BIOLOGICAL CONTROL OF *PHYTOPHTHORA* ROOT ROT OF CITRUS SEEDLINGS AND CUTTINGS

by

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DECLARATION

I, Abraha Okbasillasie Abraham, declare that the results have not been submitted in part or as a whole to another University and except where the work of others is indicated or acknowledged in the text, are the results of my own investigation.

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I certify that the above statement is correct

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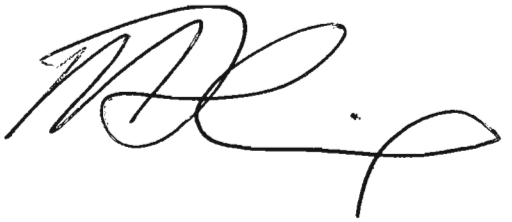

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ABSTRACT

With an increasing realization that many agrochemicals are hazardous to animals and humans, came the desire to replace these chemical agents with biological approaches that are more friendly to the environment and human health. Microorganisms play an important role in plant disease control, as naturally occurring antagonists. Microorganisms may also have beneficial effects on plant development when applied to plant roots. Research efforts worldwide have recorded successes in biological control and growth stimulation on many crops, particularly when using members of the genera *Bacillus* and *Trichoderma*. Their use on citrus rootstock could be advantageous to nurserymen and growers in reducing the incidence of seedling mortality and increasing production.

To achieve these objectives, laboratory and tunnel experiments were conducted to develop effective biocontrol agents for citrus seedlings and cuttings.

Nineteen out of 23 *Trichoderma* isolates tested *in vitro* against *Phytophthora parasitica* sp showed antagonistic activity by hyperparasitism and four out of eight *Bacillus* isolates resulted in antagonism by forming inhibition zones. The positive *in vitro* activity of *Trichoderma* and *Bacillus* isolates on *Phytophthora* provided motivation step for further trials in the greenhouse to evaluate their biological control activity on citrus seedlings and cuttings.

A greenhouse trial was carried out to evaluate the biological control potential of 23 *Trichoderma* isolates (drenched at 5 x 10^5 spores / ml) and two *Bacillus* isolates (drenched at 1 X 10^6 or 1 X 10^8 colony forming units (CFU) / ml) to suppress *Phytophthora parasitica* sp. of rough lemon (*Citrus jambhirini* Lush.) seedlings. Five isolates of *Trichoderma* (AA12, AA5, *Trichoderma harzianum* (AA16), SY3F and Eco-T®) were highly effective in suppressing *Phytophthora* root rot, with AA12 providing the best control. The *Bacillus* isolates also suppressed the pathogen but were not as effective as the *Trichoderma* isolates. This trial was used to test for growth stimulation activity by some of the biocontrol agents.

To verify these results, a further trial was carried out to evaluate growth stimulation capabilities in the absence of any pathogen. *Trichoderma* Isolates AA13 and AA17 caused no

change in seedling growth, while other *Trichoderma* and *Bacillus* isolates had an inhibitory effect on the seedling growth. This trial indicated that the biocontrol activity was affected by inoculum densities, and as a result *in vitro* sporulation capacity was evaluated. *Trichoderma* Isolate AA16 was the largest spore producer, followed by Eco-T®. Spore production was lowest from *Trichoderma* isolates AA4 and AA12.

Growth stimulation responses of *Trichoderma* Isolates AA4, AA16, Eco-T® and SYN6 were further studied at four different doses (1×10^3, 1×10^4, 5×10^5 or 1×10^6 spores / ml) on rough lemon and trifoliate orange seedlings. Trifoliate oranges responded positively to 1×10^4 and 5×10^5 spores / ml of Eco-T®, but rough lemon responded negatively to all dosages of the *Trichoderma* isolates applied. This indicates that the inoculum density responses may be host specific. Higher population density of 1×10^6 spores / ml of all tested *Trichoderma* isolates had a stunting effect on seedling growth of both species.

Based on the positive results of individual applications of some *Trichoderma* and *Bacillus* isolates, of the biological control agents on rough lemon seedlings against *Phytophthora parasitica* in an earlier greenhouse trial, their combined effect in the control of the pathogen was performed. Before carrying out a greenhouse trial, activities of the isolates to be combined were evaluated *in vitro*. This trial showed that *Trichoderma* Isolates AA16 and Eco-T® were compatible. *Trichoderma* isolates AA16 and Eco-T® were also found to be compatible with *Bacillus* Isolates B77, B81 and PHP. As a result, further *in vivo* trials were conducted.

The tunnel trials were carried out as two separate experiments:
In the first experiment, a combination of two *Trichoderma* Isolates AA16 and Eco-T® was conducted assayed at 5×10^5 or 1×10^6 spores / ml, on rough lemon seedling, and cuttings and trifoliate orange and sour orange seedlings. A combination of *Trichoderma* isolate AA16 and Eco-T® at 5×10^5 spore / ml increased significantly the new flush biomass of rough lemon cuttings compared to AA16 alone, but was not different from Eco-T® alone. The combination of AA16 and Eco-T® achieved no change of biomass of rough lemon and trifoliate orange seedlings. The combination of AA16 and Eco-T® did not increase the root biomass of sour orange compared to AA16 or Eco-T® alone. The combination of AA16 and Eco-T® at higher doses (1×10^6 spores / ml) showed significantly better suppression of *Phytophthora* root rot of

rough lemon cuttings but did not show disease suppression in all seedling species verities tested.

In a second experiment, individual and combined effects of *Trichoderma* isolates (drenched at 5 X 10^5 spores / ml) with *Bacillus* isolate (drenched at 1 X 10^6 colony forming units (CFU) / ml) for suppression of *Phytophthora* root rot on rough lemon and trifoliate orange seedlings was performed. The combination of *Trichoderma* Isolate AA16 and *Bacillus* Isolate B81 increased root biomass on rough lemon seedlings compared to the combination of *Trichoderma* AA16 or *Bacillus* PHP but was not significantly different to *Trichoderma* AA16 alone. *Bacillus* PHP combined with *Trichoderma* AA16 or singly had no effect on rough lemon seedlings. Combining *Trichoderma* Eco-T® and with *Bacillus* B81 or PHP did not increase biomass of rough lemon seedlings compared to *Trichoderma* Isolate Eco-T® alone. There was no statistically significant differences in the effects of the combinations of the *Trichoderma* and *Bacillus* isolates compared to their individual applications on the biomass of trifoliate oranges.

This study established the antagonistic potential of several South African isolates of *Trichoderma* and *Bacillus* as a viable alternative to agrochemicals for controlling *Phytophthora parasitica*. The growth stimulation capabilities of *Trichoderma* isolates in terms of seedling development was also demonstrated.

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INTRODUCTION

Phytophthora infections of citrus, caused by a number of *Phytophthora* spp., are probably the most widespread and serious diseases of citrus crops at all stages of development. There are at least six *Phytophthora* spp. capable of infecting citrus plants, but the greatest reduction in fruit yield may be attributed to the following two spp.:

1. *Phytophthora parasitica* Dast. (mostly in tropical and subtropical regions)
2. *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian (mostly in temperate climates) (Timmer & Menge, 1988).

Phytophthora has a long history as a major problem in citrus production regions throughout the world. Outbreaks of the disease occurred in groves in Australia in 1860 and later in Florida in 1952 (Broadbent, 1977). In South Africa considerable loses to the citrus industry are caused by root pathogens including *Phytophthora* spp. (Kotze, 1984). On Elabered Estate Farm, Eritrea, the pathogen is a threat, although no formal assessment has been done (Menghisteab, personal communication, 2001). During field visits to farms in KwaZulu-Natal, *Phytophthora* species was observed to be a threat to citrus tree health. The need for research into the control of this pathogen was apparent.

Citrus rootstocks have limited resistance to *Phytophthora* attack (Broadbent *et al.*, 1971). Similarly, chemical control has enjoyed only limited success in controlling the disease. Difficulties may arise from the lack of suitable application methods or alternatively, the lack of effective chemicals, or persistence and accumulation of chemicals in natural ecosystems. Breeding for resistance has not been particularly successful, possibly because the development of resistant cultivars is difficult and time consuming. Furthermore, developing a new variety having with appropriate fruit quality and disease resistance is difficult.

Therefore, alternative control strategies are required to control or reduce the pathogen to economic threshold levels. Biological control is an alternative. Members of genera *Bacillus* and *Trichoderma*, which are common residents of the soil and rhizosphere environment, have been identified as potential biocontrol agents (Papavizas, 1985; De Freitas *et al.*, 1997).

The objective of this study was to isolate and screen *Trichoderma* and *Bacillus* isolates for the suppression of *Phytophthora* root rot of citrus rootstocks. The findings of this study will enable further selection, screening and use of *Trichoderma* and *Bacillus* spp. as biological control and growth enhancing agents for citrus trees.

References

BROADBENT, P. (1977) *Phytophthora* diseases of citrus. *Proceedings of the International Society of Citriculture* **3**, 986-998.

BROADBENT, P. FRASER, L.R. & WATERWORTH, Y. (1971) The reaction of seedlings of citrus spp. and related genera to *Phytophthora citrophthora. Proceedings of the Linnean Society of New South Wales* **96**, 119-127.

DE FREITAS, J.R., BANERJEE, M.R. & GERMIDA, J.J. (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). *Biology and Fertility of Soils* **24**, 358-364.

KOTZE, J.M. (1984) Root rot in citrus. *The Citrus and Subtropical Fruit Journal* **604**, 18-21.

MENGHISTEAB, E. (2001) Personal communication. Pest Control Unit. Elabered Agro Industrial Estate, Eritrea.

PAPAVIZAS, G.C. (1985) *Trichoderma* and *Gliocladium*: Biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

TIMMER, L.W. & MENGE, J.A. (1988) *Phytophthora*-induced diseases, in *Compendium of Citrus Diseases* (Whiteside, J.O., Garnsey, S.M. & Timmer, L.W. Eds.). APS Press, Minnesota, USA. PP 22-25

CHAPTER 1

CITRUS CROPS

1.1 INTRODUCTION

Citrus belongs to the family Rutacaeae and sub-family Aurantioidae (Davies & Albrigo, 1994). The crop is ubiquitous with production in over 100 countries on six continents. Furthermore, citrus is the most important tree fruit crop in the world, with current world production far exceeding that of all deciduous tree fruits (apple, pears, peaches, plums, etc.). The area planted to citrus is estimated at two million hectares (Saunt, 1990). Citrus is grown primarily between the latitudes 40° N to 40° S (Davies & Albrigo, 1994). The majority of commercial citrus production, however, is restricted to two narrower belts in the sub-tropics, roughly between 20 and 40° N and S of the Equator (Castle, 1987; Saunt, 1990). Most citrus orchards worldwide consist of budded trees that combine favourable attributes of the scion and rootstock through grafting (Davies & Albrigo, 1994). See the details concerning rootstocks in Section 1.1.3.

1.1.1 The History and Origin of Cultivated Citrus

Citrus are some of the oldest cultivated tree crops (Ray & Walheim, 1980). Castle (1987) stated that earliest mention of citrus was in Chinese literature dated about 2201 to 2205 B.C., which shows that citrus trees have been cultivated for over 4000 years.

The center of origin of citrus is believed to be Southern Asia from eastern Arabia, east to the Philippines, and from the Himalayas, south to Indonesia. Within this large region, northern India and northern Burma were believed to be the primary center of origin, but recent evidence suggests that Yunnan province in South-central China may be as important due to the diversity of species found and the systems of rivers that could have provided dispersal to the South (Spiegel-Roy & Goldschmidt, 1996). However, the exact origin of citrus, and its ancestral types and systematics, are still unknown.

Extensive movement of the various types of citrus probably occurred within the general area of origin before recorded history. Many types of citrus are believed to have moved west to Arab areas, such as Oman, Persia, Iran, and even Palestine before Christ (Spiegel-Roy & Goldschmidt, 1996). Movement of citrus to Africa from India probably occurred between AD 700-1400 (Davies & Albrigo, 1994). Ray & Walheim (1980) characterized the movement of citrus to the west in one statement saying, "citrus followed the Cross". The report by Spiegel-Roy & Goldschmidt (1996) supported this idea and stated that the establishment of mission stations by the Roman Catholic Church aided the movement of citrus to North America. These missions established plantings of various fruit, including citrus, (particularly limes and oranges), which were introduced to South America by the Spanish and Portuguese settlers, and missionaries accompanying them.

The reviews of Bruke (1967), Jackson (1991), Davies & Albrigo (1994) and Spiegel-Roy & Goldschmidt (1996) are useful discussions of citrus, origins and movement.

Major types of citrus include (Davies & Albrigo, 1994):

Citron (*Citrus medica* L.)
Sour orange (*Citrus aurantium* L.)
Lime (*Citrus aurantifolia* Swingle)
Lemon (*Citrus limon* Burmann)
Sweet- orange (*Citrus sinensis* [L.] Osbeck)
Shaddock or Pummelo (*Citrus grandis* [L.] Osbeck)
Grapefruit (*Citrus paradisi* Macf)
Mandarin (*Citrus reticulata* Blanco)
Kumquat (*Fortunella margarita* [lour.] Swingle)
Trifoliate orange (*Poncirus trifoliate* [L.] Raf.)

Citrus is largest evergreen fruit crop in world trade. Its internal structure and long shelf life have facilitated its large-scale export as fresh fruit. Processed juice products have also become increasingly important worldwide (Spiegel-Roy & Goldschmidt, 1996). Ray & Walheim (1980) mentioned that citrus, best known for its Vitamin C content, is generally perceived as a kind of health food.

1.1.2 Nomenclature

The term "citrus" originated from the Latin form of 'Kedros', which is a Greek word denoting trees like cedar, pines and cypress. As the smell of citrus leaves and fruits is reminiscent of that of cedar, the name citrus has been applied to the citron at first and from then the name was used for whole citrus crop species (Spiegel-Roy & Goldschmidt, 1996).

Citrus species are of tropical and subtropical origin (Davies & Albrigo, 1994). Plants within the Aurantioidae family are unusual because the fruits are hesperidium berries (a single enlarged ovary surrounded by a leathery peel) and contain specialized structures, the juice vesicles (sacs). Furthermore, many species contain polyembryonic seeds.

The taxonomic situation of tribes, sub-tribes, genera and species within Aurantioidae is controversial, complex and sometimes confusing and therefore, there is no clear reproductive separation among species. Citrus and many related genera hybridize readily and have done so in the wild for centuries (Swingle & Reece, 1967).

The detailed discussions of taxonomy and taxonomic groups in citrus have been reviewed by several authors: Swingle (1948), Hodgson (1967), Swingle & Reece (1967), and Webber *et al.* (1967).

1.1.3 Citrus Rootstocks and their Characteristics

Historically citrus trees have been propagated from seedlings, due to the ease with which seeds can be propagated, and their convenience for transport as citriculture has expanded worldwide (Ray & Walheim, 1980; Castle, 1987; Saunt, 1990; Davies & Albrigo, 1994; Spiegel-Roy & Goldschmidt, 1996; Castle & Gmitter, 1999).

These authors identify the following disadvantages of propagation by seedlings:

1. Seedlings are thorny and have a relatively long juvenile period, which delays the first commercial harvest.

2. Once they enter their bearing phase, cropping can be erratic. Therefore, it takes longer to obtain a positive cash flow.

Despite these disadvantages, citrus seedlings were commonly used in much of the world until the mid-1800s (Castle, 1987). Root rot or foot rot (*Phytophthora* spp.) was recognized as a major disease of trees in the Azores in 1842, an event which initiated the practice of budding trees onto tolerant rootstocks. As *Phytophthora* spread to all major producing countries, so too did the need to use budded trees as a means of combating the disease (Castle, 1987; Saunt, 1990). *Phytophthora* foot rot spawned a search for resistant rootstocks, and initially sour orange became dominant. However, difficulty was encountered with this rootstock in South Africa and Australia. Trees declined within a few years after planting because of a disease identified as Citrus Tristeza Virus (CTV). As a result rough lemon (*C. jambhiri* Lush.) rootstock became popular in both countries and was adopted with considerable success (Castle, 1987; Saunt, 1990). Rootstocks are now widely recognized for their favourable effects on tree health and horticultural traits. The subject has been reviewed by several authors: Batchelor & Rounds (1948), Webber (1948), van Broembsen (1984), Castle (1987), Lee (1988), Castle & Gmitter (1999).

Davies & Albrigo (1994) noted that rootstock selection is a major consideration in citrus growing operations, given that more than 20 horticultural and pathological characteristics are influenced by it (see Table. 1.1.3A-C). They also pointed out that there is no perfect rootstock. The choice of rootstock should be based on the most important limiting factor(s) to production in a particular region, local climate and soil conditions, cultivars and intended use (fresh or processed) of the crop and disease situation(s).

The detailed description of the major citrus rootstocks related to their nursery characteristics, influence on tree growth, production and fruit quality, and response to various disease and soil factors are listed in Tables 1.1.3. A-C.

Table 1.1.3 A Nursery characteristics of citrus rootstocks (Van Barlow *et al.*, 1997)

Rootstock	Remarks
Lemon types	
Rough lemon	Seeds are highly nucellar and germinate well. Seedlings have good vigour; scion buds force easily and grow rapidly, especially in warm and hot climates.
Volckameriana	Similar to rough lemon except seedlings are more variable and culling should be more strict.
Macrophylla (Alemow)	Seeds are polyembryonic but seedlings are variable, moderately vigorous, and bud and force easily.
Mandarin types	
Cleopatra mandarin	Seeds are highly nucellar and germinate well. Seedlings are problematic in the nursery, not always growing vigorously. They are not easy to bud, and buds are difficult to force. Sometimes nurserymen have to cut seedlings back and wait for new growth on which to bud.
Empress mandarin	Seed are highly nucellar, but do not always germinate readily; seedlings are moderately vigorous, sometimes difficult to bud and slow to grow thereafter.
Rangpur	Seed are highly nucellar and germinate readily. Seedlings are vigorous, easy to handle, bud readily and force easily.
Orange types	
Sweet orange	Seeds are 70 to 85% nucellar but this varies with cultivar. Seedlings are thorny, bushy and vigorous and generally easy to bud and force.
Trifoliate orange	Seeds are about 90% nucellar and may require chilling for best germination. Seedling have low germination to moderately vigorous, are very thorny, and small-flowered cultivars are bushy, making them difficult to bud. Seedlings respond to prolonged day length, but generally go dormant outdoors in the autumn. Flying Dragon seedlings must be culled carefully because of the large number of off-types.
Trifoliate hybrids	
Carrizo and Troyer citrange	Both Citranges are excellent nursery plants with highly nucellar seed, and both produce uniform, vigorous, unbranched seedlings that are easy to bud and force.
Swingle citrumelo	Seeds are about 90% nucellar with an excellent germination percentage. Seedlings are very vigorous, uniform, upright and easy to bud, but forcing may be erratic with some buds not breaking.
Yuma citrumelo	Seedlings are very uneven in height due to the very high percentage of zygotic seed (40-45%) germination. Careful selection is necessary to obtain nucellar seedlings which are more uniform.

Table 1.1.3. B Characteristics of rootstock cultivars suitable for use in South Africa: influence of rootstock on tree growth, production and fruit quality (After van Barlow *et al.*, 1997)

Factor	Rough lemon	Volcka-meriana	Cleopatra mandarin	Empress mandarin	Troyer/ Carrizo citrange	Trifoliate	X639 hybrid	Swingle citrumelo
Tree performance								
Tree growth	Vigorous	Vigorous	Moderate *1	Moderate +	Moderate	Slow	Moderate +	Moderate +
Final tree size	Large	Large	Large	Large	Medium +	Medium	Medium	Medium +
Cold hardiness	Poor	Poor	Fair	Fair	Good	Excellent *2	Good	Good
Longevity	Fair	?	Good	Fair	Good	Good	?	?
Productivity and quality								
Yield per tree	Good	Good	Satisfactory	Satisfactory	Good	Good	Good	Good
Fruit size	Good	Good	Fair	Satisfactory	Good	Satisfactory	Good	Good
Fruit maturity	Early	Early	Mid	Mid	Mid	Mid-late	Mid	Mid
TSS %	Low	Low	Good	Good	Good	Excellent	Good	Good
Acid %	Low	Low	Moderate	Moderate +	Moderate +	High	Moderate	Moderate
Rind thickness	Fair +	Fair +	Fair	Fair	Thin	Very thin	Thin	Thin
Creasing	Light	Light	Light	Light	Moderate +	Severe	Moderate +	Moderate +
Rind texture	Coarse	Coarse	Intermediate	Intermediate	Smooth	Smooth	Smooth	Smooth
Rind colour development	Intermediate	Intermediate	Intermediate	Intermediate	Early	Early	Early	Late

Note:

i. Variance above or below the category given is indicated where necessary by + or -, **i.e. + means more; - means less.**

ii. The above ratings are generalisations and therefore cannot hold true for all circumstances or conditions.

iii. A question mark (?) indicates that insufficient information is available at present to provide a response rating.

*1 Very vigorous in subtropical climates.

*2 Not good in subtropical climates.

Table 1.1.3.C Characteristics of rootstock cultivars for use in Southern Africa: response to various disease and soil factors

Factor	**Rough lemon**	**Volcka-meriana**	**Cleopatra mandarin**	**Empress mandarin**	**Troyer/ Carrizo citrange**	**Trifoliate**	**X639 hybrid**	**Swingle citrumelo**
Factor								
Exocortis	Tolerant	Tolerant	Tolerant	Tolerant	Sensitive	Sensitive	Sensitive	Tolerant ?
Tristeza	Tolerant	Poor	Tolerant	Tolerant	Tolerant	Resistant	Tolerant	Tolerant
Blight	Poor	Poor	Good	?	Interm /Poor	Poor	?	Intermediate ?
Phytophthora	Susceptible	Susceptible	Intermediate	Intermediate	Tolerant +	Tolerant +	Tolerant	Tolerant +
Citrus nematode	Susceptible	Susceptible	Susceptible	Tolerant (?)	Tolerant	Tolerant +	Tolerant ?	Tolerant +
Soil conditions								
Poor drainage	Sensitive +	Sensitive	Sensitive	Sensitive	Intermediate	Tolerant	?	Tolerant
High clay	Sensitive	Sensitive	Intermediate	Intermediate	Intermediate	Tolerant	Intermediate	Intermediate
High sand	Tolerant	Tolerant	Intermediate +	Intermediate +	Sensitive	Sensitive +	?	Intermediate +
Replant	Sensitive	Sensitive	Sensitive	Sensitive	Intermediate	Tolerant	Intermediate	Tolerant
High chloride / salt	Intermediate	Intermediate	Tolerant	Intermediate	Sensitive	Sensitive +	Intermediate ?	Intermediate
High calcium / pH	Tolerant	Tolerant	Tolerant	?	Sensitive	Sensitive +	?	Intermediate ?
Drought	Tolerant	Tolerant	Tolerant	Tolerant	Intermediate	Sensitive	?	Tolerant

Note:

i. Variance above or below the category given is indicated where necessary by + or -, **i.e. + means more; - means less.**

ii. The above ratings are generalisations and therefore cannot hold true for all circumstances or conditions.

iii. A question mark (?) indicates that insufficient information is available at present to provide a response rating.

After van Barlow *et al.* (1997)

1.1.4 Citrus Fruits

1.1.4.1 International perspective

Citrus fruits are produced all around the world. Major production areas are Brazil, USA, China and Mexico (see Table 1.1.4.1 for details).

Table 1.1.4.1 World citrus production and utilization 2000/01 (thousands of tons)

	Total citrus	**Exports (fresh fruit)**	**% total exports (fresh fruit)**	**Processed**	**% total Processed**
World	89071	9423	11.0	27439	31.0
Northern Hemisphere	63569	7705	9.0	15754	18.0
USA	14049	1084	1.0	10969	12.0
Mediterranean Region	17779	5243	6.0	3025	3.0
Greece	1229	423	0.5	307	0.3
Italy	3011	232	0.3	1362	2.0
Spain	5401	2859	3.0	704	1.0
Israel	630	194	0.2	314	0.5
Morocco	965	393	0.5	40	0.04
Egypt	2508	226	0.3	115	0.1
Cyprus	193	98	0.1	40	0.05
Turkey	1902	449	0.5	102	0.1
Mexico	6143	277	0.3	728	1.0
Cuba	884	42	0.04	671	1.0
China	8783	164	0.2	202	0.2
Japan	1504	5	0.005	119	0.1
Southern Hemisphere	25502	1718	2.0	11686	13.0
Argentina	2808	413	0.5	1136	1.0
Brazil	16498	154	0.2	9846	11.0
Uruguay	339	115	0.1	54	0.06
Australia	563	181	0.01	190	0.2
South Africa	1527	757	1.0	457	0.5

(FAO, 2004)

Note

1. Production values given reflect the quantities sold through formal marketing channels; they do not reflect the informal sector or home consumption. This results in underestimates of production in countries with strong informal marketing sectors.

2. The term "citrus fruits" is taken to include: oranges, tangerines, lemons, limes and grapefruits.
3. Exports of fresh fruits represent roughly 11% of the total production of citrus fruits.
4. Processed fruits represent roughly 30% of the total production of citrus fruits.
5. The table considered data from 2000/01 because the data for 2001/02 were preliminary.

The data presented in Table 1.1.4 are abstracted from FAO production data 2004. The editor of the FAO data comments in the NOTES ON THE TABLE, "In general, it appears that the estimates refer to crops grown in fields and market gardens for sale, thus excluding crops cultivated in home gardens or small family gardens mainly for household consumption. Production from family and other small gardens not included in the current statistical surveys and consequently not included in the tables of the FAO Data (2004).

For these reasons, regional, continental and world totals are far from representative of the total production of the various kinds of citrus cultivated, but nevertheless provide an indicator as to the importance of citrus in world trade.

Citrus fruits have many uses and are consumed as fresh fruit or utilized for processed products and by-products (citrus essential oils, D-limonene, citrus pulp pellets), and medicinal products (Davies & Albrigo, 1994; FAO, 2004). The editor of FAO (2004) noted that citrus are the leading fruit crop in terms of value in international trade. The editor also noted that there are many companies, cooperatives, growers, and citrus processors involved in citrus fruit and citrus juice industries, which contribute in the global economy and human life. In South Africa, for example, the citrus industry is one of the largest agricultural industries in the country in terms of export earnings (Anon, 2004). In 1995, the South African citrus industry exported 43 million cartons of citrus to 60 countries, with a gross value of R1.6 billion. The editor also reported that agriculture not only plays a major role in the economic growth and development of the country, but also plays a distinct role in improving the economic and social options of rural people, and consequently, in improving their quality of life. Approximately 14 % (5681700 people) of the economically active population of South Africa are employed in agriculture, of which the citrus industry alone employs approximately 97000

people. The South African citrus industry plays a vital role in the economy and social welfare of the country and is a trendsetter in global fresh fruit exports.

1.2 THE ECONOMIC IMPACT OF *PHYTOPHTHORA* SPP. ON GLOBAL CITRUS PRODUCTION

1.2.1 Introduction

The major fungal diseases of citrus include root rot, collar rot, foot rot and gummosis. Fungal pathogens such as *Rhizoctonia solani* Kuhn and *Pythium* spp. occasionally cause some damage but *Phytophthora* spp. can kill citrus trees (Klotz, 1978). Most major citrus production areas of the world have experienced *Phytophthora* gummosis foot rot and root rot problems.

Phytophthora diseases of citrus are soil or seed-borne and are distributed throughout the world, wherever citrus crops are grown (Erwin & Ribeiro, 1996). Broadbent (1977) reviewed the impact of *Phytophthora* on the citrus industry of Australia and commented that root rot has been very costly to New South Wales growers over the past 100 years. The first outbreak occurred following excessive rains of 1860 and 1864. Thousands of trees were destroyed in the Hills district near Sydney, which was at the time, the main citrus area of New South Wales. The second major outbreak occurred in the inland irrigation district of the Murrumbidgee Irrigation Area (M.I.A.) between 1935 and 1942, with lesser losses occurring in the Murray River Settlements. During these years, excessive rainfall enhanced *Phytophthora* damage to citrus trees on heavy soils with faulty drainage. By 1942, nearly 50% of the citrus plantings in the M.I.A. were out of production. No orchard remained completely unaffected, and new plantings were extremely difficult to establish. The third outbreak followed seasons of high rainfall in coastal N.S.W., between 1948 and 1951. Surveys showed that 98 000 of 276 000 trees in the metropolitan area were dead or unlikely to recover.

Kotze (1984) reported that root pathogens cause considerable losses to the citrus industry in South Africa. According to surveys carried out in 1980, root rot was a serious problem in 11% of citrus trees of the most important citrus growing areas of the Transvaal. However, a large number of apparently healthy trees had infected roots and produced low yields of undersized fruits. Hough & Esselene (1992) also considered root rot to be one of the most serious

problems in the citrus industry in South Africa, causing yield reduction of over 50% in some orchards. Themann & Werres (1995) estimated the loss due to *Phytophthora* spp. alone to be up to 80% in nurseries.
In Elabered Agro Industrial estate, Eritrea, one of the biggest commercial farms has suffered a *Phytophthora* disease problem, and this one of their top concerns (Menghisteab, pers. comm. 2001).

In California and Florida, *Phytophthora* spp. cause substantial root damage in an estimated 8 to 20% of orchards (Graham & Menge, 1999). Brown rot can also cause extensive losses, with 30 to 90% loss of crop occurring in Florida citrus groves in 1953 (Broadbent, 1977). Broadbent (1977) indicated that disease losses due to root rot are unclear because the relationship between root damage and yield loss is not directly proportional. Nevertheless, yield losses from fibrous root rot, foot rot and gummosis in the United States have been estimated to range from about 3 to 6% / year, or $76 million / year, without fungicidal control treatments. These losses do not include yield losses due to *Phytophthora* brown rot of fruits, which varies widely with climatic conditions from year to year. Overall, losses due to *Phytophthora* spp. are much more prevalent in some years in certain locations, because these diseases are particularly damaging under wet or flooded conditions.

The *Phytophthora* disease problem should not be underestimated, as it initiated the change of the world citrus propagation from seedling to budded trees. The problem is far from solved. Klotz (1978) stated that most present day citrus nursery men and growers are acquainted with the destructiveness of the disease which attacks all parts of a citrus tree at any growth stage. The life expectancy of citrus trees is in the order of 50 years, and individual specimens in healthy situation often live 100 years or more (Jackson, 1999) provided that diseases such as *Phytophthora* are controlled.

1.2.2 The genus *Phytophthora* spp.

1.2.2.1 Taxonomy

The name of the genus *Phytophthora* is derived from the Greek that literally means Phyto (plant) and Phthora (destroyer). It belongs to the family Pythiaceae and order Peronosporales (Erwin & Ribeiro, 1996).

The most common and important *Phytophthora* spp. that attack citrus are *P. parasitica* Dast. and *P. citrophthora* (R.E.SM. & E.H.SM.) Leonian. *P. parasitica* is widespread in most citrus areas and causes foot rot, gummosis, and root rot. It seldom causes infection high up the trunk. *P. citrophthora* causes gummosis and root rot. It attacks aerial plant parts more frequently than *P. parasitica* and is more commonly the cause of brown rot (Timmer & Menge, 1988).

Fruits are principally attacked by *P. citrophthora*, *P. parasitica*, *P. hibernalis* (Carne) and occasionally by *P. syringae* (Kelb) (Broadbent, 1977; Timmer & Menge, 1988). *P. citricola* (Sawada) is widespread and prevalent in citrus orchards in Western Australia (Doepel, 1966), and is active from autumn until spring. *P. cactorum* (Lebert and Cohn Schroet.) and *P. palmivora* (Butler) are highly pathogenic on roots and stems of citrus seedlings. It is commonly the cause of brown rot epidemics in Florida and probably the cause of brown and foliage blights in other subtropical areas of the world (Graham *et al.*, 1998). For details of their distribution and taxonomy, see the C.M.I. Description of pathogenic fungi and bacteria for *P. citrophthora*, *P. nicotianae* var. *parasitica, P. hibernalis*, *P. syringae*, *P. cactorum* and *P. citricola*, No. 33, 35, 31, 32, 111 and 114 respectively by Waterhouse & Waterston (1964a-f).

Phytophthora spp. have different inoculum types: mycelia, chlamydospores, sporangia, zoospores, and oospores. A detailed discussion on the inoculum types has been covered by the following authors: Khew & Zentmyer (1973), Timmer & Menge (1988), Mitchelle & Kannwischer-Mitchell (1992), Erwin & Ribeiro (1996). The life cycle of *P. citrophthora* has not been well studied, but *P. parasitica* has been reviewed by Graham & Timmer (1994), see Fig.1.2.2.2.

1.2.2.2 The Disease Cycle and Epidemiology

Phytophthora spp. are endemic in the soil of citrus orchards from most areas. Infection usually occurs by means of zoospores, which are released from sporangia produced in infected roots. Zoospores are attracted to wounds or to the zone of elongation of root tips, which are extremely susceptible to pathogen infection. Zoospores are probably attracted to the zone of elongation of new roots by nutrients, which are naturally exuded from this root zone (Timmer & Menge, 1988; Graham & Menge, 1999). Chemotaxis of zoospores to the root can be an important factor in pathogenesis (Zentmyer, 1961; Zentmyer *et al.*, 1994). Upon contact with a root, zoospores encyst, germinate and then infect along the zone of elongation. Once the fungus has entered the root tip, the infection may advance in the cortex, resulting rot of the entire rootlet. Severe outbreaks are associated with prolonged periods of wet weather. The cycle can repeat itself as long as conditions are favourable and susceptible tissue is available (Timmer & Menge, 1988; Graham & Menge, 1999). Penetration of young leaves and fruits can occur without wounding. Fruit and leaf lesions on citrus are confined to the lower 50 cm of trees unless secondary inoculum is splashed from infected low-hanging lesions to the higher tree parts (Gerlach *et al.*, 1976). Brown rot epidemics are usually restricted to areas where rainfall coincides with the early stages of fruit maturity (Graham & Menge, 1999). The pathogen requires a wound or natural growth crack for infection of suberized tree trunks (Timmer & Menge, 1988) and foot rot or gummosis occurs when zoospores or other propagules are splashed onto the trunk above the bud union. Infection occurs through wounds or natural cracks in the bark when moisture is present on or around the base of the trunk. This condition is favoured by high soil moisture, heaping of soil against trunks, deep planting, low budding and cultivation injury (Whiteside, 1971).

Phytophthora spp. usually survive unfavorable periods as chlamydospores or oospores in soil, or as hyphae or sporangia in decayed roots or other organic matter (Timmer & Menge, 1988). *Phytophthora* grows actively at temperatures between 10^0C and 35^0C within an optimum of 26^0C (Erwin & Ribeiro, 1996). Similarly, citrus root growth ceases at soil temperatures of below 13^0C or above 36^0C (Davies & Albrigo, 1994). Thus, both the pathogen and citrus root development require similar soil temperatures. The pathogen causes the most damage during

summer months when optimal growth conditions occur and there is an abundance of feeder roots and elevated soil moisture. Infection is also aided by abundant soil moisture and is most severe in fine- textured soils where drainage is impeded (Strauss & Labuschagne, 1994). Root infection and trunk gummosis occur in waterlogged soils and cause reduced growth and eventual death of citrus (Gerlach *et al.*, 1976). This condition occurs when fields are flood-irrigated or when trees are grown in heavy, compacted soils with poor drainage, because water in irrigation canals is frequently infested with either *P. citrophthora* or *P. parasitica* (Joubert & Labuschagne, 1998).

High soil moisture increases infection mainly because of the increased formation of sporangia and the improved conditions for zoospore release, motility, and movement to the infection site. Disease development is usually more severe on soils with restricted drainage and soil pH between 6.0 and 6.5. Stress from either excess moisture or low moisture can also increase susceptibility of some hosts to infection (Zentmyer *et al.*, 1994; Erwin & Ribeiro, 1996).

Phytophthora spp. can be disseminated in several ways, including soil movement on nursery stocks, irrigation water and infected root pieces. Irrigated citrus often suffers from the biggest problems as runoff water can carry the pathogen into canals, streams or rivers. Water from these sources may then contaminate previously uninfected areas. The fungus may be carried in soil on farm equipment. Occasionally seeds taken from infected fruits are infectious (Graham & Timmer, 1994; Zentmyer *et al.*, 1994; Graham & Menge, 1999).

