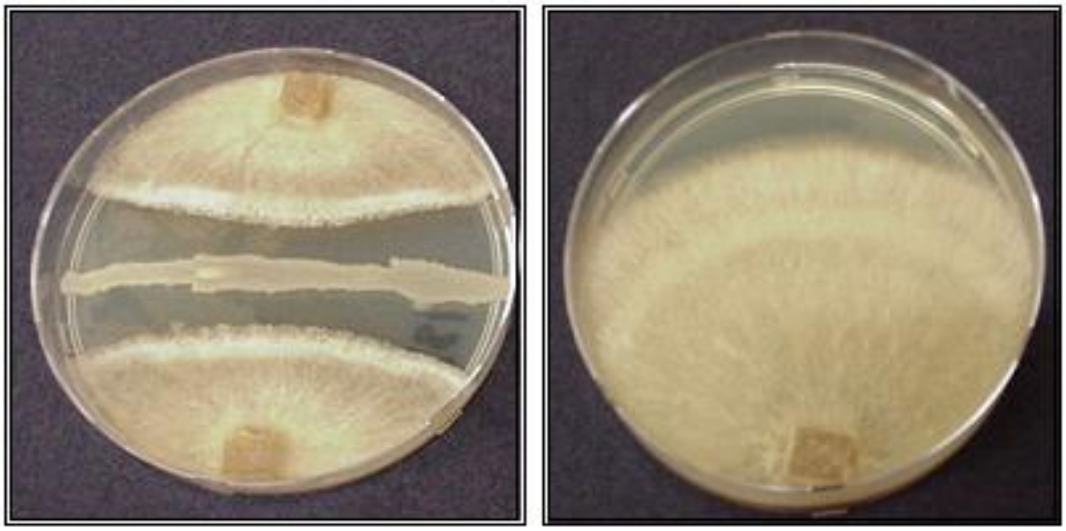

Fig 3.4A *In vitro* inhibition of *R. solani* by *Bacillus* Isolate B81 (left) compared to a *R. solani* alone control plate (right) on Day 6 after incubation at 28^0C on PDA agar plates.

Fig 3.4B *In vitro* inhibition of *R. solani* by *Bacillus* Isolate B81 (left) compared to a *R. solani* alone control plate (right) on Day 14 after incubation at 28^0C on PDA agar plates. Zone of inhibiton remained fairly constant on Day 14 compared to inhibition zone observed on Day 6.

This class of *Trichoderma* isolates were characterised by concentric growth on agar plates with lots of spores formed on all sections of the agar plates. *Trichoderma harzianum* SYN and Eco-T® were given a score of 2 with no concentric mycelial growth on agar plates. *T. harzianum* SYN formed spores on all sections of the agar plates while Eco-T® exhibited less dense mycelial growth with sparse and minimal spore formation on agar plates. *T. atroviride* SY3A exhibited weak cellulase activity with a score of 3. Dense mycelial growth and spores were seen on all sections of the agar plates.

Among the *Bacillus* isolates, only Isolate B81 produced cellulase on CMC agar medium with 55mm average diameter of zones of clearance on agar medium after 3 days of incubation. None of the *Bacillus* isolates produced cellulase on the milled filter paper agar medium, although growth did occur on all plates.

3.3.3.3 Extracellular lipase production

All the *Trichoderma* isolates tested positive for lipase production on solid agar medium. A visible precipitate due to the formation of calcium salt of the lauric acid was seen on all sections of the plates inoculated with the *Trichoderma* isolates and Eco-T® after 4 days of incubation (Fig 3.5). Calcium salt precipitate was less dense on agar plates with Eco-T® compared to the rest of the *Trichoderma* isolates. Spore formation was poor for all *Trichoderma* isolates and Eco-T® on agar medium used for lipase detection.

Only *Bacillus* isolates B77 and B81 produced lipase on the solid agar medium. However Isolate B69 did grow on the agar medium, but no calcium salt precipitate was observed after 4 days of incubation. Calcium salt precipitate was less dense on agar plates with B81 compared to plates with B77.

3.3.3.4 Extracellular proteinase production

Among the *Trichoderma* isolates screened, only *T. atroviride* SYN6 produced protease on solid agar medium. The rest of the isolates did grow on the medium but no extracellular protease activity was detected.

All the *Bacillus* isolates produced protease. Using SAS (SAS, 1987) to perform an ANOVA on diameters of zones of clearance, significant differences ($P<0.002$) were found between the *Bacillus* isolates (Table 3.1). The largest zone of clearance was recorded by B81 followed by

B77 and B69. Growth on the agar medium appeared to be directly related to the size of the zones of clearance. Isolate B81 grew best among the three *Bacillus* isolates and recorded the largest zone of clearance compared to B77 (Fig 3.6) and B69.

3.3.3.5 Extracellular amylase production

All the *Trichoderma* isolates produced evidence of extracellular amylase activity on solid medium. Agar plates were completely clear indicating utilization of soluble starch. Good growth and sporulation was observed for all isolates.

Of the *Bacillus* isolates, only B81 produced extracellular amylase (Table 3.1). Good growth was observed on agar medium incorporated with 0.2% soluble starch. Isolates B69 and B77 did grow on the agar medium but no amylase production was detected. Average diameter of zones of clearance (85mm) was recorded for Isolate B81.

3.3.3.6 Extracellular pectinase production

None of the *Trichoderma* isolates or the *Bacillus* isolates produced pectinase. Mycelial growth accompanied with very poor sporulation was observed for all *Trichoderma* isolates. Minimal growth was observed for all *Bacillus* isolates.

3.3.4 Production of siderophore

All *Trichoderma* isolates and Eco-T® produced siderophores on solid CAS-agar medium. ANOVA using SAS (Version 6.12) showed that there were no differences in zones of clearance between *Trichoderma* sp. SY2F, *T. atroviride* SY3A, *T. pseudokoningii* SYN4, and *T. atroviride* SYN6 ($P>0.05$) but these isolates were different from *T. harzianum* SYN and Eco-T® ($P<0.05$) (Table 3.1). However, *T. harzianum* SYN was different from Eco-T® ($P<0.0001$). The largest zone of clearance was recorded by *Trichoderma* sp. SY2F, while the least was recorded by Eco-T®. Good growth and sporulation were observed on all agar plates except Eco-T®, which showed minimal growth but good sporulation.

All three *Bacillus* isolates produced siderophores. No difference was found between B69 and B81 ($P>0.2$) but these two isolates were different from B77 ($P<0.004$ and $P<0.001$) respectively (Table 3.1). The largest zone of clearance was recorded by B81 followed by B69.

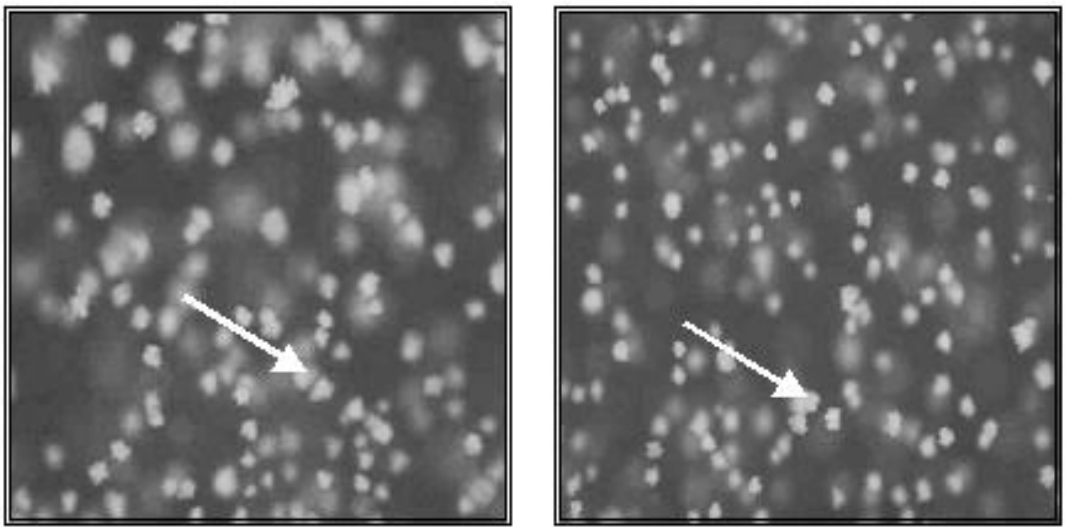

Fig 3.5 Evidence of lipase activity by *T. atroviride* SY3A (left) and *T. harzianum* SYN (right) on solid agar medium supplemented with Tween 20 (Sorbitan monolaurate) as a lipid substrate after incubation at 28^0C for 4 days. Formation of calcium salt crystals (arrowed) in agar medium is indicative of lipase activity.

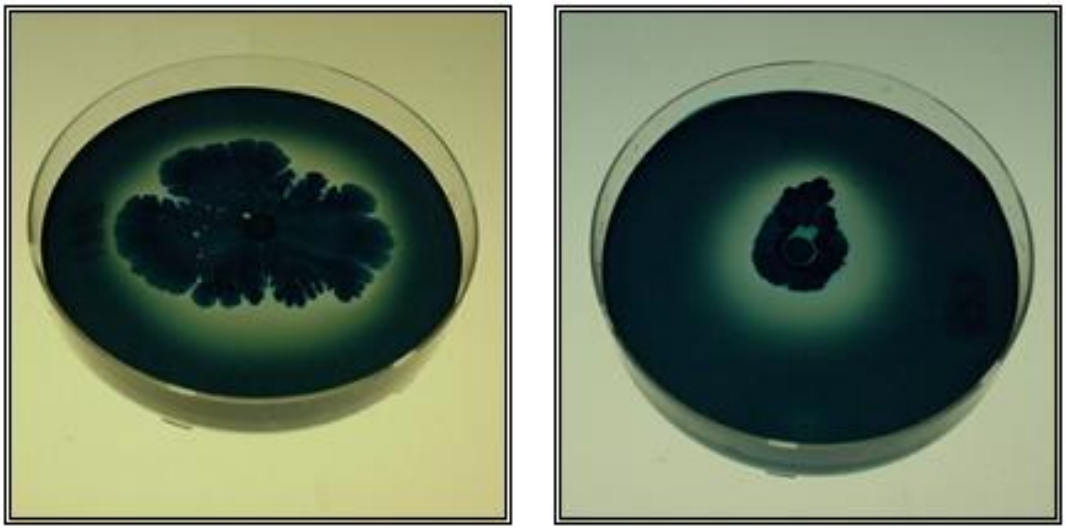

Fig 3.6 Evidence of extracellular proteinase activity by *Bacillus* Isolate B81 (left) and Isolate B77 (right) on solid agar medium supplemented with 8% (w/v) gelatine as the protein substrate, after incubation at 28^0C for 3 days. Clear zones around bacterial colonies is indicative of protease enzyme activity.

Table 3.1. Selected biochemical traits of fungal and bacterial isolates determined on solid agar medium. All isolates were negative for pectinase production

Fungi/Bacteria	Chitinase production §	Cellulase production (on CMC agar)	Cellulase production (on milled filter paper) §	Lipase production	Protease production	Amylase production	Pectinase production	Siderophore production
Trichoderma sp. SY2F	+	+ (C)	+	+	–	+ (C)	–	+ [80mm][a]
T. atroviride SY3A	+	+ (C)	+	+	–	+ (C)	–	+ [79mm][a]
T. pseudokoningii SYN4	+	+ (C)	+	+	–	+ (C)	–	+ [76mm][a]
T. atroviride SYN6	+	+ (C)	+	+	+	+ (C)	–	+ [78mm][a]
T. harzianum SYN	+	+ (C)	+	+	–	+ (C)	–	+ [69mm][b]
Eco-T	+	+ (C)	+	+	–	+ (C)	–	+ [49mm][c]
Bacillus spp B69	–	–	–	–	+ [20mm][c]	–	–	+ [29mm][a]
Bacillus spp B77	–	–	–	+	+ [36mm][b]	–	–	+ [19mm][b]
Bacillus spp B81	–	+ [55mm]	–	+	+ [68mm][a]	+ [85mm]	–	+ [32mm][a]

§ Zones of clearing scored on a scale of 1-4

(C) Complete clearance of agar plate

[ ][a] Average diameter of zones of clearance or yellow halo with respect to siderophore production. Values with different superscripts are significantly different ($P<0.05$)

– No detectable enzyme activity

3.3.5 In vitro *interaction between* Trichoderma *and* Bacillus *isolates*

Trichoderma and *Bacillus* isolates did not inhibit each other in all possible combinations or interactions tested (Fig 3.7).

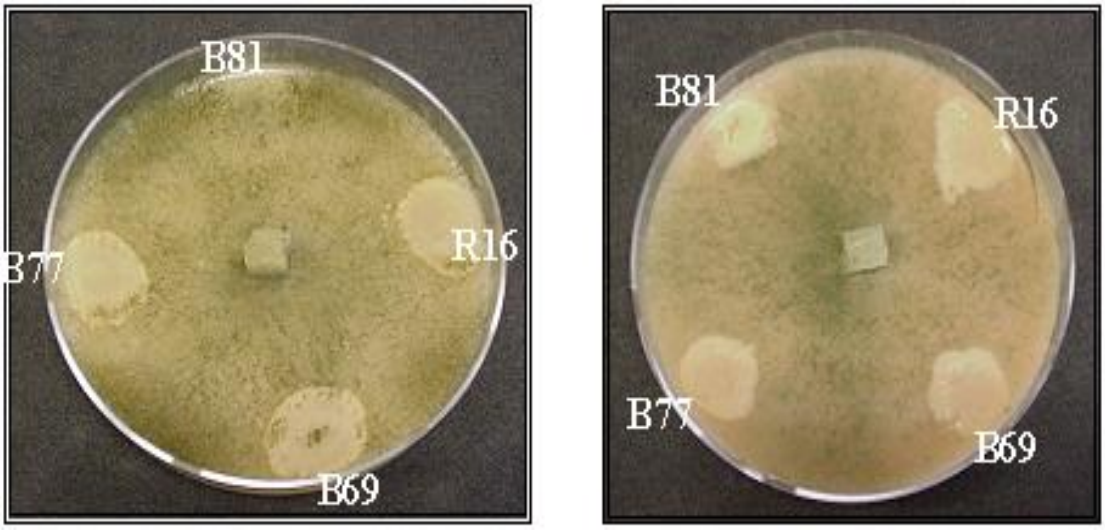

Fig 3.7 *In vitro* interactions between *T. atroviride* SYN6 (left), *T. atroviride* SY3A (right) and four *Bacillus* isolates (R16, B69, B77 and B81). No inhibition was found between any of the *Trichoderma* and the *Bacillus* isolates 5 days after inoculation at 28^0C. R16 was not used in this study.

3.4 Discussion

Understanding the modes of action of biological control agents (BCAs) in relation to their antagonistic effects on plant pathogens is vital for the optimisation and implementation of biological control systems. Modes of action of BCAs are usually difficult to ascertain *in vivo*. Therefore, *in vitro* bioassays and ultrastructure studies are useful tools in determining the possible or most probable mechanisms.

Antagonism of a pathogen through the production of an antimicrobial substance has been extensively reported for both fungal and bacterial BCAs (Ghisalberti *et al*., 1990; Leifert *et al*., 1995; Asaka and Shoda, 1996). In this regard, Eco-T® was the only fungus and *Bacillus* spp. B69 and B81 the only bacterial isolates that showed antimicrobial activity against *R. solani*.

Antibiotics produced by BCAs *in vitro* in most instances have been regarded as the principle compounds responsible for biological control *in vivo* (Leifert *et al*., 1995). Of the *Bacillus*

isolates screened, only Isolate B69 and B81 inhibited the growth of *R. solani*. *Rhizoctonia solani* overgrew the inhibition zones produced by Isolate B69 after 6 days of incubation suggesting that the antifungal compound produced could be fungistatic. Inhibition zones produced by Isolates B81 remained constant after 14 days of incubation suggesting that the antifungal compound produced was fungicidal in nature. *Bacillus* spp. have been reported to produce an array of antibiotics *in vitro* against several plant pathogens (Leifert *et al*., 1995; Asaka and Shoda, 1996). Although antibiotic production *in vitro* alone cannot be regarded as sufficient proof of the involvement of antibiotics in biological control *in vivo*, it is regarded as a useful tool for pre-screening potential BCAs *in vitro*.

Mycoparasitism of fungal plant pathogens is one of the mechanisms harnessed in the control of plant diseases (Zhang *et al*., 1999). *In vitro* bioassays coupled with ESEM ultrastructure studies indicated that all the *Trichoderma* isolates appeared to actively parasitise the *R. solani* mycelium. However, the degree of mycoparasitism and cell wall lysis differed among isolates. Cell wall disruption and lysis of *R. solani* mycelium was much more extensive in the case of *T. atroviride* SY3A and *T. harzianum* SYN coincide with mycelial browning which was attributed to a Maillard type reaction. These two isolates were the only ones to produce the brownish discolouration *in vitro*. Cell wall lysis and disruption appeared to be associated with the browning of the mycelium where mycoparasitism had occurred but whether the browning discolouration is directly linked to the mycoparasitic action of these two *Trichoderma* isolates is not clear.

Various extracellular enzymes such as protease, chitinase, cellulase and 1,3-β-glucanase have been implicated in the biological control of plant pathogens (Elad, 1996; El-Tarabily *et al*., 1996; Calistru *et al*., 1997; Menendez and Godeas, 1998). Evidence of extracellular enzyme activity was observed in the ultrastructure study where *Trichoderma* hyphae, particularly from *T. atroviride* SY3A and *T. harzianum*, appeared to etch grooves into the *R. solani* mycelium indicating that partial cell wall degradation had taken place. This finding points towards lytic enzymes being involved in the mycoparasitic process. Evidence to support this were the enzyme bioassays showing chitinase enzyme activities for all the *Trichoderma* isolates. Four *Trichoderma* isolates exhibited strong chitinase activity *in vitro*, and were given the highest possible rating. However, when viewed under ESEM, two isolates *T. atroviride* SY3A and *T. harzianum* SYN showed more pronounced hyphal degradation than *T. pseudokoningii* SYN4 and *T. atroviride* SYN6. Elad *et al*. (1982) held that extracellular lytic enzymes, 1,3-β-

glucanase and chitinase produced by *T. harzianum* were involved in cell wall degradation of *R. solani*. Several authors have since reported that chitinase and glucanase enzymes are involved in the mycoparasitic process of *Trichoderma* species (Lima *et al*., 1997; Thrane *et al*., 1997; Menendez and Godeas, 1998). Mixtures of chitinolytic enzymes and 1,3-β-glucanase have been found to have more significant effect on phytopathogenic fungi than either of the classes of enzymes used in isolation (Lorito *et al*., 1993). This could point to the evidence of destructive mycoparasitism exhibited by *T. atroviride* SY3A and *T. harzianum* during *in vitro* studies. None of the three *Bacillus* species produced chitinase enzyme *in vitro*. This discounts the action of chitinase production as a possible mechanism of action by the *Bacillus* isolates.

All the *Trichoderma* isolates showed evidence of cellulase activity on CMC agar and cellulose (milled filter paper) supplemented agar medium suggesting that the cellulolytic enzymes produced were able to degrade both soluble and non-soluble forms of cellulose. Of the *Bacillus* isolates, only B81 showed evidence of cellulase enzyme on CMC agar *in vitro*. None of the *Bacillus* isolates were able to degrade insoluble cellulose *in vitro*. *Trichoderma* species are known to synthesise cellulase enzymes (Thrane *et al*., 1997), which have also been shown to hydrolyse β-1,4-glucans (De Marco *et al*., 2003). Glucans is one of the structural components of filamentous fungal cell walls such as *Rhizoctonia* spp. and hence would be expected to be susceptible to cellulase activity. Nevertheless, cellulose is commonly present in the environment and in nature. Hydrolysis of cellulose by the cellulase enzymes into other simple compounds is potentially beneficial to BCAs as means of maintaining their saprophytic and parasitic metabolic needs (De Marco *et al*., 2003). It has also been reported that mutants of *T. harzianum* with increased cellulase production had a greater competitive saprophytic ability than the wild type strain (Ahmad and Baker, 1987). The cellulase enzyme produced by the *Bacillus* Isolate B81 *in vitro* might possibly not perform the same function as the *Trichoderma* isolates would since no activity was found on the milled filter paper agar medium. Non-soluble cellulose is more likely to accumulate in soil than soluble cellulose.

Lipids and proteins are integral structural components of fungal cell walls (Hunsley and Burnett, 1970). The *Bacillus* isolates as well as *T. atroviride* SYN6 were the only organisms exhibiting protease activity *in vitro*. Proteases have been reported to be involved in the biological control of plant pathogens (Elad and Kapat, 1999; De Marco *et al*., 2003). *T. harzianum* T39 protease enzyme was found to reduce conidial germination of *Botrytis*

cinerea, reduced the incidence of disease development on bean leaves and possibly deactivate *B. cinerea* hydrolytic enzymes that are responsible for plant tissue necrosis (Elad and Kapat, 1999). It has also been suggested that endoproteinase from *Bacillus megaterium* can inactivate extracellular enzymes activities of *R. solani* (Bertagnolli *et al*., 1996). The latter could be the case for *T. atroviride* SYN6 and the *Bacillus* spp. on *R. solani*. Similarly, De Marco and Felix (2002) reported that purified protease enzyme produced by *T. harzianum* 1051 affected *Crinipellis perniciosa* (Stahel) Singer *in vitro*. Extracellular protein precipitate from *Bacillus subtilis* AF1 was found to retard the growth of *Aspergillus niger* van Tieghem (Podile and Prakash, 1996).

All of the organisms evaluated, with the exception of *Bacillus* sp. B69, all produced lipase *in vitro*. Sivan and Chet (1989) hypothesised that a possible synergistic action of protease, lipase and polysaccharides as an essential component of fungal cell wall degradation. Similarly, results presented by Lorito *et al*. (1993) indicated that mixtures of hydrolytic enzymes with complementary modes of action may increase *in vitro* antifungal activity. The extracellular enzymes produced by the *Trichoderma* and *Bacillus* isolates may complement each other when used in combination to enhance antibiosis and cell wall lysis of *R. solani.*

Only *Bacillus* Isolate B81 produced amylase *in vitro* whereas all the *Trichoderma* isolates showed positive for the amylase test. *Trichoderma* species have been reported to produce amylase (Calistru *et al*., 1997). However, there is no report of the presence of starch in fungal cell walls (De Marco *et al*., 2003), which makes the role of amylase produced by the *Trichoderma* and *Bacillus* Isolate B81 uncertain. Electron microscopy studies with purified amylase from *T. harzianum* 1051 on *C. perniciosa* revealed that the purified amylase enzyme had no effect on *C. perniciosa* cell walls (Azevedo *et al*., 2000). Starch is readily available and widely distributed in nature, hence the amylase enzyme produced could be useful in breaking down starch present within the vicinity of the BCAs to simple products such as glucose to support their metabolic needs (De Marco *et al*., 2003). It also indicates zero ability to parasitize plants.

None of the *Trichoderma* or *Bacillus* isolates were positive for pectinase production and hence it is speculated that pectinase enzyme plays no role, if any, in the biological control activity of the *Trichoderma* and *Bacillus* isolates. However, it has been suggested that pectinolytic and cellulolytic enzymes are needed for saprophytic activity (Barbosa *et al*., 2001).

Siderophore production has been recognised extensively as a major contributing factor towards achieving effective biological control (Kloepper *et al*., 1980; Loper, 1988). Iron is an essential micronutrient required by microorganisms as a co-factor (Leong, 1986). Siderophore production was detected *in vitro* for all *Trichoderma* and *Bacillus* isolates. Siderophores are low molecular weight compounds which have high affinity for Fe(III) (Schwyn and Neilands, 1987). These molecules are usually produced when available iron concentrations are low (Barghouthi *et al*., 1989) since it is needed for microbial/fungal metabolic processes (Press *et al*., 2001). The *Trichoderma* and *Bacillus* isolates would therefore be able to show competitive advantage for iron in an iron-limiting environment compared to *R. solani*, which does not produce a siderophore under this condition. This deprives the *R. solani* of Fe(III) which is vital for its metabolic functions, hence limiting the growth of the pathogen. However, in situations where iron is readily available in soil, the antagonistic activity and hence the biological control due to siderophore production becomes less important (Montealegre *et al*., 2003).

