Assessment of some antagonists to fungal plant pathogens and development of methods for their large-scale cultivation

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ABSTRACT

Wood fibre waste (WFW) of paper mill origin and composted fish waste were investigated for use as media for the cultivation or carriage of biological control agents active against plant pathogens, with a view to producing suppressive alternatives to chemical fungicides.

Potential biological control agents were isolated from crop-soil or from the rhizosphere and rhizoplane of plants. Some 67 bacterial isolates of more than 100 cultures assessed were antagonistic to one or more fungal plant pathogens *in vitro*. Most antagonists were identified as *Bacillus* or *Pseudomonas* spp. Isolates of *Pseudomonas corrugata, Bacillus megaterium, B. thuringiensis, B. polymyxa, B. pumilus, B. mojavensis*, and *Lysobacter antibioticus* showing particular potential in a pot trial against *Sclerotinia minor* and *S. sclerotiorum* formed the focus of further developments as agents of biological control.

The optimal growth conditions of the selected bacterial antagonists and of a fungal biological control *Trichoderma* sp. (Td_{22}), were assessed *in vitro* prior to their cultivation in WFW- or fish waste compost, or in amended WFW without composting. All tested bacterial antagonists grew well at neutral pH and at temperatures between 25°C and 30°C. Td_{22} grew optimally at pH between 5.0 and 6.0 and at 25°C. This fungus had an advantage over the bacterial isolates of being cellulolytic, raising the possibility of its cultivation in cellulose-based materials, such as WFW.

The selected antagonists were cultivated in composted WFW amended (20% w/w) with millet seed and enriched with ammonium nitrate-based mineral salts solution. The initial pH of the mix was adjusted to approximately neutral for the cultivation of the bacterial agents, no pH adjustment was needed for the cultivation of Td_{22} as the initial pH of the mix was around 4.5-5.0. All antagonists showed excellent growth response in this mix, reaching densities up to ~10^{10} colony forming units (cfu)/g dry weight mix after 14 days of incubation under sterile conditions. Lower population densities of between ~10^7 and ~10^9 cfu/g for Td_{22}, or of between ~10^8 and 10^9 cfu/g for the bacterial antagonists were achieved under non-sterile conditions.

The efficacy of the suppressive mix in controlling *S. minor* was evaluated in pot trials. Td_{22} grown in this mix consistently protected lettuce plants from attack by *S. minor*. The degree of protection was correlated with the level of the suppressive mix amendment, with 100% protection being observed after four weeks at an amendment rate of 20% v/v (compost:soil mix). Soil inoculated with *S. minor* only (control treatment) showed 0% survival after the same interval. Pre-incubation of the fungus in the compost:soil mixture for four or more days prior to planting appeared to improve its disease control. Long-term storage of the Td_{22} suppressive mix was also demonstrated by the protection of pyrethrum plants from attack by *S. minor* following storage of the mix at ambient temperatures for 4.5 months. The efficacy of the selected bacterial antagonists in disease suppression was less consistent. Although the percentages of healthy seedlings/plants increased relative to controls following application of compost-grown bacterial antagonists (at a rate of 5% v/v), these increases were not statistically significant ($p>0.05$) in most cases.

In an attempt to eliminate the need for sterilizing compost material used to cultivate biological control agents, the potential of utilizing the different optimal growth temperatures of normal compost biota at elevated temperature relative to those of biological control agents was investigated. Bacterial antagonists were inoculated into mature self-heating fish waste compost immediately following its rapid cooling from 52-55°C to ambient temperatures and supplementation with amendments favouring the antagonists. All antagonists reached high numbers after 14 days incubation, with two (*P. corrugata* and *L. antibioticus*) reaching ~10^{10} cfu/g dry weight. In both cases most of the indigenous compost microbiota were excluded, the antagonists being the dominant biota found following isolation. In a glasshouse trial, they also significantly ($p<0.05$) protected lettuce plants from attack by *S. minor*, the degree of protection ranging from 40 to 50% relative to the control treatments. When applied at the rate of 12.1 tonnes/hectare in a field trial, the effectiveness of these antagonists was comparable to that of a commercially available biological control agent (Companion) applied as a cell suspension, but was not significant statistically ($p>0.05$) compared to control treatments.

The effectiveness of selected antagonists (Td_{22}, *P.corrugata*, and *L. antibioticus*, prepared as cell suspensions) in protecting zucchini leaves from downy mildew incidence

was also investigated, with a view to developing standardised 'compost teas' for the control of leaf pathogens. All but one antagonist (*L. antibioticus*) applied singly or in combination provided a significant ($p<0.05$) protection to the zucchini leaves at two weeks after the pathogen introduction, ranging from ~22% (bacterial) to 83% (Td_{22}) protection relative to the pathogen-only control. Co-inoculation of Td_{22} with *P. corrugata*, *L. antibioticus*, or a combination of Td_{22} with both bacteria resulted levels of control lower than those provided by Td_{22} alone. This could be attributed to the lower concentrations of the most active agent (Td_{22}) and/or to the antagonism between the bacteria and fungus as noted *in vitro*. The survival or establishment of these antagonists on the leaf surface was very poor, resulting in declining levels of protection after two weeks, suggesting that re-application of these antagonists is needed to provide a reliable disease control.

The molecular weights of two antimicrobial compounds produced by *P. corrugata* strain SAJ6 were determined to be 554 and 580. Other characteristics of these antibiotics were also elucidated, although determination of their structures was beyond the scope of this study. Siderophore activity by these compounds was eliminated from contention, since inhibition of *S. minor* was not affected by the presence of high levels of $FeCl_3$ in growth medium.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and that to the best of my knowledge and belief, this thesis contains no copy or paraphrase of material previously published or written by any other person, except where due reference is made in the text of this thesis.

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Publication

A proportion of this thesis has been published. The following refereed publication has been included in a pocket on the inside back cover of the thesis.

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Chapter 1

General Introduction

1.1 Background

It has been well documented that long-term application of chemical pesticides to control plant diseases has been to the detriment of the soil and water environments. In developing countries where appropriate controls are often lacking, application of toxic compounds has been particularly excessive. This has lead to the accumulation of such compounds or their residues in the soil, with resultant contamination of agricultural crops, waterways, milk, and meat.

Pesticides frequently kill biota beyond target pathogens including beneficial mycorrhizal fungi and plant growth promoting rhizobacteria (DeCeuster and Pauwels, 1995). High pesticide usage also leads to increased pathogen resistance resulting in yet higher pesticide usage to kill the same pathogen (Jutsum *et al.*, 1998; Margot, 2003; Beever, 2003). Chemical pollutants including pesticides have also been found to have adverse effects on reproduction and developmental processes in variety of animals including humans (Machala and Vondracek, 1998; Miyamoto and Klein, 1998; Poovala *et al.*, 1998). There are other causes of concern relating to pesticide use. As an example, methyl bromide is widely considered to be one of the best broad spectrum pesticides, but has been found to be a significant greenhouse gas (Ristaino and Thomas, 1997) and is due to be phased out in developed countries by 2005 (De Ceuster and Hoitink, 1999). Alternatives have been proposed, but none appear to be as effective (De Ceuster and Hoitink, 1999).

Due to these detrimental effects, reduced pesticide usage in agricultural practice has been championed with marked effect (Perin-Garnier, 1998; Mmochi and Mberek, 1998). In anticipation of such future reduction, Painuly and Dev (1998) proposed some possible alternative control measures, such as biological control, use of pathogen-resistant plant varieties, and integrated pest management.

Many agents of microbial biological control of plant pathogens, or of plant growth promotion have been identified, although only a few have been successfully adopted into farming practices (Weller, 1988; Fravel *et al.*, 1998; Nameth, 2002; Fravel and Lazarovits, 2003). In large part this is due to lack of competitiveness of potential agents with normal soil or root-biota under field conditions. Lack of commercial competitiveness against chemical pesticides has also slowed the adoption of biological control measures. An example of the early and successful use of integrated biological control is that of the application of biological control strains of *Trichoderma harzianum* after soil fumigation and prior to crop planting (De Ceuster and Pauwels, 1995).

The use of compost as a possible substrate or a carrier for biological control agents has been intensively researched (e.g. see the review by Hoitink *et al.*, 1993; Hoitink and Gardener, 2003). The present project was inspired by the findings of Vidhyasekaran *et al.* (1997[a]), Gazoni *et al.*, (1998), and Nakasaki *et al.* (1998) who were able to grow and maintain bacterial antagonists of plant pathogens in low-cost solid matrices. These reports hold considerable potential for value-adding to compost and for providing a low-cost biological control material to less wealthy countries.

1.2 Aims of the project

The main objective of this project was to assess the effectiveness of antagonists in controlling plant pathogens and to establish methods for their large-scale cultivation in low-cost materials. Substrates examined with a view to producing suppressive alternatives to chemical-based fungicides were composted and non-composted wood fiber waste and fish waste compost. The following specific aspects were explored:

1. The screening, isolation, identification, and assessment of potential bacterial and fungal antagonists to plant pathogens.
2. The *in vitro* determination of optimum growth conditions of specific fungal and bacterial antagonists, with a view to their large-scale production using low-cost materials available in Tasmania.
3. The examination of wood fiber waste of paper mill origin and mature fish waste compost as substrates or carriers for the cultivation and storage of potential biological control agents.
4. The manipulation of the cool-down phase of composting to the advantage of mesophilic inocula.

5. The evaluation of the ability of antagonists cultivated in the above low-cost substrates to protect lettuce plants and pyrethrum plants from attack by *S. minor* in glasshouse trials and in a field trial.
6. The evaluation of suspensions of the antagonist to protect zucchini leaves from downy mildew infection, with a view to the development of designer 'compost teas'.
7. Preliminary screening and characterization of active compounds produced by a selected bacterial antagonist [*Pseudomonas corrugata* (strain SAJ6)] with a view to elucidating the mechanism by which this antagonist controls the fungal pathogen (*S. minor*).

Chapter 2

Literature Review

2.1 Introduction

Losses in productivity and quality of valuable crops due to attack by pathogens are widely reported, often with severe effect, as reviewed by Chakraborty *et al.*, (1998), De Ceuster and Hoitink (1999) and Edwards *et al.* (2001). To deal with this problem, a wide range of disease control measures from traditional crop rotation (Défago, 2003) to use of organic additives (Lazarovits *et al.*, 2003) have been applied.

Crop rotation is intended to lead to starvation-death of plant pathogens by preventing them from making contact with their host plants for an extended period (Cook, 1990; Défago, 2003). However the benefits of crop rotation may be negated by the ability of many pathogens to switch hosts or to grow saprophytically, enabling their survival (Coley-Smith, 1979). The effectiveness of rotation may be improved by the addition of organic materials, particularly compost, to the soil (Nelson and Boehm, 2002; Hoitink and Gardener, 2003; Lazarovits *et al.*, 2003). Various reasons have been proposed to explain this; the additive may improve soil thermal or water-holding properties, and improved nutrient levels may result in healthier, more robust plants, or may favour the competitors or antagonists of plant pathogens. Although crop rotation coupled with the addition of organic amendment is still used, these two approaches are less favoured for disease control in modern farming systems, their being largely replaced by integrated disease control to enable continuous cropping (Cook, 1990; Yamada, 2001; van Bruggen and Termorshuizen, 2003).

Biological control of plant pathogens has a long history. Our understanding of naturally suppressive soil is well established (Schneider, 1982; Becker *et al.*, 1997) and knowledge of its effectiveness has provided the stimulus for research on biological control, which according to Whipps (1997) and Peng *et al.* (1999), is attributable to a combination of physicochemical characteristics of the soil and the effect of antagonists. Negative interactions between pathogens and their antagonists (biological control agents) include parasitism (with or without lysis), competition, and antibiosis, as outlined in

Section 2.5. These negative interactions *in-vitro* have been widely used as indicators of potential biological control activity in isolation strategies.

2.2 Plant defense mechanisms against plant pathogens

Plants are constantly exposed to potentially pathogenic microorganisms yet only occasionally do these pathogens infect the plants to cause disease (Agrios, 1997; Hirt, 2002). This is because plants possess an array of active and passive defense mechanisms (e.g. Lamb *et al.*, 1989; Dixon and Lamb, 1990; Agrios, 1997). When in contact with pathogenic microorganisms, a multi-component cascade of responses is triggered in plants, aimed at inhibiting or limiting pathogen attack. These include the production of inhibitors, such as phytoalexins or other chemicals, the formation of physical barriers (by deposition of lignin, callose, or strengthening the cell wall by increased cross-linking), the elicitation of the hypersensitive response, the elevated expression of hydrolytic enzymes, or the elicitation of non-specific, multigenic resistance, as reviewed or reported by e.g. Bolwell and Wojtaszek (1997), Tuzun and Bent (2000), Bélanger and Benhamou (2003), and Adikaram and Abayasekara (2003). These plant defence mechanisms are briefly described as follows:

2.2.1 The production of inhibitors

Phytoalexins (low molecular weight antimicrobial compounds) are common inhibitors produced by plants in response to pathogen attack (Paxton, 1981; Anon, 1999; and Adikaram and Abayasekara, 2003). Several classes of phytoalexins, such as isoflavanoids, sesquiterpenoids, and indolics (from legumes, potatoes, and brassicas, respectively) have been reviewed by Glazebrook (1996). Other compounds reported to be accumulated within the plant cell wall in response to infection include β-1,3-glucan callose (Aist, 1983), hydroxyproline-rich glycoprotein (Benhamou *et al.*, 1991), silicon oxides (Stein *et al.*, 1993), and phenolics (Bélanger and Benhamou, 2003).

The synthesis of phytoalexins involves a series of sequential reactions: The plasma membrane of plant cell will respond to the chemicals released by fungal pathogens by producing hydrogen peroxide. This triggers a chemical message to the nucleus of the infected cell, resulting in the transcription of mRNA encoding the production of phytoalexins (Anon, 2002). The presence of these compounds in the plant tissue will

contribute to the inhibition of further ingress by the pathogens, as reviewed by Kuć (1995), Hammond-Kosack and Jones (1996) and Adikaram and Abayasekara (2003).

2.2.2 The formation of physical barriers

The first barrier encountered by a plant pathogen is the plant cell wall, one that is relatively easily penetrated by most pathogens (Schäfer, 1994). However the plant will quickly respond to such infection by cell wall modifications to make it more effective, as reviewed by Hammerschmidt and Nicholson (2000), Wharton (2001), and Bélanger and Benhamou (2003). Such modifications include the lignification and deposition of phenolic compounds (Nicholson and Hammerschmidt, 1992; Lee *et al.*, 2001), with the effect of providing a toxic barrier having increased mechanical strength, making it less susceptible to the degrading enzymes (Ride, 1978; Wharton, 2001).

2.2.3 Elicitation of the hypersensitive response

Rapid induction of a localized hypersensitive response (HR) is an important mechanism by which plants resist fungal and bacterial pathogen attack (Goodman and Novacky, 1994; Baillieul *et al.*, 1995; Lam, 2003). The HR is considered to be a component of programmed cell death, and is it is coordinated with other defense mechanisms such as deposition of antimicrobial compounds at the infection site, fortification of the cell walls, and expression of defense-related genes in the surrounding tissue (Goodman and Novacky, 1994; Hammon-Kosack and Jones, 1996). All these mechanisms are aimed at inhibiting further colonization of plant tissues by depriving pathogens of water or nutrients.

The HR will occur in plants possessing a single-dominant gene encoding the production of molecules (receptors) specifically binding with counterparts produced by the invading pathogens (Halterman and Martin, 1997). The production of such compounds by pathogens is encoded by an avirulence (*avr*) gene (Knogge *et al.*, 2003), with their binding triggering the expression of the HR (Keen, 1990). Because activation of the HR in plants requires recognition of gene expression by the pathogen, this defence mechanism is often referred to as a gene-for-gene relationship (Keen, 1990). If an invading pathogen does not carry an *avr* gene, the plant host will fail to activate the HR leading to infection, as reviewed by Hammond-Kosack and Jones (1996) and Hutcheson

(1998). More recently Lam (2003) reported that reactive oxygen species and caspase-like protease were essential components of the HR.

2.2.4 Elevated expression of hydrolytic enzymes

Over-expression of plant genes encoding for hydrolytic enzymes as a response to pathogen attack, has been reported by many workers (e.g. Ye *et al.*, 1990; Maurhofer, 1994a). These enzymes directly lyse the invading fungal hyphae and/or release oligisaccharides from the hyphae, eliciting further defense responses, as reviewed by Tuzun and Bent (2000). Some hydrolytic enzymes, such as chitinase and β-1,3-glucanase have been reported to accumulate in plants following pathogen challenge (Pan *et al.*, 1992; Tang *et al.*, 2003). Increased activity and levels of these enzymes has also been reported to be correlated with the induction of systemic resistance in plants (Schneider and Ullrich, 1994; Tang *et al.*, 2003). Some plants, such as barley (Ignatius *et al.*, 1994), tobacco (Lusso and Kuć, 1995), potato (Wegener *et al.*, 1996), cabbage (Tuzun *et al*, 1997), grape (Busam *et al.*, 1997), and tomato (Bettini *et al.*, 1998) have been reported to produce these enzymes constitutively and at higher levels in disease-resistant than in susceptible plants. This attribute confers a capacity to detect and respond to invading pathogens more quickly than can susceptible plants (Tuzun *et al.*, 1997).

2.2.5 Non-specific control of plant pathogens

Where the HR is triggered by a single gene product of the pathogen, non-specific (horizontal) resistance responses tend to be multigenic in origin. This topic has been reviewed by Tuzun and Bent (2000). According to Simmonds (1991) there is a tendency for plants having multigenic resistance to encounter a greater variety of pathogens or pathogen races than those only harboring specific resistance genes.

2.3 Definition of biological control

Biological control has been defined in different ways in different disciplines. In entomology it has been defined as "the action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence" (DeBach, 1964). In plant pathology we have: "the decrease of inoculum or the disease-producing activity of a pathogen accomplished through one or more organisms, including the host plants but excluding (intervention by) man" (Baker, 1987[a])

and "the use of organisms (antagonists) to reduce disease incidence or an attack on crop species by certain pathogens" (Lucas, 1998). These definitions have in common the use of living organisms (natural antagonists of unwanted pathogens) to control the growth of pathogens. An extension of this definition, accepted in microbiology, extends to human introduction of the biological control agents into soil for the purpose of inhibiting the growth of indigenous pathogens.

2.4 Isolation of biological control agents

This topic has been the subject of numerous reviews (Baker, 1987[a]; Baker and Dunn, 1990; Campbell 1994; Utkhede, 1996; Mathre *et al.*, 1998; Olckers, 1999; Akhtar and Malik, 2000; and Soytong *et al.*, 2001). Potential biological control agents are frequently isolated from the rhizosphere or rhizoplane of plants, this improving the potential for their subsequent survival and competitive ability on re-exposure to the root habitat (Weller, 1988; Larkin and Fravel, 1998).

Biological control agents have also been isolated from suppressive soils (Campbell, 1994; Van Loon, 1999), from a parasitized pathogen (Metcalf, 1997), or by accidental contamination of media (Campbell, 1994). Weller (1984) demonstrated that fluorescent pseudomonads isolated from wheat roots previously grown in soil suppressive to take-all disease, provided greater protection to plants than those isolated from non-suppressive soil. Use of media favouring the identification of fluorescence has increased the rate of obtaining potential agents of biological control (Weller 1988; Lucas 1998).

2.5 Maximizing biological control effectiveness in the field

In order to achieve maximum effectiveness of biological control agents in the field, it is important to gather information on their host specificities (Kloepper, 1996) and on the life cycles of the pathogens (Whipps, 1997). Ideally the antagonist is isolated from the same plant it is targeted to protect as its ability to competitively colonize the growing root is then already established. Once root colonization takes place, the antagonist will prevent or reduce the capacity of other microorganisms (including pathogens) from colonizing the plant roots, leading to reduced disease incidence (Kloepper, 1996; Pieterse *et al.*, 2003). In relation to optimal timing of application, antagonists will be most effective if applied at the appropriate stage of plant growth (usually immediately prior to

germination) or at the most susceptible stage of the life cycle of the pathogens (Whipps, 1997).

2.6 Mechanisms of biological control

In nature, antagonists appear to control target pathogens by parasitism, cell lysis, antibiosis, competition and induced resistance in host plants (Schroth and Becker, 1990; Lievans *et al.*, 2001). These mechanisms are briefly discussed as follows:

2.6.1 Parasitism or lysis

Parasitism may be defined as utilization of a host as a source of energy following attack by a parasite (Atlas and Bartha, 1993). The term mycoparasite is used if both the parasite and its host are fungi (Chet *et al.*, 1997). Barnett and Binder (1973) divided mycoparasitism into destructive parasitism, where the parasites tend to kill the host or destroy components of the host in their interaction, and balanced parasitism where the parasites live in relative harmony with their host. Such division appears to be artificial and unhelpful because fungi will display a spectrum of activities between the two extremes. Manocha *et al.* (1990) found that hydrolytic enzymes and toxins were involved in the killing a host fungus (destructive parasitism), although the production of these compounds has not been demonstrated in the case of balanced parasitism (Jeffries, 1997). In such cases the parasites tend to produce specialized infection structures, which are used to absorb nutrients from their hosts (Manocha *et al.*, 1990; Jeffries, 1997). Mycoparasitism of plant pathogens has been reviewed recently by Benhamou and Chet, (1993); Chet *et al.*, (1997) and Schoeman *et al.*, (1999).

2.6.2 Competition

Competition occurs when two or more species live together and strive for the same limited resources of nutrients, light, or space (Atlas, 1995). Species with a higher growth rate or greater capacity to adapt to prevailing conditions will outcompete competitors in the same ecological niche. Nutrient supply (including micronutrients such as iron) provides the greatest source of competition in the soil environment (Chet *et al.*, 1997). Examples of biological control agents with capacity to outcompete other microbiota in habitats having limited iron availability are given in Section 2.6.5.

2.6.3 Antibiosis

Antibiosis has been variously defined as limited to the interaction of microbiota with antibiotics, or as a negative interaction in which the growth of one microorganism is inhibited by another due to the production of diffusable antibiotics, toxic molecules or volatiles, and lytic enzymes (Baker and Griffin, 1995). The effect on the target organisms may be either lethal or inhibitory to growth. Dijksterhuis *et al.* (1999) found the growth of *Fusarium oxysporum* was inhibited by both cell-free filtrate and by living cells of *Paenibacillus polymyxa* indicating antibiosis in this interaction. Likewise *Agrobacterium radiobacter* produces an effective antibiotic, 'agrocin 84' against its competitor *A. tumefaciens* (Penyalver and Lopez, 1999; Johnson and DiLeone, 1999). In a recent study, the sequence of events leading to the inhibition of *Penicillium digitatum* by antibiotics produced by *Verticillium lecanii* was elucidated by Benhamou and Brodeur (2000), using electron microscopy and gold cytochemical analysis.

2.6.4 Induced resistance in the host plants

Pathogenic attack on plants may trigger a range of physiological and biochemical protective responses as reviewed by Buell (1999), including the triggering of systemic signaling pathways resulting in production of hydrogen peroxide (leading to the hypersensitivity response) or intermediates such as salicylic acid (Delaney *et al*, 1994; Thomma *et al.*, 1998; Bol *et al.*, 2003), jasmonic acid (Thomma *et al.*, 1998; 2000; Pieterse *et al.*, 2003), or phytoalexins (Adikaram and Abayasekara, 2003). Signalling pathways may be induced artificially by exposure to compounds such as DL-β-amino butyric acid (BABA), dipotassium hydrogen orthophosphate (K_2HPO_4) (Reuveni *et al.*, 1994, 1998; Pajot *et al.*, 2001) or benzo(1,2,3)thiadiazoie-7-carbothioic acid-S-methyl ester (Bigirimana and Hofte, 2002).