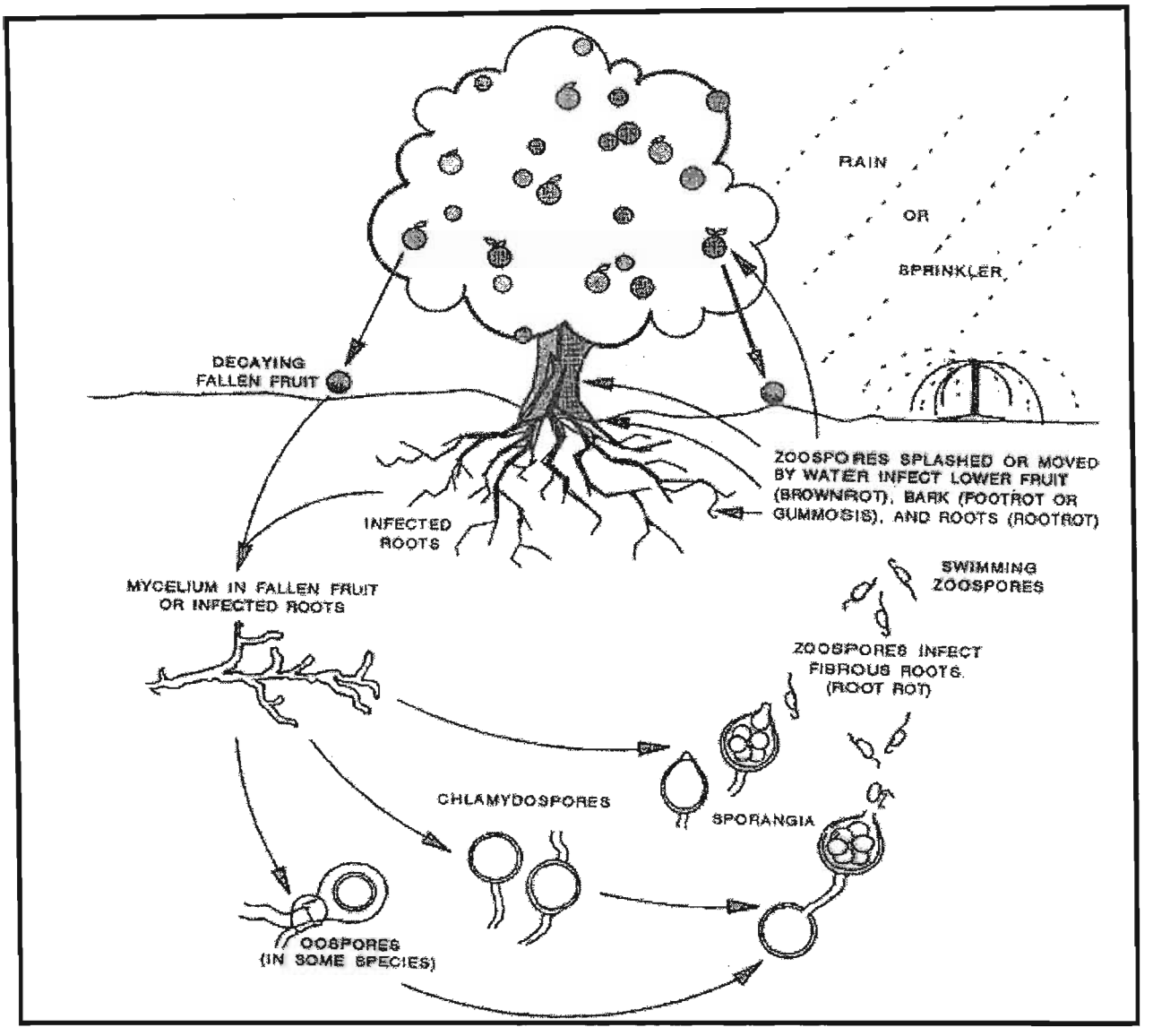

Figure 1.2.2.2 The disease cycle of *Phytophthora* diseases affecting the roots, bark and fruit of citrus trees (after Graham & Menge, 1999).

1.2.3 The Disease

Citrus trees may be susceptible at any growth stage to *Phytophthora* spp. It causes seed rot or pre-emergence rot. Damping-off caused by *Phytophthora* spp. (Fig. 1.2.3.A) is similar to that caused by *Rhizoctonia* and *Pythium* spp. The pathogen also causes decay of fibrous roots. The cortex turns soft, becomes somewhat discolored, and appears water soaked. The fibrous roots slough their cortex, leaving only the white thread-like stele, which gives the root system a stringy-like appearance. Root rot also occurs on susceptible rootstocks in bearing orchards, where damage causes tree decline as the production of new fibrous roots cannot keep pace with root death (Timmer *et al.*, 1988). The tree is then unable to maintain adequate uptake of water and mineral nutrients. Carbohydrate reserves in the root are depleted by repeated *Phytophthora* attacks, resulting in the reduction of fruit size and production, loss of leaves, and twig dieback of the canopy (Graham & Menge, 1999) (Fig. 1.2.3.B). Badly affected trees have pale green leaves with yellow veins, typical of girdling symptoms (Timmer, 1977). Infected trees often bear a heavy crop of poor quality fruit because of the girdling effect on the phloem. The leaves turn yellow and drop prematurely, especially with highly susceptible rootstocks. Trees affected with crown rot show severe yellowing of foliage, followed by decay in the underlying wood. The diseased wood is clearly defined from the healthy portions of the wood. The cambium beyond the diseased areas becomes yellow and gummosis occurs. Citrus gums are water-soluble and disappear after heavy rains, but persist on the trunk under dry conditions. Longitudinal cracking of the bark occurs (Erwin & Ribeiro, 1996) (Fig. 1.2.3.C-E).

Nursery trees and young orchard trees can be girdled and killed. Large trees may be killed but are usually only partially girdled, and the injury causes a decline of the canopy, with defoliation, twig-die-back, and short, stunted growth flushes. On susceptible rootstocks, lesions may occur below the soil line, and canopy symptoms may develop without obvious damage to the above ground portion of the trunk. This disease is referred to as crown rot (Timmer, 1977). Dry root rot cankers, caused by *Fusarium solani*, are confined to larger roots, crowns, and the trunk below the bud union. Unlike *Phytophthora* induced lesions, the *Fusarium* lesions do not ooze gum (Graham & Menge, 1999). Infection of fruit by *Phytophthora* spp. produces a decay in which the affected area is firm, light brown, leathery, and not sunken below the adjacent rind. White mycelium may form on the rind surface under

humid conditions on orchard fruit, which are on or near the ground (Fig. 1.2.3.F-G). Most infected fruits soon abscise, but those that are harvested may not show symptoms until after they have been held in storage for a few days. If infected fruit is packed, brown rot may spread to adjacent fruit in the container. In storage, infected fruit has a characteristic pungent, rancid odor (Graham & Menge, 1999).

Figure 1.2.3.A
Seedling death (damping-off) in a citrus nursery caused by *Phytophthora parasitica* (Olsen *et al.* 2001).

Figure 1.2.3.B
Tree decline characteristic of *Phytophthora citrophthora* (Vock *et al.*, 1997).

Figure 1.2.3.C
Typical gummy lesions caused by *Phytophthora parasitica* in citrus bark (Olsen *et al.*, 2001).

Figure 1.2.3.D

Typical symptoms of an active lesion caused by *P. parasitica.* Note the dark, discolored infected tissue that extends into the light-colored, disease-free trunk tissue (Vock *et al.*, 1997).

Figure 1.2.3.E

Collar rot of *Phytophthora nicotianae* (Vock *et al.*, 1997).

Figure 1.2.3.F

Fruit rot characteristics of *Phytophthora citrophthora* (Vock *et al.*, 1997).

Figure 1.2.3.G

Fruit rot characteristics of *Phytophthora citrophthora* under favorable conditions (Vock *et al.*, 1997).

1.3 INTEGRATED MANAGEMENT OF PHYTOPHTHORA DISEASES OF CITRUS FRUIT CROP PRODUCTION.

1.3.1 Disease Control of *Phytophthora* spp. in Citrus Fruit Production

Phytophthora diseases have multiple inoculum sources, (i.e., seed, soil) and dissemination mechanisms, so their control requires a holistic approach. Some of the control measures are presented in Table 1.3.1 indicating authors involved in the recommendations.

Table 1.3.1 Integrated management of Phytophthora diseases of citrus fruit crop production [1]

	T	R	E	A	T	M	E	N	T	S
Authors	**Seed**	**Debris Tillage Sanitation**	**Soil / Medium Solarization**	**Resistant Cultivars**	**Fungicides**	**Fert.**	**Bio-Ctrl.**	**Irr. H_2O**	**Impl.**
1							√		
2				√					
3						√			
4					√				
5			√						
6		√		√	√				
7	√		√	√	√	√		√	√
8	√	√		√	√			√	
9	√			√	√				√
10	√		√					√	√
11	√			√					
12			√		√				
13				√				√	
14			√		√			√	
15	√	√		√	√			√	√
16				√	√				
17						√		√	
18	√	√		√	√			√	√
Authors	**Seed**	**Debris Tillage Sanitation**	**Soil / Medium Solarization**	**Resistant Cultivars**	**Fungicides**	**Fert.**	**Bio-Ctrl.**	**Irr. H_2O**	**Impl.**
	T	R	E	A	T	M	E	N	T	S

Key to Reviewed Control Measures

Seed:	Use either certified clean seed or treat seed with an effective measures.
Debris:	Elimination of debris especially *Phytophthora* spp. on debris
Tillage:	Avoid soil compaction
Sanitation:	Use of proper sanitation to reduce disease
Soil / Medium solarization:	Use of physical sterilization or sterilized media
Resistant Cultivars:	Use resistant cultivars
Fungicides:	Use fungicides to control *Phytophthora* diseases
Fert.:	Use of fertilization to reduce effects of *Phytophthora* damage
Bio-Ctrl:	Apply a biocontrol agent to control *Phytophthora* spp.
Irr. H_2O:	Proper monitoring and treatment of irrigation water
Impl.:	Use an appropriate working implements and vehicles to avoid

[1]Continued on next page

Table 1.3.1 (Continued): Numbered Authors[1]

1. Sztejnberg *et al.* (1988); Bicici *et al.* (1992); Fang & Tsao (1992; 1995a; b); Tsao *et al.* (1996)
2. Broadbent *et al.* (1971a); Grimm & Hutchison (1977); Broadbent & Gollnow (1992); Graham (1995); Matheron *et al.* (1998); Castle & Gmitter (1999).
3. Klotz *et al.* (1958); von Broembsen & Deacon (1997)
4. Farih *et al.* (1981); Laville & Chalandon (1981); Davis (1982); Chatenet *et al.* (1988); Menge (1988); OHR *et al.* (1992); Schutte (1994); Ippolito *et al* (1996a; 1996b); Munnecke (1977); Erkilic & Canihos (1999)
5. Continella & Cartia (1993)
6. Burnett *et al.* (1977)
7. Erwin & Ribeiro (1996)
8. Graham & Menge (1999)
9. Graham & Timmer (1994)
10. Hough (1988)
11. Lee *et al.* (1999)
12. Le Roux *et al.* (1991; 1993)
13. Ohr & Menge (2001)
14. Roxburgh *et al.* (1993)
15. Timmer & Menge (1988)
16. VanDerweyen (1988)
17. Wheaton *et* al. (1999)
18. Zentmyer *et al.* (1994)

1.4 BIOLOGICAL CONTROL OF PLANT PATHOGENS

1.4.1 Introduction

Control of plant pathogens has been accomplished largely with the use of chemicals. Chemicals have provided a means of reducing plant disease. Over time this has proved to have negative side effects such as development of resistance by pathogens, high cost, and negative effects on beneficial microorganisms (Utkhede, 1992), as well as environmental, soil, and water pollution (Akhtar, 1998). It is widely accepted that there is a need to replace toxic and polluting chemicals with less dangerous chemicals or, preferably, cultural practices and biological control (Walker & Morey, 1999). Biological control is seen as a favourable choice because alternative methods of disease control, such as the development of resistant plant cultivars has often been too slow, and economic pressure on land use has limited some of the traditional cultural techniques of control (Burger, 1988).

Researchers in academic institutions and private companies have increased their efforts to develop non-chemical controls (Chet, 1990). The use of biological control systems to control plant diseases and improve plant growth has been thoroughly investigated. Biological control of soil-borne pathogens by introduced microorganisms has been studied for over 60 years (Weller, 1988), and accordingly biological control of soil-borne diseases have received increased attention as an attractive alternative (Jensen *et al.*, 2000).

Biological control of soil-borne plant diseases may be effectively achieved through a fundamental understanding of the ecological relationships of the diverse microbial populations and biological control agents in the soil rhizosphere (Huang, 1992). The important characteristic necessary for the acceptance and effectiveness of a biological control agent is its ability to survive in an environment foreign to its origin (Nemec *et al.*, 1996). In addition, an organism must be able to successfully colonize plant roots during the period that protection against pathogens would be useful (Chao *et al.*, 1986).

As shown in Table 1.3.1, limited use has been made of biological control relative to chemical control. However, the Table also shows that some successes have been achieved and that there

is a much potential for developing biological agents for the control of *Phytophthora* diseases of citrus. Large numbers of different microorganisms are commonly found in the soil including bacteria, fungi, actinomycetes, protozoa and algae of these, bacteria and fungi are by far the most common type of soil microorganisms (Glick, 1995). The objective of this study will be to focus on *Trichoderma* spp. and *Bacillus* spp as biological control agents of *Phytophthora* spp.

1.4.1.1 *Trichoderma* spp. as Biological Control and Plant Growth Promoting Agents

The genus *Trichoderma* was discovered by Persoon and was clearly delimited by Harz in 1871 (Cook & Baker, 1983). The form genus *Trichoderma* is a commonly encountered member of the family Moniliaceae, form class Deuteromycetes and subdivision Deuteromycotina (Alexopoulos & Mims, 1979). *Trichoderma* has highly ramified conidiophores that bear conidia singly or in groups. The conidia are small, smooth-walled and hyaline. Colonies of *Trichoderma* are fully white to green in colour. Its members are generally found in all soils including forest humus layers (Samuels, 1996).

Trichoderma spp. are antagonistic towards numerous plant pathogens (Eveleigh, 1985; Harman & Lumsden, 1990) and it is one of the most studied fungi species, which have been shown to control various plant pathogens (Papavizas, 1985) and the genus has been subjected to extensive investigation for several years.

Selected strains of *Trichoderma* spp. have been shown to suppress several plant diseases such as *Pythium* spp. (Chet *et al.*, 1981), *Rhizoctonia solani* Kühn (Elad *et al.*, 1980; Harman *et al.*, 1981; Lewis & Papavizas, 1987) and *Fusarium* spp. (Cook & Baker, 1983; Sivan & Chet, 1986). Some examples of successful biological control of plant pathogenic field and postharvrest diseases by *Trichoderma* spp. are provided in Table 1.4.1.1.

Table 1.4.1.1 Examples of successful biological control of plant pathogenic diseases in different crops by *Trichoderma* spp.

Crop	Disease / Pathogen	Antagonist	Authors
Radish, beans	*R. solani*	*T. hamatum*	Chet & Baker (1981)
Peas	*Pythium* spp.	*T. hamatum*	Chet & Baker (1981)
Beans	*Sclerotium rolfsii*	*T. hamatum*	Chet & Baker (1981)
Gerbera	*Phytophthora* root rot	*T. hamatum* & *T. viridae*	Orlikowski (1995)
Tomato	*Fusarium oxysporium*	*T. harzianum*	Datnoff *et al.* (1995)
Citrus	Green mould	*Trichoderma* sp.	Wilson & Pusey (1985)
Strawberry	Botrytis rot	*Trichoderma* sp.	Tronsmo & Dennis (1983)
Pine	Decay	*Trichoderma* sp.	Wilson & Pusey, (1985)
Pepper seedlings	*R. solani*	*Trichoderma* sp.	Lewis & Lumsden (2001)
Pepper plants	*Phytophthora capsici*	*T. harzianum*	Sid Ahmed *et al.* (1999)
Cucumber	*Botrytis cinerea* *Sclerotinia sclerotiorum* *Sphaerotheca fusca*	*T. harzianum*	Elad (2000)
	Rhizoctonia solani	*Trichoderma* isolate	Askew (1991)
	Rhizoctonia solani	*Trichoderma* species	Yobo *et al.* (2004)

Important reviews on *Trichoderma* spp. are listed as follows: Danielson & Davey (1973a, b, c); Papavizas (1985); Chet (1987a,b); Papavizas & Lewis (1989); Chet (1990); Lewis & Papavizas (1991).

Biocontrol agents usually do not totally kill pathogens under practical conditions. But in many cases, *Trichoderma* may successfully replace common fungicides. Moreover, while many chemicals are degraded after a short time, *Trichoderma* survives and even multiplies in soil and in the plant rhizosphere (Chet, 1987a).

Suggested mechanisms of biocontrol include: antibiosis, lysis, competition and mycoparasitism (Chet & Baker, 1980; Papavizas, 1985; Sid Ahmed *et al.*, 1999). The

antagonistic activity of species of *Trichoderma* is the result of different mechanisms often occurring concurrently (Chet, 1987a). *Trichoderma* spp. also induce systemic resistance (Martinez *et al.*, 1999). Cellulase produced by *Trichoderma harzianum* was found to have an eliciting effect, triggering peroxidase and chitinase activity to produce systemic acquired resistance (SAR), with the production of ethylene and salicyclic acid. These products have an eliciting effect, stimulating resistance mechanisms in plant roots (Martinez *et al.*, 1999). Meyer *et al.* (1998) also investigated induced systemic resistance in tomatoes, lettuce, beans, and tobacco. *T. harzianum* T39 application at sites spatially separated from *B. cinerea* inoculation resulted in a 25-100% reduction of disease symptoms. This reduction was caused by a delay or suppression of lesion formation. Given the spatial separation of both microorganisms, this effect was attributed to the induction of systemic resistance by *T. harzianum* T39. Some examples on this topic are provided by: Lorito *et al.* (1998), Yedidia *et al.* (1999), Bolar *et al.* (2000), Howell *et al.* (2000). More detailed information on the above mechanisms is provided by the following authors: Wright (1956), Dennis & Webster (1971), Hadar *et al.* (1979), Harman *et al.* (1980), Chet *et al.* (1981), Alexander (1982), Cook & Baker (1983).

Trichoderma spp. are known not only for their biological control activity, but for their plant growth promoting activity as well (Table 1.4.1.2) *Trichoderma* directly affects plants, it can live in their roots and enhances plant germination (Chet, 1987b; Baker, 1988). Chet (1990) reported that pepper seed treated with fungus germinated two days earlier than untreated controls. Chang *et al.* (1986) and Chet (1987a) also reported that *Trichoderma* enhances plant growth and flower production. This was supported by Chet (1990) who showed flowering of periwinkle was accelerated and that number of blooms / plants on chrysanthemums was increased. The heights and weights of other plants were also greater in soils infested with *Trichoderma harzianum.*

Table 1.4.1.2 Examples of successful plant growth promoters in different crops in the absence of pathogens by *Trichoderma* spp.

Crop	Growth promoters	Authors
Tomato, tobacco	*Trichoderma* spp.	Windham *et al.* (1986)
Cucumber, pepper seedlings	*T. harzianum*	Inbar *et al.* (1994)
Cucumber plants	*T. harzianum* T-203	Yedidia *et al.* (2001)
Cabbage, cucumber, Eucalyptus seedlings	*T. harzianum* KMD and G. *virens* MM1	Omarjee (2002)

Growth stimulation by *Trichoderma* sp. could be the result of:

1. Production of plant growth hormones, which may, in turn, increase the growth rate or efficiency of nutrient uptake by the plant (Windham *et al.*, 1986)
2. The control of minor pathogens in the rhizosphere (Chet, 1987b; 1990)
3. Solubilizations of phosphates and micronutrients (Altomare *et al.*, 1999).
4. Vitamin production or conversion of materials to a form used to the plant (Barber & Lynch, 1977).
5. Nutrient release from soil or organic matter (Barber & Lynch, 1977).
6. Increase uptake and translocation of less-available minerals (Inbar *et al.*, 1994)
7. Induced growth response (Windham *et al.*, 1986).
8. The destruction of plant allelopathogenic chemicals (Laing, pers. comm. 2004).

This positive effect of the antagonists on plant growth and flower production can serve as a valuable factor in promoting *Trichoderma* as a biocontrol agent (Chet, 1987a).

1.4.1.2 *Bacillus* spp. as Biological Control and Plant Growth Promoting Agents

The genus *Bacillus* belongs to the family Bacillaceae. *Bacillus* spp. are rod-shaped and generally motile bacteria. The motility is an advantage since it enables the bacteria to

scavenge more efficiently for limited nutrients excreted from root hairs (Brock & Madigna, 1991) and may also assist in their ability to colonize the rhizosphere of newly developed plant roots.

Members of the genus *Bacillus* are common residents of the soil and rhizosphere environment (Holl & Chanway, 1992; Mazzola, 1999). *Bacillus* was also reported as a dominant genus in soil by Mahaffee & Kloepper (1997). This suggests that the genus *Bacillus* occurs naturally in the root zone of many plants and hence their application as biological agents or plant growth prompters is valid.

The genus *Bacillus* has been considered less effective as rhizosphere colonists than fluorescent Pseudomonads (Millus & Rothrock, 1993). Thus *Pseudomonas* strains have been intensively investigated as biological control agents with regard to the production of anti-microbial metabolites (Thomashow, 1996). However, there is a growing list of examples that suggest that selected *Bacillus* species can successfully colonize roots after being introduced as seed inoculants by (Adejumo *et al.*, 1999). The perceptions of its potential slowly changing as a result to the potentials of *Bacillus* sp. are recognized as a result *Bacillus* sp. are receiving increased attention.

Advantages of *Bacillus* sp. include:

1. The ability to form resistant endospores. This inherently improves shelf life (Emmert & Handelsman, 1999).
2. Their ability to produce a multitude of broad-spectrum antibiotic compounds (Rytter *et al.*, 1989; Mavingui & Heulin, 1994).
3. They can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources (Glick, 1995).

Bacillus spp. have been used for many years in attempt to control plant pathogens and increase plant growth (Turner & Backman (1991), Holl & Chanway (1992), Manero *et al.* (1996), Kim *et al.* (1997).

The ability of a microorganism to colonize the rhizosphere is essential for selected bacteria to function as biological control agents of soil-borne plant pathogens (Bent & Chanway, 1998). Failure to adequately colonize roots may account for the unreliability of many biological control agents (Knudsen *et al.*, 1997) and is therefore an essential criterion in screening and selecting biocontrol agents (Millus & Rothrock, 1993). It is essential that rhizosphere colonization follows bacterial inoculation. Root colonization reflects the capacity of bacteria to multiply and keep pace with the growing roots in field soil (Kloepper *et al.*, 1992).

Bacillus spp. play a crucial role in plant disease control and suggested mechanisms are:

1- Competitive antagonists of the invading pathogen through completion for nutrients and suitable niches on the root surface (Larkin & Fravel, 1998; O'Sullivan & O'Gara, 1992).
2- Antibiosis by broad-spectrum antibiotic activities and are able to suppress more than one pathogen (Emmert & Handelsman, 1999). For instance, several strains of *Bacillus subtilis* show anti-fungal action by producing anti-fungal volatiles (Fiddaman & Rosal, 1994).
3- Synthesis enzymes that can hydrolyze the cell wall of some fungal pathogens (Mauch *et al.*, 1988).
4- Induced resistance (Liu *et al.*, 1995).

Some successful examples of biological control of plant pathogenic field and postharvest diseases by *Bacillus* spp. are provided in Table 1.4.1.3.

Table 1.4.1.3 Examples of successful biological control of plant pathogenic diseases by *Bacillus* spp.

Crop	Disease / Pathogen	Antagonist	Authors
Apples seedlings	*Phytophthora cactorum*	*Bacillus subtilis*	Utkhede (1984)
Cotton	*Fusarium* wilt	*Bacillus subtilis*	Zhang *et al.* (1996)
Geranium leaf	Geranium rust	*Bacillus subtilis*	Rytter *et al.* (1989)
Beans	*R. solani* AG-4	*Bacillus subtilis*	Turner & Backman (1991)
Wheat	*R. solani* AG8	*Bacillus subtilis* L324-92	Kim *et al.* (1997)
Wheat	*Pythium* root rot	*Bacillus subtilis* L324-92	Kim *et al.* (1997)
Wheat	Take-all	*Bacillus cereus* A47 and *Bacillus subtilis* M908	Ryder *et al.* (1999)
Wheat	*Rhizoctonia* root rot	*Bacillus cereus* A47 and *Bacillus subtilis* M908	Ryder *et al.* (1999)
Loblolly pine	Fusiform rust	*Bacillus pumilus*	Enebak & Carey (2000)
Tomato	*P. infestans*	*Bacillus subtilis* MB1600 and MB 1205	Knox *et al.* (2000)
Stone fruits	Brown rot	*Bacillus subtilis*	Pusey & Wilson (1984)
Strawberry leaves	*Botrytis cinerea*	*Bacillus mycoides*	Guetsky *et al.* (2002)
Cucumber	*Pythium aphanidermatum*	*Bacillus subtilis* BACT-O	Utkhede *et al.* (1999)
Apples	*Penicillium expansum* *Botrytis cinerea*	*Bacillus subtilis*	Leibinger *et al.* (1997)
Cucumber plants	Anthracnose Angular leaf spot Cucurbit wilt disease	*Bacillus pumilus* INR7 *and B. subtilis* GB03	Raupach & Kloepper (1998)

Many rhizobacteria have been reported to stimulate plant growth (Schroth & Hancock, 1982). Plant growth-promoting rhizobacteria (PGPR's) hold great promise as potential agriculture and forestry inoculants and, if effective, could reduce or eliminate the use of toxic or environmentally damaging chemical fertilizers and pesticides (Bent & Chanway, 1998). PGPR's have been shown to enhance tree seedling growth in the nurseries and at reforestation sites. In some cases, they improve the survival of out-planted seedlings (Bent & Chanway, 1998). Microbial populations respond to plant growth through the influence of root exudates

(Curl & Truelove, 1986). Some successful examples of plant growth promotion by *Bacillus* spp. are provided in Table 1.4.1.4.

Table 1.4.1.4 Examples of successful plant growth promoters in the absence of pathogen by *Bacillus* spp.

Crop	Growth promoters	Authors
Peanuts	*Bacillus subtilis*	Turner & Backman (1989)
Cereals	*Bacillus subtilis*	Merriman *et al.* (1974)
Carrots	*Bacillus subtilis*	Merriman *et al.* (1974)
Pepper seedlings	*Bacillus subtilis*	Broadbent *et al.* (1971b)
Tomato seedlings	*Bacillus subtilis*	Broadbent *et al.* (1971b)
Apple trees	*Bacillus* spp.	Caesar & Burr (1987)
Tomato and pepper seedlings	*Bacillus* spp.	Yobo (2000)

Plant growth-promoting rhizobacteria (PGPR) promote plant growth in several ways:

1. Solubilization of insoluble compounds such as phosphates, making them available to plants in a usable form (Kumar & Narula, 1999).
2. Production of phytohormones such as indol-3-acetic acid (IAA) that can enhance various stages of plant growth (Mahaffee & Kloepper, 1997; Monier *et al.*, 1998).
3. Defence of the plant from attack or infection by plant pathogens, by producing antimicrobial compounds such as antibiotics, and many other compounds against plant pathogens (Schippers *et al.*, 1995).
4. Induce resistance (Liu *et al.*, 1995).
5. Synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells, denying iron to plant pathogens (Brown, 1974).
6. Synthesis some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Brown, 1974).

From this review, it can be concluded that the genus *Phytophthora* colonizes and initiates disease on nearly all parts of the host plant and causes serious economic losses in citrus by reducing the life span of tree and fruit size. Efforts to control *Phytophthora* spp. by fungicides have achieved little success may be due to resistance development of the pathogen against chemicals. Breeding for resistance has not been particularly successful, possibly because the development of resistant citrus cultivars is time consuming. Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing negative impacts towards human health and the environment. Biological control has been proposed as an alternative to synthetic fungicides, and considerable success has been achieved by utilizing antagonistic microorganisms. For the purpose of control of *Phytophthora* spp., *Trichoderma* and *Bacillus* isolates may be useful as target antagonists due to their ability to survive in the rhizosphere, and to improve tree health and increase fruit production.

1.5 REFERENCES

ADEJUMO, T.O., IKOTUN, T. & FLORINI, D.A. (1999) Biological control of *Protomycopsis phaseoli*, the causal agent of leaf smut of cowpea. *Journal of Phytopathology* **147**, 371-375.

AKHTAR, M. (1998) Biological control of plant parasitic nematodes by neem products in agricultural soil. *Applied Soil Ecology* **7**, 219-223.

ALEXANDER, M. (1982) *Introduction to Soil Microbiology*. John-Wiley and Sons, New York, USA.

ALEXOPOULOS, C.J. & MIMS, C.W. (1979) *Introductory Mycology*. John Wiley and Sons, New York, USA.

ALTOMARE, C., NORVELL, W.A., BJORKMAN, T. & HARMAN, G.E. (1999) Solubilization of phosphate and micronutrients by the plant-growth-promoting and biolocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology* **65**, 2926-2933.

ANON, (2004) South African Citrus Industry. Citrus Research Programme, http://www.up.ac.za.acadamic/fabi/citrus/sa.html, 26/06/2004.

ASKEW, D.J. (1991) *Trichoderma* in the control of damping-off in containerised seedlings. MSc. thesis, University of Natal, Pietermaritzburg, South Africa.

BAKER, R. (1988) *Trichoderma* spp. as plant-growth stimulants. *CRC Critical Reviews in Biotechnology* **7**, 97-106.

BARBER, D.A. & LYNCH, J.M. (1977) Microbial growth in the rhizosphere. *Soil Biology and Biochemistry* **9**, 305-308.

BATCHELOR, L.D. & ROUNDS, M.B. (1948) Choice of rootstocks, *in The Citrus Industry, Vol. 2, Production of the Crop* (BATCHELOR, L.D. & WEBBER, J.H., Eds.). University of California Press, Berkeley, USA, pp. 169-220.

BENT, E. & CHANWAY, C.P. (1998) The growth-promoting effects of bacterial endophytes on lodgepole pine are partially inhibited by the presence of other rhizobacteria. *Canadian Journal of Microbiology* **44**, 980-988.

BICICI, M., DEDE, Y. & CINAR, A. (1992) *Trichoderma* species against gummosis disease in lemon trees, in *Biological Control of Plant Diseases, Progress and Challenges for the Future* (TJAMOS, E.C. PAPAVIZAS, G.C. & COOK, R.J., Eds.). Plenum Press, New York, USA, pp. 193-196.

BOLAR, J., NORELLI, J.L., WONG, K. -W., HAYES, C.K., HARMAN, G.E. & ALDWINCKLE, H.S. (2000) Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigour. *Phytopathology* **90**, 72-77.

BROADBENT, P. & GOLLNOW, B. I. (1992) Selecting disease tolerant citrus rootstocks for Australia. *Proceedings of the International Society of Citriculture* **2**, 758-764.

BROADBENT, P. (1977) *Phytophthora* diseases of citrus. *Proceedings of the International Society of Citriculture* **3**, 986-998.

BROADBENT, P., FRASER, L.R. & WATERWORTH, Y. (1971a) The reaction of seedlings of citrus spp. and related genera to *Phytophthora citrophthora*. *Proceedings of the Linnean Society of New South Wales* **96**, 119-127.

BROADBENT, P., BAKER, K.F. & WATERWORTH, Y. (1971b) Bacteria and actinomycetes antagonistic to root pathogens in Australian Soils. *Australian Journal of Biological Sciences* **24**, 925-944.

BROCK, T.D. & MADIGNA, M.T. (1991) *Biology of microorganisms*, 6th ed., Prentice Hall, New Jersey, USA.

BROWN, M.E. (1974) Plant growth substances produced by microorganisms of soil and rhizosphere. *Journal of Applied Bacteriology* **35**, 443-451.

BRUKE, J.H. (1967) The commercial citrus regions of the world, in *The Citrus Industry, Vol.1, History, World Distribution, Botany & Varieties* (REUTHER, I.W., WEBBER, H.J. & BATCHELOR, L.D., Eds.). University of California Press, Berkely, USA.

BURGER, M.N. (1988) The scope of fungi in biological control, in *Fungi in Biological Controls Systems* (BURGER, M.N., Ed.). Manchester University Press, Manchester, UK.

BURNETT, H.C., NEMEC. S. & GONSALVES, D. (1977) Attempts to control young tree decline. *Proceedings of the International Society of Citriculture* **3**, 891-894.

CAESAR, A.J. & BURR, T.J. (1987) Growth promotion of apple seedlings and rootstocks by specific strains of bacteria. *Phytopathology* **77**, 1583-1588.

CASTLE, W.S. & GMITTER, F.G. (1999) Rootstock and scion selection, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L.W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 21-34.

CASTLE, W.S. (1987) Citrus rootstocks, in *Rootstock for Fruit Crops* (ROM, R.C. & CARLSON, R.F., Eds.). John Wiley and Sons, New York, USA, pp. 361-399.

CHANG, Y.-C., CHANG, Y.-C., BAKER, R., KLEIFELD, O. & CHET, I. (1986) Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. *Plant Disease* **70**, 145-148.

CHAO, W.L., NELSON, E.B., HARMAN, G.E. & HOCH, H.C. (1986) Colonization of the rhizosphere by biological control agents applied to seeds. *Phytopathology* **76**, 60-65.

CHATENET, B., MERCER, R. & PAVIOT, J. (1988) Summary of six year trials with fosetyl-Al for the control of *Phytophthora* diseases of citrus. *Proceedings of the Sixth International Citrus Congress*, Tel Aviv, Israel.

CHET, I. & BAKER, R. (1980) Induction of suppressiveness to *Rhizoctonia solani* in soil. *Phytopathology* **70**, 994-998.

CHET, I. & BAKER, R. (1981) Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive of *Rhizoctonia solani*. *Phytopathology* **71**, 286-290.

CHET, I. (1987a). *Trichoderma*-application, mode of action, and potential as biocontrol agent of soil-borne plant pathogenic fungi, in *Innovative Approaches to Plant Disease Control* (CHET, I., Ed.). John Wiley and Sons, New York, USA, pp. 137-160.

CHET, I. (1987b) *Innovative approaches to plant disease control*. Wiley-Interscience, New York, USA.

CHET, I. (1990) Biological control of soil-borne plant pathogens with fungal antagonists in combination with soil treatments, in *Biological Control of Soil-borne Plant Pathogens* (HORBY, D., (Ed.), COOK, R.J., HENIS, Y., KO, W.H., ROVIRA, A.D., SCHIPPERS, B. & SCOTT, P.R.). CAB International, Wallingford, UK, pp. 15-25.

CHET, I., HARMAN, G.E. & BAKER, R. (1981) *Trichoderma hamatum* its hyphal interaction with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology* **7**, 29-38.

CONTINELLA, G. & CARTIA, C. (1993) Effects of soil solarization on weed control and seedling development in a citrus nursery (RABE, E., Ed.). *Proceedings of the fourth World congress of the International Society of Citrus Nurserymen, South Africa*, pp. 188-196.

COOK, R.J. & BAKER, K.F. (1983) The nature and practice of biological control of plant pathogens. APS Press, St. Paul, Minnesota, USA.

CURL, E.A. & TRUELOVE, B. (1986) *The rhizosphere*. Springer-Verlag, New York, USA.

DANIELSON, R.M. & DAVEY, C.B. (1973a) The abundance of *Trichoderma* propagules and the distribution of species in forest soils. *Soil Biology and Biochemistry* **5**, 485-494.

DANIELSON, R.M. & DAVEY, C.B. (1973b) Non-nutritional factors affecting in the growth of *Trichoderma* in culture. *Soil Biology and Biochemistry* **5**, 495-504.

DANIELSON, R.M. & DAVEY, C.B. (1973c) Carbon and nitrogen nutrition of *Trichoderma*. *Soil Biology and Biochemistry* **5**, 505-515.

DATNOFF, L.E., NEMEC, S. & PERNEZNY, K. (1995). Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus interaradices*. *Biological Control* **5**, 427-431.

DAVIES, F.S. & ALBRIGO, L.G. (1994) *Citrus*. CAB International, Wallingford, UK.

DAVIS, R.M. (1982) Control of *Phytophthora* foot rot with systemic fungicides, metalaxyl and phosetyl aluminium. *Plant Disease* **66**, 218-220.

DENNIS, C. & WEBSTER, J. (1971) Antagonistic properties of species-groups of *Trichoderma* I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society* **57**, 25-39.

DOEPEL, R.F. (1966) *Phytophthora* species on citrus in western Australia. *Plant Disease Reporter* **50**, 494-496.

ELAD, Y. (2000) Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection* **19**, 709-714.

ELAD, Y., CHET, I., & KATAN, J. (1980) *Trichoderma harzianum:* a biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathology* **70**,119-121.

EMMERT, E.A.B. & HANDELSMAN, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. FERMS *Microbiology Letters* **171**, 1-9.

ENEBAK, S.A. & CAREY, W.A. (2000) Evidence for induced systemic protection to fusiform rust in loblolly pine by plant growth-promoting rhizobacteria. *Plant Disease* **84**, 306-308.

ERKILIC, A & CANIHOS, Y. (1999) Determination of the effect of fosetyl-Al against citrus gummosis disease caused by *Phytophthora citrophthora* (Smith and Smith) Leonian. *Tropical Journal of Agriculture and Forestry* **23**, 419-424.

ERWIN, D.C. & RIBEIRO, O.K. (1996) *Phytophthora diseases worldwide.* APS Press, St. Paul, Minnesota, USA.

EVELEIGH, D.C. (1985). *Trichoderma*, in *Biology of Industrial Microorganisms* (DEMAIN, A.L. & SOLOMON, N.A., Eds.). Benjamin, California, USA, pp. 487-507.

FANG, J.G. & TSAO, P.H. (1992) Mechanisms of biocontrol of *Phytophthora* root rot by *Penicillium funiculosum.* (Abstr.). *Phytopathology* **82**, 1119

FANG, J.G. & TSAO, P.H. (1995a) Evaluation of *Pythium nunn* as a potential biocontrol agent against *Phytophthora* root rots of azalea and sweet oranges. *Phytopathology* **85**, 29-36.

FANG, J.G. & TSAO, P.H. (1995b) Efficacy of *Penicillium funiculosum* as a biological control agent against *Phytophthora* root rots of azalea and citrus. *Phytopathology* **85**, 871-878.

FAO Commodities and Trade Division (2004) Citrus fruit fresh and processed annual statistics. CCP:CI /ST/2002. Food and Agricultural Organization of the United Nations, Rome, Italy.

FARIH, A., MENGE, J.A., TSAO, P.H. & OHR, H.D. (1981) Metalaxyl and efosite aluminum for control of *Phytophthora* gummosis and root rot on citrus. *Plant Disease* **65**, 654-657.

FIDDAMAN, P.J. & ROSSAL, S. (1994) Effect of substrate on the production of anti-fungal volatiles from *Bacillus subtilis*. *Journal of Applied Bacteriology* **76**, 395-405.

GERLACH, W.W.P., HOITINK, H.A.J. & SCHMITTEHENNER, A.F. (1976) *Phytophthora citrophthora* on *Pieris japonica*: infection, sporulation, and dissemination. *Phytopathology* **66**, 302-308.

GLICK, B.R. (1995) The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology* **41**, 109-117.

GRAHAM, J.H. & MENGE, J.A. (1999) Root diseases, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L.W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 126-134.

GRAHAM, J.H. & TIMMER, L.W. (1994) *Phytophthora* diseases of citrus, Fact Sheet pp-155. Florida Cooperative Extension Services, University of Florida, USA.

GRAHAM, J.H. (1995) Root regeneration and tolerance of citrus rootstocks to root rot caused by *Phytophthora nicotianae*. *Phytopathology* **85**, 111-117.

GRAHAM, J.H., TIMMER, L.W., DROUILLARD, D.L. & PEEVER, T.L. (1998) Characterization of *Phytophthora* spp. causing outbreaks of citrus brown rot in Florida. *Phytopathology* **88**, 724-729.

