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Biological control of *Pythium* wilt and root rot in hydroponically grown lettuce

By

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Submitted to the Faculty of Natural and Agriculture Sciences

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In partial fulfillment of the requirements for

the degree of M.Sc.

UNIVERSITY OF PRETORIA

PRETORIA OCTOBER 2005

ACKNOWLEDGEMENTS

I sincerely thank the following individuals and institutions that made this study possible:

Prof. Nico Labuschagne, my supervisor, for his valuable advice, guidance and support throughout this study.

The University of Pretoria, Hydrotech and Biological Control Products for financial support of the project.

Hydrotech for technical suppport and providing lettuce seedlings for the *in vivo* experiments.

Marie Smith for assistance and advice on statistical analysis of data.

My parents for making it all possible through their constant encouragement, advice, love and support. I dedicate this thesis to them.

All the students in the Plant Pathology division of the department for their friendship and eagerness to lend a helping hand

My Lord and Saviour in whom I trust.

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BIOLOGICAL CONTROL OF *PYTHIUM* WILT AND ROOT ROT IN HYDROPONICALLY GROWN LETTUCE

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RESUMè

A number of techniques were used to obtain a variety of bacterial and fungal species antagonistic to *Pythium* - F group in hydroponic systems. Isolations were made from roots of 'escape' lettuce plants in a commercial hydroponic gravel system as well as *Pythium* mycelium exposed to the hydroponic solution. Seventy four bacterial and eighteen fungal isolates were obtained and were screened for *in vitro* activity against *Pythium* by means of the dual culture method. Twenty-two bacterial isolates rendered between 10.8 and 48 % inhibition and ten fungal isolates rendered between 24.3 and 54 % inhibition of *Pythium* mycelial growth.

Potential biocontrol agents were screened in a static aquaculture system on butterhead lettuce seedlings in the greenhouse prior to evaluation in a re-circulating gravel bed hydroponic system in the greenhouse and field, for both growth promoting and biocontrol ability. Significant increases of between 689 % and 922 % in total fresh yield were obtained from plants preventatively treated with isolates JH49, JH41, JH83, JM6R and JM16W. The eight best performing isolates were further evaluated for biocontrol activity against *Pythium* as well as growth promotion on butter head lettuce in a re-circulating

gravel bed hydroponic system in the greenhouse. Significant increases of 1.5 % - 63.5 % and 0.9 % - 38.8 % in total fresh yield were obtained from plants evaluated for growth promotion and *Pythium* control, respectively. Based on their performance five of the eight isolates were selected for evaluation in a re-circulating gravel bed hydroponic field system. Treatment with *Pseudomonas fluorescens* and *Bacillus subtilis* significantly increased fresh leaf weight of lettuce plants in comparison with the untreated control indicating effective suppression of *Pythium*.

Of the isolates that were previously evaluated against *Pythium* wilt and root rot of lettuce in a hydroponic system (Chapters 2 and 3), 6 bacteria and 2 fungi were most effective. The following possible modes of action of these isolates, were investigated, namely competition, production of inhibitory substances and induced resistance. The root colonizating ability of the isolates was also assessed. Competition between the isolates and the pathogen were confirmed by testing for siderophore and hydrolytic enzyme production. Five of the isolates produced siderophores much faster than the rest, demonstrating that these isolates were able to take-up iron from the media at a faster rate, thus indicating a significant competitive ability. Antibiotic production by the isolates was confirmed *in vitro* by means of the dual culture technique. Of the eight isolates screened, only one isolate showed *in vitro* inhibition of the pathogen. This result was confirmed by a TLC assay, where fluorescent bands were formed by the same isolate, indicating the presence of phenolic compounds. These compounds were separated by HPLC. Analysis of total soluble and cell wall phenolic levels in *Pythium* infected and non-infected plants treated and untreated with the biocontrol isolates did not render conclusive results. Three of the eight isolates were able to colonize 100% of the lettuce roots.

CHAPTER 1

GENERAL INTRODUCTION

Lettuce (*Lactuca sativa L.*) is, according to Valenzuela *et al.* (2002), the most popular amongst the salad vegetable crops. This crop, a member of the sunflower or Compositae Family, is native to the Mediterranean Basin. Leafy types have been cultivated for over 2,500 years. Lettuce was grown by ancient Greeks, and later the Moors developed different varieties. Lettuce is low in nutrients and energy. One kilogram of lettuce contains 95% water, 56 calories, 3.9 g protein, 0.3 g fat, 0.086 g calcium, 0.022 g iron, 0.0014 g vitamin A and 0.054 g ascorbic acid (Valenzuela *et al.*, 2002)

Hydroponic lettuce production involves intensive cultivation practices, which result in a high value and quality product (Valenzuela *et al.*, 2002). These growing operations are mostly conducted in greenhouses. Leafy and semi-head cultivars are grown and are usually planted at a density of 20 plants per square meter (m^2). One to three week-old seedlings are transplanted and the time from transplant to harvest ranges from 4 to 7 weeks. High technology systems exist wherein the nutrient solution is aerated or circulated and where there is precise control of the nutrient solution.

Globally there has been a growing interest in the use of hydroponic or soilless techniques for producing greenhouse horticultural crops. While most vegetable crops have traditionally been grown in soil, the hydroponic production processes have increased over the past twelve years (Jensen, 1991).

Hydroponics by definition means “water-working”. In practical use it means growing plants without soil, in a water and nutrient solution with a suitable inert medium if needed. These soilless media offer the potential of higher yields and quality, better control of nutrients, and reduction of soil-borne pathogens associated with soil media (Paulitz *et al.*, 1992). Hydroponics thus allows more efficient production of plants.

By growing plants hydroponically it can be ensured that every plant gets its exact requirements of water and nutrients in order to cultivate healthy plants. The most commonly grown hydroponic crops are cucumbers, flowers, tomatoes, peppers and lettuce. As a cultivation system, hydroponics is environmentally friendly and has become a well-established plant growing system (Jensen, 1991).

According to Stanghellini and Rasmussen (1994), the motivating force underlying the development of hydroponics was the more efficient production procedure and the avoidance of root diseases. Cultivation in hydroponics has resulted in a decrease in root-infecting microorganisms although root diseases still occur and at times can result in losses greater than in soil (Stanghellini and Rasmussen, 1994). Paulitz *et al.* (1992) states that the absence or low levels of other competing organisms in soilless systems favour pathogenic organisms such as *Pythium* species. This pathogen is a classic pioneer colonizer, which do not compete well with other microbes (Paulitz *et al.*, 1992).

Most of the devastating root diseases in the 800 hectares of hydroponically grown crops in South Africa, especially re-circulating systems, are caused by *Pythium* and *Phytophthora* species (Gull, 2003; Lewis, 1998; Thompson and Labuschagne, 2001). This is particularly true in surveys performed in South Africa by Labuschagne *et al*. (2002), and Gull *et al*. (2004), which confirmed the presence of pathogenic *Pythium* species on various hydroponically grown crops in South Africa. They identified *Pythium* groups F, HS, and T, and *Pythium irregulare* as the main *Pythium* spp. attacking lettuce. From all the species/groups isolated by Gull (2003), *Pythium* F- group was isolated most frequently from the greatest variety of crops and proved to be the most pathogenic. Most of the hydroponic farms in South Africa have some degree of *Pythium* infection, which have, in some cases, caused up to 60 % crop losses (A.H. Thompson – personal communication).

Re-circulating nutrient systems create an ideal environment for *Pythium* species to spread and infect roots and stems causing major losses for the

farmer (Gold and Stanghellini, 1985). *Pythium* can be introduced into hydroponic systems from infested water sources, contaminated soil, dirty tools, infected plant material or naturally infested peat-based propagation media. Once introduced into the system, their control is very difficult and sometimes the grower is forced to destroy the whole crop (Rankin and Paulitz, 1994). It was found by Menzies *et al.* (1996), that all *Pythium* species, except *Pythium* HS Group, *Pythium parvum* and *Pythium intermedium* develop zoospores and spread through the hydoponic system by means of the circulating nutrient solution. These zoospores are flagellated asexual swimming spores that can procreate easily in warm conditions and readily move in water enabling the pathogen to transmit its spores very quickly throughout the hydroponic system and infect the roots of plants (Paulitz, 1997). Zoospores have been implicated as the primary life stage responsible for the spread of the pathogen and for finding the infection sites (Stanghellini and Miller, 1997). As soon as zoospores are released into the hydroponic nutrient solution, they have limited time to infect a susceptible plant. The zoospores are attracted to germinating seeds or roots where they encyst and penetrate the host. The spores will remain viable as long as the moisture content and temperature are favourable (Martin and Loper, 1999).

Pythium cause pre-emergence and post-emergence damping-off on a wide range of crops. According to Boland (2004) and Paulitz (1997), the main symptoms include zones of root-tip browning, stubbiness, proliferation of roots, expansive root browning or yellowing, seedling rots, stem rotting, wilting at mid-day and collapse of the plant if the pathogen moves into the crown region. *Pythium* also infect mature plants and cause necrotic lesions on fine feeder roots and root tips, which the plant need for efficient uptake of nitrogen and other nutrients. *Pythium* can infect roots of lettuce produced in hydroponic systems and significantly reduce plant growth and yield without developing symptoms (Stanghellini and Rasmussen, 1994; Uthede *et al*., 2000). It is therefore often difficult for producers to determine if their lettuce crop is suffering from *Pythium* root infection. Once introduced into the system, the control of these pathogens is very difficult and sometimes the grower is forced to destroy the crop (Martin and Loper, 1999).

The challenges for producers in managing these diseases are increasing. Consumer demand for year-round production of fresh vegetables with reduced or no pesticide / fungicides residue continues to grow following concerns over the potential impact of disease management on the environment and on consumer health (Punja and Uthede, 2003). It was stated by Cook and Baker (1983), that the most widely used control measure for suppressing soilborne diseases are the use of environmentally hazardous fungicidal treatment of seed, seedling or soils. Problems encountered when using these environmentally hazardous fungicides include development of pathogen resistance; inability of seed treated products to protect the roots of mature plants; rapid degradation of the chemicals and a requirement for repeated application. These factors have prompted producers to search for alternative methods to combat fungal diseases.

According to Sutton (1995), communities of indigenous microorganisms in cropping systems are vast and relatively unexploited reservoirs of antagonists that can suppress plant pathogens in developing crops. Efforts to improve the efficacy or consistency of biocontrol of *Pythium* damping-off are focused on the identification of superior antagonists by screening naturally occurring microorganisms (Sutton, 1995).

Cook and Baker (1983) provided the basic principle guiding the search for biocontrol agents: 'Look where the disease does not occur but should be expected because the disease occurs elsewhere in the area, all environmental conditions are favourable, and the pathogen has been introduced' (Paulitz, 1997).

Biocontrol systems are highly dynamic and can involve growth and development of the host, infection cycles and serial dispersal of the pathogen, quantitative shifts in populations of the biocontrol agents and indigenous organisms. Biocontrol agents are living organisms and are sensitive to fluctuations in environmental conditions such as temperature, moisture, pH, etc. (Paulitz, 1997). Microorganisms introduced into a crop to control a disease must therefore be able to interact appropriately with the pathogen, the

host, and other organisms under the prevailing microclimatic conditions. Potential antagonists should therefore be evaluated for disease suppression under a range of environmental conditions favouring disease, in order for the selected biocontrol agents to be effective under the full spectrum of field conditions where disease constrains crop production (Martin and Loper, 1999).

Pythium oligandrum and *Pythium nunn* are non-pathogenic and aggressive primary colonizers of organic matter. Repeated field evaluations in Florida showed certain isolates of *P. oligandrum* to significantly reduced damping–off on tomatoes caused by *Pythium ultimum* and *Pythium aphanidermatum* Martin and Semer, 1992)

Prevention of infection, reduction in colonization of host tissue, or reducing sporulation of the pathogen, can each provide a level of disease control through the use of biological control agents. Many antagonistic microorganisms exist naturally on or near plant surfaces as epiphytes or saprophytes, using nutrients available in the respective niches. Research to elucidate whether these organisms could potentially be used as biological control agents to combat diseases has intensified over the past 20 years and this has led to the commercial development of several registered microbial agents for vegetable crop disease control (Table 1). Following their initial discovery, the commercial development of these biological control agents is challenging. Information therefore needs to be acquired in terms of the products efficacy and mode(s) of action of the agent, as well as on the survival, spread and potential toxicity to non-target species (Punja and Uthede, 2003).

Biocontrol agents possess mechanisms that allow them to either cure or prevent disease development disrupting some stage of the life cycle of the pathogen (Elad, 2000; Brimner and Boland, 2003; Punja and Uthede, 2003). One of the strategies used to control pathogens is mycoparatism, where a fungus directly attacks and feeds on other fungi, resulting in the direct destruction or lysis of propagules and structures. The most widely studied

fungi in this regard are different species of *Trichoderma*. Biocontrol isolates of *Trichoderma harzianum* produced a number of different chitinases and glucanases in culture that degrade major components of cell walls of plant pathogenic fungi (Larkin *et al*., 1998; Elad, 2000; Zamir and Uthede, 2003).

Antibiosis refers to the destruction or inhibition of the competitive ability of the pathogen by a metabolic product of the antagonist, such as specific toxins, antibiotics or enzymes. This interaction can result in suppression of activity of the pathogen or destruction of its propagules (Larkin *et al*., 1998). Species of *Trichoderma* and *Gliocladium*, which are currently registered biological control products, are known to produce several antibiotics with broad-spectrum antimicrobial activity (Zamir and Uthede, 2003).

Nutrients from roots and seeds support microbial growth and other activities in the spermo - and rhizosphere (Bellows, 1999). Competition for resources such as carbon, nitrogen, iron or trace elements in soil environments is vital to the ability of any particular organism to increase in numbers and consequently to reduce the density or activity of other organisms, including plant pathogens (Elad and Chet, 1987). Root colonization (the ability to build populations on or around roots) is a reflection of the ability of microorganisms to compete for ecological niches in the highly competitive rhizosphere. Highly competitive biocontrol agents have the ability to rapidly colonize plant surfaces, creating an effective 'living barrier' to subsequent pathogen invasion. When present on site, the mechanism of antagonism might be competition for nutrients or space, siderophore production, antibiosis, production of hydrolytic enzymes or other active substances. According to Larkin *et al*. (1998) competition can be an effective biocontrol mechanism when the antagonist is present in sufficient quantities at the correct time and location and can utilize limited resources more efficiently than the pathogen.

Biocontrol agents may also induce plant physiological processes that lead to the activation of plant defense mechanisms such as the hypersensitive response, production of phytoalexins or synthesis of lytic enzymes such as chitinase and glucanase (Droby and Chalutz, 1994). Production of peroxidase,

a phenol oxidase enzyme that oxidise phenolics to quinones, has also been reported (Yedidia *et al*., 2000). Quinones are often more toxic to micro-organisms than the original phenols and release highly reactive free radicals, which increase the rate of polymerization of phenolic compounds into lignin like substances. These substances are often deposited in cell walls and papillae and interfere with further growth and development of pathogens (Bellows, 1999; Elad, 2000).

Another more indirect biocontrol mechanism is the mobilization of nutrients in the soil, a process that makes compounds in the soil more available for plant uptake, resulting in increased general health and disease resistance (Bellows, 1999).

In general, epidemics cannot be satisfactory simulated in the laboratory, growth chamber, or greenhouse, and biocontrol tests done under these conditions should be interpreted accordingly. Biocontrol tests under controlled conditions can serve effectively for use in preliminary screening of organisms and the subsequent evaluation in the field (Linderman *et al.*, 1983).

The purpose of this study was to select and evaluate potential antagonistic bacteria and fungi through *in vitro* and *in vivo* screening techniques for their ability to inhibit or reduce *Pythium* root rot of lettuce in hydroponic systems. Furthermore, the mode(s) of action of the most effective organisms were investigated.

Table 1. Commercially available biological control agents for suppression of soilborne diseases caused by *Pythium* spp. (Martin and Loper, 1999; McSpadden Gardener and Fravel, 2002).

PRODUCT	BIOCONTROL AGENT	TARGET PATHOGEN	HOST PLANT
Intercept (Soil Technologies, USA)	*Burkholderia cepacia*	*Pythium* and *Fusarium* spp. Nematodes	Cotton, maize, vegetables
Deny (CTT Corporation, CA)	*Burkholderia cepacia*	*Pythium* and *Fusarium* spp *Rhizoctonia solani*	Vegetables and field crops
Mycostop (Kemira Agro Oy, Finland)	*Streptomyces griseoviridis* K-61	*Pythium* spp *Fusarium* spp.	Ornamentals and vegetables
Primastop (Kemira Agro Oy, Finland)	*Gliocladium catenulatum*	*Pythium* spp.	Vegetables
Soilgard (Thermo Trilogy Corp., Columbia, MD)	*Gliocladium virens* GL-21	*Pythium ultimum* *Rhizoctonia solani*	Bedding Plants
Polygandrum (Vyskumny ustav rastinnej, Piestany Slovak Republic)	*Pythium oligandrum*	*Pythium ultimum*	Sugar beet
ANTI-FUNGUS (Grondortsmettingen De Ceuster, Belgium)	*Trichoderma* sp.	*Pythium* spp. and others	Various
TY (Mycontrol, Israel)	*Trichoderma* spp.	*Pythium* sp. *Rhizoctonia solani* *Sclerotium rolfsii*	Vegetables and field crops
F-Stop (BioWorks, USA)	*Trichoderma harzianum*	*Pythium ultimum* *Rhizoctonia solani* *Fusarium* spp.	Vegetables and field crops
Actinovate	*Streptomyces lydicus*	Soilborne diseases	Greenhouse and nursery crops
BioJect Spot-Less	*Pseudomonas aureofaciens*	Dollar spot Anthracnose *Pythium aphanidermatum* Michrochium patch	Turf and others
Companion	*Bacillus subtilis* *B.lichenformis* *B. megaterium*	*Rhizoctonia* *Pythium* *Fusarium* *Phytophthora*	Greenhouse and nursery
Deny	*Burkholderia cepacia*	*Rhizoctonia* *Pythium* *Fusarium*	Alfalfa,barley, beans, clover, cotton, peas, grain, sorghum, vegetables crops and wheat
Intercept	*Burkholderia cepacia*	*Rhizoctonia solani* *Pythium* sp. *Fusarium*	Maize, vegetables, cotton

Mycostop	***Streptomyces griseoviridis***	***Fusarium* spp.,*Alternaria brassicola, Phomopsis* spp.,*Botrytis* spp., *Pythium* spp., *and Phytophthora.***	**Field, ornamental and vegetable crops**
Primastop	***Gliocladium catenulatum***	**Soilborne pathogens that cause seed, root, stem rot**	**Ornamental, vegetables and tree crops**
Rootshield Plant Shield T-22 Planter box	***Trichoderma harzianum***	***Pytium* spp. *Rhizoctonia solani* *Fusarium* spp.**	**Tree, shrubs, transplants, all ornamentals, cabbage, tomato and cucumber**
Soilgard	***Gliocladium virens (a.k.a. Trichoderma virens)***	***Rhizoctonia solani* *Pythium* spp.**	**Ornamental and food crop plants grown grown in greenhouses, nurseries and homes**
T-22G and T-22HB (BioWorks, USA)	***Trichoderma harzianum***	***Pythium* spp. *Rhizoctonia solani* *Sclerotium homeocarpe* *Fusarium* spp.**	**Various**

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CHAPTER 2

ISOLATION AND *IN VITRO* SCREENING OF POTENTIAL BIOCONTROL AGENTS AGAINST *PYTHIUM* F- GROUP

1. ABSTRACT

A combination of techniques was used to obtain a maximum number and variety of bacterial and fungal species antagonistic to *Pythium* F-group in hydroponic systems. Isolations were made from roots of 'escape' lettuce plants in a commercial hydroponic gravel system and *Pythium* mycelium exposed to the hydroponic solution. Seventy four bacterial and eighteen fungal isolates were obtained and were screened for *in vitro* activity against *Pythium* by means of the dual culture method. Twenty-two bacterial isolates rendered between 10.8 and 48 % inhibition and ten fungal isolates rendered between 24.3 and 54 % inhibition of *Pythium* mycelial growth.