Selection of BCAs based on *in vitro* production of extracellular enzymes, siderophores and antibiotics and other metabolites can be regarded as a useful screening procedure to reduce the large number of isolates at an initial stage for further testing *in vivo* (Kloepper *et al*., 1992). Moreover, given that the *Trichoderma* and *Bacillus* isolates were positive for most of the traits tested, coupled with the antibiotic action of *Bacillus* isolates B81 and B69 and mycoparasitic nature of the *Trichoderma* isolates, these traits are worth noting during preliminary *in vitro* screening and selection criteria for potential BCAs, although other traits such as 1,3-β-glucanase associated with biological control were not screened for. The presence of any of the metabolites tested does not guarantee any *Trichoderma* or *Bacillus* isolates as a BCA and neither does its absence guarantee that it is not a BCA. Ultimately, *in vivo* and field testing is required to ratify the choice and selection of BCAs.

The use of two groups of organisms together has been proposed as one approach to improve plant growth and enhance biological control (Darmwal and Gaur, 1988; Duffy *et al*., 1996; El-Tarabily *et al*., 1996; Larkin and Fravel, 1998). Benefits of using such combinations for plant growth promotion and biological control include increase in crop yield and mineral uptake, (e.g., nitrogen and phosphorus) (Darmwal and Gaur, 1988; Jisha and Alagawadi, 1996), increase in biological control through complementary mechanisms of action over single organisms (Duffy *et al*., 1996), increase in consistency (Larkin and Fravel, 1998) and a

decrease in variability of biological control (Guetsky *et al*., 2001) under diverse environmental conditions.

Increased biological control and plant growth promotion could be achieved by a combination of the *Trichoderma* and *Bacillus* isolates. The *Trichoderma* isolates parasitized *R. solani* hyphae and also produced cell wall degrading enzymes such as chitinase and cellulase (active on non-soluble cellulose) which were not produced by the *Bacillus* isolates. The combined activity of these mechanisms with the antifungal compound(s) produced by *Bacillus* Isolates B69 and B81 could increase the spectrum of activity of these two groups of organisms, hence leading to a possible synergistic effect rather than antagonism. Moreover, *Trichoderma* lives in the soil as a saprophyte, colonises the bulk soil as well as the rhizosphere of host plants (Duffy *et al*., 1996) while *Bacillus* mostly lives in the rhizosphere of host plants. Hence these two organisms could be said to occupy different and complementary niches. *Trichoderma* sources nutrients through its saprophytic activities as well as from its activities in the rhizosphere (Duffy *et al*., 1996). Compared to *Trichoderma* spp., *Bacillus* spp. will more likely to be active in the rhizosphere region. The potential of using the *Trichoderma* and *Bacillus* isolates exist even though the evidence presented in this chapter indicated that they did not inhibit each other *in vitro*. Because of the differences in mechanisms of action, and the different and complementary niche occupancy, which could lead to different ecological requirements by *Trichoderma* and *Bacillus* spp., the authors postulate that mixtures of these two groups of organisms could lead to a possible increase in plant growth as well as enhance biological control.

Results presented in this chapter suggest that a better understanding of fungal and bacterial interactions that enhance or detract from biological control (Handelsman and Stabb, 1996) could be useful in the implementation of applied biological control systems.

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CHAPTER FOUR

Comparison of single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates on plant growth promotion and biological control of *Rhizoctonia solani* damping-off

"...Increased yield is desirable but not essential to demonstrate efficacy…" Schroth and Becker, 1990.

K.S. Yobo[1], M.D. Laing[1] and C.H. Hunter[2]

[1]*Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

[2]*Discipline of Microbiology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

Abstract

Six *Trichoderma* isolates, *T. atroviride* SY3A, *T. atroviride* SYN6, *T. harzianum* SYN, *T. pseudokoningii* SYN4, an unidentified strain *Trichoderma* sp. SY2F and a commercial strain, *T. harzianum* (Eco-T®) and three unidentified *Bacillus* isolates, *Bacillus* sp. B69, *Bacillus* sp. B77 and *Bacillus* sp. B81, were tested *in vivo*, singly and each in combination for plant growth promotion and biological control of *Rhizoctonia solani* damping-off. Greenhouse trials indicated that combined inoculations of *T. atroviride* SYN6 and *Bacillus* B69 gave the highest plant growth promotion of bean seedlings in terms of seedling dry biomass (43.0% over uninoculated control). Rhizotron studies supported these findings where it was shown that root biomass and root area were increased. However, results obtained for bean yield trials were erratic and had no correlation with the seedling trials (P = 0.87 and P = 0.35). No increase was obtained in protein or fat content of bean seed for any of the selected isolates/combinations screened in two yield trials. In the biological control trials, single inoculations of Eco-T®, *T. atroviride* SY3A, and *T. harzianum* SYN gave the highest percentage survival of cucumber plants in the greenhouse. None of the *Trichoderma* + *Bacillus* combinations were better than the single inoculations of Eco-T®, *T. atroviride* SY3A, and *T. harzianum* SYN. The best combination was *T. atroviride* SY3A + *Bacillus* B81 a with plant stand of 61.1%, which was equal to the performance of *T. harzianum* SYN. The performances, particularly of *Bacillus* B69 and B81 were enhanced when combined with *T. atroviride* SY3A, *T. atroviride* SYN6, *T. harzianum* SYN or Eco-T®. The performance of each of the combinations was better than each of the *Bacillus* isolates used alone. This study showed that there is potential in using mixtures of *Trichoderma* and *Bacillus* spp. for improving plant growth and biological control.

Key words: biological control; cucumber; dry bean; plant growth promotion

4.1 Introduction

Increases in crop yield and plant disease control have been observed following seed and/or seedling treatments with either *Trichoderma* or *Bacillus* species (Inbar *et al*., 1994; Podile and Prakash, 1996; Kim *et al*., 1997a; Utkhede *et al*., 1999; Harman, 2000; Rabeendran *et al*., 2000). When inoculated, these organisms act via a series of mechanisms to control plant pathogens leading to a decrease in disease levels with a corresponding increase in crop yield (Fravel, 1988; Benhamou and Chet, 1993; Podile and Prakash, 1996; Elad, 2000).

The genus *Trichoderma* belongs to the Deuteromycetes (Samuels, 1996) class of fungi and have been exploited as biological control agents against a range of plant pathogenic fungi due to their antagonistic properties towards plant pathogens (Papavizas, 1985; Chet, 1987). Some strains of *Trichoderma* have been widely used as biological control agents as well as plant growth promoters (Ousley *et al*., 1994a; Harman, 2000; Rabeendran *et al*., 2000). *Bacillus* spp. are gram-positive bacteria and numerous strains have shown biological control activity on a wide range of crops (Cook and Baker, 1983; Leifert *et al*., 1995; Podile and Prakash, 1996; Utkhede *et al*., 1999). Several isolates have also been found to promote plant growth (Probanza *et al*., 1996) and in some cases increase nodulation in legumes (Podile, 1995). These *Bacillus* spp. strains are appealing candidates as inoculants for biological control against plant pathogens due to their ability to form endospores that are tolerant to heat and desiccation, giving them extended shelf lives compared to other biological control agents such as *Pseudomonas* spp. (Petras and Casida, 1985; Young *et al*., 1995).

Several reports in the literature indicate that combinations of biological control agents and plant growth promoting rhizobacteria can increase disease suppression (Guetsky *et al*., 2002), improve crop yields and enhance nutrient uptake by plants (Alagawadi and Gaur, 1988; Alagawadi and Gaur, 1992) over single organism inoculations. For example, Alagawadi and Gaur (1992) reported that combined inoculations of *Azospirillum brasilense* and *Pseudomonas striata*, or *Bacillus polymyxa*, improved nitrogen and phosphorus uptake and consequently increased sorghum grain yield compared to organisms inoculated individually. In a separate study, Jisha and Alagawadi (1996) reported an increase in sorghum (*Sorghum bicolor* L. Moench) yield when inoculated with combined formulations of *B. polymyxa* or *P. striata*, and *T. harzianum* Rifai. However, information pertaining to combined inoculations of *Trichoderma* and *Bacillus* species on plant growth and especially on disease control appears

to be very sparse, even though both *Bacillus* and *Trichoderma* species are well known for their biological control and plant growth promoting properties.

In this study, we tested the hypothesis that combinations of selected *Trichoderma* and *Bacillus* isolates could enhance disease control and/or improve seedling growth, establishment and overall yield. Using six *Trichoderma* and three *Bacillus* isolates as a model, the hypothesis was tested under greenhouse conditions to evaluate the effect of single and dual inoculation of these two groups of organisms on plant growth promotion of dry beans and biological control of *Rhizoctonia solani* Kühn damping-off on cucumbers.

4.2 Materials and Methods

4.2.1 Fungal and bacterial strains

Six *Trichoderma* isolates were used in this study. These are: *T. atroviride* P. Karsten (Isolate SY3A); *T. harzianum* Rifai (Isolate SYN); *T. pseudokoningii* Rifai (Isolate SYN4), *T. atroviride* P. Karsten (Isolate SYN6), Isolate SY2F (unidentified strain) and a commercial strain, Eco-T® (active ingredient, a strain of *T. harzianum*). Three unidentified *Bacillus* species were used in this study, Isolates B69, B77 and B81.

4.2.2 Formulation of Trichoderma *isolates in kaolin*

The *Trichoderma* isolates were all grown and formulated according to a protocol used for the commercial production of Eco-T® (*T. harzianum*). Kaolin is used as a carrier and each of the formulated isolates contains approximately 10^8 spore.g^{-1} (Dr Mike Morris, Plant Health Products, (Pty) Ltd., Nottingham Road, Pietermaritzburg, Republic of South Africa).

4.2.3 Preparation of Bacillus *cell suspensions*

Bacillus isolates were cultured separately in 250ml conical flasks containing 100ml of sterilized tryptone soy broth (Merck) medium. Each flask was inoculated with a loopful of *Bacillus* spp. isolate cultured on tryptone soy agar (Merck) (30^0C, 48h). Three replicates were made for each isolate and incubated at 30^0C for 72h in a water bath shaker at 150rpm (GFL® 1083, Labortechnik). Cell suspensions were centrifuged at 9000 x *g* for 20min (Beckman J2 HS centrifuge). Cell pellets were then resuspended and washed twice with sterile distilled water. Final cell pellets were diluted with sterile distilled water (approximately 500ml). Cell numbers were adjusted to approximately 10^9 cfu.ml^{-1} for each of the *Bacillus* isolate as

determined by dilution plating and the optical density of each *Bacillus* sp. cell suspension was read at 540nm (MILTON ROY Spectronic 301 spectrophotometer). This procedure was repeated each time fresh cell suspensions were needed.

4.2.4 Seeds

Dry bean (*Phaseolus vulgaris* L.) cv PAN 148 and cucumber (*Cucumis sativus* L.) cv Ashley seeds were used for growth promotion and biological control studies respectively. Untreated dry bean seeds were obtained from Pannar Seeds (Pty) Ltd., Greytown, Republic of South Africa. Cucumber seeds were obtained from Starke Ayres Seed Company Ltd., Pietermaritzburg, Republic of South Africa. The fungicide treated cucumber seeds were washed eight times with distilled water to reduce fungicide residues and air-dried before use.

4.2.5 Seed treatment procedures

(a) Trichoderma *isolates*

Six *Trichoderma* sp. kaolin formulations (4g) containing (approximately 10^8 spores.g^{-1}) were mixed separately with 2% (w/v) sterile carboxymethyl cellulose (CMC) sticker suspensions (20ml) in 100ml glass beakers to form a slurry. Sterile distilled water was used for the CMC sticker suspensions. Seeds were then added to each slurry suspension, mixed and allowed to soak for 30min. The treated seeds were placed in sterile 90mm Petri dishes and air-dried on a laminar flow bench 12-18h.

(b) Bacillus *isolates*

Four grams of kaolin was added to three 100ml glass beakers. To each beaker 20ml of 2%(w/v) sterile carboxymethyl cellulose (CMC) sticker made up with each *Bacillus* sp. cell suspension was added and mixed to form a slurry. Seeds were then added to each slurry suspension, mixed and allowed to soak for 30min. The treated seeds were placed in a sterile 90mm Petri dish and air-dried on a laminar flow bench for 12-18h.

(c) With mixtures of Trichoderma-Bacillus *suspension*

For combined inoculation of *Trichoderma* and *Bacillus* isolates, 20ml of 2% (w/v) sterile carboxymethyl cellulose (CMC) sticker was made from a *Bacillus* sp. cell suspension and mixed with 4g of *Trichoderma* sp. formulation to form a slurry. Seeds were then added to the slurry, mixed and allowed to soak for 30min. The treated seeds were placed in sterile 90mm

Petri dishes and air-dried on a laminar flow bench for 12-18h. This procedure was repeated for each *Trichoderma-Bacillus* combination.

4.2.6 Plant growth promotion of dry bean (Phaseolus vulgaris L.) *cv PAN 148*

4.2.6.1 Seedling trials

Treated dry bean seeds (24 cells per tray) were planted into Speedling® 24 trays filled with composted pine bark. A total of 28 treatments, with three replicates, made up of six *Trichoderma* and three *Bacillus* isolates in their respective combinations, were planted. Control seeds were treated with kaolin only. Three replicate trays were established for each treatment. The trays were watered with tap water and placed in a germination room at 20-24^{0}C for 2 days. The trays were subsequently moved to a polycarbonate greenhouse tunnel maintained at 22-26^{0}C. Trays were arranged in a randomised block design and irrigated three times daily by microjet overhead irrigation (Inverted mini wobbler, Sennenger, U.S.A). The irrigation water was maintained at 20^{0}C by means of a temperature controlled heating system (Pro Heat 2000 Plus, Republic of South Africa) and was supplemented with NPK soluble fertilizer [3:1:3(38) complete] (Ocean Agriculture, Mulders Drift, Republic of South Africa) at a rate of 1g.l^{-1}. Germination and growth of seedlings was monitored for 5 weeks. Seedlings were harvested at their base at soil level, placed in a paper bag and dried at 70^{0}C for 48h to determine the total dry weight of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated and results pooled for statistical analysis.

4.2.6.2 Rhizotron studies

One *Trichoderma* isolate (*T. atroviride* SYN6), three *Bacillus* isolates (B69, B77 and B81) and a combination of *T. atroviride* SYN6 and *Bacillus* isolate B69 were assessed in rhizotrons for their effect on root and shoot growth of dry bean. Briefly, the rhizotrons were made out of two plexiglass (100 x 150mm) plates held together with butterfly screws and separated by a silicone tube spacer (15mm). The nature and design of the rhizotrons was similar to those described by James *et al*. (1985). Using a small scoop, the rhizotrons were filled with Umgeni sand that had been previously sifted (2mm pore size sieve) and steam pasteurised (100^{0}C at 40 pounds pressure) for 60min. Dry bean seeds were treated as previously described (Section 4.2.5) and for each treatment, four rhizotrons were planted with one seed per rhizotron. Each rhizotron was covered with aluminium foil to prevent daylight from

reaching the roots and then watered with tap water and left in a germination room for 2 days. They were then moved into a growth chamber maintained at 18-25^{0}C and 60% relative humidity (Controlled Environment Research Unit, University of KwaZulu-Natal, Republic of South Africa). A 12h daylight period was maintained with a light intensity of 302.03 PAR $\mu mol^{-1}.m^2.s^1$ being achieved. Seedlings were monitored for 5 weeks. On germination, each seedling was watered daily (25ml) with NPK soluble fertilizer [3:1:3(38) complete] at a rate of 0.5g.l^{-1}. The volume of water was increased to 50ml per rhizotron after 2 weeks and subsequently to two waterings a day (mornings and evenings) from the third week till the end of the experiment. The experiment was repeated twice and results pooled for statistical analysis.

(a) Root area measurements (Image Analysis)

Replicate seedlings from each treatment and rhizotron were harvested at the base of the plant after 5 weeks of growth. The roots were carefully washed five times in basins containing tap water and then placed in plastic bags and refrigerated until root area measurements could be performed. Root samples from replicate treatments were finely spread on a scanner and covered with a piece of graph paper to allow for calibration of the system. Images were then captured, calibrated, manipulated and then root area measurements taken using Soft Imaging System (SIS®) 3.0 image analysis software. Four measurements were made per replicate root sample and the mean area measurement determined.

(b) Shoot and root dry biomass measurements

Roots and shoots of seedlings from each rhizotron were both dried at 70^{0}C for 48h in an oven and then their respective dry weight were determined (OHAUS Precision Plus, model 34BL99, Dynamics Corporation of America, New Hartford, Connecticut, USA).

4.2.6.3 Dry bean yield trials

Two successive greenhouse trials were established to determine the effect of *Trichoderma* and *Bacillus* isolates as well as their respective combinations on dry bean yields. Plastic growing bags (5*l* volume) were filled with approximately 4*l* of composted pine bark growing medium. Dry bean seeds were treated as previously described (Section 4.2.5). A total of 28 treatments, including the control, were planted. The treatments comprised of six *Trichoderma* sp. and three *Bacillus* sp. isolates assessed individually and in combinations. For each treatment two seeds were planted into each of four plastic bags (four replicates per treatment) giving a total

of 112 plastic bags. The bags were arranged in a randomised block design in a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. The plastic bags were drip irrigated twice a day. The irrigation water was supplemented with NPK soluble fertilizer [3:1:3(38) complete] at a rate of 1g.l^{-1} and plant growth was monitored until fruiting. To avoid possible competition between plants, seedlings were thinned to one plant per plastic bag, 1 week after germination. Bean pods were allowed to mature and dry completely before being harvested. The experiment was repeated once.

(a) Harvesting of dry bean pods

Bean pods from each plant were harvested separately. The pods were shelled and seeds from each plant were then weighed separately (OHAUS Precision Plus, model 34BL99, Dynamics Corporation of America, New Hartford, Connecticut, USA).

(b) Determination of percentage protein and fat contents of bean seeds

Percentage protein and fat contents of the dry beans were determined for the following nine treatments: *T. atroviride* SY3A; *T. pseudokoningii* SYN4; *T. atroviride* SY3A + *Bacillus* B77; *T. atroviride* SY3A + *Bacillus* B81; *T. pseudokoningii* + *Bacillus* B81; *T. atroviride* SYN6 + *Bacillus* B69; *T. atroviride* SYN6 + *Bacillus* B77, and the untreated control. Percentage protein was analysed in a LECO FP2000 Nitrogen Analyser (LECO Corporation, Michigan, USA) using the AOAC International (2002) methods of analyses. A Buchi 810 Soxhlett Fat Extractor (Buchi Laboratoriums-Technik AG, Posfach, Germany) was used for fat extraction and the percentage fat was calculated on the gravimetric analysis using the AOAC International (2002) methods of analyses.

4.2.7 Biological control of R. solani *damping-off*

(a) Growth and preparation of R. solani

Rhizoctonia solani (PPRI Accession number 03212) previously isolated from diseased cabbage (*Brassica oleracea* L. var. *capitata*) seedlings (Chapter Two) was sub-cultured onto V8 agar medium and the agar plates were incubated at 26±1^{0}C until fully colonized.

(b) Greenhouse seedling trial

Cucumber seeds were treated as previously described (Section 4.2.5). Speedling® 24 trays were half filled with composted pine bark (Potting Mix, Gromed). Pathogen inoculation was

achieved by placing a 4mm square V8 agar plug of *R. solani* in the centre of each cell directly on top of the growth medium. The cells were then filled and the treated seeds planted. Controls using seeds coated solely with kaolin were also established. Disease free control trays received 4mm agar plugs with no *R. solani*, whereas diseased control trays received plugs with *R. solani*. Three replicate trays were established for each treatment. The trays were watered and left in the germination room at 20-24^{0}C for two days. The trays were then moved to a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. Treatments were arranged in a randomised block design with 29 treatments and three replicates. Trays were irrigated three times a day by microjet overhead irrigation (Inverted mini wobbler, Sennenger, U.S.A). The water used was maintained at 20^{0}C using a temperature controlled heating system (Pro Heat 2000 Plus, Republic of South Africa) and contained NPK soluble fertilizer [3:1:3 (38)] complete at a rate of 1g.l^{-1}. Seedling survival was rated after 4 weeks. The plant material was then harvested at their base at soil level and subsequently dried at 70^{0}C for 48h to determine the total dry weight of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated once.

4.2.8 Statistical analysis

A general linear model (GLM) was used to run an ANOVA on all data collected. If the ANOVA was significant, ($P<0.05$) the means were separated using the Students Newman Keul's test using SAS (1987). Where necessary, multivariate cluster analysis was used in grouping the performance of all treatments based on variables measured.

4.3 Results

4.3.1 Plant growth promotion

4.3.1.1 Seedling trials in the greenhouse tunnels

Table 4.1 reveals that a combined inoculation of *T. atroviride* SYN6 and *Bacillus* B69 recorded the highest averaged seedling dry biomass of dry beans (33.1g.plot^{-1}) which was significantly greater ($P < 0.05$) than 19 of the 28 treatments including the uninoculated control. This was closely followed by single inoculations of *Bacillus* B77, B69 and a combined inoculation of Eco-T® + *Bacillus* B69 with mean dry seedling biomasses of 32.2, 31.2 and 28.9g.plot^{-1}, respectively. Single inoculation of *Bacillus* B77 gave significantly higher dry seedling biomass (32.2g.plot^{-1}) than the uninoculated control (23.1g.plot^{-1}) as well

as single inoculations of all *Trichoderma* isolates, except Eco-T® and *T. pseudokoningii* SYN4 (Table 4.1).

Only four treatments, *T. atroviride* SYN6 + *Bacillus* B69, Eco-T® + *Bacillus* B69, single inoculations of *Bacillus* B77 and B69 produced over 20% increase in seedling dry biomass (43.4, 25.3, 39.3 and 34.9%, respectively) over the uninoculated control (Table 4.1). The remainder of the single treatments and/or combinations recorded increases between 1-17% over the uninoculated control, except *T. pseudokoningii* SYN4, *T. harzianum* SYN, *T. atroviride* SY3A + *Bacillus* B81 and Eco-T® + *Bacillus* B81 which recorded mean dry seedling biomass lower than the uninoculated control (Table 4.1).

Cluster analysis was used to group all isolates, their combinations and control treatment according to performance *in vivo* on increase/decrease seedling growth. Using Ward's cluster analysis method (SAS, 1987), a four-cluster solution was developed based on average seedling dry biomass per plot. This is shown on the dendogram presented in Fig. 4.1. The clusters used were to ensure that the isolates, their combinations and control treatment were exclusively classified in one of the defined clusters.

Table 4.1 Dry biomass of bean seedling as influenced by single and dual inoculations of *Trichoderma* and *Bacillus* isolates in Speedling®24 trays grown under greenhouse conditions after 4 weeks.

Isolates/Combinations/Treatments	Mean dry seedling biomass plot^{-1} (g) after 4 weeks [a]		% Dry seedling biomass plot^{-1} (% of uninoculated control) after 4 weeks [*]
Uninoculated Control	23.12	cd	100.00 [0]
Trichoderma spp. SY2F	23.14	cd	100.95 [0.95]
T. atroviride SY3A	23.33	cd	100.91 [0.91]
T. pseudokoningii SYN4	25.46	abc	110.12 [10.12]
T. atroviride SYN6	23.66	d	102.34 [2.34]
T. harzianum SYN	22.97	cd	99.35 [- 0.65]
Eco-T®	26.82	abcd	116.65 [16.65]
Bacillus B69	31.21	abc	134.99 [34.99]
Bacillus B77	32.21	ab	139.32 [39.32]
Bacillus B81	24.51	cd	106.01 [6.01]
Trichoderma spp. SY2F + *Bacillus* B69	25.34	bcd	109.60 [9.60]
Trichoderma spp. SY2F + *Bacillus* B77	23.59	cd	102.03 [2.03]
Trichoderma spp. SY2F + *Bacillus* B81	24.34	cd	105.28 [5.28]
T. atroviride SY3A+ *Bacillus* B69	24.44	cd	105.70 [5.70]
T. atroviride SY3A+ *Bacillus* B77	27.05	abcd	117.00 [17.00]
T. atroviride SY3A+ *Bacillus* B81	23.09	cd	99.87 [- 0.13]
T. pseudokoningii SYN4 + *Bacillus* B69	26.87	abcd	116.22 [16.22]
T. pseudokoningii SYN4 + *Bacillus* B77	25.98	bcd	112.37 [12.37]
T. pseudokoningii SYN4 + *Bacillus* B81	23.51	cd	101.69 [1.69]
T. atroviride SYN6 + *Bacillus* B69	33.16	a	143.45 [43.45]
T. atroviride SYN6 + *Bacillus* B77	23.67	cd	102.38 [2.38]
T. atroviride SYN6 + *Bacillus* B81	25.38	bcd	109.78 [9.78]
T. harzianum SYN + *Bacillus* B69	25.35	bcd	109.65 [9.65]
T. harzianum SYN + *Bacillus* B77	25.38	bcd	109.78 [9.78]
T. harzianum SYN + *Bacillus* B81	23.71	cd	102.55 [2.55]
Eco-T®+ *Bacillus* B69	28.99	abcd	125.39 [25.39]
Eco-T®+ *Bacillus* B77	25.16	bcd	108.82 [8.32]
Eco-T®+ *Bacillus* B81	22.99	cd	99.44 [- 0.56]
F-ratio	3.50		
P-level	0.0001		
% CV	10.31		
Significance	* * *		

[a] Values followed by different letters within a column are significantly different (Students Newmans Keul's test, P = 0.05); [*] Values in parentheses indicates percentage increase/decrease of seedling dry biomass over uninoculated control; ***, Significant at $P \leq 0.001$.