GRIMM, G.R. & HUTCHISON, D.J. (1977) Evaluation of citrus spp. relatives and hybrids for resistance to *Phytophthora parasitica* Dastur. *Proceedings of the International Society of Citriculture* **3**, 863-865.

GUETSKY, R., SHTIENBERG, D., ELAD, Y., FISCHER, E. & DINOOR, A. (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**, 976-985.

HADAR, Y., CHET, I. & HENIS, Y. (1979) Biological control of *Rhizoctonia* damping-off with wheat bran culture of *Trichoderma harzianum Phytopathology* **69**, 64-68.

HARMAN, G.C., CHET, I. & BAKER, R. (1981) Factors affecting *Trichoderma harzianum* applied to seeds as a biocontrol agent. *Phytopathology* **71**, 569-572.

HARMAN, G.E. & LUMSDEN, R.D. (1990) Biological disease control, in *The Rhizosphere* (LYNCH, J.M., Ed.). John Wiley, Chichester, UK.

HARMAN, G.E., CHET, I. & BAKER, R. (1980) *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. *Phytopathology* **70**, 1167-1172.

HODGSON, R.W. (1967) Horticultural varieties of citrus in *The Citrus Industry, Vol. 1, History, World Distribution, Botany and Varieties* (REUTHER, W., BATCHELOR, L.D. & WEBBER, H.D., Eds.). University of California Press, Berkely, USA, pp. 431-591.

HOLL, F.B. & CHANWAY, C.P. (1992) Rhizosphere colonization and seedling growth promotion of lodgepole pine by *Bacillus polymyxa*. *Canadian Journal of Microbiology* **38**, 303-308.

HOUGH, A. & ESSELEN, F. (1992) Citrus rootstock reaction to *Phytophthora* root rot. *Citrus Journal* **2**, 40-43.

HOUGH, A. (1988) Management of *Phytophthora* in citrus nurseries. *Proceedings of the Sixth International Citrus Congress* (GOREN, R. & MENDEL, K., Eds.). Tel Aviv, Israel.

HOWELL, C.R., HANSON, L.E., STIPANOVIC, R.D. & PUCKHABER, L.S. (2000) Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology* **90**, 248-252.

HUANG, H.C. (1992) Ecological basis of biological control of soil-borne plant pathogens. *Canadian Journal of Plant Pathology* **14**, 86-91.

INBAR, J., ABRAMSKY, M. COHEN, D. & CHET, I. (1994) Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *European Journal of Plant Pathology* **100**, 337-346.

IPPOLITO, A., NIGRO, F. & LIMA, G. (1996a) Effectiveness of fosetyl-Al and metalaxyl against *Phytophthora* root rot in sour orange grafted with clementine. *Proceedings of the International Society of Citriculture* **1**, 389-393.

IPPOLITO, A., NIGRO, F. & LIMA, G. (1996b) Influence of the scion on the response of sour orange susceptibility of sour orange rootstocks to experimentally induced *Phytophthora* gummosis and root rot. *Proceedings of the International Society of Citriculture* **1**, 385-388.

JACKSON, L.K. (1991) *Citrus growing in Florida*. University of Florida Press, Florida, USA.

JACKSON, L.K. (1999) Citrus cultivation, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L.W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 17-20.

JENSEN, B., KNUDSEN, I.M.B. & JENSEN, D.F. (2000) Biological seed treatment of cereals with fresh and long-term stored formulations of *Clonostachys rosea*: biological efficacy against *Fusarium culmorum*. *European Journal of Plant Pathology* **106**, 233-242.

JOUBERT, D. & LABUSCHAGNE, N. (1998) Effect of soil compaction on *Phytophthora nicotianae* root rot in rough lemon troyer citrange citrus seedlings. *African Plant Protection* **4**, 123-128.

KHEW, K.L. & ZENTMYER, G.A. (1973) Chemotactic response of zoospores of five species of *Phytophthora*. *Phytopathology* **63**, 1511-1517.

KIM, D.-S., COOK, R.J. & WELLER, D.M. (1997) *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. *Phytopathology* **87**, 551-558.

KLOEPPER, J.W., MCINORY, J.A. & BOWEN, K.L. (1992) Comparative identification by fatty acids analysis of soil, rhizosphere, and geocarposphere bacteria of peanuts (*Arachis hypogaea* L.). *Plant and Soil* **139**, 85-90.

KLOTZ, L.J., DEWOLFE, T.A. & WONG, P.P. (1958) Decay of fibrous roots of citrus. *Phytopathology* **48**, 616-622.

KLOTZ, L.J. (1978) Fungal, bacterial, and nonparasitic diseases and injuries originating in the seedbed, nursery, and orchard, in *The Citrus Industry, V. IV, Crop Protection* (REUTHER, W. CALAVAN, E.C. & CARMAN, G.E. Eds.). University of California, Division of Agricultural Sciences, USA, pp. 1-9.

KNOX, O.G.G., KILLHAM, K. & LEIFERT, C. (2000) Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. *Applied Soil Ecology* **15**, 227-231.

KNUDSEN, I.M.B., HOCKENHULL, J., FUNK JENSEN, D., GERHARDSON, B., HOKEBERG, M., TAHVONEN, R., TEPERI, E., SUNDHEIM, L. & HENRIKSEN, B. (1997) Selection of biological control agents for controlling soil and seed-borne diseases in the field. *European Journal of Plant Pathology* **103**, 775-784.

KOTZE, J.M. (1984) Root rot in citrus. *The Citrus and Subtropical Fruit Journal* **604**, 18-21.

KUMAR, V. & NARULA, N. (1999) Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum* mutants. *Biology and Fertility of Soils* **28**, 301-305.

LAING, M.D. (2004). Personal communication. Department of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

LARKIN, R.P. & FRAVEL, D.R. (1998) Efficacy of various fungal and bacterial biocontrol organisms for control of *Fusarium* wilt of tomato. *Plant Disease* **82**, 1022-1028.

LAVILLE, E.Y. & CHALANDON, A.J. (1981) Control of *Phytophthora* gummosis in citrus with foliar sprays of fosetyl - Al, a new systemic fungicide. *Proceedings of the International Society of Citriculture* **1**, 346-349.

LE ROUX, H.F., BESTER, J.J. &. PRETORIUS, M.C. (1993) Effect of systemic and non-systemic fungicides on *Phytophthora nicotianae* resistant to furalaxyl in sand-bark medium (RABE, E., Ed.). *Proceedings of fourth World Congress of the International Society of Citrus Nurserymen*, South Africa, pp. 216-222.

LE ROUX, H.F., WEHNER, F.C., KOTZE, J.M. & GRECH, N.M. (1991) Combining fosetyl-Al trunk injection or metalaxyl soil drenching with soil application of aldicarb for control of citrus decline. *Plant Disease* **75**, 1233-1236.

LEE, A.T.C. (1988) Guidelines for the production of container grown citrus in South Africa. South African Cooperatives Citrus Exchange Bull, Pretoria, South Africa.

LEE, R.F., LEHMAN, P.S. & NAVARRO, L. (1999) Nursery practices and certification for bud wood and rootstock, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L. W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 35-46.

LEIBINGER, W., BREUKER, B., HAHN, M. & MENDGEN, K. (1997) Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. *Phytopathology* **87**, 1103-1110.

LEWIS, J.A. & LUMSDEN, R.D. (2001) Biological control of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Protection* **20**, 49-56.

LEWIS, J.A. & PAPAVIZAS, G.C. (1987) Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping-off. *Plant Pathology* **36**, 438-446.

LEWIS, J.A. & PAPAVIZAS, G.C. (1991) Biocontrol of plant diseases: the approach for tomorrow. *Crop Protection* **10**, 95-105.

LIU, L., KLOEPPER, J.W. & TUZUN, S. (1995) Induction of systemic resistance in cucumber against *Fusarium* wilt by plant growth-promoting rhizobacteria. *Phytopathology* **85**, 695-698.

LORITO, M., WOO, S.L., GARCIA FERNANDEZ, I., COLUCCI, G., HARMAN, G.E., TORO, J.A., FILIPPONE, E., MUCCIFLORA, S., LAWRENCE, C.B., ZOINA, A., TUZUN, S., & SCALA, F. (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proceedings of the National Academy of Science* **95**, 7860-7865.

MAHAFFEE, W.F. & KLOEPPER, J.W. (1997) Temporal changes in the bacterial communities in soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). *Microbial Ecology* **34**, 210-213.

MANERO, F.J.G., ACERO, N., LUCAS, J.A. & PROBANZA, A. (1996) The influence of native rhizobacteria on European alder (*Alnus glutinosa* (L.) Gaertn.) growth II. Characterisation and biological assays of metabolites from growth promoting and growth inhibiting bacteria. *Plant and Soil* **182**, 67-74.

MARTINEZ, C., BESNARD, O. & BACCOU, J.C. (1999). Stimulating plant's natural defence mechanisms: Organic cellulase and protease: two examples of elicitor substances. *Phytopma* No.521, 16-19 (Abstract).

MATHERON, M.E., WRIGHT, G.C. & PORCHAS, M. (1998) Resistance to *Phytophthora citrophthora* and *P. parasitica* and nursery characteristics of several citrus rootstocks. *Plant Disease* **82**, 1217-1225.

MAUCH, F., MAUCH-MANI, B. & BOLLER, T. (1988) Antifungal hydrolase in peat tissue. II. Inhibition of fungal growth by combination of chitinase suppressive and ß-1,3-gluconases. *Plant Physiology* **88**, 936-942.

MAVINGUI, P. & HEULIN, T. (1994) *In vitro* chitinase and anti-fungal activity of a soil, rhizosphere populations of *Bacillus polymyxa*. *Soil Biology and Biochemistry* **26**, 801-803.

MAZZOLA, M. (1999) Transformation of soil microbial community structure and *Rhizoctonia*-suppressive potential in response to apple roots. *Phytopathology* **89**, 920-927.

MENGE, J.A. (1988) The use of systemic fungicides metalaxyl and efosite aluminium in citrus nurseries. *Proceedings of the Sixth International Citrus Congress*, (GOREN, R. & MENDEL, K., Eds.). University of California, USA, pp. 169-174.

MENGHISTEAB, E. (2001). Personal communication. Pest Control Unit. Elabered Agro Industrial Estate, Zoba Anseba, Eritrea.

MERRIMAN, P.R., PRICE, R.D., KOLLMORGAN, J.F., PIGGOTT, T. & RIDGE, E.H. (1974) Effects of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. *Australian Journal of Agricultural Research* **25**, 219-226.

MEYER, G.DE, BIGIRIMANA, J. ELAD, Y. & HOFTE, M. (1998) Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. *European Journal of Plant Pathology* **104**, 279-286.

MILLUS, E.A. & ROTHROCK, C.S. (1993) Rhizosphere colonization of wheat selected soil bacteria over disease environments. *Canadian Journal of Microbiology* **38**, 303-308

MITCHELLE, D.J & KANNWISCHER-MITCHELL, M.E. (1992) *Phytophthora*, in *Methods for Research on Soil-bornePathogenic Fungi* (SINGLETON, L.L., MIHAIL, J.D. & RUSH. C.M., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 31-38.

MONIER, C., BOSSIS, E., CHABANET, C. & SAMSON, R. (1998) Different bacteria can enchance the micropropagation response of *Cotoneaster lacteus* (Rosaceae). *Journal of Applied Microbiology* **85**, 1047-1055.

MUNNECKE, D. E. (1977) Methyl bromide for control of soil-borne fungal pathogens. *Proceedings of the International Society of Citriculture* **3**, 853-856.

NEMEC, S., DATNOFF, L.E. & STRANDBERG, J. (1996) Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. *Crop Protection* **15**, 735-742.

O'SULLIVAN, D.J. & O'GARA, F. (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbial Review* **56**, 662-676.

OHR, H.D. & MENGE, J.A. (2001) Citrus *Phytopthora* root rot. UC pest management guidelines, http://www.ipm.ucdavis.edu /PMG/ r107100111.html, 12/22/01.

OHR, H.D., MURPHY, M., MENGE, J., CAMPBELL, S., JOHNSON, E. & O'CONNELL, N. (1992) Growth response to chemical treatment of three citrus rootstocks planted in soil infested with *Phytophthora parasitica*. *Proceedings of the International Society of Citriculture* **2**, 862-864.

OLSEN, M., MATHERON, M., MCCLURE, M. & XIONG, Z. (2001) Diseases of Citrus in Arizona. http://ag.arizona.edu/pubs/diseses/az1154/ on 7/21/2001.

OMARJEE. J. (2002) Studies on the application of biocontrol agents for the control of seedling diseases. MSc thesis, University of Natal, Pietermaritzburg, South Africa.

ORLIKOWSKI, L.B. (1995). Studies on the biological control of *Phytophthora cryptogea* Pethybr. et Laff. II. Effectiveness of *Trichoderma* and *Gliocladium* spp. in the control of *Phytophthora* foot rot of gerbera. *Journal of Phytopathology* **143**, 341-343.

PAPAVIZAS, G.C. & LEWIS, J.A. (1989) Effects of *Gliocladium* and *Trichoderma* on damping-off and blight of snapbean caused by *Sclerotium rolfsii* in greenhouse. *Plant Pathology* **38**, 277.

PAPAVIZAS, G.C. (1985) *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

PUSEY, P.L. & WILSON, C.L. (1984) Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant Disease* **68**, 753-756.

RAUPACH, G.S. & KLOEPPER, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**, 1158-1168.

RAY, R. & WALHEIM, L. (1980) *Citrus how to select, grow and enjoy*. Fisher Publishing, USA.

ROXBURGH, C.K., BESTER, J.J., LE ROUX, H.F. & VAN DER WALT, H.P. (1993) Guidelines for the control of *Phytophthora* in Southern African Nurseries (RABE, E., Ed.). Proceedings of fourth World Congress of the International Society of Citrus Nurserymen, South Africa, pp. 203-208.

RYDER, M.H., YAN, Z., TERRACE, T.E., ROVIRA, A.D., TANG, W. & CORRELL, R.L. (1999) The use of strains of *Bacillus* isolated in China to suppress take-all and *Rhizoctonia* root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. *Soil Biology and Biochemistry* **31**,19-29.

RYTTER, J.L., LUKEZIC, F.L., CRAIG, R. & MOORMAN, G.W. (1989) Biological control of geranium rust by *Bacillus subtilis*. *Phytopathology* **79**, 367-370.

SAMUELS, G.J. (1996). *Trichoderma*: a review of biology and systematics of the genus. *Mycological Research* **100**, 923-935.

SAUNT, J. (1990) *Citrus varieties of the world*. Sinclair International, Norwich, UK.

SCHIPPERS, B., SCHEFFER, R.J., LUGTENBERG, B.J.J. & WEISBEEK, P.J. (1995) Biocoating of seeds with plant growth-promoting rhizobacteria to improve plant establishment. *Outlook on Agriculture* **24**, 179-185.

SCHROTH, M.N. & HANCOCK, J.G. (1982) Disease-suppressive and root colonizing bacteria. *Science* **216**, 1376-1381.

SCHUTTE, G.C. (1994) The timing of fosetyl-Al (Aliette) treatments for *Phytophthora* root rot control in the summer rainfall regions. *Citrus Journal* **4**, 20-21.

SID AHMED, A., PEREZ-SANCHEZ, C., EGEA, C. & CANDELA, M.E. (1999) Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathology* **48**, 58-65.

SIVAN, A. & CHET, I. (1986) Biological control of *Fusarium* spp. in cotton, wheat, and muskmelon by *Trichoderma harzianum*. *Journal of Phytopathology* **116**, 39-47.

SPIEGEL-ROY, P. & GOLDSCHMIDT, E. E. (1996) *Biology of citrus*. Cambridge University Press, UK., pp. 4-44

STRAUSS, J. & LABUSCHAGNE, L. (1994) Effect of *Fusarium solani* and *Phytophthora nicotianae* individually and in combination on rough lemon seedlings at different soil moisture levels. *Applied Plant Sciences* **8**, 14-18.

SWINGLE, W.T. & REECE, P.C. (1967) The botany of citrus and its wild relatives, in *The Citrus Industry, Vol. 1, History, World Distribution, Botany and Varieties* (REUTHER, W., BATCHELOR, L.D. & WEBBER, H.D., Eds.). University of California Press, Berkely, USA, pp. 190-340.

SWINGLE, W.T. (1948) Botany of citrus and its wild relatives of the orange subfamily, in *The Citrus Industry, Vol. 1, History, Botany and breeding* (WEBBER, H.J. & BATCHELOR, L.D., Eds.). University of California Press, California, pp. 129-174.

SZTEJNBERG, A., OHR, H.D. & TSAO, P.H. (1988) Screening *Aspergillus* and *Penicillum* spp. for potential biological control of *Phytophthora* gummosis of citrus. *Phytopathology* **78**, 1557 (Abstract.).

THEMANN, K. & WERRES, S. (1995) Experience with diagnostic techniques to detect *Phytophthora* species in water, in *Federal Biological Research Center for Agriculture and Forestry, Institute for Plant Protection in Horticulture*, Braunschweig, Germany, pp. 83-87.

THOMASHOW, L.S. (1996) Biological control of plant root pathogens. *Current Opinions in Biotechnology* **7**, 343-347.

TIMMER, L.W. & MENGE, J.A. (1988) *Phytophthora*-induced diseases, in *Compendium of Citrus Diseases* (WHITESIDE, J.O., GARNSEY, S.M. TIMMER, L.W., Eds.). APS Press, Minnesota, USA, pp. 22-25

TIMMER, L.W. (1977) Preventive and curative trunk treatments for control of *Phytophthora* foot rot of citrus. *Phytopathology* **67**, 1149-1154.

TIMMER, L.W., SANDLER, H.A., GRAHAM, J.H. & ZITKO, S.E. (1988) Sampling citrus orchards in Florida to estimate population of *Phytophthora parasitica*. *Phytopathology* **78**, 940-944.

TRONSMO, A. & DENNIS, C. (1983) The use of *Trichoderma* species to control strawberry fruit rots. *Netherlands Journal of Plant Pathology* **83**, 449-455.

TSAO, P.H., FANG, J.G., SZTEJNBERG, A. & DAFT, G.C. (1996) Biological control with fungi antagonistic against *Phytophthora* root rot of citrus. *Proceedings of the International Society of Citriculture* **1**, 394-396.

TURNER, J.T & BACKMAN, P.A. (1991) Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Disease* **75**, 347-353.

TURNER, J.T. & BACKMAN, P. (1989) Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Disease* **75**, 347-353.

UTKHEDE, R.S. (1984) Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Canadian Journal of Botany* **62**, 1032-1035.

UTKHEDE, R.S. (1992) Biological control of soil-borne pathogens of fruit trees and grapevines. *Canadian Journal of Pathology* **14**, 100-105.

UTKHEDE, R.S., KOCH, C.A. & MENZIES, J.G. (1999) Rhizobacterial growth and yield promotion of cucumber plants inoculated with *Pythium aphanidermatum*. *Canadian Journal of Plant Pathology* **21**, 265-271.

VAN BARLOW, G., BESTER, H., DU PLOOY, C.P., MARAIS, L., SMITH, G.P., WAHL, J.P., BARRY, G., BURDETTE, S.A., KRAJEWISKI, A., MOORE, I.G. & VAN VUUREN, F. (1997) Citriculture establishment, in *Outspan's Integrated Production Guidelines for Export Citrus,* South Africa, pp. 22-24.

VAN BROEMBSEN, L. (1984). Citrus rootstocks. *The Citrus and Subtropical Fruit Journal* **610**, 6-12.

VANDERWEYEN, A. (1988) Citrus or *Phytophthora* nursery? in *Proceedings of the Sixth International Citrus Congress* (GOREN, R. & MENDEL, K., Eds.). Tel Aviv, Israel, pp. 165-168.

VOCK, N., OWEN-TURNER, J., SMITH, D. & MAYERS, P. (1997) *Citrus Information Kit.* Queensland Horticultural Institute Centre for Subtropical fruit- Maroochy Research Station, Australia, pp. 17-18, 23.

VON BROEMBSEN, S.L. & DEACON, J.W. (1997) Calcium interference with zoospore biology and infectivity of *Phytophthora parasitica* in nutrient irrigation solutions. *Phytopathology* **87**, 522-528.

WALKER, G.E. & MOREY, B.G. (1999) Effects of chemical and microbial antagonists on nematodes and fungal pathogens of citrus roots. *Australian Journal of Experimental Agriculture* **19**, 629-637.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964a) *Phytophthora cactorum.* Descriptions of pathogenic fungi and bacteria 111. C.M.I., Kew.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964b) *Phytophthora citrophthora.* Descriptions of pathogenic fungi and bacteria 33. C.M.I., Kew.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964c) *Phytophthora hibernalis.* Descriptions of pathogenic fungi and bacteria 31. C.M.I., Kew.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964d) *Phytophthora nicotianae var. parasitica.* Descriptions of pathogenic fungi and bacteria 35. C.M.I., Kew.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964e) *Phytophthora syringae.* Descriptions of pathogenic fungi and bacteria 32. C.M.I., Kew.

WATERHOUSE, G.M. & WATERSTON, G.M. (1964f) *Phytophthora citricola.* Descriptions of pathogenic fungi and bacteria 114. C.M.I., Kew.

WEBBER, H.J. (1948) Rootstocks: their characteristics and reactions in *The Citrus Industry, Vol. 2, Production of the Crop* (BATCHELOR, L.D. & WEBBER, H.J., Eds.). University of California Press, Berkely, USA, pp. 69-161.

WEBBER, H.J., REUTHER, W. & LAWTON, H.W. (1967) History and development of the citrus industry, in *The Citrus Industrys, Vol. 1, History, World Distribution, Botany and Varieties* (REUTHER, W., WEBBER, H.J., & BATCHELOR, L.D., Eds.). University of California Press, Berkely, USA, pp. 1-39.

WELLER, D.M. (1988) Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26**, 379-407.

WHEATON, T.A., CASTLE, W.S. WHITNEY, J.D. & TUCKER, D.P.H. (1999) Horticultural practices for citrus health, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L.W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 49-58.

WHITESIDE, J.O. (1971) Some factors affecting the occurrence and development of foot rot on citrus trees. *Phytopathology* **61**, 1233-1238.

WILSON, C.L. & PUSEY, P L. (1985) Potential for biological control of postharvest plant diseases. *Plant Disease* **69**, 375-378

WINDHAM, M.T., ELAD, Y. & BAKER, R. (1986) A mechanism of increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**, 518-521.

WRIGHT, J.M. (1956) Biological control of a soil-borne *Pythium* infection by seed inoculation. *Plant and Soil* **8**, 132-140.

YEDIDIA, I., BENHAMOU, N., & CHET, I. (1999) Induction of defense responses in cucumber plants (*Cucumis sativas* L.) by the biocontrol agent *Trichoderma harzianum. Applied and Environmental Microbiology* **65**, 1061-1071.

YEDIDIA, I., SRIVASTVA, A.K., KAPULNIK, Y. & CHET, I. (2001) Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil* **235**, 235-242.

YOBO, K.S. (2000) Utilization of *Bacillus* spp. as plant probiotics. MSc. thesis, University of Natal, Pietermaritzburg, South Africa.

YOBO, K.S., LAING, M.D., HUNTER, C.H. & MORRIS, M.J. (2004) Biological control of *Rhizoctonia solani* by two *Trichoderma* species isolated from South African composted soil. *South African Journal of Plant and Soil* **21**, 139-144.

ZENTMYER, J.A. (1961) Chemotaxis of zoospores for root exudates. *Science* **133**, 1595-1596.

ZENTMYER, J.A. MENGE, J.A. & OHR, H.D. (1994) *Phytophthora* root rot in *Compendium of Tropical Fruit Diseases* (PLOETZ, R.C., ZENTMYER, G.A., NISHIJIMA, W.T., ROHRBACH, K.G., & OHR, H.D., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 76-79.

ZHANG, J., HOWELL, C.R. & STARR, J.L. (1996) Suppression of *Fusarium* colonization of cotton roots and *Fusarium* wilt by seed treatment with *Gliocladium virens* and *Bacillus subtilis*. *Biocontrol Science and Technology* **6**, 175-187.

CHAPTER 2

IN VITRO EVALUATION OF *TRICHODERMA* AND *BACILLUS* ISOLATES AS BIOCONTROL AGENTS AGAINST *PHYTOPHTHORA* SP.

ABSTRACT

Nineteen *Trichoderma* and four *Bacillus* species, tested on dual cultures of PDA plates in the *in vitro* trial against *Phytophthora* sp., resulted in antagonism. The *Trichoderma* in this experiment functioned by hyperparasitism, with no antibiosis (identified by inhibition zones) occurring. The *Bacillus* isolates resulted in inhibition zones. Thus, different effects were noted for the two biological control species used in this test. Both *Trichoderma* and *Bacillus* spp. were antagonistic towards *Phytophthora* sp. These results are promising in terms of the potential biological control of *Phytophthora* root rot of citrus.

2.1 INTRODUCTION

The genus *Phytophthora* is unique among pathogenic fungi. It colonizes and initiates disease on nearly all parts of the host plant (Malajczuk, 1983) and causes serious economic losses in many crops, including citrus (Erwin & Ribeiro, 1996). Efforts to control *Phytophthora* spp. by fungicides have achieved little success (Umaerus *et al.*, 1983) and the development of resistant cultivars is a time consuming process (Burger, 1988). Furthermore, the use of fungicides, besides being expensive and involving risks to the environment are not totally effective and may lead to the appearance of resistant strains of the pathogens (Bruin & Edgington, 1980; Akhtar, 1998). Many chemicals may also lose their usefulness due to revised safety regulations (Emmert & Handlesman, 1999) and concerns over non-target effects and negative effects on beneficial microorganisms (Utkhede, 1992). Thus, there is a need for new solutions to plant disease problems that provide effective control while minimizing negative impacts towards human health and the environment (Cook *et al.*, 1996).

Biological control of soil-borne plant pathogens involves the use of microorganisms (Curl & Truelover, 1986), ideally those found in the rhizosphere (Skinner & Carr, 1976). Rhizosphere-associated microorganisms play a significant role in plant growth and development (Curl & Truelover, 1986) as well as disease control.

Some of the commonly used biological control agents used against soil-borne plant pathogens are *Trichoderma* and *Bacillus* spp. *Trichoderma* spp. are widely distributed and occur in nearly all soils and natural habitats (Papavizas, 1985). They control disease by a number of mechanisms including antibiosis, lysis, competition and mycoparasitism (Chet & Baker, 1980; Papavizas, 1985; Sid Ahmed *et al.*, 1999), as well as inducing resistance (Yedidia *et al.*, 1999; Howell *et al.*, 2000). *Trichoderma* spp. has been widely used in biological control studies against wide range diseases of numerous crops (Elad *et al.*, 1980; Harman *et al.*, 1981; Cook & Baker, 1983; Hadar *et al.*, 1984; Sivan & Chet, 1986; Tronsomo, 1986; Lewis & Papavizas, 1987; Elad & Kapat, 1999). *Trichoderma* spp. also have the ability to enhance plant growth (Chet, 1987b; Yedidia *et al.*, 2001) by the production of growth-stimulating factors (Windham *et al.*, 1986), the solubilization of essential nutrients (Altomare *et al.*, 1999), control of minor plant pathogens (Chet, 1987b) and production of vitamins (Barber & Lynch, 1977).

Bacillus spp. are also widely used biological control agents (Rytter *et al.*, 1989; Huang *et al.*, 1992; Leifert *et al.*, 1995; Kim *et al.*, 1997; Ryder *et al.*, 1999; Enebak & Carey, 2000; Knox *et al.*, 2000). *Bacillus* spp. are the most common bacteria associated with the root rhizosphere of field crops (De Freitas *et al.*, 1997). They produce an endospore that enables them to survive during unfavorable environmental conditions (Prescott *et al.*, 1993) providing a longer shelf life, which favors their commercialization as biological control products (Adegumo *et al.*, 1999).

Many *Bacillus* spp. are known to produce antimicrobial substances (Dijksterhuis *et al.*, 1999) that inhibit some fungal plant pathogens (Silo-Suh *et al.*, 1994). *Bacillus* spp., as with *Trichoderma* spp., are also able to promote plant development through plant growth regulator production (Srinivasan *et al.*, 1996), mineral nutrient solubilization (Kumar & Narula, 1999) and disease suppression (Kim *et al.*, 1997; Duffy, 2000).

Research work on biological control of soil-borne fungal diseases has increased rapidly in recent years. However, relatively few successful studies have been reported on the biocontrol of root rots caused by *Phytophthora*, for example, *Phytophthora capsici* of tomato (Sid Ahmed *et al.*, 1999), *Phytophthora cinnamomi* of avocado (Kelley, 1976) and *Phytophthora* rot and crown rot of apples (Smith *et al.*, 1990). Attempts to control *Phytophthora* root rot of citrus by *Trichoderma* and *Bacillus* spp., to our knowledge, have not been reported.

The purpose of this research was to screen *Trichoderma* and *Bacillus* isolates, using *in vitro* techniques, to establish if any of these isolates has potential for the control of *Phytophthora* root rot of citrus.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of *Phytophthora* isolate

A *Phytophthora* strain was isolated from naturally infested plant material using a modification of Grimm & Alexander's (1973) leaf-disk baiting technique. Infected citrus plant roots were placed in 500ml glass beakers and made up to 500ml with sterile distilled water. A citrus plant root suspension was allowed to settle. Six 10mm^2 leaf piece sections of rough lemon (*Citrus jambhiri* Lush) were floated on the surface of water in a beaker. Beakers were covered and kept for four days at room temperature. Leaf sections were then removed and plated on pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and hymaxazol (PARPH) medium. The PARPH agar was made up of basal and biocidal ingredients. The basal medium used consisted of 17g Difco cornmeal agar and 900ml of distilled water, autoclaved at 121^{0}C for 15min. The biocidal ingredients were 1.0g quintozene (PCNB), 0.5g hymexazol and the antibiotics pimaricin (2.0g), ampicillin (1.25g) and rifampicin (0.1g). These were mixed in 100ml of sterilized (autoclaved at 121^{0}C for 15min) distilled water and added to the autoclaved basal medium. The plates were incubated in darkness at 25^{0}C for seven days. *Phytophthora* isolates1 were identified by characteristic mycelia and spore structure (Figure 2.2.1) and morphologically identified as *Phytophthora parasitica* (*Phytophthora* Isolate 1).

Phytophthora Isolate 1 was obtained from rough lemon rootstock root tips of orange trees at the Ukulinga Research Farm (30^0 22′ 54″ E and 29^0 36′ 10″ S). This isolate was used in pathogenicity screening on rough lemon seedlings (Section 2.2.2). Other isolates, Isolates 2 and 3, were isolated from dam water, and root tips of rough lemon rootstock orange trees, respectively, found at Waterford Farm, Richmond (30^0 56′ 52.39″ E and, 29^0 45′ 45.03″ S).

Phytophthora isolates were stored in sterilized distilled water (15ml / 25ml of a rubber-lined screw-cap glass vial) and in autoclaved sand in a 100ml rubber-lined screw-cap glass vial at room temperature for further work.

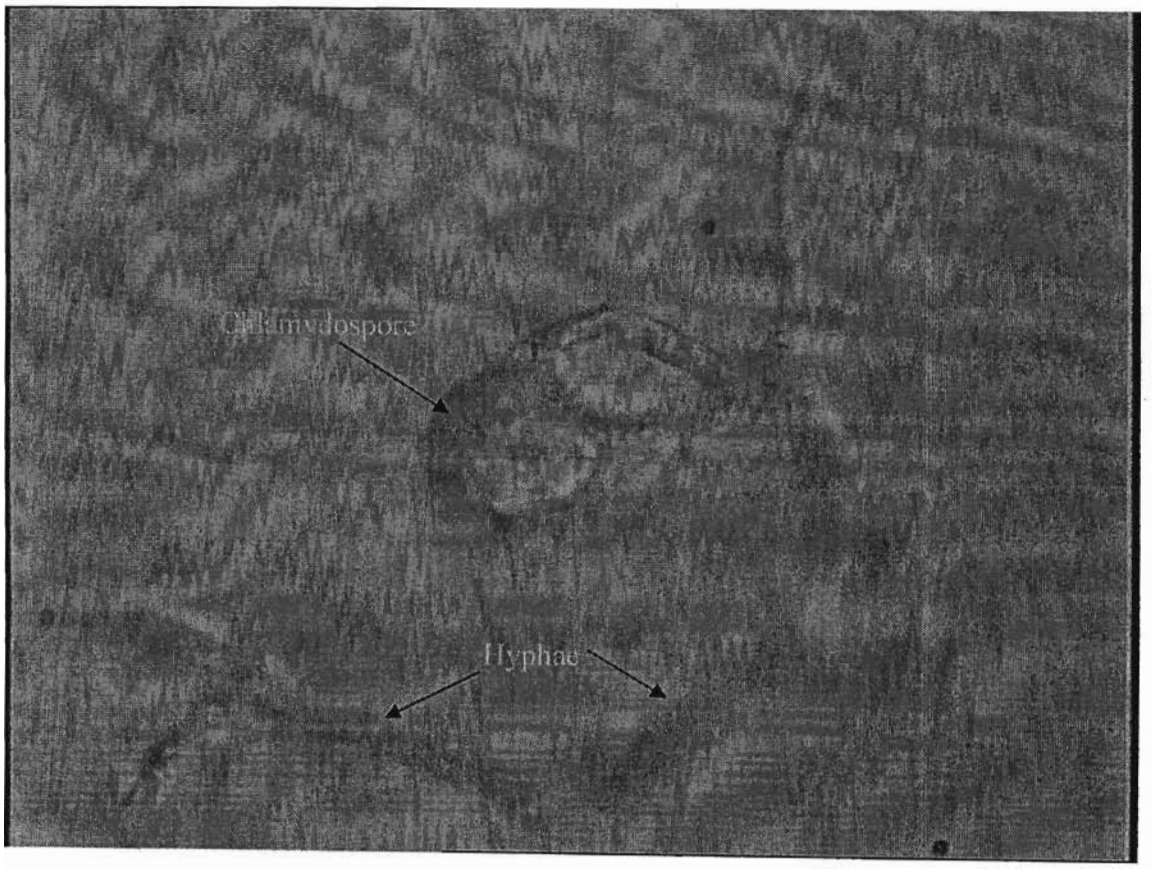

Figure 2.2.1 Micrograph of *Phytophthora* Isolate 1 (*Phytophthora parasitica*) from Ukulinga farm Pietermaritzburg (40X)

2.2.2 Pathogenicity testing of *Phytophthora* isolate on rough lemon seedlings (*Citrus jambhiri* Lush).

As a pilot trial it was important to evaluate the pathogenicity of the *Phytophthora* isolate. Rough lemon seedlings were grown from seeds collected from trees in Pietermaritzburg, KwaZulu-Natal (30^0 22′ 54.43″ E and 29^0 36′ 28″ S). Seeds were removed from the fruit and the mucilaginous coating was removed by washing in sterile distilled water to enhance germination. Seeds were sown into sterile Speedling® 24 cavity trays which had been steam sterilized at 70^0C for 30 minutes, dipped in Plazdip[1]® and filled with sterile composted pine bark medium. Trays were watered and put in a germination room at approximately 24^0C for two days. They were then moved to a polycarbonate-covered tunnel. Temperature in the tunnel ranged from 15-30^0C and relative humidity, 60-80%. Trays were irrigated three times a day with overhead sprinkler for a period of six minutes, with soluble fertilizers (NPK: 3:1:3 (38) Ocean Agriculture[2]).

In preparation for inoculation, citrus seedlings of approximately the same height and at three true leaf stage were transplanted into sterile new trays as described above. *Phytophthora* inoculation was achieved by placing a colonized 4mm diameter block bulked up on V8 agar (200.0ml of V8 juice, 3.0g calcium carbonate, 20.0g agar and 8000ml distilled water, mixed and autoclaved at 121^0C for 15min). Agar block colonized with mycelium of *Phytophthora* Isolate 1 was placed up side down at the base of each seedling. Six seedlings were used per treatment. The assessment was based on the physical appearance of the seedlings, i.e., wilting, dying and those with no symptoms considered as healthy. Seedlings in each category were counted and the percentage taken for the evaluation. Phytophthora Isolate 1 was found to cause seedling production loss of 66.67 % compared to the uninoculated control. Based on this result, *Phytophthora* Isolate 1 was chosen for further work in this study. Pathogenicity of the remaining *Phytophthora* isolates was not established.

[1] Starke Ayres (PTY) Ltd., P.O.Box 4005, Willowton, Pietermaritzburg, KwaZulu Natal 3201
[2] Ocean Agriculture (PTY) Ltd., P.O.Box 741, Muldersdrift 1747

2.2.3 Isolation of *Trichoderma* isolates

Trichoderma isolates were isolated using the basal medium *Trichoderma* selective medium (TSM) that consisted of the following components: 0.2g $MgSO_4$, 0.9g K_2HPO_4, 0.15g KCl, 1.0g NH_4NO_3, 3.0g glucose, 0.15g rose bengal and 20g agar. These constituents were added to 950ml-distilled water and autoclaved at 121^0 C for 15min. The biocidal ingredients were 0.25g chloramphenicol (crystallised), 0.2g quintozene (PCNB, wettable powder), 0.2g captab (Kaptan 517, wettable powder) and 1.2ml propamocarb (Previcur, solution concentrate). These were mixed with 50ml of sterilized distilled water and added to the autoclaved basal medium (Askew & Laing, 1993).

Media (soil, composted pine bark and plant root samples) were collected from different sites in Pietermaritzburg, KwaZulu-Natal. Each of the soil and composted pine bark samples were subjected to a dilution series of 10^{-1}-10^{-5}. A 0.1ml aliquot from each of the dilutions was applied onto TSM agar plates and spread on the agar surface with a glass rod. Three replicates were prepared for each dilution. Soil, plant roots and composted pine bark were also plated directly on the medium of three replicated plates. All plates were incubated in the dark at 25^0C for seven days.

Potential *Trichoderma* isolates were identified based on characteristic color (typical green/slight yellow-green color) and growth habit. Mycelia from colony edges were transferred to plates of V8 agar.