2. INTRODUCTION

The initial selection strategy of target sites to isolate potential antagonists from is crucial to ensure that the most effective biocontrol agent is obtained. In general, selecting antagonists from culture collections are not suitable because they will contain organisms adapted to high nutrient growth conditions and are unlikely to survive in nature (Campbell, 1986). Antagonists should also not be isolated from heavily diseased plants or crops, for there is nothing in the environment that is preventing the pathogen from affecting the plant (Campbell, 1986). The best source to obtain soilborne biocontrol agents is from places where the disease occur in patches and the spread has been restricted. Alternatively where apparently healthy plants occur amidst otherwise heavily diseased crops. The pathogen might be subjected to infections by mycoparasites, which may reduce disease incidence. Sampling sites should represent the conditions where the potential biocontrol agent will be used. They should also be in a similar climatic zone on the same or similar host plants and subject to normal agricultural practices (Campbell, 1986).

Knowledge of the environment in which biocontrol systems will function is critical to effectively understand the modes of action or factors that influence efficacy or product performance. The effectiveness of a microbial strain as a biocontrol agent will depend on the antagonists' ability to survive in the same ecological environment as the target pathogen. Occupation of the same niches, toleration of adverse conditions in the same manner, and multiplication under similar environmental conditions are of primary importance (Völksch and May, 2001).

According to Krauss (1996), it is easy to isolate potential biocontrol agents but these isolates must first be screened, discarding the majority and selecting the most promising strains. Field trials are the most realistic approach but it is expensive and can only be employed for a pre-selected number of antagonists. Campbell (1986), states that the first screening should be easy to conduct, cheap and rigorous. The technique should fail at least 99.9% of organisms and must be able to handle a large number of potential isolates to have a chance of success.

Effective bacterial and fungal biological control agents with activity against pathogenic fungi often synthesize a variety of antifungal metabolites and enzymes (Nautiyal, 1997). Two-component screening (e.g. dual cultures of a candidate antagonist and a pathogen on agar) is exclusively related to interaction studies. Potential antagonists are typically ranked according to their *in vitro* ability to inhibit the growth of the pathogen, which is evident through the formation of an inhibition zone on the agar (Knudsen *et al*, 1997).

In this chapter, different isolating techniques and *in vitro* dual culture assays were used to obtain and screen bacterial and fungal species antagonistic to *Pythium* F-group in hydroponic systems.

3. MATERIALS AND METHODS

3.1 Isolation of potential antagonists from the roots of 'escape' plants

Samples of butter head lettuce (*Lactuca sativa* L. var. *capitata* L (Nadine)) plants were obtained from a commercial gravel bed hydroponic system (Hydrotec (Pty) Ltd, Gauteng, South Africa). Ten healthy looking plants were randomly sampled

from symptomatic *Pythium* infected lettuce plants growing in gravel beds. Plants were separately placed in plastic bags and transported in iceboxes to the laboratories. From each lettuce plant 1 g of the roots was taken and placed into 9 ml sterile Ringer's solution (Merck) in a test tube. The tubes were vortexed for 10 min and the resulting suspension serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) before plating on nutrient agar (NA) (Merck) containing cycloheximide (10 ml per litre of a 0.1 % solution) (SIGMA) and Kings B medium (KB) (Biolab, Merck) for the isolation of bacteria and on potato dextrose agar (PDA) (Biolab) with chloramphenicol (250 mg/l) for fungal isolation (Baker and Cook, 1974).

3.2 Isolation of potential biocontrol agents from the rhizosphere by means of a mycelial baiting procedure

For the purpose of isolating micro-organisms which attack *Pythium* mycelium, a mycelial baiting procedure was used as described by Scher and Baker (1980). The technique consist of two strips of nylon monofil M-5 (Nybolt, Switzerland)(30 mm x 30 mm) being placed on solidified V8 juice agar (Roux and Botha, 1997) in Petri plates (90 mm). A 5-mm diameter disc from a culture of *Pythium* F- group was placed in the middle of the plate. The *Pythium* F-group isolate was previously isolated from hydroponically grown lettuce and the pathogenicity confirmed by Gull (2003). The isolate was grown on V8 juice agar and preserved in water (Gull, 2003).

After four days incubation at 25 ^{0}C the pathogen had overgrown the strips (Scher and Baker, 1980). The cellophane strips were aseptically removed from the agar and placed into a chamber comprising a hollow pvc pipe (5 cm diameter x 8 cm long), covered at both ends with nylon mesh, through which water can flow freely. One strip was placed into each of eight chambers, which were submerged into the recirculating nutrient solution of a commercial gravel bed hydroponic system (Hydrotec). After six days, the nylon strips were removed. *Pythium* mycelium recovered from four of the eight nylon strips were plated on NA and PDA as described in 3.1. The remaining nylon strips were vortexed for 30 seconds in 9 ml Ringers' solution before a dilution series were made (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) for plating on NA, KB and PDA.

3.3 Pure Cultures

From each dilution series 0,1 ml was plated out on NA and KB for the isolation of bacteria and PDA to obtain fungi and incubated at 25 ^{0}C in the dark for four days (Sfalanga *et al*., 1999). Selection of isolates on KB medium was based on fluorescence of the colonies under UV light. Representative bacterial and fungal colonies were grouped according to the size and colony/spore/mycelium morphology, purified and maintained on KB, NA or PDA at 25 ^{0}C and preserved through freeze-drying (Smith and Onions, 1994; Goszczynska and Serfontein, 2000) for further testing. All isolates were subcultured and maintained on NA medium for bacterial isolates and PDA medium for fungal isolated.

3.4 Primary screening of potential biocontrol isolates:

The potential biocontrol agents were screened *in vitro* for antagonistic activity against *Pythium* F-group by means of the dual culture technique (Walker *et al*., 1998).

3.4.1 Screening of potential biocontrol bacterial isolates:

A single colony of each bacterial isolate was used to inoculate dual culture PDA plates by producing three 20 mm streaks of inoculum at three different sides of a 90 mm-diameter Petri plate (Fig 2.1). Mycelium discs were aseptically punched with a corkborer from the periphery of actively growing, three day old *Pythium* F-group cultures, grown on PDA, and placed in the centre of each dual culture plate. Four replicate plates were used for each test organism.

3.4.2 Screening of potential biocontrol fungal isolates:

Mycelium discs (5 mm-diameter) were punched from five-day-old cultures of the various fungal isolates and placed on the edge of the dual culture PDA plates. Mycelium plugs (5 mm-diameter) of three-day-old cultures of *Pythium* F-group were placed on the edge of the plates directly opposite the test isolates (potential antagonistic fungi) (Fig 2.1). Four replicate plates were used for each test organism.

A PDA Petri plate inoculated with *Pythium* alone served as control. The plates were incubated for two days at 25 ^{0}C in the dark prior to visual assessment. Isolates that showed inhibitory activity against *Pythium* were selected (Anith *et al.*, 2003). The *Pythium* growth radius (R1) on the control plate as well as the *Pythium* growth radius (R2) on the biocontrol inoculated plate was measured. The percentage growth inhibition was determined for each biocontrol agent with the following formula: (R1-R2) / R1 x 100 (Skidmore and Dickinson, 1976).

4. RESULTS

4.1 Isolations

A total of 74 bacterial and 18 fungal isolates were selected on the basis of colony/culture size, colour and morphology.

Of these 62. 2 % of the bacterial and 50% of the fungal isolates originated from the root isolations of the lettuce plants whereas 37.8 % bacterial and 50% of the fungal isolates originated from the *Pythium* mycelium traps.

4.2 Dual culture assay

Of the 74 bacterial isolates screened for competition or antibiotic activity by means of the dual culture assay, 22 produced inhibitory zones against *Pythium* F-group ranging between 10.8 and 47,6 % inhibition (Table 2. 1 (a)). The isolates JH41, JH49, JH62, JH69, JH83, JM6R and JM17W showed particularly well-developed inhibitory zones against *Pythium* F-group (Fig 2.2).

From the 18 fungal isolates that were screened for competition or antibiotic activity against *Pythium* F-group, 10 produced inhibition zones ranging between 24.3 and 54 % (Table 2.1 (b)). The isolates JH4, JM42R, JM41R, and JH90 showed distinct activity against the *Pythium* culture (Fig 2.3).

5. DISCUSSION

Finding promising antagonistic organisms with potential to control pathogens is the first step towards the development of effective biological control. These organisms must be ecologically fit to survive, become established and function within the

particular conditions of the ecosystem (Larkin *et al.*, 1998). In this study, isolating from the rhizoplane and rhizosphere of lettuce in a *Pythium* infested re-circulating hydroponic gravelbed system resulted in the isolation of 32 microbial agents showing *in vitro* antagonistic activity against *Pythium* F – group. The methods employed to isolate antagonistic agents against a specific pathogen play an important part in the successful identification of potential biocontrol agents. According to Williams and Asher (1996), several investigations have shown that fluorescent *Pseudomonas* spp. inhibitory to *Gaeumannomyces graminis* were up to 25 times more abundant on roots infected with this pathogen than on roots not exposed to the pathogen. This suggests that not only should the pathogen be present but also that the potential biocontrol isolate should be obtained from within the rhizospere of the target crop. The biocontrol agents currently registered for management of diseases caused by *Pythium* spp., are naturally occurring isolates that have been obtained from agricultural systems (Martin and Loper, 1999).

Root samples and *Pythium* baiting samples were taken from the *Pythium* infected beds of a commercial hydroponic system to obtain potential biocontrol agents with the ability to function in the same environmental niche as the pathogen. The *Pythium* baiting isolation technique specifically isolate bacterial and fungal isolates that are able to attach to /attack the *Pythium* mycelium while the root sampling isolating technique ensures the isolation of bacterial and fungal isolates with the ability to colonise the roots of the host plant.

In the current study most of the NA, PDA and KB agar plates had 50 or more colonies which all looked similar when small. Pure cultures where made from randomly selected colonies. According to Broadbent *et al.* (1971), the odds of finding effective bacterial antagonists on dilution plates could be improved by avoiding brightly coloured colonies, which are known not to have useful antagonistic qualities.

According to Baker and Cook (1974), the greatest number of colonies can be expected to be *Bacillus*, *Streptomyces*, or *Pseudomonas* spp. The bacterial and fungal isolates that are most likely to be used are those that can easily be

established as laboratory cultures. Agents are often eliminated early in the screening process if they are difficult to culture.

Some microorganisms excrete metabolites that inhibit the growth of fungal pathogens. These organisms differ in their inhibitory activity. According to Williams and Asher (1996), potential biocontrol agents can produce a range of antifungal metabolites in culture, such as antibiotics (pyrrolnitrin, pyoluteorin and phenazine), siderophores, hydrogen ions and gaseous products (ethylene, hydrogen cyanide and ammonia). The *in vitro* dual culture assay is a qualitative indication of the inhibitory activity of a bacterium against a fungus (Knudsen *et al*, 1997). In the current study this assay produced clear, visible results such as inhibition or lysis of the pathogen and the assay was relatively easy and quick to perform with a large number of isolates.

An *in vitro* screening system that provides repeatable and reliable results in short periods of time is an important step for isolation of efficient antagonists for plant disease management. In dual culture plate assays, however, there is no involvement of the host plant. In real field conditions the host plant plays an important role in supporting the introduced antagonist. Several authors have reported a poor correlation between results of dual culture assay and that of *in vivo* assays (Baker, 1968; Schroth and Hancock. 1981; Wang and Baker, 1984; Hultberg, *et al*., 2000). Jubina and Girija (1998), used several bacterial antagonists against *Phytophthora capsici* in black pepper after initially screening the isolates by means of the dual culture procedure. Their studies revealed that one of the *Bacillus* isolates showing poor inhibition of the fungal pathogen in the dual culture assay exhibited the highest disease suppression in the *in vivo* biological control assay. However, according to Jackson *et al.* (1997), dual culture agar techniques should be included because *in vivo* screening can be too severe, rejecting potentially useful candidates. For the above reasons, all isolates in the current study showing even a slight inhibition of *Pythium* F- group was selected for further *in vivo* evaluation where the involvement of the host plant, pathogen and possible antagonist would be expected to give a more realistic result.

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Table 2.1(a) *In vitro* inhibition of mycelial growth of *Pythium* F-group by bacterial isolates on potato dextrose plates, after two days incubation at 25 ^{0}C.

Isolate	% Inhibition*
JH83	30.2
JH41	47.6
JH49	37.0
JH69	32.4
JH62	28.0
JM2R	43.7
JM17W	28.5
JM26W	33.8
JM5W	29.5
JM28W	22.4
JM16W	22.4
JM2W	24.6
JM9W	20.0
JM2R	28.2
JM15R	33.3
JM8W	27.5
JM13R	17.1
JM7W	22.5
JM39R	11.0
JM15W	10.8
JM32R	11.3
JM37R	21.8

*Percentage inhibition was determined with the following formula:
(R1-R2) / R1 x 100
R1: *Pythium* growth radius on control plate
R2: *Pythium* growth radius on biocontrol inoculated plate

Table 2.1 (b) *In vitro* inhibition of mycelial growth of *Pythium* F-group by fungal isolates on

Potato dextrose agar, after two days incubation at 25 ^{0}C.

Isolates	% Inhibition*
JH1	40.5
JM43R	54.0
JM6BO	54.0
JM41R	45.9
JM42R	48.6
JH4	4.9
JH5	29.7
JM40R	37.8
JH90	24.3
JM2BO	32.4

*Percentage inhibition was determined with the following formula:

(R1-R2) / R1 x 100

R1: *Pythium* growth radius on control plate

R2: *Pythium* growth radius on biocontrol inoculated plate

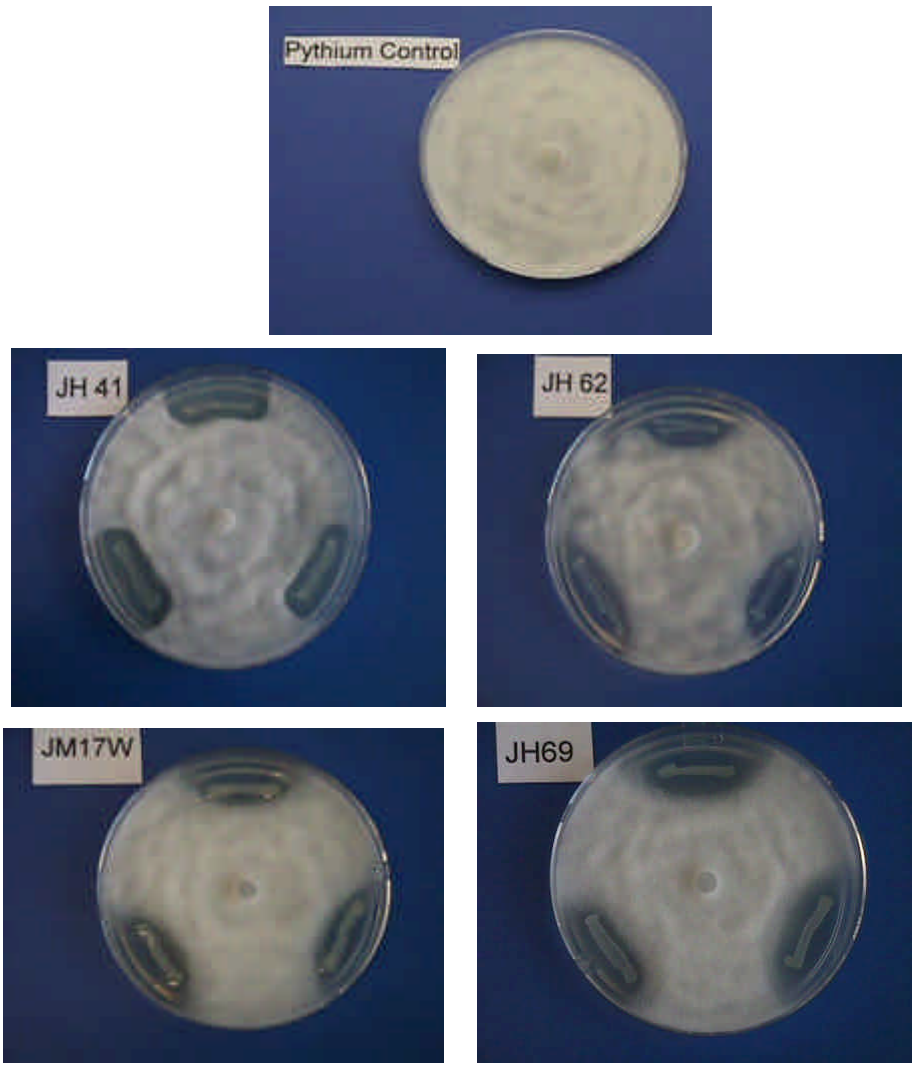

Figure 2.2 Dual culture assay of bacterial isolates against *Pythium* F-group on potato dextrose agar plates with isolates showing inhibition of the fungus.

Chapter 3

IN VIVO SCREENING OF BACTERIAL AND FUNGAL ISOLATES FOR GROWTH PROMOTION OF LETTUCE SEEDLINGS AND ANTAGONISTIC ACTIVITY AGAINST ROOT ROT AND WILTING CAUSED BY *PYTHIUM* F- GROUP

1. ABSTRACT

Potential biocontrol agents were screened in a static aquaculture system on butterhead lettuce (*Lactuca sativa* L. var. *capitata* L) seedlings in the greenhouse prior to evaluation in a re-circulating gravel bed hydroponic system in the greenhouse and field. Growth promoting and biocontrol ability were determined. Significant increase of between 688.9 % and 922.2 % in total fresh mass was obtained with plants preventatively treated with bacterial isolates JH49 (not identified), *Sphingomonas paucimobilis* (JH41), *Pseudomonas fluorescens* (JH83), *Burkholderia cepacia* (JM6R) and *Bacillus subtilis* (JM16W). The eight best performing isolates were further evaluated for biocontrol activity against *Pythium* F- group as well as growth promotion on butter head lettuce in a re-circulating gravel bed hydroponic system in the greenhouse. Significant increases of between 1.6 % and 63.5 % in total fresh mass were obtained from plants evaluated in the growth promotion test. Preventatively treated plants resulted in an increase of between 1.0 % and 38.9 % in total fresh mass. Performance-based criteria were used to select five of the eight isolates for subsequent evaluation in a commercial scale re-circulating gravel bed hydroponic system in the field. Controls consisted of untreated beds as negative control and *Pythium* infected beds as positive control. A commercial fungicide (potassium phosphonate) and commercial biological control product (*Trichoderma*) was also included for comparative purposes. Treatment with two bacterial isolates namely *Pseudomonas fluorescens* and *Bacillus subtilis* respectively resulted in significant increases in lettuce yield compared to the *Pythium* infected control and *Trichoderma* treatments.