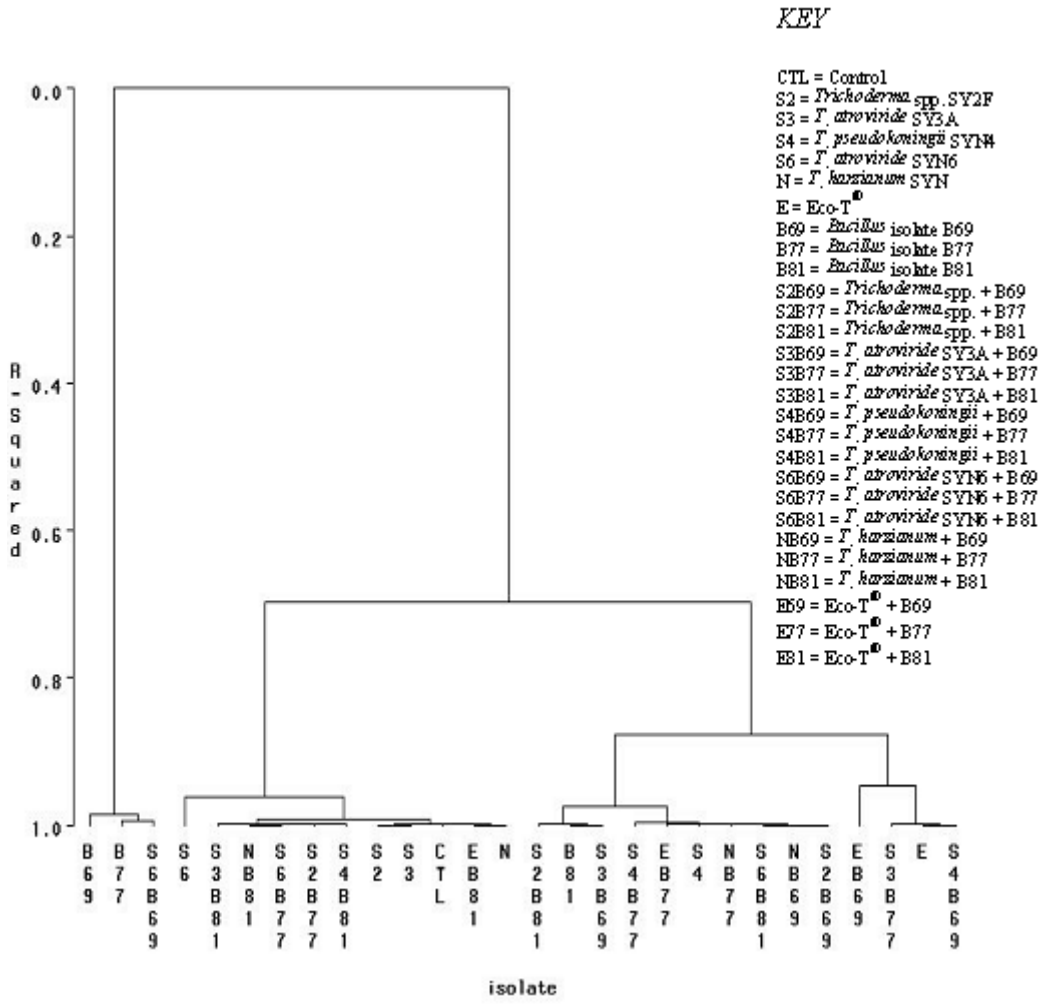

Fig 4.1 Dendogram of isolates/combinations and treatment groupings according to percentage bean seedling dry biomass 4 weeks after planting in the greenhouse

Table 4.2 presents the number of cluster groupings, mean seedling dry biomass values, percentage increase/decrease over control treatment with the F-ratio and corresponding significance level.

Cluster 1 contains eleven members and includes the uninoculated control. This group of treatments recorded the lowest seedling dry biomass.

Table 4.2 Cluster groupings and members of each group of treatments with the corresponding mean percentage dry seedling biomass

Cluster Number	Cluster Members	% Dry seedling weight per plot (% of control) after 4 weeks
1	Control (uninoculated)	100.00
1	Eco-T® + *Bacillus* B81	99.44
1	*T. harzianum* SYN	99.35
1	*T. harzianum* SYN + *Bacillus* B81	102.55
1	*Trichoderma* spp. SY2F	100.95
1	*Trichoderma* spp. SY2F + *Bacillus* B77	102.03
1	*T. atroviride* SY3A	100.91
1	*T. atroviride* SY3A + *Bacillus* B81	99.87
1	*T. pseudokoningii* SYN4+ *Bacillus* B81	101.69
1	*T. atroviride* SYN6	93.35
1	*T. atroviride* SYN6 + *Bacillus* B77	102.38
2	*Bacillus* B81	106.01
2	Eco-T® + *Bacillus* B77	108.82
2	*T. harzianum* SYN + *Bacillus* B69	109.65
2	*T. harzianum* SYN + *Bacillus* B77	109.78
2	*Trichoderma* spp. SY2F + *Bacillus* B69	109.60
2	*Trichoderma* spp. SY2F + *Bacillus* B81	105.28
2	*T. atroviride* SY3A + *Bacillus* B69	105.70
2	*T. pseudokoningii* SYN4	110.12
2	*T. pseudokoningii* SYN4 + *Bacillus* B77	112.37
2	*T. atroviride* SYN6 + *Bacillus* B81	109.78
3	Eco-T®	116.65
3	Eco-T® + *Bacillus* B69	134.32
3	*T. atroviride* SY3A + *Bacillus* B77	117.00
3	*T. pseudokoningii* SYN4 + *Bacillus* B69	116.22
4	*Bacillus* B69	134.99
4	*Bacillus* B77	139.32
4	*T. atroviride* SYN6 + *Bacillus* B69	143.45
F-ratio		144.62
P-value		0.0001
% CV		2.71
Significance		* * *

Cluster 2 contains ten treatments and is characterized by isolates and combinations with 5-12% increase in seedling dry biomass over the uninoculated control. Cluster 3 is made up of only four members and was considered to have achieved a moderate performance while Cluster 4 contains three members and were the best performing treatments in terms of seedling dry biomass and percentage values compared to the uninoculated control as presented in Table 4.2 and Fig 4.2.

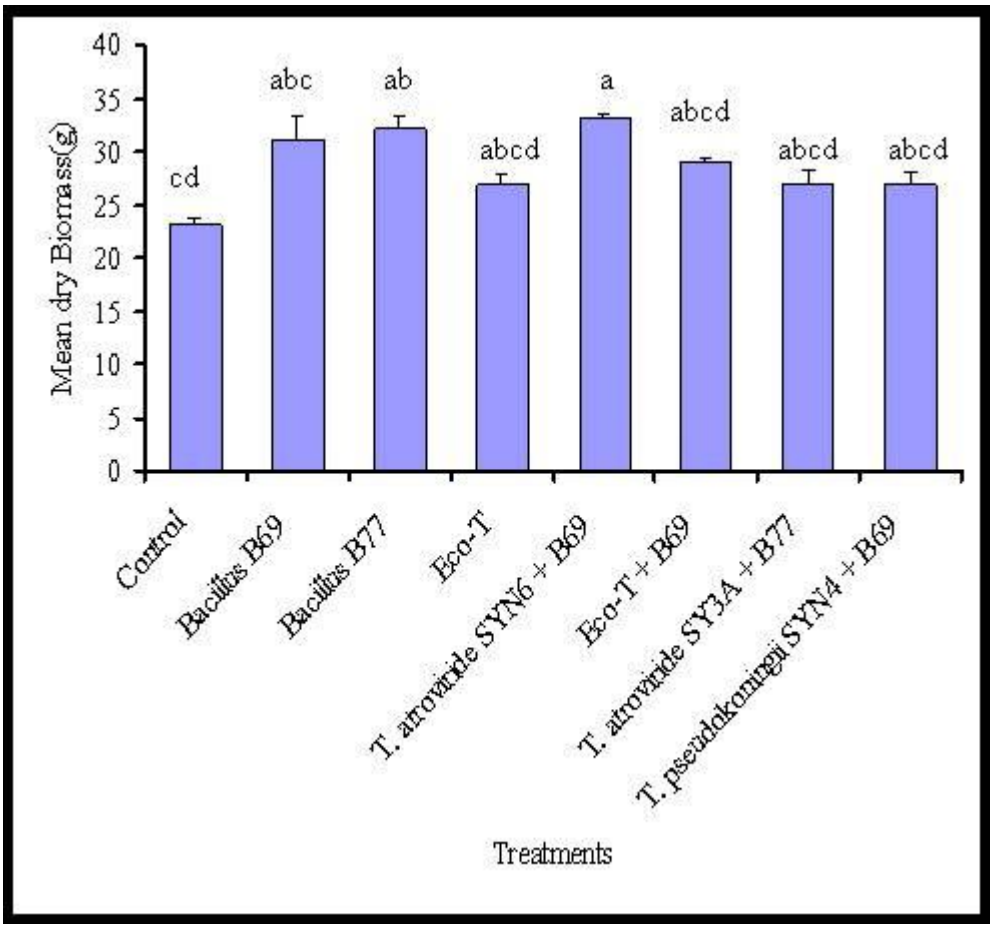

Fig 4.2 Graphical representations of the best performing treatments (members of cluster 3 and 4 groupings) compared to uninoculated control on dry biomass of bean seedlings after 4 weeks of growth in the tunnels. Bar values for each treatment with the same letter do not differ significantly according to Student Newman Keul's test ($P < 0.05$).

4.3.2 Growth promotion studies in rhizotrons

The *Bacillus* and *Trichoderma* isolates and combinations selected for the rhizotron studies were based on the results obtained from the *in vivo* greenhouse seedling studies. The data in Table 4.3 reveals an increase in the shoot and root dry biomass and root area of bean seedlings arising from inoculations with *Bacillus* B69, *Bacillus* B77, *T. atroviride* SYN6, and a combination of *T. atroviride* SYN6 and *Bacillus* B69. Maximum shoot dry biomass was obtained in a combined inoculation of *T. atroviride* SYN6 and *Bacillus* B69. This treatment showed significant ($P \leq 0.05$) increase in the shoot dry biomass over the uninoculated control and *Bacillus* B81, but did not differ significantly from *Bacillus* B69, *Bacillus* B77 and *T. atroviride* SYN6 inoculations.

Table 4.3 Dry shoot and root biomass and root area of bean seedlings as influenced by single and dual inoculations of *Trichoderma* and *Bacillus* isolates in rhizotrons grown under growth chamber conditions after 5 weeks.

Isolates/Combinations/ Treatments	Mean dry shoot biomass (g) after 5 weeks	% Dry shoot biomass (% of uninoculated control) after 5 weeks	Mean dry root biomass (g) after 5 weeks	% Dry root biomass (% of uninoculated control) after 5 weeks	Mean root area (mm^2) after 5 weeks	% Root area (% of uninoculated control after 5 weeks
Uninoculated control	2.47 b	100 [0]	1.40 ab	100 [0]	17476.20 a	100 [0]
Bacillus B69	3.48 ab	140.89 [40.89]	1.59 ab	113.57 [13.57]	23639.52 a	135.27 [35.27]
Bacillus B77	3.29 ab	133.20 [33.20]	1.63 ab	116.43 [16.47]	20931.60 a	119.77 [19.77]
Bacillus B81	2.53 b	102.43 [2.43]	1.20 b	85.71 [- 14.71]	23240.08 a	132.98 [32.98]
T. atroviride SYN6	3.14 ab	127.13 [27.13]	1.60 ab	114.23 [14.23]	23308.74 a	133.37 [33.37]
T. atroviride SYN6 + *Bacillus* B69	3.96 a	160.32 [60.32]	1.91 a	136.43 [36.43]	23352.40 a	133.62 [33.63]
F-ratio	3.41		2.75		2.43	
P-value	0.02		0.05		0.07	
% CV	19.73		18.58		14.13	
Significance	* *		*		ns	

[a] Values followed by different letters are significantly different (Students Newmans Keul's test, P = 0.05).
[*] Values in parentheses indicates percentage increase/decrease of shoot and root dry biomass or root area over uninoculated control; ns = Not significant (P > 0.05)

However, all the inoculation treatments, except for *Bacillus* B81, showed an increase in dry root biomass compared to the uninoculated control but were not significant (P > 0.05) (Table 4.3 and Fig 4.3). The combined inoculation of *T. atroviride* SYN6 and *Bacillus* B69 gave the highest dry root biomass of all the treatments. This treatment was the only treatment that showed significant (P < 0.05) increase in root dry biomass over *Bacillus* B81 inoculation.

The root area measurements was maximal for *Bacillus* B69 followed by dual inoculation of *T. atroviride* SYN6 + *Bacillus* B69 and single inoculations of *T. atroviride* SYN6 and *Bacillus* B81 (Table 4.3). However, none of these treatments were significantly different (P > 0.05) from the uninoculated control (Table 4.3).

In all cases, except the dry root biomass, the dual inoculation of *T. atroviride* SYN6 + *Bacillus* B69 was better than any of the bacterial or fungal inoculants used in isolation (Fig 4.4 and Fig 4.5).

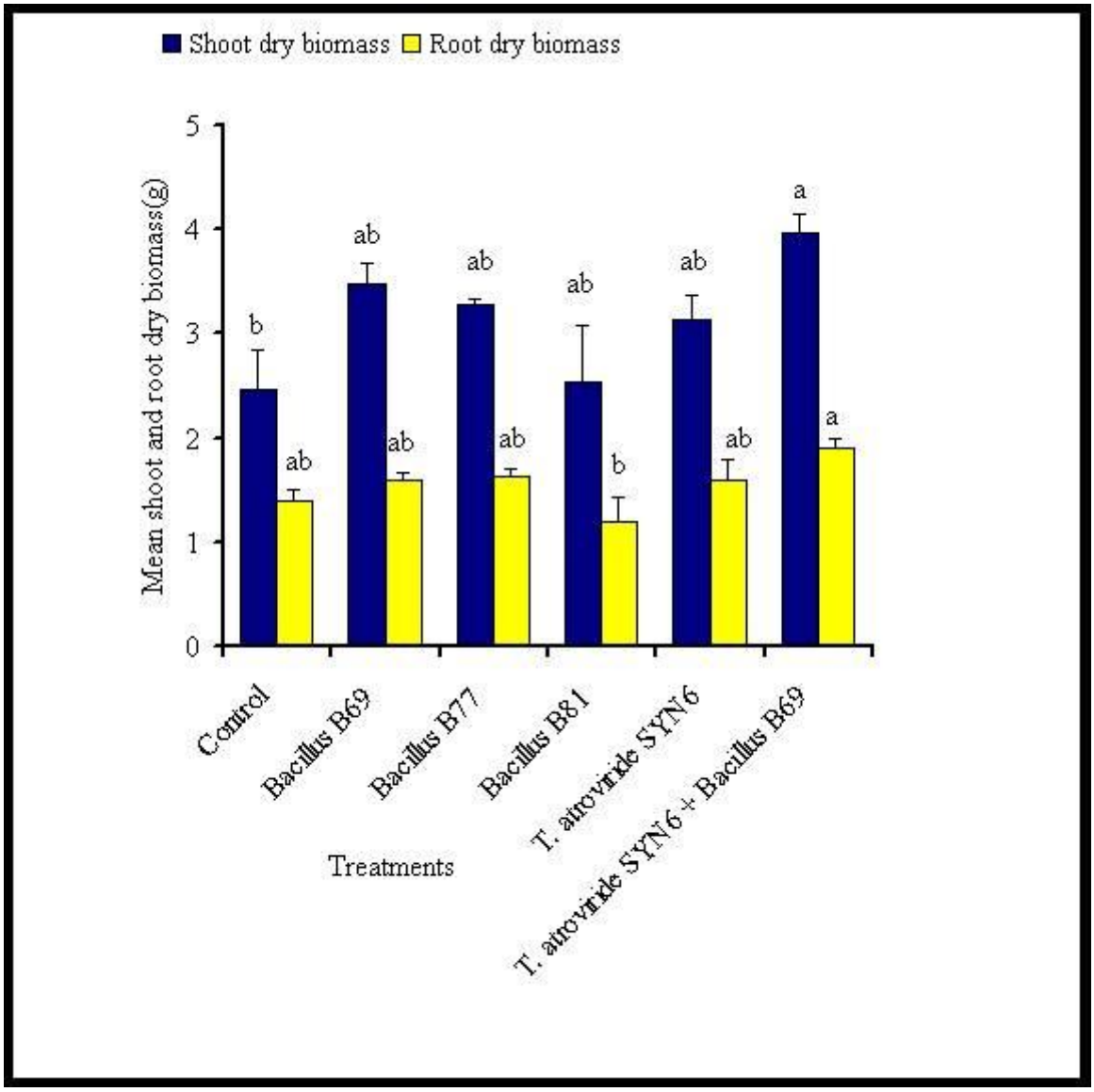

Fig 4.3 Effect of single and dual inoculations of *Trichoderma* and *Bacillus* isolates on dry shoot and root biomass of bean seedlings grown in rhizotrons under growth chamber conditions for 5 weeks. Bar values for each treatment with same letter do not differ significantly according to Student Newman Keul's test ($P > 0.05$).

Fig 4.4 Effect of dual inoculation of *T. atroviride* SYN6 + *Bacillus* B69 on 3 week old bean seedlings compared to single inoculation of *Bacillus* B69 and uninoculated control

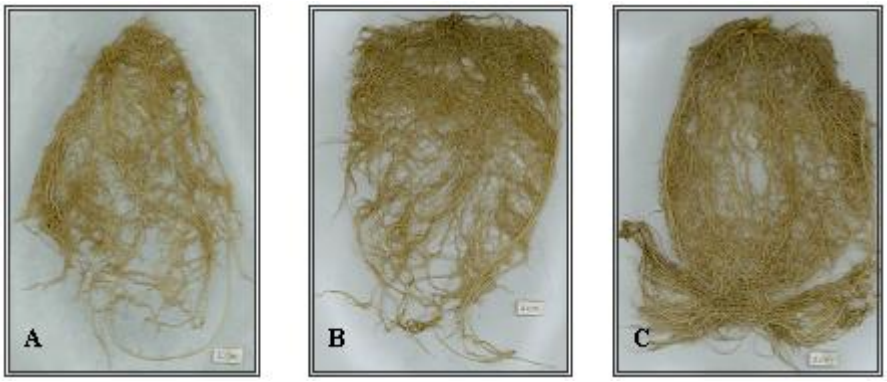

Fig 4.5 Enhanced root development with application of *Bacillus* B69 (B) and *T. atroviride* SYN6 + *Bacillus* B69 (C) after 5 weeks compared to uninoculated control (A).

4.3.3 Dry bean yield trial in tunnels

The mean bean yield and percentage increases or decreases over the control for Trial 1 and Trial 2 are presented below in Table 4.4 and Table 4.5. Each of the tables contains four cluster groupings, each group made up of isolates, combinations or treatments with similarities in response pattern.

Table 4.4 Cluster groupings of bean yield Trial 1 and members of each group of treatments with the corresponding mean yield and percentage increase or decrease (indicated in parentheses) over uninoculated control

Cluster Number	Cluster Members	Mean weight (g) of bean yield	% Bean yield (% of uninoculated control)
1	Uninoculated control	63.71	100.00 [0]
1	*Trichoderma* spp. SY2F + *Bacillus* B77	45.87	72.00 [-28]
1	Eco-T® + *Bacillus* B77	45.17	70.90 [-29.10]
1	*Trichoderma* spp. SY2F + *Bacillus* B81	62.04	97.38 [-2.62]
1	*Trichoderma* spp. SY2F + *Bacillus* B69	63.46	99.61 [-0.39]
1	*T. atroviride* SY3A + *Bacillus* B69	65.39	102.64 [2.64]
1	*T. pseudokoningii* SYN4+ *Bacillus* B69	63.80	100.14 [0.14]
1	Eco-T®	64.16	100.71 [0.71]
1	*T. atroviride* SYN6 + *Bacillus* B69	61.93	97.21 [-2.79]
2	*T. atroviride* SYN6 + *Bacillus* B77	69.39	108.92 [8.92]
2	*T. atroviride* SY3A + *Bacillus* B81	69.80	109.56 [9.56]
2	*T. pseudokoningii* SYN4+ *Bacillus* B81	70.80	111.13 [11.13]
2	*T. atroviride* SY3A	78.86	123.78 [23.78]
2	*T. atroviride* SYN6 + *Bacillus* B81	67.39	105.78 [5.78]
2	*T. pseudokoningii* SYN4+ *Bacillus* B77	68.32	107.24 [7.24]
2	*T. pseudokoningii* SYN4	76.21	119.62 [19.62]
2	*T. atroviride* SY3A + *Bacillus* B77	69.09	108.44 [8.44]
2	*Bacillus* B69	68.19	106.98 [6.98]
3	*T. atroviride* SYN6	53.96	83.13 [-16.87]
3	*T. harzianum* SYN + *Bacillus* B81	53.98	84.73 [-15.27]
3	Eco-T® + *Bacillus* B81	59.33	93.13 [-16.87]
3	*T. harzianum* SYN + *Bacillus* B77	53.19	83.49 [-16.51]
3	*T. harzianum* SYN + *Bacillus* B69	55.16	86.58 [-13.42]
3	*Bacillus* B81	58.96	92.54 [-7.46]
3	Eco-T® + *Bacillus* B69	52.46	82.34 [-17.66]
3	*Bacillus* B77	57.87	90.83 [-9.17]
4	*Trichoderma* spp. SY2F	43.66	68.53 [-31.47]
4	*T. harzianum* SYN	43.95	68.98 [-31.02]
F-ratio		70.02	
P-level		0.0001	
% CV		4.64	
Significance		* * *	

Table 4.5 Cluster groupings of bean yield Trial 2 and members of each group of treatments with the corresponding mean yield and percentage increase or decrease (indicated in parentheses) over uninoculated control

Cluster Number	Cluster Members	Mean weight (g) of bean yield	% Bean yield (% of uninoculated control)
1	*Trichoderma* spp. SY2F + *Bacillus* B81	68.07	119.74 [19.74]
1	*Trichoderma* spp. SY2F + *Bacillus* B69	65.96	116.02 [16.02]
1	Eco-T®	78.10	137.38 [37.38]
2	Uninoculated control	56.85	100.00 [0]
2	*T. atroviride* SYN6	55.30	97.27 [-2.73]
2	*Bacillus* B69	60.56	106.52 [6.52]
2	*T. atroviride* SY3A + *Bacillus* B81	58.83	103.48 [3.48]
2	*T. pseudokoningii* SYN4 + *Bacillus* B81	59.05	103.86 [3.86]
2	*T. atroviride* SY3A	61.67	108.47 [8.47]
2	*Bacillus* B81	55.32	97.31 [-2.69]
2	Eco-T® + *Bacillus* B77	56.12	98.72 [-1.28]
2	Eco-T® + *Bacillus* B69	58.04	102.09 [2.09]
2	*T. pseudokoningii* SYN4 + *Bacillus* B69	56.21	98.87 [-1.13]
2	*T. atroviride* SY3A + *Bacillus* B77	57.63	101.37 [1.37]
3	*Trichoderma* spp. SY2F	34.83	61.26 [-38.73]
3	*T. harzianum* SYN	46.53	81.84 [-18.16]
3	*T. harzianum* SYN+ *Bacillus* B81	44.27	77.87 [-22.13]
3	Eco-T® + *Bacillus* B81	45.53	80.09 [-19.91]
3	*T. harzianum* SYN+ *Bacillus* B77	47.38	83.34 [-16.66]
3	*T. atroviride* SYN6 + *Bacillus* B81	43.92	77.26 [-22.74]
3	*T. pseudokoningii* SYN4+ *Bacillus* B77	47.58	83.69 [-16.31]
3	*Bacillus* B77	43.39	76.32 [-23.68]
3	*T. atroviride* SYN6 + *Bacillus* B69	47.30	83.20 [-16.80]
4	*Trichoderma* spp. SY2F + *Bacillus* B77	51.69	90.92 [-9.08]
4	*T. atroviride* SYN6 + *Bacillus* B77	52.76	92.81 [-7.19]
4	*T. harzianum* SYN + *Bacillus* B69	48.56	85.42 [-14.58]
4	*T. atroviride* SY3A + *Bacillus* B69	50.76	89.29 [-10.71]
4	*T. pseudokoningii* SYN4	48.80	85.84 [-14.16]
F-ratio		55.65	
P-value		0.0001	
%CV		6.22	
Significance		* * *	

The data in Tables 4.4 reveal that members in Cluster 2 were the best performers in Trial 1 followed by members in Cluster 1, Cluster 3 and Cluster 4 respectively, while in Table 4.5 (Trial 2) members belonging to Cluster 1 were the best performers followed by members in Cluster 2, Cluster 4 and Cluster 3 in that order. Increases or decreases in yield between treatments in the two separate trials were very disparate. For example, compared to the uninoculated control, *Trichoderma* spp. SY2F + *Bacillus* B81, *Trichoderma* spp. SY2F + *Bacillus* B69 and Eco-T® + *Bacillus* B69 reduced yield in Trial 1 but increased yield in Trial 2 by 19.7, 16.0 and 2.0% respectively in contrast to decreases in yield of -2.6, -0.3 and -17.6% in Trial 1.