All isolates were stored in sterilized distilled water, and slants of potato dextrose agar (PDA) and V8 media (15ml / 25ml rubber-lined screw-capped glass vial) at room temperature. The isolation was done from different sources and different areas where *Trichoderma* additions had not been made. The *Trichoderma* isolates obtained are presented in the Table 2.1. *Trichoderma* isolates AA2 and AA16 were identified as *Trichoderma virens* (PPRI 7531) and *Trichoderma harzianum* (PPRI 7530) respectively by the ARC-Plant Protection Research Institute, Mycology Division, Pretoria, South Africa.

Table 2.1 *Trichoderma* isolates obtained from different sources

Trichoderma isolates	Source	Collection Area
AA1, AA2, AA3, AA4, AA6, AA7, AA8, AA9, AA10, AA11 and AA14	Valencia trees (rough lemon rootstocks) and soils	Waterford Farm, Richmond, KwaZulu-Natal, South Africa
AA5	Under table of general seedling nursery.	Sunshine Seedling, KwaZulu-Natal, South Africa.
AA12 and AA13	Soil of Valencia tree (rough lemon rootstock)	Agricultural campus of Pietermaritzburg, KwaZulu-Natal, South Africa.
AA15and AA16	Lemon (rough lemon rootstock) farm	Ukulinga, Pietermaritzburg, KwaZulu-Natal, South Africa.
AA17	Seedling mix (composted pine bark)	Agricultural campus of Pietermaritzburg, KwaZulu-Natal, South Africa.

2.2.4 Isolation of *Bacillus* isolates

Root materials and associated rhizosphere soils were collected from various sites at citrus farms in KwaZulu-Natal, RSA. Root samples of rough lemon root tips were harvested by carefully pulling the plant from the soil and shaking off the excess soil. Root material was then placed in a plastic bag and stored at 4^0C until further processing. Approximately 1.0g of rhizosphere soil/root samples was suspended in 99.0ml of sterile ¼ strength Ringer's Solution and shaken vigorously for two min. The suspension was then heat treated at 80^0C for 15min in a water bath to destroy vegetative cells. A dilution series of 10^{-1}-10^{-6} of the suspension was prepared and 0.1ml plated out on duplicate plates of Tryptone Soy Agar (TSA) using the pour plate technique. Agar plates were incubated for three days at 25^0C, after which representative colonies were arbitrarily selected and streaked onto fresh TSA plates to obtain single colonies. Sub-cultures were made from the resulting colonies on 10% (w/v) TSA agar slants and after incubation at 25^0C for 48 hours, were stored at 5^0C for further work. Morphological and Gram staining procedures were employed to verify that the isolates were Gram-positive endospore formers. Five isolates were obtained and numbered as A1-A5 as presented in Table 2.2.

Table 2.2 *Bacillus* isolates obtained from different sources

Isolate	Source used	Rootstock	Collection area
A1	Rhizosphere soils	Rough lemon	Ukulinga Research Farm, Pietermaritzburg, KwaZulu-Natal, South Africa.
A2	Rhizosphere soils	Rough lemon	Richmond Citrus Farm, KwaZulu-Natal, South Africa.
A3	Rhizosphere soils	Rough lemon	Agricultural campus of Pietermaritzburg, KwaZulu-Natal, South Africa.
A4	Speedling® tray of pine bark medium	Pine seedling	SAPPI Nursery, Richmond, KwaZulu-Natal, South Africa.
A5	Under growing table	-	Sunshine Nursery, KwaZulu-Natal, South Africa.

2.2.5 *In vitro* reaction of selected *Trichoderma* isolates against a *Phytophthora* isolate

Seventeen isolates of *Trichoderma* as shown in Table 2.1 were screened along with five isolates obtained from Sackey Yobo[3] (SY3F, SY2F, SYN4, SYN6, and TN) and one *Trichoderma* isolate was obtained from Plant Health Products[4] (Eco-T®). *Trichoderma* isolates were evaluated for *in vitro* activity against the *Phytophthora* Isolate 1 (Section 2.2.2) using the dual bioassay method. The interactions were studied in Petri plates (90mm diameter) containing potato dextrose agar (PDA). The *Phytophthora* and *Trichoderma* isolates were grown on V8 agar, incubated at 25^{0}C. A 4mm in diameter agar plug was taken from the edge of an actively growing *Phytophthora* isolate colony was transferred to each plate and a similar size plug of *Trichoderma* isolate, cut in the same manner, was placed on the opposite side of each plate. Pates were incubated at 25^{0}C in darkness. Each pair was replicated three times.

[3] K.S. Yobo, Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, RSA

[4] Plant Health Products (Pty) Ltd., P.O.Box 207, Nottingham Road, 3280

After five days cultures were scored for degrees of activity using the rating system of Bell *et al.* (1982) on a scale of 1-5:

Class1 = *Trichoderma* completely over-grew the pathogen and covered the entire medium surface.

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

Class 3 = *Trichoderma* and *Phytophthora* each colonized 50% of the medium surface and neither organism appeared to dominate the other.

Class 4 = *Phytophthora* colonized at least two thirds of the medium surface and appeared to withstand encroachment by *Trichoderma* and

Class 5 = *Phytophthora* completely dominated *Trichoderma*, overgrew it, and occupied the entire medium surface.

An isolate of *Trichoderma* was considered to be antagonistic/(hyperparasitic) if the mean score was ≤ 2, but not antagonistic /(hyperparasitic) if the number was ≥ 3.

2.2.6 *In vitro* reaction of selected *Bacillus* isolates against *Phytophthora* isolate

Five isolates were obtained as described in Section 2.2.4, Table 2.2. An additional three *Bacillus* isolates, i.e., B69, B77 and B81 were obtained from the Department of Plant Pathology, University of KwaZulu-Natal, South Africa. Isolates were cultured up in sterile Tryptone Soy Broth (TSB) 250ml flasks and were incubated at 30^0C for four days at and agitated in a water bath shaker (120rpm (G.F.L. 1083, Labortechnik, Germany). After four days, the TSB containing *Bacillus* was transferred into sterile centrifuge tubes and centrifuged (Beckman J2-HS centrifuge, USA) at 9000rpm for 20min. After centrifugation, the supernatent was removed. The settled pellet wasresuspended in 100.0ml sterile distilled water. Aliquot, (0.1ml) were transferred onto sterile paper discs and immediately placed at the sides of PDA plates, with a 4mm plug of *Phytophthora* Isolate 1 (grown on V8 agar medium) occupying the center of the plate and incubated at 25^0C for two days.

The treatments were evaluated for evidence of inhibition of *Phytophthora parasitica*.

2.3 RESULTS

Nineteen of the *Trichoderma* isolates showed a high level of antagonism (rating ≤ 2) as is reflected in Figure 2.3.2. Four of the *Bacillus* isolates (A1, B69, B77 or B81) tested showed inhibitory reactions against *Phytophthora* Isolate 1 (*Phytophthora parasitica*) root rot of citrus.

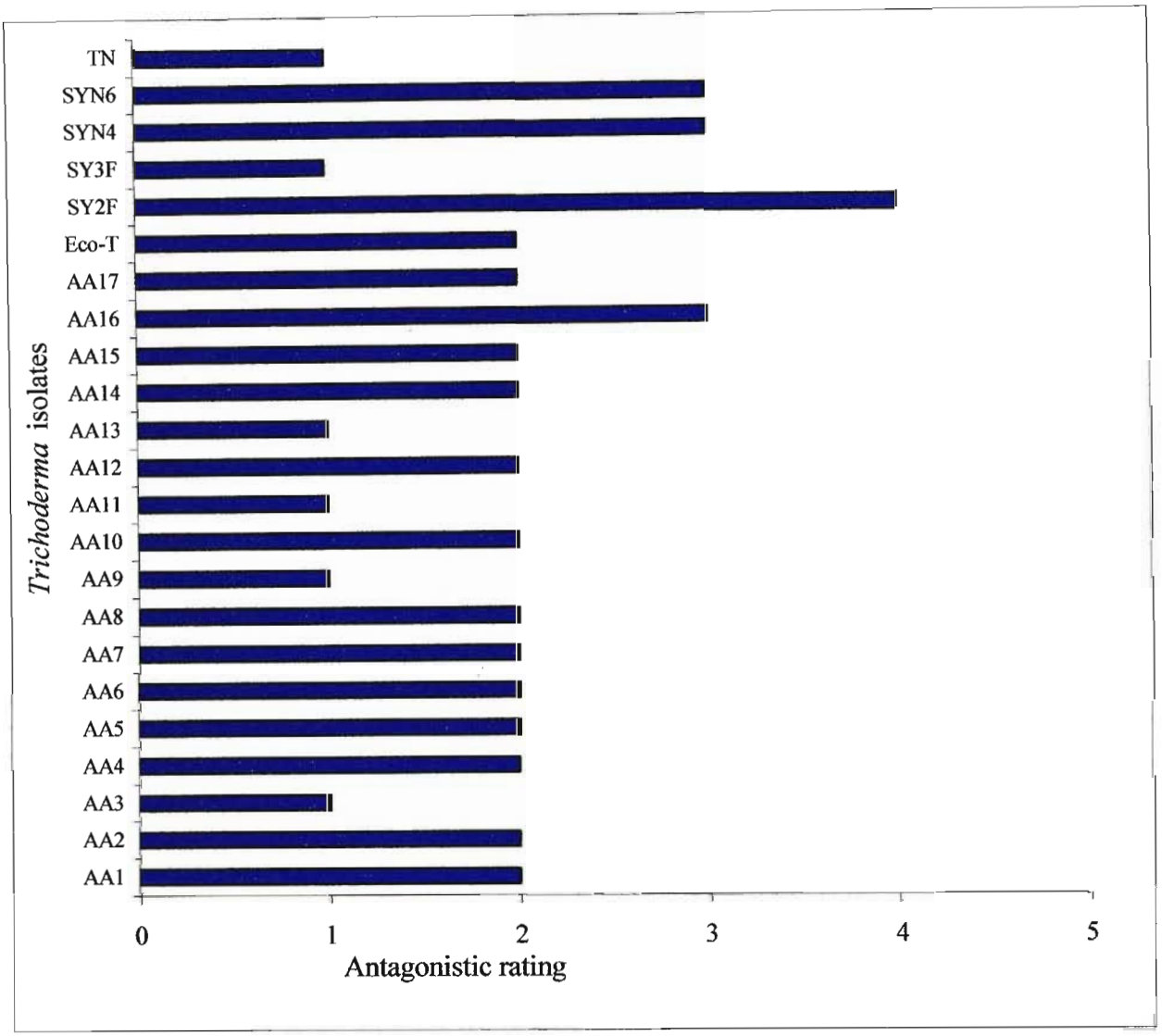

Figure 2.3.1 Antagonistic reactions of a series of *Trichoderma* isolates against *Phytophthora* Isolate 1 *in vitro*. Antagonistic rating of isolates of *Trichoderma* with means of three (rounded to nearest whole number) in antagonism classes 1-5: 1 = *Trichoderma* completely overgrew the pathogen and covered the entire medium surface; 2 = *Trichoderma* overgrew at least two thirds of the medium surface; 3 = *Trichoderma* and *Phytophthora* each colonized 50% of the medium surface and neither organism appeared to dominate the other; 4 = *Phytophthora* colonized at least two- thirds of the medium surface and appeared to withstand encroachment by *Trichoderma* and 5 = *Phytophthora* completely dominated *Trichoderma*, overgrew it, and occupied the entire medium surface. All the isolates of *Trichoderma* are given with their corresponding rating.

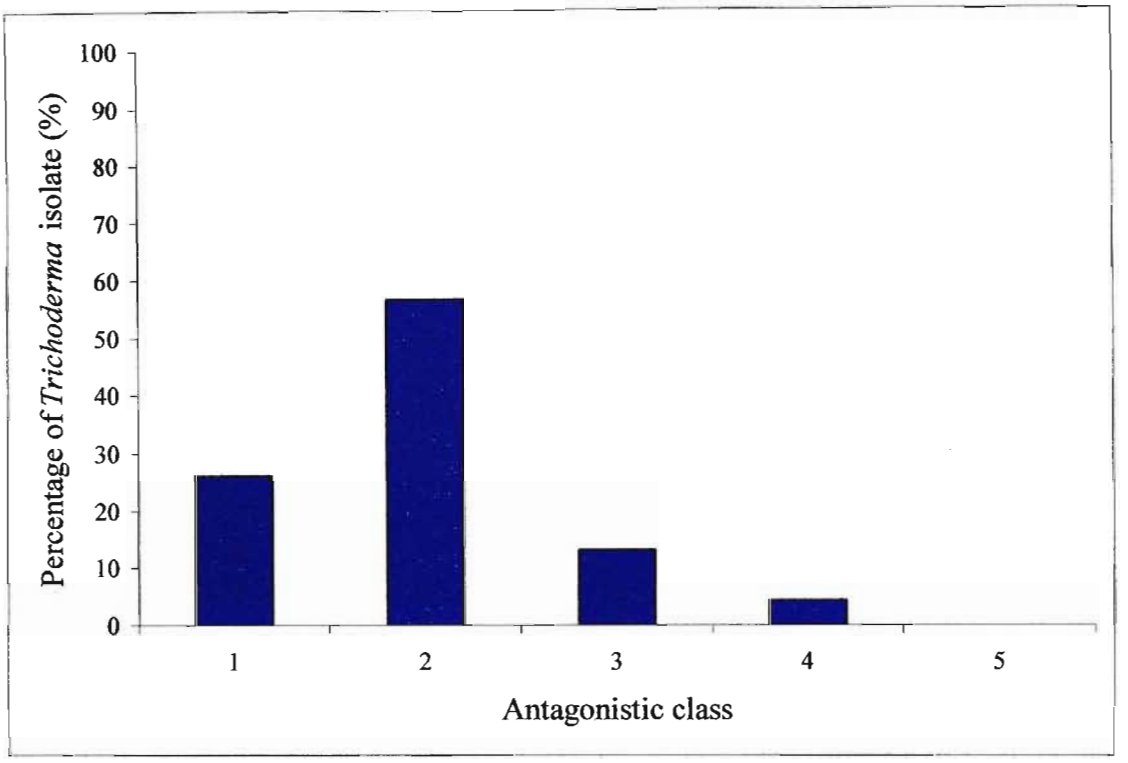

Figure 2.3.2 Percentage antagonistic activities of a series of *Trichoderma* isolates against *Phytophthora* Isolate 1 *in vitro*. Percentage of isolates of *Trichoderma* with means (rounded to nearest whole number) in antagonism classes 1-5: 1 = *Trichoderma* completely overgrew the pathogen and covered the entire medium surface; 2 = *Trichoderma* overgrew at least two thirds of the medium surface; 3 = *Trichoderma* and *Phytophthora* each colonized 50% of the medium surface and neither organism appeared to dominate the other; 4 = *Phytophthora* colonized at least two thirds of the medium surface and appeared to withstand encroachment by *Trichoderma* and 5 = *Phytophthora* completely dominated *Trichoderma*, overgrew it, and occupied the entire medium surface.

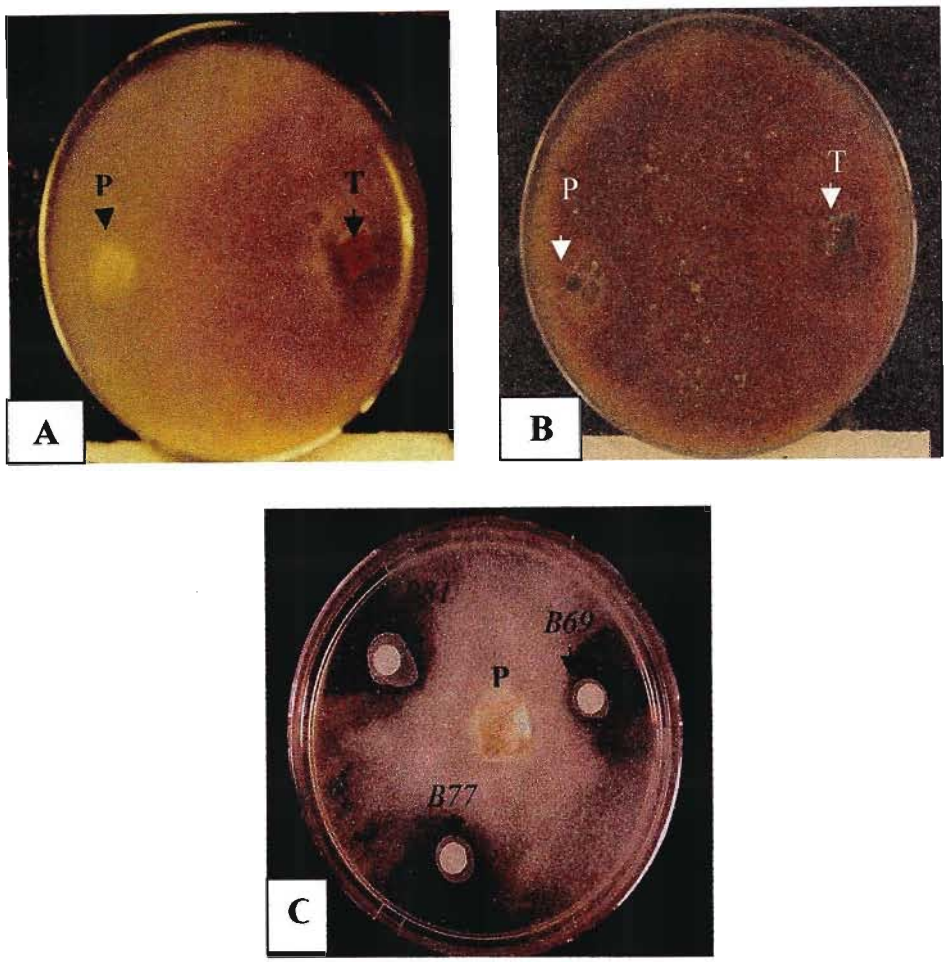

Figure 2.3.3 *In vitro* antagonistic reactions of *Trichoderma* isolate and *Bacillus* isolates against *Phytophthora parasitica*, plated in dual culture PDA medium and incubated at 25^0C. Figures 2.3.3A-B show the antagonistic activity of *Trichoderma* (T) Isolate (AA3) against *Phytophthora parasitica* (P) by after 24hours and 48 hours, respectively. Figure 2.3.3C shows inhibition of *Phytophthora parasitica* (P) by *Bacillus* Isolates B81, B77 and B69 after 48 hours incubation.

2.4 DISCUSSION

Bell tests are used as a primary screening method to select potential biocontrol agents for further testing (Merriman & Russel, 1990). *In vitro* tests were conducted on a medium and at a temperature where both the *Trichoderma* spp. and *Phytophthora* sp. grew well in the laboratory. In most natural sites of biological control, supply of nutrients may be limiting. Temperature may also fluctuate during different seasons either favoring the antagonist or the pathogen at any one time. This is a simplistic approach to understanding a small component of biological systems in disease control. However, controlling a large sector of the environment, excluding other soil microflora and supplying a uniform food base, temperature, moisture, and light should yield useful information on the degree of antagonistic variability within *Trichoderma*. Furthermore, *In vitro* provides an indication of the diversity of ability among soil-borne pathogens to resist antagonism (Bell *et al.*, 1982).

The mean Bell ratings of each candidate biocontrol agent evaluate the general antagonistic ability of that isolate in a series of pathogen X antagonist combinations. Overall performance of *Trichoderma* isolates can be compared with other isolates. Isolates with the lowest mean Bell rating (Figure 2.3.1) show the greatest antagonistic potential.

Bell ratings are based on the microbial antagonism interactions of antibiosis and hyperparasitism. Overgrowth of *Phytophthora* isolates by the candidate *Trichoderma* isolates was observed. Antibiosis can be easily identified by the formation of zones of inhibition (Smith *et al.*, 1990). The *Trichoderma* isolates tested did not show this phenomenon.

The potential of *Trichoderma* strains as biocontrol agents has been well documented (Elad *et al.*, 1980; Cook & Baker, 1983; Papavizas, 1985; Chet, 1987a, 1987b; Harman & Taylor, 1990). Askew & Laing (1994) found that 74% of the *Trichoderma* isolates tested were antagonistic against *Rhizoctonia solani in vitro*. In this study 19 out of 23 *Trichoderma* isolates tested were antagonistic to *Phytophthora* root rot of citrus *in vitro*. Differences in colony morphology and diversity of isolate collection sites suggest that all isolates were unique strains.

Many *Bacillus* strains are known to suppress fungal growth *in vitro* by production of one or more antifungal compounds (Katz & Demain, 1977; Silo-Suh *et al.*, 1994; Leifert *et al.*, 1995). The potential of *Bacillus* strains as biocontrol agents is also well known (Dunleavy, 1955; Utkhede & Gaunce, 1983; Utkhede, 1984; Capper & Campbell, 1986; Huang *et al.*, 1992; Podile & Prakash, 1996; Kim *et al.*, 1997; Emmert & Handelsman, 1999; Enebak & Carey, 2000; Knox *et al.*, 2000). For example, *Bacillus* spp. Strain L324-92 possesses an *in vitro* antibiotic activity against isolates of *Gaeumannomyces graminis* (Sacci) Arx & Oliver var. *tritici* as well as *Rhizoctonia* and *Pythium* spp. (Kim *et al.*, 1997).

In this study, four of the eight of the *Bacillus* isolates tested caused inhibition reactions against *Phytophthora* Isolate 1, the causal agent of root rot of citrus as shown in Figure 2.3.3C. Inhibition zones could be the result of antimicrobial production by the *Bacillus* isolates. Antibiotics are commomly produced as secondary metabolites during the stationary phase of growth but it also appears that in some *Bacillus* species antibiotics are produced during the growth phase (Madigan *et al.*, 1997). Four of the *Bacillus* isolates tested did not show inhibition zones of the pathogen.

In vitro screening fails to take into account the amount of natural inoculum present in the soil, nor the effects of physical, chemical and biological properties in the soil. Primarily it identifies antagonists that are antibiotic producers and those with a hyperparasitic nature, and will not select antagonists that have other modes of action such as nutrient competition (Wisniewski *et al.*, 1991). Hence, *in vitro* screening does not give an accurate indication of the level of biological control given by these isolates (Merriman & Russel, 1990).

Therefore, the positive *in vitro* activity of *Trichoderma* and *Bacillus* isolates on *Phytophthora* sp. only provided an initial step for further *in vivo* testing under greenhouse conditions to evaluate the *Trichoderma* isolates and selected *Bacillus* isolates as biological control and growth enhancement agents on citrus.

2.5 REFERENCES

ADEGUMO, T.O., IKOTUN, T. & FLORINI, D.A. (1999) Biological control of *Protomycopsis phaseoli*, the causal agent of leaf smut of cowpea. *Journal of Phytopathology* **147**, 371-375.

AKHTAR, M. (1998) Biological control of plant parasitic nematodes by neem products in agricultural soil. *Applied Soil Ecology* **7**, 219-223.

ALTOMARE, C., NORVELL, W.A., BJORKMAN, T. & HARMAN, G.E. (1999) Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology* **65**, 2926-2933.

ASKEW, D.J. & LAING, M.D. (1993) An adapted selective medium for the quantitative isolation of *Trichoderma* species. *Plant Pathology* **42**, 686-690.

ASKEW, D.J. & LAING, M.D. (1994) The *in vitro* screening of 118 *Trichoderma* isolates for antagonism to *Rhizoctonia solani* and an evaluation of different environmental sites of *Trichoderma* as sources of aggressive strains. *Plant and Soil* **159**, 277-281.

BARBER, D.A. & LYNCH, J.M. (1977) Microbial growth in the rhizosphere. *Soil Biology and Biochemistry* **9**, 305-308.

BELL, D.K., WELLS, H.D. & MARKHAM, C.R. (1982) *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology* **72**, 379-382.

BRUIN, G.C. & EDGINGTON, L.V. (1980) Induced resistance to ridomil of some Oomycetes. *Phytopathology* **70**, 459-460.

BURGER, M.N. (1988) The scope of fungi in biological control, in *Fungi in Biological Control Systems* (BURGER, M.N., Ed.). Manchester University Press, Manchester,UK, pp. 1-18.

CAPPER, A.L. & CAMPBELL, R. (1986) The effect of artificially inoculated antagonistic bacteria on the prevalence of take-all disease of wheat in field experiments. *Journal of Applied Bacteriology* **60**, 155-160.

CHET, I. & BAKER, R. (1980) Induction of suppressiveness to *Rhizoctonia solani* in soil. *Phytopathology* **70**, 994-998.

CHET, I. (1987a) *Innovative approaches to plant disease control*. John Wiley and Sons, New York, USA, pp. 379.

CHET, I. (1987b) *Trichoderma*-application, mode of action, and potential as biocontrol agents of soil-borneplant pathogenic fungi, in *Innovative Approaches to Plant Disease Control* (CHET, I., Ed.). John Wiley & Sons, New York, USA, pp. 137-160.

COOK, R.J. & BAKER, K.F. (1983) The nature and practice of biological control of plant pathogens. APS Press, St. Paul, Minnesota, USA.

COOK, R.J., BRUCKART, W.L., COULSON, J.R. GOETTEL, M.S., HUMBER, R.A., LUMSDEN, R.D., MADDOX, J.V., MCMANUS, M.L., MOORE, L., MEYER, S.F., QUIMBY, P.C., STACK, J.P. & VAUGHAN, J.L. (1996) Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biological Control* **7**, 333-351.

CURL, E.A. & TRUELOVER, B. (1986) *The rhizosphere*. Springer-Verlag, New York, USA.

DE FREITAS, J.R., BANERJEE, M.R. & GERMIDA, J.J. (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). *Biology and Fertility of Soils* **24**, 358-364.

DIJKSTERHUIS, J., SANDERS, M., GORRIS, L.G.M. & SMID, E.J. (1999) Antibiosis plays a role in the context of direct interaction during antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. *Journal of Applied Microbiology* **86**, 13-21.

DUFFY, B. (2000) Combination of pencycuron and *Pseudomonas fluorescens* Strains 2-79 for integrated control of *Rhizoctonia* root rot and take-all of spring wheat. *Crop Protection* **19**, 21-25.

DUNLEAVY, J. (1955) Control of damping-off of sugarbeet by *Bacillus subtilis*. *Phytopathology* **45**, 252-258.

ELAD, Y. & KAPAT, A. (1999) The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. *European Journal of Plant Pathology* **105**, 177-189.

ELAD, Y., CHET, I. & KATAN, J. (1980) *Trichoderma harzianum:* a biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathology* **70**, 119-121.

EMMERT, E.A.B. & HANDELSMAN, J. (1999). Biocontrol of plant disease: a gram-positive perspective. FERMS *Microbiology Letters* **171**, 1-9.

ENEBAK, S.A. & CAREY, W.A. (2000) Evidence for induced systemic protection to *Fusarium* rust in loblolly pine by plant growth promoting rhizobacteria. *Plant Disease* **84**, 306-308.

ERWIN, D.C. & RIBEIRO, O.K. (1996) *Phytophthora diseases worldwide*. APS Press, St. Paul, USA.

GRIMM, G.R. & ALEXANDER, A.F. (1973) Citrus leaf pieces as traps for *Phytophthora parasitica* from soil slurries. *Phytopathology* **63**, 540-541.

HADAR, Y., HARMAN, G.C. & TAYLOR, A.G. (1984) Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. *Phytopathology* **74**, 106-110.

HARMAN, G.E., CHET, I. & BAKER, R. (1981) Factors affecting *Trichoderma harzianum* applied to seeds as a biocontrol agent. *Phytopathology* **71**, 569-572.

HARMAN, G.E. & TAYLOR, A.G. (1990) Development of an effective biocontrol seed treatment system in *Biological Control of Soil-bornePlant Pathogens* (HORNBY, D., COOK, R.J., HENNIS, Y., KO, W.H., ROVIRA, A.D. SCHIPPERS, B., SCOTT, P.R., Eds.). CAB International, Wallingford, UK, pp. 415-426.

HOWELL, C.R., HANSON, L.E., STIPANOVIC, R.D. & PUCKHABER, L.S. (2000) Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology* **90**, 248-252.

HUANG, Y., WILD, B.L. & MORRIS, S.C. (1992) Postharvest biological control of *Penicillium digitatum* decay on fruit by *Bacillus pumilus*. *Annals of Applied Biology* **102**, 367-372.

KATZ, E. & DEMAIN, A.L. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. *Bacteriological Review* **41**, 449-474.

KELLY, W.D. (1976) Evaluation of *Trichoderma harzianum*-impregnated clay granules as a biocontrol for *Phytophthora cinnamomi* causing damping-off of pine seedlings. *Phytopathology* **66**, 1023-1027.

KIM, D.-S., COOK, R.J. & WELLER, D.M. (1997) *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. *Phytopathology* **87**, 551-558.

KNOX, O.G.G., KILLHAM, K. & LEIFERT, C. (2000) Effects of increased nitrate availability on the control of plant pathogenic fungi by the soil bacterium *Bacillus subtilis*. *Applied Soil Ecology* **15**, 227-231.

KUMAR, V. & NARULA, N. (1999) Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum* mutants. *Biology and Fertility of Soils* **28**, 301-305.

LEIFERT, C., LI, H., CHIDBUREE, S., HAMPSON, S., WORKMAN, S., SIGEE, D., EPTON, H.A.S. & HARBOUR, A. (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. *Journal of Applied Bacteriology* **78**, 97-108.

LEWIS, J.A. & PAPAVIZAS, G.C. (1987) Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping off. *Plant Pathology* **36**, 438-446.

MADIGAN, M.T., HARLEY, J.P. & KLEIN, D.A. (1997) *Microbiology, 2nd ed.,* Wm. VCH, New York, USA, pp. 459-462.

MALAJCZUK, N. (1983) Microbial antagonism to *Phytophthora*, in *Phytophthora*: Its B*iology, Taxonomy, Ecology, and Pathology* (ERWIN, D.C., BARTNICKI-GARCIA, S. & TSAO, P.H., Eds.). APS Press, St. Paul, Minnesota, pp. 197-214.

MERRIMAN, P. & RUSSEL, K. (1990) Screening strategies for biological control, in *Biological Control of Soil-borne Plant Pathogens* (HORNBY, D., Ed.). CAB International, Wallingford, UK.

PAPAVIZAS, G.C. (1985) *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

PODILE, A.R. & PRAKASH, A.P. (1996) Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. *Canadian Journal of Microbiology* **42**, 533-538.

PRESCOTT, L.M., HARLEY, J.P. & KLEIN, D.A. (1993) *Microbiology, 2nd ed.,* Wm.C. Brown Publishers, UK, pp. 459-462.

RYDER, M.H., YAN, Z., TERRACE, T.E., ROVIRA, A.D., TANG, W. & CORRELL, R.L. (1999) The use of strains of *Bacillus* isolated in China to suppress take-all and *Rhizoctonia* root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. *Soil Biology and Biochemistry* **31**, 19-29.

RYTTER, J.L., LUKEZIC, F.L., CRAIG, R. & MOORMAN, G.W. (1989) Biological control of geranium rust by *Bacillus subtilis*. *Phytopathology* **79**, 367-370.

SID AHMED, A., PEREZ-SANCHEZ. C. & CANDELA, M.E. (1999) Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathology* **48**, 58-65.

SILO-SUH, L.A., LETHBRIDGE, B.J., RAFFEL, S.J., HE, H., CLARDY, J. & HANDELSMAN, J. (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Applied and Environmental Microbiology* **60**, 2023-2030.

SIVAN, A. & CHET, I. (1986) Biological control of *Fusarium* spp. in cotton, wheat, and muskmelon by *Trichoderma harzianum*. *Journal of Phytopathology* **116**, 39-47.

SKINNER, F.A. & CARR, J.G. (1976) *Microbiology in Agriculture, Fisheries and Food*. Academic Press, New York, USA, pp. 37-38.

SMITH, V.L., WILCOX, W.F. & HARMAN, G.E. (1990) Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology* **80**, 880-885.

SRINIVASAN, M., PETERSON, D.J. & HOLL, F.B. (1996) Influence of indoleacetic-acid-producing *Bacillus* isolates on the nodulation of *Phaseolus vulgaris* by *Rhizobium etli* under gnotobiotic conditions. *Canadian Journal of Microbiology* **42**, 1006-1014.

TRONSOMO, A. (1986) Use of *Trichoderma* spp. in biological control of necrotrophic pathogens, in *Microbiology of the Phyllosphere* (FOKKEMA, N.J. & VAN DEN HEUVELS, J., Eds.). Cambridge University Press, Cambridge, UK, pp. 348-362.

UMAERUS, V., UMAERUS, M., ERJEFALT, L. & NILSSON, B.A. (1983) Control of *Phytophthora* by host resistance: problems and progress, in *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology* (ERWIN, D.C., BARTNICKI-GARCIA, S. & TSAO, P.H., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 315-326.

UTKHEDE, R.S. & GAUNCE, A.P. (1983) Inhibition of *Phytophthora cactorum* by a bacterial antagonist. *Canadian Journal of Botany* **61**, 3343-3348.

UTKHEDE, R.S. (1984) Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Canadian Journal of Botany* **62**, 1032-1035.

UTKHEDE, R.S. (1992) Biological control of soil-borne pathogens of fruit trees and grapevines. *Canadian Journal of Pathology* **14**, 100-105.

WINDHAM, M.I., ELAD, Y. & BAKER, R. (1986) A mechanism for increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**, 518-521.

WISNIEWSKI, M., BILES, C., DROBY, S., MCLAUGHLIN, R., WILSON, C. & CHALUTZ, E. (1991) Mode of action of the postharvest biocontrol yeast, *Pichia guilliemnondii*. I. Characterization of attachment to *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* **39**, 245-258.

YEDIDIA, I., BENHAMOU, N. & CHET, I. (1999) Induction of defense response in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology* **65**, 1061-1070.

YEDIDIA, I., SRIVASTA, A.K., KANULNIK, Y. & CHET, I. (2001) Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil* **235**, 235-242.

CHAPTER 3

IN VIVO BIOLOGICAL CONTROL OF *PHYTOPHTHORA* SP. ON ROUGH LEMON (*CITRUS JAMBHIRINI* LUSH.) SEEDLINGS

ABSTRACT

Trichoderma and *Bacillus* isolates were evaluated in greenhouse trials for their ability to suppress *Phytophthora* root rot of rough lemon (*Citrus jambhirini* Lush.) seedlings in a sterile medium. Seedling height, root collar diameter, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and percentage survival were used as measures of biocontrol activity. *Trichoderma* and *Bacillus* isolates were grown on V8 agar and nutrient broth, respectively. [*Trichoderma* isolates were applied in drench form at 5 x 10^5 spores / ml, while the *Bacillus* isolates were drenched at 1 x 10^6 or 1 x 10^8 colony forming units (CFU) / ml]. Rough lemon seedlings were transplanted into Speedling® trays filled with sterile pine bark media to which the antagonist was applied two days prior to inoculation as a 4 mm diameter plug of *Phytophthora* Isolate 1 grown on V8 agar. Five isolates of *Trichoderma* (AA12, AA5, AA16, SY3F and Eco-T®) were highly effective in suppressing *Phytophthora* root rot, with AA12 providing the best control. Several *Bacillus* isolates also caused suppression of the pathogen but were not as effective as the *Trichoderma* isolates. This study clearly demonstrated the antagonistic potential of several isolates of *Trichoderma* and *Bacillus* against *Phytophthora* root rot of rough lemon seedlings under greenhouse conditions.

3.1 INTRODUCTION

Phytophthora root rot is a major problem in citrus nurseries and orchards worldwide (Timmer & Menge, 1988). The most common species are *P. parasitica* and *P. citrophthora* (R.E. Sm & E.H.- S m.) Leonian. *Phytophthora* spp. causing root rots have a major impact on plant growth, resulting in destructive yield reductions (Timmer & Menge, 1988). For example, *Phytophthora* infected Cleopatra mandarin trees showed approximately 35% reduction in dry root mass compared with healthy control trees (Roxburgh *et al.*, 1993) causing significant and unacceptable losses in terms of crop yield (Kotze, 1984; Hough, 1992; Themann & Werres,

1995; Graham & Menge, 1999). The control of *Phytophthora* root rot in citrus is thus of utmost importance. Biological control is considered an environmentally safe alternative control for this problem. Due to the high value of the citrus crop, relatively expensive or complex control measures such as biocontrol may also be economically feasible (Campbell, 1986).

The purpose of this investigation was to test the efficacy of various *Trichoderma* and *Bacillus* isolates in the biological control of *Phytophthora* root rots of citrus in planting mixes under greenhouse conditions.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of plant materials

Plant material preparation was carried out as previously described in Chapter Two, Section 2.2.2, where seedlings of similar height at the three leaf stage were transplanted into Speedling® 24s trays cut in half (12 cells). Temperature in the tunnel ranged from 15-30^{0}C and relative humidity, 60-80%. Trays were irrigated three times a day with overhead sprinkler for a period of six minutes, with soluble fertilizers (NPK: 3:1:3 (38) Ocean Agriculture).

3.2.2 Preparation of biocontrol agents and a *Phytophthora* isolate

All twenty three *Trichoderma* isolates in Chapter Two *in vitro* screening were used together with two promising *Bacillus* isolates in this study. The *Trichoderma* isolates were grown up on V8 agar. Seven days later, spores were washed from the agar surface using sterile distilled water and filtered through sterile gauze surgery cloth into a sterile Erlenmeyer flask. The spore concentrations of *Trichoderma* isolates were determined using a counting chamber viewed under a compound microscope and then adjusted to $5x10^5$ spores /ml. The two *Bacillus* isolates for this study were prepared as previously described in Chapter Two, Section 2.2.6. In this case, a dilution series was prepared from the resuspended (nutrient agar/*Bacillus* water) pellet to determine overall CFU's. This suspension was then adjusted $1x10^6$ colony forming units (CFU) / ml and $1x10^8$ CFU / ml, respectively for the use in study.

Phytophthora inoculation was done by placing a four mm diameter plug of *Phytophthora* 1, colonized V8 agar upside down at the base of each seedling. Treatments were then applied two days later at a volume of three ml / seedling (Table 3.2.1). The trial was done using a completely randomized block design each treatment with 12 seedlings being replicated three times. Positive control (inoculated with *Phytophthora parasitica*) and negative control (untreated control) were also included.