2. INTRODUCTION

A potential biocontrol organism's ability to interact, colonize and protect roots and seeds can only be determined if the host plant is included in screening

assays (Nelson, 1991; Cook and Baker, 1983; Kloepper, 1991). The rhizosphere is relatively rich in nutrients released by the roots. Microbial communities living in the rhizosphere differ from those living in other environments (Cocking, 2003). Seed and root-infecting pathogens are often highly dependent on exudates to initiate plant infections. The ability of antagonists to metabolize these exudates may be an important step in biocontrol processes (Nelson, 1991). By extending the screening program to include plants growing in natural substrates, other mechanisms such as induced resistance and plant growth promotion can be evaluated. Edaphic or nutritional factors such as root exudates and plant residues will also be present in the system (Nelson, 1991).

In vivo screening employs placement of the pathogen and potential biocontrol agent on the host. This type of screening is superior to *in vitro* screening because the isolate's ability to survive on the host is evaluated. Modes of action other than antibiosis are selected and a direct assessment is obtained of biocontrol potential among individual organisms (Campbell, 1986; Nelson and Craft, 1992). *In vivo* screening of potential antagonists increases the chances of selecting the best agents but is usually more expensive and labour intensive. It is therefore important to eliminate ineffective or slightly effective antagonistic agents before testing in the field (Weller and Cook, 1985).

Campbell (1986), states that organisms should be screened under the most realistic conditions by employing normal agricultural practices. *In vivo* pre-screening assays are usually small-scale simulations, which resembles specific, natural conditions that has the ability to predict expression of biological control traits in more complex ecosystems (Nelson and Craft, 1992). They are carried out in the laboratory or greenhouse but do not take climatic fluctuations into account, which play an important role in the field (Kraus, 1996). Field trials are therefore the most reliable test and are important in verifying that the organisms work outside the laboratory or greenhouse (Campbell, 1989; Sutton and Peng, 1993; Kraus, 1996).

The aim of this study was to obtain the best performing biocontrol or growth promoting agents by screening selected isolates in a static aquaculture system on butterhead lettuce seedlings in the greenhouse prior to evaluation in a re-circulating gravel bed hydroponic system in the greenhouse and field.

3. MATERIALS AND METHODS

3.1 Inoculum preparation

3.1.1 Pythium inoculum

Pythium F – group cultures obtained from preserved cultures (Chapter 2) were grown on V8 juice agar Petri plates (90 mm diameter) for three days at 25 ^{0}C in the dark. One *Pythium* Petri plate culture was blended in 1000 ml sterile water for five seconds with a Waring blender (Jenkins and Averre, 1983; Moulin *et al*., 1994) and mixed with 4 l of the nutrient solution specified in 3.2. The 250 ml plastic cups were filled with the *Pythium* / nutrient mix on the day of inoculation.

3.1.2 Biocontrol agent inoculum

(*i) Bacteria*

Bacterial isolates (Chapter 2) were cultured in 250 ml Erlenmeyer flasks containing 400 ml Nutrient broth (NB) (Merck). After 48 h shake-incubation at 25 ^{0}C, cells were harvested in 50 ml test tubes by centrifugation at 20 rpm. The pellet was dissolved in 250 ml sterile quarter strength Ringer's (Merck) solution. The bacterial cell concentration was determined with a Petroff-Hausser counting chamber (Korsten and De Jager, 1995). The final cell concentration was adjusted to 4 x 10^9 cfu/ml. From this suspension 2 ml was added to each 250 ml plastic container to obtain 10^6 cfu/ml in the nutrient solution.

(ii) *Fungi*

Fungal isolates were grown on potato dextrose agar (PDA)(Merck) in the dark for five days at 25 ^{0}C. Spores were harvested in sterile quarter strength Ringer's solution and counted in a haemocytometer (Korsten and De Jager, 1995). The final spore concentration was adjusted to 4 x 10^7 cfu/ml. From this

suspension 2 ml was added to each 250 ml plastic container to obtain a 10^4 cfu/ml concentration in the nutrient solution.

3.2 Static aquaculture bio-assay

Two hundred and sixty pathogen free butterhead lettuce seedlings *Nadine* (*Lactuca sativa* L. var. *capitata* L.) were grown in polystyrene seedling trays in steam-pasteurized Canadian peat moss. After four weeks the seedlings were pulled from the trays and the growth media washed from the roots. These seedlings were then transferred to 250 ml plastic containers, one seedling per container. Each container was filled with a nutrient solution consisting of 0.45 g Agrosol® 'O 3:2:8 (Fleuron, Braamfontein), 0.15 g Micromix ® (Fleoron), 1^{-1} sterile water, with pH of 7.0 and 0.3 g $Ca(NO_3)_2$ and had a lid with a 10 mm diameter hole in the center. Plants were suspended through the hole in the lid whilst supported with a strip of foam rubber, while their roots were submerged in the nutrient solution. The nutrient solution was replaced on a weekly basis. The plants were placed in a greenhouse with the temperature ranging between 25 ^{0}C and 35 ^{0}C (Fig 3.1). Plants were inoculated one week after transplanting into the static aquaculture system when they were well established and free of any disease symptoms.

Biocontrol isolates were divided into four groups, which consisted of 6 - 8 isolates. Inoculum of the biocontrol isolates and pathogen was added to the nutrient solution in each plastic container. To evaluate preventative biocontrol activity the inoculum of the biocontrol agent was added six days before adding the pathogen inoculum (Sanogo and Moorman, 1993). To evaluate curative ability, inoculum was added six days after the pathogen inoculum. For growth promotion evaluation, uninfected plants were treated with only the antagonistic inoculum. *Pythium* infected plants and plants treated with sterile V8 juice agar served as the infected and uninfected controls (Cherif *et al.*, 1994). Pathogen and biocontrol agent inoculum was added to the respective treatments once a week for the following three weeks. Five replicate plants were used for each treatment. Three weeks

after inoculation, plants were removed from the containers, their roots and shoots separated and fresh mass determined.

3.3 Identification of the most promising biocontrol agents

The bacterial isolates that showed the best growth promotion activity and/or biocontrol activity in the previous assay (3.2) were characterized by evaluating their physical characteristics (configuration of the colony, cell shapes and arrangements) and their metabolic capabilities (Norris and Ribbons, 1971).

The KOH - test was used to determine the cell-wall structure of each bacterial isolate followed by phase contrast microscopy to determine morphology and mobility (Norris and Ribbons, 1971). Bacterial isolates were inoculated in Hugh - Leifson's medium (Hugh and Leifson's, 1953; Krieg and Holt, 1984) to determine their ability to oxidize or ferment glucose (Norris and Ribbons, 1971). Oxidase activity was determined by means of cytochrome oxidase test (Norris and Ribbons, 1971). Further identification was achieved using API 50 CH, API 20NE and API 20E (Biomerieux, France).

The fungal isolates were identified microscopically based on morphological features (Rifai, 1969).

3.4 Re-circulating gravel bed hydroponic system in the greenhouse

Eight of the most promising isolates, previously screened in the static aquaculture bio-assay system (3.2) were further tested in a re-circulating gravel bed hydroponic system in the greenhouse at 25 – 30 °C for their growth promoting ability and biocontrol of *Pythium* root infection on butter head lettuce plants.

The re-circulating gravel bed hydroponic system comprised of ten units, each with a 100 l reservoir, supplying nutrient solution with the same composition as in (3.2), to three pvc troughs, 13 cm wide, 10 cm deep and 250 cm long, positioned at an incline of 1:13 (Fig 3.2). The troughs were

filled with 9.5 mm diameter granite chips to a dept of 5 cm. The nutrient solution was constantly circulated to the top-end of the troughs by means of an IDRA 300 l h^{-1} submersible pump. The solution flowed through the gravel and returned to the reservoir by gravity flow. The nutrient solution was replaced once a week and the pH and electrical conductivity were maintained at 6.9 and 2.1 mS/cm respectively.

3.4.1 Evaluation of biocontrol activity

A hundred and thirty pathogen free butterhead lettuce (*Lactuca sativa* L. var. *capitata* L.) seedlings were grown in polystyrene seedling trays in steam-pasteurized Canadian peat moss. At the four-leaf-stage, the roots of 210 butter head lettuce seedlings were suspended in *Pythium* inoculum for two days as described in 3.1.1. To serve as source of inoculum seven of these pre-inoculated seedlings were planted at the top of each trough. The remainder of each through was planted with seven un-inoculated plants spaced 20 cm apart (Jenkins and Averre, 1983) (Fig 3.2). 100 ml of the previously prepared *Sphingomonas paucimobilis* (JH41), *Pseudomonas fluorescens* (JH83), *Bacillus cereus* (JM2R), *Burkholderia cepacia* (JM6R), *Brucella* spp (JM17W), *Bacillus subtilis* (JM16W), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) inoculum (as prepared in 3.1.2(i) and (ii)) was added to each reservoir to obtain a 10^6 cells/ml final concentration for the bacteria and a 10^4 spores/ml for the fungi.

Four weeks after the experiment was initiated, the butter head lettuce plants were harvested and the roots rinsed in clean water. The roots and shoots of each plant were separated and weighed. The seven pre-inoculated plants at the top of each through were evaluated as a separate set to give an indication of the curative effect of each treatment while the remaining seven plants in each through were used to determine the preventative effect of each treatment. The pathogen inoculated control consisted of seven lettuce seedlings, pre-infected with *Pythium* at the top and seven uninfected lettuce seedlings at the bottom of each trough. The uninfected control consisted of fourteen uninfected lettuce plants in each trough. Three replicate troughs were used per treatment.

Root tips were excised from each plant, plated on BNPRA selective medium (Roux and Botha, 1997) and incubated at 25 ^{0}C for three to six days. *Pythium* growth was verified under a compound microscope at 10x magnification. The number of root pieces rendering *Pythium* growth was recorded.

3.4.2 Evaluation of growth promotion ability

Pathogen free butterhead lettuce (*Lactuca sativa* L. var. *capitata* L.) seedlings were grown in polystyrene seedling trays in steam-pasteurized Canadian peat moss. After four weeks, fourteen uninfected butter head lettuce seedlings were transferred to each trough of the re-circulating gravel bed hydroponic system. Inoculum of the biocontrol isolates was added to the nutrient solution in each reservoir on a weekly basis as described under 3.3.1. For comparative purposes a *Pythium* infected control was included. However, all the plants treated with biocontrol isolates were *Pythium* free.

Four weeks after the start of the experiment, plants were harvested and the roots rinsed in clean water. The roots and shoots of each plant were subsequently separated, and weighed. The pathogen inoculated control consisted of seven lettuce seedlings which were pre-infected with *Pythium*, at the top and seven uninfected lettuce seedlings at the bottom of each trough. The uninfected control consisted of fourteen uninfected lettuce plants in each trough. Three replicate troughs were used per treatment.

3.5 Re-circulating gravel bed hydroponic system in the field

Five of the most promising isolates, previously screened in a re-circulating gravel bed hydroponic system in the greenhouse (3.4) were subsequently tested in a re-circulating gravel bed hydroponic system in the field at 10 °C – 35 °C as potential antagonists against *Pythium* root infection on butter head lettuce plants under simulated commercial conditions.

The hydroponic field system comprised of eighteen units, each with a 500 l reservoir, supplying nutrient solution to a bed that was divided into two equal sides (25 m long, 10 cm deep and 75 cm wide). Two units were used

per treatment. The beds were filled with 9.5 mm diameter granite chips to a dept of 5 cm. The nutrient solution consisting of 460 g Hydrogrow, 250 g Ca $(NO_3)_2$ and 57 ml nitric acid per 500 l was constantly circulated through the gravel in the beds by means of an Eden 140G Q400 - 2800 l h^{-1} submersible pump, returning to the reservoir by gravity flow.

Pathogen free butterhead lettuce (*Lactuca sativa* L. var. *capitata* L) seedlings were grown in polystyrene seedling trays in steam-pasteurized Canadian peat moss. After four weeks, seedlings were transplanted into the beds with 20 cm spacing between plants (Jenkins and Averre, 1983). Each bed contained between 400 – 600 lettuce plants (Fig. 3.3).

The following treatments were included:

a. A commercial fungicide, Phytex (K-phosphonate, 200g a.i.l^{-1}, soluble concentrate (SL)) (Horticura, Gezina), at 1 ml / l water
b. A commercial biocontrol compound Biotricho (Stimuplant cc, Pretoria), at 200 g/ 500 l
c. Untreated control
d. *Pythium* infected control
e. *Pseudomonas fluorescens* (JH83) at 10^6 cells/ml nutrient solution
f. *Bacillus cereus* (JM2R) at 10^6 cells/ml nutrient solution
g. *Burkholderia cepacia* (JM6R) at 10^6 cells/ml nutrient solution
h. *Trichoderma* T1 (JM41R) at 10^4 cells/ml nutrient solution
i. *Trichoderma* T2 (JM6BO) at 10^4 cells/ml nutrient solution

One litre of previously prepared *Pseudomonas fluorescens* (JH83), *Bacillus cereus* (JM2R), *Burkholderia cepacia* (JM6R), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) inoculum (3.1.2(i) and (**i**)) were added to the designated reservoir to obtain a 10^6 cells/ml final concentration for the bacterial inoculum and a 10^4 spores/ml for the fungal inoculum. *Pythium* F – group cultures were grown on V8 juice agar (Roux and Botha, 1997) Petri plates (90 mm) for three days at 25 °C in the dark. *Pythium* culture plates were placed under the gravel: two at the top, two in the middle and two at the bottom of each bed on both sides to provide pathogen inoculum. Except

for the untreated control, all beds were treated with the pathogen inoculum. Two replicate beds were used per treatment. The commercial chemical product (Phytex) and the commercial biocontrol product (Biotricho) were included for comparative purposes The nutrient solution and all treatments were replaced once a week and the pH and electrical conductivity were maintained at 5.8 and 2.1 mS/em, respectively.

Five weeks after planting a sub - sample of 24 plants per treatment (12 plants per replicate bed) was harvested before the commercial scale harvest commenced. For the small scale assessment twelve plants were randomly selected per bed, removed from the gravel and their roots rinsed in clean water. The shoots and roots of each plant was separated and weighed individually. Sub – samples of roots were taken from each treatment and transported to the laboratory for assessment of *Pythium* incidence.

The commercial scale harvest was performed by workers from Hydrotec (Gauteng, S. Africa) (Fig. 3.4). The remaining lettuce shoots from each treatment were cut from the roots, packed into crates and the total mass of plants per bed was determined. The amount of plants per bed was counted two weeks before harvest. The following equation was used to determined average mass / plant:

$$\frac{\text{Total mass per gravel bed}}{\text{Total amount of plants per gravel bed}}$$

3.6 *Pythium* incidence

3.6.1 Pythium incidence in the nutrient solutions

Samples of nutrient solution (250 ml each) were taken from each reservoir in the greenhouse and field hydroponic systems to determine the incidence of *Pythium* in the water by means of the citrus leaf disc baiting procedure of Grimm and Alexander (1973). Citrus leaf discs (5-mm-diameter) were punched from the leaves of rough lemon seedlings obtained from a citrus nursery, sprayed with 70 % ethanol, rinsed in sterile water and subsequently floated on the surface of the individual water samples for 24 h in the dark. For each treatment, ten citrus leaf discs were plated on of BNPRA selective

media (Roux and Botha, 1997) in 90 mm Petri plates and incubated at 25 ^{0}C. After five days, *Pythium* growth from the citrus leaf discs was microscopically assessed and the number of discs rendering *Pythium* recorded.

3.6.2 Pythium incidence in roots

Roots were sampled from each treatment after harvest and rinsed clean in sterile distilled water. No further surface-disinfestation was applied in accordance with Stanghellini and Kronland (1986). Ten root tip segments (10 mm in length) per plant were excised and plated on BNPRA selective media (Roux and Botha, 1997) and incubated at 25 ^{0}C for five days. The plates were examined under a compound microscope (plates were inverted) and the number of root pieces rendering *Pythium* growth recorded.

3.7 Statistical analysis

Data were statistically analyzed using the statistical program GenStat (2000). Treatments were separated using Fisher's protected ttest least significant difference (LSD) at the 1 %, 5 % and 10 % level of significance (Snedecor and Cochran, 1980). One-way analysis of variance (ANOVA) was used to test for differences between treatments.

4. RESULTS

4.1 Static aquaculture experiments

The growth promotion effect of selected isolates on lettuce plants evaluated in the static aquaculture system is presented in Table 3.1. Pathogen free plants treated with isolates JH83, JM6R, JM16W, JM2W, JM2R, JM28W, JM37R, JM32R, JM13R, JM8W, JM7W, JH4, JM43R showed an increase of between 0.4% and 44.4% in fresh leaf mass and a significant increase of between 3.6 % and 73.4 % in fresh root mass when compared to the untreated control (Fig 3.6; Fig 3.7). There were, however, no significant differences ($P< 0.05$) among any of the treatments in any of the four groups in terms of the increased fresh leaf mass because of the variation between replicates as reflected by the large standard error of the means (Table 3.1). As this was intended as a screening experiment these tendencies were

recorded and compared with follow-up experiments in the gravel bed systems.

The effect of selected isolates on preventatively treated lettuce plants subsequently inoculated with *Pythium* in the static aquaculture systems are presented in Table 3.2. Treatment of plants with isolates JH49, JH41, JH83, JM6R and JM16W resulted in a significant increase ($P< 0.05$) of between 688.9 % and 922.2 % in fresh leaf mass and 8.4 % and 130.2 % in fresh root mass when compared to the *Pythium* infected control (Fig 3.8; Fig 3.9). In all four potential biocontrol groups a significant difference of between 58.1 % and 207.8% was obtained from the untreated control when compared to the *Pythium* infected control indicating the affectivity of the *Pythium* inoculum.

The effect of selected isolates on curatively treated lettuce plants evaluated in the static aquaculture system is presented in Table 3.3. Treatment of *Pythium* infected plants with isolates JH41, JH83, JM6R, JH69 and JM17W resulted in an increase of between 174.6 % and 311.1 % in fresh leaf mass and a significant increase of between 11.8 % and 36.6 % in fresh root mass when compared to the *Pythium* infected control (Fig 3.10; Fig 3.11). There were, however, no significant differences ($P< 0.05$) among any of the treatments in any of the four groups in terms of the increased fresh leaf mass because of the large standard error of means.

4.2 Identification of selected biocontrol agents

Results of the characterization and identification of the selected bacterial biocontrol isolates are summarized in Table 3.4. All of the isolates were Gram negative and motile except for JM16W and JM2R, which were Gram-positive, not motile and produced endospores. The Gram-negative isolates were able to oxidize the glucose in the Hugh-Leifson's medium but only JM17W were able to ferment the glucose without oxygen. All of the isolates were cytochrome oxidase positive except for JM16W, which were negative. Gram-positive isolates were identified as *Bacillus subtilis* (JM16W) and *Bacillus cereus* (JM2R) by means of the API 50 CH test kit. The Gram-

negative isolates were identified as *Burkholderia cepacia* (JM6R), *Pseudomonas fluorescens* (JH83) and *Sphingomonas paucimobilis* (JH41) by means of the API 20 NE test kit and JM17W was identified as *Brucella* spp. by means of API 20 E (Table 3.4).