Dual inoculations of *T. atroviride* SY3A + *Bacillus* B77 and *T. atroviride* SY3A + *Bacillus* B81 were consistently better in the two Trials than the single inoculations of *Bacillus* B77 and B81. However this increase was not better than single inoculation of *T. atroviride* SY3A that consistently increased yield in the two trials (Table 4.4 and 4.5). Most of the combined inoculations, however, gave a lower yield than the uninoculated control.

Single inoculations of Eco-T®, *T. atroviride* SY3A and *Bacillus* B69 consistently increased yield in Trial 1 and 2 respectively compared to the uninoculated control. Increases of 0.7, 23.7 and 6.9 and 37.3, 8.4, and 6.5% respectively were observed in Trial 1 and 2 for the three isolates compared to the uninoculated control (Table 4.4 and 4.5).

Nonparametric analysis using the cluster groupings showed that there was no correlation between the performance of the isolates/combinations and/or treatments in the seedling trial and the two yield trials (Table 4.6). Similarly, no correlation was found between the two yield trials as shown in the Chi Square (χ^2) table below.

Table 4.6 Chi Square (χ^2) test of association between: (1) seedling performance and yield performance, and (2) yield Trial 1 and 2 performances of isolates/combinations and/or treatments in the greenhouse

	Chi Square (χ^2)	P- value	Significance
Seedling Trial vs Yield Trial 1	4.05	0.87	ns
Seedling Trial vs Yield Trial 2	9.99	0.35	ns
Yield Trial 1 vs Yield Trial 2	13.66	0.13	ns

ns = Not Significant (P > 0.05)

4.3.4 Determination of percentage protein and fat contents of bean seeds

The data in Table 4.7 shows that no significant increase in percentage protein and fat content of bean seeds was observed between the two yield trials for the selected isolates/combinations and/or treatments.

Table 4.7 Percentage protein and fat content of bean seed for selected isolates/combinations and/or treatments from yield Trials 1 and 2.

Isolate/Combination/Treatment	Yield Trial 1		Yield Trial 2	
	% Protein	% Fat	% Protein	% Fat
Uninoculated Control	22.25 a	0.83 a	23.08 a	0.74 a
Eco-T®	23.25 a	0.79 a	23.35 a	0.76 a
T. atroviride SY3A	23.38 a	0.83 a	24.10 a	0.80 a
T. atroviride SY3A + *Bacillus* B77	22.75 a	0.81 a	23.53 a	0.81 a
T. atroviride SY3A + *Bacillus* B81	24.57 a	0.75 a	22.79 a	0.86 a
T. pseudokoningii SYN4	22.10 a	0.75 a	22.22 a	0.87 a
T. pseudokoningii SYN4+ *Bacillus* B81	23.83 a	0.75 a	23.56 a	0.81 a
T. atroviride SYN6 + *Bacillus* B69	23.49 a	0.78 a	23.39 a	0.83 a
T. atroviride SYN6 + *Bacillus* B77	22.04 a	0.73 a	21.38 a	0.86 a
F-ratio	0.98	1.28	0.75	1.13
P-value	0.48	0.31	0.65	0.39
%CV	6.53	7.19	9.12	8.99
Significance	ns	ns	ns	ns

[a] Values followed by the same letter within a column are significantly different (Students Newmans Keul's test, $P > 0.05$); ns = Not significant ($P > 0.05$)

4.3.5 Biological control of R. solani *damping-off*

Percentage seedling survival for the controls ranged from 36.1% for the diseased control to 98.6% for the disease free control ($P < 0.0001$) (Table 4.8). The mean dry seedling biomass yield for the diseased control was 40.0% of the yield obtained for the disease free control ($P < 0.0001$).

Table 4.8 Seedling survival and dry biomass of cucumber as influenced by single and dual inoculations of *Trichoderma* and *Bacillus* isolates in the greenhouse after 4 weeks of growth

Isolate/Combination/Treatment	Mean number of surviving seedlings after 4 weeks [a]	% Seedling survival after 4 weeks	Mean dry biomass after 4 weeks [a]	% Dry biomass after 4 weeks (% of disease free control)
Disease free control	23.67 a	98.68	18.59 a	100
Diseased control	8.67 h	36.13	7.44 ij	40.02
Trichoderma spp. SY2F	10.67 fgh	44.46	8.70 hij	46.80
T. atroviride SY3A	15.33 c	63.88	13.30 c	71.54
T. pseudokoningii SYN4	10.50 fgh	43.75	8.05 hij	43.30
T. atroviride SYN6	12.00 defgh	50.00	9.99 efghi	53.74
T. harzianum SYN	14.67 cd	61.13	13.15 c	70.74
Eco-T®	18.83 b	78.46	16.61 b	89.35
Bacillus B69	10.50 fgh	43.75	8.82 ghij	47.44
Bacillus B77	8.83 h	36.79	8.42 hij	45.29
Bacillus B81	11.83 defgh	49.29	9.78 efghi	52.61
Trichoderma spp. SY2F + *Bacillus* B69	10.17 fgh	42.36	7.34 ij	39.48
Trichoderma spp. SY2F + *Bacillus* B77	11.33 efgh	47.21	9.01 fghij	48.47
Trichoderma spp. SY2F + *Bacillus* B81	11.00 efgh	45.83	7.42 ij	39.91
T. atroviride SY3A+ *Bacillus* B69	13.00 cdefg	54.17	11.40 cdefg	61.32
T. atroviride SY3A+ *Bacillus* B77	10.77 fgh	44.88	8.20 hij	44.11
T. atroviride SY3A+ *Bacillus* B81	14.67 cd	61.13	12.57 cd	67.62
T. pseudokoningii SYN4 + *Bacillus* B69	9.83 gh	40.96	7.72 hij	41.53
T. pseudokoningii SYN4 + *Bacillus* B77	11.67 defgh	48.63	9.42 fghij	50.67
T. pseudokoningii SYN4 + *Bacillus* B81	10.00 fgh	41.67	7.77 hij	41.80
T. atroviride SYN6 + *Bacillus* B69	14.16 cde	59.04	12.65 cd	68.05
T. atroviride SYN6 + *Bacillus* B77	8.67 h	36.13	6.65 j	35.77
T. atroviride SYN6 + *Bacillus* B81	14.17 cde	59.04	13.28 c	71.44
T. harzianum SYN + *Bacillus* B69	12.83 cdefg	53.46	10.33 defgh	55.57
T. harzianum SYN + *Bacillus* B77	10.33 fgh	43.04	8.88 ghij	47.77
T. harzianum SYN + *Bacillus* B81	13.33 cdef	55.54	10.58 defgh	56.91
Eco-T®+ *Bacillus* B69	12.83 cdefg	53.46	11.55 cdef	62.13
Eco-T®+ *Bacillus* B77	10.83 fgh	45.13	8.66 hij	46.58
Eco-T®+ *Bacillus* B81	13.17 cdefg	54.88	11.97 cde	64.39
F-ratio	22.12		24.81	
P-level	0.0001		0.0001	
% CV	9.42		9.64	
Significance	* * *		* * *	

[a] Values followed by different letters within a column are significantly different (Students Newmans Keul's test, $P < 0.05$); ***, Significant at $P \leq 0.001$.

The commercial product, Eco-T®, substantially reduced pre- and post-emergence damping-off caused by *R. solani*. Compared to the diseased control, Eco-T® significantly increased seedling survival from 36.1% to 78.4% and dry shoot biomass from 40.0% to 89.3% of the biomass yield of the disease free control (Table 4.8). None of the three *Bacillus* and Eco-T® combinations were better than Eco-T® alone in terms of seedling survival and dry shoot biomass. Although not significantly different, Eco-T® + *Bacillus* B69 and Eco-T® + *Bacillus* B81 recorded better percentage seedling survival and dry shoot biomass than a single application of *Bacillus* B69 and B81 respectively (Table 4.8).

Compared to the diseased control, applications of *T. atroviride* SY3A and *T. harzianum* SYN significantly increased percentage seedling survival from 36.1% to 63.8 and 61.1% and dry shoot biomass from 40.0% to 71.5 and 70.7% respectively. No combinations of these two *Trichoderma* isolates with the *Bacillus* isolates produced better percentage seedling survival and dry shoot biomass compared to the two *Trichoderma* isolates alone. However, combinations of *T. atroviride* SY3A + *Bacillus* B69, *T. atroviride* SY3A + *Bacillus* B81 and *T. harzianum* SYN + *Bacillus* B69, *T. harzianum* SYN + *Bacillus* B81 were better than the *Bacillus* isolates alone, although the results were not significantly different (Table 4.8).

The best combinations were *T. atroviride* SY3A + *Bacillus* B81, *T. atroviride* SYN6 + *Bacillus* B69 and *T. atroviride* SYN6 + *Bacillus* B81 with percentage seedling survival of 61.13, 59.04 and 59.05% respectively. Although percentage seedling survival of *T. atroviride* SY3A + *Bacillus* B81 was not higher than *T. atroviride* SY3A alone, percentage seedling survival achieved by *T. atroviride* SYN6 + *Bacillus* B69 and *T. atroviride* SYN6 + *Bacillus* B81 was higher than *T. atroviride* SYN6 and *Bacillus* B69 and B81 alone. Similarly, percentage dry shoot biomass yield of the disease free control was significantly superior for *T. atroviride* SYN6 + *Bacillus* B69 and *T. atroviride* SYN6 + *Bacillus* B81 than each of the isolates used alone (Table 4.8).

In all cases, either used alone or in combination with the *Bacillus* isolates, *Trichoderma* spp. SY2F and *T. pseudokoningii* SYN4 did not enhance disease control (Table 4.8).

A four-cluster solution using Ward's cluster solution was developed and treatments were exclusively classified in one of the defined clusters as shown in Fig 4.6 and Table 4.9. Table 4.9 reveals that members belonging to Cluster 4 were the best and contained two treatments, the disease free control and Eco-T®. Next best in performance is Cluster 3, which was made up of nine treatments. *T. atroviride* SY3A and *T. harzianum* SYN were the single treatments found in this group. Third in performance is Cluster 1, which was also made up of nine members. This contained the highest number of single bacterial and fungal treatments. Majority of the combined treatments with *Bacillus* B77 were found in this Cluster grouping. Cluster 2 contains treatments that performed poorly. This Cluster was made up of nine members, with the diseased control in this group.

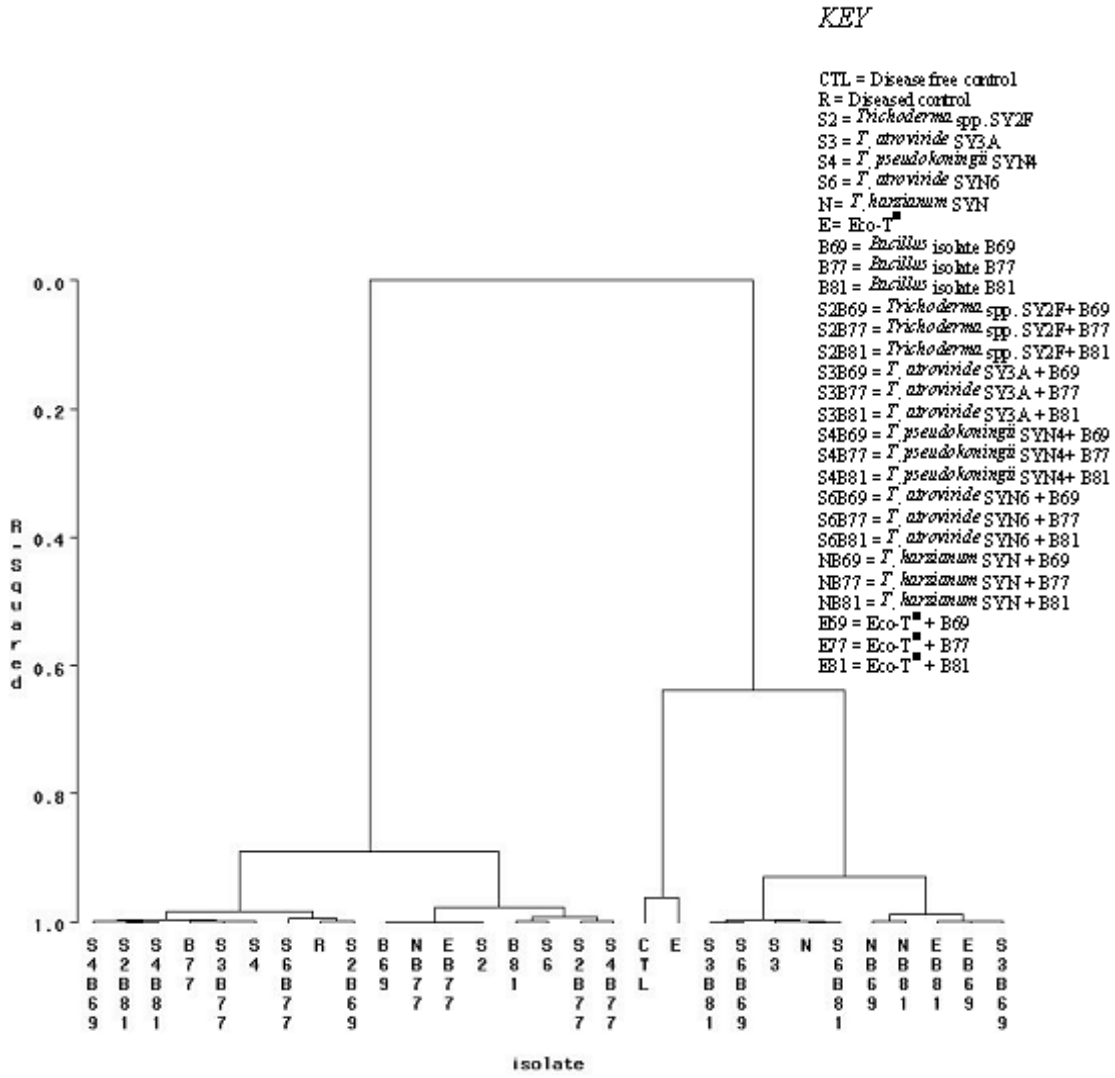

Fig 4.6 Dendogram of treatment groupings according to percentage seedling survival and dry biomass 4 weeks after planting in the greenhouse

Table 4.9 Cluster groupings and members of each group of treatments with the corresponding mean percentage seedling survival and mean dry biomass (g)

Cluster Number	Cluster Members	Mean seedling survival (%)	Mean dry biomass (g)
1	*Bacillus* B69	43.75	8.82
1	*Bacillus* B81	49.29	9.78
1	Eco-T® + *Bacillus* B77	45.13	8.66
1	*T. harzianum* SYN + *Bacillus* B69	53.46	10.33
1	*T. harzianum* SYN + *Bacillus* B77	43.04	8.88
1	*Trichoderma* spp. SY2F	44.46	8.70
1	*Trichoderma* spp. SY2F + *Bacillus* B77	47.21	9.01
1	*T. pseudokoningii* SYN4 + *Bacillus* B77	48.63	9.42
1	*T. atroviride* SYN6	50.00	9.99
2	*Bacillus* B77	36.79	45.29
2	Diseased control	36.13	7.44
2	*Trichoderma* spp. SY2F + *Bacillus* B69	42.36	7.34
2	*Trichoderma* spp. SY2F + *Bacillus* B81	45.83	7.42
2	*T. atroviride* SY3A + *Bacillus* B77	44.88	8.20
2	*T. pseudokoningii* SYN4	43.75	8.05
2	*T. pseudokoningii* SYN4 + *Bacillus* B69	40.96	7.72
2	*T. pseudokoningii* SYN4 + *Bacillus* B81	41.67	7.77
2	*T. atroviride* SYN6 + *Bacillus* B77	36.13	6.65
3	Eco-T® + *Bacillus* B69	53.46	11.55
3	Eco-T® + *Bacillus* B81	54.88	11.97
3	*T. harzianum* SYN	61.13	13.15
3	*T. harzianum* SYN + *Bacillus* B81	55.54	10.58
3	*T. atroviride* SY3A	63.88	13.30
3	*T. atroviride* SY3A + *Bacillus* B69	54.17	11.40
3	*T. atroviride* SY3A + *Bacillus* B81	61.13	12.57
3	*T. atroviride* SYN6 + *Bacillus* B69	59.04	12.65
3	*T. atroviride* SYN6 + *Bacillus* B81	59.04	13.28
4	Disease free control	98.63	18.59
4	Eco-T®	78.46	16.61
F-ratio		71.27	121.67
P-level		0.0001	0.0001
% CV		8.76	7.43
Significance		* * *	* * *

4.4 Discussion

The major objective of this Chapter was to investigate whether the potential of plant growth promotion and biological control could be enhanced through combined applications of selected *Trichoderma* and *Bacillus* spp. isolates. From the growth promotion seedling trials it was demonstrated that a combined application of *T. atroviride* SYN6 and *Bacillus* B69 achieved the highest bean seedling dry biomass (+43.4%) compared to the uninoculated control. This result was later confirmed in rhizotron studies in pasteurised sand with *T. atroviride* SYN6 + *Bacillus* B69 giving the highest shoot and root dry biomass. However, these growth improvements were not reflected in the two successive dry bean yield trials conducted in this study, nor were increases in protein or fat content in bean seeds observed.

Biological control trials in the greenhouse demonstrated that single inoculations of Eco-T®, *T. atroviride* SY3A and *T. harzianum* SYN significantly reduced *R. solani* damping-off. A general trend indicates that dual inoculations of *Trichoderma* and *Bacillus* isolates tend towards an increased suppression of damping-off better than single inoculations of the *Bacillus* isolates. This increase was consistent with combinations of either *T. atroviride* SY3A, Eco-T®, *T. atroviride* SYN6 or *T. harzianum* SYN with *Bacillus* B69 and B81.

Different mechanisms of action for the *Trichoderma* and *Bacillus* isolates may explain why some combinations of the two organisms increased plant growth and disease control than some single inoculations. The growth promotion results are in agreement with studies by Jisha and Alagawadi (1996) who demonstrated that mixtures of a strain of *Bacillus polymyxa* and *T. harzianum* increased the growth of sorghum better than each organism used alone. The *Trichoderma* and *Bacillus* isolates used in this study were primarily selected for biological control purposes, except *Bacillus* B77 that was previously shown to enhance plant growth. One would therefore not expect the selected *Trichoderma* and *Bacillus* isolates to exhibit marked plant growth promotion. The increases in bean seedling growth by selected *Trichoderma* and *Bacillus* isolates and their combinations were attributed to possible factors such as increased mineral uptake, siderophore production and/or possible production of plant growth promoters. For instance, Kumar and Dube (1992), reported that inoculation of chickpea (*Cicer arietinum* L.) and soybean [*Glycine max* L. (Merr.)] seeds with siderophore-producing fluorescent pseudomonads resulting in early seed germination, increase growth and yield. All the *Trichoderma* and *Bacillus* isolates used in this trial produced siderophores *in vitro* as one of the biological control traits (Chapter Three) and might have contributed to an increase in seedling growth. The findings from rhizotron studies suggests that there may be some plant growth promoting trait(s) which lead to increased root areas such as cytokinin production, regulation of ethylene in roots and solubilization of nutrients such as phosphorus (Tien *et al*., 1979; Arshad and Frankenberger, 1991; Glick, 1995; Jisha and Alagawadi, 1996).

Reports on *Trichoderma* and *Bacillus* as plant growth promoters have mainly been focussed on seedling growth and development where their performances have been found to vary in consistency (Kleifeld and Chet, 1992; Ousley *et al*., 1993; 1994a; Shishido *et al*., 1995; Rabeendran *et al*., 2000). In this study, increase in growth in the bean seedling trial was found to be consistent with increase in growth in the rhizotron trials.

Plant growth promoting rhizobacteria (PGPR) have been reported to increase crop yields (Kloepper *et al*., 1989; Jisha and Alagawadi, 1996; Harman, 2000; Mathre *et al*., 2000). However, results obtained during the yield trials were erratic and by no means conclusive, which raises the question whether increase in seedling performance by PGPR translates into actual increase in crop yield. The erratic performance by the isolates and/or combinations in the yield trials could partly be accounted for by some factors, which were thought to have a direct effect on yield. A possible inhibitory effect of the *Trichoderma* and *Bacillus* isolates to one another was ruled out, as this was not observed during the seedling trials in the Speedling trays and rhizotron. Moreover, greenhouse conditions provide a favourable environment for plant growth. An optimal environment is more likely to mask the effect of the *Trichoderma* and *Bacillus* isolates. It is more likely that the fungal and bacterial effect on plant growth could be seen under periods of stress as found in the field (Rabeendran *et al*., 2000). Erratic and inconsistent performances of bacterial PGPR have been reported under field conditions (Schroth and Becker, 1990). Although Kloepper *et al*. (1989) reported increase in yield as a result on inoculation of bacterial PGPR to a range of crops, decreases in yield were also common in trials.

A significant observation in this study was that plant growth promotion/seedling vigour appeared to have little or no corresponding effect on yield. Schroth and Becker (1990) noted that, "early growth promotion often is not accompanied by higher yield". They explained that early plant growth triggered by fungal and bacterial plant growth promoters may not have a corresponding increase in yield due to physiological stress caused by factors such as lack of water, nutrients and unfavourable temperatures. Furthermore, these factors may have a "levelling" effect on the treatments and hence affect plant yield. The above explanation by Schroth and Becker (1990) further clarifies the non-correlation effect observed between the seedling trial and the yield trials and also between the two yield trials. It is therefore necessary that growth measurements be taken throughout the duration of a yield/growth promotion trial, as "increased yield is desirable but not essential to demonstrate efficacy" (Schroth and Becker, 1990). The authors therefore maintain that increase in seedling vigour could be attributed to an increase in mineralization as a result of *Trichoderma* and *Bacillus* inoculations. It is therefore important to take into consideration the effect of these isolates on plant nutrition (Chapter Five).

Various *Trichoderma* and *Bacillus* spp. have been reported as being able to successfully control several plant pathogens (Rytter *et al*., 1989; Kim *et al*., 1997a; Koch, 1999; Utkhede *et al*., 1999; Zhang *et al*., 1999; Lewis and Lumsden, 2001). The results obtained for the biological control trials support the finding of Koch (1999) and Lewis and Lumsden (2001), both of which demonstrated that *Trichoderma* sp. formulations were able to reduce damping-off caused by *R. solani*. Our results indicate that none of the *Trichoderma* and *Bacillus* isolates competitively controlled *R. solani* damping-off better than the commercial *Trichoderma* strain Eco-T®. Only two of the *Trichoderma* isolates, *T. atroviride* SY3A and *T. harzianum* SYN gave percentage plant stands of 64 and 61% compared to 78% plant stand by Eco-T®. These three *Trichoderma* isolates were shown to be hyperparasitic against *R. solani in vitro* and also exhibited chitinase activity and siderophore production (Chapter Three). These mechanisms are all thought to contribute to biological control (Hadar *et al*., 1979; Kumar and Dube, 1992; Menendez and Godeas, 1998). Enhanced biological control by Eco-T compared to other *Trichoderma* isolates may have resulted from the additive action of antibiosis, as it was the only *Trichoderma* isolate that inhibited *R. solani in vitro*, suggesting the production of an anti-inhibitory compound. None of the *Bacillus* isolates were able to achieve a 50% plant stand. The highest plant stand (49%) was recorded by *Bacillus* B81. Both *Bacillus* B69 and B81 inhibited *R. solani in vitro* and also produced siderophores (Chapter Three).