Application of plant hormones has also frequently been found to result in an enhanced level of resistance (Boller, 1991; Pieterse *et al.*, 1996, 2000; Thomma *et al.*, 2000). The role of ethylene seems more ambiguous, in some cases it is apparently involved in disease development (Lund *et al.*, 1998), in others it is associated with disease resistance (Knoester *et al.*, 1998; Bol *et al.*, 2003; Pieterse *et al.*, 2003).

Some rhizobacteria, such as pseudomonad species have been reported to have the capacity to accumulate salicylic acid on the root surface of plants, triggering the systemic acquired resistance (SAR) signalling pathway in those plants (Mayer *et al.*, 1992; Visca *et*

al., 1993; Gupta *et al.*, 2000). In other cases, substances such as lipopolysaccharide (from the outer membrane of *Pseudomonas fluorescens*) have also been demonstrated to play an important role in eliciting SAR (Leeman *et al.*, 1995[a,b]; van Loon *et al.*, 1998). Srivastava *et al.* (2001) describe the induction of SAR in chickpeas against attack by *Macrophomina phaseolina*, the protection provided by a *P. fluorescens* isolate when applied in combination with 2,6 dichloroisonicotinic and o-acetylacilic acid.

2.6.5 Combined mechanisms of antagonism by specific biological control agents

Many microorganisms display a combination attack on pathogens (Chet *et al.*, 1997; Lievens *et al.*, 2001). Specific examples of microorganisms having this capacity are provided by representatives of the fungal genera *Trichoderma* and *Gliocladium* and by bacteria of the genus *Pseudomonas*.

Trichoderma spp.

Indication of parasitism by *Trichoderma* spp. on fungal pathogens was first noticed by Wiendling (1932). Since then the mechanism of parasitism by these fungi has been the subject of numerous investigations. Chet *et al.* (1981) suggested that the parasitic attack was initiated by the growth of hyphae towards chemical attractants produced by the hosts (positive chemotropism). Following contact, the fungi attach and bind to specific host lectins (Inbar and Chet, 1992) with subsequent penetration and degradation of the host cell wall, involving extracellular β-1,3 glucanase and chitinase (Elad *et al.*, 1982; Ridout *et al.*, 1988). This results in lysis and death of the fungal host. Some *Trichoderma* spp. supplement this parasitic activity by inducing SAR in the host plants (Elad, 2000) and/or antibiosis against the fungal pathogen (Jaworski *et al.*, 1999; Rebuffat *et al.*, 2000; and Humphris *et al.*, 2001).

Gliocladium spp.

Lewis *et al.* (1991) suggested the involvement of the enzymes β-1,4 glucanase, protease, β-1,3 glucanase, and chitinase in the destruction of the host cell by *Gliocladium*. The sequence of infection is similar to that of *Trichoderma* spp; with chemotrophic growth towards the host, followed by attachment and production of hydrolitic enzymes (Chet *et al.*, 1997). The ability of some species of *Gliocladium* to also produce antibiotics

has been reported (Howell, 1982). For example, *G. virens* can produce gliovirin, which was suspected to be responsible for the growth inhibition and death of *Pythium ultimum* (Howell *et al.* 1993; Howell and Stipanovic, 1995).

***Pseudomonas* spp.**

At least three mechanisms of disease control (induced SAR, competition and antibiosis) by *Pseudomonas* spp. have been reported. Many pseudomonads produce iron-chelating siderophores that allow them to outcompete plant pathogens in an iron-limited environment (Kloepper *et al.*, 1980[a]; Leong, 1986; Neilands and Leong, 1986; Schippers *et al.*, 1995). Reports demonstrating the efficacy of siderophore-producing pseudomonads in suppressing plant pathogens include those of various antagonists of *Fusarium* spp. (Raaijmakers *et al.*, 1995[b]; Leeman *et al.* 1996) and of *Ps. fluorescens* antagonistic to *Fusarium moniliforme*, *F. graminearum* and *Macrophomina phaseolina* (Pal *et al.*, 2001).

Antibiotics produced by *Pseudomonas fluorescens* include pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrol], effective against fungi associated with cotton seedling disease, except that caused by *Pythium ultimum* (Howell and Stipanovic, 1979; 1980), and pyoluteorin (4,5-dichloro-1 *H*-pyrrol-2-yl-2,6-dichlorohydroxyphenyl ketone), produced by strain CHAO and effective against *P. ultimum* (Maurhofer *et al.*, 1994). The role of other antibiotics, such as phenazine, has been reported by Thomashow and Weller (1998). The synthesis and regulation of phenazine production has been reviewed by Whitehead and Salmond (2000).

2.7 Naturally suppressive soil

Naturally suppressive soil has been widely identified and known for more than 100 years (Hornby, 1983). The occurrence of these soils is partly due to continuous monoculture cropping for many years (Schippers, 1992; Weller and Thomashow, 2003). The change in soil properties from conditions that favor pathogens to ones that naturally suppress them appears to be attributable to the soil microbiota (Alabouvette, 1998), particularly since this suppressiveness is usually lost if the long term monoculture cropping is interrupted or if the soil is fumigated or heat sterilized (Parke, 1995; Weller and Thomashow, 2003). A clear conclusion is that the suppressive effect of the soil is largely due to the presence of microbial antagonists in the soil, which suppress the

pathogens so that the ability of those pathogens to cause disease is lacking (Van Loon, 1999; Weller and Thomashow, 2003).

The decline of take-all disease of wheat (caused by *Gaeomannomyces graminis* var *tritici*) following serial planting is a classic example of the occurrence of suppressive soil (Parke, 1995; Weller and Thomashow, 2003). This continuous cropping has been speculated by Weller and Thomashow (1999; 2003) to increase population of non-pathogenic microorganisms well adapted to growth in the wheat root rhizospere. These microorganisms, particularly bacterial antagonists of fungal pathogens, colonize the root surface (rhizoplane), utilize the root exudates, and limit the fungus in its attack (Van Loon, 1999; Pieterse *et al.*, 2003). Bacteria, particularly *Pseudomonas* spp., (Raaijmakers and Weller, 1998; Weller and Thomashow, 1999; 2003) and fungi such as *Phialophora* spp.(Zriba *et al.*, 1999) have been reported to play important roles in the suppression of take-all disease. According to Raaijmaker and Weller (2001) and Weller and Thomashow (2003) 2,4-diacetylphloroglucinol produced by a strain of *Pseudomonas* is responsible for the suppression of take-all disease by this bacterium.

2.8 Strategies for achieving biological control

Indirect and direct strategies have been implemented to achieve biological control of plant pathogens. The indirect approach relies on amendment of organic into soil to enhance the activity of indigenous soil microbiota, particularly beneficial antagonists (Lazarovits *et al.*, 2003). This is often achieved by adding specific organic materials to the soil, such as chitin, which stimulates the growth of beneficial *Streptomyces* and *Trichoderma* (Chen *et al.*, 1999). Alternatively the resistant responses in young plant seedlings (or seeds) may be stimulated by drench or surface-application of non-virulent strains related to the pathogenic species (Chet *et al.*, 1997; Heil, 2001; Adikaram and Abayasekara, 2003) (Section 2.6.4), or of unrelated non-pathogenic rhizobacteria (Ahn *et al.*, 2002), or by spraying with chemicals as indicated above (Section 2.6.4).

In the direct approach, a potential antagonist (or a mix of antagonists) is/are introduced to soil, seeds or seedlings with a view to directly inhibiting or attacking the pathogen (Cook and Baker, 1983; Stewart, 2003). Rapid establishment and proliferation of the inoculated antagonist(s) in an appropriate ecological niche is required to provide biological control (Chet *et al.*, 1997). Once an antagonist has become part of the dominant

biota in that niche, it will hopefully exclude the pathogens or prevent them from attacking the colonized plants.

2.9 Obstacles in the development and application of biological control agents

The application of biological control in the field has been hampered for a number of reasons. Difficulties include handling, packaging, and delivery (Vidhyasekaran *et al*, 1997[a]), strict government regulation (Lucas, 1998), establishment in the field (Weller, 1988), inconsistent performance (Weller, 1988; Lucas, 1998) and questionable competitiveness with commercially-available chemical agents of control (Nameth, 2001). These obstacles are briefly discussed below.

2.9.1 Difficulties in handling, packaging, and delivery

Being living organisms, the biological control agents are more difficult to handle, package, and deliver than chemical alternatives (Fravel and Lazarovits, 2003). This is because their viability may be variable following storage in their carriers or substrates (Drahos, 2001). They may also be genetically unstable, which may result in loss of potency during long-term storage (Duffy and Defago, 2000). Also problematic is the relative bulk of storage material where application to the soil rather than the seed is envisaged. This is exemplified by application rates of chemical fungicides (kg/ha) relative to many biological alternatives (tonnes/ha).

2.9.2 Government regulations

The use of biological control agents in field applications (in the USA) has been strictly regulated by government as reviewed by Lethbridge (1989), Lucas (1998), and Fravel and Lazarovits (2003). This is partly due to unknown health risks following field applications (Mabbayad and Watson, 2000) since some of these agents are suspected of producing toxins that may be harmful to animals or humans (Mabbayad and Watson, 2000), *Fusarium pallidoroseum* being a specific example. Cook *et al.* (1996) identified potentially adverse non-target effects of microorganisms as biological control agents in the field regardless of whether they were native, naturally occurring, or genetically modified. These were competitive displacement of a non-target beneficial microorganism, allergenicity, toxigenicity and pathogenicity to non-target organisms, particularly animals

or humans. The development of new strains, which may produce one or more of the above non-target effects as a result of horizontal gene transfer from the introduced antagonists, was also of concern. These potential adverse non-target effects in the field are briefly reviewed as follows:

Competitive displacement

Competitive displacement could become an issue for the introduced antagonists in the rhizosphere if they displace mycorrhizal fungi or rhizobia, which are important to the health of the colonized crop (Mathre *et al.*, 1999).

Allergenicity

Fungal biological control agents can be of concern because fungal spores can be allergenic (Cook *et al.*, 1996). This was identified as a worker-safety issue (not a public issue) by Cook *et al.* (1996), and the danger must contribute to the difficulties in obtaining registration for new commercial biological control agents.

Toxigenicity

As noted in Section 2.6.3, toxic compounds may be involved in the protection of plants from attack by pathogens, but they may also be produced following application of biological control agents. These compounds are normally secondary metabolites produced when the pathogens or their antagonists have made sufficient growth at the location where they are applied (Cook *et al.*, 1996). According to Mathre *et al.* (1999) these compounds may also be toxic to non-target organisms in addition to the pathogens. In such cases the biological control agents may produce a negative rather than positive response by crops in the field.

Pathogenicity

Some microorganisms are opportunistic pathogens of humans and these would need to be excluded from commercial consideration in any screening process. Examples are *Aspergillus ochraceus* and *Burkholderia cepacia*, both being pathogenic to humans (Mathre *et al.*, 1999; Parke, 2000). Therefore, before being approved for large-scale or for commercial purposes, any biological control agent should pass through a stringent testing procedure, such as that outlined by Paulitz and Linderman (1989), or reviewed by Walsh *et al.* (2001).

As a result of the above stringencies or safety issues, commercial registration of a biological control often takes years to obtain (Templeton, 1982). An example is the development of *Colletrotichum gloeosporioides* f. sp. *aeschynomene* for the control of northern joint vetch (*Aeschynomene virginica*), which took about 13 years from its discovery to registration for use in the USA (Templeton, 1982; Makowski and Mortenson, 1992).

2.9.3 Difficulties in establishing biological control agents in the field

Field establishment of biological control agents has always been problematic as their viability is determined by complex and variable environmental factors. Most notable of these are adverse physical conditions (especially soil moisture and pH) and biotic factors (especially competition with indigenous biota, appropriate plant hosts and availability of soil nutrients) (Weller, 1988; Campbell, 1994; Stockwell *et al.*, 1998). To function in the field, the inoculated biological control agents must not only survive, but also reach a significant cell density in the root rhizosphere (Bull *et al.*, 1991; Raaijmakers *et al.*, 1995; Latour *et al.*, 1999). Therefore many of biological control agents that show a good disease control in glasshouse trials fail to be effective in the field.

2.9.4 Inconsistent performance of biological control agents

Inconsistent performance of biological control agents in the field is also a major obstacle in their development and acceptance (Baker, 1987[a]; Jutsum *et al.*, 1988; and Campbell, 1994; Fravel and Lazarovits, 2003). Possible reasons for this are loss of ecological competence (Weller, 1988; Campbell, 1994), variability in root colonization by the biological control agents (Weller, 1988), and the narrow spectrum of disease control of the antagonists (Janisiewicz, 1996). These are briefly discussed as follows:

Loss of ecological competence

The ability of a bacterium to compete and survive in nature has been referred to as its 'ecological competence' (Schroth *et al.*, 1983). Such competence has contributions from a number of gene-regulated traits, loss of one or more of which may lead to reduced competitiveness in the rhizosphere. Schroth *et al.* (1983) and Caesar and Burr (1987) have suggested that inconsistent performance resulting from spontaneous mutations may be attributed to repeated sub culture *in vitro* where selective pressure is absent. An example

is provided by the reported loss of ability of *Pseudomonas fluorescens* to produce antimicrobial metabolites and extracellular enzymes resulting from mutations in single regulatory genes (Natsch *et al.* (1994). Likewise Dufy and Défago (2000) noted that spontaneous mutation in one of two genes of *P. fluorescens* responsible for regulating the production of secondary metabolites, resulted in a significant reduction in antimicrobial effectiveness. Alternative reasons for the loss of competitiveness by biological control agents have been suggested to be attributable to viral infection, conjugation, or transformation (Kloepper, 1996). Evidence for interspecies signaling that may affect the rhizosphere competence of a biological control agent was demonstrated by Fedi *et al.* (1997) who found that diffusable factors produced by the phytopathogen *Pythium ultimum* repressed the expression of a gene that was important for the survival of a *Ps. fluorescens* strain in the sugarbeet rhizosphere.

Variability in root colonization ability

Colonization of plant roots by introduced antagonists is important in achieving disease control of root pathogens, which will be enhanced if the antagonists can reach high population densities or become well established in that zone (Schroth and Baker, 1990). This, according to Kloepper (1996) is not easy to achieve, because root colonization is a competitive process and is affected by the characteristics of the introduced antagonists and the plant hosts.

The ability of antagonists to colonize plant roots has been reported to vary among species (Scher *et al.*, 1988; Weller, 1988), from plant to plant or even from root to root on a given plant (Weller, 1984; Loper *et al.*, 1985; Bahme and Schroth, 1987). For instance, a significant variation in the population (by a factor of 10-100) of an introduced plant growth promoting rhizobacteria (PGPR) was reported on the root systems of potato or sugarbeet seedlings (Loper *et al.* 1984).

Narrow spectrum of disease control

Root colonization by antagonists has often been found to be host specific (Schroth and Becker, 1990; Chanway *et al*, 1991; Fravel and Lazarovits, 2003), a factor that contributes to inconsistent performance of biological control agents in the field. Unlike most chemical pesticides, biological control agents generally have a relatively narrow spectrum of disease control (Janisiewicz, 1996). When applied singly in the presence of non-target or target pathogens having high genetic diversity, the biological control agent

may exhibit inconsistent performance or appear to be ineffective in pathogen control (Mazzola *et al.*, 1995). Backman *et al.* (1994) found that the efficacy of a commercial biological control *Bacillus subtilis* was inconsistent (ineffective) in the presence of *Rhizoctonia solani* (the target pathogen) and *Pythium ultimum* (a non-target pathogen). Both are the causative agents of damping-off in soybeans, cotton and peanut (Backman *et al.*, 1994; Dorrance *et al.*, http://ohioline.osu.edu/ac-fact/0025.html).

2.9.5 Questionable competitiveness with commercial chemical control

According to Gianessi (1998), farmers in the U.S.A spend approximately US$8 billion p.a. on synthetic chemical pesticides, as against tens of millions of dollars p.a. on biological products to control plant pathogens, indicating that the biological control market is not yet significantly competitive with chemical pesticides. Disadvantages of biological control agents, which may contribute to their lack competitiveness in the market have been described by Gianessi, 1998; Nameth (2001):

- they are more difficult to apply than chemicals,
- they generally have a narrower target range as discussed in Section 2.9.4,
- their effects on the pathogens are slow,
- they do not eradicate the pathogens or rescue the host from infection,
- they often have a short shelf-life if not properly stored,
- they are more expensive and less effective than chemical pesticides,
- field application may require specialized equipment,
- compatibility with other chemical pesticides is lacking.

2.10 Overcoming obstacles in the development of biological control agents

Strategies to improve the success of biological control agents include improved methods of preparation (Kloeper and Schroth, 1981), isolation from the rhizosphere zone where antagonism is needed (Weller, 1988), improved bioassay methods (Rhodes *et al.*, 1987), the use of mixed cultures (Raupach and Kloepper., 1998), and genetic engineering to produce strains with a wide range of disease control (Rissler, 1991). These are briefly discussed as follows:

2.10.1 The use of improved preparation methods

The handling, storing and delivery of liquid cell suspensions of biological control agents is often difficult (Kloeper and Scroth, 1981; Vidhyasekaran *et al.*, 1997[a,b]) so many attempts have been made to improve these aspects for commercial production. In bacterial formulations, there is less need for concern of the viability of sporulating *Bacillus* spp. than that of other bacteria, because of their ability to survive high temperatures and low moisture.

Agents utilized in formulations for assisting the longevity or application of biological control agents include methyl cellulose powder (Suslow, 1980), gum (Kloepper and Schroth, 1981), mineral soil (Chao and Alexander, 1984) and talc (Vidhyasekaran *et al.*, 1997[b]; Gazoni *et al.*, 1998; Amer and Utkhede, 2000). Kloeper and Schroth (1981) developed a formulation of talc and 20% xanthan gum able to maintain effectiveness and population density of plant growth promoting rhizobacteria for two months at 4°C. The maintenance of antagonists for five months or more in powder formulations amended with carboxy methyl cellulose has been reported by Vidhyasekaran *et al.* (1997[a]) and Vidhyasekaran *et al.* (1997[b]). De Ceuster and Hoitink (1999) demonstrated the effectiveness of compost formulations in maintaining biological control agents for a 'reasonable' period of time. However, no such formulation has been produced commercially or in large-scale, indicating a need for further development.

To avoid the problem of loss of ecological competence of cultures resulting from repeated sub-culture, freeze-drying of biological control agents in protective rehydration media has been reported, this allowing indefinite storage (Abadias *et al*, 2001) although the cost could be prohibitive.

2.10.2 Focusing on the screening of potential biological control agents from the root rhizosphere or rhizoplane

The root rhizosphere and rhizoplane are nutrient-rich zones that are easily colonised by soil microbiota. According to Schroth and Hancock (1981) and Schroth and Hancock (1982) about 10% of the cultivable bacteria in this zone are potential biological control agents. As previously discussed, isolation of biological control agents from the rhizosphere will increase the possibility of screening and selecting effective biological control agents and the isolation of good root colonizers (Weller, 1988; Lucas, 1998).

2.10.3 The need for improved bioassay methods

Following the isolation of potential biological control agents, an appropriate bioassay for their screening is needed. The bioassay would ideally be designed so that it simulates natural conditions. Until recently, screening of biological control agents has invariably relied on *in vitro* dual culture assays (Andrews, 1985; Weller, 1985; Campbell, 1986; Campbell, 1994; Berg *et al.*, 2000), with selection on the basis of the presence of inhibition zones between the antagonist candidates and the tested pathogens. According to Renwick *et al.* (1991) and Hebbar *et al.* (1992) the result of this assay is often misleading as the degree of inhibition varies with the medium used. The antagonists producing the greatest inhibition zones on agar plates do not necessarily perform biological control in the field application, because the relationship between the two results is very poor (Gamard and Deboer, 1995; Avis *et al.*, 2001). However, these assays are still in use due to their simplicity and rapidity (Campbell, 1994; Berg *et al.*, 2000).

In an attempt to better match natural conditions, Rhodes *et al.* (1987) screened isolates by co-inoculating the pathogens and the antagonist on a piece of potato tuber, comparing the efficacy of the antagonists against appropriate controls. This assay was also applied by Schisler and Slininger (1994) and Elson *et al.* (1997) to screen antagonists inhibitory to *Fusarium spp.* and *Helminthosporium solani*, respectively. Similarly Cheah *et al.* (1996) successfully tested some antagonists of *Botrytis cinerea* from wounded kiwifruit on the basis of their ability to inhibit the development of lesions following their co-inoculation with the pathogen. These techniques can be expected to have only limited applicability for assessing biological control in the root rhizosphere, where different plant/microbial interactions are involved.

2.10.4 The use of mixed cultures

The narrow spectrum of disease control of biological control agents and the variability of their antagonism can be countered to some degree by the development and application of mixed cultures of antagonists (Janisiewicz, 1988). Mixtures of fungal agents (Paulitz *et al.*, 1990; Budge *et al.*, 1995; Datnoff *et al.*, 1995), bacterial agents (Pierson and Weller, 1994; Raaijmaker *et al.*, 1995; Schisler *et al.* 1997; Raupach and Kloepper, 1998) or combinations of the two (Lemanceau and Alabouvette, 1991; Duffy and Weller, 1995; Duffy *et al.*, 1996) have been reported to improve the success of biological control in the field where more than one pathogen frequently exists. A factor

that must be considered in the development of mixed cultures of antagonists is potential incompatibility of the co-inoculants, since the antagonists may also inhibit each other in addition to the target pathogens (Leeman *et al.*, 1996).

Raupach and Kloepper (1998) suggested several strategies in the development of mixed cultures of compatible antagonists, including the use of mixtures having different plant colonization patterns, different target pathogens, different mechanism of disease control, or different requirements for environmental conditions. These strategies appear to be sensible to improve the spectrum of disease control of the antagonists, providing a degree of redundancy in achieving the desired outcome.

A potential drawback of a biological control product composed of a mixture of antagonist strains, is that the production and registration cost of the mixed cultures may be more expensive than that composed of a single strain (Schisler *et al.*, 1997). However, the advantages of using mixed cultures, such as a better plant colonization, better adaptation to the environmental changes, a larger number of pathogen-suppressive mechanisms, or a wider spectrum of disease control, may outweigh this drawback (Backman *et al.*, 1997).

2.10.5 The use of genetic engineering for the production of biological control agents

Screening of microorganisms in laboratory or glasshouse scale experiments to protect crops from pathogens is a simple process having reasonable prospects for a successful outcome. However, to isolate an agent having a broad range of disease control and the ability to control the pathogens consistently and effectively in the field can be very time consuming. The use of genetic engineering techniques may provide a more direct route to achieving superior biological control agents, a topic that has been reviewed by Mathre *et al* (1998) and Walsh *et al.*, (2001). The possibility of combining several beneficial traits in the one biological control agent has been demonstrated by a number of researchers, examples being:

- the transfer of genes encoding endotoxin production from *Bacillus thuringiensis* to *Pseudomonas fluorescens*, the recombination strain then showing some toxicity to root cutworm of corn (Gorlach, 1994)
- the modification of strains of *P. putida* endowing them with the ability to inhibit soil fungi (Thomson *et al.*, 1995; De Leij *et al.*, 1995).