Both fungal isolates (JM41R and JM6BO) were identified as *Trichoderma* species based on morphological characteristics.

4.3 Re-circulating gravel bed hydroponic system in the greenhouse

Results on the growth promotion, preventative and curative effect of selected isolates on lettuce plants evaluated in the greenhouse hydroponic system is presented in Table 3.5.

4.3.1 *Evaluation for growth promotion ability*

Treatment of pathogen - free lettuce plants with *S. paucimobilis* (JH41), *B. cereus* (JM2R), *Trichoderma* T1 (JM41R), *Trichoderma* T2 (JM6BO) and *B. subtilis* (JM16W) resulted in a significant growth increase ($P< 0.05$) of between 20 % and 63.5 % in total fresh mass when compared to the uninfected control (Fig 3.12).

4.3.2 *Evaluation for preventative biocontrol ability*

Plants treated preventatively with *B. cepacia* (JM6R), *B. cereus* (JM2R), *P. fluorescens* (JH83), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) showed significant yield increases ($P < 0.05$) of between 1.0 % and 38.9 % in total fresh mass when compared to the *Pythium* infected control (Fig 3.13).

4.3.3 *Evaluation for curative biocontrol ability*

Plants treated curatively with *B.cepacia* (JM6R), *B. cereus* (JM2R), *P. fluorescens* (JH83), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) showed yield increases of between 35.9 % and 62.2 % in total fresh mass when compared to the *Pythium* infected control (Fig 3.14). The results however did not show any significant differences between treatments at $P <$

0.05 because of the large variation between replicates as reflected by the standard error of the means.

4.3.4 Determining Pythium incidence

Treatment with *B. cepacia* (JM6R) and *P. fluorescens* (JH83) reduced *Pythium* incidence in the water and in the roots from 100 % to 20 % (Table 3.6).

4.4 Re-circulating gravel bed hydroponic system in the field

4.4.1 Small scale assessment

Results of the small scale assessment of a sub-sample of 24 plants per treatment, is presented in Table 3.7. Plants treated with *Trichoderma* T1 (JM41R), *P. fluorescens* (JH83), *B. cepacia* (JM6R), *B. cereus* (JM2R) and Phytex showed a total yield increase of between 10.7% and 16.1% when compared to the *Pythium* infected control (Fig 3.15). There were, however, no significant differences ($P< 0.05$) between treatments because of the large variation between replicates as reflected by the standard error of means. *Pythium* incidence in the water and roots of all treatments except the uninfected control were between 80 % and 100 %. The impact of *Pythium* infection was clearly visible when the infected control was compared with the uninfected control (Fig 3.5).

4.4.2 Commercial scale assessment

Results of the commercial scale assessment are presented in Table 3.8. Plants treated with *B. cepacia* (JM6R), *B. cereus* (JM2R), *P. fluorescens* (JH83), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 showed a significant ($P< 0.05$) increase in total yield (Fig 3.16). The best results were obtained with *P. fluorescens* (JH83) and *B. cereus* (JM2R) rendering a yield of 241.3 g and 248.9 g respectively. The *Pythium* free control had a yield mass of 8.9 % more than the *Pythium* infected control.

5. DISCUSSION

The screening procedures used in this study were designed to eliminate ineffective isolates and select effective isolates whilst moving systematically

from an artificial environment to a final assessment under commercial conditions in the field.

Five of the thirteen biocontrol isolates that showed growth promotion activity on lettuce plants in the static aquaculture bio-assay also caused a significant increase in the growth of *Pythium* free lettuce when tested in the greenhouse re-circulating hydroponic gravel system. The five isolates were identified as *S. paucimobilis* (JH41), *B. cereus* (JM2R), *Trichoderma* T1 (JM41R), *Trichoderma* T2 (JM6BO) and *B. subtilis* (JM16W). Microbial agents are known to exert additional beneficial effects such as growth and yield promotion (Zheng *et al*., 2000). Van Peer and Schippers (1989), found that bacterial growth in the hydroponic nutrient solution and the rhizosphere developed rapidly due to substrates released by roots and this resulted in increased plant growth of tomato, cucumber, lettuce and potato in hydroponic systems.

In a study by Ousley *et al.* (1993), four week old lettuce seedlings showed a consistent increase in fresh mass of up to 54 % when treated with *Trichoderma*. *Bacillus, Pseudomonas* and *Burkholderia* species are also well-known plant growth promoting bacteria that increase plant yield of commercially important crops (Burdman *et al*., 2000).

The data on fresh leaf mass obtained from the growth promotion and curative tests in the static aquaculture bioassay where not statistically significant but were still used, together with results obtained from the preventative tests, as an indication of possible biocontrol activity. Statistically results could have been improved by including more replicates per treatment. In our experience, we found this screening technique too time- and space consuming to include more replicates. The screening procedure might be improved by using seedling trays or pots that can contain 5 or more plants.

Based on the results obtained in the static aquaculture bioassay system, *B. cepacia* (JM6R), *B. cereus* (JM2R), *P. fluorescens* (JH83), *S. paucimobilis* (JH41), *B. subtilis* (JM16W), *Brucella* Spp. (JM17W), *Trichoderma* T1 (JM41R)

and *Trichoderma* T2 (JM6BO) were selected for evaluation in the greenhouse re-circulating hydroponic gravel system.

Bacillus cepacia (JM6R), *B. subtilis* (JM16W), *B. cereus* (JM2R), *P. fluorescens* (JH83), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) caused a significant increase in total yield of lettuce plants in the greenhouse re-circulating hydroponic gravel system when treated preventatively. These results correlated well with the results obtained in the static aquaculture system (Table 3.9). The same five isolates caused an increase in total yield of lettuce plants when treated curatively although the treatments did not differ significantly from each other. It is a common phenomenon that the biocontrol treatments in screening experiments show a certain trend, which might not be significant but can still be used for screening purposes (Romeiro *et al*, 2000). The five most promising isolate were thus further evaluated in a re-circularing hydroponic field system where the best biocontrol activity against *Pythium* root rot where obtained with *B. cereus* (JM2R) and *P. fluorescens* (JH83) (Table 3.9).

The amount of plants to be evaluated for each treatment in the hydroponic field system made it practically impossible to weigh all the shoots and roots separately. A small-scale assessment on a sub-sample of randomly selected plants was performed to give an indication of the effect the treatments had on yield. In the small-scale assessment, treatment with *B. cereus* (JM2R), *P. fluorescens* (JH83), *B. cepacia* (JM6R) and *Trichoderma* T1 (JM41R) showed an increase in the total fresh mass of butterhead lettuce. However, this trend was not significant because of the high variance between replicates. This was probably due to the fact that there were only two repeats per treatment with 12 plants sampled from each. The results did however, correlate well with the commercial harvest where the 600 - 800 plants evaluated per treatment resulted in significant differences in the total fresh mass per treatment. The highest yields were obtained with lettuce plants treated with *B. cereus* (JM2R) and *P. fluorescens* (JH83).

Lettuce plants in the untreated control off all the trials had a greater total fresh mass when compared to the *Pythium* infected control indicating the

effectiveness of the *Pythium* inoculum. The static aquaculture bioassay system was an ideal environment for *Pythium* infection because of the totally immersed roots in the static nutrient solution and the high temperatures, which were maintained. The lettuce plants that were treated with *Pythium* only were severely infected and some plants died. This is in accordance with observations by Jenkins and Averre (1983), who stated that severe root rot and subsequent plant death can occur in just a few days when the entire root system is exposed to the inoculum of the pathogen.

The decrease in *Pythium* incidence in the nutrient solution of the greenhouse hydroponic system could be ascribed to the weekly replacement of the nutrient solution thus removing most of the *Pythium* zoospores from the system. *Pythium* incidences in the lettuce roots of preventatively and curatively treated plants were however very high, indicating that effective *Pythium* infection occurred. The nutrient solution in a re-circulating hydroponic system creates an ideal environment for *Pythium* zoospores to spread and infect roots (Gold and Stanghellini, 1985). The flagellated zoospores procreate easily under warm conditions and move easily in water enabling the pathogen to transmit its spores very quickly through the system (Paulitz, 1997).

The importance of field screening has already been demonstrated by a number of studies (Deacon, 1991). Lumsden and Lewis, (1989) also prefer screening antagonists in the field. Zheng *et al*. (2000) and Sutton and Boland, (2004), states that microbial agents can provide direct protection of the host by suppressing pathogenic *Pythium* spp. in the root zone or within roots of hydroponic crops. *Pseudomonas, Bacillus* and *Trichoderma* species have been implicated as potential biocontrol agents against antagonistic *Pythium, Sclerotinia* and *Rhizoctonia* species on radish, alfalfa, sugar - beet, peas and beans in screening tests performed by Williams and Asher (1996).

Pseudomonas fluorescens has been indicated in several tests as effective biological control agents against *Pythium* root rot of lettuce, tomatoes and cucumbers in hydroponic systems (Schmidt, *et al*., 2004; Van Peer and Schippers, 1989; Paulitz, 1997). Fluorescent *pseudomonas* grows rapidly and

effectively colonizes the rhizosphere (Hultberg *et al*., 2000). Uthede *et al*. (2000), demonstrated the biocontrol activity of *B. subtilis* on hydroponically grown lettuce infected with *P. aphanidermatum*. The mechanisms by which these biocontrol agents operate are however not well known. Paulitz, *et al*. (1992), states that more efficient antagonists could be selected if the mechanism of the biocontrol agent-host-pathogen interaction was better understood.

In the current study isolates that performed best in the static aquaculture systems, performed well in the greenhouse hydroponic system and also showed biocontrol activity against *Pythium* root rot in the hydroponic field system. The results indicate that preliminary screening in the static aquaculture bio-assay systems could predict to some extent, the potential of an antagonist when applied under field conditions. Considerable time was saved by relying on results from the first two screening tests and not evaluating all isolates in the field.

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Treatments	Fresh leaf mass per plant (g)[a]	Fresh root mass per plant (g)[a]	Increase in fresh leaf mass (%)[b]	Increase in fresh root mass (%)[b]
Group 1				
Uninfected control	12.16	4.60	-	-
JH49	9.78	3.46	-19.6	-24.8
JH41	10.02	3.48	-17.6	-24.3
JH83	12.78	3.72	5.1	-19.1
JM6R	13.74	3.86	13.0	-16.1
JH62	7.28	3.56	-40.1	-22.6
JH69	8.42	3.54	-30.8	-23.0
S.E.[1]	5.655	1.150	45.06	38.21
LSD (0.05)[2]	0.521	0.362	0.344	< 0.001
Group 2				
Uninfected control	5.54	2.78	-	-
JM16W	8.00	4.82	44.4	73.4
JM2W	6.24	2.88	12.6	3.6
JM2R	8.26	4.34	49.1	56.1
JM9W	4.42	2.54	-20.2	-8.6
JM28W	5.88	3.74	6.1	34.5
JM5W	4.70	3.02	-15.2	8.6
JM17W	4.20	3.24	-24.2	16.5
JM26W	4.78	4.20	-13.7	51.1
S.E.[1]	3.339	3.070	45.06	38.21
LSD (0.05) [2]	0.452	0.322	0.344	< 0.001
Group 3				
Uninfected control	14.32	4.10	-	-
JM39R	13.86	4.90	-3.2	19.5
JM37R	15.50	4.98	8.2	21.5
JM32R	17.82	5.66	24.4	38.0
JM13R	14.62	4.30	2.10	4.9
JM8W	16.12	6.48	12.6	58.0
JM7W	16.96	5.34	18.4	30.2
S.E.[1]	3.677	3.000	45.06	38.21
LSD (0.05)[2]	0.591	0.321	0.344	< 0.001
Group 4				
Uninfected control	14.32	4.10	-	-
JH4	14.38	4.76	0.4	16.1
JM6BO	12.74	5.12	-11.0	24.9
JM41R	11.80	5.14	-17.6	25.4
JH1	12.30	4.10	-14.1	0.0
JM43R	15.30	5.18	6.8	26.3
JM42R	13.62	3.84	-4.9	-6.3
S.E.[1]	3.694	1.160	45.06	38.21
LSD (0.05) [2]	0.738	0.523	0.344	< 0.001

Group 2

Group 4

Treatments	Fresh leaf mass per plant (g)[a]	Fresh root mass per plant (g)[a]	Increase in fresh leaf mass (%)[b]	Increase in fresh root mass (%)[b]
Group 1				
Infected control	1.26	2.20	-	-
Uninfected control	11.20	4.60	788.9	109.1
JH49	9.94	4.30	688.9	95.5
JH41	12.88	4.44	922.2	101.8
JH83	11.36	4.22	801.6	91.8
JM6R	11.98	3.70	850.8	68.2
JH62	4.36	3.34	246.0	51.8
JH69	1.24	2.74	-1.6	24.5
S.E.[1]	4.290	4.090	214.30	55.25
LSD (0.05) [2]	0.001	0.032	<0.001	<0.001
Group 2				
Infected control	1.80	2.62	-	-
Uninfected control	5.54	2.10	207.8	-19.8
JM16W	14.23	6.03	690.6	130.2
JM2W	5.68	3.16	215.6	20.6
JM2R	11.50	4.82	538.9	84.0
JM9W	6.06	2.84	236.7	8.4
JM28W	5.32	4.20	195.6	60.3
JM17W	7.45	4.15	313.9	58.4
JM26W	6.40	5.76	255.6	119.8
S.E.[1]	3.614	1.099	214.30	55.25
LSD (0.05) [2]	0.001	0.02	<0.001	<0.001
Group 3				
Infected control	9.06	3.70	-	-
Uninfected control	14.32	4.10	58.1	10.8
JM39R	9.52	5.08	5.1	37.3
JM37R	11.48	4.74	26.7	28.1
JM32R	9.32	4.12	2.9	11.4
JM13R	7.46	2.94	-17.7	-20.5
JM8W	7.42	3.66	-18.1	-1.1
JM7W	7.26	2.94	-19.9	-20.5
S.E.[1]	4.385	1.670	214.30	55.25
LSD (0.05) [2]	0.719	0.041	<0.001	<0.001
Group 4				
Infected control	9.06	3.70	-	-
Uninfected control	14.32	4.10	58.1	10.8
JH4	14.06	4.58	55.2	23.8
JM6BO	13.98	4.86	54.3	31.4
JM41R	9.70	5.64	7.1	52.4
JH1	11.00	4.18	21.4	13.0
JM43R	11.92	3.88	31.6	4.9
JM42R	12.46	4.28	37.5	15.7
S.E.[1]	3.066	0.874	214.30	55.25
LSD (0.05) [2]	0.183	0.121	<0.001	<0.001

Group 1

Group 2

Group 3

Group 4

Treatments	Fresh leaf mass per plant (g)[a]	Fresh root mass per plant (g)[a]	Increase in fresh leaf mass (%)[b]	Increase in fresh root mass (%)[b]
Group 1				
Infected control	1.26	2.20	-	-
Uninfected control	11.20	4.60	788.9	109.1
JH49	1.30	1.70	3.2	-22.7
JH41	3.46	1.92	174.6	-12.7
JH83	3.98	2.54	215.9	15.5
JM6R	5.18	1.86	311.1	-15.5
JH62	1.76	1.62	39.7	-26.4
JH69	4.12	2.46	227.0	11.8
S.E.[1]	3.224	3.160	152.80	35.29
LSD (0.05) [2]	0.348	0.512	0.053	0.027
Group 2				
Infected control	1.80	2.62	-	-
Uninfected control	5.54	2.10	207.8	-19.8
JM16W	2.52	3.24	40.0	23.7
JM2W	2.20	2.93	22.2	11.8
JM2R	2.74	3.48	52.2	32.8
JM9W	2.56	1.94	42.2	-26.0
JM28W	2.50	2.95	38.9	12.6
JM17W	4.96	3.58	175.6	36.6
JM26W	1.98	2.88	10.0	9.9
S.E.[1]	2.021	0.734	152.80	35.29
LSD (0.05) [2]	0.431	0.654	0.053	0.027
Group 3				
Infected control	9.06	3.70	-	-
Uninfected control	14.32	4.10	58.1	10.8
JM39R	10.16	4.14	12.1	11.9
JM37R	13.50	4.92	49.0	33.0
JM32R	10.30	4.58	13.7	23.8
JM13R	8.36	2.98	-7.7	-19.5
JM8W	6.96	2.46	-23.2	-33.5
JM7W	7.24	2.50	-20.1	-32.4
S.E.[1]	4.982	1.550	152.80	35.29
LSD (0.05) [2]	0.445	0.063	0.053	0.027
Group 4				
Infected control	9.06	3.70	-	-
Uninfected control	14.32	4.10	58.1	10.8
JH4	11.22	4.28	23.6	15.7
JM6BO	11.96	4.00	32.0	8.1
JM41R	11.20	4.26	23.6	15.1
JH1	10.26	4.04	13.2	9.2
JM43R	7.32	3.56	-19.2	-3.8
JM42R	10.72	3.34	18.3	-9.7
S.E.[1]	3.677	1.100	152.80	35.29
LSD (0.05) [2]	0.789	0.053	0.053	0.027

Group 2

Table 3.4. Characteristics and identification of bacterial isolates tested for growth promotion and biocontrol activity in the current study

Isolate code	Cell shape	Movement	Gram +/-	Hugh-Leifson's test	Cytochrome oxsidase activity	API test	Identity
JM16W	Rod (Endospores)	Not Motile	+	NA	-	API 50 CH	*Bacillus subtilis*
JM2R	Rod (Endospores)	Not Motile	+	NA	+	API 50 CH	*Bacillus cereus*
JM6R	Rod	Motile	-	Oxidative	+	API 20 NE	*Burkholderia cepacia*
JH83	Rod	Motile	-	Oxidative	+	API 20 NE	*Pseudomonas fluorescens*
JH41	Rod	Motile	-	Oxidative	+	API 20 NE	*Sphingomonas paucimobilis*
JM17W	Rod	Motile	-	Fermentative	+	API 20 E	*Brucella* spp.