Using bacterial/bacterial and fungal combinations to improve biological control has been suggested and studied (Duffy *et al*., 1996; Raupach and Kloepper, 1998; Guetsky *et al*., 2002). To the best of our knowledge, reports on the feasibility of combining *Trichoderma* and *Bacillus* spp. to improve biological control is sparse or absent. Although limited studies have been carried out on the combined effect of *Trichoderma* and *Bacillus* spp. on plant growth that has been cited in this study, there is still a lack of information on the combined effect of these two organisms on biological control. Our results indicate that none of the combinations were better than Eco-T®, *T. atroviride* SY3A and *T. harzianum* SYN. The best combination was *T. atroviride* SY3A + *Bacillus* B81 with a plant stand of 61.1% which was equal in performance to *T. harzianum* SYN. The performances, particularly of *Bacillus* B69 and B81, were enhanced by combinations with *T. atroviride* SY3A, *T. atroviride* SYN6, *T. harzianum* SYN or Eco-T®. This suggests that the *Trichoderma* isolates were largely responsible for the control of *R. solani* damping-off in this study. This suggests a possible

synergism between these two organisms leading to a better biological control than the two *Bacillus* isolates used alone.

The *in vitro* compatibility test between the *Trichoderma* and *Bacillus* isolates (Chapter Three) appears to have some predictive value for the biological control of *R. solani* damping-off by combinations of these two organisms. This probably accounts for the enhanced performances of *Bacillus* B69 and B81 in combinations with *T. atroviride* SY3A, *T. atroviride* SYN6, *T. harzianum* SYN or Eco-T®. These *Trichoderma* and *Bacillus* isolates did not inhibit each other *in vitro* (Chapter Three). Hence, improved performances of these combinations *in vivo* are consistent to some extent with the *in vitro* compatibility test. Such consistency was also reported by De Boer *et al*. (1999) using combinations of fluorescent *Pseudomonas* spp. to control fusarium wilt on radish (*Raphanus sativus* L.). Although *in vitro* compatibility tests may predict the feasibility of using two organisms together, this may not apply to all combinations as factors such as competition is difficult to test *in vitro*.

Eco-T® is the only registered *Trichoderma* product available to farmers in South Africa for soil application. Attempts to find superior *Trichoderma* isolates to augment Eco-T® were not altogether successful. Although *T. atroviride* SY3A and *T. harzianum* SYN significantly reduced damping-off in the greenhouse, the performance of these two *Trichoderma* isolates was not better than the existing Eco-T® product under the conditions in which the isolates were screened and tested for efficacy. This however does not demerit the aims of this study as the *Bacillus* sp. isolates showed improved plant growth over Eco-T®. Eco-T® was originally isolated from spent composted pine bark while the *Trichoderma* isolates used in this work were isolated from composted soil in a display garden. Askew and Laing (1994) demonstrated in their study that *Trichoderma* isolates selected from composted pine bark were more aggressive against *R. solani* than those isolated from soil and other sources.

Applications of *Trichoderma* and *Bacillus* spp. isolates increased dry bean yield in some treatments but were not consistent. Mechanisms for this were not established and this requires further investigation to understand the inconsistencies observed in bean yield Trials 1 and 2.

The results presented here suggest that there are possibilities of enhancing biological control of plant diseases and/or increase seedling growth and establishment through mixtures of *Trichoderma* and *Bacillus* spp. Several reports have shown that individual *Trichoderma* and

Bacillus spp. could suppress plant pathogen activities as well as promote plant growth. A combination of these two organisms, as shown in this study, could lead to an increase in disease suppression and plant growth. The likelihood of this combination performing maximally will depend on the modes of action and compatibility of the intended isolates to be combined. Complementary modes of action between these two organisms, if exploited, could lead to increased synergism and activity especially under variable environmental conditions (Raupach and Kloepper, 1998), and in situations where more than one plant pathogen exists. Mixtures of these two organisms could be beneficial to organic farming (Raupach and Kloepper, 1998) or could be used in conjunction with a reduced rate of fungicide applications. The *Bacillus* isolates were the main contributors to growth promotion in this study as with the *Trichoderma* isolates during the biological control trials. Essentially, combinations of *Trichoderma* and *Bacillus* spp. isolates did not antagonise each other *in vitro* (Chapter Three). Hence, the different niche occupancy by these two organisms when used as a mixture could aid in sourcing of nutrients and controlling possible minor and major plant fungal plant pathogens leading to improved plant growth and biological control.

In practice, *Trichoderma* and *Bacillus* spp. are easier to formulate than other organisms such as fluorescent pseudomonads due to production of spores by the *Trichoderma* and *Bacillus* spp. The feasibility of producing the two organisms as a mixture and as a commercial product may not be feasible due to a high production and registration cost that may be involved compared to the cost incurred by producing a single strain (Schisler *et al*., 1997).

This work in part supports the hypothesis put forward in Chapter Three that combinations of these two organisms could result in a possible additive effect, leading to enhanced plant growth and biological control.

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CHAPTER FIVE

Effects of single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates on growth, photosynthetic efficiency and mineral uptake of dry bean seedlings (*Phaseolus vulgaris L.*) grown under shadehouse conditions

"…When plants are grown under optimal conditions, expression of growth promotion is unlikely, whereas under suboptimal conditions, enhanced growth can be achieved…" ..Rabeendran et al., 2000.

K.S. Yobo[1], M.D. Laing[1] and C.H. Hunter[2]

[1]*Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

[2]*Discipline of Microbiology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

Abstract

A shadehouse pot trial was conducted to study the efficiency of single and dual inoculations with selected *Trichoderma* isolates and *Bacillus* isolates in increasing plant growth, photosynthetic efficiency and mineral uptake in dry bean plants (*Phaseolus vulgaris* L.) grown in composted pine bark potting medium under low nutrient conditions. Results showed that all the plant treatments inoculated with *Trichoderma* spp. and/or *Bacillus* spp. had higher Fv/Fm values (photosynthetic efficiency) than the unfertilized control. Fertilized control plants exhibited the highest photosynthetic efficiency compared to all other treatments. Linear regression analysis showed a significant ($P = 0.008$) decrease in Fv/Fm values for the unfertilized control during the course of the trial. Of the treatments, only *Bacillus* B69 showed a significant increase ($P = 0.02$) in Fv/Fm values over the growth period. The fertilized control plants and the *Trichoderma-Bacillus* treated plants all showed significant increase in dry shoot biomass compared to the unfertilized control. Increases in dry shoot biomass as high as 126% were recorded for *Bacillus* B77. *Trichoderma-Bacillus* dual inoculations recorded higher mean dry shoot biomass compared to the single *Trichoderma* inoculations, however, these increases were not significant ($P > 0.05$). The greatest degree of nodulation was observed in *Trichoderma-Bacillus* treated plants whereas the fertilizer control exhibited the lowest. Increase in nitrogen concentrations were observed in leaves of plants inoculated with *Trichoderma-Bacillus* isolates but not significantly better than the fertilized control. Only *T. atroviride* SY3A significantly increased phosphorus concentrations compared to the unfertilized control. The rest of the *Trichoderma* and *Bacillus* treatments did not significantly increase phosphorus concentrations, and in some instances, the values were lower than the unfertilized control. The fertilizer control plants had the highest nitrogen and phosphorus concentrations and were significantly different from all treatments. No significant differences in potassium and calcium concentrations were found among *Trichoderma-Bacillus* treatments and also between *Trichoderma-Bacillus* treatments and unfertilized control treatment.

Key words: *Bacillus*; dry bean; photosynthetic efficiency; mineral uptake; *Trichoderma*

5.1 Introduction

In addition to their biological control properties, various *Trichoderma* and *Bacillus* spp. have been reported to increase plant growth and seedling establishment. Several possible growth promoting mechanisms such as production of plant hormones (Baker, 1988), increase in nutrient uptake (Jisha and Alagawadi, 1996), control of minor pathogens (Inbar *et al*., 1994) and release of soil nutrients and minerals (Ousley *et al*., 1994a) have all been suggested to explain the phenomenon behind enhanced plant growth.

Mineral elements such as Nitrogen (N), Phosphorus (P), Potassium (K), and Calcium (Ca) play key roles in plant nutrition and growth (Engelhard, 1989; Marschner, 1995). Deficiencies in these elements, particularly N and K, can severely retard plant growth. Pronounced senescence of older leaves is symptomatic of N deficiency whereas chlorosis and necrosis of leaves and stems results from severe K deficiency (Marschner, 1995). Shoot growth may be stunted in P deficient plants, resulting in a decrease in shoot-root ratio (Marschner, 1995). Calcium plays an essential role in regulating many physiological processes that influence both growth and responses to environmental stress, as well as maintaining cell wall structure and integrity (Marschner, 1995; Fernandez *et al*., 2000). Considering the importance of N, P, K and Ca with regards to their roles in plant growth and nutrition, certain microbial and fungal inoculants have been investigated to discern their role in mineral and nutrient uptake in plants. For example, Jisha and Alagawadi (1996) reported that single and dual inoculations of *Pseudomonas striata*, or *Bacillus polymyxa*, with *Trichoderma harzianum* Rifai increased N and P uptake in sorghum. Similarly, Alagawadi and Gaur (1992) reported an increase in N and P uptake in sorghum using single and dual inoculations of *Azospirillum brasilense* and *B. polymyxa* or *P. striata* with and without nitrogen fertilizer and rock phosphate. *Trichoderma viride* Pers.:Fr. and *Bacillus subtilis* have also been shown to enhance growth and nutrient uptake (N, P and K) of deodar cedar (*Cedrus deodara* Roxb. ex D. Don) (Bisht *et al*., 2003). Yedidia *et al*. (2001) observed that Ca levels in cucumber plant roots increased when inoculated with *T. harzianum* and grown in an aseptic hydroponic growth system. These observations support the assertion that microbial inoculants can promote plant growth by enhancing mineral uptake (Jisha and Alagawadi, 1996; Bisht *et al*., 2003).

Within this thesis (Chapter 4), it has been postulated that growth promotion of dry bean seedlings when inoculated with *Trichoderma* and *Bacillus* treatments could be attributed to

increased N uptake/assimilation. This hypothesis was tested using selected *Trichoderma* and *Bacillus* isolates, singly and in combinations, applied to dry bean seeds grown in nutrient limited growth medium under shadehouse conditions. Nutrient uptake of N, P, K and Ca was evaluated as well as other growth parameters such as shoot dry weight, nodule numbers and plant efficiency (photosynthetic efficiency).

5.2 Materials and Methods

5.2.1 Fungal and bacterial isolates

Four *Trichoderma* isolates was used in this study. These are: *T. atroviride* P. Karsten (Isolate SY3A); *T. atroviride* P. Karsten (Isolate SYN6), *Trichoderma* sp. SY2F (unidentified strain) and a commercial strain of *T. harzianum* (Eco-T®). Three unidentified *Bacillus* isolates were used in this study, *Bacillus* Isolate B69, B77 and B81.

5.2.2 Formulation of Trichoderma *isolates in kaolin*

This was carried out as previously described in Chapter 4 (Section 4.2.2.).

5.2.3 Preparation of Bacillus *cell suspension*

This was carried out as previously described in Chapter 4 (Section 4.2.3.).

5.2.4 Seeds

Dry bean (*Phaseolus vulgaris* L.) cv PAN 148 was used in this study. Untreated dry bean seeds were obtained from Pannar Seeds (Pty) Ltd., Greytown, Republic of South Africa.

5.2.5 Seed treatment procedures

This was performed as previously described in Chapter 4 [Sections 4.2.5 (a), (b) and (c)]. In addition to single inoculations of *Trichoderma* and *Bacillus* isolates, combinations of *T. atroviride* SY3A + *Bacillus* Isolate B69, *T. atroviride* SYN6 + *Bacillus* Isolate B69, *Trichoderma* sp. SY2F + *Bacillus* Isolate B81 and Eco-T® + *Bacillus* Isolate B69 were used/evaluated in this trial.

5.2.6 Seedling trials in the shadehouse

Seedling trials were carried out in pots kept under shadehouse conditions. Trials were established in mid-October (2002)(Spring) and were run until late December. Treated dry bean seeds (Section 5.2.5.) were planted into 18cm diameter pots (approximately 2.3*l* volume) filled with composted pine bark growing medium (CPB). Two seeds were planted per pot and were thinned to one plant per pot after germination. The trial consisted of 13 treatments, including two control treatments, one control treatment with nutrients and the second without nutrients. Each treatment was replicated in triplicate. Pots were arranged in a complete randomised block design.

Each pot was hand watered with tap water on the first day of planting. After the first day, one of the control treatments received water supplemented with NPK soluble fertilizer [3:1:3(38) Complete] (Ocean Agriculture, Muldersdrift, Republic of South Africa) at a rate of 1g.l^{-1} daily, while the rest of the treatments along with a second unfertilized control treatment received tap water only for the duration of the trial. Plants were watered once daily and growth of plants were monitored for 40 days.

5.2.6.1 Photosynthetic efficiency measurements

The effect of *Trichoderma* and *Bacillus* isolates and their combinations on plant growth was assessed indirectly by measuring the photosynthetic efficiency using a Plant Efficiency Analyser (PEA) (Hansatech, England). Readings were taken over a period of 4 weeks, starting 4 days after germination (Day 12) followed by three subsequent weekly readings (Days 19, 26 and 35 respectively). All programmed parameters in the PEA were determined but only Fv/Fm ratio (Fm = Transient maximum emission yield of dark adapted samples; Fo = Emission yield before the onset of actinic light; Fv = Fm ~ Fo), which is a measure of photosystem II efficiency was considered for this trial. A decrease in Fv/Fm ratio is considered a good indication of photo-inhibitory damage in plants subject to stress. Readings of Fv/Fm ratio from 0.8 indicates optimum efficiency of light usage; values between 0.7-0.8 indicates that plants are not stunted and efficiency of photosystem II is almost maximum while 0.6-0.7 indicates slightly depressed photosynthetic efficiency (Dr Isa Bertling, 2003 Personal Communication).

5.2.6.2 Dry shoot biomass measurements

Plants were harvested at their base at soil level on the 40th day after planting, placed in a paper bag and dried at 70^{0}C for 48h. The total dry weight of each plant per plot was determined using a tabletop laboratory scale (OHAUS Precision Plus, Model TP2KS, OHAUS Corporation, USA). Only above-ground stems and leaves were weighed.

5.2.6.3 Root nodule counts

Roots of plants were carefully washed under running tap water and the number of nodules determined for each plant. Nodules on each plant's roots were counted at least three times and mean number of nodules determined. The appearance of the roots for each plant was recorded.

5.2.6.4 Mineral content of plant leaves

For mineral content determination, all the leaves of dried bean plants were ground separately using a Waring Commercial Blender (Model 34BL99, Dynamics Corporation of America, USA). The blender was cleaned thoroughly and dried between treatments.

(a) Ashing and treatment of the ground plant material

Approximately 0.5g of ground leaf samples were weighed separately into a wide-form porcelain crucible (about 20ml capacity) and labelled. These were placed in a cold muffle furnace heated to 500^{0}C for two hours. The ashed samples were removed and placed in a desiccator to cool. Samples were moistened with distilled water and 10ml of 4M HCL/HNO_3 acid mixture was slowly added. The mixture was then digested on a sand bath for 20min at room temperature. After digestion, samples were transferred onto filter paper (Whatman No. 1) with distilled water and into a 250ml volumetric flask using a glass rod to prevent losses and made up to volume (250ml) with distilled water. The resulting solutions were analysed for P, K and Ca content using the Handbook of Standard Soil Testing Methods (1990). Phosphorus was measured using UV-visible Spectrometry (Varian Spectrophotometer, Varian, Australia). Potassium and calcium were measured using a flame photometer (Varian Spectra AA-200 Spectrophotometer, Varian, Australia).

(b) Kjeldahl analysis for nitrogen content

For determination of N content, 0.5g of ground plant samples were weighed into 100ml separate Kjeldhal flasks. Into each flask, approximately 0.15g of K_2SO_4 was added followed by 0.5ml of $HgSO_4$ from a burette to catalyse the acid. Using an automatic dispenser, 10ml of concentrated H_2SO_4 was added into each flask and heated in a fume cupboard. Flasks were occasionally swirled and their position changed to allow samples to digest equally. After 2-3h of heating, the digested solutions were allowed to cool, 10ml of distilled water added onto each flask and allowed to cool again. The solutions were transferred into 250ml volumetric flasks and made up to volume with distilled water. This solution was used for analysis of N (measured in the form of ammonia) using the Handbook of Standard Soil Testing Methods (1990). Ammonia was measured using an ORION Specific ion electrode (model Orion-9512) coupled to LABOTEC specific ion meter (model LABION). Ammonia concentrations of samples (mV) were read against a standard curve and N content of samples calculated.

5.2.7 Statistical analysis

All experiments were repeated once and results pooled together for statistical analysis. SAS (1987) was used to run linear regression on the photosynthetic efficiency data. A general linear model (GLM) was also used to run an ANOVA on all data collected. If the ANOVA was significant, ($P<0.05$) the means were separated using the Students Neuman Keul's test. Data for dry plant biomass were transformed (arctangent dry shoot biomass (g)/plant) prior to analysis. No statistical analysis was performed on the root nodule numbers due to the nature of the data.

5.3 Results

5.3.1 Seedling trials in the shadehouse

5.3.1.1 Photosynthetic efficiency measurements

Linear regression equations for increase/decrease of Fv/Fm values during the growth period for the fertilized control and the unfertilized control were found to be significantly different ($P = 0.34$ and $P = 0.008$ respectively). Linear regression equations of Fv/Fm values of dry bean plants for all treatments except *Bacillus* B69, which showed a significant increase ($P = 0.02$) while the unfertilized control showed a significant decrease ($P = 0.008$), showed no significant changes over a four week period (Table 5.1).

Only the unfertilized control treatment resulted in a steady decrease in Fv/Fm values over the course of the trial (Table 5.2).

Table 5.1 The effect of selected *Trichoderma* and *Bacillus* isolates and combinations thereof, on photosynthetic efficiency of dry bean plants grown in composted pine bark

Equation	Intercept	Slope	RSD	Adjusted R^2	P-value
Overall	0.733 (0.010)	- 0.00 (0.004)	0.052	0	0.96^{ns}
Fertilized Control	0.782 (0.016)	0.006 (0.006)	0.022	0	0.34^{ns}
Unfertilized control	0.730 (0.022)	- 0.026 (0.008)	0.031	0.47	0.008**
Trichoderma sp. SY2F	0.689 (0.053)	0.015 (0.019)	0.075	- 0.04	0.46^{ns}
T. atroviride SY3A	0.764 (0.027)	- 0.018 (0.010)	0.038	0.17	0.10^{ns}
T. atroviride SYN6	0.742 (0.036)	- 0.00 (0.013)	0.050	- 0.06	0.55^{ns}
Eco-T®	0.777 (0.038)	- 0.018 (0.014)	0.054	0.06	0.22^{ns}
Bacillus B69	0.691 (0.020)	0.019 (0.007)	0.028	0.36	0.02*
Bacillus B77	0.722 (0.038)	0.002 (0.014)	0.053	- 0.09	0.87^{ns}
Bacillus B81	0.724 (0.027)	0.002 (0.010)	0.038	-0.09	0.84^{ns}
Trichoderma sp. SY2F + *Bacillus* B81	0.759 (0.025)	- 0.01 (0.009)	0.036	0	0.36^{ns}
T. atroviride SY3A + *Bacillus* B69	0.707 (0.031)	0.01(0.011)	0.043	0	0.34^{ns}
T. atroviride SYN6 + *Bacillus* B69	0.699 (0.028)	0.02 (0.010)	0.039	0.22	0.07^{ns}
Eco-T® + *Bacillus* B69	0.744 (0.027)	0.001 (0.010)	0.039	- 0.10	0.91^{ns}

*, ** Significantly different at $P < 0.05$ and $P \leq 0.01$; ^{ns} Not significant ($P > 0.05$)

In all of the weekly measurements, the Fv/Fm values for the fertilized control was significantly different ($P < 0.05$) compared to the unfertilized control (Table 5.2). No significant differences were found between the unfertilized control and the fungal or bacterial treatments in the first and second week measurements. However, an increase in Fv/Fm values in third week for plants treated with all three *Bacillus* isolates and combinations of *T. atroviride* SY3A + *Bacillus* B69, *T. atroviride* SYN6 + *Bacillus* B69 and Eco-T® + *Bacillus* B69 were significantly different from the unfertilized control (Table 5.2). These significant differences were not apparent during the fourth week of measurements with only plants treated with *Trichoderma* sp. SY2F and *T. atroviride* SYN6 + *Bacillus* B69 maintaining the significant difference over the unfertilized control (Table 5.2).

Table 5.2 Photosynthetic efficiency values (Fv/Fm values) of dry bean plants treated with selected *Trichoderma* and *Bacillus* isolates and grown under shadehouse conditions in composted pine bark

Treatments	Photosynthetic efficiency measurements (Fv/Fm values)			
	Week 1	Week 2	Week 3	Week 4
Fertilized Control	0.78 a	0.80 a	0.81 a	0.81 a
Unfertilized control	0.71 b	0.66 b	0.65 b	0.63 b
Trichoderma sp. SY2F	0.76 ab	0.64 b	0.74 ab	0.77 a
T. atroviride SY3A	0.76 ab	0.70 ab	0.74 ab	0.70 ab
T. atroviride SYN6	0.76 ab	0.69 ab	0.73 ab	0.71 ab
Eco-T®	0.77 ab	0.71 ab	0.74 ab	0.70 ab
Bacillus B69	0.71 b	0.72 ab	0.78 a	0.75 ab
Bacillus B77	0.75 ab	0.67 ab	0.77 a	0.72 ab
Bacillus B81	0.73 ab	0.71 ab	0.76 a	0.72 ab
Trichoderma sp. SY2F + *Bacillus* B81	0.76 ab	0.72 ab	0.74 ab	0.73 ab
T. atroviride SY3A + *Bacillus* B69	0.74 ab	0.68 ab	0.78 a	0.74 ab
T. atroviride SYN6 + *Bacillus* B69	0.74 ab	0.70 ab	0.79 a	0.77 a
Eco-T® + *Bacillus* B69	0.76 ab	0.71 ab	0.77 a	0.75 ab
F-ratio	3.09	2.20	3.15	2.80
P-level	0.008	0.05	0.007	0.01
% CV	2.95	6.51	4.94	6.25
Significance	**	*	**	**

*, ** Significantly different at $P \leq 0.05$ and $P \leq 0.01$ respectively.
Values followed by different letters are significantly different (Students Newmans Keul's test, $P \leq 0.05$).
Fv/Fm values from 0.8 indicates optimum efficiency of light usage; values between 0.7-0.8 indicates that plants are not stunted and efficiency of photosystem II is almost maximum; values between 0.6-0.7 indicates slightly depressed photosynthetic efficiency.

Dual inoculations of *Bacillus* B69 with either *T. atroviride* SY3A, *T. atroviride* SYN6 or Eco-T® did not have any significant increase on Fv/Fm readings compared to single fungal inoculations although values for the combined inoculations were slightly higher than the single fungal inoculations during the third and fourth week readings (Table 5.2). A slightly different result was obtained for dual inoculation of *Trichoderma* sp. SY2F and *Bacillus* B81. Although not significant, the Fv/Fm value for the fourth week measurements for the single fungal inoculation was slightly higher than single inoculation of *Bacillus* B81 and dual inoculations of *Trichoderma* sp. SY2F and *Bacillus* B81 (Table 5.2).

5.3.1.2 Dry shoot biomass measurement

The fertilized control plants did not show any obvious sign of stress compared to unfertilized control plants, which showed evidence of stress and leaf senescence (Fig. 5.1). Dry biomass of fertilized control plants was significantly ($P < 0.0001$) different from the unfertilized control (Table 5.3).

Fig. 5.1 Growth comparison between fertilized (left) and unfertilized (right) control plants under shadehouse conditions 35 days after planting.

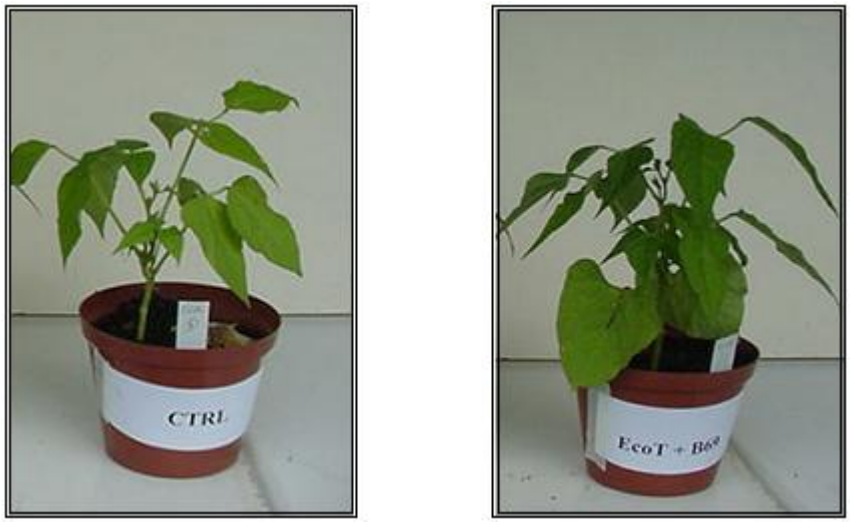

Fig. 5.2 Growth comparison between unfertilized control plant (left) and plant treated with combination of Eco-T® + *Bacillus* B69 (right) 35 days after planting. Treated plants showed more vigorous growth and were healthier than control plants.