- the insertion of a gene encoding the production phenazine-1-carboxylate from one strain of *Pseudomonas* to another producing ploroglucinol, extending its control of take-all disease to *Rhizoctonia* root rot (Huang *et al.*, 1997)
- the manipulation of *Bacillus thuringiensis* strains to extend their ability to produce more than one type of toxin (Genetic Engineering Newsletter, 2000)
- the transformation-insertion and expression of a gene for a cytolytic protein toxin (lethal to mosquito and black fly larvae) from *B. thuringiensis* into the genome of *Enterobacter gergoviae* (Kuzina *et al.*, 2002).

These examples show that genetic engineering can widen the spectrum of disease control of antagonists and may lead to a reduced cost of production and registration (Section 2.10.4) for commercial use. This approach may also avoid the disadvantages of biological control products (Section 2.9.5). A major problem relating to the use of genetic engineering techniques however is the inherent or perceived dangers associated with them, requiring major risk assessment before such strains are released to the environment (Hagedorn and Hagedorn, 1998). The public perception of the dangers associated with the use of manipulated microorganisms in food crops also needs consideration.

2.11 Bacteria as biological control agents

Pseudomonas spp. and *Bacillus* spp. have been the main focus of the studies for biological control development because of their association with the root rhizosphere, their ease of growth, their ability to control a wide range of pathogens, their frequent ability to promote the growth of the plant being colonized (section 2.12) and in the case of *Bacillus* spp., their ease of storage. A compilation of information relating to proposed biological control agents is given in Table 2.1.

2.12 Plant growth promoting rhizobacteria (PGPR)

Kloepper and Schroth (1978) defined the plant growth promoting rhizobacteria (PGPR) as rhizobacteria that colonize plant roots and exhibit beneficial effects, such as growth promotion. The beneficial effect to the plant can either be direct or indirect as reviewed by Kloepper *et al.* (1999) and Wall (2000).

Table 2-1: Bacterial biological control agents suggested as having potential for field application

Biological control bacterium	Target pathogen	Infection	Mechanism	References
Agrobacterium rhizogenes K84	Pathogenic *A. rhizogenes* Pathogenic agrobacteria	Galling Crown gall	Antibiosis Production of hydroxamate iron chelator	McClure *et at.* (1998); Penyalver and Lopez (1999) Penyalver *et al.* (2001)
A. radiobacter and *A. vitis*	Tumorgenic *Agrobacterium*	Crown gall	-	Bazzi *et al.* (1999)
A. radiobacter G12A	*Globodera pallida*	Potato disease	-	Hackenberg *et al.* (1999)
Alcaligenes sp. *MFA1*	*Fusarium* sp. *F. oxysporum* f.sp. *dianti*	Wilt Wilt	Siderophore production Siderophore production	Yuen *et al.* (1985) Yuen and Schroth (1986)
Alcaligenes sp.	Plant parasite nematodes	Root pathogen	-	Siddiqui and Mamood (1999)
Bacillus subtilis A13	*Rhizoctonia solani* *Sclerotium rolfsii* *F. oxysporum* f. sp. *dianti*	Damping-off and wire-stem Root pathogen Wilt	Antibiosis & plant growth promotion Antibiosis Antibiosis	Broadbent *et al.* (1971) Merriman *et al.* (1974) Yuen *et al.* (1985)
B. subtilis GB03	*Rhizoctonia spp.* and *Fusarium* spp. *Colletotrichum orbiculare* *Pseudomonas syringae* *Erwinia tracheiphila*	Damping-off Wilt Antrachnose Angular leaf spot Wilt	Antibiosis Antibiosis Induced systemic resistance Induced systemic resistance Antibiosis	Koch *et al.* (1998) Raupach and Kloepper, 1998)

Table 2-1: (Continued)

Biological control bacterium	Target pathogen	Infection	Mechanism	References
B. subtilis (strain BB)	*Xanthomonas campestris*	Black rot	-	Wulff *et al.* (2002)
B. subtilis	*Botrytis cinerea* *Rhizoctonia solani* AG2-2 *Phytophthora nicotianae* *Meloidogyne incognata*	Lesion Large-patch in grass Root pathogen Root galling	- Antibiosis - -	Tatagiba *et al.*, (1998) Nakasaki *et al.* (1998) Grosch and Grote (1998) Hoffmannhergarten *et al.* (1998)
B. cereus	*Rhizoctonia solani* *Pseudomonas solanacearum* *Meloidogyne incognata*	Damping-off Wilt Root galling	Antibiosis Antibiosis -	Pleban *et al.* (1997) and Gazoni *et al.* (1998) Sunaina *et al.* (1997) Jonathan *et al.* (2000)
Enterobacter agglomerans	*Phytophthora cactorum*	Crown and root rot	-	Utkhede and Smith (1997)
E. cloacae.	*Pythium ultimum.*	Seed rot and Damping-off	Competition	Van Dijk and Nelson (1998; 2000)
E. cloacae S11	*Fusarium sambucinum*	Potato tuber dry rot	-	Schisler *et al.* (2000)
E. cloacae WBMH-3-CMr	Insect pest	-	Gut colonization	Watanabe *et al.* (2000)
E. gergoviae	*Mosquito & black fly larvae*	-	Toxin production	Kuzina *et al.* (2002)
Erwinia aphidicola	Pea aphid	-	Gut infection	Harada and Ishikawa (1997)

Table 2-1: (Continued)

Biological control bacterium	Pathogen	Infection	Mechanism	References
E. herbicola Eh1087	*Erwinia amylovora*	Fire blight	Antibiosis	Kearns and Mahanty (1998)
	Botrytis cinerea	Powdery mildew	Lysis and competition	Bryk *et al.* (1998)
	Penicillium expansum	Powdery mildew	Lysis and competition	
E. herbicola Eh1087	*Erwinia amylovora*	Fire blight	Antibiosis	Kearns and Mahanty (1998)
	Botrytis cinerea	Powdery mildew	Lysis and competition	Bryk *et al.* (1998)
	Penicillium expansum	Powdery mildew	Lysis and competition	Bryk *et al.* (1998)
Flavobacterium sp.	*Phytophthora cactorum*	Root Rot	-	Alexander and Stewart (2001)
Pseudomonas fluorescens	*Fusarium udum*	Wilt	Antibiosis	Vidhyasekaran *et al.* (1997[b])
	Erwinia carotovora	Soft rot disease	Antibiosis	Elhendawy *et al.* (1998)
	Rhizoctonia solani	Damping off	Antibiosis	Gazoni *et al.* (1998); Nielsen *et al.* (1998)
	Fusarium sp.	Wilt	-	Larkin and Fravel (1998)
	Pythium ultimum	Damping-off	Antibiosis	Nielsen *et al.* (1998)
P. fluorescens WCS374	*Fusarium oxysporum* f.sp. *raphani*	Wilt	Induced systemic resistance	Leeman *et al.* (1995[a,b])
P. fluorescens CHAO	*Pythium ultimun*	Damping-off	Antibiosis	Schmidlisacherer *et al.* (1997)
	Thielaviopsis basicola	Black root rot	-	Troxler *et al.* (1997)
	Rhizoctonia solani	Damping-off	-	Vavrac *et al.* (1997)
	Fusarium oxysporum	Crown and root rot	-	Duffy and Defago (1997)

Table 2-1: (Continued)

Biological control bacterium	Pathogen	Infection	Mechanism	References
P. fluorescens 41	*Xanthomonas malvacearum,* *Rhizoctonia solani* *Fusarium vasinfectum* *Verticillium dahliae*	- Damping-off - -	Antibiosis Antibiosis Antibiosis Antibiosis	Safiyazov *et al.* (1995)
P. fluorescens strain 220	*Pseudocercosporella herpotrichoides*	Eyespot pathogen of cereals	Antibiosis	Clarkson and lucas (1997)
P. fluorescens strain F113	*Pythium* sp.	Damping-off	Antibiosis	Moenneloccoz *et al.* (1998)
P. fluorescens strain 68-28	*Fusarium oxysporum f.*sp. *radicislycopersici*	Root rot	Induced systemic resistance	Mpiga *et al.* (1997)
P. fluorescens CR330D	*Agrobacterium tumefaciens*	Crown gall	Antibiosis	Khmel *et al.* (1998)
P. fluorescens 5.014	*Pythium ultimum*	Damping-off	Antibiosis	Hultberg *et al.* (2000)
P. fluorescens DR54	*Pythium ultimum*	Damping-off	Antibiosis	Thrane *et al.* (2000)
P. fluorescens 4-92	*Macrophomina phaseolina*	Charcoal rot disease	Induced systemic resistance	Srivastava *et al.* (2001)
P. fluorescens Pf1	*Cercosporidium personatum* *Puccinia arachidis* *Fusarium oxysporum f.*sp. *cubense*	Leaf spots Rust Wilt	Antibiosis Antibiosis Antibiosis	Meena *et al.* (2002) Meena *et al.* (2002) Rajappan *et al.* (2002)
P. fluorescens WCS417r	*Fusarium* sp.	Wilt	Induced systemic resistance	Duijff *et al.* (1998)

Table 2-1: (Continued)

Biological control bacterium	Pathogen	Infection	Mechanism	References
P. putida	*S. minor and S. sclerotiorum*	Lettuce drop	Antibiosis	Oedjijono *et al.* (1993)
P. putida stain 89B-27	*Colletotrichum orbiculare*	Anthracnose	Induced systemic resistance	Liu *et al.* (1995[a,b])
P. putida strain *V14i*	*Pyricularia oryzae*	Rice Blast disease	Induced systemic resistance	Krishnamurthy and Gnanamanickam (1998)
P. putida WCS 358	*Fusarium* sp.	Wilt	Induced systemic resistance	Leeman *et al.* (1996)
P. putida BTP1	*Pythium aphanidermatum*	Root rot	Antibiosis	Ongena *et al.* (2000)
P. aureofaciens B-4117	*Agrobacterium tumefaciens*	Crown gall	Antibiosis	Khmel *et al.* (1998)
Pasteuria penetrans	*Meloidogyne incognata*	Root gall	Parasitism	Weibelzahfulton *et al.* (1996); Duponnois *et al.* (2000); Jonathan *et al.* (2000); Giannakou *et al.* (2002); and Talavera *et al.* (2002)
	Meloidogyne javanica	Root gall	Parasitism	Gowen *et al.* (1998)
	Meloidogyne sp.	Root knot	Parasitism	Siddiqui and Mahmood (1999).
Serratia liquifaciens	*Fusarium oxysporum* f.sp. *dianti*	Wilt	Lysis	Sneh (1981)
	Botrytis cinerea	Fruit rot in grapes	Antibiosis	Whiteman and Stewart (1998)

Table 2-1: (Continued)

Biological control bacterium	Pathogen	Infection	Mechanism	References
S. marcescens 90-166	*F. oxysporum* f.sp. *cucumerinum*	Wilt	Induced systemic resistance	Liu *et al.* (1995[b])
S. marcescens 9M5	*Magnaporthe poae*	Summer patch disease on Kentucky bluegrass	-	Kobayashi *et al.* (1995)
S. marcescens	*Pythium ultimum* *Rhizoctonia solani* *S. minor*	Damping-off Damping-off Lettuce drop	- - chitinase & β-1,3-glucanase production	Burla *et al.* (1996) El-Tarabily *et al.* (2000)
S. marcescens Bn10	Noxious insects	-	-	Sezen *et al.* (2001)
S. plymuthica R1GC4	*Pythium aphanidermatum* *Pytium ultimum*	Root rot Damping off	- Induced systemic resistance	Mccullagh *et al.* (1996) Benhamou *et al.* (2000)
S. plymuthica	Various pathogenic fungi		Antibioisis, production of lytic enzymes, or growth promotion to plants	Kalbe *et al.* (1996)
S. plymuthica HRO-C48	*Verticillium dahliae* *Phytophthora cactorum*	Wilt Root rot	Chitinase production Chitinase production	Kurze *et al.* (2001) Kurze *et al.* (2001)
Streptomyces diastatochromogenes PosSSII	*S. scabies*	Potato scab	-	Liu *at al.* (1995[c])

Table 2-1: (Continued)

Biological control bacterium	Pathogen	Infection	Mechanism	References
S. hygroscopicus	*Sclerotinia homoeocarpa*	Dollar spot on the phylloplane of *Poa pratensis*	-	Hodges *et al.* (1993)
	Bipolaris sorokiniana	Leaf spot on the phylloplane of *Poa pratensis*	-	
S. lydicus WYEC108	*Pythium ultimum*	Root and seed rot	Antibiosis	Yuan and Crowford (1995)
	Rhizoctonia solani	Root and seed rot	Antibiosis	Yuan and Crowford (1995)
S. scabies PonR	*S. scabies*	Potato scab	antibiosis	Neeno-Eckwall *et al.* (2001)
Streptomyces sp. strain 93	*Pythium* sp.	Damping-off	-	Jones and Samac (1996)
Streptomyces sp. strain 385	*Fusarium* sp.	Wilt	-	Singh *et al.* (1999)
Streptomyces sp strain DSMZ 12424	*P. ultimum*	Damping-off	Antibiosis, siderophore production	Berg *et al.* (2001)
	Rhizoctonia. solani	Damping off		Berg *et al.* (2001)
Streptomyces sp.	*Pratylenchus penetrans*	Root lesion	-	Samac and Kinkel (2001)
S. violascens	*Phytophthora cinnamomi*	Root rot	Antibiosis	Eltarabily *et al.* (1996)
S. violaceusniger YCED-9	*Fusarium* sp.	Wilt	Antibiosis and hydrolytic enzyme production	Trejoestrada *et al.* (1998)
	Phytophthora spp.	Root rot		
S. *violaceusniger G10*	*F. oxysporum f.*sp. *cubense*	Wilt	Antibiosis	Getha and Vikineswary (2002)
S. viridodiastaticus	*S. minor*	Lettuce drop	Antibiosis	El-Tarabily *et al.* (2000)

Rhizobacteria facilitate direct effects by the fixation of nitrogen or the provision of plant growth promoting substances (Kapulnik, 1991; Wall, 2000). Alternatively, some strains of PGPR also have the capability to induce systemic resistance to the plant being colonized (Kloepper, 1993; Kloepper *et al.*, 1999). In this mechanism, the PGPR stimulate the plant's defenses, which lead to a reduced level of disease due to attack by pathogens (Kloepper *et al.*, 1996).

In terms of growth promotion, the effectiveness of PGPR probably lies in their shortening the susceptible stage of colonized plants, for example by enhancing seedling germination rate, leading to a reduced period of susceptibility for pre-emergence damping-off (Kloepper *et al.*, 1999).

PGPR strains may benefit plants indirectly by attacking the plant pathogens, leading to the provision of protection to plants from these pathogens (Kloepper, 1991; Kloepper, 1996; Kloepper *et al.*, 1999; and Wall, 2000). Many species of soil borne pathogens, such as *Aphanomyces* spp, *Fusarium* spp., *Gaeumannomyces graminis*, *Phytophthora* spp., *Pythium* spp., *Sclerotium rolfsii*, and *Rhizoctonia solani* have been demonstrated to be controlled by specific strains of PGPR as reviewed by Weller (1988, Schippers (1988), and Kloepper (1991). The mechanisms of this control is discussed elsewhere.

Bacillus subtilis strain A13 and related strains GB03 and GB07 isolated by Broadbent *et al.* (1971) were the first PGPR marketed in the USA under the trade names of Quantum®, Kodiak®, and Epic®, respectively. Strain GB03 was found to be effective in controlling cotton disease caused by *Rhizoctonia spp.* and *Fusarium spp* (Backman *et al.*, 1994). This strain was also found to induce growth promotion in cucumber (Koch *et al.*, 1998; Raupach and Kloepper, 1998).

2.13 Fungi as biological control agents

Many fungal species, particularly *Trichoderma* spp., have been known to control plant pathogens with various modes of action, such as parasitism, antibiosis, induced systemic resistance, or competition. Reported potential fungal antagonists of plant pathogens are shown in Table 2.2.

Table 2-2: Fungal biological control agents suggested as having potential for field application

Biological control fungus	Target pathogen	Infection	Mechanism	Reference
Ampelomyces quisqualis	*Oidium sp.* *Erysiphe sp.* *Sphaerotheca sp.* *Podosphaera sp.* *Leveillula sp.*	Powdery mildew Powdery mildew Powdery mildew Powdery mildew Powdery mildew	Parasitism Parasitism Parasitism Parasitism Parasitism	Sztejnberg *et al.* (1989); Chet *et al.* (1997); and (http://www.nysaes.cornell.edu/ent/biological control/pathogens/ampelomyces.html)
Coniothyrium minitans	*Botrytis sp.*		Parasitism	Turner and tribe (1976)
	Pythium ultimum.	Damping-off	Parasitism	Whipps *et al* (1993)
	Sclerotinia minor	Lettuce drop (root rot)	Parasitism	Turner and Tribe (1975); Whipps *et al.* (1993).
	S. sclerotiorum	Wilt in sunflower or lettuce drop (root rot)	Parasitism	Whipps (1991); Budge *et al.* (1995); Mcquilken and Whipps (1995); Mclaren *et al.* (1996); Budge and Whipps (2000).
	Sclerotium cepivorum	Onion white rot	Parasitism	McLean and Stewart (2000)
C. minitans A69	*S. minor and S. sclerotiorum*	Root rot	Parasitism	Stewart (2003)
Corticium sp.	*Rhizoctonia solani*	Damping-off	-	Odvody *et al.* (1977); Lewis and Papavizas (1980)
Epicoccum nigrum	*Monilinia laxa*	Twig blight	Antibiosis	Madrigal *et al.* (1994); Madrigal and Melgarejo (1995).
	Sclerotinia sclerotiorum	White mold in bean	Antibiosis	Zhou *et al.* (1996)

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
E. purpurascens	*Botrytis cinerea*	Grey mold	-	Falconi and Mendgen (1994)
	M. fructigena	Blossom blight	-	Falconi and Mendgen (1994)
	Penicillium expansum	Powdery mildew	-	Falconi and Mendgen (1994)
	M. fructicola	Blossom blight	-	Wittig *et al.* (1997)
	S. sclerotiorum	White mold in bean	-	Huang *et al.* (2000)
Fusarium graminearum	*S. sclerotiorum*	White mold in bean	-	Bolan and Inglis (1989)
F. lateritium	*S. sclerotiorum*	Lettuce drop (root rot)	-	Sitepu and Wallace (1984)
F. oxysporum	*F. oxysporum* f.sp. *lycopersici*	Wilt	-	Larkin and Fravel (1998)
F. oxysporum 101-2	*F. oxysporum* f.sp.*batatas*	Wilt	Induced systemic resistance	Shimizu *et al.* (2000)
F. pallidoroseum	A range of weeds	-	Mycotoxin production	Mabayad and Watson (2000)
F. solani	*S. sclerotiorum*	Root rot in cauliflower	-	Gupta and Agarwala (1990)
Gliocladium catenulatum	*S. sclerotiorum*	White rot, root rot	Parasitism	Huang (1978)
	Fusarium sp.	Wilt	Parasitism	Huang (1978)
	Alternaria alternata		Parasitism & antibiosis	Turhan (1993)
G. fimbriatum	*R. solani*	Damping-off	Antibiosis (mycotoxin production)	Weindling (1937, 1941)

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
G. roseum	*A. alternata*	-	Parasitism & antibiosis	Turhan (1993)
	P. ultimum	Damping-off	Parasitism	Steinmetz and Schonbeck (1994)
	Botrytis squamosa	Leaf blight	-	James and Sutton (1996)
	Septoria tritici.	Leaf blotch of wheat	-	Parello *et al.* (1997)
G. virens	*Sclerotium rolfsii*	Damping-off, Root rot	Parasitism	Mukherjee *et al.* (1995); Harris and Lumsden (1997)
	R. solani	Damping-off	Parasitism	
	P. ultimum	Damping-off	Antibiosis	Harris and Lumsden (1997)
Paecilomyces lilacinus	*Meloidogyne javanica*	Root galling	-	Alraddad (1995); Frietas *et al.* (1995); Siddiqui *et al.* (2000)
	Rhizoctonia sp.	Stem rot	-	Cartwright and Benson (1995)
	Meloidogyne incognata	Root-knot disease	-	Mittal *et al.* (1995)
P. lilacinus 251	*Meloidogyne javanica*	Root galling (root-knot)	-	Holland *et al.* (1999)
P. marquandii	*Meloidogyne hapla*	Root galling	-	Chen *et al.* (2000)
P. fumosoroseus	Various species of insect, *such as Argyresthia conjugella*	-	-	Vanninen and Hokkanen (1997)
Penicillium citrinum	*S. minor*	Lettuce drop (root rot)	-	Akem and Melouk (1989)

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
P. frequentans	*Monilinia laxa*	Twig blight	-	De Cal and Sagasta (1990); Pascual *et al.* (2000)
P. nigricans	*S. cepivorum*	White rot in onion	Parasitism	Uthkede and Rahe (1980)
Pythium carolinianum	Larvae of *Culex quinquefaciatus* and *Aedes albopictus*	-	Parasitism	Su *et al.* (2001)
P. nunn N3	*Pythium ultimum*	Damping-off	-	Lifshitz (1984); Paulitz and Baker (1987)
P. oligandrum	*P. ultimum*	Damping-off	Parasitism	Walther and Gindrat (1987); Abdelzaher *et al.* (1997); and Holmes *et al.* (1998)
	Fusarium oxysporum f.*sp. radicislycopersici*	Crown and rot rot	Induced systemic resistance	Benhamou *et al.* (2001)
P. periplocum	*B. cinerea*	Grey mold	Parasitism	Paul (1999)a
P. radiosum	*B. cinerea*	Grey mold	Parasitism	Paul (1999)b
Sporidesmium sclerotivorum	*S. minor*	Lettuce drop (root rot)	Parasitism	Ayers and Adam (1979); Adam and Ayers (1981); and Mischke (1998).
	Botrytis sp.	Grey mold	Parasitism	Mischke (1998)
	Monilinia sp.	Twig blight	Parasitism	Mischke (1998)
	S. sclerotiorum	Lettuce drop, white rot	Parasitism	del Rio (2001)

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
Talaromyces flavus	*Verticillium dahliae*	Wilt	-	Nagtzaam *et al.* (1998); Soesanto (2000)
	S. sclerotiorum	Lettuce drop, white rot	Parasitism	Huang *et al.* (2000)
Trichoderma hamatum	*Pythium spp.*	Damping-off	Parasitism	Harman *et al.* (1980)
	R. solani	Damping off	Parasitism	Harman *et al.* (1980)
	P. ultimum	Damping-off	Parasitism	Inglis and Kawchuk (2002)
T. hamatum 382	*Rhizoctonia sp.*	Damping-off	Induced systemic resistance	Nelson *et al.* (1983); Kwok *et al.* (1987); Han *et al.* (2000); Krause *et al.* (2001)
T. hamatum TMCS-3	*S. sclerotiorum*	Lettuce drop, white rot	Parasitism	Gracia-Garza *et al.* (1997)
T. harzianum	*S. rolfsii*	Damping-off, root rot	Parasitism	Wells *et al.* (1972); Mishra *et al.* (2000); and El-Katatny *et al.* (2000).
	Sclerotinia trifoliorum		Parasitism	Wells *et al.* (1972)
	B. cinerea	Grey mold	Parasitism	Wells *et al.* (1972)
	S. sclerotiorum	Lettuce drop, white rot	Parasitism	Dandurand *et al.* (2000)
	Fusarium oxysporum	Damping-off	-	Mousseaux *et al.* (1998)
T. harzianum TrH40	*Botryodiplodia theobromae*	Stein end rot	Antibiosis & parasitism	Sivakumar *et al.* (2000)
	Colletotrichum gloeosporoides	Anthracnose	Antibiosis & parasitism	
	Gliochepaloirichum microchlamidosporum	Brown spot	Antibiosis & parasitism	

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
T. harzianum T39	*Spanerotheca fusca*	Powdery mildew	Induced systemic resistance	Elad *et al.* (1998)
	B. cinerea	Grey mold	Induced systemic resistance	Elad *et al.* (1998); Dik and Elad (1999); Dik *et al.* (1999); Elad (2000)
T. koningii	*Protomycopsis phaseoli*	Leaf smut	Parasitism	Adejumo *et al.* (1999)
	S. rolfsii	Damping-off, root rot	Parasitism	Tsahouridou and Thanassoulopoulos (2001)
T. koningii T8	*Pythium sp.*	Damping-off	Parasitism	Hadar *et al.* (1984)
T. koningii S8	*Fusarium verticillioides*	Stalk necrosis	Parasitism	Danielson and Jensen (1999)
T. koningii Tr5	*S. cepivorum*	White rot	Parasitism	Metcalf and Wilson (2001)
T. polysporum	*B. cinerea* *G. graminis var. tritici* *P. cactorum*	Grey mold Take-all Root rot	Antibiosis Antibiosis Antibiosis	Dennis and Webster (1977) Maas and Kotze (1987) Lederer *et al.* (1992)
T. pseudokoningii	*B. cinerea*	Grey mold, fruit rot	Antibiosis	Dennis and Webster (1977); Tronsmo and Raa (1977); Tronsmo and Dennis (1977).
	Neovosia indica	Karnal bunt of wheat	Antibiosis	Amer *et al.* (1998)

Table 2-2: (Continued)

Biological control fungus	Pathogen	Infection	Mechanism	References
T. viride	*Stereum purpureum*	Silver leaf disease	-	Dubos and Ricard (1974)
	Fusarium sp.	Wilt	-	Locke *et al.* (1985)
	R. solani	Damping-off, sheath blight	Parasitism, antibiosis	Locke *et al.* (1985); Krishnamurthy *et al.* (1999)
	S. rolfsii	Damping-off-root rot	-	Hoynes *et al.* (1999); Mathivanan *et al.* (2000)

2.14 Commercial biological control agents

At present, at least 50 products of biological control agents (both fungal and bacterial) are commercially available (Fravel and Lazarovits, 2003)(Table 2.3) after more than six decades of intensive research in this field. Of these, less than a dozen are well known and applied in commercial nurseries (Nameth, 2002).