Table 3.2.1 Treatments used to assess *in vivo* activity of various selected biocontrol agents for the control of *Phytophthora* Isolate 1 on rough lemon (*Citrus jambhirini* Lush.) seedlings

Treatment	Biocontrol isolate	Biocontrol concentration
1	Negative control only	0
2	Positive control only	0
3	AA1	$5x10^5$ spores/ml
4	AA2	$5x10^5$ spores/ml
5	AA3	$5x10^5$ spores/ml
6	AA4	$5x10^5$ spores/ml
7	AA5	$5x10^5$ spores/ml
8	AA6	$5x10^5$ spores/ml
9	AA7	$5x10^5$ spores/ml
10	AA8	$5x10^5$ spores/ml
11	AA9	$5x10^5$ spores/ml
12	AA10	$5x10^5$ spores/ml
13	AA11	$5x10^5$ spores/ml
14	AA12	$5x10^5$ spores/ml
15	AA13	$5x10^5$ spores/ml
16	AA14	$5x10^5$ spores/ml
17	AA15	$5x10^5$ spores/ml
18	AA16	$5x10^5$ spores/ml
19	AA17	$5x10^5$ spores/ml
20	SY2F	$5x10^5$ spores/ml
21	SY3F	$5x10^5$ spores/ml
22	SYN6	$5x10^5$ spores/ml
23	SYN4	$5x10^5$ spores/ml
24	TN	$5x10^5$ spores/ml
25	Eco-T ®	$5x10^5$ spores/ml
26	B77	$1x10^6$ CFU/ml
27	B77	$1x10^8$ CFU/ml
28	B81	$1x10^6$ CFU/ml
29	B81	$1x10^8$ CFU/ml

CFU = colony forming units

Treatment evaluation

Four months after inoculation, seedlings were extracted from the trays and the roots were washed to remove the pine bark. The growth parameters, i.e., seedling height and root collar diameter were taken / seedling, while the other parameters, i.e., fresh shoot weight, dry shoot weight, fresh root weight and dry root weight were measured / replicated plot (12 seedlings). Roots and shoots of seedlings were then separated and dried at 80°C for 72 hrs before being weighed. Treatments that elicited growth parameters significantly better than the diseased control were attributed to biological control arising from the biological control from the *in vivo* trials.

The measures given in the table and graphs are the means values.

Statistical analysis

Data were analyzed statistically using one-way analysis of variance (ANOVA) and then the Least Significant Difference test (LSD) using Genstat statistical package (Steel & Torrie, 1980).

3.3 RESULTS

Table 3.3.1 *In vivo* evaluation of biological activity of biological control agents on the control of *Phytophthora* root rot of rough lemon seedlings

Treatment[a]	PH (cm)	PD (mm)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	% S
Uninoculated	14.7	3.5	43.54c[b]	14.91c	34.15e	9.83e	97.22
Inoculated	9.8	2.8	32.28a	10.14a	16.04a	5.30a	77.77
AA1	13.5	3.6	42.33b	15.05c	29.64d	8.70d	97.22
AA2	13.0	3.4	42.58b	13.67b	21.72b	5.93a	94.44
AA3	14.5	3.4	33.55a	11.69a	23.64b	6.77b	80.55
AA4	13.5	3.2	35.37a	11.44a	23.30b	5.86a	91.66
AA5	14.0	3.7	54.49e	18.39d	29.74d	8.52d	91.66
AA6	14.5	3.5	43.03c	16.07c	27.63d	7.43c	91.66
AA7	13.6	3.3	41.28b	14.03b	26.64c	6.91b	88.89
AA8	11.7	3.2	34.71a	11.84a	20.07b	5.88a	86.11
AA9	13.2	3.3	41.32b	13.35b	21.21b	5.81a	94.44
AA10	13.3	3.6	49.58d	16.63c	27.20c	7.72c	100.00
AA11	12.6	3.3	39.42b	12.77b	22.28b	6.23b	94.44
AA12	14.3	3.7	50.84d	17.88d	31.85e	9.14d	97.22
AA13	13.6	3.5	37.06a	12.33a	21.00b	6.11b	88.89
AA14	13.7	3.4	46.44c	15.39c	27.07c	7.03b	94.44
AA15	12.3	3.0	35.38a	11.50a	17.23a	4.83a	94.44
AA16	13.9	3.9	49.72d	17.34d	29.91d	8.40d	100.00
AA17	12.6	3.2	40.18b	13.73b	22.89b	6.33b	94.44
ECO-T®	14.3	3.7	46.41c	16.81c	29.48d	7.91c	88.89
SY2F	13.5	3.3	40.60b	12.68b	21.60b	5.19a	88.89
SY3F	14.4	3.7	53.77e	17.56d	31.69e	8.16d	94.44
SYN4	13.7	3.3	40.86b	12.60b	21.72b	5.91a	91.67
SYN6	14.4	3.6	42.97c	14.55b	25.67c	7.24c	88.89
TN	13.1	3.2	37.62b	12.11a	22.70b	6.33b	86.11
B77 (a)	13.8	3.6	39.90b	13.58b	23.61b	6.98b	72.22
B77 (b)	14.2	3.3	41.04b	13.81b	23.48b	6.16b	94.44
B81 (a)	14.1	3.4	37.60b	14.38b	25.53c	6.82b	91.66
B81 (b)	12.4	3.8	38.86b	14.34b	25.07c	6.68b	75.00
LSD Value	2.63	0.56	5.267	2.344	3.819	1.103	19.406
P Value	NS	NS	<0.001***	<0.001***	<0.001***	<0.001***	NS
% CV	11.9	9.9	8.0	10.5	9.4	11.1	10.5

NB: PH, PD, SFW, SDW, RFW and RDW were taken four months after treatment. The wet biomass was weighed by separating the roots and shoots and the dry biomass was weighed after drying at 80^{O} C for 72 hrs.

[a] *Bacillus* isolates (B77 (a), B81 (a) and B77 (b), B81 (b) were drenched at 1 x 10^6 or 1 x 10^8 CFU / ml respectively). *Trichoderma* isolates were drenched at 5 x 10^5 spores / ml onto rough lemon seedling 24 hrs before a 4mm diameter *Phytophthora* plug was placed at the base of the seedling.

[b]Means within columns sharing the same letters are not significantly different (P<0.05) as determined by least significant difference (LSD). (a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml.

* = 0.05 Significant, ** = 0.01 highly significant, *** = 0.001 Very highly significant.

Abbreviation	Name
PH	- Plant Height (cm)
PD	- Plant Diameter (cm)
SFW	- Shoot Fresh Weight (g)
SDW	- Shoot Dry Weight (g)
RFW	- Root Fresh Weight (g)
RDW	- Root Dry Weight (g)
% S	- Percentage Survival

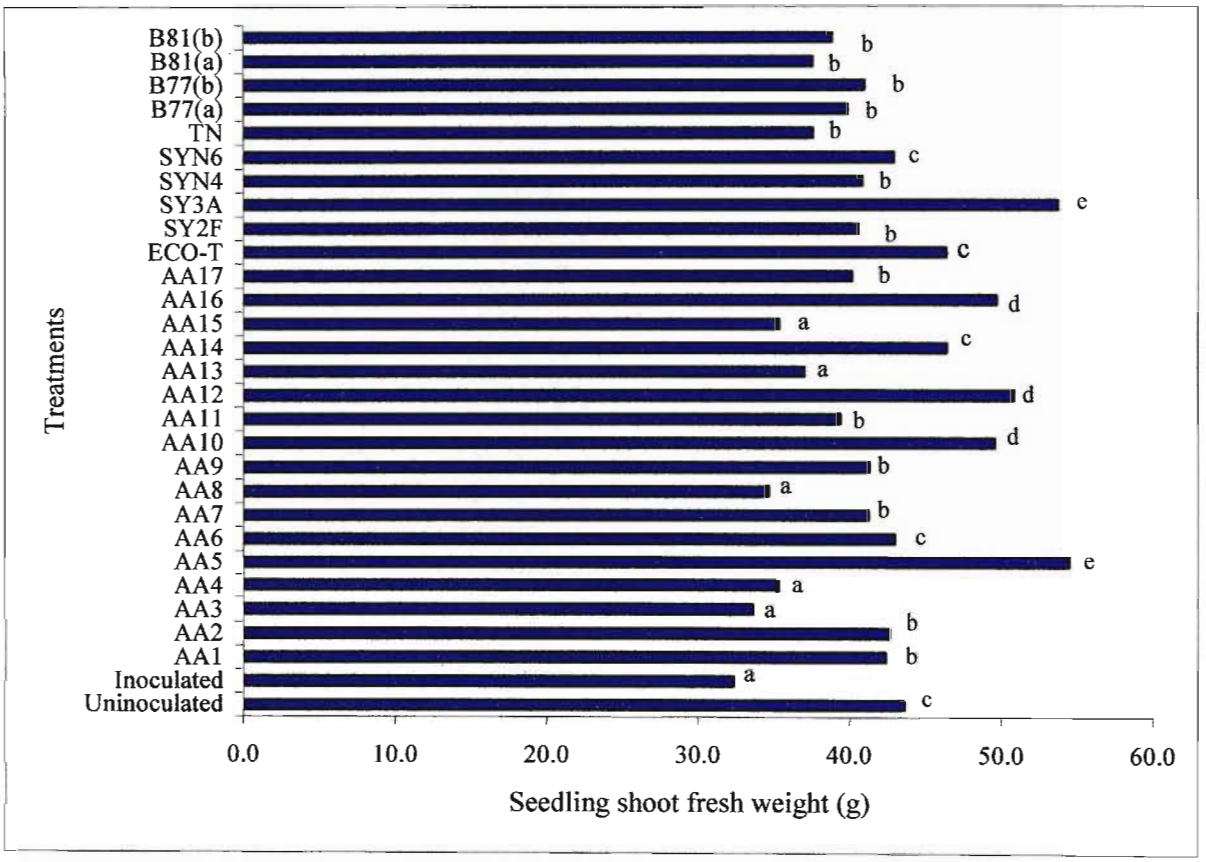

Figure 3.3.1 Response of seedling shoot fresh weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units.

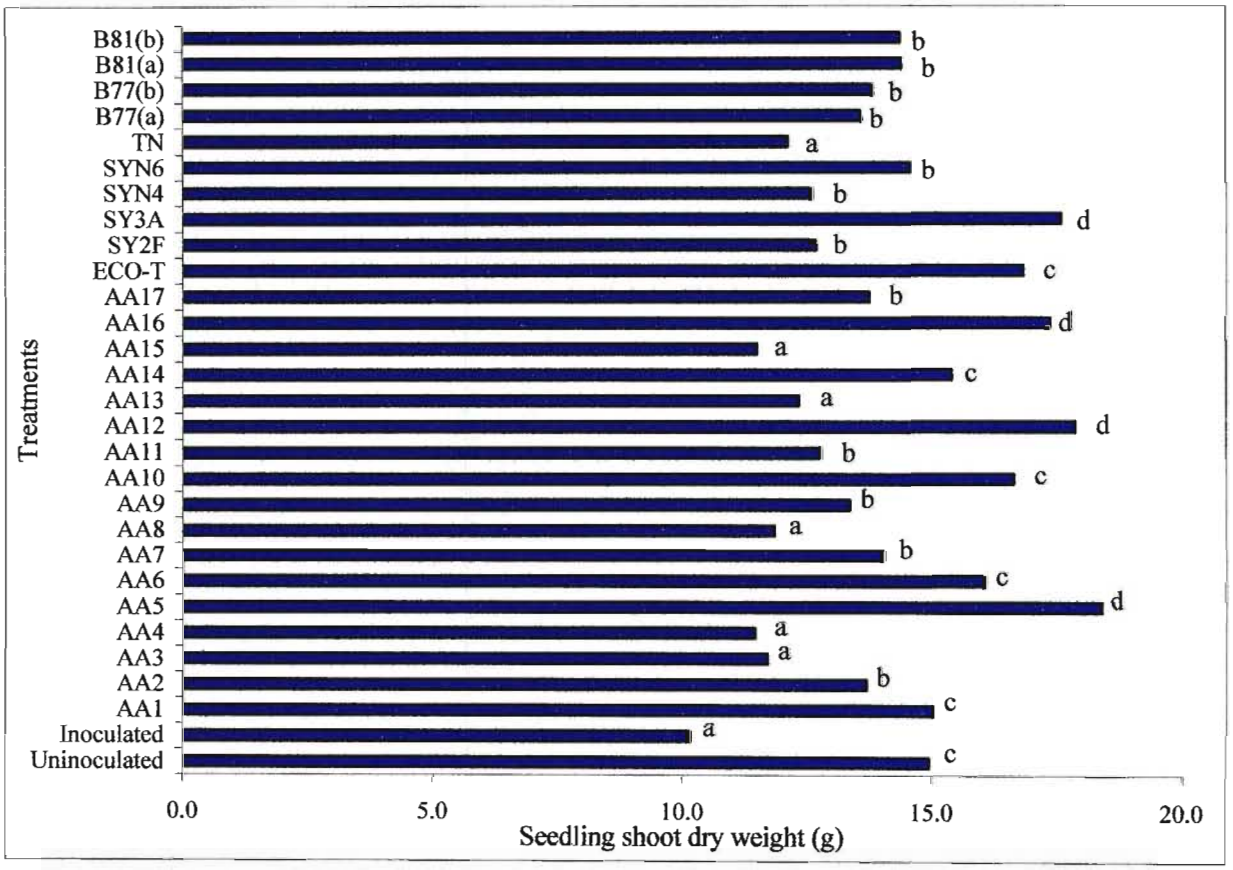

Figure 3.3.2 Response of seedling shoot dry weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units.

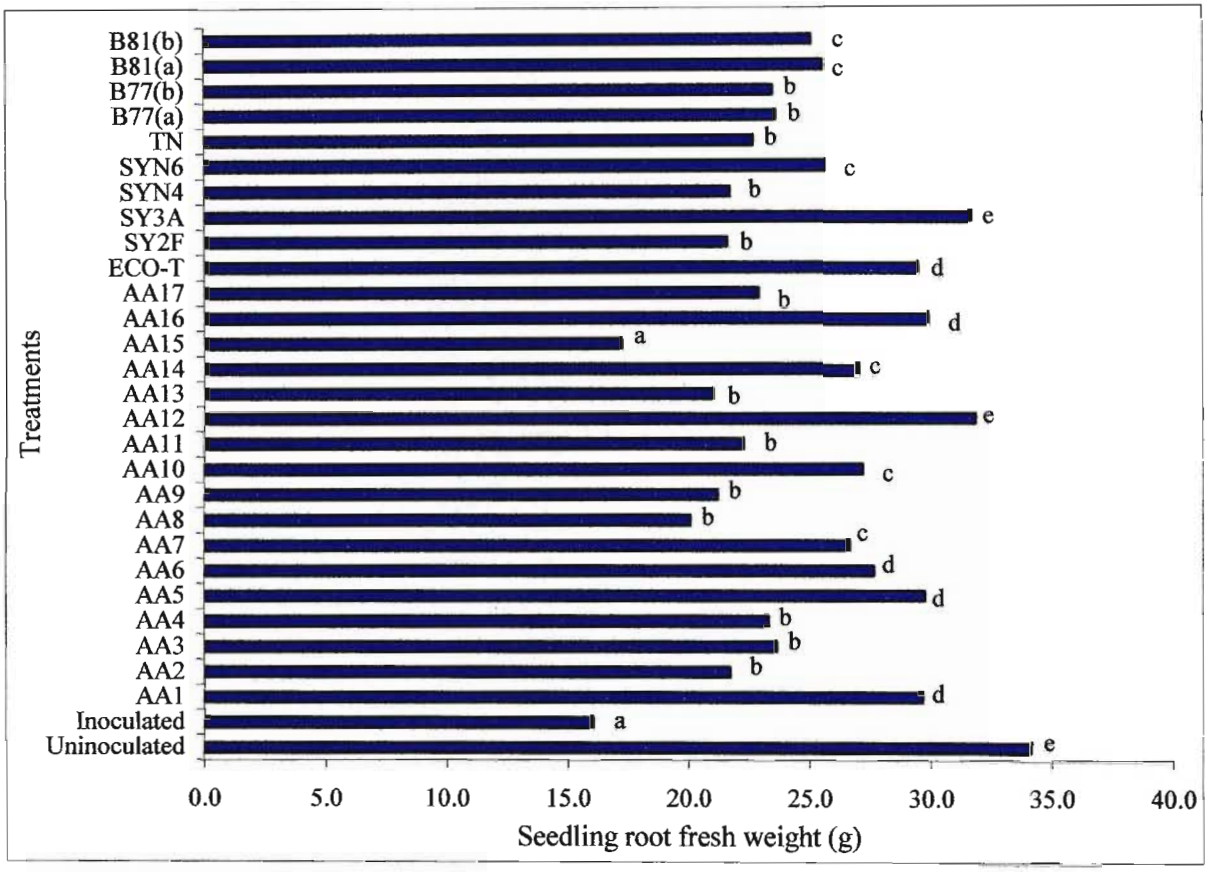

Figure 3.3.3 Response of seedling root fresh weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units.

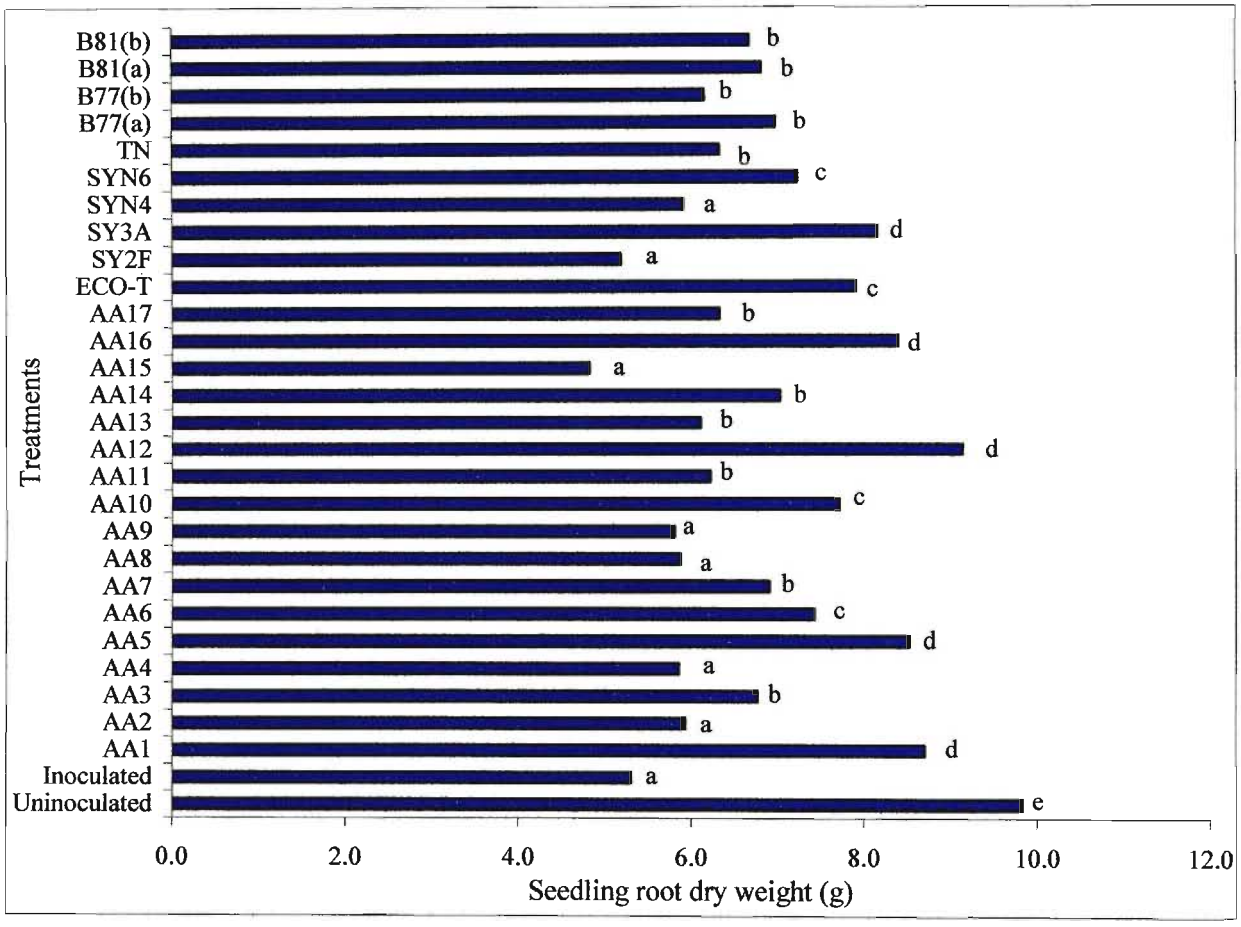

Figure 3.3.4 Response of seedling root dry weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units.

3.4 DISCUSSION

Little is known about the interaction between *Phytophthora* spp. and antagonistic fungi and bacteria.

Laboratory and greenhouse studies of the nature of pathogenic *Phytophthora* spp. in the presence of antagonistic fungi such as *Pythium nunn* (Lifsh., Stangh. & Baker) and *Penicillium funiculosum* (Thom.) (Fang & Tsao, 1995a, 1995b) or T. *harzianum* and *Gliocladium* spp. (Smith *et al.*, 1990) as well as *Bacillus* isolate (B8) (Utkhede & Gaunce, 1983), and *Bacillus subtilis* (Utkhede, 1984) have demonstrated the biocontrol capacity of these fungi and bacteria. Utkhede (1984) found that *B. subtilis* produced antibiotics antagonistic to *P. cactorum* (the causal agent of apple crown rot) *in vitro* as well as providing significant reductions of infection on seedlings under controlled environments. Smith *et al.* (1990) reported that seedlings treated with *Trichoderma* and *Gliocladium* spp. resulted in significant reductions in root damage and increase plant weight compared with seedlings exposed to *P. cactorum*, in the greenhouse trial.

Results from trials showed that the parameters of PH and PD did not reflect significant differences as a result of the different treatments. Hence, they were not useful in ascertaining success of biological control activity, whereas the other parameters were.

Responses of the parameter Seedling SFW showed that four *Trichoderma* isolates (AA6, AA14, SY2F and SYN6) were not significantly different to the uninoculated control. Five *Trichoderma* isolates (AA3, AA4, AA8, AA13, and AA15) were not significantly different to the inoculated control. Three *Trichoderma* isolates (AA10, AA12 and AA16) were significantly better than the uninoculated control. This could have been due to growth stimulatory effects of the biocontrol agents. Such activity has been documented to promote growth of vegetable crops (Paulitz *et al.*, 1986; Windham *et al.*, 1986; Inbar *et al.*, 1994; Yedidia *et al.*, 2001). *Trichoderma* Isolates SY3F and AA5 performed the best according to this parameter. *Bacillus* isolates (at both dosages) were significantly better than the inoculated control but not as good as the uninoculated control or the nine *Trichoderma* isolates.

Results from the parameter Seedling SDW showed that five *Trichoderma* isolates (AA1, AA6, AA10, AA14 and Eco-T) were not significantly different to the uninoculated control. Six *Trichoderma* isolates (AA3, AA4, AA8, AA13, AA15, and TN) were not significantly different to the inoculated control. Four *Trichoderma* isolates (AA5, AA12 and SY3F) performed significantly better than the uninoculated control, possibly due to plant growth stimulation. *Bacillus* isolates (at both dosages) were significantly better than the inoculated control but not as good as the uninoculated control or the nine *Trichoderma* isolates. *Trichoderma* isolates AA5 and SY3F were consistently significantly better than the uninoculated control and the *Trichoderma* Isolates AA3, AA4, AA13 and AA15 were not consistently significantly different to the inoculated control when the SFW and SDW parameters were measured.

Results from the parameter Seedling RFW showed that two *Trichoderma* Isolates (AA12 and SY3F) were not significantly different to the uninoculated control. One *Trichoderma* isolate (AA15) was not significantly different to the inoculated control. Four *Trichoderma* isolates (AA5, AA12 and SY3F) were significantly better than the uninoculated control, possibly of plant growth promotion. *Bacillus* Isolate B81 (at both dosages) was better than B77 (at both dosages) and was good as *Trichoderma* isolates (AA7, AA10, AA14 and SYN6). *Trichoderma* Isolate AA5 was significantly worse than the uninoculated control. *Trichoderma* Isolates AA5 and SY3F were consistently significantly better than the inoculated control and the *Trichoderma* Isolate AA15 was not consistently significantly different to the inoculated control when the SFW, SDW and RFW parameters were measured. The SFW, SDW and RFW of the measured parameters showed some consistency in determining the best performing isolates.

Response to the parameter Seedling RDW showed that all the *Trichoderma* isolates and *Bacillus* isolates (at both dosages) performed worse than the uninoculated control. The responses to *Trichoderma* isolates (AA1, AA5, AA12, AA16 and SY3F) were highly significant compared to the inoculated control. Seven *Trichoderma* isolates (AA2, AA4, AA8, AA9, AA15, SY2F and SYN4) were not significantly different to the inoculated control. *Bacillus* isolates (at both dosages) were significantly better than the inoculated control but not

as good as the uninoculated control or the seven *Trichoderma* isolates. *Trichoderma* Isolates AA5 and SY3F were consistently better than inoculated control. The *Trichoderma* Isolate AA15 was not significantly different to the inoculated control when the SFW and RFW as well as SDW and RDW parameters. These parameters show some consistency in determining the best or worst performing isolates.

Variation in effectiveness of different isolates of biological control agents has been previously documented (Henis *et al.*, 1984). The first possible reason for the difference in the effectiveness of biological agents in suppressing *Phytophthora* root rot is the ability of introduced antagonists to establish and proliferate in the soil or growing substrates (Lewis & Papavizas, 1984). A second possible reason may be the ability of these isolates to act as mycoparasites/hyperparasites as indicated in the Bell test in Chapter Two. A third possibility may be the ability of isolates to make use of numerous mechanisms of biocontrol.

The *Trichoderma* isolates (AA12, AA5, AA16, SY3F and Eco-T) that resulted suppression of *Phytophthora parasitica in vivo* having Bell ratings between 1 and 3 as in Chapter Two; Figure 2.3.1, showed correlation with the *in vitro* test. Conversely, some of the *Trichoderma* isolates (AA3, AA4, AA11, AA13, AA15, AA17 and TN), with high *in vitro* antagonistic activity (ratings of 1 and 2) could not suppress *Phytophthora* root rot and did not correlate with the *in vivo* results, based on growth parameters. This could have been due to weak rhizosphere establishment and proliferation. Therefore, to avoid discarding some potential biological control isolates, a rapid root colonization bioassay as used by Silva *et al.* (2003) could prove useful.

Two *Bacillus* isolates were selected based on their *in vitro* microbial activity against *Phytophthora* root rot of citrus. Based on the results of parameters measured, it appeared that the two *Bacillus* isolates caused effective suppression of *Phytophthora* root rot. There was no significant difference between the two doses applied ($1x10^6$ and $1x10^8$ colony forming units (CFU) / ml). The two *Bacillus* isolates performed in the greenhouse as expected from the *in vitro* results. This indicates a correlation between *in vitro* and *in vivo* tests. Authors such as Leifert *et al.* (1995), Knudsen *et al.* (1997), Melissa *et al.* (2001) and Walker *et al.* (2002)

showed that such correlation does not always exist. In most of the parameters measured (SFW, RFW, SDW and RDW) *Bacillus* isolates were less effective than *Trichoderma* isolates.

This study established the antagonistic potential of *Trichoderma* and *Bacillus* agents against *Phytophthora* root rot of citrus seedlings under greenhouse conditions, as well as giving an indication of growth stimulation activity. These results indicate that biocontrol could be a viable option as an alternative control strategy in controlling this important citrus disease and would be useful for nurserymen propagating citrus rootstocks under greenhouse conditions to combat the pathogen.

3.5 REFERENCES

CAMPBELL, R. (1986) The search for biological control agents against plant pathogen: A pragmatic approach. *Biological Agriculture and Horticulture* **3**, 317-327.

FANG, J.G. & TSAO, P.H. (1995a) Efficacy of *Penicillium funiculosum* as a biological control agent against *Phytophthora* root rots of azalea and citrus. *Phytopathology* **85**, 871-878.

FANG, J.G. & TSAO, P.H. (1995b) Evaluation of *Pythium nunn* as a potential biocontrol agent against *Phytophthora* root rots of azalea and sweet orange. *Phytopathology* **85**, 29-36.

GRAHAM, J.H. & MENGE, J.A. (1999) Root diseases, in *Citrus Health Management* (TIMMER, L.W. & DUNCAN, L.W. Eds.). APS Press, St. Paul, Minnesota, USA, pp. 126-134.

HENIS, Y., LEWIS, J.A. & PAPAVIZAS, G.S. (1984) Interactions between *Sclerotium rolfsii* and *Trichoderma* spp.: relationship between antagonism and disease control. *Soil Biology and Biochemistry* **16**, 391-395.

HOUGH, A. (1992) Citrus rootstock reaction to *Phytophthora* root rot. *Citrus Journal* **2**, 40-43.

INBAR, J., ABRAMSKY, M., COHEN, D. & CHET, I. (1994) Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *European Journal of Plant Pathology* **100**, 337-346.

KNUDSEN, I.M.B., HOCKENHULL, J., JENSEN, D.F., GERHARDSON, B., HOKEBERG, M., TAHVONEN, R., TEPERI, E., SUNDHEIM, L. & HENRIKSEN, B. (1997) Selection of biological control agents for controlling soil and seed borne diseases in the field. *European Journal of Plant Pathology* **103**, 775-784.

KOTZE, J.M. (1984) Root rot in citrus. *The Citrus and Subtropical Fruit Journal* **604**, 18-21.

LEIFERT, C., LI, H., CHIDBUREE, S., HAMPSON, S., WORKMAN, S., SIGEE, D., EPTON, H.A.S. & HARBOUR, A. (1995) Antibiotic production and biocontrol activity of *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. *Journal of Applied Bacteriology* **78**, 97-108.

LEWIS, J.A. & PAPAVIZAS, G.C. (1984) A new approach to stimulate population proliferation of *Trichoderma* spp. and other potential biocontrol fungi introduced into natural soils. *Phytopathology* **74**, 1240-1244.

MELISSA, J.B., DE LEIJ, F.A. & LYNCH, J.M. (2001) The effect of root exudates on rhizosphere microbial populations, in *The Rhizospher: Biochemistry and Organic Substances at Soil-Plant Interface*. Marcel Dekker, Inc., New York, USA, pp. 95-126.

PAULITZ, Y., WINDHAM, M. & BAKER, R. (1986) Effects of peat vermiculite mixes containing *Trichoderma harzianum* on increased growth response of radish. *Journal of the American Society of Horticultural Science* **111**, 810-814.

ROXBURGH, C.K., BESTER, J.J., LE ROUX, H.F. & VAN DER WALT, H.P. (1993) Guidelines for the control of *Phytophthora* in Southern African Nurseries (RABE, E., Ed.). *Proceedings of fourth World Congress of the International Society of Citrus Nurserymen*, South Africa, pp. 203-208.

SILVA, H.S.A., ROMEIRO, R.D.S. & MOUNTEER, A. (2003) Development of a root colonization bioassay for rapid screening of rhizosphere for potential biocontrol agents. *Journal of Phytopathology* **151**, 42-46.

SMITH, V.L., WILCOX, W.F. & HARMAN, G.E. (1990) Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology* **80**, 880-885.

STEEL, R.G.D. & TORRIE, J.H. (1980) *Principles and procedures of statistics*: a biometrical approach, 2nd ed., McGraw-Hill, inc., New York, USA.

THEMANN, K. & WERRES, S. (1995) Experience with diagnostic techniques to detect *Phytophthora* species in water, in *Federal Biological Center for Agriculture and Forestry, Institute for Plant Protection in Horticulture*, Braunschweig, Germany, pp. 83-87.

TIMMER, L.W. & MENGE, J.A. (1988) *Phytophthora*-induced diseases, in *Compendium of Citrus Diseases* (WHITESIDE, J., GARNSEY, S.M. AND TIMMER, L.W., Eds.). APS Press, St. Paul, Minnesota, USA, pp. 22-24.

UTKHEDE, R.S. & GAUNCE, A.P. (1983) Inhibition of *Phytophthora cactorum* by a bacterial antagonist. *Canadian Journal of Botany* **61**, 3343-3348.

UTKHEDE, R.S. (1984) Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Canadian Journal of Botany* **62**, 1032-1035.

WALKER, R., ROSSALL, S. & ASHER, M. (2002) Colonization of the developing rhizosphere of sugar beet seedlings by potential biocontrol agents applied as seed treatments. *Journal of Applied Microbiology* **92**, 228-237.

WINDHAM, M.T., ELAD, Y. & BAKER, R. (1986) A mechanism of increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**, 518-521.

YEDIDIA, I., SRIVASTA, A.K., KANULNIK, Y. & CHET, I. (2001) Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil* **235**, 235-242.

CHAPTER 4

EFFECTS OF BIOCONTROL ISOLATES OF *TRICHODERMA* AND *BACILLUS* ON PLANT GROWTH

ABSTRACT

Trichoderma and *Bacillus* isolates were evaluated in greenhouse trials for their ability to enhance the growth of rough lemon (*Citrus jambhirini* Lush.) seedlings in sterile pine bark medium. The seedlings were grown in composted pine bark media and transplanted into new Speedling® trays at the three leaf stage for treatment. *Trichoderma* and *Bacillus* isolates were grown on V8 agar and nutrient broth, respectively. *Trichoderma* isolates were applied in drench form at 5 x 10^5 spores / ml, while the *Bacillus* isolates were drenched at 1 x 10^6 or 1 x 10^8 colony forming units (CFU) / ml. Four months later, seedling height, root collar diameter, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight were measured as indicators of growth stimulation activity.

None of the isolates screened exhibited as growth stimulatory response. Depending on the growth parameters measured treatments were shown to either stunt or inhibit growth or have no significant effect on the growth promotion compared to the control. Therefore, considering effects of population density of the biocontrol agents during growth stimulation evaluation is important.

4.1 INTRODUCTION

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by root pathogens (Podile & Prakash, 1996). *Trichoderma* spp. have been identified as antagonists of other fungi (Papavizas, 1985) and have been shown to be effective biocontrol agents of several soil-borne plant pathogenic fungi under both greenhouse (Chet, 1987; Yedidia *et al.*, 2001) and field conditions (Sivan *et al.*, 1987; Sivan & Chet, 1993). Furthermore, in some cases the application of *Trichoderma* to pathogen-free soils has resulted in increased plant growth.

Responses to application of *Trichoderma* spp. are characterized by increased germination percentage, plant height and dry weight and a shorter germination time in vegetables. *Trichoderma*-induced increased growth has been reported for several plant species, including bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), pepper (*Capsicum annun* L.), periwinkle, (*Vinca minor*), and petunia (*Petunia hybrida*) (Baker *et al.*, 1984; Chang *et al.*, 1986; Paulitz *et al.*, 1986; Windham *et al.*, 1986; Baker, 1989; Kleifeld & Chet, 1992; Inbar *et al.*, 1994; Yedidia *et al.*, 2001).

Bacillus spp. have also been shown to have potential for biocontrol (Dunleavy, 1955; Utkhede & Gaunce, 1983; Utkhede, 1984; Capper & Campbell, 1986; Podile & Prakash, 1996). *Bacillus* spp. have been used for many years in an attempt to control plant pathogens and increase plant growth (Turner & Backman, 1991; Holl & Chanway, 1992; Manero *et al.*, 1996; Kim *et al.*, 1997).

Plant growth enhancement by bacteria and fungi (Brown, 1974a) may involve: control of minor pathogens (Kloepper & Schroth, 1981; Suslow & Schroth, 1982; Elad *et al.*, 1987), production of plant hormones Chang *et al.*, 1986; Windham *et al.*, 1986), production of vitamins or the conversion of nonutilizable elements to forms useful to growth of the plant (Brown, 1974b; Barber & Lynch, 1977; Baker, 1989), minor nutrient element release from soil or organic matter (Brown, 1974b; Barber & Lynch, 1977; Altomare *et al.*, 1999) and increased uptake and translocation of minerals useful to the plant (Brown, 1974b; Okon & Kapulnik, 1986).

On the basis of the above literature and indications from the results of Chapter Three, it deemed appropriate to investigate whether some of the *Trichoderma* and *Bacillus* isolates identified in this research would have a direct effect on growth of citrus seedlings when no disease pressure was present.

4.2 MATERIALS AND METHODS

Trials were set-up according to the materials and methods described in Chapter Three except that the seedlings were not inoculated with any pathogen.