Table 5.3 Effect of single and dual inoculation of selected *Trichoderma* and *Bacillus* isolates on dry biomass of of dry bean plants (expressed as arctangent of dry shoot biomass (g)/plant) and root nodule formation 40 days after planting in composted pine bark potting medium under shadehouse conditions

Treatments	Dry shoot biomass	Nodules numbers	Arrangements of nodules/ Comments
Fertilized Control	1.49 a (832.98)	± 41.75	S&C; Healthy roots
Unfertilized Control	1.08 c (0)	± 188.50	S&C; Not much root development
Trichoderma sp. SY2F	1.25 b (61.26)	± 235.75	S&C; Healthy roots
T. atroviride SY3A	1.26 b (68.59)	± 215.25	S&C; Tiny nodules, healthy roots
T. atroviride SYN6	1.30 b (89.53)	± 203.50	S&C; Healthy roots
Eco-T®	1.32 b (110.47)	± 290.75	S&C; Healthy roots
Bacillus B69	1.32 b (119.90)	± 260.25	S&C; Healthy roots
Bacillus B77	1.33 b (126.70)	± 260.50	S&C; Healthy roots
Bacillus B81	1.31 b (101.85)	± 327.75	S&C; Healthy roots
Trichoderma sp. SY2F + *Bacillus* B81	1.26 b (74.87)	± 229.00	S&C; Healthy roots
T. atroviride SY3A + *Bacillus* B69	1.31 b (110.99)	± 264.25	S&C; Healthy roots
T. atroviride SYN6 + *Bacillus* B69	1.29 b (91.10)	± 214.25	S&C; Healthy roots
Eco-T® + *Bacillus* B69	1.32 b (115.18)	± 298.00	S&C; Healthy roots
F-ratio	5.34		
P-level	0.0002		
%CV	5.07		
Significance	***		

± Nodule counts based on average of three counts from each plant. ± indicates 5 more or less nodules on value indicated on Table 5.3 above
*** Significantly different at $P \leq 0.0002$
( ) Percentage increase of dry biomass above unfertilized control calculated using the actual (non-transformed values) dry biomass values.
S&C Nodules are arranged in singles and clumps.
Values followed by different letters are significantly different (Students Newmans Keul's test, at $P \leq 0.05$).

Moreover, fungal and bacterial treated plants showed more vigorous growth than the unfertilized control (Fig. 5.2). However, no significant differences were found among fungal and bacterial treatments and their combinations.

Plants treated with *Trichoderma* and *Bacillus* isolates showed enhanced greenness compared to the unfertilized control. Compared to the unfertilized control, there was a general increase in dry shoot biomass of bean plants treated with single and dual inoculations of *Trichoderma* and *Bacillus* isolates (Table 5.3). Fertilized control plants exhibited the highest percentage increase of dry shoot biomass (832.9%) over the unfertilized control plants followed by *Bacillus* B77 (126.7%) (Table 5.3). The least increase in dry shoot biomass, however was recorded for *Trichoderma* sp. SY2F (61.2%). Moreover, dry biomass of plants treated with single and dual inoculation of *Trichoderma* and *Bacillus* isolates were significantly different from the unfertilized control (Table 5.3). Plants inoculated with combinations of *Trichoderma* and *Bacillus* isolates had higher dry shoot biomass than the *Trichoderma* isolates alone but were not significantly different.

5.3.1.3 Root nodule counts

The nodule counts numbers were lowest for the fertilized control (± 42) followed by the unfertilized control (± 189) (Table 5.3). Plants treated with *Trichoderma* and *Bacillus* isolates, either singly or in combination produced higher nodule counts than either of the controls. Mean nodule counts for fungal or bacterial treated plants were all above ± 200, with the highest number of nodules recorded on plants treated with *Bacillus* B81 (± 328) (Table 5.3).

5.3.1.4 Mineral contents of plant leaves

The effect of *Trichoderma* and/or *Bacillus* isolates application on mineral content in leaves of dry bean plant is as shown in (Table 5.4).

The fertilizer control had the highest concentration of K in plant leaves and was significantly different from the remaining 12 treatments. No significant increase and differences were found among *Trichoderma* and *Bacillus* treatments, either single application or in combination and neither was there any significant increase nor differences between the unfertilized control and the fungal or bacterial treatments (Table 5.4). No significant increase or differences were found among all treatments for Ca content in leaves (Table 5.4).

A 175.6% increase in N content was recorded by the fertilized control over the unfertilized control ($P < 0.0001$) (Table 5.4). Moreover, all of the *Trichoderma* and/or *Bacillus* treatments, excluding *T. atroviride* SYN6 and *Bacillus* B77 recorded N contents that were significantly greater than the unfertilized control. No significant differences were found among the remaining fungal and bacterial treatments. Single *Trichoderma* and *Bacillus* inoculations were as effective as dual inoculations (Table 5.4). The highest N content recorded by a microbial treatment was *Bacillus* B81, which achieved a 99.9% increase in N content over unfertilized control, which was still 75.7% less than the N content of the fertilized control.

The level of P content in the fertilized control leaves was significantly ($P < 0.0001$) higher than the unfertilized control. Phosphorus content was increased from 2.8mg.g^{-1} in unfertilized control plant leaves to 6.7mg.g^{-1} in fertilized control leaves, representing a 138.5% increase in P content over the unfertilized control (Table 5.4). Phosphorus contents in five treatments

Table 5.4. Effect of single and combined inoculations of selected *Trichoderma* and *Bacillus* isolates on N, P, K and Ca concentrations ($mg.g^{-1}$ dry weight) in leaves of dry bean plant growth under shadehouse conditions

Treatments	Nitrogen concentration ($mg.g^{-1}$) of ground leave sample	Phosphorus concentration ($mg.g^{-1}$) of ground leave sample	Potassium concentration ($mg.g^{-1}$) of ground leave sample	Calcium concentration ($mg.g^{-1}$) of ground leave sample
Fertilized Control	28.01 a [175.69]	6.75 a [138.52]	23.33 a	10.79 a
Unfertilized Control	10.16 e [0]	2.83 cd [0]	14.50 b	13.79 a
Trichoderma sp. SY2F	14.71 cd [44.78]	2.43 d [-14.13]	13.83 b	17.84 a
T. atroviride SY3A	19.26 bc [89.57]	4.03 b [42.40]	13.65 b	15.97 a
T. atroviride SYN6	12.96 de [27.56]	2.55 d [-9.89]	14.61 b	15.95 a
Eco-T®	19.96 b [96.46]	3.50 bc [23.67]	14.25 b	13.31 a
Bacillus B69	18.39 bc [81.00]	3.23 bcd [14.13]	13.77 b	12.10 a
Bacillus B77	12.09 de [19.00]	2.47 d [-12.72]	14.01 b	15.59 a
Bacillus B81	20.31 b [99.90]	3.43 bc [21.20]	15.24 b	14.58 a
Trichoderma sp. SY2F + *Bacillus* B81	19.44 bc [91.34]	3.40 bc [20.14]	14.37 b	14.50 a
T. atroviride SY3A + *Bacillus* B69	18.56 bc [82.68]	2.38 d [-15.90]	13.95 b	14.53 a
T. atroviride SYN6 + *Bacillus* B69	18.39 bc [81.00]	2.50 d [-11.66]	13.72 b	13.34 a
Eco-T® + *Bacillus* B69	19.61 bc [93.01]	3.45 bc [21.91]	14.10 b	14.29 a
F-ratio	20.35	38.12	14.54	1.12
P-level	< 0.0001	< 0.0001	< 0.0001	0.42
% CV	8.00	8.07	6.433	17.82
Significance	***	***	***	ns

Values followed by different letters within a column are significantly different (Student Newmans Keul's test, $P \leq 0.05$).
*** Significantly different at $P \leq 0.0001$; ns Not significant at $P > 0.05$.

[ ] Percentage increase or decrease in N and P concentrations above and below unfertilized control.

(*Trichoderma* sp. SY2F, *T. atroviride* SYN6, *Bacillus* B77, and *T. atroviride* SYN6 + *Bacillus* B69) were lower than the unfertilized control (Table 5.4). Although increases in P content were recorded for the rest of the fungal and bacterial treatments, either singly or in combination, only concentrations in *T. atroviride* SY3A (4.0mg.g^{-1}) was significantly better than the unfertilized control (2.8mg.g^{-1}) (Table 5.4).

Although *Bacillus* B69 and *T. atroviride* SY3A caused an increase in P contents in leaves, a combination of the two yielded a lower P content than the unfertilized control and each of the isolates used. Likewise, a combination of *T. atroviride* SYN6 and *Bacillus* B69 did not improve P content compared to the unfertilized control or the single inoculation of each of the isolates. However, a combination of *Trichoderma* sp. SY2F + *Bacillus* B81 yielded P content which was significantly better than *Trichoderma* sp. SY2F used alone (Table 5.4). Similarly, a combination of Eco-T® + *Bacillus* B69 yielded an increase in P content which was better than *Bacillus* B69 used alone but not significantly different (Table 5.4). Moreover, treatments with higher phosphorus contents than the unfertilized control appeared to have more and well-developed leaves. Typical examples were found in plants treated with Eco-T® + *Bacillus* B69 and the fertilized control (Fig. 5.1 and Fig. 5.2).

5.4 Discussion

Inoculation of agricultural crops with selected *Trichoderma* and *Bacillus* spp. have been reported to increase plant growth (Inbar *et al*., 1994; Podile, 1995; Rabeendran *et al*., 2000). Most of these reports have focused on increases in plant growth enhancement during the growth cycle of the crop.

To the best of our knowledge, no studies has been done to investigate the effect of *Trichoderma* and *Bacillus* spp. and their combinations on mineral nutrition/uptake, photosynthetic efficiency and vigour of dry bean plants grown in composted pine bark under fertilizer stress. The trial design, with the appropriate controls, allowed us to study the direct effect of four *Trichoderma* and three *Bacillus* isolates and their respective combinations on growth response of dry bean plants.

With regards to Fv/Fm values, the unfertilized control showed decreasing Fv/Fm ratio over time. As expected, this resulted in a corresponding decrease in photosynthetic efficiency. Initially, unfertilized control plants may access mineral nutrients present in potting mix, but

these deplete over time. The decrease in nutrient availability correlates with the decrease in photosynthetic efficiency (Fv/Fm) values recorded by the unfertilized control. However, the fertilized control showed a fairly constant Fv/Fm ratio, which depicts that nutrients were not limited.

All the *Trichoderma* and/or *Bacillus* inoculants showed similar trends. The mean Fv/Fm values were greater than the unfertilized control but as expected, the Fv/Fm values were not as good as the fertilizer control. Moreover, the Fv/Fm ratios did not show a decrease over time and in some instances, it increased over time. Although the Fv/Fm ratio trend was not consistent, several isolates or combinations thereof produced Fv/Fm ratios which were significantly better than the unfertilized control but not significantly different from the fertilised control.

The Fv/Fm values for the fertilized control plants were consistently higher than the unfertilized, fungal and bacterial treated plants. This result was expected and supports the assertion that the nutritional status of plants influences their photosynthetic efficiency. The steady decrease in Fv/Fm values for the unfertilized control plants could be explained by an increase in leaf senescence during the course of the trial arising from nutrient depletion. Leaves of unfertilized plants turned a yellowish colour by the end of the trial. In general, all the inoculated dry bean plants exhibited more vigorous growth than the unfertilized control plants and this effect was attributed primarily to the *Trichoderma* and *Bacillus* treatments. Similar findings have been demonstrated by Inbar *et al*. (1994) who reported that cucumber (*Cucumis sativus* L.) seedlings treated with an isolate of *T. harzianum* were much more developed, showed vigorous growth and had higher chlorophyll content than untreated control plants.

Analysis of dry shoot biomass revealed significant increase in growth of all plants treated with *Trichoderma* and *Bacillus* isolates compared to the unfertilized control. Moreover, the increase in plant growth was prominent in both single and dual inoculations. This further strengthens the apparent role of the *Trichoderma* and *Bacillus* isolates in plant growth promotion as demonstrated by other authors (Inbar *et al*., 1994; Shishido *at al*., 1995; Probanza *et al*., 1996; Harman, 2000). The low number of nodules formed by the fertilized control plants was not unexpected since nodule formation and nitrogen fixation is typically suppressed by the presence of a readily available inorganic nitrogen source. The unfertilized control plants as well as the plants treated with *Trichoderma* and *Bacillus* isolates all

produced higher numbers of nodules compared to the fertilized control plants. Although no specific trend in nodule numbers was apparent for inoculated plants, the unfertilized control plants were consistently found to have lower numbers of nodules compared to the *Trichoderma* and/or *Bacillus* treated plants. Since a microbially active potting medium was used in the trial it is possible that activities of resident nodule-forming bacteria, such as rhizobia, were stimulated leading to increased nodulation. A possible explanation is that root growth stimulation promoted by *Trichoderma* and *Bacillus* isolates increases surface area for infection by the resident rhizobia. This phenomenon warrants further investigations as it is possible that other factor(s) or mechanisms could be responsible for the increased nodulation of the *Trichoderma* and *Bacillus* inoculated plants. Harman *et al*. (1981) observed no significant increase in nodulation when *T. hamatum* (Bon.) Bain. and *Rhizobium* spp. were co-inoculated onto bean seeds compared to single rhizobial inoculation. However, increased nodulation was observed by Sapatnekar *et al*. (2001) when a mixed culture of *T. viride*, *Aspergillus niger* van Tieghem, *A. fumigatus* Fresen. and *Chaetomium fumicola* Cooke, supplemented with four levels of superphosphate, was applied to green gram (*Vigna radiata* L.) cv. S8 under field conditions. In a separate study, Alagawadi and Gaur (1988) observed an increase in nodulation when *B. polymyxa* and Cicer *Rhizobium* (F75) were co-inoculated onto chickpea (*Cicer arietinum* L.).

Inoculations with *Trichoderma* and *Bacillus* singly, or in combination, substantially increased nitrogen content of dry bean compared to the unfertilized control. This result was supported by, and correlated to leaf greenness and overall increase in plant biomass. Normal/increased nitrogen levels in plants enhance growth with a corresponding delay in leaf senescence (Marschner, 1995). Jisha and Alagawadi (1996) suggested that mixtures of *T. harzianum* and *B. polymyxa* acted synergistically in increasing nitrogen uptake by sorghum and that the findings were significantly higher than each of the organisms used alone. Bisht *et al*. (2003) also reported enhanced nitrogen contents in plant parts inoculated with *T. viride* and *B. subtilis*. Our findings suggest that no significant synergistic interactions occurred between the *Trichoderma* and *Bacillus* isolates with respect to nitrogen uptake. The results presented support the hypothesis that increased plant growth in dry bean seedlings was due to increased nitrogen uptake/assimilation (Chapter 4). This hypothesis is supported by the improvement in nitrogen content and dry shoot biomass in plants inoculated with the *Trichoderma* and/or *Bacillus* isolates in the absence of inorganic fertilizer.

Increases in phosphorus levels in plants as a result of *Trichoderma* and *Bacillus* spp. inoculations have been reported (Jisha and Alagawadi, 1996; Gaikwad and Wani, 2001; Bisht *et al*., 2003). In our study, increase in phosphorus content was observed in some plants treated with *Trichoderma* and/or *Bacillus* isolates compared to unfertilized control plants. Although decreases in phosphorus contents were observed in some *Trichoderma* and/or *Bacillus* – plant interactions, these did not affect the photosynthetic efficiencies (Fv/Fm values), growth and plant establishment of the fungal and bacterial treated plants. This result suggests that the phosphorus contents/levels were not limiting enough to cause any major effect on the plant, although some *Trichoderma* and *Bacillus* treated plants appears to have more and better developed leaves (eg. Eco-T® + *Bacillus* B69) than the unfertilized control. Likewise, the fertilized control plants had more and better developed leaves compared to any of the treatments. Reduction in the number of leaves is recognised as one of the symptoms of phosphorus deficiency in plants (Lynch *et al*., 1991). Hence, we suggest that the most limiting factor in dry bean plants in the absence of inorganic fertilizer appears to be nitrogen. Potassium concentrations, although very high in the fertilizer control due to daily supply of NPK fertilizer, were very similar in all other treatments. Calcium concentrations in all treatments were also very similar and showed no significant differences between treatments. Hence, potassium and calcium contents in leaves appeared to have no significant effect on photosynthetic efficiency. *Bacillus* B77 consistently improved seedling growth and development (Chapters 4 and 5). However, it caused the lowest nitrogen concentration compared to the rest of the *Trichoderma* and/or *Bacillus* treatments and a negative phosphorus concentration compared to the unfertilized control. Due to its consistent effect on seedling growth and development, we suggest that other unknown factor(s) other than mineralization contribute substantially to the growth promoting abilities of *Bacillus* B77 which could be more prominent than the mechanism of mineralization. This warrants further research to determine other possible mechanism(s) of growth promotion by *Bacillus* B77.

The results presented here show that growth promotion by the *Trichoderma* and *Bacillus* isolates was clearly expressed under nutrient limiting conditions. This result supports the hypothesis by Rabeendran *et al*. (2000) who postulated that growth promotion by *Trichoderma* spp. is most likely to be expressed or achieved in plants grown under suboptimal conditions rather than under optimal growth conditions. It is therefore desirable to screen plant growth promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) under suboptimal growth conditions because screening under optimal conditions could mask

the potential of these organisms. Furthermore, Fv/Fm values (photosynthetic efficiency) and using the Plant Efficiency Analyser (PEA) could be a useful tool for determining the effectiveness of PGPR and PGPF under suboptimal growth conditions.

The potential of using *Trichoderma* and/or *Bacillus* spp. to enhance plant growth and establishment in soils low in mineral elements exist. However, the effect of environmental conditions needs to be ascertained as the efficacy and performance of biological control agents, PGPRs and PGPFs, are affected under varied environmental conditions (Guetsky *et al*., 2002).

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CHAPTER SIX

Evaluation of the integrated control of *Rhizoctonia solani* damping-off in cucumber seedlings with selected *Trichoderma* and *Bacillus* isolates in conjunction with Rizolex® (tolclofos-methyl) under greenhouse conditions

"…On consumer safety, there must be no compromise - consumer safety is key and the aim must be to reduce exposure to chemical residues as far as possible…" Urech, 2000.

K.S. Yobo[1], M.D. Laing[1] and C.H. Hunter[2]

[1]*Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209, Pietermaritzburg, Republic of South Africa*

[2]*Discipline of Microbiology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X 01, Scottsville 3209. Pietermaritzburg, Republic of South Africa*

Abstract

Selected *Trichoderma* and *Bacillus* isolates were tested with reduced concentrations of Rizolex® (tolclofos-methyl) as an integrated control system against *Rhizoctonia solani* damping-off on cucumber seedlings. *In vitro* bioassays with three different concentrations (0.01, 0.1 and 0.25g.l^{-1}) of Rizolex® showed that *Trichoderma* isolates were less sensitive to 0.01 and 0.1g.l^{-1} than 0.25g.l^{-1} Rizolex® while *Bacillus* isolates were not affected by any of the three concentrations. A greenhouse seedling trial in Speedling® trays with the aforementioned Rizolex® concentrations indicated that only the reduced Rizolex® concentrations control and Eco-T® (commercial *Trichoderma* isolate) recorded significant (P = 0.001 and P = 0.04 respectively) relationships between increasing Rizolex® concentrations and disease control achieved by the integrated system. In a second greenhouse study with a 0.1g.l^{-1} Rizolex® concentration, integration with single and dual inoculations of *Trichoderma* and *Bacillus* resulted in a better control than the *Trichoderma* and *Bacillus* treatments used alone. As high as 86% control, was achieved by integrating 0.1g.l^{-1} Rizolex® with *T. harzianum* SYN which was significantly better than the 0.1g.l^{-1} Rizolex® control (P = 0.001). A greenhouse pot trial using *T. atroviride* SY3A + *Bacillus* B81 with or without Rizolex® (0.1g.l^{-1}) on three cucumber cultivars showed increased seedling survival by each of the three cultivars to the treatments used. These results indicate that there is a possibility of using reduced concentrations of Rizolex® in combination with *Trichoderma* and/or *Bacillus* to control *R. solani* damping-off in an effort to curtail heavy use of fungicides on greenhouse crops. In all cases, synergistic effects of adding 0.1g.l^{-1} Rizolex® with *Trichoderma* and *Bacillus* treatments were observed.

Keys words: cucumber; cultivar; damping-off; integrated control; Rizolex®

6.1 Introduction

Chemical control of plant diseases is an indispensable component of crop protection. However, the widespread use of agrochemicals is increasingly being questioned due to various negative effects on the environment arising from soil, river and ground water contamination. For example, the risk associated with pesticide residues in food has led to the introduction of strict regulations governing the use of chemical pesticides in agriculture (Becker and Schwinn, 1993). As a result, the production and application of compounds such as methyl bromide (MeBr) have been banned (Raupach and Kloepper, 2000).

Biological and chemical control systems have widely been used in an attempt to curtail yield losses as a result of plant diseases caused by several plant pathogens. However, each of these systems has limitations and demerits such as toxic effects on human and animals (chemical control) and inconsistencies and variabilities in performance (biological control) (Becker and Schwinn, 1993; Guetsky *et al*., 2001). Various reports in the literature indicate that a number of biological control agents can control plant pathogens with efficiencies equal to that of available chemical pesticides (Koch, 1999). However, one of the most serious shortfalls of biological control is that the control rendered is not always equivalent to the chemical control or the efficacy is not consistent (Guetsky *et al*., 2001). In order to overcome these effects, integration of chemical control (reduced levels of chemicals) and biological control has been sought. Such practices and successful disease control regarding integration of chemical and biological control systems has been reported by several authors (Henis *et al*., 1978; Elad *et al*., 1993; Conway *et al*., 1997; Raupach and Kloepper, 2000). For example, Kaur and Mukhopadhyay (1992) successfully controlled 'chickpea wilt complex' using a combined application of *T. harzianum* Rifai and either of three fungicides (Vitavax-200, Ziram or Bavistin). Similarly, Elad *et al*. (1993) effectively controlled cucumber grey mould using *T. harzianum* in combination with Ronilan 50 WP, Resec WP, Rovral 50 WP or Silvacur WP.

Tolclofos-methyl has been reported to control bottom rot disease of lettuce caused by *R. solani* Kühn (Coley-Smith *et al*., 1991). It is also being used in South Africa under the trade name Rizolex® to control damping-off caused by *R. solani* on vegetable seedlings and tuber crops such as potato (*Solanum tuberosum* L.). The aim of this study was to determine the efficacy of single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates in combination with Rizolex® (tolclofos-methyl) as an integrated system to control damping-off caused by *R. solani* on cucumber seedlings. In addition, the effect of a dual inoculation of

one *Trichoderma* and one *Bacillus* isolate with and without Rizolex® on three cucumber cultivars were assessed.

6.2 Materials and Methods

6.2.1 Fungal, bacterial isolates and fungicide

Four *Trichoderma* and two *Bacillus* isolates were used in this study. These are: *T. atroviride* P. Karsten (Isolate SY3A), *T. atroviride* P. Karsten (Isolate SYN6), *T. harzianum* Rifai (Isolate SYN) and a commercial strain, Eco-T® (active ingredient *T. harzianum*). The *Bacillus* isolates were *Bacillus* Isolate B69 and B81. Rizolex® WP (tolclofos-methyl) (Philagro South Africa (Pty) Ltd., Menlo Park, Republic of South Africa) was used in this experiment.

6.2.2 Formulation of Trichoderma *isolates in kaolin*

This was as described in Chapter 4 under Section 4.2.2.

6.2.3 Preparation of Bacillus *cell suspensions*

This was as described in Chapter 4 under Section 4.2.3.

6.2.4 Seeds

Cucumber (*Cucumis sativus* L.) cv Ashley seeds were obtained from Starke Ayres Seed Company Ltd., Pietermaritzburg, Republic of South Africa. In addition, two cucumber cultivars, Cumlaude RZ® and 22-27 RZ® were donated by Film Flex (Pty) Ltd., Republic of South Africa. The fungicide treated cucumber seeds were washed eight times with distilled water to reduce fungicide residues and air-dried before use.