Table 2-3: Commercially available bacterial and fungal biological control agents (Powell *et al.*, 1990; Fravel *et al.*, 1998; Nameth, 2002; Fravel and Lazarovits, 2003).

Biological control organisms	Target disease/pathogens	Product name
Ampelomyces quisqualis	Powdery mildew caused by various fungi	AQ 10
Agrobacterium radiobacter	Crown gall disease caused by *A. tumefaciens*	Galltro 1-A, Norbac 84-C
Bacillus subtilis GB03	*Rhizoctonia solani, Pythium sp.*, and *Fusarium sp.*	Companion and Kodiak®
B. thuringiensis	Various insect diseases	Dipel, Gnatrol®
Coniothyrium minitans	*Sclerotinia minor and S. sclerotiorum*	Contans WG, KONI
Candida oleophita	*Botrytis spp.* and *Penicillium spp.*	Aspire
Cryptococcus albidus	*B. cinerea* and *P. expansum*	Yield*Plus*
Gliocladium catenulatum	*Pythium sp., Rhizoctonia solani., Botrytis sp.*, and *Diymella sp.*	Primastop
Gliocladium viren strain GL-21	*Pythium sp.* and *Rhizoctonia sp.*	SoilGuard
Peniophora (Phlebiopsis) gigantea	*Heterobasidium annosum*	PgSuspension, Rot stop
Pseudomonas fluorescens	*P. ultimum, Rhizoctonia solani*	Dagger G
Pythium oligandrum	*P. ultimum*	Polygandron
Streptomyces griseoviridis strain K61	*Fusarium sp., Alternaria sp., Phomopsis sp., Botrytis sp., Pythium sp.*, and *Phytophthora sp.*	Mycostop

Trichoderma harzianum strain T-22	*Rhizoctonia solani, Pythium sp., Fusarium sp.*, and *Sclerotinia homeocarpa*	PlantShield
T. viride	*Fusarium sp., Pythium sp., Rhizoctonia sp., Macrophomina phaseolina,* and *phytophthora sp.*	Ecofit
Mix of *T. harzianum* and *T. polysporum*	Various infecting fungi	Bionab-TWP
Mix of *T. harzianum* and *T. viride*	*Chondrosterum purpureum*	Trichodowels, Trichoject, Trichoseal
Trichoderma sp.	*Chondrosterum purpureum*	BIBAB-T

Among these, PlantShield that contains *T. harzianum* strain T-22 is the most commonly used in the greenhouse industries (Nameth, 2002), partly because of its wide range of disease control.

2.15 The possible use of compost as a carrier or growth medium for biological control agents

Compost is a humus-like substance produced from organic wastes by living organism (microorganisms or earthworms) in the process of decomposition. High temperature composting is the most common form utilized commercially (Hoitink *et al.*, 1996), but earthworm composting and mid-temperature static pile composting (Diaz *et al.*, 1993) are also employed. Mature compost is rich in humic substances and microbial biomass (Chen and Inbar, 1993; Hoitink and Gardener, 2003).

There have been some reports of possible use of compost as a carrier or growth medium for bacterial and fungal biological control agents (e.g. by Hoitink, 1990; Nakasaki *et al.*, 1998; Ramona and Line, 2002). Advantages of compost as a carrier or growth medium are:

- its low relative cost, (commercial synthetic media are considerably more expensive),
- its natural antagonism to plant pathogens, particularly noted for compost extracts (compost teas),

- its provision of slow-release nutrients at rates better matching the requirements of plants than do inorganic fertilizers, and
- its provision to the soil of improved water-holding capacity, improved porosity, improved tilth, and decreased evaporative water loss in the field.

Suppression of plant pathogens by compost amendment has been reported by e.g. Cook and Baker (1983); Hoitink and Kuter (1986); Hoitink and Fahy (1986); Gorodecki and Hadar (1990); Chung and Hoitink (1990); Hadar and Gorodecki (1991); Hardy and Sivasithamparam (1991) Hoitink *et al.* (1993); Nakasaki *et al.* (1998); Ramona and Line (2002); and Hoitink and Gardener (2003). The mechanisms of compost suppression of plant pathogens have been reviewed by Hoitink *et al.* (1997) and include both biotic and abiotic factors. Microorganisms involved in suppressive activity include *Bacillus* spp., *Pseudomonas* spp., *Flavobacterium* spp. (Hoitink *et al.*, 1991) or fungal antagonists, such as *Gliocladium virens* (Hoitink and Fahy, 1986) and *Trichoderma* spp. (Chung and Hoitink, 1990; Hoitink and Gardener, 2003). Biological control biota arise as serendipitous colonists during cool-down of maturing compost, and therefore the quality of suppressive effectiveness can be variable.

Inoculation of specific antagonists into compost following peak heat has been slow to develop, and until recently largely unsuccessful (Hoitink, 1990). Although Hoitink (1990) patented a method of antagonist inoculation into matured bark composts, he admitted that the method cannot be applied as it stood, because 'the temperature and the degree of compost maturity may vary according to environmental factors, the raw material being composted, and the composting system used'. Nakasaki *et al.* (1998) re-vitalized this area of research with the demonstration of effective manipulation of grass-clipping compost by the inoculation of a *Bacillus subtilis* strain at the outset of composting. The resultant spores were able to survive the hot stage to be present in high numbers in mature compost, the resultant mix being found to be consistently effective in the control of *Rhizoctonia solani*, the causative agent for the large patch in turf grass.

2.16 Conclusions

The potential for biological control of plant pathogens has received a major boost as a result of the imminent phasing out of methyl bromide, probably the most effective

broad-spectrum biocide. Specific problems needing attention in the area of biological control relate to the viability of biological control agents prior to application, the cost of biological formulations relative to chemical control and difficulties faced in the commercialization process. These difficulties have conspired in the past to limit the number of available biological control agents on the market.

Chapter 3

Isolation, screening and identification of biological control agents inhibitory to plant pathogens

3.1 Abstract

Potential biological control agents were isolated from mature compost and from the rhizosphere of crop plants found at the University of Tasmania farm as well as from lettuce farms in Bali-Indonesia. Commercially available biological control agents plus a laboratory contaminant showing significant antagonism to a plant pathogen were also included in this study. Some 67 isolates of more than 100 cultures assessed showed antagonistic activity against one or more tested fungal pathogens (*Sclerotinia minor*, *S. sclerotiorum*, *Fusarium* spp., and *Rhizoctonia solani*) *in vitro*. Most of these antagonist isolates were identified as *Bacillus* or *Pseudomonas* spp. In a non-replicated glasshouse screening trial, eight of these isolates (subsequently identified as *Pseudomonas corrugata*, *Bacillus megaterium*, *B. polymyxa*, *B. mojavensis*, *B. pumilus*, *B. thuringiensis*, *Exiguobacterium acetylicum*, and *Lysobacter antibioticus*) continued to show effective inhibition of *Sclerotinia minor* and *S. sclerotiorum*, resulting in their further study.

3.2 Introduction

The first stage in the development of biological control agents encompasses the isolation, screening and identification of agents antagonistic to plant pathogens. As previously noted (Chapter 2), biological control agents are abundant in the soil, particularly in the rhizosphere or rhizoplane of plant roots. Although only a small portion of microbiota present in the root region of plants and capable of inhibiting pathogens may be cultivable (Sorensen, 1997), this portion continues to provide a rich source of antagonists to plant pathogens (Weller, 1988).

Biological control agents effective against root pathogens should ideally be isolated from the root or rhizosphere of the specific crop intended for protection since they are already closely associated with and well adapted to the prevailing environmental conditions (Weller, 1988; Cook, 1993; Larkin and Fravel, 1998). Several improved

screening methods to identify likely antagonist candidates have been reviewed in Chapter 2. An important consideration in the selection of a screening method is that should be related as closely as possible to field conditions. In the present study, the isolates showing *in vitro* antagonism to selected plant pathogens were subsequently screened in a non-replicated glasshouse pot trial prior to select the best-performing isolates.

The primary objective of the present study was to isolate, screen, and identify potential bacterial antagonists to a range of fungal pathogens, including *S. minor*, *S. sclerotiorum, Fusarium* spp. and *Rhizoctonia solani.*

3.3 Materials and Methods

3.3.1 Sample collection and isolation of potential antagonists

Samples of soil, or roots of lettuces, wheat, and barley were obtained from the University of Tasmania farm 30 km North of Hobart, or from other local market gardens. Isolations were also made from commercially available mature compost.

Samples of soils, plant roots, or compost (5.0 g) were added to 100 mL of sterile saline (0.85% NaCl) in sterile plastic bags and stomached for 15 to 20 minutes. Serial dilutions (0.1 mL) were spread onto potato dextrose agar (PDA, Appendix 1C) or trypticase soya agar (TSA, Appendix 1B) and incubated at 25°C for 2-5 days. Colonies appearing on 10^{-4}–10^{-6} dilution plates were purified and sub-cultured on fresh TSA or PDA. For regular use, these isolates were stored on PDA or TSA at 4°C. For storage, some potential antagonists were cryo-preserved in the trypticase soya broth (TSB, Appendix 1B minus agar) amended with glycerol (at 30% of the final concentration) at –70°C.

Additional cultures were isolated from lettuce farms in Bali, Indonesia, or obtained from the stock culture collection of the School of Agricultural Science, University of Tasmania. A fungal antagonist (*Trichoderma* sp., isolate Td_{22}), known to be effective against sclerotia-forming fungal pathogens, was kindly provided by Dr. Dean A. Metcalf.

3.3.2 Fungal pathogens

Fungal pathogens (*Sclerotinia minor*, *S. sclerotiorum*, *Fusarium* sp., and *Rhizoctonia solani*) were obtained from the stock culture collections of the School of Agricultural Science, University of Tasmania. For regular use, these pathogens were sub-cultured on fresh PDA. For long-term storage, *S. minor* was grown in sterile moist millet seeds, *S. sclerotiorum* in sterile moist barley seeds, while *R. solani* and *Fusarium* sp were grown on PDA or in sterile distilled water. All cultures were stored at 4°C.

3.3.3 *In vitro* dual culture assay for screening potential antagonists

Isolates reported in Section 3.3.1 were spot inoculated in duplicate onto the periphery of PDA or TSA plates (corresponding to the medium on which they were isolated). Plugs (1 cm^2) of 48 hour old fungal pathogen on PDA were then placed in the center of the plates followed by incubation at 25°C for two to seven days, after which time the inhibition zones between antagonists and pathogens were measured from three different angles with averages recorded. Isolates producing inhibition zone were sub-cultured on fresh PDA or TSA for further study.

3.3.4 Pot trials for screening potential biological control agents

Bacterial antagonists showing significant antagonistic activity against fungal pathogens *in vitro* (Section 3.3.3) were further screened against *S. minor* or *S sclerotiorum* in a non-replicated glasshouse trial with a view to selecting a limited number of potential antagonists for further study. For this trial, 48 hour-old bacterial antagonists were suspended in sterile 0.85% saline to give cell densities of ~10^8 cell/mL (determined by OD readings at 540nm). Roots of two weeks old lettuce seedlings were washed and suspended in the antagonist suspensions for five minutes prior to planting the seedlings in 15 cm diameter pots (four per pot) of steam-sterilized potting mix (Appendix 1A). After acclimatization for one week the surface 20 mm of potting mix was inoculated with fungal pathogen (*S. minor* grown on millet seeds or *S. sclerotiorum* grown on barley seeds) at the rate of 2 g seeds/pot. Pots were maintained in the glasshouse for up to eight weeks until disease incidence could be assessed. Uninoculated pots and pots inoculated with the pathogen served as controls.

3.3.5 Identification of bacterial biocontrol agent candidates

Most bacterial antagonists reported in Section 3.3.1 were identified to genus level or higher on the basis of their morphological characteristic and other tests indicated in Bergey's Manual of Systematic Bacteriology (Krieg *et al.*, 1984; Holt *et al.*, 1994). Those showing particular potential as biological control agents were further characterised on the basis of 16S rDNA sequence analysis.

3.3.5.1 Identification of bacterial antagonists

The following tests were performed to identify the antagonists to genus level using Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

3.3.5.1.1 Gram stain and cellular morphology

Gram staining was performed according to the method of Madigan *et al.* (1997), using 24-48 hour TSA cultures. The bacterial cells were fixed on a glass slide, stained with crystal violet solution for one minute, washed with water, and covered with Lugol's iodine solution for 30 seconds. Following this the cells were exposed to acetone-alcohol for several seconds, washed with water, and counterstained with safranin for one minute. The stained cells were washed with water, dried, and examined under oil immersion (x1000 magnification).

3.3.5.1.2 Spore stain

Spore staining was undertaken using stationary-phase cultures of large rod shaped Gram-positive bacterial isolates only. A loopful of isolate was fixed smear on a slide, covered with a strip of filter paper and flooded with malachite green to avoid dryness over a boiling water bath for five minutes. The slide was then washed in a stream of gently running water, covered by 0.5% aqueous safranin for five minutes, washed again, and examined under oil immersion (x1000 magnification).

3.3.5.1.3 Motility

A drop of 24 hour-old TSB culture was placed on a glass slide and covered with a cover slip. Motility was examined using phase contrast microscopy (x1000 magnification). Bacteria were considered to be motile if one or more cells moved in a direction different from others.

3.3.5.1.4 Flagella stain

The method of Heimbrook *et al.* (1989) was used. A loopful of motile bacteria (24 hour old TSA) taken from the edge of a colony was suspended in drops of sterile saline solution on a glass slide, covered with a cover slip, and allowed to dry for ~15 minutes. Several drops of flagella stain (Appendix 1E) were then added through one edge of the cover slip and left for several minutes before examination under oil immersion (x1000 magnification).

3.3.5.1.5 Oxidative/fermentative test

A loopful of culture was stab-inoculated to the base of a tube containing Hugh and Liefson medium (Appendix 1F) (Collins and Lyne, 1984) and incubated at 25°C for 24-48 hours. Oxidative metabolism was indicated by either no acid being associated with growth, or acid production being restricted to the top third of tube, with a change of colour from green to yellow. Fermentative metabolism was indicated by the production of acid to the base of the tube.

3.3.5.1.6 Oxidase test

This test was performed on Gram-negative isolates only. A loopful of bacteria was smeared onto a piece of filter paper previously moistened with freshly prepared oxidase reagent (1% aqueous *NNN'N'*-tetramethyl-*p*-phenylene diamine dihydrochloride). A positive test was indicated by the development of a purple color within 10-15 seconds as a result of oxidation of the oxidase reagent by cytochrome oxidase.

3.3.5.1.7 Catalase test

This test was for Gram-positive bacteria only. A loopful of 24 hour old culture was smeared onto a glass slide to which was added two drops of 3% H_2O_2. Bubbles of oxygen gas generated from the enzymatic degradation of H_2O_2 indicated a positive result.

3.3.5.1.8 Casein hydrolysis

Isolates were streak-inoculated onto skim milk agar overlaying TSA and incubated for 4-7 days at 25°C. A positive result was indicated by a clear zone around the growth (Sneath, 1986).

3.3.5.1.9 Starch hydrolysis

Starch agar medium (Appendix 1G) was streak-inoculated with loopfuls of the tested isolates, followed by incubation for 3-7 days at 25°C, after which plates were flooded with iodine. Hydrolysis of starch was indicated by a clear zone around the growth.

3.3.5.1.10 Urease

A loopful of culture was streaked onto urease agar (Appendix 1H) and incubated at 25°C for 3-7 days. A colour change from colourless to pink indicated a positive result.

3.3.5.1.11 Methyl red test

A loopful of culture was inoculated into glucose-peptone broth and incubated at 25°C for 24-48 hours. One or two drops of methyl red were then added to the culture. A positive result (produced by a pH of 4.2 or lower) was indicated by a red colour.

3.3.5.1.12 Voges-Proskauer test

A loopful of culture was inoculated into glucose-peptone broth and incubated at 25°C for 24-48 hours. To the culture was added 1 ml of 40% KOH and a knife point of creatine. The mixture was shaken well and allowed to stand for 10 minutes. The formation of a pink to red colour indicated a positive result (the production of diacetyl from acetoin, indicative of the possession of the butylene glycol pathway) (Smibert and Krieg, 1981).

3.3.5.1.13 Citrate utilization

Koser's citrate broth (Appendix 1U) was inoculated with a low cell-density of bacteria. A positive result was indicated by the development of turbidity following incubation at 25°C for 24-48 hours.

3.3.5.1.14 Indole formation

Peptone water medium in 5-mL Bijoux bottles was inoculated with a loopful of culture and incubated for 24-48 hours at 25°C. A few drops of Kovac's reagent (5g of *p*-dimethylaminobenzaldehyde in a mixture of 75 ml of amyl alcohol and 25 ml of concentrated sulphuric acid) were then added to the culture, which was briefly shaken and allowed to stand for 10 minutes. A positive result was indicated by the formation of a pink to red colour indicating the production of indole from tryptophan (Collins and Lyne, 1984).

3.3.5.1.15 UV fluorescence

The test assisted the identification of *Pseudomonas* species. Isolates incubated for 24-48 hours on Kings B medium were exposed to UV light. Pyocyanin production was indicated by a blue fluorescence; other fluorescent pigments were yellow.

3.3.5.1.16 Levan production

Plates of nutrient agar (Appendix 1I) containing 4% sucrose were streak-inoculated with the isolates and incubated at 25°C for 5-7 days. A positive result was indicated by colonies appearing highly mucoid and often dome-shaped.

3.3.5.1.17 Gelatin hydrolysis

The isolates were stab-inoculated into tubed nutrient gelatin medium (Appendix 1T), incubated for seven days, and examined daily. A positive result was indicated by liquefaction around the growth (Collins and Lyne, 1984).

3.3.5.2 Molecular 16s rDNA sequencing for the identification of bacterial isolates

Some potential bacterial antagonists were identified by sequencing components of their 16S rDNA and comparing these with counterparts in a clone library of known bacteria (http://www.ncbi.nlm.nih.gov). This method is described as follows:

3.3.5.2.1 DNA extraction

Two loopfuls of cells of each isolate were suspended in 0.3 mL of saline-EDTA buffer (Appendix 1J) in 1.5 mL Eppendorf tubes, to which was added 30 µL 20% sodium dodecyl sulphate, followed by gentle mixing to lyse the cells. For Gram-positive bacteria, 30 µL (10mg/mL) lysozyme was also added and the mix incubated at 37°C for two hours. One volume of chloroform:isoamylalcohol (24:1) was subsequently added to the lysate and thoroughly mixed to form a milky emulsion prior to centrifugation at 21,000 *g* for five minutes. The upper aqueous phase was removed and placed in a new 1.5 mL Eppendorf tube for further extraction.

3.3.5.2.2 Prep-A-gene purification

The aqueous extract described in Section 3.3.5.2.1 was combined with 1 mL of binding buffer (Appendix 1K) plus 10 µL of binding matrix (Bio-Rad), and vortexed for a few seconds. After incubation at room temperature for 30 minutes, the extract was centrifuged at 21,000 *g* for one minute, the supernatant discarded, and 500 µL binding buffer added to re-suspend the pellet with vortexing. This suspension was subsequently re-centrifuged at 21,000 *g* for one minute and the supernatant discarded.

The pellet was washed in 500 µL washing buffer (Appendix 1L), centrifuged at 21,000 *g* for one minute and the supernatant discarded. This was repeated twice. To re-suspend and elute the DNA from the binding matrix, 50 µL of sterile 'milli Q' water was added and the mix incubated for 30 minutes at 30°C followed by centrifugation at 21,000 *g* for three minutes. The supernatant (approximately 45 µL) was carefully removed and placed in a new 1.5 mL Eppendorf tube. This DNA sample was stored frozen at –20°C until use.

3.3.5.2.3 Polymerase chain reaction using the HotStart Mastermix PCR kit, Qiagen

The purified DNA (5 µL), PCR Mastermix (25 µL, Appendix 1M), primer 10F (0.5 µL, Appendix 1N), primer 1492R (0.5 µL, Appendix 1N), and 'milli Q' water (19 µL) were mixed in a PCR tube and placed in a thermocycler (PerkinElmer) equipped with the 'Hotstart' program. The program consisted of: an initial denaturation at 94°C for 15 minutes; 30 cycles of denaturing at 94°C for one minute with annealing at 52°C for one

minute; extension at 72°C for 1.5 minutes; and final extension at 72°C for ten minutes. The success of the PCR reaction was checked by agarose electrophoresis at 80 V in a 1% agarose gel containing 1μg/mL ethidium bromide in TAE buffer (Appendix 1O). Five μL of PCR product was mixed with 1 μL of loading dye (Appendix 1P), and loaded into a well of agarose gel. Electrophoresis was undertaken for 30-45 minutes followed by visualization of bands under UV transillumination. Purification of the PCR product followed once bands of the correct size were visualized.