NA = not applicable

Treatments	Fresh leaf mass per plant (g)[a]	Fresh root mass per plant (g)[a]	Total fresh mass per plant (g)[a]	Increase in fresh leaf mass (%)[b]	Increase in fresh root mass (%)[b]	Increase in total fresh mass (%)[b]
Growth promotion						
Uninfected control	117.9	35.0	152.9	-	-	-
JM16W	152.3	33.2	185.5	29.2	-5.1	21.3
JH41	149.3	34.2	183.5	26.6	-2.3	20.0
JM6R	107.7	29.5	137.2	-8.7	-15.7	-10.3
JM2R	154.3	40.6	194.9	30.9	16.0	27.5
JH83	125.5	29.8	155.3	6.4	-14.9	1.6
JH41R	149.3	35.0	184.3	26.6	0.0	20.5
JH6BO	206.6	43.4	250.0	75.2	24.0	63.5
S.E.[1]	120.60	28.20	136.90	9.43	8.00	8.21
LSD (0.05)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Preventative[c]						
Infected control	145.5	31.3	176.8	-	-	-
JM16W	179.1	29.9	209.0	23.1	-4.5	18.2
JH41	140.6	32.8	173.4	-3.4	4.8	-1.9
JM6R	149.8	28.7	178.5	3.0	-8.3	1.0
JM2R	160.0	28.8	188.8	10.0	-8.0	6.8
JH83	169.9	29.1	199.0	16.8	-7.0	12.6
JH41R	209.8	35.7	245.5	44.2	14.1	38.9
JH6BO	186.6	32.0	218.6	28.2	2.2	23.6
S.E.[1]	19.60	2.28	19.91	13.47	7.27	11.26
LSD (0.05)[2]	0.007	0.020	0.005	0.007	0.022	0.005
Curative[c]						
Infected control	88.9	25.4	114.3	-	-	-
JM16W	156.2	27.7	183.9	75.7	9.1	60.9
JH41	116.8	30.9	147.7	31.4	21.7	29.2
JM6R	145.8	27.4	173.2	64.0	7.9	51.5
JM2R	151.4	28.6	180.0	70.3	12.6	57.5
JH83	131.9	25.7	157.6	48.4	1.2	37.9
JH41R	154.8	30.6	185.4	74.1	20.5	62.2
JH6BO	130.0	25.3	155.3	46.2	-0.4	35.9
S.E.[1]	25.49	2.79	25.57	28.67	10.97	22.39
LSD (0.05)[2]	0.065	0.132	0.051	0.065	0.132	0.051

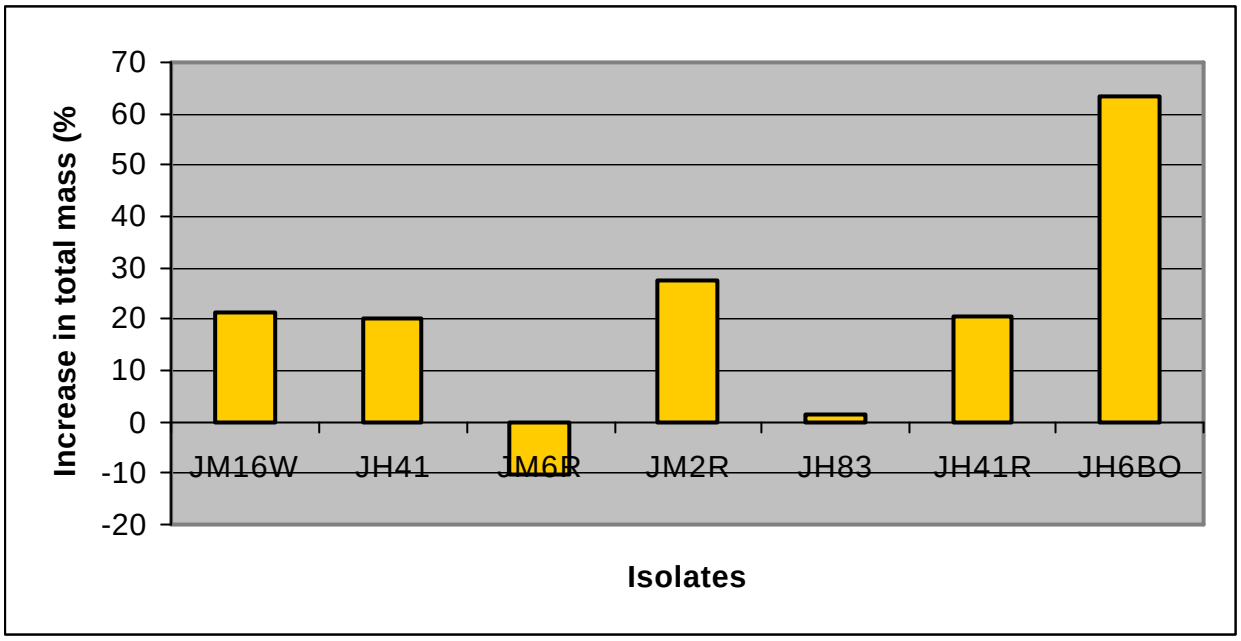
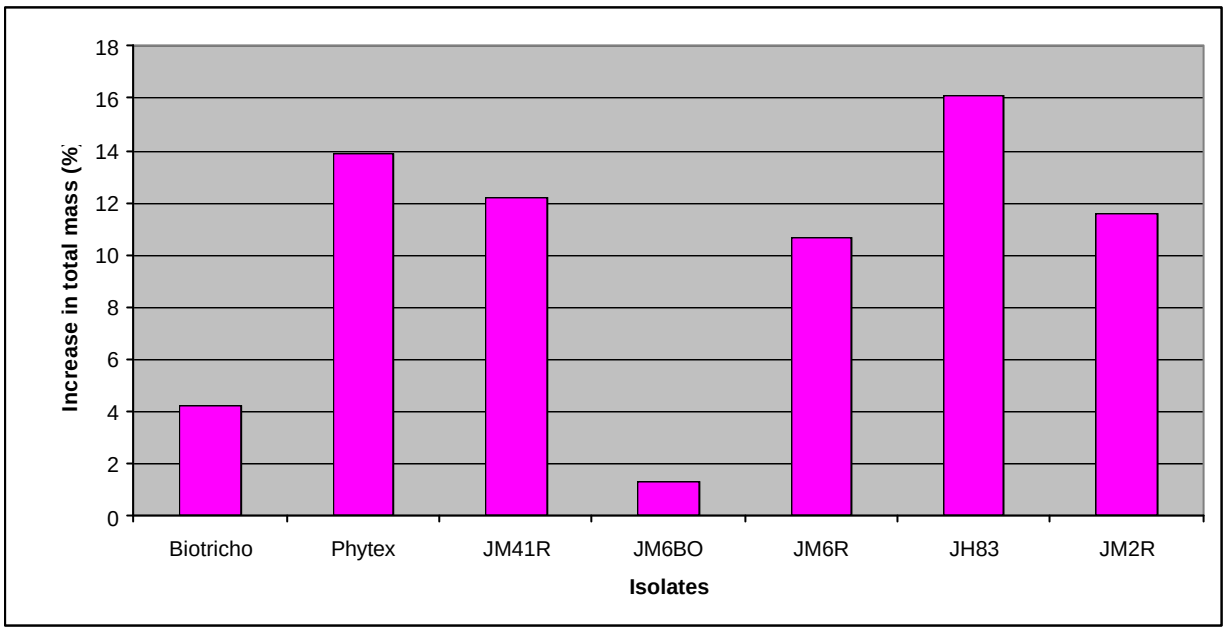


Table 3.6. *Pythium* incidence (%) in the nutrient solution 2 weeks (first reading) and 5 weeks (second reading) after transplanting lettuce plants into the re-circulating hydroponic system in the greenhouse. The *Pythium* incidence (%) in the roots was determined at harvest.

Potential biocontrol agent	First reading (nutrient solution)	Second reading (nutrient solution)	*Pythium* incidence on roots
JM6R	100	20	20
JH83	100	20	20
JM16W	100	80	60
JM2R	100	80	70
JH41	100	60	60
JM41R	100	100	100
JM6BO	100	100	100
JM17W	100	100	100

Table 3.7 Effect of biocontrol treatments and a commercial fungicide on yield of *Pythium* inoculated butterhead lettuce in a re-circulating gravel bed hydroponic system in the field (Small scale assessment of sub-sample of 24 plants per treatment).

Treatments	Fresh leaf mass per plant (g)[a]	Fresh root mass per plant (g)[a]	Total fresh mass per plant (g)[a]	Increase in fresh leaf mass (%)[b]	Increase in fresh root mass (%)[b]	Increase in total fresh mass (%)[b]
Semi-commercial						
Uninfected control	140.8	108.4	249.2	-8.2	15.0	0.61
Infected control	153.4	94.3	247.7	-	-	-
Biotricho	152.7	105.4	258.1	-0.5	11.8	4.2
Phytex	181.8	100.3	282.1	18.5	6.4	13.9
JM41R	173.3	104.7	278.0	13.0	11.0	12.2
JM6BO	145.3	105.5	250.8	-5.3	11.9	1.3
JM6R	167.7	106.4	274.1	9.3	12.8	10.7
JH83	165.1	122.5	287.6	7.6	29.9	16.1
JM2R	160.5	116.0	276.5	4.6	23.0	11.6
S.E.[1]	29.27	5.03	32.07	20.04	15.63	12.90
LSD (0.05)[2]	0.202	0.208	0.197	0.352	0.316	0.189



Treatments	Total fresh mass per plant (g)[a]
Commercial	
Uninfected control	227.7
Infected control	209.1
Biotricho	234.7
Phytex	232.5
JM41R	208.4
JM6BO	191.7
JM6R	224.8
JH83	241.3
JM2R	248.9
S.E.[1]	21.37
LSD (0.05)[2]	0.021

Table 3.9 Comparison of the activity of potential biocontrol and/or growth promotion isolates in static and a re-circulating gravel bed hydroponic system.

Isolates	Static Aquaculture System	Re-circulating gravel bed hydroponic system			
		Greenhouse		Field	
		Growth Promotion	Preventative /Curative	Small scale	Commercial
Burkholderia cepacia (JM6R)	+	-	+	+	-
Bacillus subtilis (JM16W	+	+	-	-	-
Bacillus cereus (JM2R)	+	+	+	+	+
Pseudomonas fluorescens (JH83)	+	-	+	+	+
Brucella spp. (JM17W)	+	-	-	-	-
Sphingomonas paucimobilis (JH41)	+	+	-	-	-
Trichoderma T1	+	+	+	-	-
Trichoderma T2	+	+	+	-	-

+ = Showed activity;
- = Showed no activity.

CHAPTER 4

MODES OF ACTION INVOLVED IN THE INHIBITION OF *PYTHIUM* F- GROUP

1. ABSTRACT

Of the isolates that were previously evaluated against *Pythium* wilt and root rot of lettuce in a hydroponic system (Chapters 2 and 3), six bacteria and two fungi were most effective. The following possible modes of action of these isolates were investigated: competition for nutrients, production of inhibitory substances and induced resistance. The root colonizing ability of the isolates was also assessed. Competition between the isolates and the pathogen were confirmed by testing for siderophore and hydrolytic enzyme production. Five of the isolates produced siderophores much faster than the rest, demonstrating that these isolates were able to take-up iron from the media at a faster rate, thus indicating a significant competitive ability. Antibiotic production by the isolates was confirmed *in vitro* by means of the dual culture technique. Of the eight isolates screened, only one isolate showed *in vitro* inhibition of the pathogen. This result was confirmed by a TLC assay, where fluorescent bands were formed by the same isolate, indicating the presence of phenolic compounds. These compounds were separated by HPLC. Analysis of total soluble and cell wall phenolic levels in *Pythium* infected and non-infected plants treated and untreated with the biocontrol isolates did not render conclusive results. Three of the eight isolates were able to colonize 100% of the lettuce roots.

2. INTRODUCTION

Biological control is a nonchemical control measure that has been reported in several cases to be as effective as chemical control (Guetsky *et al*., 2002). Saprophytic bacteria, yeast and filamentous fungi are common inhabitants of plant surfaces where some, through various mechanisms, have the ability to alter growth of pathogens and reduce diseases (Shtienberg, 2001). According to Guetsky *et al*. (2002), it has been shown that the efficacy of biological control

can occasionally be inadequate and variability in results may be high. To overcome this problem and enhance the efficacy of biological control, a better understanding of the mechanisms involved in biocontrol is necessary. There are several mechanisms involved in biocontrol which include, among others, competition, antibiosis, root colonization and induced resistance (Guetsky *et al*., 2002).

Droby and Chalutz, (1994), defined 'competition' as niche overlap, where there is simultaneous demand on the same resources by two or more microbial populations. All microorganisms need nutrients for multiplication and development of populations. Competition for limited nutrients on plant surfaces is an important mechanism of biological control against pathogens that depend on external nutrients for their population development (Shtienberg *et al*., 2001).

Pathogens may gain access into plant tissue by direct penetration of fungal hyphae through the cuticle and epidermis of the plant, others enter through wounds, senescing host tissue or natural openings such as stomates and lenticels. These areas are generally nutrient rich owing to exudation of sugars and amino acids. Biocontrol agents that can compete effectively with the pathogen to occupy these infection sites and utilize the nutrients more efficiently than the pathogen, can effectively displace the pathogen by preventing germination or infection (Punja and Uthede, 2003).

Siderophore production is another mechanism by which biocontrol agents may antagonize plant deleterious and plant pathogenic microorganisms (Buyer *et al*., 1989). Buyer *et al.* (1989) define siderophores as low molecular weight iron chelating agents produced by most bacteria and fungi under iron-limiting conditions. Siderophores are excreted, bind Fe, and are taken up by microorganisms, which use the Fe for cellular nutrition. They also state that certain pseudomonas produce siderophores to cause iron starvation of the pathogenic organisms by binding sufficient iron.

Antibiosis refers to the inhibition or destruction of a pathogen by a metabolic product of an antagonist, such as the production of a specific toxin, antibiotic or enzyme (Nakayama *et al*., 1999; Heungens and Parke, 2001). Secretion of antibiotic substances is a common phenomenon in nature. A number of antagonists have been reported to produce antibiotics *in vitro* and may have a role in protecting commodities against diseases before and after harvest (Droby and Chalutz 1994). Antibiotics, however, must be produced in sufficient quantities at the precise time of interaction with the pathogen to be effective (Larkin *et al*., 1998).

An important attribute of a successful biocontrol agent would be the ability to remain at high population density on the root surface, providing protection of the whole root for the duration of its life. Evidence has shown that root colonization by some non-pathogenic microorganisms may form a physical barrier on the root surface making it difficult for other microorganisms to penetrate or trigger a defense response in plants (Yedidia *et al*., 2000).

According to Nicholson and Hammerschmidt (1992), resistance responses are usually characterized by the early accumulation of secondary soluble and insoluble phenolic compounds that effectively isolates the pathogen at the point of infection. It is thought that rapid accumulation of toxic phenols (soluble) may result in the effective isolation of the pathogen at the original site of entrance. Associated responses include the accumulation of cell-wall appositions such as papillae and the early accumulation and modification of phenols within host cell walls as well as the synthesis and deposition of the phenolic polymer, lignin (Sauvesty *et al*., 1992). Esterification of phenols to cell wall materials has been suggested, in numerous reports, to play a major role in the expression of resistance. The presence of phenols in host cell walls is usually taken to imply an increase in resistance to fungal enzymes as well as a physical barrier against fungal penetration (De Ascensao and Dubery, 2003).

In this study, the most likely modes of action employed by eight different biocontrol agents against *Pythium* F-group was investigated and demonstrated.

3. MATERIALS AND METHODS

3.1 Isolates

Isolates of *Bacillus cereus* (JM2R), *Burkholderia cepacia* (JM6R), *Pseudomonas fluorescens* (JH83), *Sphingomonas paucimobilis* (JH41), *Bacillus subtilis* (JM16W), *Brucella* sp. (JM17W), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) were used throughout this study. Original pure cultures of all eight isolates were freeze-dried and stored at room temperature (Smith and Onions,1994; Goszczynska and Serfontein, 2000). Bacteria were revived by dissolving the cultures in 5 ml nutrient broth (NB) (Biolab, Merck) and spreading 100 µl of the solution on nutrient agar (NA) (Biolab) plates. Cultures were incubated at 25 ^{0}C in the dark for two days prior to use. Fungal cultures were revived by plating them out on potato dextrose agar (PDA) (Biolab) and incubating the cultures at 25 ^{0}C in the dark for a week and then exposing them to light for three days at room temperature to elicit spore production.

3.2 Competition

3.2.1 Production of hydrolysing enzymes

Specific media were used to determine the production of amylase, lipase, and proteinase (Norris and Ribbons, 1971). Bacterial isolates were streak inoculated in the middle of the prepared Petri plates. For fungal isolates, mycelial plugs (5 mm diameter) were punched from actively growing cultures and placed in the middle of the plates. These plates were incubated at 25 ^{0}C for three days. In all cases four replicate plates were inoculated for each isolate and the experiment was repeated three times.

3.2.1.1 Extracellular Amylases

The specific media used to determine extracellular amylase activity was prepared by adding starch to PDA medium at 0.02:1 w/v. (Norris and Ribbons, 1971). The medium was sterilized for 20 min at 121 ^{0}C. Plates were inoculated as in 3.1 and incubated at 25 ^{0}C for three days before covering the plates with Gram's jodium. The presence (+) or absence (-) of clear zones in the agar surrounding bacterial and fungal growth was recorded.

3.2.1.2 Lipase activity

The specific media used to determine lipase activity was prepared by adding 0.01% $CaCl_2$ (Fluka) to 1 % Tween - 80 (Sigma) in 1000 ml PDA medium (Norris and Ribbons, 1971). The medium was autoclaved for 20 min at 121 °C. Plates were inoculated as in 3.1 and incubated at 25 ^{0}C for three days. The presence (+) or absence (-) of clear zones in the agar surrounding bacterial and fungal growth was recorded.

3.2.1.3 Proteinase Activity

The specific media used to determine proteinase activity was prepared by adding 100 ml skimmed milk to 4.3 % w/v PDA medium (Norris and Ribbons, 1971). The medium was autoclaved for 20 min at 121 °C. Plates were inoculated as in 3.1 and incubated at 25 ^{0}C for three days. The presence (+) or absence (-) of clear zones in the agar surrounding bacterial and fungal growth was recorder.

3.2.1.4 Gelatinase Activity

The specific media used to determine proteolitic enzyme activity was prepared by adding gelatine to NB at a 0.15:1 w/v ratio and boiling the solution to dissolve. 5 ml of the heated solution was dispensed into test tubes and autoclaved at 121 °C for 20 min. The tubes were cooled down to room temperature and stab-inoculated with the various bacteria. For testing of the fungal isolates, a mycelial plug was placed inside the tubes. After three days

growth at 25 ^{0}C the samples were placed in the freezer for 30 min. The solidity (-) or liquefacation (+) of the media was recorded (Norris and Ribbons, 1971).

3.2.2 SIDEROPHORE PRODUCTION

The methods of Schwyn and Neilands (1987), and Buyer *et al.* (1989), were combined to evaluate the production of siderophores.

3.2.2.1 CAS Stock solution

Solution A was prepared by slowly adding 2 mM Crome Azurol S (CAS) (Sigma, USA) to an iron solution (1 mM $FeCl_3.6H_2O$ (AnalaR) in 10 mM HCL (Sigma)) in a 5:1 v/v ratio. 5 mM Hexadecyltrimethylammonium Bromide (HDTMA) (Fluka, USA) was dissolved in 40 ml distilled water over a hot plate to prepare solution B. Solution A was slowly added to solution B in a 3:2 v/v ratio and autoclaved to prepare the CAS stock solution.

3.2.2.2 RSM Stock solution

The following were all added together and autoclaved for 20 min at 121^{0}C to prepare the RSM Agar medium: 6.35 mM $Ca(NO_3)_2.4H_2O$ (Saarchem, South Africa); 2 mM $MgSO_4.7H_2O$ (Saarchem); 0.2 M N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) (Fluka); 0.1 M NaOH (Merck); 30 % w/v bacteriological agar (Biolab). The following solutions were autoclaved separately before being added to the RSM agar medium: 1 M KH_2PO_4 (Saarchem) at 1:0.002 v/v; 10 % Casamino acid solution (Difco, USA) at 1:0.17 v/v, and 30 % sucrose (Saarchem) at 1:0.07 v/v.

The following solutions were filter sterilized before adding to RSM agar medium to prepare the RSM stock solution: 7 x 10^{-3} M $ZnSO_4.7H_2O$ (Fluka) at 1 : 2 x 10^{-3} v/v; 0.009 M $MnSO_4.H_2O$ (Saarchem) at 1 : 2 x 10^{-3} v/v; 0.02 % W/v thiamine HCL (Sigma); and 1 x 10^{-3} % w/v biotin (Fluka).