4.3 RESULTS

Table 4.3.1 *In vivo* evaluation of biocontrol isolates of *Trichoderma* and *Bacillus* on growth of rough lemon seedlings

Treatment[a]	PH (cm)	PD (mm)	SFW (g)	SDW (g)	RFW (g)	RDW (g)
Control	13.07	4.0 b[b]	13.28 b	5.27 c	5.47 c	2.91 b
AA1	10.37	3.4 a	8.55 a	3.44 a	4.34 b	2.12 a
AA2	13.20	3.7 a	10.34 a	3.36 a	3.82 a	2.79 b
AA3	12.13	3.4 a	8.24 a	3.22 a	4.11 a	2.22 a
AA4	10.57	3.3 a	7.38 a	4.06 b	3.64 a	2.09 a
AA5	11.93	3.6 a	10.08 a	4.34 b	3.44 a	2.82 b
AA6	12.87	3.7 a	10.94 b	4.34 b	4.44 b	2.75 b
AA7	10.70	3.4 a	10.89 b	4.16 b	4.02 a	2.04 a
AA8	13.80	4.0 b	11.99 b	5.18 c	4.70 b	2.58 a
AA9	11.77	3.5 a	9.37 a	3.65 a	3.83 a	2.00 a
AA10	13.37	3.7 a	11.81 b	4.80 b	4.87 b	2.51 a
AA11	11.20	3.3 a	7.97 a	3.37 a	3.40 a	2.01 a
AA12	11.87	3.7 a	10.64 a	4.13 b	4.36 b	2.80 b
AA13	13.83	3.9 a	13.21 b	5.36 c	6.21 c	2.98 b
AA14	9.40	3.9 a	9.21 a	3.70 a	5.38 c	2.43 a
AA15	14.40	4.3 b	10.93 b	4.52 b	3.56 a	2.17 a
AA16	12.27	3.4 a	9.55 a	3.69 a	5.07 b	2.58 a
AA17	12.50	4.0 b	13.64 b	5.51 c	5.57 c	3.01 b
ECO-T®	13.10	4.0 b	12.36 b	4.83 b	5.49 c	2.81 b
SYN4	10.07	3.6 a	7.97 a	2.94 a	3.86 a	2.06 a
SY3F	11.80	3.6 a	11.78 b	4.46 b	4.58 b	2.44 a
TN	10.67	3.5 a	8.88 a	2.76 a	4.10 a	2.31 a
SY2F	12.87	3.7 a	11.51 b	3.00 a	4.24 b	2.33 a
SYN6	9.70	3.7 a	7.49 a	3.40 a	3.19 a	2.29 a
B77(a)	12.70	3.5 a	10.19 a	4.33 b	4.39 b	2.83 b
B77(b)	12.83	3.8 a	11.92 b	4.86 b	5.44 c	2.88 b
B81(a)	13.80	4.4 b	10.67 a	3.89 a	4.67 b	2.87 b
B81(b)	11.67	3.8 a	12.25 b	4.89 b	4.94 b	2.64 a
LSD	3.156	0.61	3.365	1.151	1.028	0.680
P value	NS	0.008**	0.004**	<0.001***	<0.001***	0.019*
CV%	16.0	10.0	19.6	17.1	13.8	16.6

Abbreviation	Name
PH	Plant Height (cm)
PD	Plant Diameter (cm)
SFW	Shoot Fresh Weight (g)
SDW	Shoot Dry Weight (g)
RFW	Root Fresh Weight (g)
RDW	Root Dry Weight (g)

NB: PH, PD, SFW, SDW, RFW and RDW were taken four months after treatment. The wet biomass was weighed by separating the roots and shoots and the dry biomass was weighed after drying at 80^0C for 72 hrs.

[a] *Bacillus* isolates (B77(a), B 81(a), B 77(b) and B 81(b) drenched at 1 x 10^6 or 1 x 10^8 CFU / ml or the rest *Trichoderma* isolates drenched at 5 x 10^5 spores / ml onto rough lemon seedlings.

[b] Means within columns sharing the same letters are not significantly different (P<0.05) as determined by least significant difference (LSD)

* = Significant, ** = highly significant, *** = very highly significant

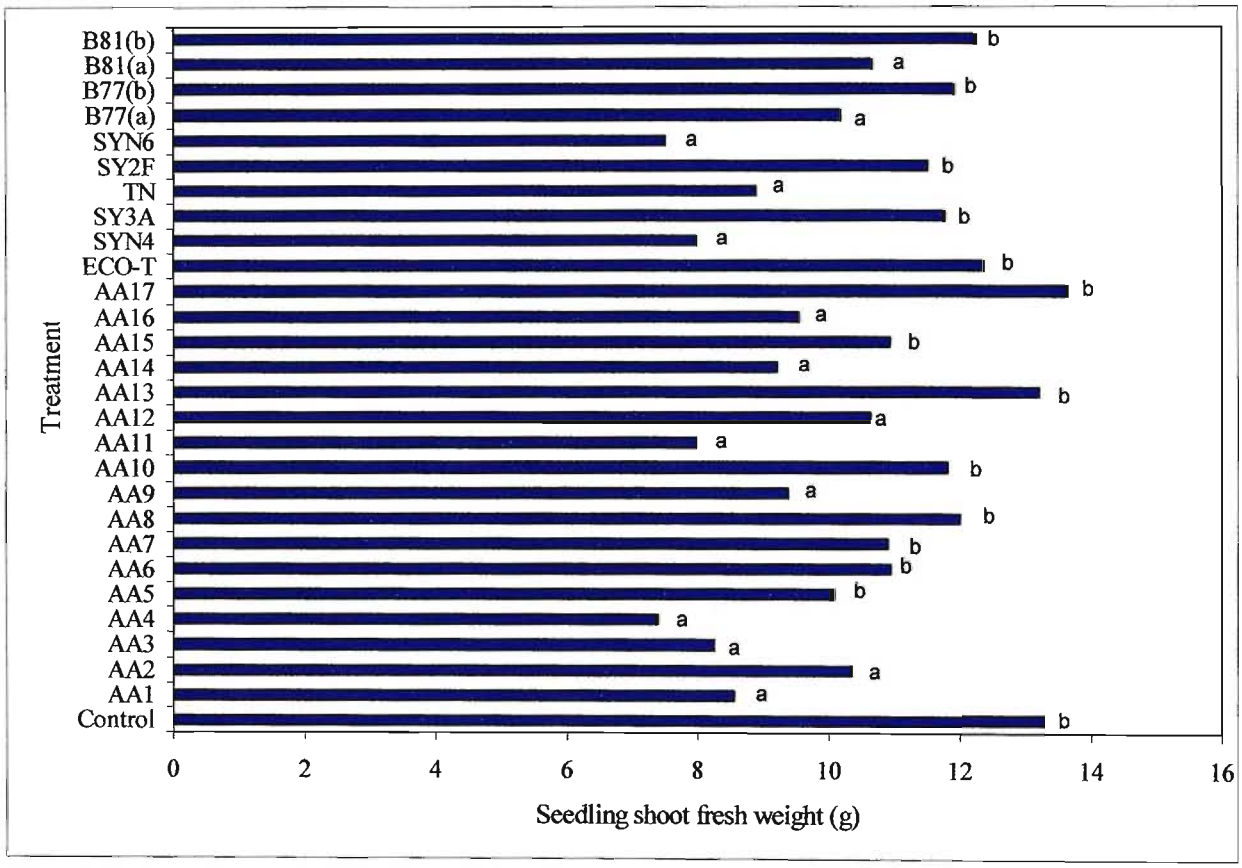

Figure 4.3.1 Response of seedling shoot fresh weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching, in the absence of any pathogen ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units

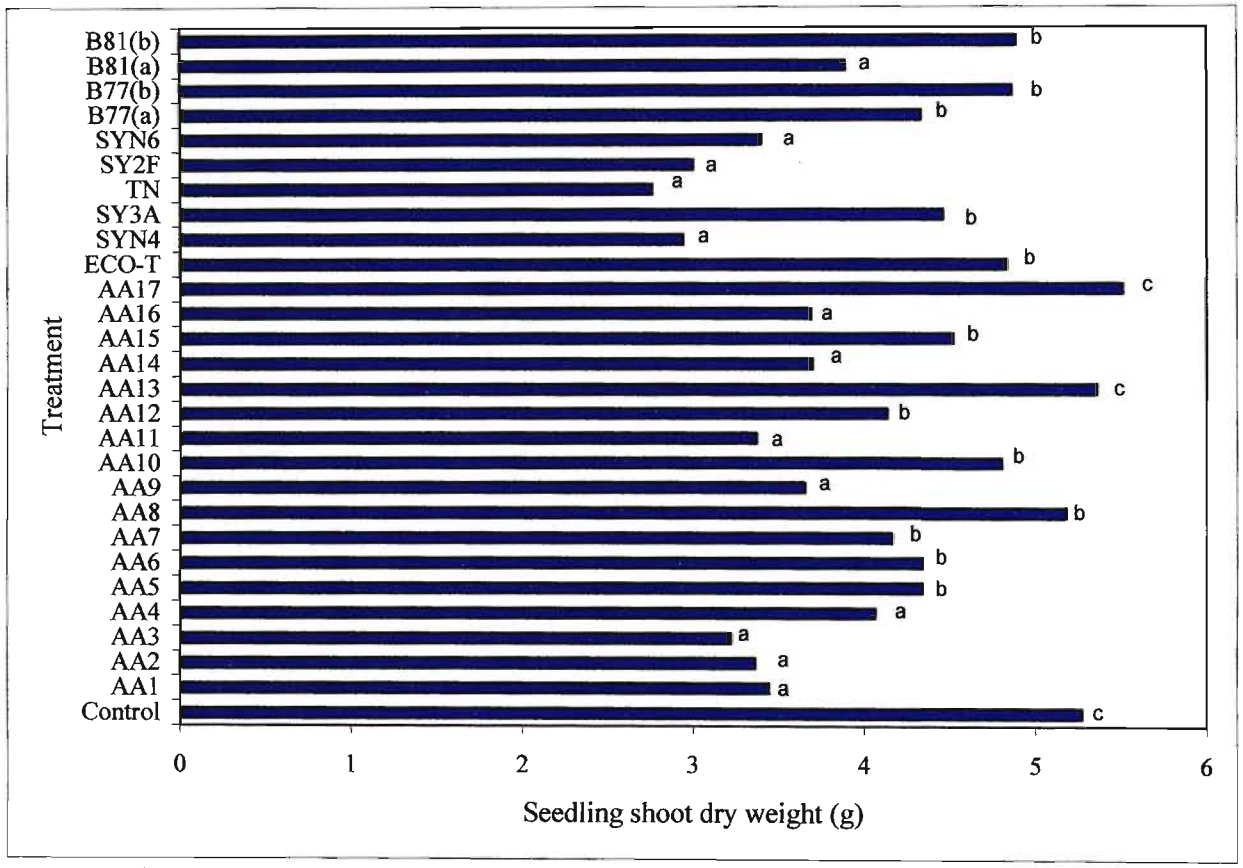

Figure 4.3.2 Response of seedling shoot dry weight (g) of rough lemon seedling to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching, in the absence of any pathogen ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units

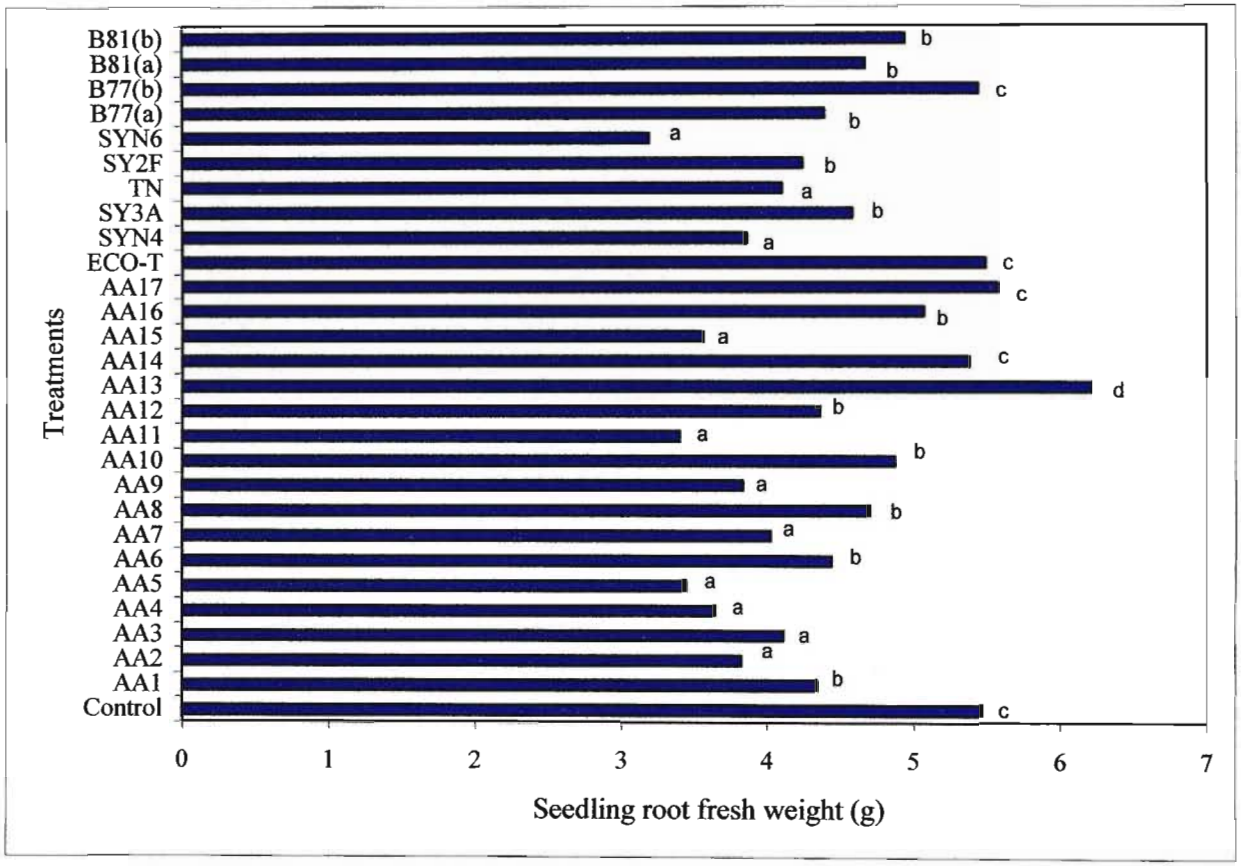

Figure 4.3.3 Response of seedling root fresh weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching, in the absence of any pathogen ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units

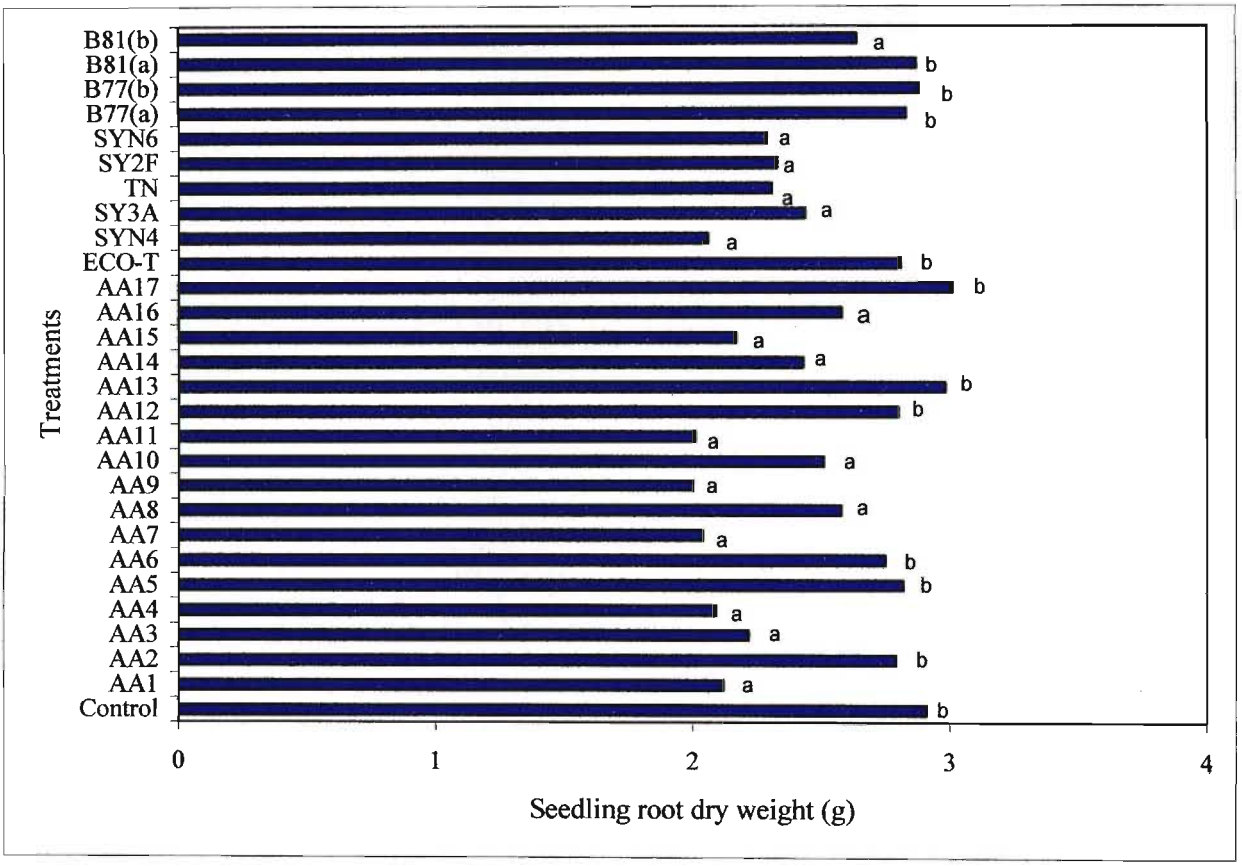

Figure 4.3.4 Response of seedling root dry weight (g) of rough lemon seedlings to treatment with *Trichoderma* or *Bacillus* isolates, applied by drenching, in the absence of any pathogen ($P < 0.05$). ((a) = 1 x 10^6 CFU / ml; (b) = 1 x 10^8 CFU / ml). CFU = colony forming units

4.4 DISCUSSION

Trichoderma spp. are known as biocontrol agents of soil-borne plant pathogenic fungi (Chet, 1987; Chet, 1990; Harman & Lumsden, 1990; Lewis & Lumsden, 2001). The increased growth response of several plants such as lettuce, radish, bean, cucumber, pepper and periwinkle under greenhouse and field conditions following application of *Trichoderma* spp. to a pathogen-free soil has also been documented (Chang *et al.*, 1986; Baker, 1989; Kleifeld & Chet, 1992; Inbar *et al.*, 1994; Ousley *et al.*, 1994; Harman, 2000; Yedidia *et al.*, 2001). *Trichoderma* application increased radish dry weight after six weeks by 150% - > 250% (Baker, 1988). Windham *et al.* (1986) reported increased root and shoot dry weights of tomatoes and tobacco seedlings in the order of 213-275% and 259-318% respectively, over the controls.

The ability of *Bacillus* spp. to reduce disease caused by soil-borneplant pathogenic fungi is well known (Utkhede & Gaunce, 1983; Utkhede, 1984; Turner & Backman, 1991; Holl & Chanway, 1992; Kim *et al.*, 1997; Utkhede *et al.*, 1999; Guetsky *et al.*, 2002). A *Bacillus subtilis* strain (BACTO-0) increased shoot growth by 9% and weight of cucumber plants by 29% compared with the *Phytophthora aphanidermatum* inoculated controls. The same strain increased fruit yield by 14% and fruit number by 50% in cucumber plants compared with the *P. aphanidermatum* inoculated controls (Utkhede *et al.*, 1999). *Bacillus subtilis* Strain A13, was found to be inhibitory *in vivo* to several plant pathogens as well as showed improvement in growth of many plant species in steamed and natural soils (Broadbent *et al.*, 1977; Yuen *et al.*, 1985). As a seed treatment, this strain increased the yield of carrots by 48%, and oats by 33% (Merriman *et al.*, 1974). It also appeared to improve plant growth both indirectly by suppressing pathogens and possibly also by more direct effects (Broadbent *et al.*, 1977).

Results from trial showed that the parameter of PH did not reflect significant difference for different treatments. Hence, it was not useful in ascertaining success of biological control activity, whereas the other parameters were.

Results from the parameter Seedling PD showed that four *Trichoderma* isolates (AA8, AA15, AA17 and Eco-T) and *Bacillus* B81 (a) were not significantly different to the control.

However, *Bacillus* B 81(a) performed significantly better than *Bacillus* B 81(b) and *Bacillus* B77(a) and B77(b).

Response of the parameter seedling SFW showed that 13 *Trichoderma* isolates (AA5, AA6, AA7, AA8, AA10, AA12, AA13, AA14, AA15, AA17, Eco-T, SYN4, and SY2F) and *Bacillus* Isolates B81(b) and B77(b) were not significantly different to the control. The rest of the isolates performed significantly poorer than the control

Results of the parameter Seedling SDW showed that two *Trichoderma* Isolates (AA13 and AA17) were not significantly different to the control. The rest of the *Trichoderma* isolates performed significantly worse than the control. *Bacillus* isolates (at both dosages) were significantly better than control. *Bacillus* Isolates B81(a) at lower concentration was significantly poorer than the B77(a) and (b), B81(a).

Response to the parameter Seedling RFW showed that one *Trichoderma* Isolate AA13 was significantly better than the control. Three *Trichoderma* isolates (AA14, AA17 and Eco-T) were not significantly different to the control. *Bacillus* Isolates B77(b) was significantly better than B77(a), B81(a) and B81(b), but not as good as *Trichoderma* Isolate AA13.

Results from the parameter Seedling RDW showed that 10 isolates (AA2, AA5, AA6, AA12, AA13, AA17, Eco-T, SYN4, B77(a), B77(b) and B81(b)) were not significantly better than the control.

In this study, *Trichoderma* Isolate AA13 showed growth promotion in the parameter Seedling RFW but was not confirmed in the RDW and it may have been due to moisture content. *Trichoderma* Isolate AA17 caused no effect as a growth promoter on rough lemon seedling growth based on all parameters except for the plant height. Other isolates of *Trichoderma* had negative effects on the growth of these seedlings in all parameters measured except for the plant height compared to the Control (Table 4.3.1). A similar finding was reported by Lynch *et al.* (1991) on lettuce when applying *Trichoderma*. This response could be due to influences associated with the population density of the biocontrol agents or host factors of the rough

lemon seedlings (Baker, 1988). Alternatively, growth promotion may be masked due to optimum crop husbandry being used and may only be apparent under stressed growth conditions such as low soil moisture or nutrient poor conditions.

Several *Trichoderma* isolates (AA12, AA5, AA16, SY3F and Eco-T) caused suppression of *Phytophthora parasitica in vivo* based on the all parameters measured except the PH and PD (Table 3.3.1). All caused stunting or no significant effect on the seedlings in absence of any pathogen. Baker (1988) found that peat-bran formulation providing 10^8 colony forming units (CFU) g^{-1} soil induced stunting of eggplants. Such negative activity was also reported by McFadden & Sutton (1975). Similarly high population densities of *Trichoderma* spp. in the field inhibited germination of maize (Baker, 1988) and the possible reason for this may be nutrient competition or release of microbial compounds that inhibit germination. Conversely, some *Trichoderma* isolates that showed no effect on the growth of seedlings in the absence of any pathogen did not perform well as biocontrol agents. A possible reason may be that they are weaker competitors and therefore do not perform well in the presence of competing pathogens.

None of the isolates screened exhibited as growth stimulatory response. Depending on the growth parameters measured treatments were shown to either stunt or inhibit growth or have no significant effect on the growth promotion compared to the control. Therefore, considering effects of population density of the biocontrol agents during growth stimulation evaluation is important.

4.5 REFERENCES

ALTOMARE, C., NORVELL, W.A., BJORKMAN, T. & HARTMAN, G.E. (1999) Solubilization of phosphate and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Applied and Environmental Microbiology* **65**, 2929-2933.

BAKER, R. (1988) *Trichoderma* spp. as plant-growth stimulants. *CRC Critical Reviews in Biotechnology* **7**, 97-106.

BAKER, R. (1989) Improved *Trichoderma* spp. for promoting crop productivity. *Trends in Biotechnology* **7**, 34-38.

BAKER, R., ELAD, Y. & CHET, I. (1984) The controlled experiment in the scientific method with special emphasis on biological control. *Phytopathology* **74**, 1019-1021.

BARBER, D.A. & LYNCH, J.M. (1977) Microbial growth in the rhizosphere. *Soil Biology and Biochemistry* **9**, 305-308.

BROADBENT, P., BAKER, K.F., FRANKS, N. & HOLLAND, J. (1977) Effect of *Bacillus* spp. on increased growth of seedlings in steamed and in nontreated soil. *Phytopathology* **67**, 1027-1034.

BROWN, M.E. (1974a) Plant growth substances produced by microorganisms of soil and rhizosphere. *Journal of Applied Bacteriology* **35**, 443-451.

BROWN, M.E. (1974b) Seed and root bacterization. *Annual Review of Phytopathology* **12**, 181-197.

CAPPER, A.L. & CAMPBELL, R. (1986) The effect of artificially inoculated antagonistic bacteria on the prevalence of take-all disease of wheat in field experiments. *Journal of Applied Bacteriology* **60**, 155-160.

CHANG, Y.-C., CHANG, Y.-C., BAKER, R., KLEIFELD, O. & CHET, I. (1986) Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. *Plant Disease* **70**, 145-148.

CHET, I. (1987) *Trichoderma*–application, mode of action, potential as biocontrol agent of soil-borneplant pathogenic fungi, in *Innovative Approaches of Plant Disease Control* (CHET, I., Ed.). John Wiley and Sons, New York, USA, pp. 137-160.

CHET, I. (1990) Biological control of soil-bornepathogens with fungal antagonists in combination with soil treatments, in *Biological Control of Soil-bornePathogens* (HORNBY, D., COOK, R.J., HENNIS, Y., KO, W.H., ROVIRA, A.D. SCHIPPERS, B., SCOTT, P.R., Eds.). CAB Publishing House, New York, USA, pp. 15-25.

DUNLEAVY, J. (1955) Control of damping-off of sugarbeet by *Bacillus subtilis*. *Phytopathology* **45**, 252-258.

ELAD, Y., CHET, I. & BAKER, R. (1987) Increased growth response of plants induced by rhizobacteria antagonists to soil-borne pathogenic fungi. *Plant and Soil* **98**, 325-330.

GUETSKY, R., SHTIENBERG, D., ELAD, Y., FISCHER, E. & DINOOR, A. (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**, 976-985.

HARMAN, G.E. & LUMSDEN, R.D. (1990) Biological disease control in *The Rhizosphere* (LYNCH, J.M., Ed.). Wiley Interscience, Chichester, UK, pp. 259-2980.

HARMAN, G.E. (2000) Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* **84**, 377-393.

HOLL, F.B. & CHANWAY, C.P. (1992) Rhizosphere colonization and seedling growth promotion of lodgepole pine by *Bacillus polymyxa*. *Canadian Journal of Microbiology* **38**, 303-308.

INBAR, J., ABRAMSKY, M., COHEN, D. & CHET, I. (1994) Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *European Journal of Plant Pathology* **100**, 337-346.

KIM, D.-S., COOK, R.J. & WELLER, D.M. (1997) *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. *Phytopathology* **87**, 551-558.

KLEIFELD, O. & CHET, I. (1992) *Trichoderma*-plant interaction and its effect on increased growth response. *Plant and Soil* **144**, 267-272.

KLOEPPER, J.W. & SCHROTH, M.N. (1981) Plant-growth promoting rhizobacteria and plant growth under gnotobiotic conditions. *Phytopathology* **71**, 642-644.

LEWIS, J.A. & LUMSDEN, R.D. (2001) Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Protection* **20**, 49-56.

LYNCH, J.M., WILSON, K.L., OUSLEY, M.A. & WHIPPS, J.M. (1991) Response of lettuce to *Trichoderma* treatment. *Letters in Applied Microbiology* **12**, 59-61.

MANERO, F.J.G., ACERO, N., LUCAS, J.A. & POBANZ, A. (1996) The influence of native rhizobacteria on European Alder (*Alnus glutinosa* (L.) Gaerth.) growth II. Characterization and biological control assays of the metabolites from growth promoting and growth inhibiting bacteria. *Plant and Soil* **182**, 67-74.

MCFADDEN, A.G. & SUTTON, J.C. (1975) Relationships of populations of *Trichoderma* spp. in soil to disease in maize. *Canadian Journal of Plant Science* **55**, 579.

MERRIMAN, P.R., PRICE, R.D., KOLLMORGEN, J.F., PIGGOTT, T. & RIDGE, E.H. (1974) Effects of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. *Australian Journal of Agricultural Research* **25**, 219-226.

OKON, Y. & KAPULNIK, Y. (1986) Development and function of *Azospirillium*-inoculated roots. *Plant and Soil* **90**, 3-16.

OUSLEY, M.A., LYNCH, J.M. & WHIPPS, J.M. (1994) Potential of *Trichoderma* as consistent plant growth stimulators. *Biology and Fertility of Soils* **17**, 85-90.

PAPAVIZAS, G.C. (1985) *Trichoderma* and *Gliocladium*: Biology, ecology and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

PAULITZ, Y., WINDHAM, M. & BAKER, R. (1986) Effects of peat vermiculite mixes containing *Trichoderma harzianum* on increased growth response of radish. *Journal of the American Society of Horticultural Science* **111**, 810-814.

PODILE, A.R. & PRAKASH, A.P. (1996) Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. *Canadian Journal of Microbiology* **42**, 533-538.

SIVAN, A. & CHET, I. (1993) Integrated control of *Fusarium* crown root rot of tomato with *Trichoderma harzianum* in combination with methyl bromide or soil solarization. *Crop Protection* **12**, 380-386.

SIVAN, A., UCKO, O. & CHET, I. (1987) Biological control of *Fusarium* crown rot of tomato by *Trichoderma harzianum* under field conditions. *Plant Disease* **71**, 587-592.

SUSLOW, T.V. & SCHROTH, M.N. (1982) Rhizobacteria of sugar beets: Effects of seed application and root colonization on yield. *Phytopathology* **72**, 199-206.

TURNER, J.T. & BACKMAN, P.A. (1991) Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant Disease* **75**, 347-353.

UTKHEDE, R.S. & GAUNCE, A.P. (1983) Inhibition of *Phytophthora cactorum* by a bacterial antagonist. *Canadian Journal of Botany* **61**, 3343-3348.

UTKHEDE, R.S. (1984) Antagonism of isolates of *Bacillus subtilis* to *Phytophthora cactorum*. *Canadian Journal of Botany* **62**, 1032-1035.

UTKHEDE, R.S., KOCH, C.A. & MENZIES, J.A. (1999) Rhizobacterial growth and yield promotion of cucumber plants inoculated with *Pythium aphanidermatum*. *Canadian Journal of Plant Pathology* **21**, 265-271.

WINDHAM, M.T., ELAD, Y. & BAKER, R. (1986) A mechanism of increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**, 518-521.

YEDIDIA, I., SRIVASTA, A.K., KAPULNIK, Y. & CHET, I. (2001). Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil* **235**, 235-242.

YUEN, G.Y., SCHROTH, M.N. & MCCAIN, A.H. (1985) Reduction of *Fusarium* wilt of carnation with suppressive soils and antagonistic bacteria. *Plant Disease* **69**, 1071-1075.

CHAPTER 5

IN VITRO SPORULATION CAPACITY OF SELECTED *TRICHODERMA* ISOLATES AND THEIR DOSE-RESPONSE ON PLANT GROWTH ENHANCEMENT OF ROUGH LEMON *(CITRUS JAMBHIRINI* LUSH.) AND TRIFOLIATE ORANGE (*PONCIRUS TRIFOLIATE* L.) ROOTSTOCKS SEEDLINGS UNDER GREENHOUSE CONDITIONS

ABSTRACT

Trichoderma isolates were evaluated for their sporulation capacity *in vitro* by growing them on V8 medium plates incubated at 25^0C for seven days. After seven days, a single plate of each isolate was washed twice in 500ml sterile distilled water and spore concentration per ml for each suspension was determined using a counting chamber viewed under a compound microscope. *Trichoderma* Isolate AA16 produced the largest number of spores followed by Eco-T®. *Trichoderma* isolates AA4 and AA12 produced the lowest spore concentration. *Trichoderma* isolates AA4, AA16, Eco-T® and SYN6 were tested in the greenhouse for their ability to stimulate growth on rough lemon (*Citrus jambhirini* Lush.) and trifoliate orange (*Poncirus trifoliate* L.) seedlings at four different spore concentrations. *Trichoderma* isolates were grown on V8 agar, washed in sterile distilled water, filtered and then adjusted to four dosage levels (1×10^3, 1×10^4, 5×10^5 or 1×10^6 spores / ml) of which five (5ml) were drenched / seedling. Four months after treatment, root dry weight and shoot dry weight were used as measures of growth stimulation activity. Trifoliate oranges responded positively to 1×10^4 or 1×10^5 spores / ml of Eco-T® but rough lemon responded negatively to all dosages of *Trichoderma* isolates applied. The response of dosage depended on host crop. A high population density of 1×10^6 spores / ml concentration of all tested *Trichoderma* isolates had a stunting effect on rough lemon and trifoliate orange seedling growth.

5.1 INTRODUCTION

Trichoderma spp. are recognized antagonists of other fungi and have been tested extensively as potential biocontrol agents against a range of soil-borne plant pathogenic fungi (Papavizas, 1985) under both greenhouse (Yedidia *et al.*, 2001) and field conditions (Sivan & Chet, 1993). *Trichoderma* spp. may also promote plant growth when applied to pathogen-free soils. Responses to application of *Trichoderma* spp. are characterized by increased germination percentage, plant height and dry weight, a shorter germination time, earlier flowering and an increased number of blooms. *Trichoderma*-induced growth responses have been reported for several plant species (Paulitz *et al.*, 1986; Windham *et al.*, 1986; Kleifeld & Chet, 1992; Inbar *et al.*, 1994; Yedidia *et al.*, 2001).

Baker (1988) suggested that plant growth enhancement by *Trichoderma* spp. may be influenced by population densities of *Trichoderma* spp. This is supported by the report of McFadden & Sutton (1975) who noted that high densities of *Trichoderma* spp. caused stunting and in some instances inhibition of maize in the field. Establishment and proliferation of biocontrol agents in the soil and rhizosphere may influence growth responses by biocontrol agents (Lewis & Papavizas, 1984). The phenomenon of establishment and proliferation of an antagonist in soil in relation to biological control is important and deserves consideration. For effective control of soil-borne plant pathogens, a major consideration is the proliferation of the biological control agent after introduction into the soil rhizosphere (Lewis & Papavizas, 1984). Among the desirable attributes of successful antagonists is the ability to survive, grow and proliferate in the soil and rhizosphere (Lewis & Papavizas, 1984). The capacity of *Trichoderma* spp. to produce large numbers of spores is an important aspect which should be considered in terms of performance in the rhizosphere as well as its commercial potential. However, *in vitro* growth of *Trichoderma* spp. fails to take into account the amount of natural inoculum present in the soil, or the effects of physical, chemical and biological properties in the soil, seasonal conditions, and water relations (Wisniewski *et al.*, 1991).

Results from the previous experiment (Chapter Four) indicated that *Trichoderma* isolates screened *in vivo* did not exhibit plant growth promoting properties. In some instances the

application of *Trichoderma* spore suspension resulted in significant decrease in plant growth. This was attributed to the high spore count of the inoculum. It became apparent that inoculum concentration could be an important factor in selecting appropriate strains of biocontrol agent and warrant further investigation

The aims of this study were to investigate:

1. *in vitro* sporulation capacity of selected *Trichoderma* isolates and
2. determine the effect of inoculum dosage of *Trichoderma* isolates on the growth performance of rough lemon and trifoliate orange rootstock seedlings.

5.2 MATERIALS AND METHODS

5.2.1 Sporulation capacity of *Trichoderma* isolates

Four *Trichoderma* isolates (AA10, AA12, AA16 and Eco-T®) (from Chapter Three) and two *Trichoderma* isolates (AA4 and SYN6) (from Chapter Four) that caused stunting of the rough lemon seedlings were grown on V8 plates incubated at 25^0C for seven days. After seven days a single plate of each isolate was washed in 500ml sterile distilled water and spore concentration / ml for each suspension was determined using a counting chamber viewed under a compound microscope. Each treatment was repeated twice. Data were analyzed statistically using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test (Steel & Torrie, 1980).

5.2.2 Dosage response of *Trichoderma* isolates

The growth of *Trichoderma* isolates, preparation of inoculum and seedling stage as well as inoculation techniques was similar to that described in Chapter Four except that for each *Trichoderma* isolate evaluated (AA4, AA16, Eco-T® and SYN6) were tested to four dosage levels ($1x10^3$, $1x10^4$, $5x10^5$ and $1x10^6$ spores / ml). Five mls were drenched onto each seedling, three weeks after germination. Seedlings were transplanted into Speedling® 49 cavity trays for treatment. There were six replicates for each treatment, with five seedlings per replication. The trial was done using a completely randomized block design.

Although the *Trichoderma* isolates AA10 and AA12 exhibited good biological control activity, they were not included in the dosage trial because of their poor sporulation capacity with low commercial implication and interest. Four months after inoculation, seedlings were extracted from the trays and roots were washed to remove the pine bark. Roots and shoots of seedlings were then separated and dried at 80°C for 72 hrs before being weighed. The growth parameters, i.e., seedling shoot dry weight and root dry weight were used as measures of growth stimulation / plot of five seedlings. Data were analyzed statistically using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test (Steel and Torrie, 1980).

5.3 RESULTS

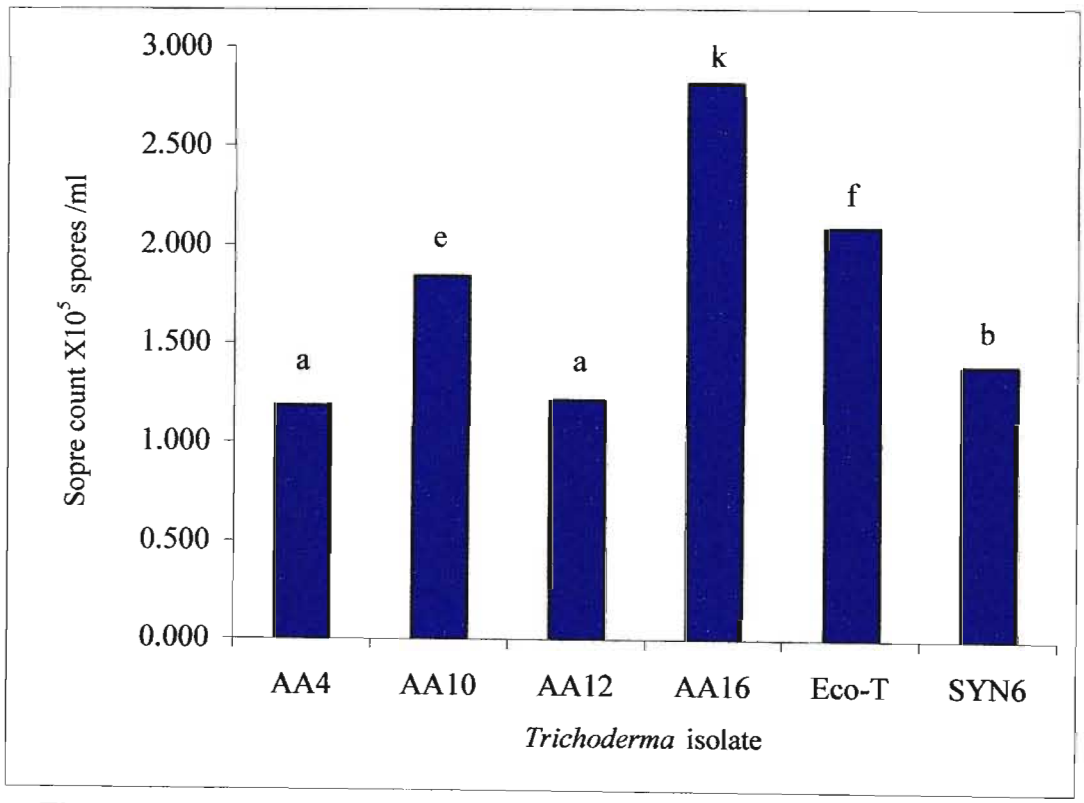

Figure 5.3.1 Spore count of six *Trichoderma* isolates grown *in vitro* after seven days, using a counting chamber under a microscope. Means within bar graphs sharing the same letters are not significantly different ($P<0.05$) as determined by the least significant difference (LSD).