3.3.5.2.4 Purification of PCR product

The PCR product (Section 3.3.5.2.3) was added to 700 μL of prep-A-gene binding buffer plus 10 μL binding matrix followed by purification as described in Section 3.3.5.2.2.

3.3.5.2.5 Sequencing reactions

An ABI Prism Big-Dye terminator cycle sequencing ready kit (Applied Biosystems) was used for the sequencing reaction. Purified PCR product (5 μL), primer 10F or 1492R (1 μL, Appendix 1N), buffer (4 μL), milli Q water (6 μL), and reaction mix (4 μL) were mixed together in a 100 μL PCR tube, and placed in a thermocycler (DNA engine model PTC-200). The thermal cycling Big-Dye program (for 30 cycles) was 96°C for 20 seconds, 50°C for 20 seconds and 60°C for four minutes.

3.3.5.2.6 Purification of sequencing reactions

The product of the sequencing reaction was transferred to a 1.5 mL Eppendorf tube, to which was added 4 μL 3 M sodium acetate (pH 4.6) plus 60 μL of cold absolute ethanol and placed on ice for 15-20 minutes prior to centrifugation at 21,000 *g* for 30 minutes. The supernatant was carefully removed with a pipette and the pellet was carefully washed by rinsing twice with 250 μL cold 70% ethanol (with centrifugation and decanting of the ethanol), followed by drying of the pellet in a vacuum centrifuge for ten minutes. The samples were analyzed by automated sequencing at the Molecular Biology Unit, School of Biomedical and Molecular Science, Griffith University, Queensland-Australia.

3.4 Results

3.4.1 *In vitro* screening of bacterial antagonists for inhibition of plant pathogens

From a total of over 100 bacterial isolates tested, 67 were found to be antagonistic *in vitro* (on TSA or PDA) to one or more plant pathogens (*S. minor*, *S. sclerotiorum*, *Fusarium* sp, or *R. solani*). Table 3-1 shows the relative *in-vitro* inhibition of four pathogenic fungi by these isolates.

Table 3-1: Relative inhibition of four fungal plant pathogens by bacterial antagonist isolates on TSA or PDA[1].

Isolate no.	Alternative code/ growth medium	Inhibition zone (mm diameter) after one week incubation at 25°C			
		S. minor	*S. sclerotiorum*	*R. solani*	*Fusarium sp*
1	C4 (TSA)	0.0 ± 0.0	1.2 ± 0.2	ND	ND
2	C6 (TSA)	0.0 ± 0.0	1.0 ± 0.0	ND	ND
3	C7 (TSA)	1.0 ± 0.0	1.0 ± 0.0	ND	0.0 ± 0.0
4	C8 (TSA)	3.3 ± 0.4	3.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
5	C9 (TSA)	3.3 ± 0.2	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
6	UT1 (PDA)	6.0 ± 0.3	5.0 ± 0.4	3.5 ± 0.2	3.7 ± 0.2
7	UT4 (TSA)	1.8 ± 0.2	2.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
8	UT5 (TSA)	0.0 ± 0.0	1.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
9	RC ant. (TSA)	5.8 ± 0.3	2.3 ± 0.2	ND	ND
10	3A (TSA)	1.0 ± 0.0	3.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
11	SAJ1 (TSA)	2.2 ± 0.2	4.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
12	SAJ2 (TSA)	5.3 ± 0.4	3.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
13	SAJ3 (TSA)	3.0 ± 0.3	5.0 ± 0.3	ND	ND
14	SAJ5 (TSA)	3.2 ± 0.3	2.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
15	SAJ6 (TSA/PDA)	9.7 ± 0.7	4.2 ± 0.3	ND	0.0 ± 0.0
16	SAJ9 (TSA)	2.0 ± 0.0	4.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
17	SAJ10 (TSA)	0.0 ± 0.0	2.7 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
18	SAJ11 (TSA)	1.0 ± 0.0	1.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
19	SAJ12 (TSA)	3.5 ± 0.4	1.7 ± .02	0.0 ± 0.0	0.0 ± 0.0
20	SBJ1 (TSA)	0.0 ± 0.0	1.8 ± 0.3	ND	0.0 ± 0.0
21	SBJ2 (TSA)	0.0 ± 0.0	1.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
22	SBJ4 (TSA)	0.0 ± 0.0	1.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
23	SBJ5 (TSA)	0.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24	SBJ7 (TSA)	6.2 ± 0.2	3.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
25	RW1 (TSA)	4.5 ± 0.2	4.3 ± 0.2	ND	0.0 ± 0.0
26	RW2 (TSA)	1.7±0.23	1.5±0.24	ND	ND
27	RW3 (TSA)	1.0 ± 0.0	5.5 ± 0.4	ND	0.0 ±0.0

Table 3-1: (Continued)

28	**RW4 (TSA)**	0.0 ± 0.0	1.3 ± 0.2	ND	0.0 ± 0.0
29	**RW6 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
30	**RW7 (TSA)**	1.0 ± 0.0	2.0±0.28	ND	ND
31	**PA (PDA)**	1.8 ± 0.2	5.0 ± 0.3	ND	ND
32	**TRA1 (TSA)**	0.0 ± 0.0	2.2 ± 0.2	ND	ND
33	**TRA2 (TSA)**	0.0 ± 0.0	1.7 ± 0.2	ND	ND
34	**TRA3 (TSA)**	0.0 ± 0.0	1.5 ± 0.2	ND	ND
35	**TRA5 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
36	**TRA7 (TSA)**	0.0 ± 0.0	2.0 ± 0.0	ND	ND
37	**TRA8 (TSA)**	1.0 ± 0.0	2.7 ± 0.2	ND	ND
38	**TRA9 (TSA)**	0.0 ± 0.0	2.8 ± 0.2	ND	ND
39	**TRA10 (TSA)**	0.0 ± 0.0	1.8 ± 0.2	ND	ND
40	**TRA11 (TSA)**	0.0 ± 0.0	3.2 ± 0.2	ND	ND
41	**TRA12 (TSA)**	0.0 ± 0.0	2.3 ± 0.2	ND	ND
42	**TRA13 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
43	**TRA14 (TSA)**	0.0 ± 0.0	3.2 ± 0.2	ND	ND
44	**SRA1 (TSA)**	0.0 ± 0.0	1.8 ± 0.2	ND	ND
45	**SRA2 (TSA)**	0.0 ± 0.0	1.8 ± 0.2	ND	ND
46	**SRA3 (TSA)**	0.0 ± 0.0	2.2 ± 0.2	ND	ND
47	**SRA7 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
48	**SRA9 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
49	**SRA10 (TSA)**	0.0 ± 0.0	2.2 ± 0.2	ND	ND
50	**SRA11 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
51	**SRA12 (TSA)**	1.0 ± 0.0	2.0 ± 0.3	ND	ND
52	**SRA13 (TSA)**	0.0 ± 0.0	3.2 ± 0.2	ND	ND
53	**SRA14 (TSA)**	0.0 ± 0.0	2.7 ± 0.2	ND	ND
54	**SRA15 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
55	**SRA16 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
56	**SRA17 (TSA)**	0.0 ± 0.0	1.0 ± 0.0	ND	ND
57	**TSA (TSA)**	0.0 ± 0.0	2.3 ± 0.2	ND	ND
58	**Bali A (TSA)**	0.0 ± 0.0	3.6 ± 0.2	ND	ND
59	**Bali C (TSA)**	2.0 ± 0.3	1.0 ± 0.0	ND	ND
60	**Bali E (TSA)**	2.6 ± 0.2	1.6 ± 0.6	ND	ND
61	**Bali F (TSA)**	2.0 ± 0.4	ND	ND	ND
62	**Bali G (TSA)**	5.6 ± 0.2	6.6 ± 0.2	ND	ND
63	**Bali H (TSA)**	1.0 ± 0.0	ND	ND	ND
64	**Bali J (TSA)**	2.6 ± 0.2	4.6 ± 0.6	ND	ND
65	**Bali K (TSA)**	2.0 ± 0.3	0.0 ± 0.0	ND	ND
66	**Bali L (TSA)**	1.0 ± 0.0	1.0 ± 0.0	ND	ND
67	**Dipel (TSA)**	1.0 ± 0.0	2.0 ± 0.0	ND	ND

[1]Inhibition zone was measured from the edge of bacterial colonies to the edge of the fungal pathogens. Each value is an average of duplicate plates, each measured from three different angles ± standard error. Some of these isolates are recorded elsewhere, hence the 'alternative code'. The medium used for the assay in brackets, column 2; PDA = Potato Dextrose Agar, TSA = Trypticase Soya Agar.
ND = Not determined

As indicated in Table 3-1, the isolates varied both in the degree and in the host range of inhibition. Most isolates failed to inhibit *R. solani* or *Fusarium* sp. in this dual culture assay. In some cases, the fungi showed a colour change of the mycelial tips (becoming light brown instead of white around the zones of inhibition (Plate 3-1A and C). The most effective isolate (UT1) was the only one found to inhibit all four of the fungi tested. This isolate came from stored cultures of the School of Agricultural Science, University of Tasmania, being originally isolated from the root region of daisy plants (*Olearia phlogopappa*).

Examples of *in vitro* antagonism by some of the bacterial antagonists and of a fungal biological control agent (*Trichoderma* sp. isolate Td_{22}) against various fungal pathogens are shown in Plate 3-1.

A microscopic observation of the *S. minor* hyphal tips when challenged with *P. corrugata* on PDA medium is presented in Plate 3-2. Some hyphal tips of this pathogen appeared to become swollen when approaching the colony of *P. corrugata* (Plate 3-2B). In some cases, lysis of hyphal tips was also observed (Plate 3-2C).

3.4.2 Screening of bacterial antagonists in a non-replicated glasshouse trial

The effectiveness of some bacterial antagonists (selected from those listed in Table 3-1) in protecting lettuce seedlings/plants from attack by *S. minor* or *S. sclerotiorum* in a glasshouse trial is presented in Table 3-2 and 3-3, respectively. Under the conditions of the trial the pathogen (*S. minor*) aggressively attacked the lettuce seedlings, killing almost all within one week. Five of the antagonists (UT1 = *Bacillus polymyxa,* SAJ6 = *Pseudomonas corrugata,* PA = *B. mojavensis,* Bali C = *Exiguobacterium acetylicum,* and Bali G = *Lysobacter antibioticus*) provided protection of 25% to 50% relative to the control treatment for up to six days after *S. minor* inoculation (Table 3-2).

The equivalent assay of potential antagonists against *S. sclerotiorum* is presented in Table 3-3. This pathogen was found to be less aggressive than *S. minor,* since disease symptoms were first observed two weeks after pathogen inoculation, instead of within days as indicated for *S. minor.*

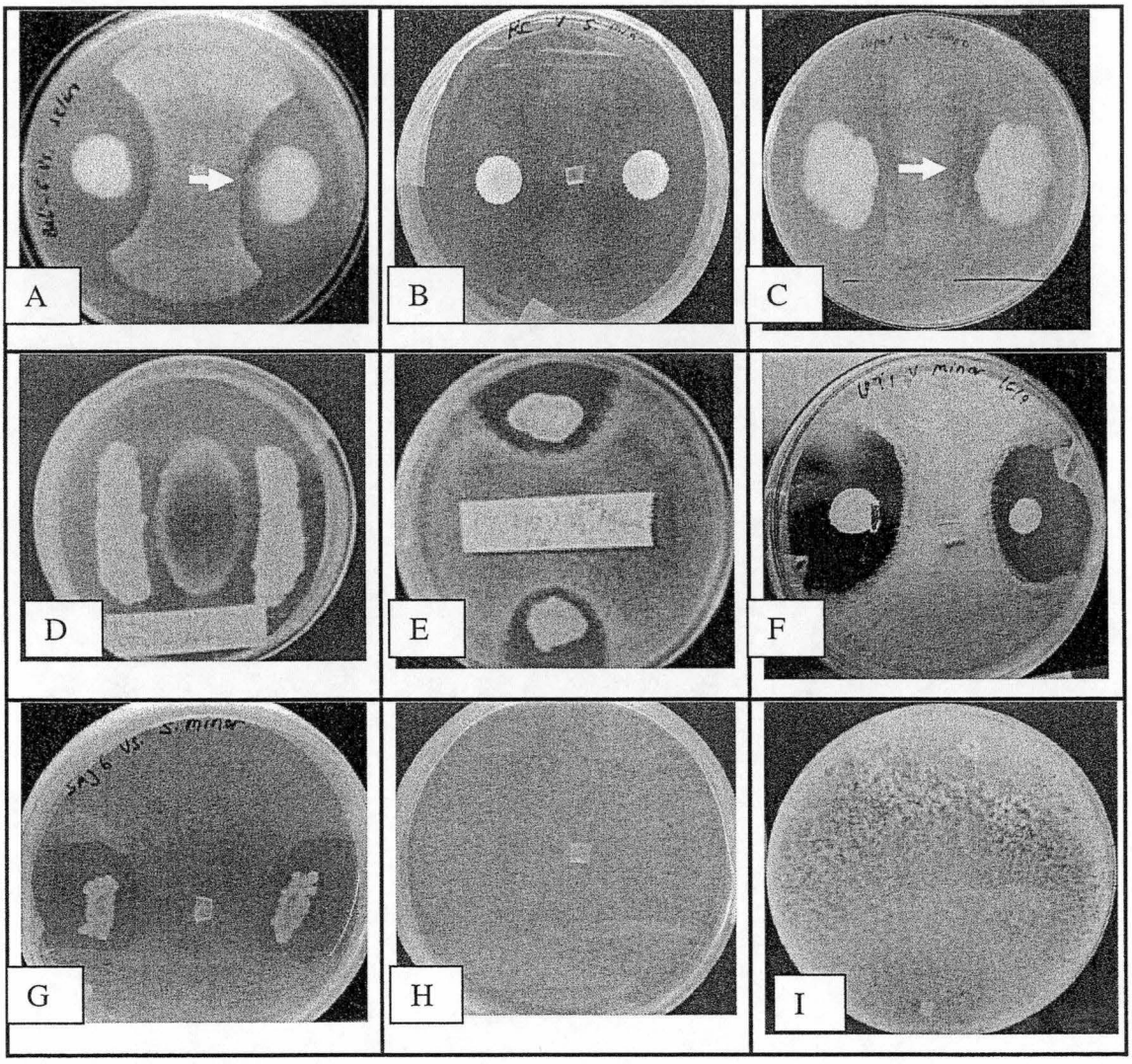


Plate 3-1: Dual culture assays of some bacterial antagonists and of a fungal biological control agent (Td_{22}) against fungal pathogens. A = *Lysobacter antibioticus* against *S. sclerotiorum*; B = *Bacillus pumilus* against *S. minor*; C = *B. thuringiensis* against *S. sclerotiorum;* D, E, F = *B. polymyxa* against *Fusarium* sp., *R. solani*, and *S. minor*, respectively; G = *Pseudomonas corrugata* against *S. minor;* H = normal growth of *S. minor*; and I = Td_{22} (bottom plug) overgrowing *S. minor* (upper plug) on pectin agar. Arrowheads in (A) and (C) show colour changes on the mycelial tips of the fungal pathogens, which appear to indicate growth abnormalities.

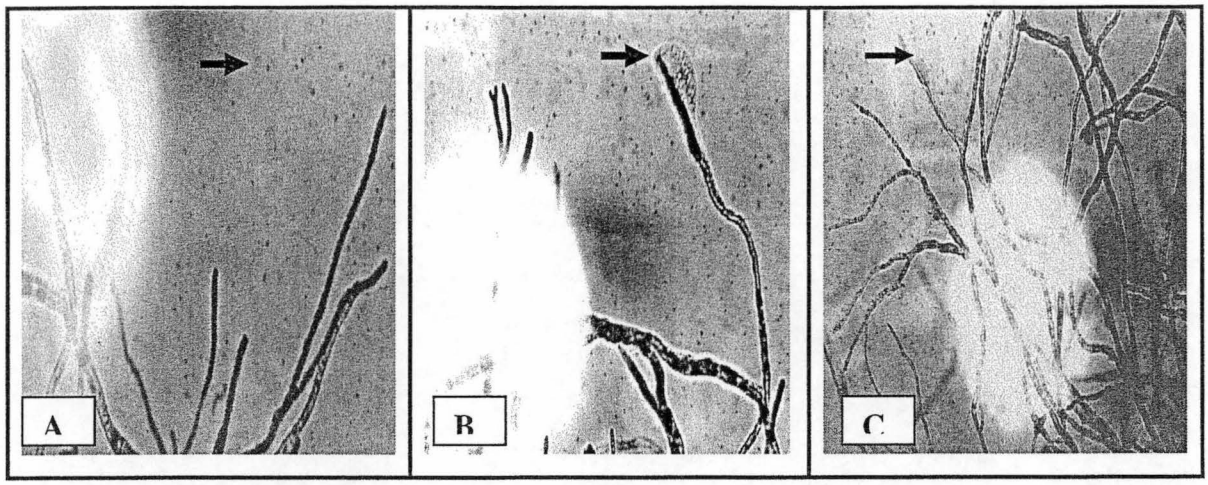


Plate 3-2: Microscopic observations of the *S. minor* hyphal tips in proximity to a colony of *P. corrugata* on potato dextrose agar. Normal growth of *S. minor* hyphae (A); A swollen hyphal tip of *S. minor* (arrowhead B) in close proximity to the zone of inhibition surrounding the colony of *P. corrugata*; In some cases lysed hyphal tips were also observed in this *in vitro* interaction (arrowhead C).

Table 3-2: Pot trial of to assess the effectiveness of some bacterial antagonists to protect lettuce seedlings from attack by *S. minor*[1]

Treatments	Percentage of healthy plants (%) Days after pathogen inoculation		
	4	5	6
C7	25	0	0
C8	50	0	0
C9	75	25	0
UT1	75	25	25
UT4	25	25	0
RC antagonist	50	0	0
3A	50	0	0
SAJ1	50	0	0
SAJ2	25	0	0
SAJ3	50	0	0
SAJ5	50	0	0
SAJ6	100	75	50
SAJ9	0	0	0
SBJ7	50	25	0
RW1	50	25	0
PA	75	50	25
Bali C	25	25	25
Bali E	25	0	0
Bali G	50	50	25

Bali J	0	0	0
Dipel	25	25	0
A0B1*	0	0	0
A0B0**	100	100	100

1. Four seedlings per pot were planted.
*Pots inoculated with *S. minor* only (control treatment)
**Nil control (neither pathogen nor antagonist was inoculated)

Table 3-3: Pot trial to assess the effectiveness of some bacterial antagonists in protecting lettuce seedlings from attack by *S. sclerotiorum*[1]

Treatments	Percentage of healthy plants (%) Weeks after pathogen inoculation		
	2	4	8
C7	100	50	0
C8	75	50	25
C9	100	25	0
UT1	75	25	25
UT4	100	0	0
UT5	50	25	0
RC antagonist	100	75	50
3A	50	50	25
SAJ1	50	25	25
SAJ2	75	50	25
SAJ3	50	0	0
SAJ5	75	50	25
SAJ6	75	50	25
SBJ1	75	50	0
SBJ2	100	100	75
SBJ4	100	50	50
SBJ5	100	75	25
SBJ7	75	50	25
RW1	50	25	0
RW3	50	50	50
RW4	75	25	0
RW6	75	50	25
PA	100	50	50
Bali A	50	0	0
Bali C	75	25	25
Bali E	50	25	0
Bali G	100	50	50
Bali J	50	25	0
Bali L	25	0	0
Dipel	100	50	25
A0B1*	25	0	0
A0B0**	100	100	100

[1]Four seedlings were planted per pot.
*Pots inoculated with *S. sclerotiorum* only (control treatment)
**Nil control (neither pathogen nor antagonist was inoculated)

The five antagonists previously noted to be effective against *S. minor* (UT1 = *Bacillus polymyxa*, SAJ6 = *Pseudomonas corrugata*, PA = *B. mojavensis*; Bali C = *Exiguobacterium acetylicum*, and Bali G = *Lysobacter antibioticus*) also protected between 25% and 50% of the lettuce seedlings/plants from *S. sclerotiorum* attack over eight weeks relative to the control treatment (A0B1) (Table 3-3). These and other antagonists showing some degree of efficacy against *S. sclerotiorum* (RC antagonist = *B. pumilus*; SBJ4 = *B. megaterium* and the commercial strain Dipel = *B. thuringiensis*) were selected for further studies.

3.4.3 Identification of the isolated bacterial antagonists

Some 54 of the 67 bacterial isolates that showed antagonistic activities *in vitro* against one or more fungal pathogens (Table 3-1) were identified at to genus level or higher. The results are presented in Table 3-4 (a-c).

A large portion of the bacterial antagonists was identified as either *Bacillus* or *Pseudomonas* spp. (Table 3-4), less frequently isolated cultures were identified as *Acinetobacter, Flavobacterium, Moraxella, Alcaligenes, Chromobacterium, Erwinia, Brevibacterium* or *Proteus* species.

Small sub-unit ribosomal gene (16S rDNA) sequence analysis allowed a more definitive identification of some major antagonists (SAJ6, PA, RC antagonist, Bali C, Bali G, Bali J, SBJ4 and the bacterium representing the commercial product Dipel), following comparison with sequences held at the Gen-Bank Nucleotide Database Library using GAPPED BLAST on-line searches (http://www.ncbi.nml.nih.gov/blast/blast.cgi) (Altschul *et al.*, 1997). Based on the results of 16S rDNA sequencing, these antagonists were most closely related to *Pseudomonas corrugata* (SAJ6), *Bacillus mojavensis* (PA), *B. pumilus* (RC antagonist), *Lysobacter antibioticus* (Bali G), *Chryseobacterium indologenes* (Bali J), *B. thuringiensis* (Dipel), and *B. megaterium* (SBJ4). The phylogenetic tree presented in Figure 3-1 shows the degree of relatedness of the antagonists with their corresponding type species.

Table 3-4a: Preliminary identification of Gram-negative bacterial antagonists.

Code	Gram reaction	O/F	Motility	Flagella position	Oxidase	UV fluorescence	Starch hydrolysis	Levan production	Gelatine hydrolysis	Pigment	Preliminary identification
C8	-	O	+	polar	+	-	+	-	-	-	*Pseudomonas* sp.
UT4	-	O	+	polar	+	-	+	-	-	-	*Pseudomonas* sp.
SAJ1	-	O	+	polar	+	-	+	-	+	-	*Pseudomonas* sp.
SAJ2	-	O	+	polar	+	-	-	+	-	-	*Pseudomonas* sp.
SAJ6	-	O	+	polar	+	-	-	+	+	-	*Pseudomonas* sp.
SAJ10	-	O	+	polar	+	+	-	+	+	-	*P. fluorescens*
SAJ11	-	O	+	polar	+	-	+	-	-	-	*Pseudomonas* sp.
RW1	-	O	+	polar	-	-	-	+	-	-	*Pseudomonas* sp.
RW3	-	O	+	polar	+	+	-	+	+	-	*P. fluorescens*
SRA3	-	O	+	polar	+	-	-	+	+	-	*Pseudomonas* sp.
SRA9	-	O	+	polar	+	+	-	+	-	-	*P. fluorescens*
SRA11	-	O	+	polar	+	-	-	+	+	-	*Pseudomonas* sp.
SRA14	-	O	+	polar	+	+	-	-	-	-	*P. fluorescens*
SRA17	-	O	+	polar	+	-	+	+	+	-	*Pseudomonas* sp.
SRA19	-	O	+	polar	+	-	+	-	-	-	*Pseudomonas* sp.
SRA20	-	O	+	polar	+	-	-	-	-	-	*Pseudomonas* sp.
SRA27	-	O	+	polar	-	-	+	+	-	-	*Pseudomonas* sp.