3.2.2.3 The RSM-CAS medium for siderophore screening

The CAS stock solution was slowly added to the RSM stock solution (1:9 v/v), to keep foam from forming, and dispensed into Perti dishes. The plates were inoculated with the eight different isolates by using the dual culture method as described in Chapter 2. Three replicate plates were used per treatment. Control plates were inoculated with only the potential biocontrol agent to determine if the presence of the pathogen would influence siderophore production. The experiment was repeated four times. Plates were incubated at 25 ^{0}C and monitored for two days for the presence or absence of yellow zones surrounding the colonies on the green plates. The yellow zones were measured after 24 h and 48 h.

3.4 PRODUCTION OF INHIBITORY SUBSTANCES (ANTIBIOSIS)

3.4.1 In vitro plate assay

The eight biocontrol agents described in 3.1 were inoculated into specific media (APM) that optimized the production of antibiotics (McKeen, *et al*, 1986). The media were prepared by adding 2 % (w/v) sucrose, 0.5 % (w/v) DL-glutamic acid, 7 x 10^{-5} $MgSO_4.7H_2O$, 5 x 10^{-5} K_2HPO_4, 6 x 10^{-5} KCl and 1 ml Trace Element Solution (0.5 % (w/v) $MnSO_4.H_2O$, 0.16 % (w/v) $CuSO_4.5H_2O$, 0.015 % (w/v) $FeSO_4.7H_2O$ in distilled water) per liter distilled water. The pH was adjusted to 6.0 – 6.2. Erlenmeyer flasks (250 ml) containing 200 ml of the prepared liquid were autoclaved at 121 ^{0}C for 20 min and allowed to cool down to room temperature. Single bacterial colonies growing on NA and fungal mycelium plugs (5 mm) growing on PDA were used to inoculate the flasks which were then shake incubated (110 rpm) at 23 ^{0}C. After seven days the cells / spores were removed by centrifuging (5 000 x g) suspensions for 20 min and filtering the supernatant through 0.22 µm pore size acetate filters for bacterial isolates and 0.4 µm pore size acetate filters for fungal isolates. Serial dilutions (6.25 %, 12.5 %, 25 %, 50 % and 100 %) were made of the filtrates.

Five holes were prepared in the agar of PDA agar plates with a sterile 5 mm diameter cork borer. From each bacterial/fungal liquid culture filtrate, 100 µm was pipetted into each hole per plate (Fig 4.4). A filtrate of sterile APM medium was used as a negative control. 5 mm mycelium plugs of *Pythium* F-Group, grown on PDA, were placed in the middle of each plate. Plates were incubated at 25 ^{0}C and monitored daily for development of inhibition zones. The presence or absence of inhibition zones was recorded. Four replicate plates were inoculated for each isolate and the experiment was repeated three times.

3.4.2 Quantification of total phenolic compounds

Total soluble phenolics in the culture filtrates of the biocontrol isolates obtained in 3.4.1 were determined by means of the Folin-Ciocalteu's Phenol reagent (Bray and Thorpe, 1954). The reaction mixture was scaled down to enable the use of 96 well ELISA-plates. The mixture comprised of: 175 µl distilled water, 5 µl liquid culture filtrate, 25 µl Folin and Cioalteu's Phenol reagent (Sigma) and 50 µl of 20 % (w/v) sodium carbonate solution (NA_2CO_3) per well. A negative control was obtained by adding all compounds excluding the liquid culture filtrate. The solution in each well was mixed with a micropipette until the yellow colour faded. Plates were incubated in a microplate incubator at 40^{0}C for 30 min.

Three wells were used per sample and the experiment was done in triplicate. Absorbency, which is an indication of the concentration of phenolic compounds in the liquid culture filtrate, was measured with a Multiskan Ascent VI. 24 354-00973 (version 1.3.1) at 690 nm. Data were calculated as gallic acid equivalent in mg/ml extract from the standard curve using an equation: $y = 1.3527x + 0.0109$ ($R^2 = 0.9989$).

3.4.3 Thin Layer Chromatography (TLC)

A comparative study was performed on the eight different culture filtrates prepared in 3.4.1 by thin layer chromatography on pre-coated Silica Gel 60 (Merck). The following solvents were tested to obtain the best separation of phenolic compounds: hexan / ethyl acetate, toluene / ethyl acetate, water, chlorophorm / methyl (9:1), acetic acid (2%), acetic acid (12%), butanol / acetic acid / water (6:1:2), acetic acid / ethyl acetate/ methanol (8:1:1) and ethyl acetate/ methanol/ water (7:2:1) of which the latter gave the best result. The TLC plate was loaded with 10 µl of each sample. Sterile APM liquid were filtered and used as control. The plates were developed with ethyl acetate/ methanol/ water (7:2:1) and left under an extractor fan overnight to remove all traces of volatile solvents. Phenolic compound separation was visualized with a 50 Hz UV lamp (256 and 365 nm).

3.4.4 High performance liquid chromatography (HPLC)

HPLC analysis was performed on the culture filtrate of *P. fluorescence* (JH83) by injecting 10 µl of the filtrate into an HPLC column. The chromatographic system consisted of Varian 9012 high pressure pumps (3 phases), a manual injector, an integrated system controller, a MALsil C18, 5 micron, reverse-phase analytical column (250 x 4.6mm, five µm particle size), and a system spectra 6000 LP UV diode array detector with an attached analysis computer and data storage system (OS/2 WARP, Thermo Separation Products). The mobile phase, consisting of water and acetonitrile, as well as the flow rate used at specific times are summarized in table 4.1.

Table 4.1 Summary of the program followed during separation of samples by means of HPLC.

Time (min)	% Acetonitrile	Flow rate
0	10	1.5
10	30	1.6
13	50	1.9
15	55	1.9
17	10	1.6

3.5 INDUCED RESISTANCE

3.5.1 Inoculum preparation

Bacterial isolates were cultured in 250 ml Erlenmeyer flasks containing 200 ml NB. Inoculation of the broth was done with a single colony of bacteria growing on NA. Cultures were shake incubated (110 rpm) at 24 ^{0}C for 48 h. The medium was then centrifuged at 5 000 rpm's for 20 min and the resulting pellet dissolved in 200 ml sterile Ringer's solution. A dilution series was made in 9 ml Ringers' solution to determine the bacterial cell concentration with a Petroff - Hausser counting chamber (Korsten and De Jager, 1995). The solution was adjusted to a final concentration of 10^6 cells/ml.

The fungal isolates were grown on PDA and incubated for five days in the dark at 25 ^{0}C and subsequently exposed to light for two days to induce spore formation. Spores were harvested by pouring 5 ml Ringers' solution on the plates and scraping the surface with a sterile plastic hockey stick. 1 ml of the spore suspension was inoculated into 9 ml sterile quarter strength Ringer's solution. Spores were counted with a haemocytometer and adjusted to obtain a concentration of 10^4 spores/ml.

Pythium F- group was grown on PDA for three days at 25 ^{0}C. Mycelial plugs (5 mm diameter) were punched with a sterile cork borer and used as pathogen inoculum.

3.5.2 In vivo inoculation

Butter head lettuce seedlings (*Lactuca sativa* L. var. *capitata* L) were grown in polystyrene seedling trays in steam-pasteurized Canadian peat moss in the nursery of a commercial hydroponic grower (Hydrotec, Gauteng, South Africa). Four weeks after planting, root tips of ten seedlings from each seedling tray were plated on BNPRA media (Roux and Botha, 1997) to determine if seedlings were *Pythium* free. For the remainder of the plants the roots of the seedlings were washed in sterile water to get rid of the growth medium and subsequently transferred to test tubes filled with sterile water. Seedlings were supported with sterile cotton wool at the top of each test tube whilst their roots were suspended in the water. For each treatment, lettuce plants were inoculated with 2 ml of the potential biocontrol agent and three *Pythium* mycelium plugs (5 mm diameter). Nine plants were used per treatment. Untreated lettuce plants, lettuce plants infected with only *Pythium* and lettuce plants treated only with the potential biocontrol agents served as controls. After two days plants were harvested, roots were excised and freeze-dried for 48 h. The dried material was ground with a pestle and mortar to a fine powder and the total soluble and insoluble phenolic compounds quantified as described below.

3.5.3 Quantification of total soluble phenolic compounds

Total soluble phenolic compounds were determined by preparing extracts from the powdered root tissue as follows: one milliliter of methanol/ acetone/ water (7:7:1) was added to 0.05 g powdered root sample, vortexed for 30 seconds, shaken on an rotary shaker (110 rpm) for 1 hour and centrifuged at 13 500 rpm (24 000 g) for 5 minutes. The extraction procedure was repeated three times. The supernatant was transferred to a clean eppendorf tube, left under an extraction fan to evaporate to 0.5 ml and readjusted to 1 ml by adding distilled water. The remaining pellet was used for the quantification of insoluble phenolics. Total soluble phenolics were quantified using the Folin-Ciocalteu's reagent (Bray and Thorpe, 1954) as described in 3.4.2 except for

the amounts of reaction mixture used. In each well, 1 µl of the sample was added to 179 µl distilled water, 25 µl Folin and Cioalteu's Phenol reagent (Sigma) and 50 µl of 20 % (w/v) NA_2CO_3.

3.5.4 Quantification of total insoluble phenolic compounds

One milliliter NaOH (0.5 N) was added to 0.01 g of the pellet obtained in 3.5.3, sealed in glass tubes and placed in warm water for 4 h at 96 ^{0}C to prepare the crude extract. The extraction mixture, which comprised of 60 µl HCl, 1 ml diethyl ether and 1 ml crude extract, were mixed together and the supernatant transferred to a clean eppendorf tube. The extraction process was repeated four times. The tubes were left under an extraction fan for the diethyl ether to evaporate completely. The resulting precipitate was dissolved in 250 µl methanol and stored at 4 ^{0}C. Total insoluble phenolics were quantified by using the Folin - Ciocalteu's Phenol reagent (Bray and Thorpe, 1954) as described in 3.4.2.

3.6 ROOT COLONIZATION BIOASSAY

3.6.1 Preparation of micro-organisms

Bacterial and fungal suspensions were prepared by growing them on NA (bacteria) and PDA (fungi) at 25 ^{0}C for two days. Cells/spores were harvested by pouring 5 ml Ringers' solution over the plate and scraped the surface with a plastic hockey stick. A dilution series was made and the cell concentration determined as described before (3.5.1). A suspension of 10^{10} cfu/ml was used for bacterial isolates and 10^8 spores/ml for fungal isolates.

3.6.2 Root colonization

The ability of eight selected organisms (3.1) to colonize lettuce plant roots were established according to the method described by Misaghi, (1990). Lettuce seeds were surface sterilized in 1 % $NaOCl_3$ and washed three times in sterile water. Eight seeds were embedded approximately 3 cm from the

centre of a 90 mm diameter Petri plate containing 25 ml of 1.2 % water agar (Fig 4.6).

Plates were turned on their sides and incubated at 25 ^{0}C in the dark and roots allowed to grow down through the agar for approximately 3 cm from the seed line. About 2 µl of the various biocontrol suspensions were introduced into the agar by inserting the tip of a pipette filled with 10 µl of the biocontrol suspension into the agar, approximately 2 mm below the tips of the roots.

Plates were again incubated on edge at 25 ^{0}C in the dark to allow the roots to grow through the inoculation point.

Root colonization was observed by studying the roots on an inverted Petri plate under a compound microscope at 10x magnification. A root colonization index was calculated for each isolate on the lettuce roots on a scale from zero (0 % of roots were colonized) to 10 (100 % of roots were colonized). Four replicate plates were inoculated for each isolate and the experiment was repeated three times.

4. RESULTS

4.1 COMPETITION

4.1.1 Hydrolytic enzyme activity

Through the formation of clear zones in starch and caseien medium and the liquefication of the gelatine rich media, *B. cereus* (JM2R) and *B. subtilis* (JM16W) proved to be able to produce extracellular amylase, gelatinase and proteinase. *P. fluorescence* (JH83) and *Brucilla spp* (JM17W) showed the ability to produce gelatinase, while *S. paucimobilis* (JH41) produced extracellular amylase and *B. cepacia* (JM6R) produced proteinase (Table 4.2). Not one of the isolates showed any lipase activity.

4.1.2 Siderophore production

All isolates tested were able to produce siderophores (Fig 4.1). The presence of *Pythium* on the dual culture plates did not influence the production of siderophores when compared to the control. *B. cereus* (JM2R), *B. cepacia* (JM6R), *P. fluorescence* (JH83), *B. subtilis* (JM16W) and *Brucilla* spp (JM17W) produced siderophores much quicker over the 24 h and 48 h period than *S. paucimobilis* (JH41) and the two *Tricoderma* species (Fig 4.2 and Fig 4.3).

4.2 PRODUCTION OF INHIBITORY SUBSTANCES

4.2.1 In vitro assay

Different substances were produced by *B. cepacia, S. paucimobilis, P. fluorescens*, *B. subtilis* and *Brucella* spp. as was evident through the colour changes in the originally colourless APM media (Fig 4.5), but only *P. fluorescence* (JH83) produced inhibitory substances. The greatest inhibition was obtained with the undiluted liquid culture filtrate. The inhibitory effect diminished at higher dilutions (Fig 4.7).

4.2.2 Production of phenolic compounds

All isolates produced phenolic compounds with the greatest amount present in the liquid culture filtrate of *Pseudomonas fluorescence* (JH83) at 9.18 E – 04 mg / ml gallic acid equivalent (Fig 4.8). High amounts of phenolic compounds were also produced by *B. subtilis* (JM16W) at 5.35 E - 04 mg / ml gallic acid equivalent. The amount of phenolic compounds produced by *S. paucimobilis* (JH41), *B. cepacia* (JM6R), *B. cereus* (JM2R), *Brucilla spp* (JM17W), *B. subtilis* (JM16W), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) ranged from 4.12 E – 04 to 6.77 E – 05 mg / ml gallic acid equivalent. Uninoculated APM was used as control and contained 5.29 E - 05 mg / ml gallic acid equivalent.

4.2.3 Compound separation with TLC

The liquid culture filtrate of *P. fluorescence* (JH83) formed three distinct blue fluorescent bands on a pre-coated silica glass plate when visualized under UV light (Fig 4.9). No bands were noticed in the control or any other sample.

4.2.4 HPLC of liquid culture filtrate of isolate JH83

The results of the HPLC analysis are presented in figure 4.8, where A is the liquid culture filtrate *P. fluorescence* (JH83) and B the uninfected APM control (Fig 4.10).

4.3 INDUCED RESISTANCE

4.3.1 Total soluble phenolic compounds produced by the roots of lettuce plants treated with Pythium F-group and potential biocontrol isolates.

The treatment of uninfected and *Pythium* infected lettuce plants with the potential biocontrol agents did not increase the total soluble and insoluble phenolic compounds produced in the roots when compared to the untreated controls. The mode of action of these isolates does not appear to induce resistance in the lettuce plants (Fig. 4.11 and Fig. 4.12), at least not based on increased levels of total phenolics.

4.5 ROOT COLONIZATION

Of the eight isolates tested, *P. fluorescence* (JH83), *Brucilla spp* (JM17W) and *Trichoderma* T1 (JM41R) colonized 100 % of the roots, whereas *B. cereus* (JM2R), *B.* cepacia (JM6R) colonized 50 % of the roots. *S. paucimobilis* (JH41) and *Trichoderma* T2 (JM6BO) colonized only the area around the inoculation point. No colonization was observed with *B. subtilis* (JM16W). Figure 4.13 shows the colonization of the middle (A) and tips (B) of the butter lettuce roots by potential biocontrol isolates as observed at 10x magnification under a compound microscope.

5. DISCUSSION

Five of the potential biocontrol isolates which were tested produced siderophores much quicker than the rest, implying that these isolates were able to utilize iron from the media at a faster rate, depriving other organisms of limited iron thereby showing a higher competitive ability. Different siderophores differ in their affinity for iron, therefore if an antagonist produces siderophores with a higher iron affinity than the pathogen, the latter could be deprived of iron and therefore its growth will be inhibited (Campbell, 1989). According to Alexander and Zuberer (1991), the excretion of siderophores by antagonistic microorganisms may stimulate plant growth by improving the iron nutrition of the plant.

Six of the isolates produced at least one of the extracellular enzymes: proteonase, amylase and gelatinase. The breakdown of complex molecules can give an organism the ability to utilize the nutrients more effectively than the pathogen and may be able to displace the pathogen (Campbell, 1989). The data obtained in this study suggests that competition for nutrients could play a role in some of the isolates ability to inhibit *Pythium* growth, but that it is probably not the only mechanism.

Antibiotic substances, effective against *Pythium* F- group, was produced by only one of the eight isolates tested namely *P. fluorescence* (JH83). The observed inhibition could have been due to one or more substances. In accordance with the results found with the *in vitro* plate assay, the same isolate produced a significant number of phenolics. According to Siqueira *et al*. (1991), it is well established that micro-organisms are capable of metabolizing and producing a range of phenolic compounds. TLC analysis of the culture filtrate of isolate JH83 showed that at least three different compounds were produced by this isolate in the current study. HPLC separation of this isolates' liquid culture filtrate as well as the corresponding control resulted in the elution of two major peaks, which did not appear in the control. Howell and Stripanovic (1980), found that their *P.*

fluorescens strain were antagonistic to *Pythium ultimum* because of the production of the antibiotic pyoluteorin. The liquid culture filtrate of the remaining isolates did not induce inhibition zones against *Pythium* F -group, however, there is still the possibility that the isolates could produce antibiotics effective against *Pythium* when grown on different substrates or at different temperatures.

The treatment of uninfected and *Pythium* infected lettuce plants with the potential biocontrol agents did not result in significant increase in total soluble or insoluble phenolic compounds as expected. This indicates that none of the isolates triggered the lettuce plant's defense response via the phenolic pathway. The mode of action of these isolates does not appear to be linked to induced resistance. Plants need time to reach the induced state. Many studies have shown that it generally takes from a few days to a week for systemic acquired resistance or induced systemic resistance to develop and then only when plants are inoculated with a dosage of bacteria that exceeds a certain threshold population size (Van Loon *et al*., 1998). Chemical and biocontrol products tested by Agostini *et al*. (2003) for induced resistance was found to be more effective when applied one day prior to pathogen inoculation. Different results might be obtained if the same phenolic compound induction experiments were conducted over an extended period of a week in stead of two days, the concentration of cells of the biocontrol isolates were optimized and lettuce roots treated with biocontrol agents before *Pythium* infection.

The eight potential biocontrol agents differed in their ability to colonize lettuce roots in the water agar assay. Bacterial cells could readily be seen swarming around the roots while mycelium of fungal isolates could be seen on the root surface and the area around the root similar to the observations by Misaghi (1990). Harris *et al*. (1997) indicated that the prior colonization of a *Capsicum* seedling by antagonistic binucleate *Rhizoctonia* inhibits subsequent colonization by *Rhizoctonia solani* and *Pythium ultimum* var. *sporangiiferum*. Rapid

colonization of roots could give an organism a competitive advantage in the rhizosphere and cause the exclusion of a pathogen.