Table 5.3.1 *In vivo* dosage response of *Trichoderma* isolates on growth of rough lemon and trifoliate orange seedlings

		Rough lemon (g)[b]		Trifoliate orange (g)	
Treatment[a]	**Dosage**	**RDW**	**SDW**	**RDW**	**SDW**
Untreated	-	3.526	14.877 c[c]	2.986 a	5.955 b
AA4	$1x10^3$	2.672	7.231 a	2.572 a	5.785 a
AA4	$1x10^4$	2.770	9.545 a	3.033 b	6.287 b
AA4	$5x10^5$	2.978	11.764 b	2.992 a	5.260 a
AA4	$1x10^6$	2.810	9.270 a	2.846 a	5.629 a
AA16	$1x10^3$	2.530	7.665 a	2.670 a	5.445 a
AA16	$1x10^4$	3.049	11.286 b	3.117 b	6.319 b
AA16	$5x10^5$	2.940	11.147 b	2.579 a	5.530 a
AA16	$1x10^6$	3.097	11.670 b	2.529 a	5.242 a
Eco-T®	$1x10^3$	2.644	8.235 a	3.030 b	6.385 b
Eco-T®	$1x10^4$	2.559	8.426 a	3.325 b	7.120 c
Eco-T®	$5x10^5$	3.063	12.821 c	3.350 b	6.831 c
Eco-T®	$1x10^6$	3.040	12.054 b	2.979 a	5.300 a
SYN6	$1x10^3$	3.201	13.590 c	2.953 a	5.629 a
SYN6	$1x10^4$	2.820	11.715 b	2.681 a	5.725 a
SYN6	$5x10^5$	3.132	8.410 a	3.356 b	6.072 b
SYN6	$1x10^6$	2.839	10.370 b	2.706 a	4.781 a
LSD	-	0.6458	2.6830	0.4799	1.1581
P value	-	NS	< 0.001***	0.003**	< 0.001***
CV%	-	19.2	22.0	14.3	17.5

Keys

RDW Root Dry Weight
SDW Shoot Dry Weight

[a] *Trichoderma* isolates drenched at four doses ($1x10^3$, $1x10^4$, $5x10^5$ and $1x10^6$ spores / ml).

[b] Dry biomass was weighed four months after treatment by separating and then dried at 80^0C for 72 hrs.

[c] Data in each column followed by different letters are significantly different (LSD, $P<0.05$). The data are means of six replicates.

* = Significant, ** = highly significant, *** = very highly significant

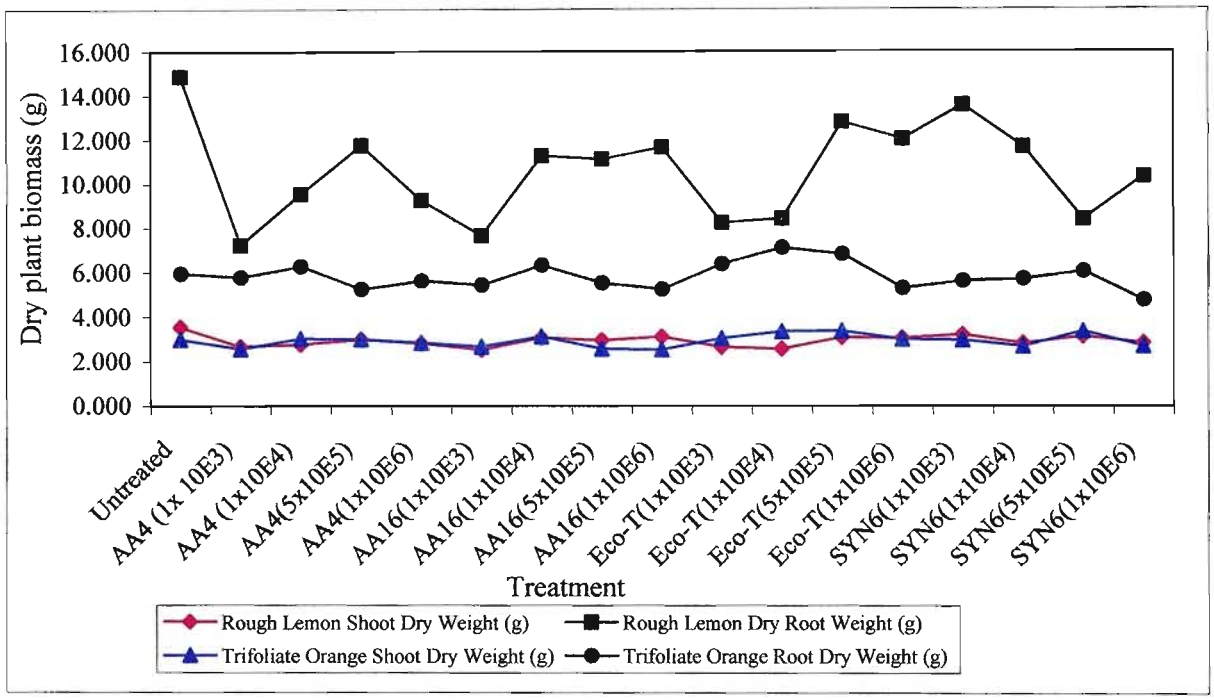

Figure 5.3.2 *In vivo* dosage response, dry weight (g) of *Trichoderma* isolates on rough lemon and trifoliate orange seedlings, applied by drenching, in the absence of any pathogen ($P < 0.05$).

5.4 DISCUSSION

Trichoderma spp. are well documented as biocontrol agents (Papavizas, 1985; Harman & Lumsden, 1990; Lewis & Lumsden, 2001) and growth stimulation agents (Chang *et al.*, 1986; Windham *et al.*, 1986; Baker, 1989; Kleifeld & Chet, 1992; Inbar *et al.*, 1994; Ousley *et al.*, 1994; Yedidia *et al.*, 2001). For effective control of soil-borne plant pathogens, antagonistic proliferation after being introduced into the soil rhizosphere is important (Lewis & Papavizas, 1984). The capacity of *Trichoderma* spp. to produce large numbers of spores is affected by soil rhizosphere environmental conditions (Wisniewski *et al.*, 1991). However, evaluation of the *Trichoderma* isolates *in vitro* gives an indication as to how it grows, and high production of spores of good biological control agent would be important for commercial purposes.

In this experiment, the *Trichoderma* isolates tested showed highly significant differences in their sporulation capacity. *Trichoderma* Isolate AA16 produced the largest spore number followed by Eco-T® while *Trichoderma* isolates AA4 and AA12 produced the lowest spore concentration. *Trichoderma* Isolate AA12, which had the best biological control against *Phytophthora* root rot of rough lemon seedlings (Chapter Three, Table 3.3.1), produced the lowest spore numbers. Thus, it is important to take into account that promising biocontrol agents *in vivo* might not necessarily have a good harvest of spores to provide an economically viable product. However, the wide range of sporulation could be utilized as a source of variation, and shows that the isolates were unique.

Activity of biocontrol agents depends greatly on pathogen and biocontrol agent densities (Adams, 1990; Mandeel & Baker, 1991; Yuen, *et al.*, 1994). There are reports suggesting the negative effect of *Trichoderma* spp. applied in the absence of a plant pathogen. For example, Baker (1988) found a peat-bran formulation at 10^8 colony forming units (CFU) per g soil caused stunting of eggplants. McFadden & Sutton (1975) reported high population densities (10^4 or 10^5 propagules / gram) of *Trichoderma* spp. caused stunting of maize seedlings in the field.

Results from trial showed that the parameter of RDW did not reflect significant differences as a result of different treatments. Hence, it was not useful in ascertaining success of biological

control activity, in the *in vivo* dose response of *Trichoderma* isolates, whereas the Seedling SDW for the rough lemon seedling was.

Results from the parameter Seedling SDW on rough lemon showed that two *Trichoderma* isolates Eco-T at 5 x 10^5 spore concentration / ml and SYN4 at 1 x 10^3 spore concentration / ml were non-significant differences in growth to the control were found.

Response to the parameter Seedling RDW on trifoliate orange showed that five treatments AA4 (1 x 10^4 spore concentration / ml), AA16 (1 x 10^4 spore concentration / ml), Eco-T (5 x 10^3 spore concentration / ml), Eco-T (1 x 10^4 spore concentration / ml) and Eco-T (1 x 10^5 spore concentration / ml) were significantly better than the control. The rest of the treatments were significantly worse than the control. The Eco-T seems to works under wider range of dosages.

Results from Seedling SDW of trifoliate orange showed that AA4 (1 x 10^4 spore concentration / ml), AA16 (1 x 10^4 spore concentration / ml), Eco-T (1 x 10^3 spore concentration / ml) and SYN6 (1 x 10^3 spore concentration / ml) were not significantly different to the control. Eco-T (1 x 10^4 spore concentration / ml) and Eco-T (5 x 10^5 spore concentration / ml) were significantly better than the control, possibly due to growth promotion in trifoliate orange seedlings.

Rough lemon seedlings responded negatively to the treatment of *Trichoderma* isolate at all doses except those observed with Eco-T® (5 X 10^5 spores concentration / ml) and SYN6 (1 X 10^3 spores concentration / ml) were not significantly different than the control on the SDW parameter. These responses support results in Chapter Four that the *Trichoderma* isolates at the tested spore concentrations did not promote seedling growth. In the case of trifoliate orange seedlings showed a significant difference between treatments in both parameters. Considering the root Seedling RDW (Table 5.3.1 and Figure 5.3.2), AA4 (1 X 10^3 spores / ml), AA16 (1 X 10^4 spores / ml), Eco-T® (1 X 10^3 spores /ml; 1 X 10^4 spores / ml and 5 X 10^5 spores / ml) and SYN6 (5 X 10^5 spores / ml) performed better than the untreated control. Other dosage levels caused a negative response. These results show that Eco-T® functions under

wide dosage levels (Table 5.3.1). Seedling roots of the trifoliate oranges responded to all *Trichoderma* isolates at different levels. The better root weight would give the plant a better chance to resist plant pathogens and better nutrient and water take-up. *Trichoderma* isolate, Eco-T® (1×10^4 spores / ml or 5×10^5 spores per ml) also caused increased Seedling SDW. The higher dosage (1×10^6 spores per ml) of the *Trichoderma* isolates applied to rough lemon and trifoliate orange seedlings caused stunting. Such negative activity was also reported in maize by McFadden & Sutton (1975).

These trials showed that different *Trichoderma* isolates have varied sporulation capacities This criterion is important to give an indication of the proliferation and establishment of the isolates although it may not be an entirely accurate representation, as the process is affected by soil microflora and physical and chemical factors. Sporulation tests also give some idea of the commercial implications in terms of manufacturing and economics provided that spore optimisation is attempted. The experiment showed that the response of the *Trichoderma* isolate, as a growth stimulant is host dependent. Spore concentrations higher than 1×10^5 spores / ml, in the absence of any pathogen caused negative effects on the tested seedling.

5.5 REFERENCES

ADAMS, P.B. (1990) The potential of mycoparasites for biological control of plant diseases. *Annual Review of Phytopathology* **28**, 59-72.

BAKER, R. (1988) *Trichoderma* spp. as plant-growth stimulants. *CRC Critical Reviews in Biotechnology* **7**, 97-105.

BAKER, R. (1989) Improved *Trichoderma* spp. for promoting crop productivity. *Trends in Biotechnology* **7**, 34-38.

CHANG, Y.C., CHANG, Y.C., BAKER, R., KLEIFELD, O. & CHET, I. (1986) Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. *Plant Disease* **70**, 145-148.

HARMAN, G.E. & LUMSDEN, R.D. (1990) Biological disease control in *The Rhizosphere* (LYNCH, J.M., Ed.). Wiley Interscience, Chichester, UK, pp. 259-2980.

INBAR, J., ABRAMSKY, M., COHEN, D. & CHET, I. (1994) Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *European Journal of Plant Pathology* **100**, 337-346.

KLEIFELD, O. & CHET, I. (1992) *Trichoderma-harzianum* interaction with plants and effect on increased growth response. *Plant and Soil* **144**, 267-272.

LEWIS, J.A. & LUMSDEN, R.D. (2001) Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Protection* **20**, 49-56.

LEWIS, J.A. & PAPAVIZAS, G.C. (1984) A new approach to stimulate population proliferation of *Trichoderma* spp. and other potential biocontrol fungi introduced into natural soils. *Phytopathology* **74**, 1240-1244.

MANDEEL, Q., & BAKER, R. (1991) Mechanisms involved in biological control of *Fusarium* wilt of cucumber with strains of non-pathogenic *Fusarium oxysporum*. *Phytopathology* **81**, 462-469.

MCFADDEN, A.G. & SUTTON, J.C. (1975) Relationships of populations of *Trichoderma* spp. in soil to disease in maize. *Canadian Journal of Plant Science* **55**, 579.

MONTESINOS, E. & BONATERRA, A. (1996) Dose-response models in biological control of plant pathogens: An empirical verification. *Phytopathology* **86**, 464-472.

OUSLEY, M.A., LYNCH, J.M. & WHIPPS, J.M. (1994) Potential of *Trichoderma* as consistent plant growth stimulators. *Biology and Fertility of Soils* **17**, 85-90.

PAPAVIZAS, G.C. (1985) *Trichoderma* and *Gliocladium*: Biology, ecology and potential for biocontrol. *Annual Review of Phytopathology* **23**, 23-54.

PAULITZ, Y., WINDHAM, M. & BAKER, R. (1986) Effects of peat vermiculite mixes containing *Trichoderma harzianum* on increased growth response of radish. *Journal of the American Society of Horticultural Science* **111**, 810-814.

SIVAN, A. & CHET, I. (1993) Integrated control of *Fusarium* crown root rot of tomato with *Trichoderma harzianum* in combination with methyl bromide or soil solarization. *Crop Protection* **12**, 380-386.

STEEL, R.G.D. & TORRIE, J.H. (1980) *Principles and procedures of statistics*: a biometrical approach, 2nd ed., McGraw-Hill, inc., New York, USA.

WINDHAM, M.T., ELAD, Y. & BAKER, R. (1986) A mechanism of increased plant growth induced by *Trichoderma* spp. *Phytopathology* **76**, 518-521.

WISNIEWSKI, M., BILES, C., DROBY, S., MCLAUGHLIN, R., WILSON, C., CHALUTZ, E. (1991) Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I. Characterization of attachment to *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* **39**, 245-258.

YEDIDIA, I., SRIVASTA, A.K., KAPULNIK, Y. & CHET, I. (2001) Effects of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant and Soil* **235**, 235-242.

YUEN, G.Y., CRAIG, M.L., KERR, E.D. & STEADMAN, J.R. (1994) Influences of antagonist population levels, blossom development stage, and canopy temperature on the inhibition of *Sclerotinia sclerotiorum* on dry edible bean by *Erwinia herbicola*. *Phytopathology* **84**, 495-501.

CHAPTER 6

CONTROL OF *PHYTOPHTHORA* ROOT ROT OF CITRUS SEEDLINGS AND CUTTINGS USING COMBINATIONS OF BIOCONTROL AGENTS

ABSTRACT

Compatibility of *Trichoderma* isolates AA16 and Eco-T® or *Trichoderma* isolates AA16 or Eco-T® and *Bacillus* isolates B77, B81 or PHP were tested *in vitro* on PDA plates incubated at 25^{0}C for five days. After five days of incubation, observations were made. All combinations were found compatible. Their separate and combined *in vivo* activity was also investigated against *Phytophthora* root rot of citrus rootstock seedlings or cuttings.

Two isolates of *Trichoderma* (AA16 and Eco-T®) were tested separately or together for suppression of *Phytophthora* root rot on rough lemon seedlings and cuttings. Four months after treatment, root dry weight and shoot dry weight were used as measures of biological control activity. A combination of AA16 and Eco-T® drenched at 5 x 10^5 spores / ml increased new flush (new shoot growth) dry biomass on rough lemon cuttings compared to AA16 alone, but was not different from Eco-T® individually. The combination of AA16 and Eco-T® caused no effect on dry weight of rough lemon and trifoliate orange seedlings. The combination of AA16 and Eco-T® did not increase the dry root weight of sour orange compared to AA16 or Eco-T® alone. The combination of AA16 and Eco-T® at a higher dosage rates (1 x 10^6 spores / ml) showed significantly better disease suppression of *Phytophthora* root rot on rough lemon cuttings, but did not cause disease suppression in seedlings tested.

In addition, *Trichoderma* and *Bacillus* isolates were also investigated individually or in combination for suppression of *Phytophthora* root rot on rough lemon and trifoliate orange seedlings in sterile pine bark media. The combination of *Trichoderma* Isolate AA16 and *Bacillus* Isolate B81 increased dry root weight of rough lemon seedlings compared to the combination of the Isolate AA16 and *Bacillus* Isolate PHP but was not different to AA16 alone. Isolate PHP combined with Isolate AA16 or singly, caused no effect on rough lemon seedlings. Combinations of *Trichoderma* Isolate Eco-T® and *Bacillus* Isolates B81 or PHP did not increase dry weight of rough lemon seedlings compared to the single application of Eco-

T®. There were no significant differences of the combinations of the *Trichoderma* and *Bacillus* isolates compared to their separate application on the dry weight of the trifoliate oranges. Generally the application of a mixture of biocontrol agents did not enhance *Phytophthora* root rot suppression compared with the same biocontrol agents applied alone.

6.1 INTRODUCTION

Biocontrol of soil-borne diseases are particularly complex because these diseases occur in the dynamic environment at the interface of the root and soil rhizosphere. The rhizosphere is subject to dramatic changes over short or longer temporal scales (Rovira, 1965, 1969; Waisel *et al.*, 1991). Therefore, integration of several control techniques may be required to increase yield and quality and minimize environmental hazards (Abd-El Moity *et al.*, 1982; Papavizas *et al.*, 1982; Locke *et al.*, 1984), which is a flexible, multidimensional approach to a range of control components such as biological, cultural, and chemical strategies. These strategies are required to hold diseases below damaging economic thresholds without damaging the ecosystem (Andrews, 1983; Papavizas & Lewis, 1988). Microorganisms functioning as biological control agents typically have a relatively narrow spectrum of activity compared with synthetic chemicals (Baker, 1991; Janisiewicz, 1996) and often exhibit inconsistent performance in practical agriculture, resulting in limited commercial use of biocontrol approaches for suppression of plant pathogens (Raupach & Kloepper, 1998). Thus, biocontrol agents may be integrated with physical or chemical treatments, often with better overall effects than would result from the use of either when applied singly (Rahe & Utkhede, 1985). *Trichoderma* combined with either chemicals or with solarization as integrated treatments have shown promising results (Harman *et al.*, 1981). For example, satisfactory control of cucumber fruit rot (*Rhizoctonia solani*) in the field was achieved by a combination of ploughing and the addition of *T. harzianum* (Lewis & Papavizas, 1980). Disease caused by *R. solani* and *Sclerotium rolfsii* was controlled by employing *T. harzianum* combined with soil solarization (Elad *et al.*, 1980; Katan, 1981).

Most approaches for biological control of plant diseases have used single biocontrol agents (Raupach & Kloepper, 1998). This may partially account for the reported inconsistent performance by biocontrol agents, because single biocontrol agents are not likely to be active

in all soil environments in which they are applied or against all pathogens that attack the host plant. A wide spectrum of pathogen control by applied antagonists largely remains an unfulfilled aim for biological control. There are several approaches to achieve this aim:

1. Alter the environment to favor the biological control agent and to disfavor competitive microflora (Janisiewicz, 1988).
2. Develop strain mixtures with superior biocontrol activity (Janisiewicz, 1988). Several strategies for forming mixtures of biocontrol agents could be envisioned including mixtures of organisms with different plant colonization patterns; mixtures of antagonists that control different pathogens; mixtures of antagonists with different mechanisms of disease suppression; mixtures of taxonomically different organisms; or mixtures of antagonists with different optimum temperatures, pHs, or moisture conditions for plant colonization (Raupach & Kloepper, 1998).

Previous studies using a combination of biological control mechanisms for plant diseases have included mixtures of fungi (Paulitz *et al.*, 1990; Budge *et al.*, 1995; Datnoff *et al.*, 1995). Earlier studies on mixtures of fungi and bacteria have been provided (Kwok *et al.*, 1987; Janisiewicz, 1988; Park *et al.*, 1988; Lemanceau & Alabouvette, 1991; Duffy & Weller, 1995; Duffy *et al.*, 1996; Janisiewicz, 1996; Leeman *et al.*, 1996; Leibinger *et al.*, 1997; Guetsky *et al.*, 2001; Guetsky *et al.*, 2002). *Trichoderma* spp. generally tolerate a lower pH than bacterial biocontrol agents and thus may protect plants better in acidic soils compared to bacteria (Cook & Baker, 1983).

Trichoderma spp. provide protection in most arid regions or later in the growing season as moisture becomes less available (Duffy *et al.*, 1996). *Bacillus* strains can be used because of their resistant endospores, which may remain viable for long periods and are tolerant to extreme temperatures (Adegumo *et al.*, 1999). Previous investigations on mixtures of bacteria have been cited (Johnson *et al.*, 1993; Pierson & Weller, 1994; Waechter-Kristensen *et al.*, 1994; Janisiewicz & Bors, 1995; Mazzola *et al.*, 1995; Raaijmakers *et al.*, 1995; Stockwell *et al.*, 1996; Raupach & Kloepper, 1998; de Boer *et al.*, 1999). Most of these reports on mixtures

of biocontrol agents showed that combining antagonists resulted in improved biocontrol. However, there are also reports of combinations of biocontrol agents that do not result in improved suppression of disease compared with the use of a single antagonist (Dandurand & Knudsen, 1993; Hubbard *et al.*, 1983; Sneh *et al.*, 1984). For example, combining a *Trichoderma harzianum* strain with a *Pseudomonas fluorescens* strain, both able to suppress root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*, did not result in better disease suppression (Dandurand & Knudsen, 1993).

Positive and negative interactions between introduced biocontrol microorganisms or between an introduced biocontrol agent and the indigenous microflora can influence their performance in the rhizosphere. For example, two groups of microorganisms that occupy the same ecological niche and have the same nutritional requirements to compete for nutrients (Fukui *et al.*, 1994; Janisiewicz & Bors, 1995; Raaijmakers *et al.*, 1995). Raaijmakers *et al.* (1995) demonstrated that siderophore-mediated competition for iron between the biocontrol agents *P. putida* WCS358 and *P. fluorescens* WCS 374 decreased colonization of the radish roots by the latter strain. Hubbard *et al.* (1983) described negative effects of endemic *Pseudomonas* spp. strains on the biocontrol agent *T. harzianum*. Another negative interaction between two populations of biocontrol microorganisms can be due to detrimental effects of secondary metabolites produced by one organism on the growth of the other (Mew *et al.*, 1994).

Biocontrol agents are affected by biotic and abiotic conditions. Because different mechanisms of control may be dissimilarly influenced by these conditions, it is possible that if multiple mechanisms are involved, under a certain set of conditions, one mechanism may compensate for the other. Therefore, biological control with multimechanisms may be achieved by using one biocontrol agent exhibiting several mechanisms or by applying more than one biocontrol agent in a mixture, provided that each of them has one (or several) distinct mechanisms (Guetsky *et al.*, 2002). Consequently, application of mixtures of introduced biocontrol agents would more closely mimic the natural situation and might widen the spectrum of biocontrol activity, enhance the efficacy and represent a more viable and reliable control (Duffy & Weller, 1995). It is an important prerequisite for the desired effectiveness of species to appear compatible for joint-inoculation of microorganisms (Li & Alexander, 1988; Baker, 1990; Raaijmakers *et al.*, 1995). Harman (2000) rejected this concept.

The objectives of this study were to determine:

1. the *in vitro* interactions between *Trichoderma* isolates and also the interactions between *Trichoderma* and *Bacillus* isolates.
2. whether *in vi*tro interactions between *Trichoderma* isolates can give a predictive value for suppression of *Phytophthora* root rot by co-inoculations of these isolates *in vivo*.
3. whether *in vitro* interactions between *Trichoderma* and *Bacillus* isolates can give a predictive value for suppression of *Phytophthora* root rot by co-inoculations of these isolates *in vivo*.

6.2 MATERIALS AND METHODS

6.2.1 Compatibility of selected *Trichoderma* isolates with Eco-T®

Trichoderma Isolate AA16 and Eco-T® (commercial product) were grown on V8 medium plates and were incubated at 25°C in darkness for seven days. After seven days, a 4mm agar plug from the edges of actively sporulating Isolate AA16 grown on V8 agar was transferred to a plate of V8 medium and tested for compatibility with Eco-T ® by placing a similar size agar plug cut in the same manner, on the opposite side of the plate. Each dual cultured bioassay pair was replicated three times. Activities were checked daily for five days. Assessment was based on the observation of the interactions of the pairs, in terms of whether they grew without inhibiting each other or whether they inhibited the performance of one another.

6.2.2 Compatibility of *Trichoderma* and *Bacillus* isolates

Trichoderma Isolates AA16 and Eco-T® were grown on V8 medium and incubated at 25°C in darkness for seven days. The *Bacillus* isolates used were B77, B81, and PHP[5]. The PHP isolate was included in this experiment after it showed an *in vitro* antagonistic activity against *Phytophthora* root rot of citrus (Isolate 1).

Similar procedures were followed for the growth and preparation of the inoculum as for the *in vitro* testing of the *Bacillus* isolates presented in Chapter Two, except that a single paper disc for each *Bacillus* isolate was placed approximately 3.5mm from the edge of each PDA plate,

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whereas the 4mm diameter agar plug of *Trichoderma* grown on V8 medium was placed at the center of the plate on a dual PDA culture medium. Each treatment was replicated three times. Activities were checked daily for five days. Evaluation was based on the observation of the interactions of the pairs of *Trichoderma* and *Bacillus* isolates, whether they inhibited the growth and performance of each other or not.

6.2.3 Combined effects of *Trichoderma* isolates or *Trichoderma* and *Bacillus* isolates on *Phytophthora* root rot citrus rootstock seedlings

Similar procedures were followed as in Chapter Three for the set-up of the trial. Differences in methods and materials used are shown in Table 6.2.1 and Table 6.2.2. Seedlings were transplanted into Speedling® 49 trays in composted pine bark media. The trial was a complete randomized block design with each treatment being replicated six times, and each replicate consisting five seedlings.

Table 6.2.1 Treatments used to determine combined effects of *Trichoderma* isolates against *Phytophthora* root rot on rough lemon, trifoliate and sour orange seedlings

		Isolate AA16		Eco-T®	
Treatments	***Phytophthora***	**Dose**	**Concentration[a]**	**Dose**	**Concentration**
1	No	0	0	0	0
2	Yes	0	0	0	0
3	Yes	5ml / plug	$5x10^5$	0	0
4	Yes	0	0	5ml / plug	$5x10^5$
5	Yes	2.5ml / plug	$5x10^5$	2.5ml / plug	$5x10^5$
6	Yes	2.5ml / plug	$1x10^6$	2.5ml / plug	$1x10^6$

[a] Spores of *Trichoderma* isolates / ml

Table 6.2.2 Treatments used to determine combined effects of *Trichoderma* and *Bacillus* isolates against *Phytophthora* root rot on rough lemon and trifoliate oranges

Treatments	*Phytophthora*	AA16	Eco-T®	PHP	B81
1	No	-	-	-	-
2	Yes	-	-	-	-
3	Yes	5ml / plug	-	-	-
4	Yes	-	5ml / plug	-	-
5	Yes	-	-	5ml / plug	-
6	Yes	-	-	-	5ml / plug
7	Yes	5ml / plug		5ml / plug	
8	Yes	5ml / plug	-	-	5ml / plug
9	Yes	-	5ml / plug	5ml / plug	-
10	Yes	-	5ml / plug	-	5ml / plug

The concentration for *Trichoderma* Isolate AA17 or Eco-T® was $5x10^5$ spores / ml and for *Bacillus* isolates PHP or B81, 1 x 10^6 CFU / ml following similar procedures as described in Chapter Three.

6.2.4 Combined effects of *Trichoderma* isolates on *Phytophthora* root rot of rough lemon stem cuttings

Stem cuttings were collected from citrus farms around Pietermaritzburg, KwaZulu-Natal, South Africa. The cuttings were dipped in a growth hormone (Seradix 3) before placing them in the rooting bed composed of 50% composted pine bark and 50% perlite media at 30^0C and misted, as regulated by a leaf wet balance. Cuttings were kept for four weeks. After four weeks the cuttings of the similar height and size of rooting (Figure 6.2.2) were transplanted into pots with a 140mm diameter. Each cutting was drenched with a total of 30ml of *Trichoderma* isolate. A pot of the same size used for the experiment was filled with composted pine bark and wetted with water. Thereafter, 1000ml of water was poured on the pot medium and a container placed underneath to collect the draining water. The drained water was measured using a graduated measuring cylinder and subtracted from 1000ml. The medium retained about 30ml of the added water and the treatment volume was based on this outcome. Treatments were set-up as shown in Table 6.2.3. The trial was a complete randomized block

design with each treatment being replicated four times, with each plot having six potted seedlings.

Table 6.2.3 Description of treatments used to determine ccombined effects of *Trichoderma* isolates against *Phytophthora* root rot on rough lemon stem cuttings

		Isolate AA16		Eco-T®	
Treatments	***Phytophthora***	**Dose**	**Concentration[b]**	**Dose**	**Concentration**
1	No	0	0	0	0
2	Yes	0	0	0	0
3	Yes	30ml / pot	$5x10^5$	0	0
4	Yes	0	0	30ml / pot	$5x10^5$
5	Yes	15ml / pot	$5x10^5$	15ml / pot	$5x10^5$
6	Yes	15ml / pot	$1x10^6$	15ml / pot	$1x10^6$

[b] Unit for isolates AA16, Eco-T® (*Trichoderma* isolates) concentration used was spores / ml

Figure 6.2.2 Rough lemon cuttings in the rooting bed before transplanting

Treatment evaluation and statistical analysis

Four months after inoculation, seedlings or cuttings were extracted from the trays or pots. Roots were washed to remove the pine bark, for all the combination trials (*Trichoderma* with

Trichoderma or *Trichoderma* with *Bacillus* isolates). Roots and shoots of seedlings were then separated and dried at 80°C for 72 hrs before being weighed. Roots and new shoot growth (new flush growth) of the cuttings were separated and dried in the same way as described for the seedlings. The data taken per plot were analyzed statistically using one-way analysis of variance (ANOVA) and the LSD (Least Significant Difference) test (Steel & Torrie, 1980).

6.3 RESULTS

In vitro interaction of *Trichoderma* Isolates AA16 with Eco-T® or *Trichoderma* Isolates AA16 or Eco-T® with *Bacillus* Isolate PHP was found to be compatible (Table 6.3.1 and Figures 6.3.1.A-C) but the *Bacillus* isolates once come into contact with the *Trichoderma* isolates

The *in vivo* separate and combined activity of *Trichoderma* isolate with other *Trichoderma* isolate, and *Trichoderma* isolate with *Bacillus* isolates against *Phytophthora* root rot of citrus rootstock seedlings/cuttings are presented in Table 6.3.2 and Figures 6.3.2-6.3.3, and Table 6.3.3 and Figure 6.3.4, respectively.

Table 6.3.1 Compatibility of *Trichoderma* isolates with each other and compatibility of *Trichoderma* isolates with *Bacillus* isolates

***Trichoderma* isolate**	***Trichoderma* isolate**	***Bacillus* isolate**	**Compatibility (Yes or No)**
AA16	Eco-T®	-	Yes
AA16	-	B77, B81, PHP	Yes
Eco-T®	-	B77, B81, PHP	Yes

This table shows that whether any of the isolates inhibit one another and the reactions are presented as either yes (no inhibition) or no (there is inhibition).

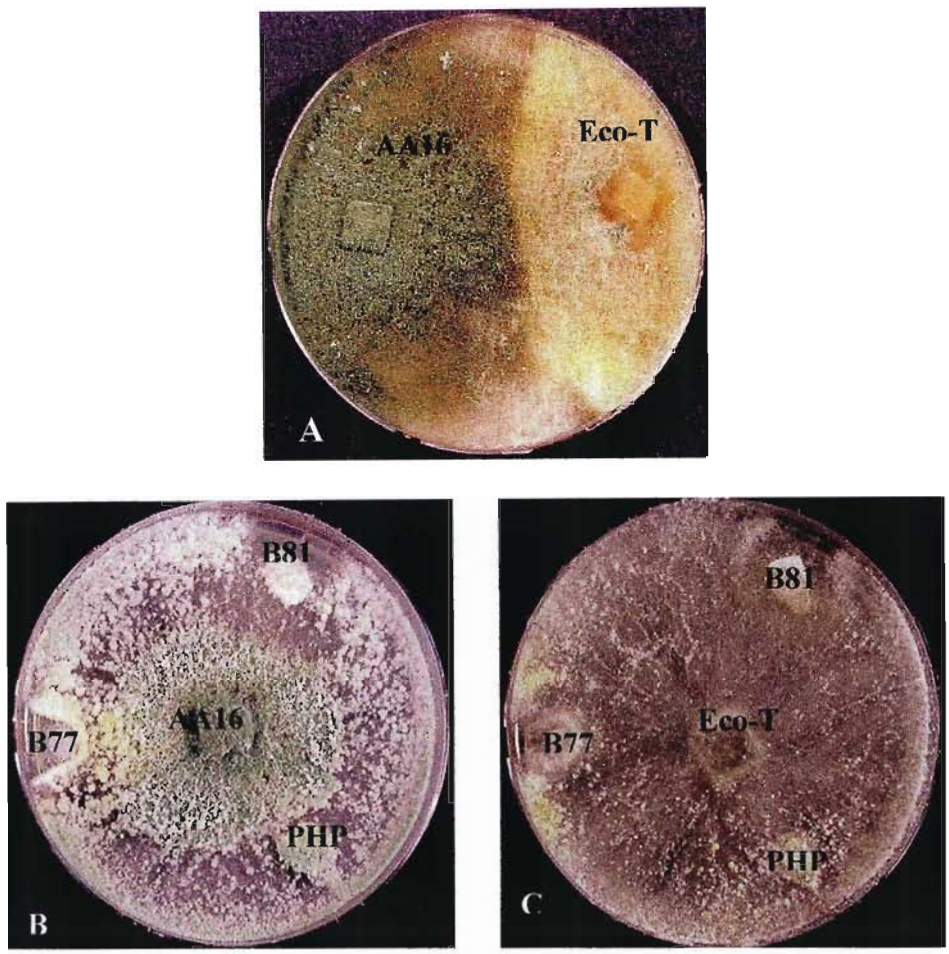

Figure 6.3.1 Compatibility of *Trichoderma* isolate with other *Trichoderma* isolates or *Trichoderma* isolate with *Bacillus* isolates. Isolates were plated in dual PDA medium and grown for five days at 25^0C. Observations were taken five days later. Figure 6.3.1.A shows the compatibility of two *Trichoderma* isolates (AA16 and Eco-T®) and Figures 6.3.1.B-C show the compatibility of *Trichoderma* isolates (AA16 or Eco-T®) with three *Bacillus* isolates (B77, B81 and PHP).

Table 6.3.2 Effects of combining *Trichoderma* isolates for control of *Phytophthora* root rot on citrus rootstocks seedlings and cuttings

Treatments[a]	Rough lemon Cuttings (g)[d]		Rough lemon Seedlings (g)		Sour orange Seedlings (g)		Trifoliate orange Seedlings (g)	
	RDW	NFSDW	RDW	SDW	RDW	SDW	RDW	SDW
Uninoculated control	6.593	11.306c[e]	3.598c	11.774c	2.613b	4.462b	3.122c	8.360e
Inoculated control	4.536	7.745a	2.529a	8.736a	2.014a	3.532a	2.697a	5.102a
AA16 (5×10^5)	5.021	10.031b	3.079b	10.495b	2.878c	4.384b	3.147c	6.234b
Eco-T® (5×10^5)	5.489	11.285c	3.175b	10.714b	2.660c	4.438b	3.177c	6.223b
AA16+Eco-T®.(5×10^5)	5.781	11.431c	3.261b	10.929b	2.356b	4.283b	3.141c	5.905b
AA16+ Eco-T® (1 $\times 10^6$)	5.786	9.331b	2.720a	8.644a	2.245a	3.878a	2.650a	5.188a
P value	NS	<0.001***	<0.001***	<0.001***	<0.001***	0.040*	<0.001***	<0.001***
LSD		1.370	0.3922	1.1651	0.386	0.6573	0.2257	0.7869
% CV		8.9	10.8	9.9	10.5	13.3	6.3	10.7

Keys

RDW	Root dry weight
NFDSW	New flush shoot dry weight
SDW	Shoot dry weight

[a] Isolates of *Trichoderma*, AA16 or Eco-T® were drenched on Speedling® trays of citrus root stock seedlings (rough lemon, trifoliate and sour orange) and rough lemon cuttings in pots, alone or in combination, 24 hrs before a 4mm diameter *Phytophthora* agar block was placed at the base of the seedling or cutting.

[d] Dry biomass was done four months after treatment by separating roots and shoots or new shoot flushes (in case of rough lemon cuttings) then dried at 80^0C for 72 hrs.

[e] Values within the same column followed by different letters are significantly different ($P< 0.05$) as determined by least significant difference (LSD).