Table 3-4a: (Continued)

Code	Gram reaction	O/F	Motility	Flagella Position	Oxidase	UV fluorescence	Starch hydrolysis	Levan production	Gelatine hydrolysis	Pigment	Preliminary Identification
SRA28	-	O	+	polar	+	-	-	+	+	-	*Pseudomonas* sp.
TRA13	-	O	+	polar	+	-	+	-	+	-	*Pseudomonas* sp.
TRA15	-	O	+	polar	-	-	+	+	+	-	*Pseudomonas* sp.
Bali A	-	O	+	polar	+	-	+	-	-	-	*Pseudomonas* sp.
Bali L	-	O	+	polar	+	+	-	-	+	-	*P. fluorescens*
UT5	-	O	-	ND	-	ND	-	ND	-	-	*Acinetobacter*
SAJ9	-	O	-	ND	-	ND	-	ND	-	-	*Acinetobacter*
SBJ7	-	O	-	ND	-	ND	-	ND	+	-	*Acinetobacter*
SRA9	-	O	-	ND	-	ND	-	ND	-	-	*Acinetobacter*
TRA15	-	O	-	ND	-	ND	-	ND	-	creamy	*Acinetobacter*
SAJ3	-	O	-	ND	+	ND	ND	ND	-	-	*Moraxella*
SBJ1	-	O	-	ND	+	ND	ND	ND	-	-	*Moraxella*
RW4	-	O	-	ND	+	ND	ND	ND	-	-	*Moraxella*
SRA1	-	O	-	ND	+	ND	ND	ND	-	-	*Moraxella*
TRA9	-	O	-	ND	+	ND	ND	ND	-	-	*Moraxella*
SAJ12	-	O	-	ND	+	ND	-	ND	+	yellow	*Flavobacterium*
SRA2	-	O	-	ND	+	ND	-	ND	+	yellow	*Flavobacterium*

Table 3-4a: (Continued)

Code	Gram reaction	O/F	Motility	Flagella Position	Oxidase	UV fluorescence	Starch hydrolisis	Levan production	Gelatine hydrolysis	Pigment	Preliminary Identification
TRA11	-	O	-	ND	-	ND	-	ND	+	yellow	*Flavobacterium*
Bali J	-	O	-	ND	-	ND	-	ND	+	Light yellow	*Flavobacterium*
SAJ5	-	F	-	ND	+	ND	ND	ND	ND	Blue/violet	*Chromobacterium*
SRA18	-	O	+	peritrichous	+	ND	ND	ND	ND		*Alkaligenes*
TRA15	-	O	+	peritrichous	+	ND	ND	ND	ND		*Alcaligenes*

Table 3-4b: Preliminary identification of Gram-positive bacterial antagonists.

Code	Gram reaction	O/F	Motility	Flagella position	Catalase	Pigment	Spore	Starch hydrolisis	Urease	Casein hydrolisis	Colony	Preliminary Identification
C1	+	O	+	ND	+	-	+	ND	ND	ND	dry	*Bacillus* sp.
C4	+	O	-	ND	+	-	+	+	ND	ND	dry	*Bacillus* sp.
C7	+	F	-	ND	+	-	+	ND	ND	ND		*Bacillus* sp.
UT1	+	F	+	ND	+	-	+	+	-	+		*Bacillus* sp.
3A	+	F	+	polar	+	-	+	+	-	+	dry	*Bacillus* sp.
RC antagonist	+	F	+	ND	+	-	+	-	-	+		*Bacillus* sp.
SAJ3	+	O	-	ND	+	-	+	-	+	+		*Bacillus* sp.

Table 3-4b: (Continued)

Code	Gram reaction	O/F	Motility	Flagella position	Catalase	Pigment	Spore	Starch hydrolysis	Urease	Casein hydrolisis	Colony	Preliminary Identification
SBJ4	+	O	-	ND	+	ND	+	+	+	+		*Bacillus* sp.
PA	+	O	ND	ND	+	-	+	-	+	+		*Bacillus* sp.
SRA 11	+	O	+	ND	+	-	+	-	+	-		*Bacillus* sp.
Bali C	+	F	+	peritrichous	+	Yellow	-	+	-	+		*Brevibacterium*
Bali E	+	F	-	ND	+	-	ND	ND	ND	ND	ND	*Bacillus* sp.
Dipel	+	F	-	ND	+	-	+	+	+	+	dry	*Bacillus* sp.

Table 3-4c: Preliminary identification of antagonists from family *Enterobacteriaceae*

Code	Gram reaction	O/F	Motility	Flagella position	Oxidase	Indol	Methyl red	Voges-Proskauer	Citrate	Preliminary Identification
C9	-	F	+	ND	-	-	+	-	-	*Proteus*?
SBJ2	-	F	+	peritrichous	-	-	-	-	+	*Erwinia*

Note:
ND: not determined
C1, C4, C7, C8, C9: Antagonists isolated from commercially available mature compost.
Dipel: A commercially available biological control agent.
SAJ and SBJ: Antagonists isolated from (pyrethrum plant) rhizospere soils from the University of Tasmania farm.
RW: Antagonists isolated from roots of wheat
SRA and TRA: Antagonists isolated from root of lettuce plants resistance to streptomycin or tetracycline, respectively.
Bali: Antagonists isolated from lettuce roots taken from market garden farms in Bali, Indonesia
PA, 3A: Antagonists isolated from laboratory contaminants.
RC antagonist, UT1 and UT5 Isolates obtained from stock culture collection of the school of Agricultural Science, University of Tasmania

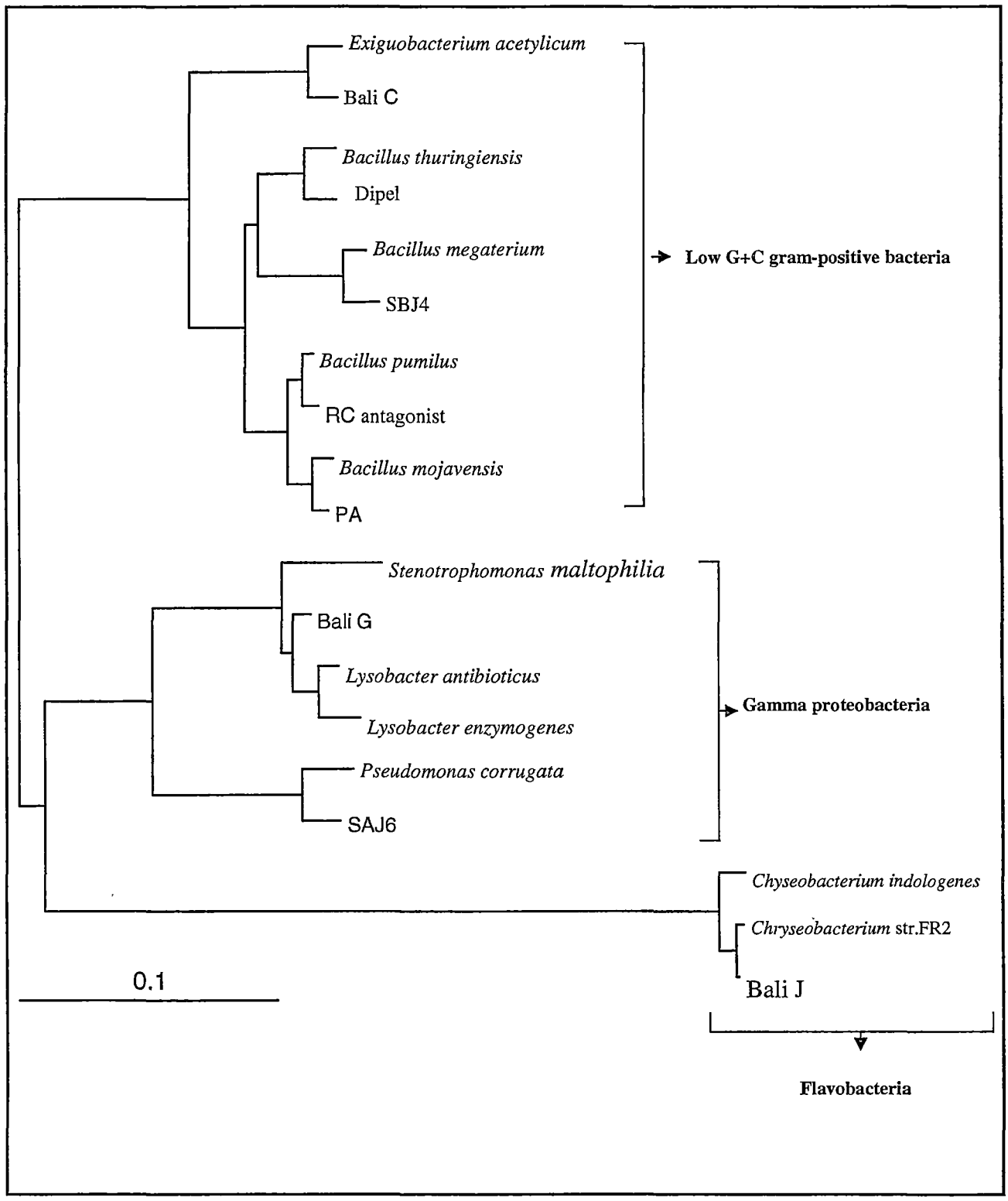


Figure 3-1: The phylogenetic relatedness of selected antagonists with their corresponding type species based on 16S rDNA sequence similarity. The bar below indicates 0.1% rDNA/rDNA difference in relatedness.

3.5 Discussion

Bacterial antagonists to *Sclerotinia minor or S. sclerotiorum* were found abundantly in soils, particularly in the root rhizosphere. In this zone they are known to play a significant mutualistic role with their plant hosts (Weller, 1988). It has been claimed that only a few of the biological control agents that exist in the soil are culturable (Sorensen, 1997), however as this study shows, there still remain an abundance of biotypes that are culturable using conventional methodology. Soil, particularly from the root rhizosphere has traditionally been the preferred source of antagonists to plant pathogens, because it is in this habitat that they are competitive and where their continued antimicrobial activity is desired. As indicated in the present study, most of the antagonists (Table 3-1) isolated from compost and plant roots (non-rhizosphere isolates) showed no disease control in the glasshouse screening trial. Surprisingly however, one antagonist (PA, Table 3-1) isolated as a laboratory agar-plate contaminant, performed well in this glasshouse trial (Table 3-3), although it failed to produce consistent results in further studies.

The use of dual culture (pathogen plus potential antagonist) assay for the initial recognition of candidate antagonists proved to be rapid and simple allowing a broad screening of potential biological control agents. Growth abnormalities of the *S. minor* pathogen in the presence of some antagonists *in-vitro*, such as colour change of hyphal tips, swollen hyphal tips or lysed hyphal tips, as indicated in Plates 3-1 and 3-2, have been reported previously. For example, Backhouse and Stewart (1989) observed swollen mitochondria, rupture of hyphal walls, and leakage of cytoplasm, leading to the death of *S. sclerotiorum* following its dual culture with *B. subtilis*. Oedjijono (1992, 1993) also reported a colour change of the hyphal tips of some pathogenic fungi in the vicinity of antagonists on various synthetic media.

In the present study, some mycelial plugs of the pathogens taken from the edge of the inhibition zone were unable to recover following sub-culture onto fresh PDA (data not shown), demonstrating that the active compounds released by some antagonists could be lethal rather than simply fungistatic. Inhibition of the fungal pathogen *in vitro* was in most cases attributed to antibiosis rather than ferric siderophore activity. Antibiosis is widely reported to be the dominant mechanism by which antagonists control pathogens (Baker and Cook, 1974), with the *in-vitro* zone of inhibition varying according to the medium used for cultivation of the pathogen/antagonist (Broadbent *et al.*, 1971; Renwick

et al., 1991; Hebbar *et al.*, 1992), and not necessarily related to the size of the bacterial colony produced (Hebar *et al.*, 1992).

The correlation between the *in-vitro* dual culture assay and the glasshouse pot trial was weak, as has been noted previously by Fravel (1988). Most of the bacterial antagonistsof plant pathogens listed in Table 3-1 failed to control the same pathogen in a glasshouse trial (Table 3-2 and 3-3), with only eight isolates being found to warrant further study. Identification of these isolates showed that most could be assigned to genera or species previously described as having biological control potential (Weller, 1988), although reports on the use of *L. antibioticus* as a biological control agent are sparse (e.g. by Hashizume *et al.*, 2001).

Possible reasons for the poor correlation between *in-vitro* assay and glasshouse trials were outlined by Baker and Cook (1974) as follows (with minor updating):

1. Compounds of the synthetic medium used in the dual culture assay may induce some tested antagonists to produce substances inhibitory to plant pathogens. These compounds however are not produced in the soil; therefore, the biological control only occurs on agar medium but not in the soil.
2. The medium used in the *in vitro* assay is more suitable for the antagonists than the pathogens, which leads to an aggressive growth of the antagonists toward the pathogens.
3. In the dual culture assay, only two microorganisms are pitted against each other without challenge (particularly to the antagonist) by a diverse soil population. Apart from direct competition between the antagonist and the indigenous microbiota, the latter may parasitise or otherwise inhibit introduced antagonists, or counteract their effect on the pathogens. It is this competition that has lead to the failure of most of the prospective antagonists to perform disease control in subsequent trials.
4. The environmental conditions of the agar used in the *in vitro* assay, such as temperature, pH, water potential, and nutrient are often adjusted to favor and optimize the growth of the antagonist and do not represent the conditions in the soil.
5. In the dual culture assay, there is a tendency to isolate only bacterial antagonists that control the pathogens through antibiosis or siderophore activity, because other mechanisms of disease control, such as parasitism or hyperparasitism are generally rare on agar. This tends to give '...a distorted emphasis of the importance of this type of antagonism in soil' (Baker and Cook, 1974).

Added to the above factors, *in-vitro* screening of antagonists will fail to detect potential biological control organisms that actively produce inhibitory compounds only in the soil but not in the presence of fungal pathogens on agar.

In this investigation more than 100 isolates were screened for antagonism to *S. minor* and *S. sclerotiorum*, using the dual culture assay, with eight of them showing a significant level of control of plant pathogens in pot trials. None has yet been assessed in the field. While dual culture assay appears to be appropriate for screening purposes, weaknesses have been reviewed e.g. by Baker and Cook (1974) and Weller (1988). As Baker and Cook (1974) noted, the chance of obtaining effective biological control agents is increased in proportion to the number of candidate organisms screened. In this regard it is interesting to note that of 3500 isolates isolated by Broadbent *et al.* (1971), 40% were effective in inhibiting one or more fungal pathogens *in vitro*, with one in ten of these (1.6% of the total isolates) being effective in field application. Similarly, Hebbar *et al.* (1992) needed to screen up to 500 isolates in order to obtain nine potential biological control agents inhibitory to *Fusarium* in maize. To isolate less than 100 antagonists inhibitory to the causative agents of take-all disease and other plant pathogens, Campbell *et al.* (1986) and Renwick *et al.* (1991) screened about 2000 primary isolates, although Földes *et al.* (2000) successfully isolated one potential antagonist to *Botrytis allii* and *B. cinerea* from only 25 isolates, although this antagonist was not field tested. The use of a non-replicated glasshouse trial to further screen and limit the number of the potential antagonists obtained from dual culture assays was found to be useful, although in retrospect a degree of replication would have helped to delineate those antagonists worthy of further study.

The genera *Bacillus* and *Pseudomonas* which were most commonly represented among the antagonist isolates (Table 3-4) are known to be common in the soil/root rhizosphere habitat and are easy to isolate due to their simple growth requirements (Krieg and Holt, 1984; Mishagi, 1990). In addition to *Bacillus* and *Pseudomonas*, bacterial antagonists belonging to *Flavobacterium, Moraxella, Chromobacterium, Acinetobacter, Alcaligenes, Erwinia,* and *Proteus* were also identified in the present study. None was field-tested.

The use of PCR methodology to examine several isolates provided a definitive phylogenetic placement, considerably more accurate than was possible using conventional methods outlined in Bergey's Manual of Systematic Bacteriology. Some bacterial

antagonists (*Bacillus thuringiensis, B. pumilus, B. mojavensis, B. megaterium*, and *Pseudomonas corrugata*) identified using the PCR method are seen to belong to the same species as some biological control agents frequently reported in the literature (e.g. by Khayami-Horani and Ateyyat, 2002; Yan *et al.*, 2002; Bacon and Hinton, 2002; Jock *et al.*, 2002; and Pandey *et al*, 2001, respectively), while others (*Lysobacter antibioticus, Exiguobacterium acetylicum,* and *Chryseobacterium indologenes*) have rarely or have never been reported as biological control agents.

3.6 Conclusions

Bacterial antagonists of *Sclerotinia minor, S. sclerotiorum, Rhizoctonia solani,* and *Fusarium* spp. were isolated from a range of sources, *viz.* soil, (including that from the root-zone of lettuces, barley, and wheat) mature compost, or as laboratory contaminants. A small portion (8 out of 67) of the antagonists isolated from the dual culture assays showed significant disease control in a non-replicated glasshouse trial, indicating dual culture assay to be a useful tool for the primary selection of potential antagonists. The antagonists obtained by dual culture were screened further in a pot trial using *S. minor* and *S. sclerotiorum* as pathogens and lettuce as indicator host, leading to the detection of three strains warranting further field investigation. The application of 16S rDNA sequencing to the identification of some of the antagonists showed some (*Bacillus thuringiensis, B. megaterium, B. pumilus, B. mojavensis,* and *Pseudomonas corrugata*) to be related to known and frequently reported biological control agents, while others (*Lysobacter antibioticus, Exiguobacterium acetylicum,* and *Chryseobacterium indologenes*) have been rarely or never reported as biological control agents.

Chapter 4

In vitro study of the optimum growth conditions for potential fungal and bacterial antagonists of plant pathogens

4.1 Abstract

The objective of this study was to investigate the optimum growth conditions of antagonists (*Trichoderma* sp. isolate Td_{22} and seven bacterial isolates) of fungal pathogens at differing initial pH of growth media and differing incubation temperatures prior to their cultivation in low-cost materials, such as wood fibre waste (WFW) or fish waste compost as reported in further studies. It was found that Td_{22} grew well in the pH range of 4.0 to 7.0 with an optimum between pH 5.0 and 6.0. The optimal temperature was 25°C, no growth was observed at 37°C or higher. This fungus was shown to utilize cellulose in the form of carboxy methyl cellulose raising the possibility for its cultivation in waste cellulosic-based materials, such as WFW. Most of the tested bacterial antagonists grew optimally between 25°C and 30°C at an initial pH of 7.0 or 8.5, and demonstrated no celluloytic activity.

4.2 Introduction

Various environmental factors, such as temperature, pH, water activity, nutrient availability, CO_2, light or a combination of these factors may have a significant effect on the growth response of microorganisms (Hurlbert, 1999). The purpose of this study was to investigate the pH and temperature optima of *Trichoderma* sp. (isolate Td_{22}) and several potential bacterial antagonists to plant pathogenic fungi as reported in the previous study, prior to their further investigation. The cellulolytic activity of Td_{22} and its growth response on pectin agar amended with antibiotics were assessed with a view to formulating a semi-selective medium for this fungus.

4.3 Materials and methods

4.3.1 Antagonists

Seven isolates of bacterial antagonists (*Bacillus megaterium, B. polymyxa, B. thuringiensis, B. mojavensis, B. pumilus, Pseudomonas corrugata,* and *Lysobacter antibioticus*) isolated in this investigation, and a fungal antagonist (Td_{22}) obtained from Dr. Dean Metcalf, were the subject of this study.

4.3.2 The effect of pH on the antagonist's growth

The effect of pH on the growth of the fungal (Td_{22}) and bacterial antagonists was studied on pectin agar medium (Appendix 1D) and in half strength nutrient broth (Appendix 1Q), respectively. The pH of these media was adjusted to pH 4.0, 5.0, 6.0, and 7.0 pH units for the fungus and to pH 5.5, 7.0, and 8.5 for the bacteria by addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH) (Metcalf, 1997). Prior to sterilization at 121°C for 15 minutes, 50 mL of half strength nutrient broth was dispensed into 125 mL side-armed flasks. The half strength nutrient broth was used because it gave the lowest optical density (OD) reading at 520nm, while still being sufficient for the rapid growth of the bacteria under study.

In the study of fungal growth response, one cube (5 x 5 mm) of a 3-day-old Td_{22} culture on pectin agar was placed in the centre of the same medium (with five replicates) but at varying pH values. The radial growth of mycelia was determined after incubation at 25°C for 48 hours.

For the bacterial antagonists, the sterile pectin medium (with four replicates) was inoculated with 100 µL of 24 hour-old bacterial cultures grown in the same medium (but full strength). The growth of the cultures at 25°C was followed over a 30 hours period by measuring optical density at 520nm (Spectronic 20) at time intervals. The doubling times and the specific growth rates of bacterial antagonists were estimated from the exponential growth rates (Appendix 2).

4.3.3 The effect of temperature on the antagonist's growth

The same media, procedure of inoculation, and measurements as indicated in Section 4.3.2 were applied. Fungal cultures were incubated at 20°C, 25°C, 37°C, and 40°C for 48 hours, with daily data collection, while the growth of the bacterial antagonists was studied at 25°C, 30°C, and 35°C.

4.3.4 Assay for the ability of Td_{22} to utilize carboxy methyl cellulose (CMC) at various pH values.

The assay for the ability of mycelial plugs of Td_{22} (from pectin agar) to degrade carboxy methyl cellulose (CMC) was conducted at 25°C in 0.1 M citrate-phosphate buffer (Cruickshank *et al.*, 1975) adjusted to pH 3.0, 4.5, 5.5, and 7.8 (four replicates of each) using a Wells-Brookfield microviscometer, model LVT. The initial CMC concentration was adjusted to give a relative viscosity reading of approximately 65 from the microviscometer. Loss of viscosity of the CMC was measured at 10 minutes intervals.

4.3.5 Assay for resistance of Td_{22} to antibiotics

Assay of resistance to streptomycin or tetracycline at concentrations of 100 µg/mL was conducted on pectin agar (pH 5.0) as described in Section 4.3.2. Inoculated medium (four replicates) was incubated at 25°C for up to 72 hours with radial growth measurements recorded daily.

4.3.6 Statistical analysis

Analysis of variance (ANOVA) of data obtained from this study was carried out using Minitab software for Windows. The significance of differences between means was further tested using the least significant different (LSD) test at a p of <0.05 following ANOVA.

4.4 Results

4.4.1 The effect of pH on the growth of antagonists

The growth response of Td_{22} measured as radial growth on pectin agar at various pH values is presented in Figure 4-1.