6.2.5 In vitro effect of Rizolex® on growth of Trichoderma *and* Bacillus *isolates*

Three concentrations of Rizolex® WP were used, viz. 0.01, 0.1 and 0.25g.l^{-1}, to evaluate the effect of Rizolex® vapour on growth of *Trichoderma* and *Bacillus* isolates.

Mycelial plugs (4mm diameter, cut from the actively growing edge of a 3 day old mycelial mat on V8 agar) of a single *Trichoderma* isolate were placed in the centre of three plastic Petri dishes (90mm diameter) containing V8 agar medium and carefully inverted. Rizolex® concentrations, (0.01, 0.1 and 0.25g.l^{-1}) were separately prepared using distilled water.

Whatman No.1 filter paper discs (90mm diameter) were separately dipped in each of the three Rizolex® concentrations and placed on the lids of the inverted plastic petri dishes. Plates were incubated for 5 days at 28^0C in the dark after which diameter of growth were measured for all treatments. Each bioassay was replicated two times. Control plates lacked Rizolex® impregnated filter discs. The bioassay was repeated for the rest of the *Trichoderma* isolates as well as *R. solani* and the experiment was repeated once.

For the *Bacillus* isolates, 0.2ml of each bacterial suspension was evenly spread on the surface of tryptic soy agar (TSA, Merck) in disposable Petri dishes using a sterile glass hockey stick. The inoculated plates were allowed to dry and then treated with Rizolex® as described above. Control plates lacked Rizolex® treatments. There were three replicates per treatment and the experiment was repeated once. Plates were incubated at 30^0C for 3 days and then compared to control plates and rated. Bacterial growth was assigned ' + ' for growth equal to control plate or ' < + ' for growth less than the control (Bauske *et al*., 1997).

6.2.6 Seed treatments procedures

This was as described in Chapter 4 under Section 4.2.5 (a), (b) and (c).

6.2.7 Integrated biological control of R. solani *damping-off*

(a) Seedling Trial 1 (with three fungicide concentrations)

Seedling trials were carried out in Speedling® trays (24 cells per tray) under greenhouse conditions. Cucumber seeds (Ashley) were treated as previously described (Chapter 4 Section 4.2.5). Speedling® trays were half filled with composted pine bark (Potting Mix, Gromed, Crammond, Republic of South Africa). Pathogen inoculation was achieved by placing 4mm square V8 agar plugs of *R. solani* in the centre of each cell directly on top of the growth medium. The cells were then filled and the treated seeds planted. Each treatment was planted into three Speedling® trays. Prior to covering of seeded trays, the trays were inoculated with the three fungicide concentrations (one tray per concentration, each cell receiving 1m*l* of fungicide) directly on top of the seed. All *Trichoderma* and *Bacillus* treatments with their respective combinations received fungicide applications. Controls using seeds coated solely with kaolin were also established. Three control treatments were established:

a) Rizolex® control trays inoculated with *R. solani* and four concentrations of fungicide (0.01, 0.1, 0.25g.l^{-1}) and full strength Rizolex® (1g.l^{-1}) (manufacturer's recommended concentration);
b) Disease free control trays that received 4mm agar plugs with no *R. solani* and no fungicide application but inoculated with 1ml of water and a
c) Diseased control tray that received *R. solani* plugs and received 1ml of water in place of a fungicide drench.

Three replicate trays were established for each treatment. The trays were left in the germination room at 20-24^{0}C for 2 days. The trays were then moved to a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. Treatments were arranged in a randomised block design with 48 treatments and three replicates. Trays were irrigated three times a day by microjet overhead irrigation (Inverted mini wobbler, Sennenger, U.S.A). The water used was maintained at 20^{0}C using a temperature controlled heating system (Pro Heat 2000 Plus, Republic of South Africa) and contained NPK soluble fertilizer [3:1:3 (38)] complete at a rate of 1g.l^{-1}. Seedling survival was rated after 4 weeks. The plant material was then harvested and subsequently dried at 70^{0}C for 48h to determine the total dry weight of seedlings per plot (tray). The experiment was repeated once.

(b) Seedling Trial 2 (with one fungicide concentration)

Cucumber seeds (Ashley) were used for this trial. Cucumber seeds were treated as previously described (Chapter 4 Section 4.2.5). Pathogen inoculation and planting procedure were as described in Section 6.2.7(a) above. Each treatment was planted into two Speedling® trays. Prior to covering of seeded trays, one tray was treated with Rizolex® (0.1g.l^{-1}) each cell receiving 1ml of Rizolex® directly on top of the seed. The second tray was inoculated with 1ml of water. Four control treatments were established:

a) Rizolex® control tray inoculated with *R. solani* and 1ml per cell of 0.1g.l^{-1} Rizolex® concentration;
b) Full strength Rizolex® (1g.l^{-1}) (manufacturer's recommended concentration) inoculated with *R. solani* and 1ml per cell of recommended concentration;
c) Disease free control tray that received 4mm agar plugs with no *R. solani* and received 1ml of water in place of a fungicide drench and a

d) Diseased control tray that received *R. solani* plugs and received 1ml of water in place of a fungicide drench.

Three replicate trays were established for each treatment. The trays were left in the germination room at 20-24^{0}C for two days. The trays were then moved to a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. Treatments were arranged in a randomised block design with 18 treatments and three replicates. Trays were further treated as described in Section 6.2.7(a) above. The plant material was then harvested at their base at soil level and subsequently dried at 70^{0}C for 48h to determine the total dry weight of seedlings per plot (tray). Only the above-ground stems and leaves were weighed. The experiment was repeated once.

(c) Cucumber cultivars trial

Three cucumber cultivars, viz; Ashley, Cumlaude RZ® and 22-72 RZ® were used. Cucumber seeds were treated as previously described (Chapter 4 Section 4.2.5). Only *T. atroviride* SY3A + *Bacillus* B81 was used in this trial. The rationale behind this trial was to evaluate the effect of combined *Trichoderma-Bacillus* treatment on cucumber cultivars and/or the response of different cucumber cultivars to *Trichoderma-Bacillus*, fungicide and *R. solani* treatments.

Prior to planting of cucumber treated seeds, V8 agar plates (20ml per plate) previously colonised with *R. solani* were used for inoculation. Each V8 agar plate colonised with *R. solani* was ground using a Waring Commercial Blender (Model 34BL99, Dynamics Corporation of America, USA). Distilled water was slowly added until the mixture was finely ground and homogenised. Approximately 200ml of distilled water was added to each ground V8 agar plate colonised with *R. solani*. The homogenous mixture was carefully mixed with approximately 3.45kg of composted pine bark growing medium. This procedure was repeated until the required amount of pathogen infested growth medium was achieved. Approximately 282.0g (about 750ml volume) of pathogen infested growth media were filled into 15cm diameter pots (about 1.550*l* volume) using a 12.5cm diameter pot. Each pot was further layered with approximately 46.0g (about 110ml volume) of composted pine bark and *Trichoderma-Bacillus* treated seeds were then planted into each pot (two seeds per pot). Two pots were planted for each cucumber cultivar and one pot was inoculated with 1ml of 0.1g.l^{-1} Rizolex® directly on top of the seed whereas the other received 1ml of water directly on top of

each seed. Seeds were then covered. Four control treatments were established as previously described under Section 6.2.7 (b) above. Each treatment was replicated five times making a total of 10 seeds per treatment. The pots were left in the germination room at 20-24^{0}C for 2 days. The pots were then moved to a polycarbonate greenhouse tunnel maintained between 22-26^{0}C. Treatments were arranged in a randomised block design with 18 treatments and five replicates. Pots were further treated as described in Section 6.2.7(a) above. Seedling survival was rated after 5 weeks. The plant material was then harvested, dried and weighed as previously described under Section 6.2.7 (b) above. The experiment was repeated once and results pooled for statistical analysis.

6.2.8 Statistical analysis

SAS (1987) was used to run linear regression on data for Seedling Trial 1. A general linear model (GLM) was used to run an ANOVA on all data collected. If the ANOVA was significant, ($P < 0.05$), the means were separated using the Students Newman Keul's test.

6.3 Results

6.3.1 In vitro effect of Rizolex® on growth of Trichoderma *and* Bacillus *isolates*

All *Trichoderma* isolates were tolerant of the three Rizolex® concentrations tested, although the growth of the *Trichoderma* isolates were reduced compared to their respective untreated controls (Table 6.1).

Table 6.1 Effects of Rizolex® concentrations (0.01, 0.1 and 0.25g.l^{-1}) on growth of *Trichoderma* and *R. solani* after 5 days of incubation at 28^{0}C

Isolates	Controls (diameter of growth in mm)	Rizolex® concentrations (g.l^{-1})/diameter of growth (mm) of respective isolates		
		0.01 g.l^{-1}	0.1 g.l^{-1}	0.25 g.l^{-1}
Eco-T®	90.00	68.25 b	60.25 b	49.25 c
T. atroviride SY3A	90.00	90.00 a	63.25 b	52.50 b
T. atroviride SYN6	90.00	90.00 a	77.25 ab	60.50 bc
T. harzianum SYN	90.00	73.50 b	56.00 b	58.00 bc
R. solani	90.00	48.25 c	0.00 c	0.00 d
F-ratio		33.21	36.15	180.71
P-level		0.0003	0.0002	< 0.0001
%CV		5.40	12.60	5.94
Significance		* * *	* * *	* * *

Values followed by different letters within a column are significantly different (Student Newman Keul's test, $P \leq 0.05$); * * * Significant at $P \leq 0.001$

Mycelial growth was reduced as the Rizolex® concentration was increased. *T. atroviride* SY3A and *T. atroviride* SYN6 both exhibited tolerance to Rizolex® at 0.01g.l^{-1}. Concentrations as high as 3.0g.l^{-1} Rizolex® did not completely inhibit the *Trichoderma* tested (data not shown). At a concentration of 0.01g.l^{-1}, Rizolex® vapour adversely reduced the growth of *R. solani*, whereas concentrations of 0.1 and 0.25g.l^{-1} completely inhibited *R. solani* growth. None of the Rizolex® concentrations affected the growth of the *Bacillus* isolates. Visually, no differences in growth were found compared to the untreated controls. Higher Rizolex® (3.0g.l^{-1}) concentrations had no effect on *Bacillus* growth (data not shown).

6.3.2 Integrated biological control of R. solani *damping-off*

(a) Seedling Trial 1 (with three fungicide concentrations)

A significant increase in the number of surviving cucumber seedlings was recorded for the Rizolex® control concentrations as concentrations were increased (Table 6.2).

Table 6.2. Relationships between cucumber (Ashley) seedling survival achieved by single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates in combinations with three Rizolex® concentrations (0.01, 0.1 and 0.25g l^{-1}) against *R. solani* damping-off

Equation	Intercept	Slope	RSD	Adjusted R^2	P-value
Overall	14.420 (0.311)	5.55 (1.129)	3.263	0.13	0.0001**
Disease free control	22.611 (0.111)	-	0.333	0.00	-
Diseased control	8.500 (0.433)	-	1.299	0.00	-
Rizolex® Full strength control (1 g.l^{-1})	20.167 (0.601)	-	1.803	0.00	-
Rizolex® controls (reduced concentrations)	13.494 (0.586)	19.95 (3.769)	1.119	0.77	0.001**
T. atroviride SY3A	16.596 (0.987)	2.44 (6.346)	1.885	-0.12	0.71ns
T. atroviride SYN6	13.198 (0.993)	1.587 (6.385)	1.896	-0.13	0.81ns
T. harzianum SYN	15.609 (0.751)	1.19 (4.825)	1.433	-0.13	0.81 ns
Eco-T®	16.637 (0.635)	10.43 (4.083)	1.213	0.411	0.04 *
Bacillus B69	12.827 (0.568)	5.61 (3.649)	1.084	0.15	0.17 ns
Bacillus B81	15.487 (0.466)	- 3.12 (2.993)	0.889	0.01	0.33 ns
T. atroviride SY3A + *Bacillus* B69	13.332 (1.218)	10.66 (7.832)	2.326	0.10	0.22 ns
T. atroviride SYN6 + *Bacillus* B69	12.169 (0.821)	3.68 (5.277)	1.567	-0.07	0.51 ns
T. harzianum SYN + *Bacillus* B69	14.609 (0.257)	1.87 (1.655)	0.492	0.03	0.30 ns
Eco-T® + *Bacillus* B69	11.189 (1.188)	11.85 (7.637)	2.268	0.15	0.16 ns
T. atroviride SY3A + *Bacillus* B81	18.036 (0.763)	0.62 (4.905)	1.457	-0.14	0.90 ns
T. atroviride SYN6 + *Bacillus* B81	13.056 (0.984)	2.78 (6.326)	1.879	-0.11	0.67 ns
T. harzianum SYN + *Bacillus* B81	14.524 (0.804)	9.52 (5.169)	1.535	0.23	0.11 ns
Eco-T® + *Bacillus* B81	11.765 (1.276)	14.46 (8.205)	2.437	0.21	0.12 ns

* , ** Significantly different at $P \leq 0.05$ and $P \leq 0.01$ respectively; ns Not significant ($P > 0.05$)

The overall general linear regression equation of surviving seedlings across all treatments shows a significant increase (P = 0.0001) in the number of surviving seedlings with increase in Rizolex® concentrations (Table 6.2). Of all single and combined fungal and bacterial treatments, only Eco-T® in combination Rizolex® concentrations showed a significant increase (P = 0.04) in the number of surviving seedlings as concentrations were increased (Table 6.2). Linear regression for dry biomass of surviving seedlings showed no significant increase in dry biomass for any of the treatments (Regression analysis not presented). However, the overall general linear regression equation for dry biomass of surviving seedlings across all treatments showed a significant increase (P = 0.01) (regression analysis not presented) in dry biomass with increasing Rizolex® concentrations.

The disease free and the full strength Rizolex® controls recorded the highest percentage of surviving seedlings (94.4 and 84.0% respectively). These results were not significantly different from each other, but were significantly different from the diseased control in which only 35.4% of seedlings survived (Table 6.3). An increase in percentage seedling survival was observed for Rizolex® controls with increasing concentration. Although percentage seedling survival at 0.01g.l^{-1} was not significantly different from the diseased control, increases at 0.1 and 0.25g.l^{-1} concentrations (68.0 and 75.7% respectively) were (Table 6.3). At 0.25g.l^{-1} Rizolex® concentration, percentage seedling survival was not significantly different from the full strength Rizolex® (1g.l^{-1}) control treatment (Table 6.3).

At 0.01g.l^{-1} Rizolex® concentration, percentage seedling survival for the disease free control was significantly different from all *Trichoderma* and *Bacillus* treatments in combination with fungicide. However, 1g.l^{-1} Rizolex® control (84.0%) was not significantly different from *T. atroviride* SY3A (73.6%), Eco-T® (68.0%) and *T. atroviride* SY3A + *Bacillus* B81 (75.0%) treatments. However, the variability of the findings were such that, these treatments were also not significantly different from the rest of the *Trichoderma* and *Bacillus* treatments or the 0.01g.l^{-1} Rizolex® control with the exception of *T. atroviride* SYN6, *Bacillus* B69, *T. atroviride* SYN6 + *Bacillus* B69 and Eco-T® + *Bacillus* B69.

Table 6.3 Seedling survival of cucumber (Ashley) as influenced by integration of single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates with three Rizolex® concentrations (0.01, 0.1 and 0.25g.l^{-1}) to control *R. solani* damping-off in the greenhouse

Isolates/Treatments/Combinations	Rizolex® concentrations (g.l^{-1})					
	0.01		0.1		0.25	
	Mean number of surviving seedlings after 4 weeks	% Seedling survival after 4 weeks	Mean number of surviving seedlings after 4 weeks	% Seedling survival after 4 weeks	Mean number of surviving seedlings after 4 weeks	% Seedling survival after 4 weeks
Disease free control	22.67 a	94.46	22.67 a	94.46	22.67 a	94.46
Diseased control	8.50 g	35.42	8.50 f	35.42	8.50 f	35.42
Rizolex® controls (reduced concentrations)	13.17 cdefg	54.88	16.33 cde	68.04	18.17 bcd	75.71
Rizolex® Full strength control (1g.l^{-1})	20.17 ab	84.04	20.17 ab	84.04	20.17 b	84.04
T. atroviride SY3A	17.67 bcd	73.63	15.17 cde	63.21	17.83 bcd	74.29
T. atroviride SYN6	12.50 defg	52.08	14.50 cde	60.42	13.17 e	54.88
T. harzianum SYN	15.17 cdef	63.21	16.67 cde	69.46	15.67 cde	65.29
Eco-T®	16.33 bcde	68.04	18.33 bc	76.37	19.00 bc	79.17
Bacillus B69	12.50 defg	52.08	14.00 cde	58.33	14.00 e	58.33
Bacillus B81	15.67 cdef	65.29	14.83 cde	61.79	14.83 de	61.79
T. atroviride SY3A + *Bacillus* B69	13.17 cdefg	54.88	14.83 cde	61.79	15.83 cde	65.95
T. atroviride SYN6 + *Bacillus* B69	12.33 efg	51.38	12.33 e	51.38	13.17 e	54.88
T. harzianum SYN + *Bacillus* B69	14.50 cdef	60.42	15.00 cde	62.50	15.00 de	62.50
Eco-T® + *Bacillus* B69	12.50 defg	52.08	13.67 de	56.96	13.67 e	56.96
T. atroviride SY3A + *Bacillus* B81	18.00 bc	75.00	18.17 bcd	75.70	18.17 bcd	75.70
T. atroviride SYN6 + *Bacillus* B81	13.50 cdef	56.25	12.67 e	52.79	14.00 e	58.33
T. harzianum SYN + *Bacillus* B81	14.50 cdef	60.42	15.67 cde	65.29	16.83 bcde	70.13
Eco-T® + *Bacillus* B81	13.00 cdefg	54.12	13.33 e	55.54	15.33 de	63.88
F-ratio	10.32		11.31		15.98	
P-level	0.0001		0.0001		0.0001	
% CV	12.67		10.58		8.69	
Significance	* * *		* * *		* * *	

Values followed by different letters within a column are significantly different (Student Newman Keul's test, $P \leq 0.05$); *** Significantly different at $P \leq 0.001$

Table 6.4 Seedling dry biomass of cucumber (Ashley) as influenced by integration of single and dual inoculations of selected *Trichoderma* and *Bacillus* isolates with three Rizolex® concentrations (0.01, 0.1 and 0.25g.l^{-1}) to control *R. solani* damping-off in the greenhouse

Isolates/Treatments/Combinations	Rizolex® concentrations (g.l^{-1})					
	0.01		0.1		0.25	
	Mean dry biomass of after 4 weeks (g)	% Dry biomass after 4 weeks (% of disease free control)	Mean dry biomass of after 4 weeks (g)	% Dry biomass after 4 weeks (% of disease free control	Mean dry biomass of after 4 weeks (g)	% Dry biomass after 4 weeks (% of disease free control
Disease free control	8.33 a	100	8.33 a	100	8.33 a	100
Diseased control	4.04 c	52.82	4.04 b	52.82	4.04 b	52.82
Rizolex® controls (reduced concentrations)	6.74 abc	80.91	7.13 ab	85.59	6.10 ab	73.23
Rizolex® Full strength control (1 g.l^{-1})	7.69 ab	92.32	7.69 a	92.32	7.69 a	92.32
T. atroviride SY3A	7.65 ab	91.84	7.25 ab	87.03	7.26 a	87.15
T. atroviride SYN6	5.54 abc	66.51	6.22 ab	74.67	5.69 ab	68.31
T. harzianum SYN	7.71 ab	92.56	8.24 a	98.92	6.94 a	83.31
Eco-T®	7.13 abc	85.59	7.20 ab	86.43	7.21 a	86.55
Bacillus B69	5.62 abc	67.47	6.31 ab	75.75	5.76 ab	69.15
Bacillus B81	7.35 ab	88.24	6.82 ab	81.87	7.33 a	87.99
T. atroviride SY3A + *Bacillus* B69	5.95 abc	71.43	7.11 ab	85.35	6.99 a	83.91
T. atroviride SYN6 + *Bacillus* B69	5.33 abc	63.99	5.55 ab	66.63	6.65 a	79.83
T. harzianum SYN + *Bacillus* B69	6.60 abc	79.23	8.08 a	96.99	7.10 a	85.23
Eco-T® + *Bacillus* B69	4.76 bc	57.14	5.99 ab	71.91	5.44 ab	65.31
T. atroviride SY3A + *Bacillus* B81	7.48 ab	89.79	8.08 a	96.99	7.13 a	85.59
T. atroviride SYN6 + *Bacillus* B81	6.42 abc	77.07	6.64 ab	79.71	6.80 a	81.63
T. harzianum SYN + *Bacillus* B81	6.63 abc	79.59	7.50 a	90.04	7.89 a	94.72
Eco-T® + *Bacillus* B81	5.05 abc	60.62	6.13 ab	73.59	6.66 a	79.95
F-ratio	3.37		2.68		3.08	
P-level	0.001		0.006		0.002	
%CV	17.49		16.71		14.86	
Significance	* * *		* *		* *	

Values followed by different letters within a column are significantly different (Student Newman Keul's test, $P \leq 0.05$); **, *** Significantly different at $P \leq 0.01$ and $P \leq 0.001$ respectively

Only one dual inoculation, *T. atroviride* SY3A + *Bacillus* B81 was significantly better than single inoculations of *T. atroviride* SYN6 and *Bacillus* B69. Seven treatments, including the Rizolex® control at 0.01g.l^{-1} concentration were not significantly different from the diseased control (Table 6.3).

At 0.1g.l^{-1} Rizolex® concentration, all single and dual inoculations of *Trichoderma* and *Bacillus* isolates were significantly different from the diseased control. The disease free control was significantly different from all other treatments except the full strength Rizolex® control (Table 6.3). Percentage seedling survival for Eco-T® and *T. atroviride* SY3A + *Bacillus* B81 were the highest among the single and dual *Trichoderma* and *Bacillus* treatments at 0.1g.l^{-1} Rizolex® concentration and were not significantly different from the 0.1g.l^{-1} Rizolex® control.

At 0.25g.l^{-1} Rizolex® concentration, all single and dual inoculations of *Trichoderma* and *Bacillus* isolates were again significantly different from both the diseased and disease free control (Table 6.3). In addition to Eco-T® and *T. atroviride* SY3A + *Bacillus* B81 treatments, three other treatments, viz, the 0.25g.l^{-1} Rizolex® control, *T. atroviride* SY3A, and *T. harzianum* SYN + *Bacillus* B81 were not significantly different from the full strength Rizolex® control.

For dry biomass, the disease free control was significantly different from the diseased control, but not significantly different from full strength Rizolex® control and the controls for the different/reduced Rizolex® concentrations (Table 6.4). Moreover, all 17 treatments recorded more than 50.0% of the dry biomass of the disease free control. The least percentage dry biomass (52.8%) was recorded by the diseased control (Table 6.4).

At 0.01g.l^{-1} Rizolex® concentration, dry biomass of only four *Trichoderma* and *Bacillus* treatments, *T. atroviride* SY3A, *T. harzianum* SYN, *Bacillus* B81 and *T. atroviride* SY3A + *Bacillus* B81 and the full strength Rizolex® control were significantly greater than the diseased control.

Also at 0.1g.l^{-1} Rizolex® concentration, only four *Trichoderma* and *Bacillus* treatments, *T. harzianum* SYN, *T. harzianum* SYN + *Bacillus* B69, *T. atroviride* SY3A + *Bacillus* B81 and *T. harzianum* SYN + *Bacillus* B81 and the full strength Rizolex® control were significantly different from the diseased control (Table 6.4) whereas, at 0.25g.l^{-1}, all treatments except for

the 0.25g.l^{-1} Rizolex® control, *T. atroviride* SYN, *Bacillus* B69 and Eco-T® + *Bacillus* B69 were significantly greater than diseased control treatment.

(b) Seedling Trial 2 (with one fungicide concentration)

Seedling survival within the disease free control was significantly better than the diseased, and Rizolex® (0.1g.l^{-1}) controls (Table 6.5). No significant difference was found between the disease free control and the full strength Rizolex® (1g.l^{-1}).