In this study, the most likely modes of actions employed by eight different biocontrol agents against *Pythium* F- group was investigated and demonstrated. It is clear that more than one mechanism is responsible for the antagonistic activity of these organisms against *Pythium*. Further studies would be required to identify the specific antibiotics involved and possible mycoparasite activity where fungal cell wall degrading enzymes such as chitinase and glucanase dissolve their fungal hosts' cell walls and penetrate the cells.

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Table 4.1 Production of hydrolytic enzymes by potential biocontrol agents determined by means of specific assays testing for the ability to break down starch, lipids, caseien and gelatine.

Isolate	Number	Amylase activity	Lipase Activity	Gelatinase activity	Proteinase activity
Bacillus cereus	(JM2R)	+	-	+	+
Bacillus subtilis	(JH16W)	+	-	+	+
Brucella spp	(JM17W)	-	-	+	-
Burkholderia cepacia	(JM6R)	-	-	-	+
Pseudomonas fluorescence	(JH83)	-	-	+	-
Sphingomonas paucimobilis	(JH41)	+	-	-	-
Trichoderma T1	(JM41R)	-	-	-	-
Trichoderma T2	(JM6BO)	-	-	-	-

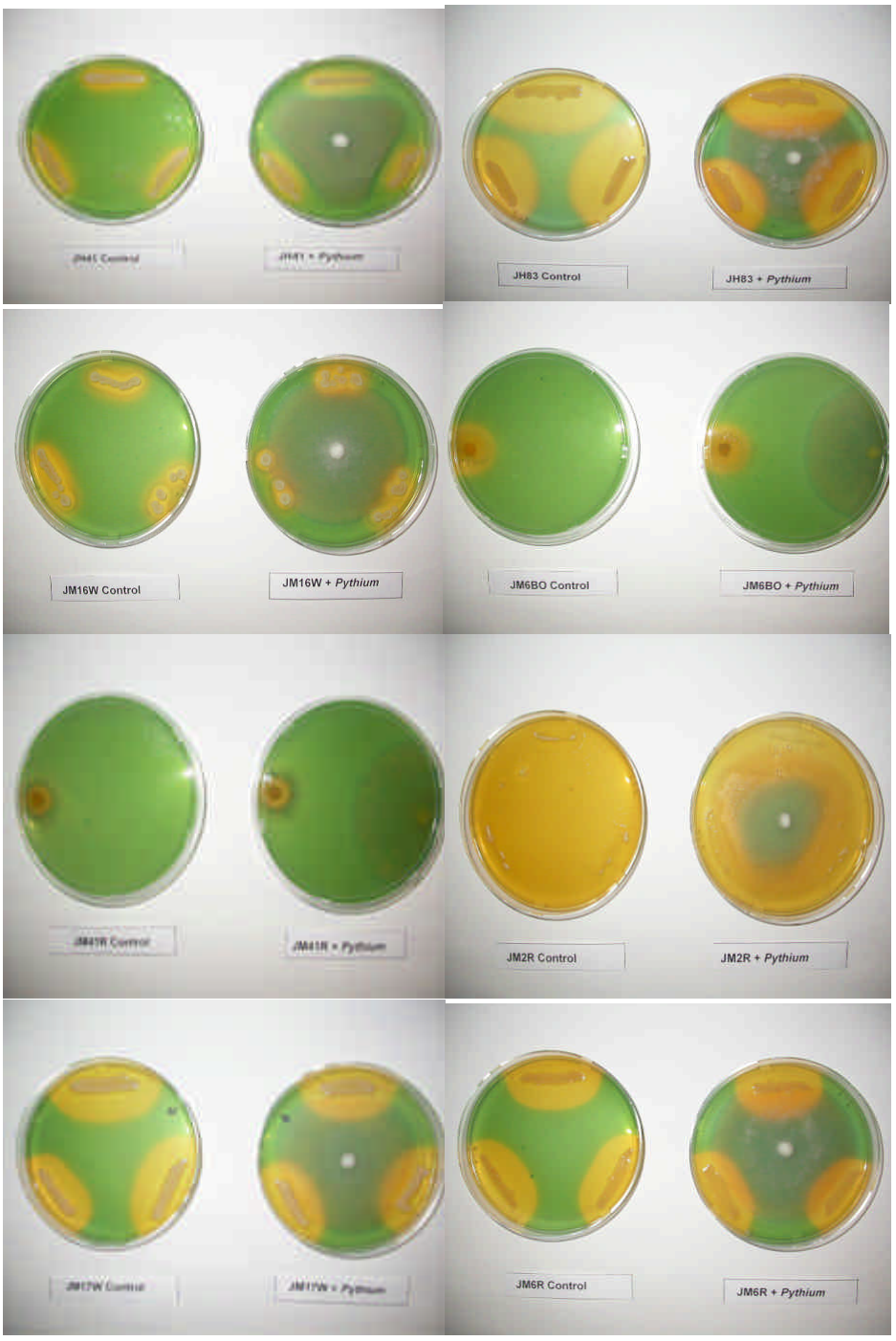


Figure 4.1 Results of siderophore production assay of potential biocontrol agents on CAS-RSM-medium (Schwyn and Neiland, 1987; Buyer *et al*. 1989). The yellow zones indicate the production of siderophores.

Figure 4.2 *In vitro* siderophore production on iron limited media by potential biocontrol agents against *Pythium* F-group after 24 h.

Figure 4.4. An illustration of the method used to determine the production of inhibitory substances by potential biocontrol agents against *Pythium* F-group on potato dextrose agar media.

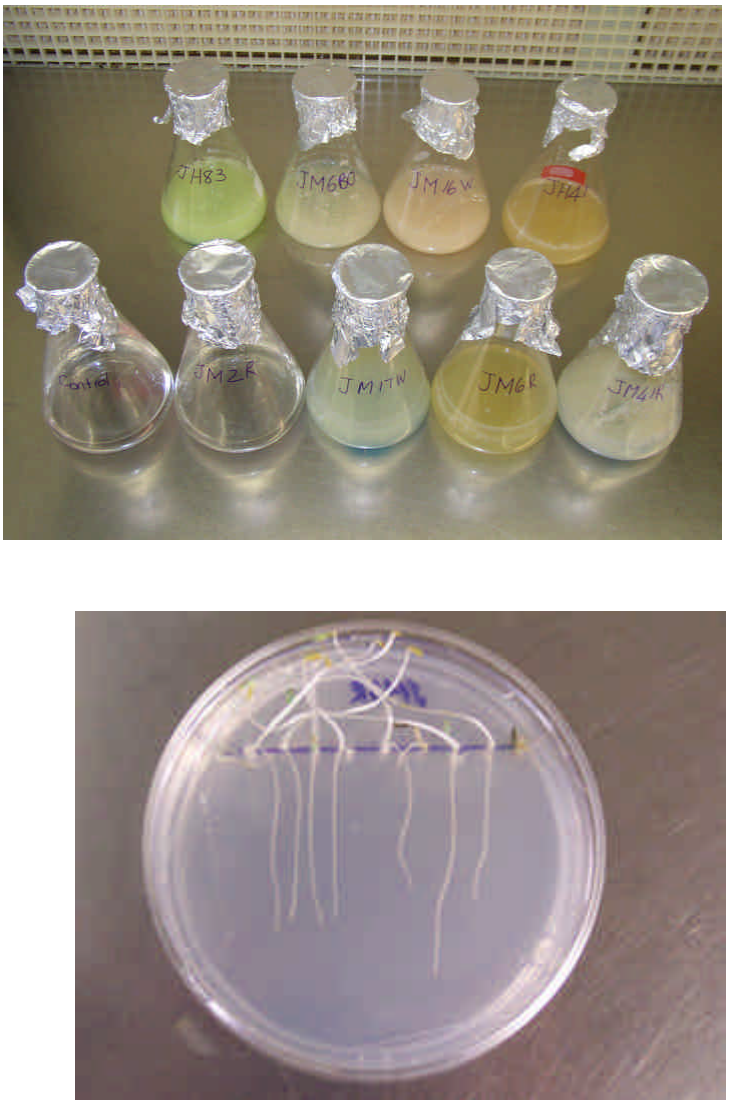


Figure 4.6 Root colonization assay with butter lettuce seedlings. Seeds were imbedded in water agar, the Petri plate incubated on edge (vertically) and the roots allowed to grow through the agar. Inoculum was added 2 mm from the root tips.

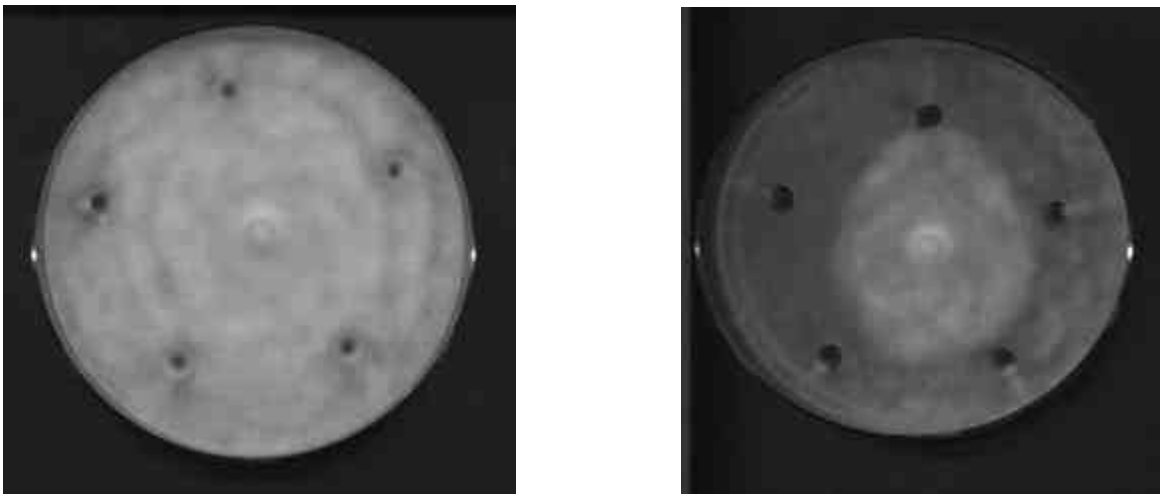
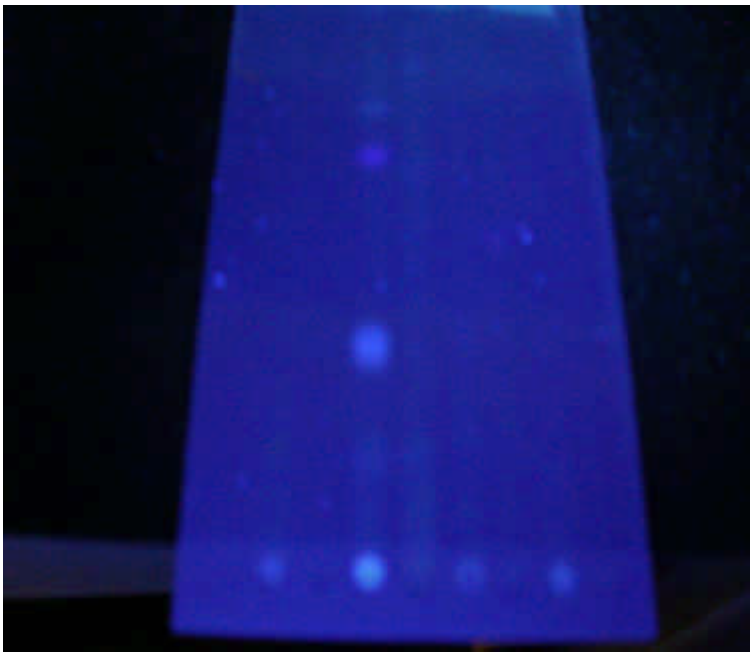


Figure 4.7 Antibiosis test (dual culture assay) of isolate JH83 against *Pythium* F-group on potato dextrose agar plates showing inhibition of the pathogen (Left = *Pythium* control; Right = isolate JH83).

Figure 4.8 *In vitro* production of total soluble phenolic compounds by potential biocontrol agents against *Pythium* F-group. Analysis was conducted on culture filtrates by means of the Folin-Ciocalteu's reagent (Bray and Thorpe, 1954).



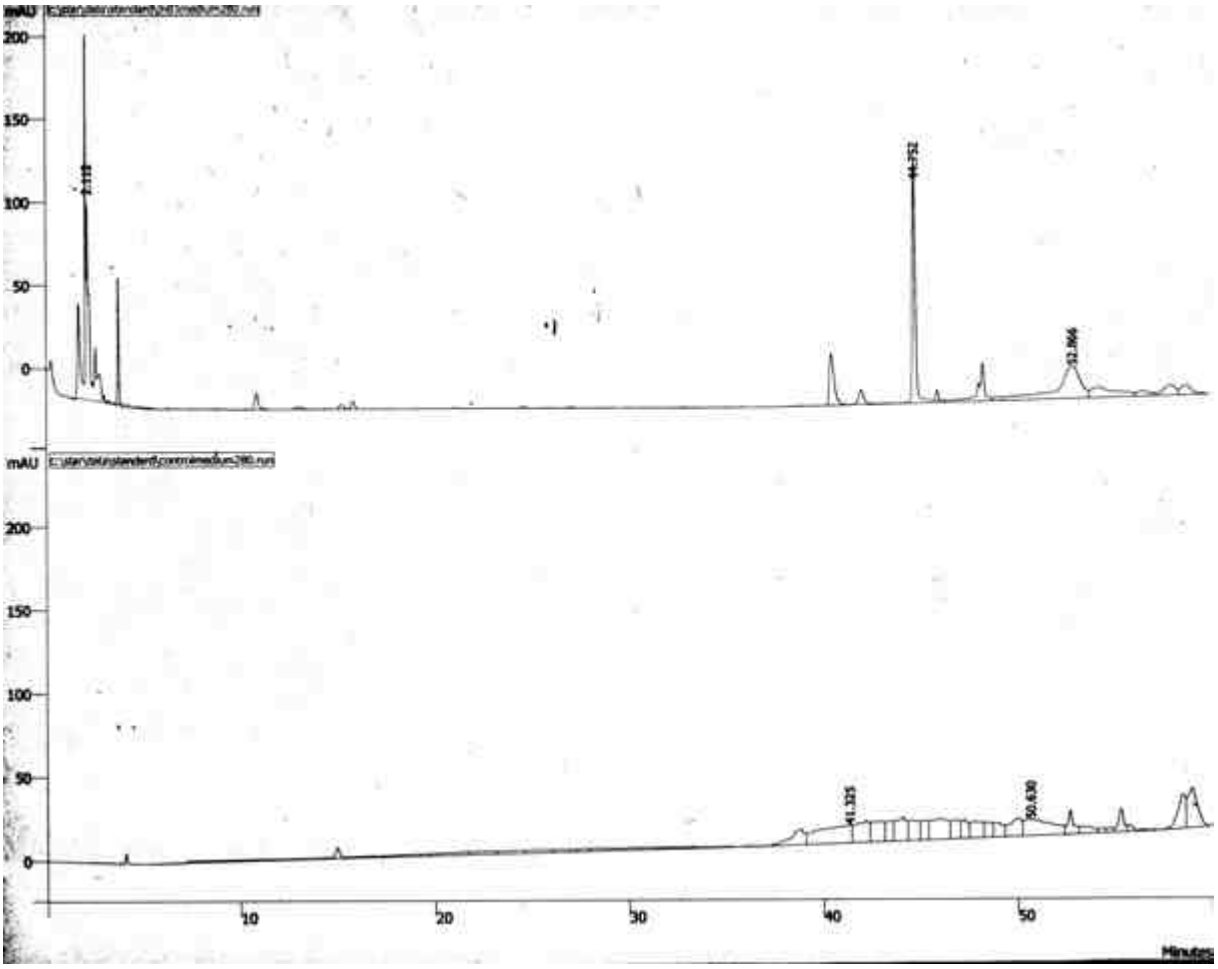


Figure 4.10 HPLC chromatogram of the liquid culture filtrate of *Pseudomonas fluorescence* (JH83) viewed at 280nm.

Figure 4.11 Quantification of total soluble phenolic compounds produced in butter head lettuce roots infected with *Pythium* F-group and treated with potential biocontrol isolates. Analysis conducted by means of the Folin-Ciocalteu's reagent (Bray and Thorpe, 1954).

Figure 4.12 Quantification of total insoluble phenolic compounds produced in butter head lettuce roots infected with *Pythium* F-group and treated with potential biocontrol isolates. Analysis conducted by means of the Folin-Ciocalteu's reagent (Bray and Thorpe, 1954).

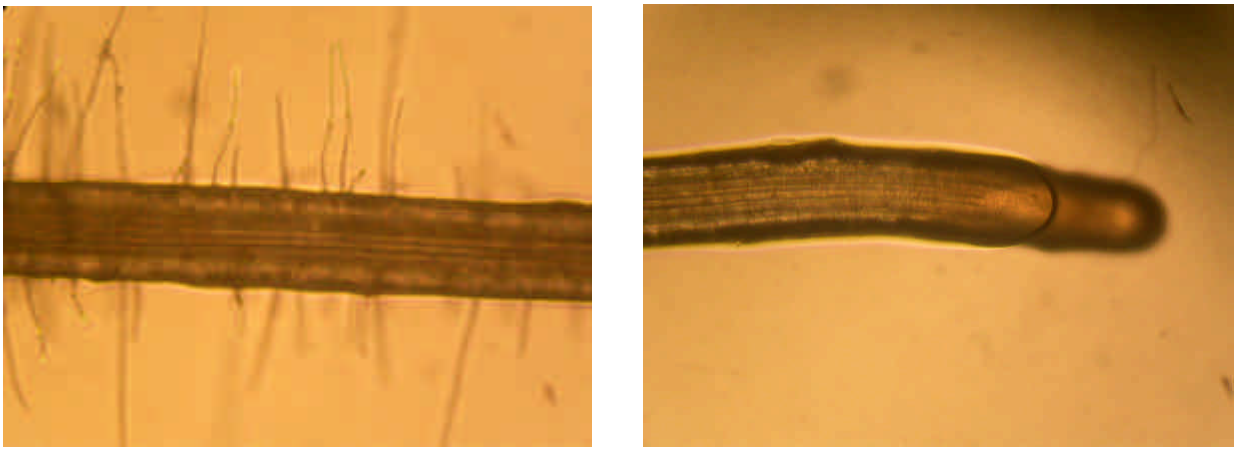


Figure 4.13.1 Root colonization assay with butterhead lettuce seedlings: control root inoculated with Ringers' solution (A: Root growing through Inoculation point; B: Root tip).