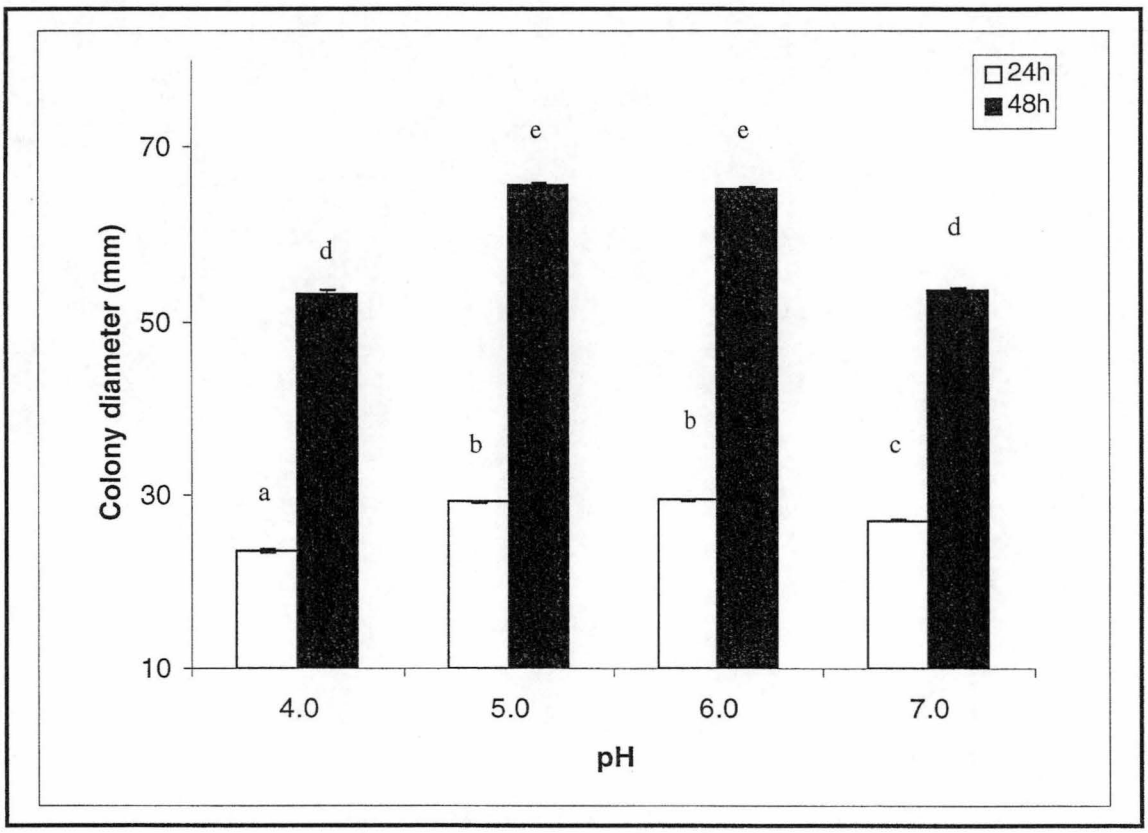


Figure 4-1: Relative radial growth of Td_{22} mycelia on pectin agar at pH 4.0, 5.0, 6.0, and 7.0 measured after 24 hours (light bars) and 48 hours (dark bars) incubation. Each bar with standard error is an average obtained from five replicate plates with three measurements on each plate. Bars with the same letter are not significant statistically at $p<0.05$.

The fungus grew well at all pH values, with optimal growth appearing to be in the range of pH 5.0 and 6.0. Radial growth at these initial pH values after 24 hours was not statistically significant ($p>0.05$) but growth on these plates was significantly different to that at initial pH values of 4.0 or 7.0.

The doubling times and the specific growth rates of the seven bacterial antagonists as a response to growth media set at different pH values are presented in Figure 4-2.

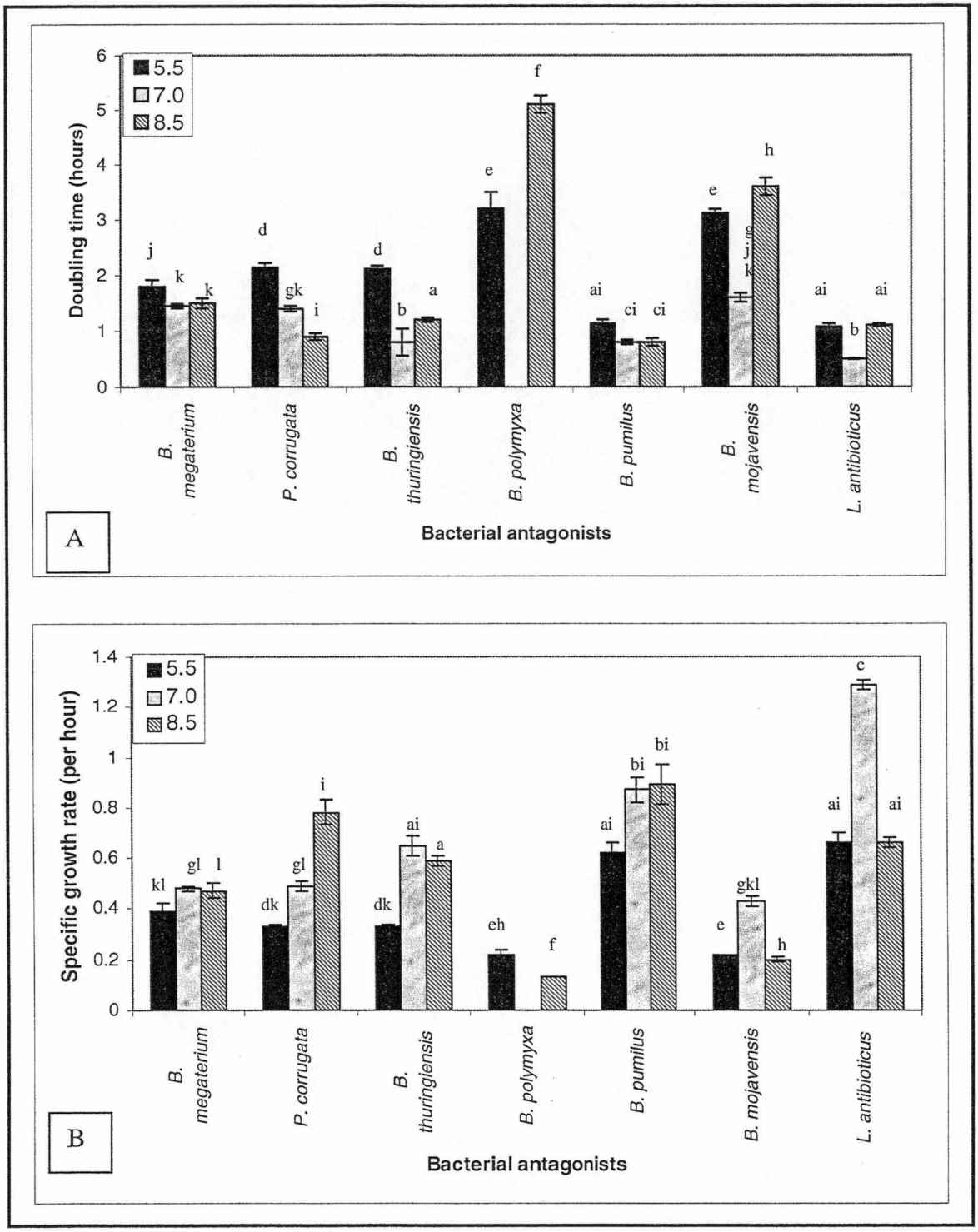


Figure 4-2: The relative doubling time (A) and specific growth rate (B) of the bacterial antagonists grown in a half strength nutrient broth with varying initial pH values. Each bar with standard error is an average of 4 replicate assays. Bars with the same letter(s) are not significant at $p<0.05$ according to the lsd test, following ANOVA.

The doubling time of these antagonists at pH 7.0, as measured within their exponential phases (after ~ 8-16 hours incubation), ranged from 0.54 hours to 1.63 hours (Figure 4-2). After 30 hours incubation, antagonists grown in either acid or alkaline media had changed the pH of their growth medium to approximately neutral. From the Figures it is apparent that three isolates have an optimal pH in the vicinity of 7.0 (*B. thuringiensis*, *B. mojaviensis* and *L. antibioticus*), two isolates indicated an optimal between pH 7.0 and 8.5 (*B. megaterium* and *B. pumilus*), *P. corrugata* showed a preference for pH 8.5, and *B. polymyxa* gave indeterminate results because of a failure to grow at pH 7.0.

4.4.2 Effect of temperature on the growth of antagonists

The relative radial growth of Td_{22} at four different temperatures after 24h and 48h incubation is shown in Figure 4-3. The most favorable temperature for this fungus was 25°C, this being significantly ($p<0.05$) better than 20°C. No growth response was observed at 37°C or above.

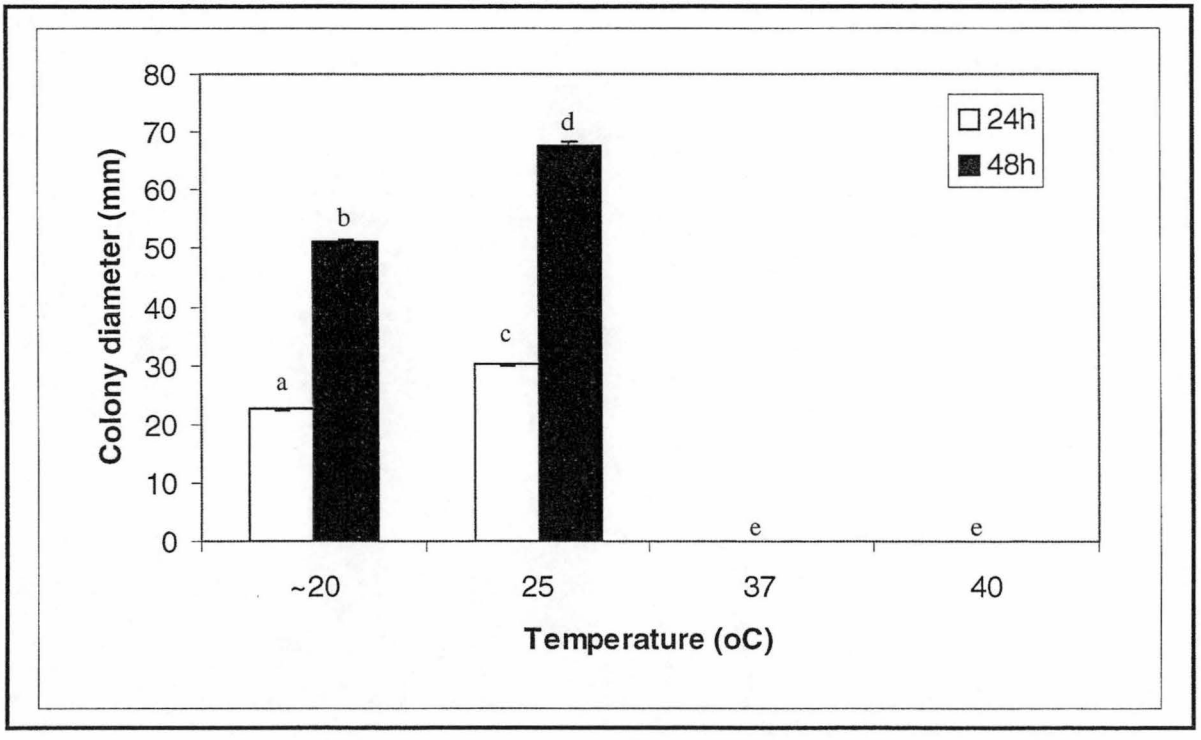


Figure 4-3: The relative radial growth of Td_{22} mycelia incubated at four different temperatures measured after 24h (light bars) and 48h (dark bars) incubation. Each bar (± standard error) represents a mean derived from five replicate plates, with three measurements of radial growth on each plate. Bars with the same letter are not significant statistically at $p<0.05$.

An incubation temperature of 25°C and medium pH between 5.0 and 6.0 was found to be most suitable for the cultivation of Td_{22}, these conditions being significantly better ($p<0.05$) than other combinations tested (Figure 4-4).

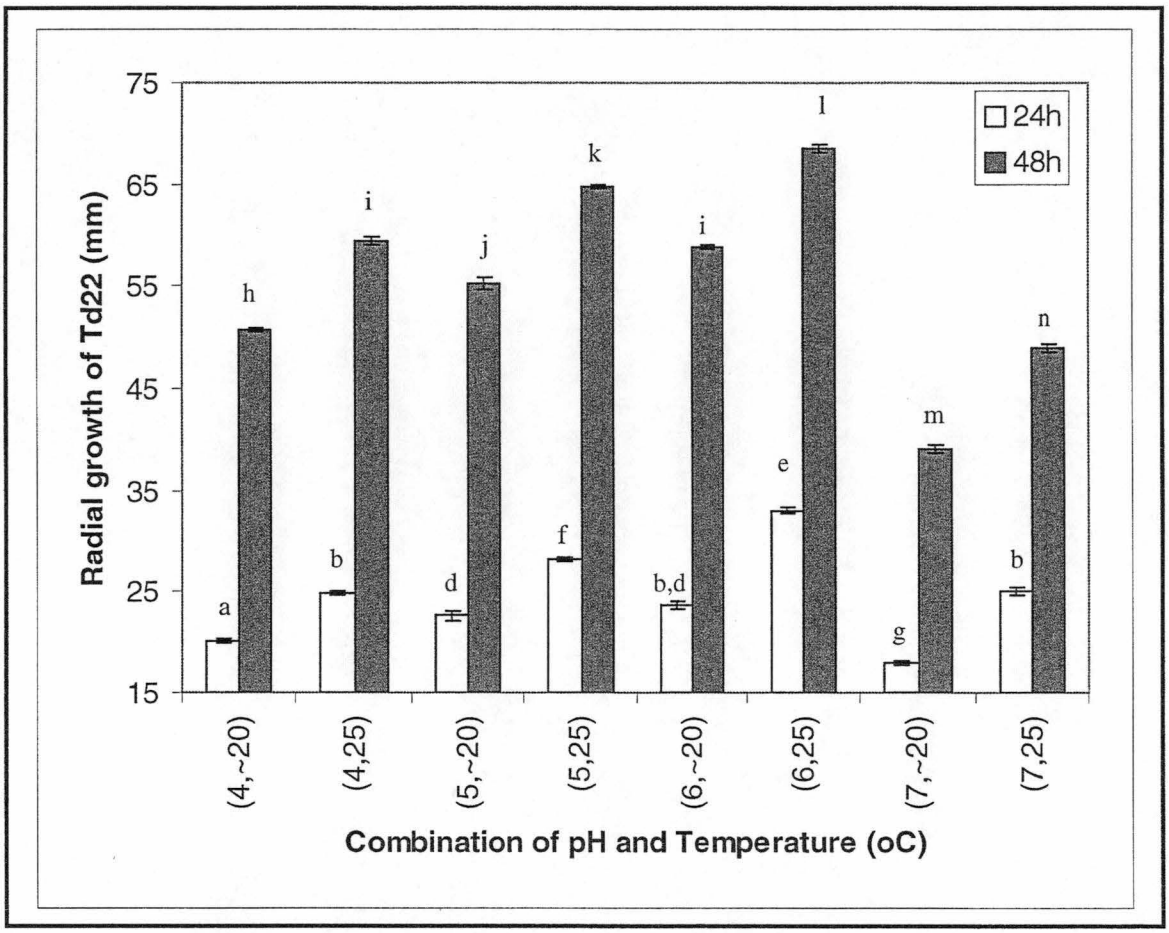


Figure 4-4: The relative radial growth of Td_{22} in a combined treatment of pH and temperature, following 24h (light bars) and 48h (dark bars) incubation. Each bar (± standard error) is an average of five plates with three measurements of radial growth /plate. Bars with the same letter(s) are not significant statistically at p level <0.05.

The growth responses of the bacterial antagonists measured as doubling time and specific growth rates at three different temperatures, and at an initial pH of 7.4, is presented in Figure 4-5.

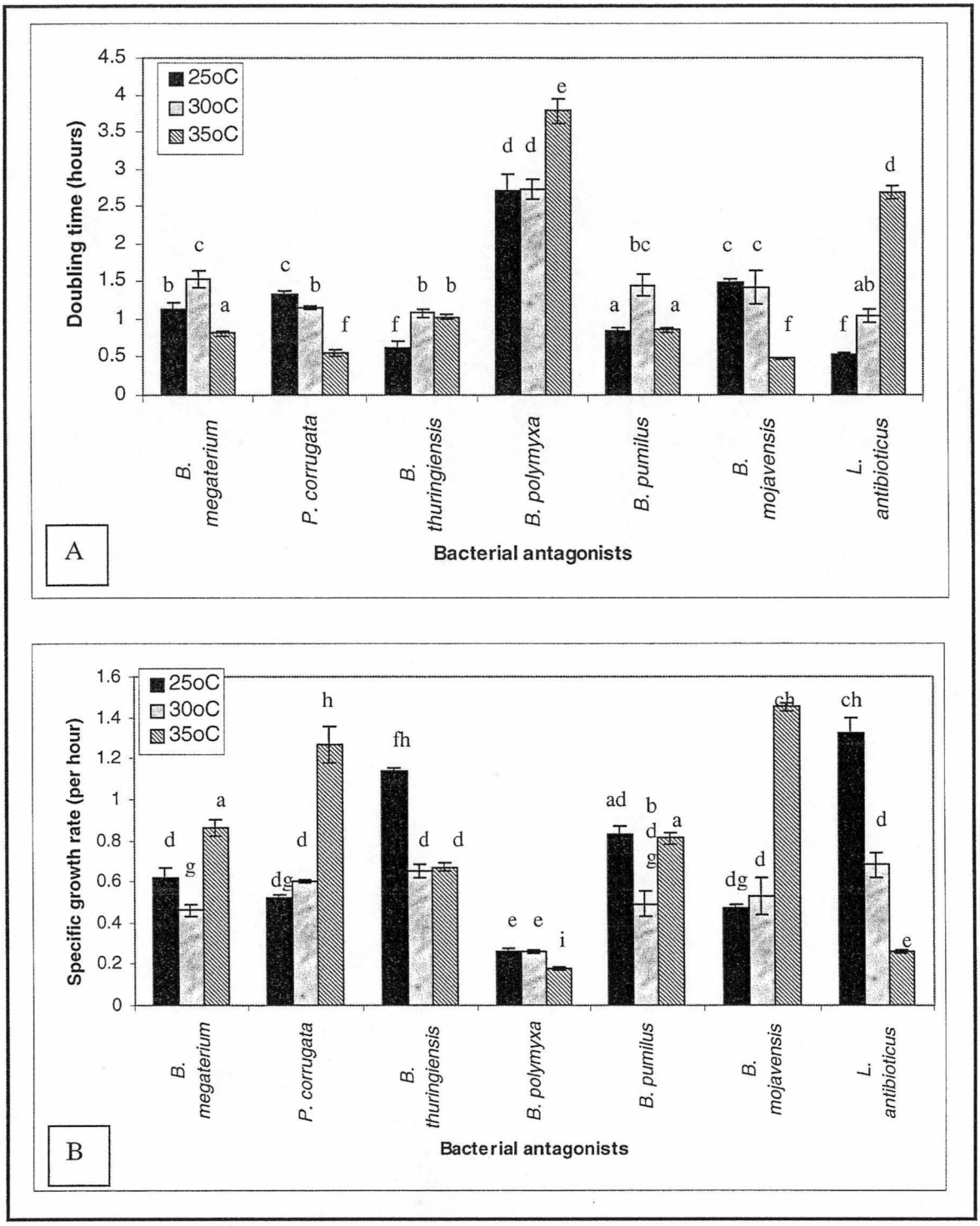


Figure 4-5: The relative doubling time (A) and the specific growth rate (B) of the bacterial antagonists grown at three different temperatures and an initial pH of 7.4. Each bar with standard error is an average of four replicate determinations. Bars with the same letter(s) are not significant at $p<0.05$ according to lsd test, following ANOVA.

L. antibioticus, B thuringiensis and *B. polymyxa* were found to have preferred growth temperatures of 25°C relative to 30°C or higher. In contrast *B. megaterium, B. mojavensis* and *P. corrugata* showed a preference of 35° over 30°C or lower. *B. pumilus* gave questionable results in this study (Figure 4-5). *L. antibioticus* and *B. polymyxa* were also found to have relatively long lag phases (of 16 hours) at 35°C. The pH of broth cultures increased over the 30 hours incubation period for all isolates except *B. polymyxa* in which case it decreased by about 0.7 pH units, indicating that with one exception, proteolytic activity (with release of ammonia) dominated fermentative metabolism over this time period. In view of these results the bacteria were again tested at 25°C and 30°C at pH 7.0, and the result are presented in Table 4-1.

Table 4-1: The relative doubling time and specific growth rates of bacterial isolates in half-strength nutrient broth at two temperatures, pH 7.0.

Bacteria	Doubling time (h)[#]		Specific growth rate (h^{-1})[#]	
	25°C	30°C	25°C	30°C
B.megaterium	1.31±0.05 cf (7.76±0.02)	1.58±0.16 cf (7.89±0.02)	0.53±0.02 f	0.45±0.05 f
P. corrugata	1.29±0.06 cf (7.7±0.02)	1.08±0.11 f (7.83±0.02)	0.54±0.03 f	0.66±0.07 a
B. thuringiensis	0.72±0.04 b (6.78±0.02)	1.01±0.06 af (7.07±0.06)	0.98±0.06 c	0.70±0.05 ab
B. polymyxa	ND	4.25±0.06 e (6.55±0.03)	ND	0.17±0.01 e
B. pumilus	0.89±0.04 a (6.79±0.03)	0.81±0.04 abf (6.93±0.01)	0.78±0.03 ab	0.86±0.04 bc
B. mojavensis	ND	1.57±0.04 c (7.2±0.03)	ND	0.44±0.01e
L. antibioticus	0.98±0.06 a (7.37±0.02)	0.96±0.05 a (7.22±0.02)	0.72±0.04 ab	0.73±0.04 ab

ND= not determined

[#]Values (± standard errors) are an average of four replicates. Values in brackets indicate the pH of the broth after 30 hours incubation. Values in the column of doubling time or specific growth rate followed by the same letter(s) are not significant at $p<0.05$ according to lsd test, following ANOVA

In this case *B. pumilus* is seen to grow preferably at 30°C relative to 25°C and *B. megaterium* grew preferably at 25°C relative to 30°C. Other results where they are evident are in line with results obtained previously, or in the case of *L. antibioticus* are indeterminate.

4.4.3 Ability of Td_{22} to utilize CMC

The ability of Td_{22} to degrade CMC in a citric phosphate buffer at various pH values is presented in Figure 4-6. Degradation of CMC was indicated by loss of viscosity as a function of time.