* = Significant, ** = highly significant, *** = very highly significant

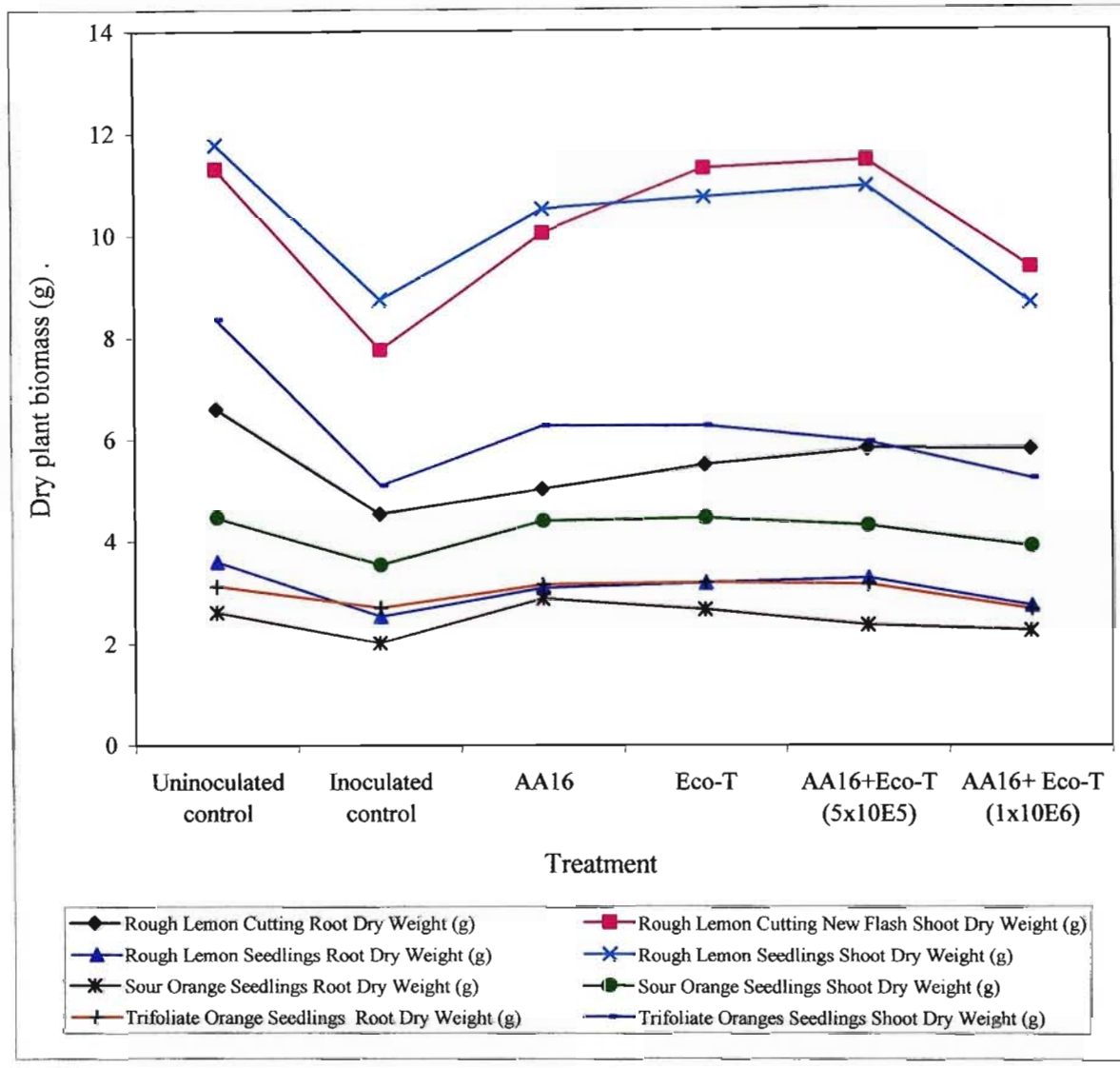

Figure 6.3.2 Response of dry weight (g) of citrus rootstocks (rough lemon seedling cuttings, sour orange seedlings and trifoliate orange seedlings) to combination treatments with two *Trichoderma* isolates, applied by drenching ($P < 0.05$).

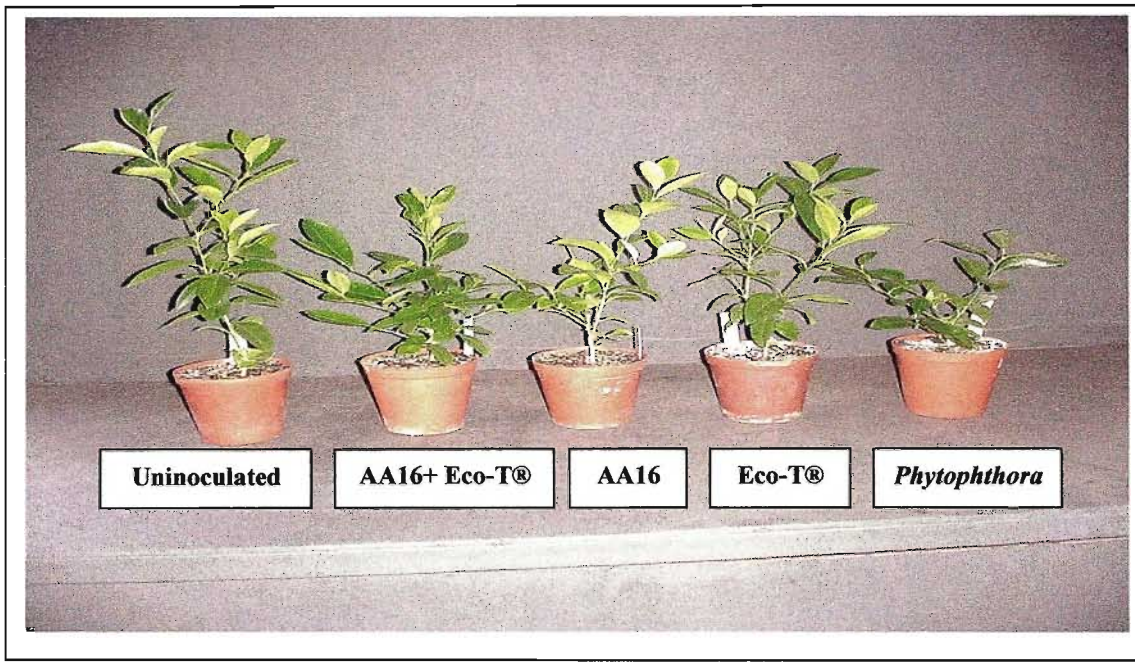

Figure 6.3.3 Single and combined effect of *Trichoderma* isolates on rough lemon stem cuttings against *Phytophthora parasitica*.

Table 6.3.3 Effects of combining *Trichoderma* and *Bacillus* isolates for control of *Phytophthora* root rot on rough lemon and trifoliate citrus rootstocks seedlings

Treatments [f]	Rough lemon (g) [g]		Trifoliate orange (g)	
	RDW	SDW	RDW	SDW
Uninoculated control	3.351d [h]	13.680c	3.084b	7.527c
Inoculated control	2.377a	9.880a	2.697a	5.202a
AA16	3.014c	11.440b	2.991b	6.900b
Eco-T®	3.105c	11.670b	3.051b	6.945b
B81	3.113c	11.010b	3.057b	7.008b
PHP	2.710b	10.280b	3.093b	7.116b
AA16 + B81	3.095c	11.500b	3.119b	7.140b
Eco-T® + B81	2.738b	10.490b	3.115b	6.893b
AA16 + PHP	2.968b	10.330b	3.102b	6.794b
Eco-T® + PHP	2.952b	11.030b	3.010b	6.901b
P value	<0.001***	<0.001***	0.026*	0.003**
LSD	0.3154	1.924	0.2714	1.0792
% CV	9.2	15.3	7.6	13.6

Keys

RDW	Root Dry Weight
SDW	Shoot Dry Weight

[f] Isolates AA16, Eco-T® (*Trichoderma*) and B81, PHP (*Bacillus* isolates) were drenched at concentrations of 5 X 10^5 spores / ml and 1 X 10^6 CFU / ml, respectively, on rough lemon seedlings or trifoliate orange onto seedlings on Speedling® trays 24 hrs before a 4mm diameter *Phytophthora* agar block was placed at the base of the seedlings.

[g] Dry biomass was taken four months after treatment by separating the roots and shoots and then dried at 80^0C for 72 hrs.

[h] Data in each column followed by different letters are significantly different ($P< 0.05$) determined by least significant difference (LSD).

* = Significant, ** = highly significant, *** = very highly significant

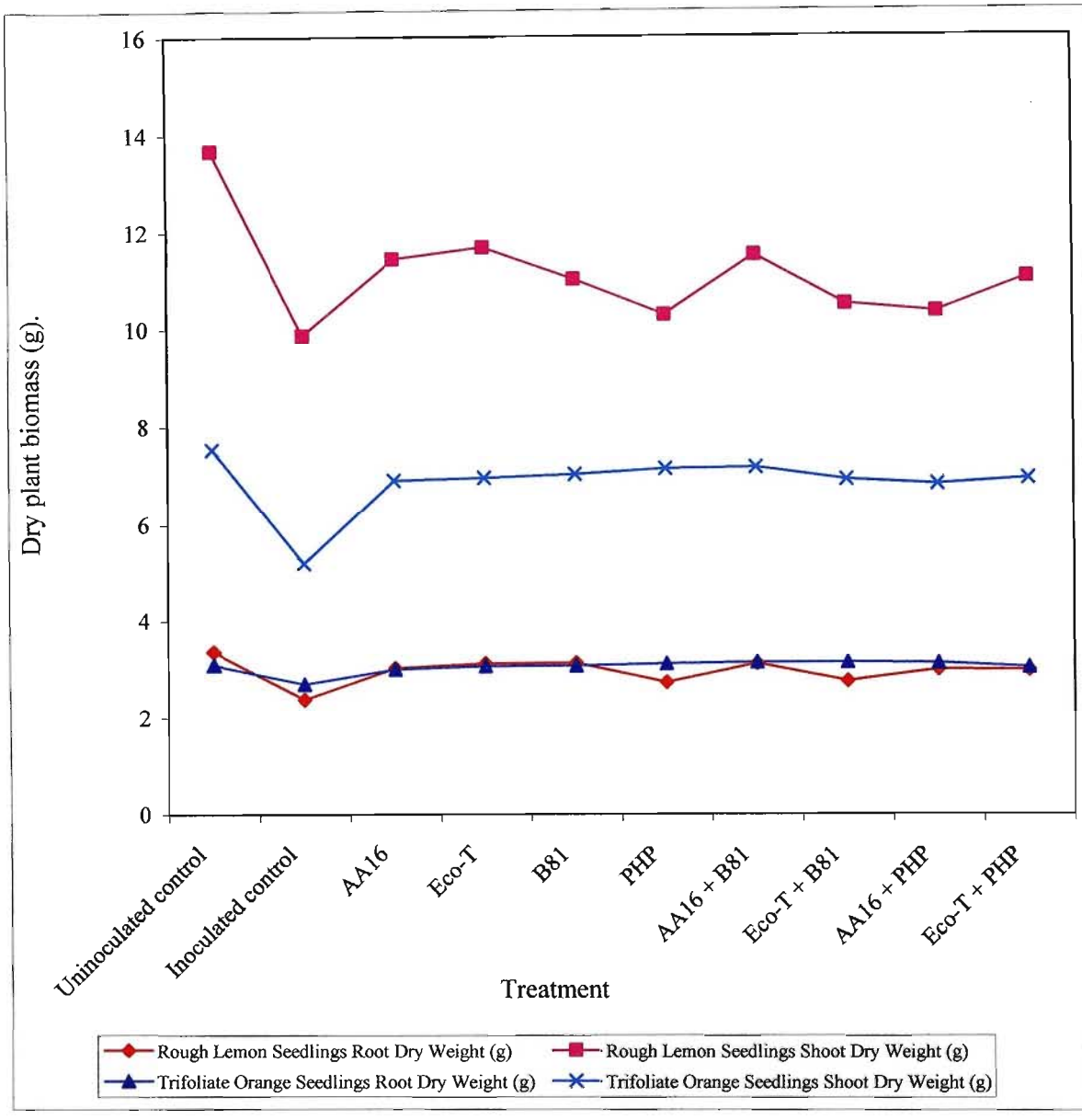

Figure 6.3.4 Response of dry weight (g) of rough lemon and trifoliate orange seedlings to treatment, combining *Trichoderma* with *Bacillus* isolates applied by drenching ($P < 0.05$).

6.4 DISCUSSION

Combining biocontrol strains has been suggested as an approach to enhance the level and consistency of control of soil-borne pathogens (Park *et al.*, 1988; Paulitz *et al.*, 1990; Lemanceau & Alabouvette, 1991; Pierson & Weller, 1994; Duffy & Weller, 1995). Increasing the diversity of biological control systems using mixtures of microorganisms may result in treatments that persist longer, are more stable in the rhizosphere and utilize a wider and more diverse spectrum of biocontrol mechanisms under a broader range of environmental conditions, which, in return, may suppress a broader range of pathogens (Pierson & Weller, 1994).

However, introducing combinations of biocontrol microorganisms does not always result in better and more consistent disease suppression, as was demonstrated by Dandurand & Knudsen (1993). Several biotic and abiotic conditions contribute to this inconsistent performance of biocontrol microorganisms (Weller, 1988). Insufficient colonization of the rhizosphere, limited tolerance to changes in environmental situations and changeable production of, or activities of, antifungal metabolites are among the most important factors (Pierson & Weller, 1994; Duffy *et al.*, 1996). Antagonism between indigenous microbial population and a biocontrol agent or between biocontrol agents applied in a mixture can also influence the performance of biocontrol agents in the rhizosphere. For example, competition for a limited carbon source in the soil can influence the result of root colonization (Raaijmakers *et al.*, 1995) and consequently disease suppression. A few strategies have been suggested to reduce variability in biological control and to increase efficacy of biological control agents:

1. Combining biocontrol agents with other measures that are less affected by the external conditions (Elad & Zimand, 1993).
2. Developing biocontrol agents that are effective under a wide range of biotic and abiotic conditions. This option appears to be difficult to achieve because biocontrol agents are living organisms that, like the pathogens, are affected by the environment.

3. Applying numerous biocontrol agents simultaneously provided they possess different ecological requirements for survival, growth, and activity (Guetsky *et al.*, 2001).

It has been demonstrated that a positive correlation exists between population size of the biocontrol agents on roots and disease reduction (Bull *et al.*, 1991; Johnson, 1994; Montesinos & Bonaterra, 1996; Smith *et al.*, 1997). The rhizosphere population density of the biocontrol agent has to attain a threshold level before reduction of disease occurs. It is possible that in combinations of biocontrol agents this threshold level is not attained by one or both agents, due to negative interactions between agents, and therefore enhanced disease suppression cannot be expected. Therefore, several authors have suggested that mixtures of introduced biocontrol agents have to be compatible in order to establish better and more reliable disease suppression (Baker, 1990; Janisiewicz & Bors, 1995; Raaijmakers *et al.*, 1995; Janisiewicz, 1996).

In this experiment the *in vitro* interaction of the two *Trichoderma* isolates tested was compatible, and no inhibition zones (Figure 6.3.1.A. The compatibility of *Trichoderma* isolates with *Bacillus* isolates was also tested *in vitro* and all *Trichoderma* and *Bacillus* isolates were also found to be compatible (Figure 6.3.1.B-C). These results are in agreement with the suggestions made on different research findings on compatibility of biocontrol agents (Baker, 1990; Janisiewicz and Bors, 1995; Raaijmakers *et al.*, 1995; Janisiewicz, 1996; Raupach & Kloeppers, 1998). Whether this had a predictive value for interactions *in vivo* remains to be investigated.

Results from trial showed that the parameter of cutting RDW did not reflect significant differences a result of different treatments. Hence, it was not useful in ascertaining success of biological control activity, in the *in vivo* combination of two *Trichoderma* isolates of rough lemon cuttings, whereas the SDW was.

Combination of AA16 with Eco-T®, which were compatible *in vitro* (Figure 6.3.1.A), caused better disease suppression on rough lemon cuttings as demonstrated by NFDSW compared to

AA16 alone but was not different from Eco-T® alone. However, a combination of AA16 and Eco-T® caused significantly lower disease suppression compared to AA16 or Eco-T® alone as shown by root the RDW of sour orange seedlings (Table 6.3.1). A combination of AA16 and Eco-T® caused no effect on dry weight of rough lemon and trifoliate orange seedlings. It seems that the disease suppression activity of AA16 or Eco-T® was diminished by their combined use. This result is in agreement with a previous suggestion by Schisler *et al.* (1997) that a mixture of biocontrol agents improves the activity on one host but may be antagonistic on a different host.

Although *Trichoderma* Isolates AA16 and Eco-T® were compatible *in vitro*, combining these isolates did not cause better disease suppression as compared to the single compatible *Trichoderma* Isolate AA16 or Eco-T® in rough lemon seedlings (Table 6.3.2, Figure 6.3.1.A). Such lack of correlation between *in vitro* and *in vivo* antagonism was demonstrated through mixing fluorescent *Pseudomonads* in wheat by Pierson & Weller (1994).

The combination of AA16 and Eco-T® isolates at higher concentrations (1 X 10^6 spores / ml) caused disease suppression of rough lemon cuttings but did not suppress disease of the tested seedlings (Table 6.3.2).

Trichoderma and *Bacillus* isolates were found to be compatible *in vitro* (Figure 6.3.1.B-C). The *Bacillus* Isolate PHP significantly lowered disease compared to *Trichoderma* Isolates AA16 or Eco-T® or *Bacillus* Isolate B81 when applied individually on rough lemon seedlings (Table 6.3.3). Combinations of AA16 and B81 did not result in better disease suppression (Table 6.3.2). These results agree with reports by Dandurand & Knudsen (1993) that showed that combinations of *P. fluorescens* 2-79 and *T. harzianum* ThzID1 neither inhibited nor enhanced biocontrol activity of the latter agent against root rot of pea caused by *Aphanomyces euteiches* f. sp. *pisi*. Combinations of AA16 and PHP or Eco-T® and PHP significantly resulted in lower disease suppression compared to AA16 or Eco-T® alone on dry root weight of rough lemon seedlings (Table 6.3.3). It appears that disease suppression activity of AA16 or Eco-T® is reduced by the presence of PHP.

The compatible combinations of Eco-T® and B81 significantly caused lower disease suppression compared to the single isolates on rough lemon RDW and SDW (Table 6.3.3). Thus, the unimproved disease suppression by combinations of Eco-T® and B81 was not consistent with their *in vitro* compatibility.

Single isolates or combinations of *Trichoderma* and *Bacillus* isolates on trifoliate orange seedlings did not cause significant control of *Phytophthora* root rot compared with their performance on rough lemon seedlings. This suggests that the response of the combination of biocontrol agents is host specific as suggested by Schisler *et al.* (1997) when two strain mixtures were used on biological control of *Fusarium* dry rot of potatoes.

Trichoderma isolates AA16 (5×10^5) and Eco-T (5×10^5) as shown in Tables 6.3.2 resulted in better performance than the uninoculated control in the RDW of sour oranges. This could have been due to contamination of treatments from inoculated controls. Such contamination could occur by insect vectors such as fungus gnats. Harris (1993) stated that there is increasing evidence to support the role of fungus gnats in disseminating fungal spores and many fungi with which these insects are associated are serious plant pathogens.

Overall, the findings of this experiment showed that combinations of *Trichoderma* isolate with other *Trichoderma* isolate (Figure 6.3.2) or combinations of *Trichoderma* isolate with *Bacillus* isolates (Figure 6.3.3) did not improve disease suppression *in vivo*, although they showed compatibility *in vitro*. Some combinations of *Trichoderma* and *Bacillus* isolates caused significantly poorer performance than their individual application.

These results suggest that the best strategy is to develop single isolate products. It is also more feasible from an economic point of view as a biocontrol product composed of mixtures of spp. has drawbacks, because producing and registering such a product will be more costly than a product composed of a single isolate (Schisler *et al.*, 1997).

6.5 REFERENCES

ABD-EL MOITY, T.H., PAPAVIZAS, G.C. & SHATLA, M.N. (1982) Induction of new isolates of *Trichoderma harzianum* tolerant to fungicides and their experimental use for control of white rot of onion. *Phytopathology* **72**, 396-400.

ADEGUMO, T.O., IKOTUN, T. & FLORINI, D.A. (1999) Biological control of *Protomycopsis phaseoli*, the causal agent of leaf smut of cowpea. *Journal of Phytopathology* **147**, 371-375.

ANDREWS, J.H. (1983) Future strategies for integrated control, in *Challenging Problems in Plant Health* (KOMMEDAHL, T. & WILLIAMS, P.H., Eds.). APS Press, St Paul, U.S.A.

BAKER, R. (1990) An overview of current and future strategies and models for biological control, *in Biological Control of Soil-bornePlant Pathogens* (HORNBY, D., COOK, R.J., HENNIS, Y., KO, W.H., ROVIRA, A.D. SCHIPPERS, B., SCOTT, P.R., Eds.). CAB International, Wallingford, UK, pp. 375-388.

BAKER, R. (1991) Diversity in biological control. *Crop Protection* **10**, 85-94.

BUDGE, S.P., MCQUILKEN, M.P., FENLON, J.S. & WHIPPS, J.M. (1995) Use of *Coniothyrium minitans* and *Gliocladium virens* for biological control of *Sclerotinia sclerotiorum* in glasshouse lettuce. *Biological Control* **5**, 513-522.

BULL, C.T., WELLER, D.M. & THOMASHOW, L.S. (1991) Relationship between root colonization and suppression of *Gaeumannomyces graminis var. tritici* by *Pseudomonas fluorescens* Strain 2-79. *Phytopathology* **81**, 954-959.

COOK, R.J. & BAKER, K.F. (1983) *The nature and practice of biological control of plant pathogens.* APS Press, St. Paul. Minnesota. USA.

DANDURAND, L.M. & KNUDSEN, G.R. (1993) Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermoshere and rhizosphere of pea. *Phytopathology* **83**, 265-270.

DATNOFF, L.E., NEMEC, S. & PERNEZNY, K. (1995) Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Gliocladium interaradices*. *Biological Control* **5**, 427-431.

DE BOER, M., VAN DER SLUIS, I., VAN LOO, L.C. & BAKKER, P.A.H.M. (1999) Combining *Fluorescent pseudomonas* spp. strains to enhance suppression of *Fusarium* wilt of radish. *European Journal of Plant Pathology* **105**, 201-210.

DUFFY, B.K. & WELLER, D.M. (1995) Use of *Gaeumannomyces graminis* var. *graminis* alone and in combination with fluorescent *Pseudomonas* spp. to suppress take-all of wheat. *Plant Disease* **79**, 907-911.

DUFFY, B.K., SIMON, A. & WELLER, D.M. (1996) Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. *Phytopathology* **86**, 188-194.

ELAD, Y. & ZIMAND, G. (1993) Use of *Trichoderma harzianum* in combination or alternation with fungicides to control cucumber grey mould (*Botrytis cinerea*) under commercial greenhouse conditions. *Plant Pathology* **42**, 324-332.

ELAD, Y., KATAN, J. & CHET, I. (1980) Physical, biological and chemical control integrated for soil-borne diseases in potatoes. *Phytopathology* **70**, 418-422.

FUKUI, R., SCHROTH, M.N., HENDSON, M. & HANCOCK, J.G. (1994) Interaction between strains of Pseudomonads in sugar beet spermospheres and their relationship to pericarp colonization by *Pythium ultimum* in soil. *Phytopathology* **84**, 1322-1330.

GUETSKY, R., SHTIENBERG, D., ELAD, Y. & DINOOR, A. (2001) Combining biocontrol agents to reduce variability of biological control. *Phytopathology* **91**, 621-627.

GUETSKY, R., SHTIENBERG, D., ELAD, Y., FISCHER, E. & DINOOR, D. (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology* **92**, 976-985.

HARMAN, G.E. (2000) Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease* **84**, 377-393.

HARMAN, G.E., CHET, I. & BAKER, R. (1981) Factors affecting *Trichoderma harzianum* applied to seeds as a biocontrol agent. *Phytopathology* **71**, 569-572.

HARRIS, M. (1993) Fungus gnats: They are more than just a nuisance. *Grower Talks* **56**, 49-59.

HUBBARD, J.P., HARMAN, G.E. & HADAR, Y. (1983) Effects of soil-borne *Pseudomonas* spp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. *Phytopathology* **73**, 655-659.

JANISIEWICZ, W.J. & BORS, B. (1995) Development of a microbial community of bacterial and yeast antagonists to control wound-invading postharvest pathogens of fruits. *Applied and Environmental Microbiology* **61**, 3261-3267.

JANISIEWICZ, W.J. (1988) Biocontrol of postharvest diseases of apples with antagonist mixtures. *Phytopathology* **73**, 655-659.

JANISIEWICZ, W.J. (1996) Ecological diversity, niche overlap, and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. *Phytopathology* **86**, 473-479.

JOHNSON, K.B. (1994) Dose-response relationships and inundative biological control. *Phytopathology* **84**, 780-784.

JOHNSON, K.B., STOCKWELL, V.O., MCLAUGHLIN, R.J., SUGAR, D., LOPER, J. E. & ROBERTS, R.G. (1993) Effects of antagonistic bacteria on establishment of honey bee-dispersed *Erwinia amylovora* in pear blossoms and on fire blight control. *Phytopathology* **83**, 995-1002.

KATAN, J. (1981) Solar heating (solarization) of soil for control of soil-bornepests. *Annual Review of Phytopathology* **19**, 211-236.

KWOK, O.C.H., FAHY, P.C., HOITINK, H.A.J. & KUTER, G.A. (1987) Interactions between bacteria and *Trichoderma hamatum* in suppression of *Rhizoctonia* damping-off in bark composted media. *Phytopathology* **77**, 1206-1212.

LEEMAN, M., DEN OUDEN, F.M., VAN PELT, J.A., CORNELLISSEN, C., MATAMALA-GARROS, A., BAKKER, P.A.H.M., & SCHIPPERS, B. (1996) Suppression of *Fusarium* wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. *European Journal of Plant Pathology* **102**, 21-31.

LEIBINGER, W., BEUKER, B., HAHN, M. & MENDGEN, K. (1997) Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. *Phytopathology* **87**, 1103-1110.

LEMANCEAU, P. & ALABOUVETTE, C. (1991) Biological control of *Fusarium* diseases by fluorescent *Pseudomonas* and nonpathogenic *Fusarium*. *Crop Protection* **10**, 279-2863.

LEWIS, J.A. & PAPAVIZAS, G.C. (1980) Integrated control of *Rhizoctonia* fruit rot of cucumber. *Phytopathology* **70**, 85-89.

LI, DE..M. & ALEXANDER, M. (1988) Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. *Plant and Soil* **108**, 211-219.

LOCKE, J.C., MAROIS, J.J. & PAPAVIZAS, G.C. (1984) Biological control of *Fusarium* wilt of greenhouse-grown chrysanthemums. *Plant Disease* **69**, 167-169.

MAZZOLA, M., FUJIMOTO, D.K., THOMASHOW, L.S. & COOK, R.J. (1995) Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by fluorescent *Pseudomonas* spp. and effect on biological control of take-all of wheat. *Applied and Environmental Microbiology* **61**, 2554-2559.

MEW, T.W., ROSALES, A.M. & MANINGAS, T.W. (1994) Biological control of *Rhizoctonia* sheath blight and blast of rice, in *Improve Plant Production with Rhizosphere Bacteria, Proceedings of the third International Workshop on Plant Growth Promoting Rhizobacteria* (RYDER, M.H., STEPHENS, P.M., BOWEN, G.D., (Eds.). Adelaide, South Australia.

MONTESINOS, E. & BONATERRA, A. (1996) Dose-response models in biological control of plant pathogens: An empirical verification. *Phytopathology* **86**, 464-472.

PAPAVIZAS, G.C. & LEWIS, J.A. (1988) The use of fungi in integrated control of plant disease, in *Fungi in Biological Control Systems* (BURGES, M.N., Ed.). Manchester University Press, Manchester, UK.

PAPAVIZAS, G.C., LEWIS, J.A. & ABD-EL MOITY, T.H. (1982) Evaluation of new biotypes of *Trichoderma harzianum* for tolerance to benomyl and enhanced biocontrol capacities. *Phytopathology* **72**, 126-132.

PARK, C., PAULITZ, T.C. & BAKER, R. (1988) Biocontrol of wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic i solates of *Fusarium oxysporum*. *Phytopathology* **78**, 190-194.

PAULITZ, T.C., AHMAD, J.S. & BAKER, R. (1990) Integration of *Pythium nunn* and *Trichoderma harzianum* isolate T-95 for the biological control of Pythium damping-off of cucumber. *Plant and Soil* **121**, 243-250.

PIERSON, E.A. & WELLER, D.M. (1994) Use of mixtures of fluorescent *Pseudomonads* to suppress take-all and improve the growth of wheat. *Phytopathology* **84**, 940-947.

RAAIJMAKERS, J.M., VAN DER SLUIS, I., KOSTER, M., BAKKER, P.A.H.M., WEISBEEK, P.J. & SCHIPPERS, B. (1995) Utilization of heterologous siderophores and rhizosphere competence fluorescent *Pseudomonas* spp. *Canadian Journal of Microbiology* **41**, 126-135.

RAHE, J.E. & UTKHEDE, R.S. (1985) Integrated biological and chemical control of sclerotial p athogens, in *Ecology and Management of Soil-bornePlant Pathogens* (PARKER, M., Ed.). APS Press, St. Paul, Minnesota, USA.

RAUPACH, G.C. & KLOEPPER, J.W. (1998) Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**, 1158-1164.

ROVIRA, A.D. (1965) Interactions between plant roots and soil microorganisms. *Annual Review of Micrbiology* **19**, 241-266.

ROVIRA, A.D. (1969) Plant root exudates. *Botanical Review* **35**, 35-57.

SCHISLER, D.A., SLININGER, P.J. & BOTHAST, R.J. (1997) Effects of antagonist cell concentration and two strain mixtures on biological control of *Fusarium* dry rot of potatoes. *Phytopathology* **87**, 177-183.

SMITH, K.P., HANDELSMAN, J. & GOODMAN, R.M. (1997) Modelling dose-response relationships in biological control: Partitioning host response to the pathogen and biocontrol agent. *Phytopathology* **87**, 720-729.

SNEH, B., DUPLER, M., ELAD, Y. & BAKER, R. (1984) Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from a Fusarium-suppressive soil. *Phytopathology* **74**, 1115-1124.

STEEL, R.G.D. & TORRIE, J.H. (1980) *Principles and procedures of statistics*: a biometrical approach, 2nd ed., McGraw-Hill, inc., New York, USA.

STOCKWELL, V.O., JOHNSON, K.B. & LOPER, J.E. (1996) Compatibility of bacterial antagonists of *Erwinia amylovora* with antibiotics used to control fire blight. *Phyotpathology* **86**, 834 -840.

WAECHTER-KRISTENSEN, B., GERTSSON, U.E., SUNDIN, P. & SERRA, G. (1994) Prospects for microbial stabilization in the hydroponic culture of tomato using circulating nutrient solution. *Acta Horticulturae* **361**, 382-387.

WAISEL, Y. ESHEL, A., & KAFKAFI, U. (1991) Plant Roots: *The Hidden Half*, New York, USA.

WELLER, D.M. (1988) Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* **26**, 379-407.

CHAPTER 7

GENERAL OVERVIEW

Biological control of soil-borne plant pathogens by introduced microorganisms has been studied for over 65 years (Baker, 1987). Currently there is a shift towards the opinion that biological control can have an important role in agriculture, such as suppression of plant diseases as well as increased plant development. *Trichoderma* and *Bacillus* spp. are among the useful biocontrol agents in plant disease control and growth promotion. Commercial products of these genera are available (Koch, 1999). It is also encouraging that a number of companies presently have programs to develop biological control agents as commercial products. The product Eco-T®, included in this research is one such a product with markets not only in South Africa but also in several East African countries such as Kenya, Uganda, Tanzania and Ethiopia.

The findings presented in this thesis are the result of evaluations of *Trichoderma* and *Bacillus* isolates and their combinations, for biological control of citrus seedlings and cuttings, and growth stimulation on citrus seedlings. Trials were carried out under greenhouse conditions and the drenching method was used as the procedure of application. It was established that:

1. During *in vitro* dual culture bioassays of *Trichoderma* or *Bacillus* and *Phytophthora* root rot of citrus 19 of the 23 *Trichoderma* showed antagonistic activity by hyperparasitism and four of the eight *Bacillus* isolates exhibiting antagonism (by forming inhibition zone), respectively. All isolates were tested *in vivo* without discarding those with poor performance from the *in vitro* trial. For example, *Trichoderma* Isolate AA16, regardless of it's Bell rating of 3 in the *in vitro* test showed good *in vivo* antagonism against *Phytophthora parasitica*. Similar observations were made by Utkhede & Sholberg (1986) on postharvest cherry diseases. Utkhede *et al.* (1999) stated that *in vitro* results could only give an indication as to what the results may be *in vivo*. Furthermore, poor results *in vitro* do not necessarily mean the agents will not be effective *in vivo*.

2. In the *in vitro* test, *Bacillus* isolates showed inhibition zones while *Trichoderma* showed overgrowth of *Phytophthora* sp., i.e., two different effects were noted. Therefore, a detailed study on the mode of actions of the biocontrol agents would increase our understanding of the mode of action of these organisms.
3. In the greenhouse study, the *Bacillus* isolates and some *Trichoderma* isolates (AA5, AA12, AA16, SY3F and Eco-T) suppressed *Phytophthora* root rot disease. *Trichoderma* isolates were more effective than *Bacillus* isolates. However, the *Trichoderma* isolates with a good level of disease control did show stunting of the seedlings in the absence of disease when the same dose of (5×10^5 spores / ml) of the same *Trichoderma* biocontrol agents were applied. This was found in later work to be a dose response. According to our results higher doses than 1×10^4 spores / ml caused stunting, even though the response of the *Trichoderma* concentrations was found to be host dependent. Rabeendran *et al.* (2000) suggested that the enhanced growth stimulation could best be achieved under sub-optimal growth conditions, while our work was done under optimal conditions.

4. A test for sporulation capacity of *Trichoderma* isolates may give an initial indication as to establishment and proliferation of the isolates in the growth medium rhizosphere, although it will be affected by the environment to which it is introduced. The sporulation test may also provide information on commercial implications in terms of manufacturing and economics. In this work it was found that *Trichoderma* Isolate AA12, which showed best *in vitro* disease suppression, resulted in the worst sporulation capacity and was discontinued for further tests. Therefore, in this kind of work it is important to start with a larger number of isolates in order to discover satisfactory potential products for commercial development and use.

5. The compatibility of the *Trichoderma* isolate with other *Trichoderma* spp. as well as the compatibility of *Trichoderma* isolates with *Bacillus* isolates *in vitro* led to their combined use *in vivo*. In greenhouse trials, the dual application of *Trichoderma* isolates with other *Trichoderma* isolates or the *Trichoderma* isolate with the *Bacillus* isolate did not enhance suppression of *Phytophthora* root rot of citrus rootstock

seedlings compared to their individual applications. This result suggests the use of a single isolate product, which may also be more feasible from an economic point of view. Schisler *et al.* (1997) suggested that producing and registering a biocontrol product composed of mixtures of spp. would be more costly than a product composed of a single isolate.

Trichoderma and *Bacillus* isolates resulted in good levels of *Phytophthora* root rot control of citrus seedlings and cuttings, and growth promotion of rootstock seedlings.

The use of such biocontrol agents without chemical pesticides, as demonstrated in this study, will be of interest to the growing organic crop industry and to the environment.

Recommendations:

- Assessment of the biological control activity of *Trichoderma* and *Bacillus* and their population establishment and proliferation in relation to the *Phytophthora* population would be of interest to study. The information would aid recommendations on the dose and frequency / ies of application / s.
- Studies on growth promotion were done under optimal conditions in this study. Work under sub-optimal conditions would be important to obtain a better understanding of the effects on nutrient elements uptake and other plant development parameters such as leaf area index.
- Field evaluation of combinations of the *Trichoderma* strain as well as *Trichoderma* and *Bacillus* strains would be of importance if greater emphasis on developing mixtures of biocontrol agents is needed. Such combinations may result in better plant colonization, be better adapted to environmental changes that occur throughout the growing season, result in a larger number of pathogen suppressive mechanisms or protect against a broader range of pathogens (Raupach & Kloepper, 1998).
- Biocontrol agents providing a good level of *Phytophthora* root rot control may be tested as curatives to already existing problems.

- Integration of biocontrol agents with proper crop husbandry activities, e.g., optimum irrigation, good quality irrigation water, fertilization, mulching may have a long lasting beneficial effect for the crops and subsequently the growers.
- During the course of this study, it was observed that seeds had a 60-65% germination rate. It would be interesting in the future work to include seed treatment with biocontrol agents and study on their influence on germination.
- Screen for *Trichoderma* isolates that exhibit plant growth promoting properties.
- Combine growth promoters with effective biological control agents.

REFERENCES

BAKER, K.F. (1987) Evolving concepts of biological control of plant pathogens. *Annual Review of Phytopathology* **25**, 67-85.

KOCH, E. (1999) Evaluation of commercial products for microbial control of soil-borne plant diseases. *Crop Protection* **18**, 119-125.

RABEENDRAN, N., MOOT, D.J., JONES, E.E. & STEWART, A.A. (2000) Inconsistent growth promotion of cabbage and lettuce from *Trichoderma* isolates. *New Zealand Journal of Plant Protection* **53**, 143-146.

SCHISLER, D.A., SLININGER, P.J. & BOTHAST, R.J. (1997) Effects of antagonist cell concentration and two-strain mixtures on biological control of *Fusarium* dry rot of potatoes. *Phytopathology* **87**, 177-183.

UTKHEDE, R.S. & SHOLBERG, P.L. (1986) *In vitro* inhibition of plant pathogens by *Bacillus subtilis* and *Enterobacter aerogenes* and *in vivo* control of two postharvest cherry diseases. *Canadian Journal of Microbiology* **32**, 963-967.

UTKHEDE, R.S., KOCH, C.A. & MENZIES, J.G. (1999) Rhizobacterial growth and yield promotion of cucumber plants inoculated with *Pythium aphanidermatum*. *Canadian Journal of Plant Pathology* **21**, 265-271.