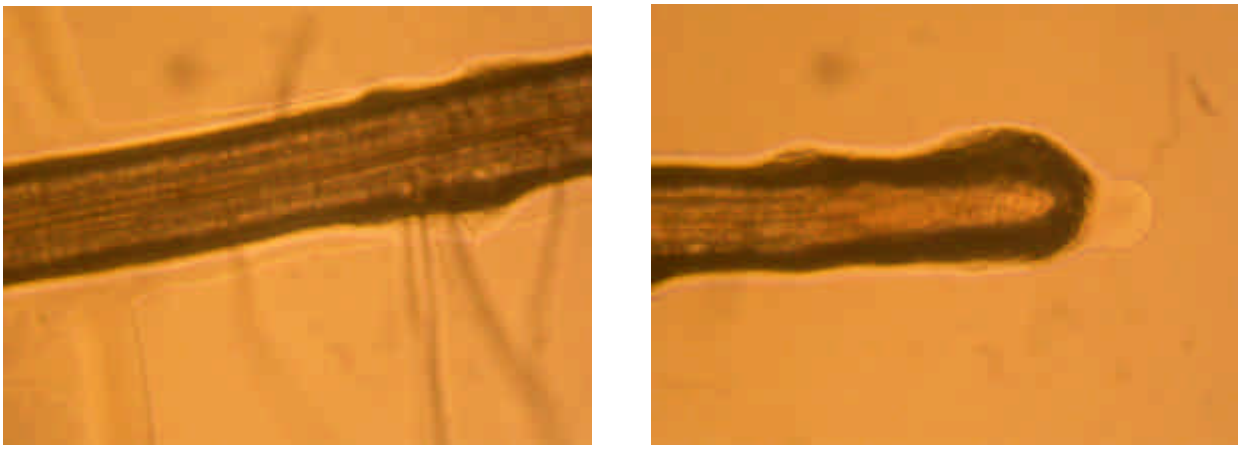


Figure 4.13.2 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Sphingomonas paucimobilis* (JH41) showing bacterial colonization. A: Root growing through inoculation point; B: Root tip.

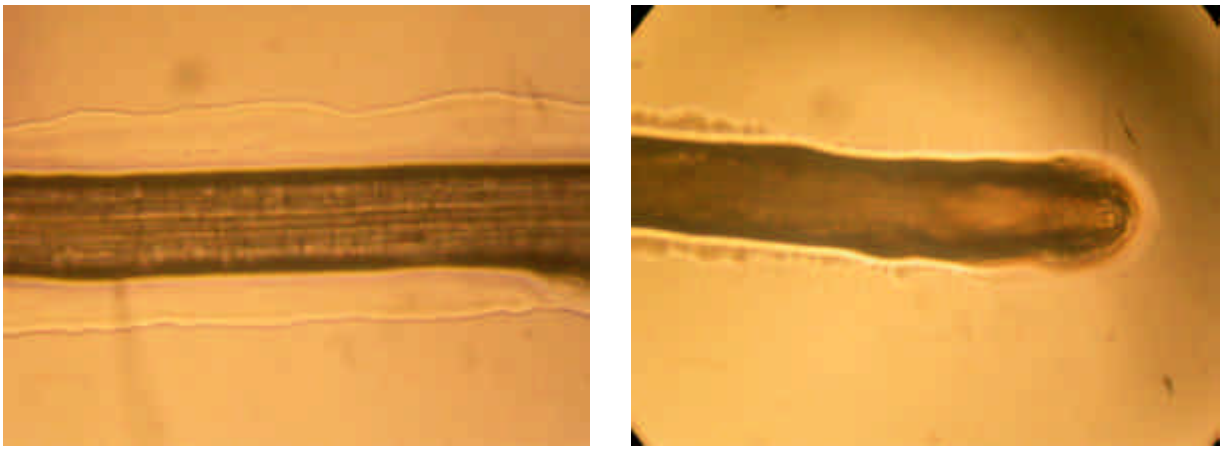


Figure 4.13.3 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Brucella* spp (JM17W). A: Root growing through inoculation point; B: Root tip.

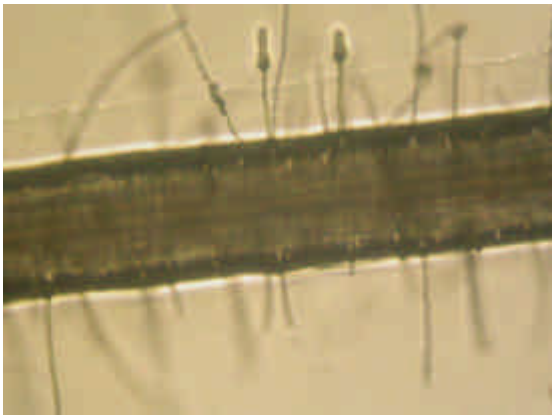


Figure 4.13.4 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Bacillus cereus* (JM2R). A: Root growing through inoculation point.

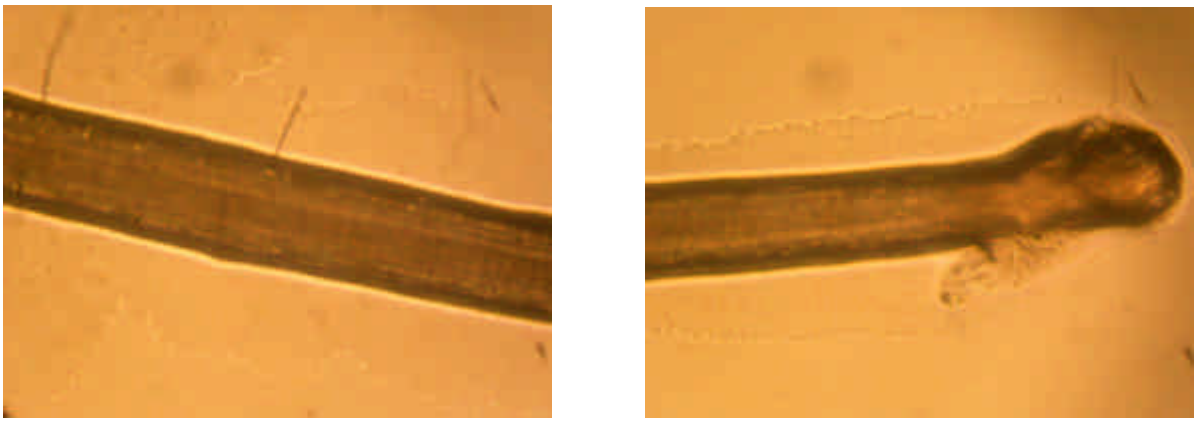


Figure 4.13.5 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Burkholderia cepacia* (JM6R). A: Root growing through inoculation point; B: Root tip.

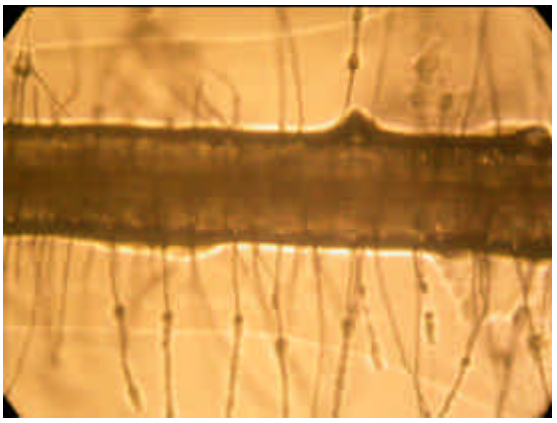


Figure 4.13.6 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Pseudomonas fluorescence* (JH83). A: Root growing through inoculation point.

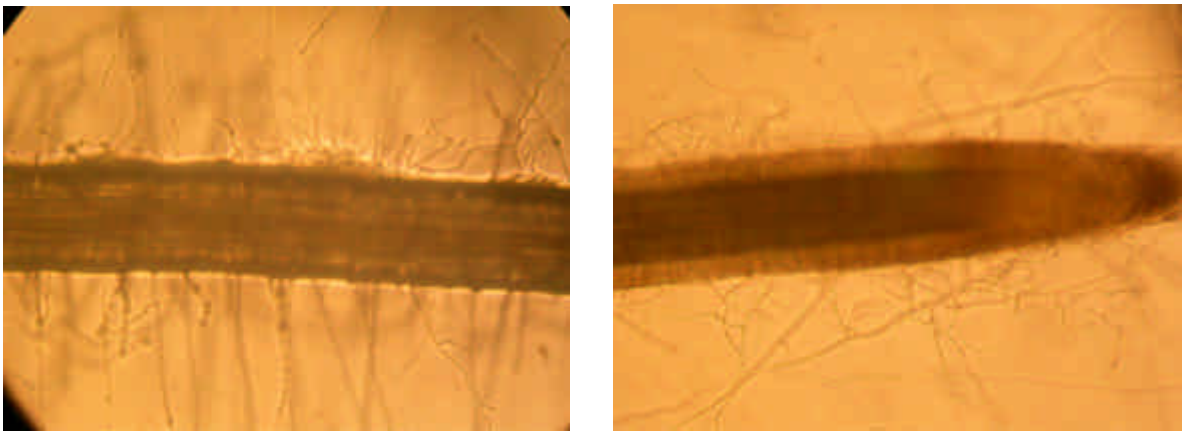


Figure 4.13.7 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Trichoderma* T1 (JM41R). A: Root growing through inoculation point; B: Root tip.

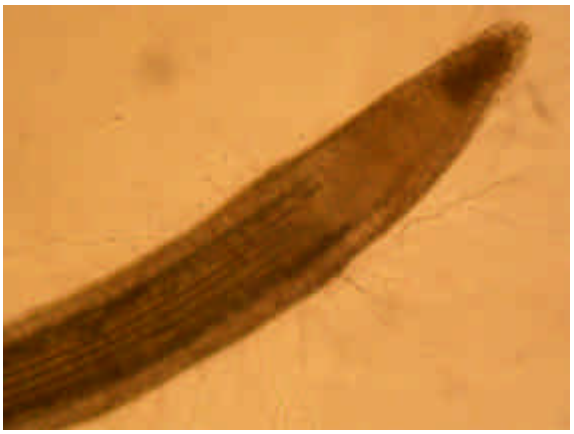


Figure 4.13.8 Root colonization assay with butterhead lettuce seedlings: root inoculated with *Trichoderma* T2 (JM6BO). B: Root tip.

CHAPTER 5

GENERAL DISCUSSION

The purpose of this study was to select and evaluate bacteria and fungi through *in vitro* and *in vivo* screening techniques for their ability to control *Pythium* root rot and promote growth of lettuce. Static aquaculture systems were used as prescreening assays to eliminate isolates that were uneffective against *Pythium* root rot on lettuce. The growth promotion and biocontrol ability of the most effective isolates were subsequently evaluated in a re-circulating hydroponic system in the greenhouse from which the best performing isolates were chosen for further evaluation in a commercial scale re-circulating gravel bed hydroponic system in the field. Furthermore, the mode(s) of action of the most effective organisms were investigated.

Pythium species cause pre- and post-emergence damping-off on a wide range of crops. Re-circulating hydroponic systems provide an ideal environment for *Pythium* to spread and infect roots, significantly reducing plant growth and yield and causing major losses to the farmer (Gold and Stanghellini, 1985; Stanghellini and Rasmussen, 1994; Uthede *et al*., 2000). The presence of *Pythium* in South African commercial re-circulating gravel bed hydroponic systems was confirmed through consistent isolation of pathogenic *Pythium* species on various hydroponically grown crops (Labuschagne *et al*., 2002; Gull *et al*., 2004). Hydroponic systems offer a unique environment for biocontrol since various parameters can be managed to favour the antagonist whilst optimal distribution of biocontrol agents are achieved through the re-circulating nutrient solution (Paulitz, 1997).

In the present study, root and water samples were taken from the *Pythium* infected beds of a commercial hydroponic system to obtain potential biocontrol agents, which have the ability to function in the same ecological environment as the pathogen. According to Völksch and May, (2001) the potential biocontrol isolates should be able to occupy the same niches, tolerate the same adverse conditions and multiply efficiently under similar environmental conditions as the

pathogen they are to control. The samples obtained in this study contained bacterial and fungal isolates that were isolated in such a way that it would be selective for organisms capable of either colonizing the rhizoplane of the host plant or attaching to /attacking the *Pythium* mycelium.

In the current study *in vitro* dual culture assays were used as a qualitative indication of the inhibitory activity of the potential biocontrol agents against *Pythium* F – group. Clear, visible results such as inhibition / lysis of the pathogen were obtained. The *in vitro* screening system provided repeatable and reliable results and was relatively easy and quick to perform with a large number of isolates. This is in accordance with Anith *et al.* (2003), who found dual culture assays to be a rapid and accurate preliminary screening technique for selecting antagonists against soil borne infection by *Phytophthora capsici* in black pepper nurseries.

Dual culture plate assays, however, does not involve the host plant so the organism's ability to interact, colonize and protect roots and seeds are not evaluated (Nelson, 1991; Cook and Baker, 1983; Kloepper, 1991). Seed and root-infecting pathogens are often highly dependent on exudates to initiate plant infections and the ability of the antagonist to metabolize these exudates is an important step in the biocontrol processes (Nelson, 1991). Field trials are the most reliable test and are important in verifying that the organisms are effective outside the laboratory or greenhouse (Campbell, 1989; Sutton and Peng, 1993; Kraus, 1996; Deacon, 1991). For this reason, isolates in the current study showing even a slight inhibition in the dual culture assays of *Pythium* F- group, was selected for *in vivo* evaluation where the host plant, pathogen and antagonist were involved.

Static aquaculture bioassays were used for *in vivo* pre-screening evaluation to indicate the biocontrol ability against *Pythium* and growth promotion of selected isolates on butterhead lettuce seedlings (Nelson and Craft, 1992). *Burkholderia cepacia* (JM6R), *Bacillus cereus* (JM2R), *Sphingomonas paucimobilis* (JH41), *Bacillus subtilis* (JM16W), *Pseudomonas fluorescens* (JH83), *Brucella* spp. (JM17W), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) showed the

most promise in the static aquaculture screening experiments. Although the growth promotion and curative data of the static aquaculture systems were not statistically significant, it was still used as an indication of potential activity against *Pythium* and growth promotion of lettuce plants.

Growth promotion, preventative and curative trials were subsequently performed with the most promising isolates in a re-circulating hydroponic gravel bed system in the greenhouse. *S. paucimobilis* (JH41), *B. cereus* (JM2R), *Trichoderma* T1 (JM41R), *Trichoderma* T2 (JM6BO) and *B. subtilis* (JM16W) caused a significant increase in the growth of *Pythium* free lettuce plants when tested in the greenhouse hydroponic system. Similarly Anith *et al*., (2003), found that their *P. fluorescens* isolate increased the shoot growth of black pepper when compared to the uninoculated control suggesting that the bacterial isolate had some plant growth promotion ability. *Trichoderma*, *Bacillus, Pseudomonas* and *Burkholderia* species are also well known plant growth promoting bacteria that increase plant yield of commercially important crops (Burdman *et al*., 2000; Ousley *et al.*, 1993).

In the current study *B. cepacia* (JM6R), *B. cereus* (JM2R), *P. fluorescens* (JH83), *Trichoderma* T1 (JM41R) and *Trichoderma* T2 (JM6BO) showed the most promise as preventative and curative treatments when tested in the greenhouse re-circulating hydroponic gravel system. In the United States *Trichoderma harzianum* and *B. cepacia*, which suppressed *Pythium* root rot on a number of vegetable crop, has been developed as commercial products (Martin and Loper, 1999).

Bacteria and fungi are common inhabitants of plant surfaces where some, through various mechanisms, have the ability to alter growth of pathogens and reduce diseases (Shtienberg *et al*., 2001). *B. cereus* (JM2R), *B. subtilis* (JM16W), *P. fluorescence* (JH83) and *Brucilla* spp (JM17W) produced at least one of the extracellular enzymes: proteonase, amylase and gelatinase. All of the isolates tested produced siderophores but *S. paucimobilis* (JH41) and the two *Trichoderma* species did so at a much slower rate. The competitive ability of an organism to breakdown complex molecules and utilize the nutrients more

effectively and quicker than the pathogen may result in the displacement of the pathogen. *Pseudomonas* spp. have been reported to produce siderophores which cause iron starvation of pathogenic organisms by binding and utilizing iron in the rhizosphere (Buyer *et al.,* 1989).

Antibiotic substances, effective against *Pythium* F- group, was obtained only from the culture filtrate of *P. fluorescence* (JH83). TLC analysis of the culture filtrate of this isolate showed that at least three different compounds were produced. It is well established that micro-organisms are capable of metabolizing and producing a range of phenolic compounds (Siqueira, *et al*., 1991). HPLC separation of the liquid culture filtrate of isolate JH83 resulted in the elution of two major peaks, which did not appear in the control. Howell and Stripanovic (1980), found that their *P. fluorescens* strain were antagonistic to *P. ultimum* because of the production of the antibiotic pyoluteorin. The liquid culture filtrate of the remaining isolates did not induce inhibition zones against *Pythium* F –group. However, these isolates might produce antibiotics effective against *Pythium* when grown on different substrates or at different temperatures. Environmental factors, such as nutrition, type and age of growth media, size or concentrations of inoculum, incubation temperature and low concentrations of the cell - free filtrates may affect the production and effectivity of antibiotics (Jackson *et al*., 1997).

None of the isolates significantly increased the total soluble or insoluble phenolic compounds of lettuce plants in the current study, indicating that induced resistance is not a probable mode of action for any of the isolates. Different results might, however, be obtained if the same experiments were conducted over a longer time period of a week in stead of only two days. Many studies have shown that it generally takes from a few days to a week for systemic acquired resistance or induced systemic resistance to develop and then only when plants are inoculated with a dosage of bacteria that exceeds a certain threshold population size (Van Loon *et al*., 1998).

An important attribute of a successful biocontrol agent is the ability to colonize the rhizosphere and remain at a high population density on the root surface,

providing protection of the root by excluding the pathogen (Yedidia *et al.*, 2000; Misaghi, 1990). In the current study the best root colonization was achieved by *P. fluorescence* (JH83), *Brucilla spp* (JM17W) and *Trichoderma* T1 (JM41R) that colonized 100 % of the roots. *B. cereus* (JM2R) and *B.* cepacia (JM6R) colonized 50 % of the roots. Fluorescens *pseudomonas* strains, used in the biocontrol of *Pythium* spp., have been shown to be efficient colonizers of tomato seedling roots (Hultberg and Waechter-Kristensen, 1998).

When evaluated under field conditions in a commercial scale hydroponic system, the best results were obtained with *B. cereus* (JM2R) and *P. fluorescens* (JH83). This is in accordance with many other reports that confirm the successful biocontrol ability of *P. fluorescens* and *B. cereus* against *Pythium* spp. (Martin and Loper, 1999). *P. fluorescens* has also been indicated as a biocontrol agent against *Botrytis cinerea* on lettuce and *Phytophthora capsici* on black peppers (Anith *et al*., 2003; Card *et al.*, 2002). *Bacillus* species have the ability to form endospores, which afford them the ability to tolerate extreme pH, temperatures and osmotic conditions giving them an advantage over other organisms. *Bacillus* species also have the ability to colonize root surfaces, increase plant growth and cause lysis of fungal mycelium (Basha and Kandasamy, 2002).

Finally, to give a better understanding of the mechanisms involved, more work needs to be done on the mode of action of the isolates that showed biocontrol ability against *Pythium* root rot and growth promotion activity on butterhead lettuce seedlings. Different combinations of biological agents should also be evaluated (Martin and Loper, 1999) as mixtures of biocontrol agents could mimic the natural situation more closely, broaden the spectrum of biocontrol activity and enhance efficacy and reliability of control (Duffy and Wellar, 1995). In conclusion, biological control of *Pythium* by naturally occurring microorganisms offers a possible means for disease management in hydroponic systems. This study demonstrated some isolation, screening and evaluation procedures that could be used to obtain successful and efficient biocontrol agents against *Pythium* root rot in hydroponically grown lettuce. This study also achieved the objective of identifying a number of effective biocontrol organisms

against *Pythium* root rot of lettuce in hydroponic systems. These organisms can now potentially be commercialized for use in hydroponic systems. For this purpose further testing will be necessary to evaluate efficacy of these isolates on a range of other lettuce cultivars as well as other crops.

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