Table 6.5 Survival and dry biomass of cucumber seedlings (Ashley) as a result of integration of *Trichoderma* and *Bacillus* treatments with Rizolex® to control damping-off caused by *R. solani* under greenhouse conditions

Isolates/Treatments/Combinations with or without Rizolex®	Mean number of surviving seedlings after 4 weeks	% Seedling survival after 4 weeks	Mean dry biomass after 4 weeks (g)	% Dry biomass after 4 weeks (% of disease free control)
Disease free control	23.67 a	98.63	9.21	100 a
Diseased control	9.83 g	40.96	4.51	48.97 d
Rizolex® control (0.1g.l^{-1})	15.83 cdef	56.96	6.34	68.84 bc
Full strength Rizolex® control (1g.l^{-1})	20.67 abc	86.13	6.88	74.70 bc
T. atroviride SY3A	16.17 bcdef	67.38	7.85	85.23 abc
T. atroviride SY3A (R)	19.17 abcde	79.88	7.16	77.74 abc
T. harzianum SYN	15.83 cdef	65.96	7.71	83.17 abc
T. harzianum SYN (R)	20.83 ab	86.79	9.25	100.43 a
Eco-T®	19.50 abcde	81.25	6.73	73.07 bc
Eco-T® (R)	20.33 abc	84.71	6.48	70.36 bc
Bacillus B81	12.17 fg	50.71	6.02	65.36 c
Bacillus B81 (R)	16.67 bcdef	69.46	6.99	75.90 bc
T. atroviride SY3A + *Bacillus* B81	15.50 def	64.58	7.88	85.56 abc
T. atroviride SY3A + *Bacillus* B81 (R)	20.67 abc	86.13	8.32	90.34 ab
T. harzianum SYN + *Bacillus* B81	14.83 ef	61.79	7.53	81.76 abc
T. harzianum SYN + *Bacillus* B81 (R)	20.17 abcd	84.04	8.23	89.36 ab
Eco-T® + *Bacillus* B81	14.50 f	60.42	7.40	80.35 ab
Eco-T® + *Bacillus* B81 (R)	19.33 abcde	80.54	8.31	90.23 abc
F-ratio	12.28		6.91	
P-level	0.0001		0.0001	
% CV	9.90		10.23	
Significance	***		***	

Values followed by different letters within a column are significantly different (Student Newman Keul's test, $P \leq 0.05$)
*** Significantly different at $P \leq 0.001$ respectively
(R) indicates *Trichoderma* and *Bacillus* and their respective combinations supplemented with 1m*l* of 0.1g.l^{-1} Rizolex®

Trichoderma and *Bacillus* treatments and their respective combinations without Rizolex® (0.1g.l^{-1}) were significantly better than the diseased control (except *Bacillus* B81) but in general term they were not as effective as the disease free or full strength Rizolex® controls with the exception of Eco-T® (Table 6.5). For example, *T. atroviride* SY3A treatment caused 67.3% seedling survival which was significantly better than the diseased control, but not different to the disease free or full strength Rizolex® controls. However, Eco-T® caused

81.2% seedling survival which was not significantly different from the disease free or full strength Rizolex® controls (Table 6.5).

A general trend was that combined applications of microbial inoculants plus 0.1g.l^{-1} Rizolex® resulted in a synergistic effect with a corresponding increase in percentage seedling survival. The increase in percentage seedling survival were comparable to the full strength Rizolex® control (Table 6.5). Moreover, percentage seedling survival was better than the *Trichoderma* and *Bacillus* and the 0.1g.l^{-1} Rizolex® control. For example, *T. harzianum* SYN and Eco-T® plus Rizolex® (0.1g.l^{-1}) gave 86.7% and 84.7% seedling survival respectively which was comparable to the full strength Rizolex® control and not different significantly from the disease free control. Moreover, percentage seedling survival was better than either of the individual components. *T. harzianum* SYN plus 0.1g.l^{-1} Rizolex® for example, caused percentage seedling survival of 86.7% which was better than *T. harzianum* SYN and the 0.1g.l^{-1} Rizolex® used in isolation. With either a single or dual inoculations of *Trichoderma* and/or *Bacillus* isolates improved percentage seedling survival were achieved with the integrated system (Table 6.5).

The diseased control exhibited a significantly lower seedling dry biomass compared to all the other treatments (Table 6.5). *T. harzianum* SYN in combination with Rizolex® (0.1g.l^{-1}) achieved the highest percentage dry biomass (100.4%) which was 0.4% higher but not significantly different to the disease free control (100.0%). With the exception of *Bacillus* B81 without 0.1g.l^{-1} Rizolex®, the percentage dry biomass for the rest of the *Trichoderma* and *Bacillus* treatments with or without 0.1g.l^{-1} Rizolex® were higher than the 0.1gl^{-1} Rizolex® control. Percentage dry biomass of *Trichoderma* and *Bacillus* treatments without 0.1g.l^{-1} Rizolex® were not significantly different from *Trichoderma* and *Bacillus* treatments with 0.1g.l^{-1} Rizolex® (Table 6.5).

(c) Cucumber cultivar trial

The results for the cucumber cultivar trial are presented in Table 6.6. Under the prevailing growth conditions, two cucumber cultivars, Ashley and 22-72 RZ® were found to germinate quicker than the Cumlaude RZ® cultivar.

The percentage seedling survival rated for the disease free control was significantly better than the diseased control for all the three cucumber cultivars tested, but not significantly

Table 6.6 Effect of *T. atroviride* SY3A + *Bacillus* B81 with or without Rizolex® on damping-off of three cucumber cultivars caused by *R. solani* under greenhouse conditions

Treatments	Cucumber Cultivars					
	Ashley		Cumlaude RZ®		22-72 RZ®	
	Mean seedling survival after 5 weeks	Mean dry biomass after 5 weeks (g)	Mean seedling survival after 5 weeks	Mean dry biomass after 5 weeks (g)	Mean seedling survival after 5 weeks	Mean dry biomass after 5 weeks (g)
Disease free control	2.00 (100) a	5.33 [100] a	1.80 (90) a	4.19 [100] a	2.00 (100) a	4.42 [100] a
Diseased control	0.90 (45) b	2.60 [48.78] b	0.50 (25) c	1.37 [32.70] c	1.00 (50) c	2.15 [48.64] b
Rizolex® control (0.1g.l^{-1})	1.80 (90) a	4.59 [86.12] a	1.70 (85) a	4.03 [96.18] a	1.70 (85) ab	3.70 [83.71] a
Full strength Rizolex® control (1g.l^{-1})	1.90 (95) a	4.69 [87.99] a	1.90 (95) a	4.38 [104.53] a	1.80 (90) ab	4.03 [91.18] a
T. atroviride SY3A + *Bacillus* B81	1.60 (80) a	4.85 [90.99] a	1.10 (55) b	2.29 [54.65] bc	1.30 (65) bc	2.10 [47.51] b
T. atroviride SY3A + *Bacillus* B81 (R)	1.90 (95) a	5.11 [95.87] a	1.50 (75) ab	3.10 [73.99] ab	1.80 (90) ab	4.28 [96.83] a
F-ratio	8.36	8.10	10.49	9.54	5.64	7.41
P-level	0.0001	0.0001	0.0001	0.0001	0.0003	0.0001
% CV	26.44	24.16	36.58	38.11	31.13	35.47
Significance	***	***	***	***	***	***

Values followed by different letters within a column are significantly different (Student Newman Keul's test, $P \leq 0.05$); *** Significantly different at $P \leq 0.001$
(R) Indicates *Trichoderma* and *Bacillus* and their respective combinations supplemented with 1m*l* of 0.1g.l^{-1} Rizolex®
( ) Values indicate percentage seedling survival calculated from actual number of surviving seedlings from two greenhouse pot trials
[ ] Values indicate percentage dry biomass of surviving seedlings calculated as a percentage of disease free control

different to the two Rizolex® controls (1g.l^{-1} and 0.1g.l^{-1} respectively) (Table 6.6). With the *T. atroviride* SY3A + *Bacillus* B81 treatment, the best performance was observed in cultivar Ashley with 95% and 80% seedling survival with and without 0.1g.l^{-1} Rizolex® respectively.

T. atroviride SY3A + *Bacillus* B81 without Rizolex® (0.1g.l^{-1}) were significantly better than the diseased control in two cultivars, viz, Ashley and Cumlaude RZ®. A general trend was that *T. atroviride* SY3A + *Bacillus* B81 plus 0.1g.l^{-1} Rizolex® resulted in increased seedling survival for all three cultivars which was comparable to full strength Rizolex® control and the disease free control (Table 6.6). These results were better than either of the individual components (*T. atroviride* SY3A + *Bacillus* B81 and 0.1g.l^{-1} Rizolex®) used alone (Table 6.6).

Percentage seedling biomass for the disease free control was significantly better than the diseased control but not significantly different to the full strength Rizolex® and 0.1g.l^{-1} Rizolex® (Table 6.6). Seedling biomass for *T. atroviride* SY3A + *Bacillus* B81 treatment without Rizolex® (0.1g.l^{-1}) was better than the diseased control for cultivars Ashley and Cumlaude RZ®. However, with 0.01g.l^{-1} Rizolex®, seedling biomass for all three cultivars were comparable to the full strength Rizolex® control and the disease free control (Table 6.6).

6.4 Discussion

In this study, *in vitro* bioassay showed that selected *Trichoderma* isolates partially tolerated a range of Rizolex® concentrations. *Bacillus* isolates were not affected. In the greenhouse, only two treatments in Seedling Trial 1 (Rizolex® control-reduced concentrations) and integration of Eco-T® with 0.1g.l^{-1} Rizolex® showed significant relationships between levels of Rizolex® concentrations and the disease control in the integrated system. In Seedling Trial 2, a trend towards improved disease control was achieved when *Trichoderma* and *Bacillus* isolates were used together with Rizolex® (0.1g.l^{-1}) compared to the isolates and Rizolex® (0.1g.l^{-1}) used alone. Seedling trials with three cucumber cultivars provided similar responses towards improved disease control when a dual inoculation of an isolate of *Trichoderma* and *Bacillus* was integrated with 0.1g.l^{-1} Rizolex®.

Chemical residue in foods is of major concern to the consumer leading to criticisms of the chemical crop protection industry (Urech, 2000). Hence, the main objective of this study was to ascertain whether reduced concentrations of Rizolex®, in combination with selected

Trichoderma and *Bacillus* isolates could be used to achieve effective control of *R. solani* damping-off comparable to the manufacturer's dosage recommendation for Rizolex®. The control achieved with 0.1g.l^{-1} Rizolex® in combination with selected *Trichoderma* and *Bacillus* treatments (*T. harzianum* SYN, Eco-T® and *T. atroviride* SY3A + *Bacillus* B81 and *T. harzianum* SYN + *Bacillus* B81) were comparable to that of the prescribed Rizolex® dosage (1g.l^{-1}). The significance of this result is that fungicide application rates could feasibly be reduced resulting in lowered fungicide residues and a possible decrease in costs per season. Similar experiments by Henis *et al*. (1978) used a combination of pentachloronitrobenzene (PCNB) and an isolate of *T. harzianum* to control *R. solani* damping-off on radish. In a separate study, Kaur and Mukhopadhyay (1992) successfully controlled "chickpea wilt complex" by integrating three fungicides (Vitavax-200, Bavistin and Ziram) separately with an isolate of *T. harzianum*.

The *in vitro* bioassay had a predictive value on whether lowered concentrations of Rizolex® and the selected *Trichoderma* and *Bacillus* isolates could be used in an integrated control system. At concentrations ranging from 0.1-3.0g.l^{-1} Rizolex® did not exhibit fungicidal or bactericidal action towards either the *Trichoderma* and *Bacillus* isolates indicating that the two disease control systems could be used together for management of plant diseases in the greenhouse. Kaur and Mukhopadhyay (1992) suggested that reduced doses of fungicide could weaken fungal pathogens thereby increasing the antagonistic activity of biological control agents when used in an integrated system. Based on this assumption, and the results obtained during *in vitro* studies, Rizolex® at a concentration of 0.1g.l^{-1} was chosen for further testing. Integrated control with this concentration proved successful and much better seedling performance was achieved than for a 0.1g.l^{-1} Rizolex® control or selected *Trichoderma* and *Bacillus* isolates used alone. This synergistic response supports the hypothesis made by Kaur and Mukhopadhyay (1992). Lowered doses of PCNB have been found to enhance the efficiency of *T. harzianum* (Chet *et al*., 1979). Weakening of the pathogen could also mean less competition between the pathogen and the biological control agents for resources.

Three cucumber cultivars, Ashley, Cumlaude RZ® and 22-72 RZ® responded to *T. atroviride* SY3A + *Bacillus* B81 inoculations with/without a 0.1g.l^{-1} Rizolex® concentration. Percentage surviving seedlings in the integrated and non-integrated disease control in both cases was better in Ashley and 22-72 RZ® cultivars than for Cumlaude RZ®. This was partly attributed to the different germination times exhibited by the different cucumber cultivars. Ashley and

22-72 RZ® germinated quicker than Cumlaude RZ®. Hence, early germination of cultivars Ashley and 22-72 RZ® might have promoted early and vigorous microbial and fungal activities in the rhizosphere through release of root exudates than would be observed in Cumlaude RZ® cultivar.

Although further trials might be needed to ascertain the integrated use of reduced Rizolex® concentrations and biological control agents such as *Trichoderma* and *Bacillus* as a disease control system in different soils, growth media and under different sets of environmental conditions, the results presented here indicate the possibility of chemical and biological integrated control systems to curb *R. solani* damping-off in the greenhouse. This work suggests the need for development and implementation of proper and workable strategies with this integration system, which will drastically reduce the amount of Rizolex® used while still maintaining an effective control of *R. solani* damping-off.

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CHAPTER SEVEN

General overview

"…Good Agricultural Practices is our way of translating all the wishful thinking on sustainable agriculture into very concrete recommendations for countries and production systems…" Fresco Louise O., 2003 (Head of FAO's Agriculture Department).

The contributions of beneficial fungal and bacterial species to the development of sustainable agriculture has been emphasised in literature (Lewis and Papavizas, 1991; Schippers *et al*., 1995). Such contributions range from control of plant diseases, increased plant growth as well as enhanced mineral uptake by plants. *Trichoderma* and *Bacillus* spp. are among the beneficial fungal and bacterial species that have been widely studied for their role in biological control of plant diseases and in plant growth promotion. Moreover, species belonging to these two genera have been commercialised and are currently available commercially (Kim *et al*., 1997a; Koch, 1999).

The findings presented in this thesis resulted from the evaluation six *Trichoderma* and three *Bacillus* isolates and their respective combinations, for biological control of *Rhizoctonia solani* Kühn damping-off on cucumber (*Cucumis sativus* L.) and growth promotion on dry bean (*Phaseolus vulgaris* L.). Experimental trials were all conducted under greenhouse and shadehouse conditions with the use of seed treatment as the method of application. It was established that:

- During *in vitro* dual culture bioassays between *Trichoderma* isolates and *R. solani*, mycelium browning of *R. solani* caused by *T. atroviride* SY3A and *T. harzianum* SYN correlated with enhanced mycoparasitism visualized using scanning electron microscopy ultrastructure studies. *Trichoderma* isolates, rated Class 1 and Class 2 on the invasion ability scale *in vitro,* exhibited the best biological control activity *in vivo*.
- None of the *Trichoderma* and *Bacillus* isolates antagonised each other *in vitro*.

- All the *Trichoderma* and *Bacillus* isolates showed activity for at least one of the following enzymes *in vitro*, viz., chitinase, cellulase, lipase, protease, amylase, pectinase and siderophore production.
- The *Bacillus* isolates tested were the most effective agents of plant growth promotion in this study whereas the *Trichoderma* isolates contributed the most effective biological control agents.
- Improved dry bean seedling growth/vigour did not correlate with crop yield studies.
- Dual inoculations of *Trichoderma* and *Bacillus* isolates to control *R. solani* damping-off were generally better than single inoculations of the *Bacillus* isolates but not significantly different to single inoculations of *Trichoderma* sp.
- Growth promotion by the *Trichoderma* and/or *Bacillus* isolates was more apparent under nutrient limiting conditions.
- Integrated control (i.e., combining chemical and biological treatments) of *R. solani* damping-off on cucumber seedlings proved successful and similar response trends were observed on three different cucumber cultivars.

Two *Trichoderma* isolates, *T. atroviride* SY3A and *T. harzianum* SYN both caused mycelium browning of *R. solani in vitro* which correlated with enhanced mycoparasitism during ultrastructure studies.

Mycoparasitism is one of the mechanisms used by *Trichoderma* spp. for biological control of plant pathogens (Tronsmo and Hjeljord, 1998). In as much as this mechanism is important, there is a lack of information in the literature detailing as to how mycoparasitism can be rapidly assessed *in vitro*. Although ultrastructure studies have been explored, the question that still remains unanswered is how to effectively screen for mycoparasites from a large pool of *Trichoderma* isolates which shows no antifungal activity against *R. solani*. In this thesis, two *Trichoderma* isolates, *T. atroviride* SY3A and *T. harzianum* SYN caused browning of *R. solani* mycelium *in vitro* on V8 juice agar medium. Ultrastructure studies showed extensive mycoparasitism by these two isolates compared to the rest of the *Trichoderma* isolates screened, which showed no browning effect. These two isolates also provided the best control of damping-off in greenhouse trials. It is therefore proposed that *R. solani* could be used as an indicator organism for screening for mycoparasitism. Ortiz and Orduz (2000) used a colonization ability scale to screen *Trichoderma* and *Gliocladium* isolates against *Attamyces* sp. *in vitro* but we are not aware of any invasive rating scale incorporating the melanistic

browning reactions as observed in this study with *R. solani*. This finding could serve as a step forward in developing more comprehensive rating and screening systems for *Trichoderma* isolates, which are mycoparasitic but show no antifungal activity during initial *in vitro* screening.

The observation that none of the *Bacillus* isolates tested inhibited the *Trichoderma* isolates *in vitro* indicates that these two groups of organisms have the potential to be used together for biological control and/or plant growth promotion applications.

A question that needs to be addressed, is to determine what the optimum combination of biological traits required from each organism are, in order to achieve a synergistic effect. Jisha and Alagawadi (1996) combined phosphate solubilizing *B. polymyxa* and cellulolytic *T. harzianum*, which resulted in increased yield and nitrogen uptake in sorghum (*Sorghum bicolor* L. Moench) compared to single inoculations of each organism. In this study, the greatest growth promotion of dry bean seedlings was achieved with dual inoculations of *T. atroviride* SYN6 + *Bacillus* B69. Both isolates were selected on the basis of their antagonism to *R. solani* rather than their growth promoting properties. Ideally, a combination of biological control and growth promoting traits would be advantageous and would result in a better biological control and plant growth promotion effects when compared to any of the organisms used alone. In this study, combinations of the *Trichoderma* and *Bacillus* spp. generally resulted in a better biological control of *R. solani* than the corresponding *Bacillus* sp. isolate used alone. Duffy *et al*. (1996) reported that a combination of *T. koningii* and *Pseudomonas fluorescens* Q29z-80, which both exhibited biological control properties against take-all on wheat increased wheat (*Triticum aestivum* L.) yield in the field compared to *P. fluorescens* alone, but the results were not significantly different from *T. koningii* applied by itself. They also reported that combinations of *T. koningii* with any one of six fluorescent *Pseudomonas* spp. resulted in a better disease control than the *Pseudomonas* spp. used alone, in growth chamber experiments. The biological properties inherent to a biological control agent (BCA) or plant growth promoting rhizobacteria (PGPR) or both, therefore contribute enormously towards a better synergy for increased biological control and plant growth promotion.

Increased seedling growth observed in dry beans under greenhouse did not necessarily translate into yield increase.

This supports the findings of Schroth and Becker (1990) who reported that seedling growth promotion does not necessarily give a corresponding increase in crop yield. This poses the question whether plant growth promotion could simply result from early germination and seedling vigour, which leads to early flowering and fruiting rather than increasing yield. In this study, increases in dry bean yield were observed for some isolates but results were not consistent in the two yield trials. Similar inconsistencies in yield studies have been reported in literature (Kloepper *et al*., 1989; Schroth and Becker, 1990). Assuming that plant growth promotion results from early germination, seedling vigour and early flowering and fruiting, then the "positive" growth promoting effect observed on dry bean could perhaps be more beneficial to crops such as cucumbers and tomato (*Lycopersicon esculentum* L.) which have shorter crop production cycles. Furthermore, the "positive" growth promoting effect could also extend the growth season of the plant as well as promote a rapid and uniform turnover of seedlings in the nursery.

Dry bean plants treated with *Trichoderma* and/or *Bacillus* spp. and grown in composted pine bark medium under nutrient limiting conditions recorded increases in growth, photosynthetic efficiency, nitrogen uptake and root nodule formation.

These findings support the hypothesis that enhanced growth promotion can best be achieved under sub-optimal growth conditions (Rabeendran *et al*., 2000). They also suggest that *in vivo* screening for plant growth promoters would be more successful under sub-optimal growth conditions thereby allowing the "cause" and "effect" of plant growth promoting bacteria or fungi on seedlings to be more easily observed. The Plant Efficiency Analyser (PEA) used to assess photosynthetic efficiency proved to be a useful tool in screening for plant growth promotion. Delays in leaf senescence and increased mineralization were expressed in terms of Fv/Fm values and this was later supported by dry leaf analysis of mineral contents. However, this study needs to be confirmed under field studies. There appeared to be a relationship between nitrogen leaf content and the degree of nodulation on roots, which in turn appeared to be influenced by *Trichoderma* and/or *Bacillus* inoculations. The mechanisms involved were not determined and require further elucidation.

Lowered dosages of Rizolex®, co-inoculated with *Trichoderma* and/or *Bacillus* spp. achieved control of *R. solani* damping-off of cucumber seedlings equivalent to recommended dosages of the fungicide.

This result corroborates the findings of Kaur and Mukhopadhyay (1992) and Elad *et al*. (1993) which showed that combinations of lowered dosages of fungicides with BCAs can be used to effectively control plant diseases. A synergistic relationship between Rizolex® and the BCAs was apparent and showed that reduced dosages of the fungicide in an integrated biological and chemical control system stressed and weakened the target plant pathogen rendering it susceptible to antagonistic attack by the BCAs (Kaur and Mukhopadhyay, 1992; Tronsmo and Hjeljord, 1998). With legislation concerning the use of agric-chemicals/fungicides in South Africa becoming more stringent, the need to apply reduced amounts of fungicides while still maintaining proper control of plant diseases has become imperative. Developing and adopting an integrated crop protection system such as this could significantly reduce the amount and frequency of fungicide applications required thereby reducing cost and adverse effects of fungicides on the environment.

A case for the dual application of *Trichoderma* and *Bacillus* spp.

With regards to the *Trichoderma-Bacillus* dual inoculation system, benefits such as increase in growth and mineralization were achieved. Also, increase in disease control was achieved with the integrated Rizolex® and dual inoculation system. Although the *Trichoderma* and *Bacillus* isolates used in this study may not be the perfect combination in dual inoculation studies, the results suggest that *Trichoderma* and *Bacillus* could be used together. Unravelling the necessary conditions under which these two organisms could be used together efficiently and effectively could help reduce inconsistencies reported with the use of single organisms. The work presented in this thesis considered seed treatment as the only method of BCA application; the question of whether different inoculation systems, such as seedling drenches, capping or broadcasting would improve biological control and growth promotion efficiencies in a dual inoculation system needs to be looked at.

The way forward?

This thesis forms the basis of *Trichoderma-Bacillus* interaction studies and proposes that with the right combinations, these two organisms can be used together to enhance plant growth and biological control of plant diseases. What remains to be resolved is how these two organisms can be formulated together as a single commercial product for use on agricultural crops. Obstacles that would need to be overcome include formulation and storage conditions, cost and shelf life. Larkin and Fravel (1998) suggested that combinations of fungi and bacteria might provide protection against plant diseases at different times and/or under different conditions. To the best of our knowledge, currently there are no registered commercial products on the market that contain fungal and bacterial mixtures. For this to become a reality, there is an urgent need for the development of appropriate selection criteria of candidate organisms and strategies towards product formulation and implementation.

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APPENDIX 1

Composition of V8 agar medium and *Trichoderma* selective medium (TSM)

V8 AGAR MEDIUM (Composition)

V8 tomato juice	200ml
$CaCO_3$	3.0g
Agar (Merck)	20.0g
Distilled water	800ml

Mix and autoclave for 15min at 121^0C.

(*TRICHODERMA* SELECTIVE MEDIUM) (TSM) (Composition)

The basal medium used consisted of the following:

$MgSO_4.7H_2O$	0.2g
K_2HPO_4	0.9g
KCl	0.15g
NH_4NO_3	1.0g
Glucose (anhydrous)	3.0g
Rose Bengal	0.15g
Agar	20.0g

The constituents were added to 900ml of distilled water and autoclaved at 121^0C for 15min.

The biocidal ingredients were:

Chloramphenicol (Crystallized)	0.25g
Quintozene (PNCB wettable powder)	0.2g
Captan (Kaptan wettable powder)	0.2g
Propamocarb-hydrochloride (Previcur, solution concentrate)	1.2ml

Mix the ingredients in 50ml of sterilized (autoclaved at 121^0C for 15min) distilled water and added to the autoclaved basal medium.