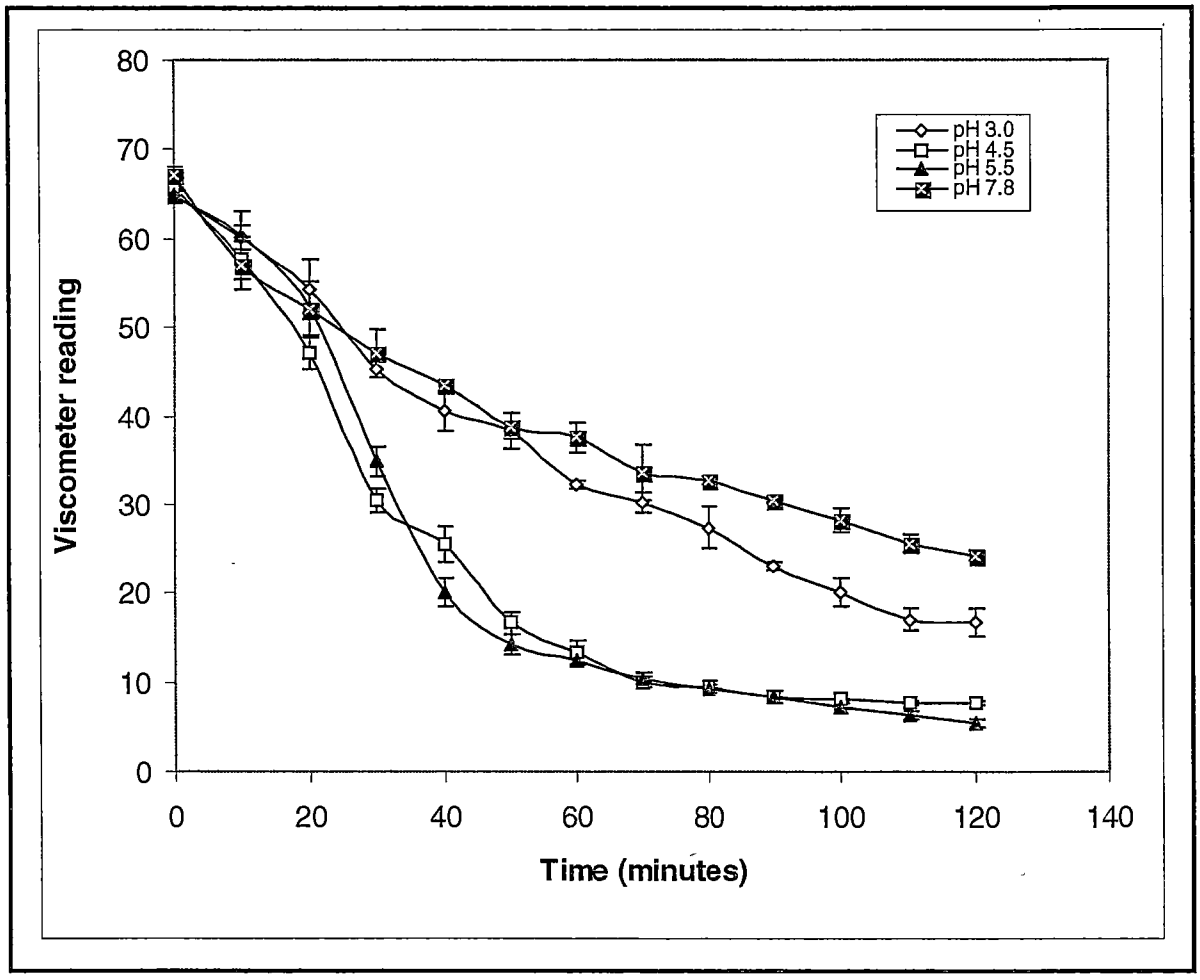


Figure 4-6: Degradation of CMC by Td_{22} in a citric phosphate buffer solution at various pH values. Each value (± standard error) is an average of four replicate assays.

The rate of CMC degradation by Td_{22} was significantly greater at pH 4.5 and 5.5 than that at pH 3.0 or 7.8 (Figure 4-6), indicating an optimal pH for cellulolysis of between pH 4.5 and 5.5, but not necessarily the optimal pH for growth of the fungus.

4.4.4 Antibiotic resistance of Td_{22}

The result of this assay is presented in Figure 4-7.

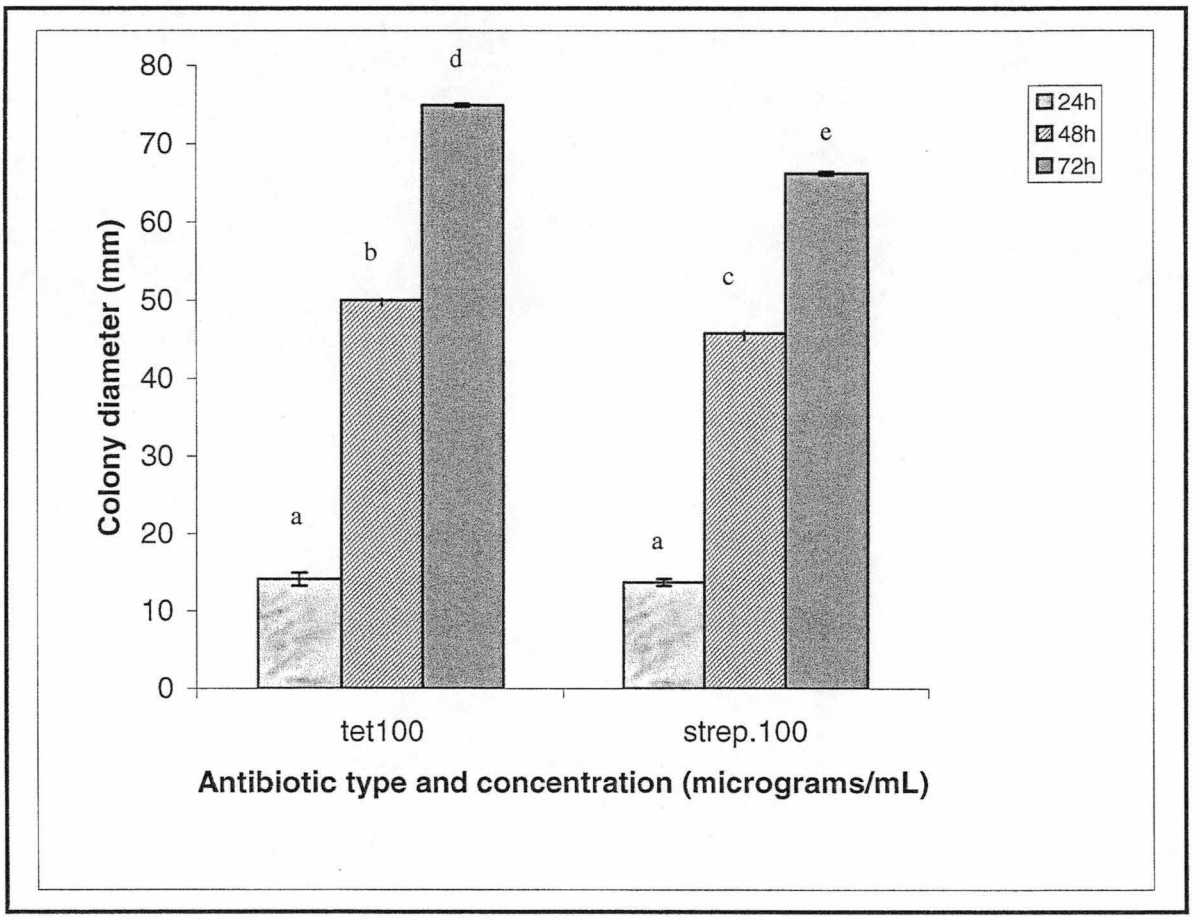


Figure 4-7: The relative radial growth of Td_{22} in pectin agar medium amended with streptomycin (strep.) or tetracycline (tet.) at concentrations of 100 µg/mL medium. Each bar (± standard error) is an average of five replicate plates with three measurements from different sides. Bars with the same letter are not significant statistically at $p<0.05$.

Td_{22} was found to grow well in the medium amended with both antibiotics (streptomycin or tetracycline) at concentrations of 100 µg/mL, indicating they could be

used in a formulation of semi selective media to suppress bacterial contaminants in the re-isolation of this fungus from non-sterile samples.

4.5 Discussion

The effect of pH of the media and incubation temperatures on the growth of fungal and bacterial antagonists was investigated with a view to optimising these factors in the utilization of low-cost substrates for their cultivation. The fungus Td_{22} grew well in the range of pH 4.0 to 7.0 with an optimum growth rate between pH 5.0 and 6.0, while the bacteria grew optimally between pH 7.0 and 8.0 (Figure 4-1, Figure 4-2, and Figure 4-4). A growth temperature of 25°C was near optimal for most isolates (Figure 4-3, Figure 4-4, Figure 4-5, and Table 4-1). Danielson and Davey (1973) reported an optimal range of temperatures of *Trichoderma* spp. of between 22°C and 34°C; incubation temperatures of bacteria isolated from soil habitats of 25°C to 30°C are well established.

A combination of 25°C and pH 5.0 and 6.0 appeared to be most suitable for the growth of Td_{22} (Figure 4-4), while pH levels around neutral combined with incubation temperatures of 25°C to 30°C appeared to be optimum for most of the tested bacterial antagonists (Table 4-1). The results obtained in Figures 4-2/4-5 and Table 4-1 were generally not statistically significant at $p<0.05$.

Trichoderma spp. have been reported to produce a range of enzymes, such as cellulases, pectinases, and chitinases (Metcalf, 1997), with the ability of the isolate Td_{22} to utilize a cellulose-substitute (CMC) being confirmed in the present study. These attributes auger well for the possible cultivation of Td_{22} in low-cost cellulosic materials such as WFW, as reported in Chapter 5.

The rate of CMC degradation appeared to be affected by the initial pH of the solution (Figure 4-6), as reported for fungi generally by e.g. Jay (1996). Optimal cellulase activity by Td_{22} of between 4.5 and 5.5 was consistent with its optimal growth rate in full-nutrient medium at pH values between 5.0 and 6.0. It was found that Td_{22} was resistant to tetracycline and streptomycin at 0.1 mg/mL, although its growth rate was slightly inhibited when compared to that grown on pectin agar medium minus antibiotics. As a result of this finding, streptomycin or tetracycline as selective agents were used in media at concentrations of between 0.06 mg/mL and 0.1 mg/mL. Metcalf (1997) has also used streptomycin at the rate of 0.05 mg/mL in selective media for *T. koningii*. The use of various antibiotics, such as Nikkomycin Z in the formulation of selective media with a

view to suppress the growth of contaminants, has been reviewed by Tariq and Devlin (1996).

4.6 Conclusions

The effect of initial pH of the medium and incubation temperature on the growth of a fungal antagonist (Td_{22}) and of several potential bacterial antagonists was assessed *in vitro*. The fungus performed best at an initial pH of the medium of between 5.0 and 6.0 and an incubation temperature of 25°C. Its ability to utilize CMC, and therefore probably cellulose, as sole C source raises the possibility of its cultivation on low-cost materials such as WFW, which is abundantly available in Tasmania and elsewhere. The bacterial antagonists grew optimally at initial pH values around 7 and between 25°C and 30°C.

Chapter 5

Growth and survival of a *Trichoderma* sp. isolate (Td_{22}) in composted and non-composted wood fibre waste of paper mill origin under sterile and non-sterile conditions

5.1 Abstract

The growth and survival of *Trichoderma* sp. (isolate Td_{22}) antagonistic to *Sclerotinia minor* and *Sclerotium cepivorum* was studied in raw wood fibre waste (WFW) of paper mill origin and in mature compost of this material, both under sterile and non-sterile conditions. In sterilized, nutrient-amended raw and composted WFW (both supplemented with 20% w/w millet seed), Td_{22} was found to grow well and reached densities in the order of ~10^{10} colony forming units (cfu)/g after 14 days incubation. In the former of these mixes, the bulk of the cfu density was in the form of spores. Lower population densities were achieved under non-sterile conditions in the compost:millet mix of between ~10^7 and ~10^9 cfu/g after the same period of incubation, depending on pre-treatment. These results indicate that WFW could provide an abundant low-cost growth medium for the large-scale cultivation of this or other biological control fungi.

5.2 Introduction

Problems related to the large-scale production of biological control agents in low cost materials including difficulties in handling, transport and storage, have been reviewed previously (Chapter 2). These problems have been largely overcome by maintenance of bacterial antagonists in carriers such as peat or vermiculite for five months or more (e.g. Vidhyasekaran *et al.* 1997[a,b]; Gazoni *et al.*, 1998). Cost of the cultivation medium, its transport and field dispersal is a critical factor in any assessment of biological control relative to chemical treatments, a problem sometimes exacerbated by the perceived need for proprietary media formulations.

The use of suppressive compost to control plant diseases has been extensively examined, with recent reviews or reports of the topic by e.g. Craft and Nelson (1996), Hointink, *et al.* (1997), Nakasaki *et al.* (1998), Hoitink and Gardener (2003), and McKellar and Nelson (2003). However, the use of compost as a growth-medium for specific microbial antagonists rather than a storage material or an agent of non-specific inhibition is recent. An indication of the potential for manipulating the microbiota of compost to advantage was provided by Ramamurthy *et al.*, (1996), who demonstrated that composting of eucalypt sawdust inoculated with the mushroom fungus *Volvariella* resulted in a product which enhanced the growth of wheat seedlings.

Norske-Skog Paper Mill Limited produces approximately 33,000 tonnes of WFW per annum, to be dumped as landfill (Ramona and Line, 2002). The potential for recycling the material by composting was demonstrated by Jackson and Line (1997), but this option has not been utilized by the company. This cellulosic waste was considered worthy of examination in view of its attributes of excellent water-holding and/or aeration capacity, its freedom from toxic elements, its free availability and its potential utilization as a source of energy and carbon by cellulose-utilizing fungi, such as the *Trichoderma* isolate Td_{22}.

The objective of the present study was to investigate the growth and survival of Td_{22} in raw WFW of paper mill origin (a product of the Norske-Skog Paper mill Company) and in a mature compost of this material, with a view to the possible low-cost, large-scale cultivation of this or other fungal antagonists.

5.3 Materials and Methods

5.3.1 The fungal antagonist *Trichoderma* sp. (Td_{22})

The origin and attributes of the fungal isolate Td_{22} has previously been described (Chapter 4). The fungus was normally maintained on moist millet seed and on citrus pectin agar at 4°C.

5.3.2 Composting of WFW

The WFW is a mix of eucalypt and *Pinus radiata*, comprising holocellulose as its primary constituent, with very low levels of metal contaminants and being deficient (from a recycling perspective) in N and P. In concentrated form it is phytotoxic to seeds, although the potential for its recycling to the field following composting was

demonstrated by Jackson and Line (1997) and Jackson (1998). Composting followed amendment with superphosphate, urea and potassium nitrate to give a C:N:P:K ratio of 35:1:0.6:0.1. Composting was for three months with monthly sample collection and assay for toxicity to radish seeds, before use of the product as a cultivation medium for Td_{22}.

5.3.3 Inoculum preparation

Td_{22} inoculum was grown in sterile citrus pectin broth medium (pH 4.5) as described in Appendix 1D minus agar, following inoculation with plugs of the fungus grown on citrus pectin agar (Appendix 1D). Flasks (500 mL) of inoculated medium (100 mL) were shaken for 7 days in a water bath at 25°C. Before use, the potency of this inoculum, measured in cfu on pectin agar, was determined by serial dilution plating (in triplicate).

5.3.4 WFW/compost preparation and inoculation (sterile conditions)

The raw WFW and the three-month old WFW compost were air-dried in a glasshouse (daytime temperatures of 25-35°C) for three weeks to minimize the indigenous microbiota. Before use as a cultivation medium for Td_{22}, seven variants of mixes (on a dry weight basis; 100°C to constant weight) were prepared as given below (Figure 5-1). Unless otherwise stated these mixes were brought to approximate field capacity moisture (amounting to ~ 1.5L/kg for the 80:20 WFW/millet seed) with a solution containing (g/L distilled water): NH_4NO_3, 5.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; $FeCl_3$, 0.01. The mixes were then dispensed into 750mL flasks (100g each) and autoclaved at 121°C for 30 minutes on each of two consecutive days. Initial pH values after autoclaving are given in Fig. 5-1. All flasks were aseptically inoculated with 10mL of Td_{22} suspension described in section 5.3.3 to give an initial density of between $3.7\log_{10}$ cfu/g and $5.5\log_{10}$ cfu/g dry mix, and incubated at 25°C for four weeks with periodic assay of growth of the fungus following dilution plating onto Oxoid potato dextrose agar (PDA) (Appendix 1C).

The relative biomass of Td_{22} under sterile conditions was assessed using the assay for chitin described by Chen and Johnson (1983). Microscopic estimation of spore numbers in the various mixes was made using a haemocytometer. All estimates of cfu/g, spores/g or chitin biomass/g are given on a dry weight basis (100°C to constant weight).

5.3.5 WFW compost preparation and inoculation (non-sterile conditions)

Air-dried WFW compost (dried to ambient moisture content under glasshouse conditions) was brought to field capacity with 2% (w/v) Phostrogen® solution (approximately 350 mL/litre of compost) and 100g quantities placed into 750mL flasks. The compost was then either briefly autoclaved at 121°C (5 minutes) prior to inoculation (compost A) or directly inoculated with Td_{22} suspension described in Section 5.3.3 (compost B) to give 5.4 $\log_{10}$ cfu/g compost. Flasks were incubated at 25°C for four weeks with periodic assay of cfu on PDA (Appendix 1C) for Td_{22} and on TSA (Appendix 1B) for bacterial microbiota. The identity of representative Td_{22} isolates was confirmed by both morphological characteristics and isoenzyme analysis of pectic enzymes as described by Cruickshank and Pitt (1987) (Appendix 3).

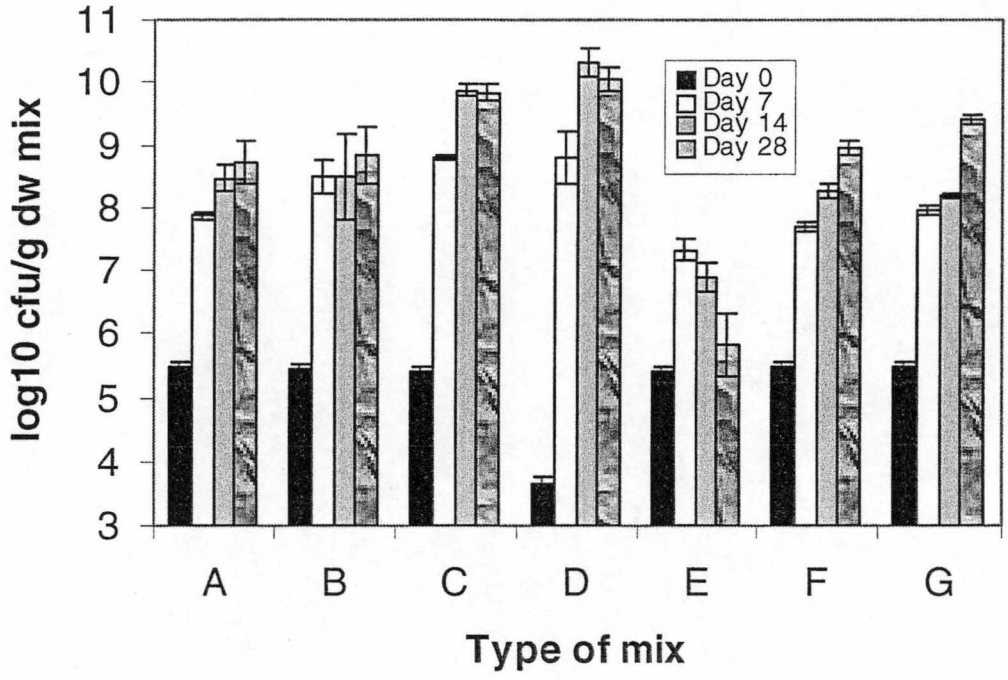
5.3.6 Statistical analysis

Analysis of variance (ANOVA) of data obtained from this study was carried out using Minitab software for Windows as described in Chapter 4.

5.4 Results

5.4.1 Growth of Td_{22} in various mixes (sterile condition)

The *Trichoderma* sp. (Td_{22}) grew well in all mixes except in 100% millet seeds (Mix E), in which cfu fell after 14 days of incubation (Figure 5-1). Significant increases in cfu generally occurred after seven days incubation, followed by moderate increases up to 14 (mixes C and D) or 28 (mixes A, B, F and G) days. Estimated cfu of Td_{22} in all mixes except for E were between 8.7 and 9.8 $\log_{10}$/g at week four. The best growth response was observed in either raw (mix D) or composted WFW (mix C) supplemented with nutrients and 20% millet seed, plateauing at 14 days incubation (at 10.3 and 9.9 $\log_{10}$ cfu/g, respectively). Both results were significantly higher than those for a nutrient supplemented compost control after 14 or 28 days. Likewise, nutrient-supplemented WFW compost containing 50% w/w millet seed or 1% w/w starch did not improve the growth (cfu) of Td_{22} over the unamended control. Replacing the mineral nutrient supplements to WFW compost with 2% w/w Phostrogen® resulted in a similar growth response (cfu) by Td_{22} (Mix A cf. Mix F). This response was independent of the initial inoculation density of Td_{22} in the mix C, with a similar response resulting from an inoculum of 3.5 $\log_{10}$ or 5.5 $\log_{10}$ cfu/g (data not shown).



Composition of the mixes:

Mix A: 100% WFW compost

Mix B: 50% WFW compost + 50% millet seed (w/w)

Mix C: 80% WFW compost + 20% millet seed (w/w)

Mix D: 80% raw WFW + 20% millet seed (w/w)

Mix E: 100% millet seeds

Mix F: 100% compost re-wetted with 2% w/v Phostrogen® solution

Mix G: 100% compost amended with 1% w/w potato starch

Figure 5-1: Growth of Td_{22} in various mixes under sterile conditions. Values (with standard errors shown) are means of three replicate determinations. The initial and final pH levels of the mixes were: A = 4.6-5.4, B = 4.8-7.5, C = 5.3-5.5, D = 5.0-5.5, E = 5.6-7.8, F = 4.9-5.6, G = 4.7-5.4.

Spores of Td_{22} with various densities were found in all mixes, except in mix E, where only hyphal fragments and lysed spores were observed. The spore density of Td_{22} in the mixes after 10 weeks incubation is presented in Table 5-1.

Table 5-1: Spore densities of Td_{22} in compost/WFW mixes after 10 weeks of incubation at 25°C.

Type of Mix*	Log_{10} total spore/g dw mix **
A	8.70 ± 0.02 a
B	8.68 ± 0.05 a
C	9.56± 0.02 b
D	9.51 ± 0.02 b
E	0.00 ± 0.00 c
F	8.42 ± 0.01 d
G	8.67 ± 0.02 a

* The constituents of the mixes are as given in Figure 5-1.

**Values followed by the same letter are not significantly different (at $p<0.05$) according to lsd test following the analysis of variance (ANOVA). Each value is an average of triplicate assays ± standard error.

As was seen in the cfu assay, mixes C and D consistently produced the highest spore densities of 9.56 ± 0.02 and 9.51 ± 0.02 log_{10} spores/g, respectively. Again starch amendment (mix G) or replacement of mineral nutrients with Phostrogen® (Mix F) did not increase the number of spores in the mix. In contrast, millet seed amendment (mixes C and D at 20% millet) resulted in spore densities almost ten-fold higher than that in the non-millet seed compost (mix A) (Table 5-1), although 50% w/w amendment of millet seed (mix B) resulted in a significantly lower spore counts than their 20% counterparts. Use of 100% millet seed (mix E) was apparently inhibitory to spore formation.

The growth of Td_{22} in mix D (80% raw WFW and 20% millet seed, showing the highest levels of cfu) was found to be consistent in a repeated experiment (Figure 5-2). Haemocytometer assay of spore numbers in this mix indicated these to be of the same order of magnitude as the cfu determined from dilution plate counts (Figure 5-2).

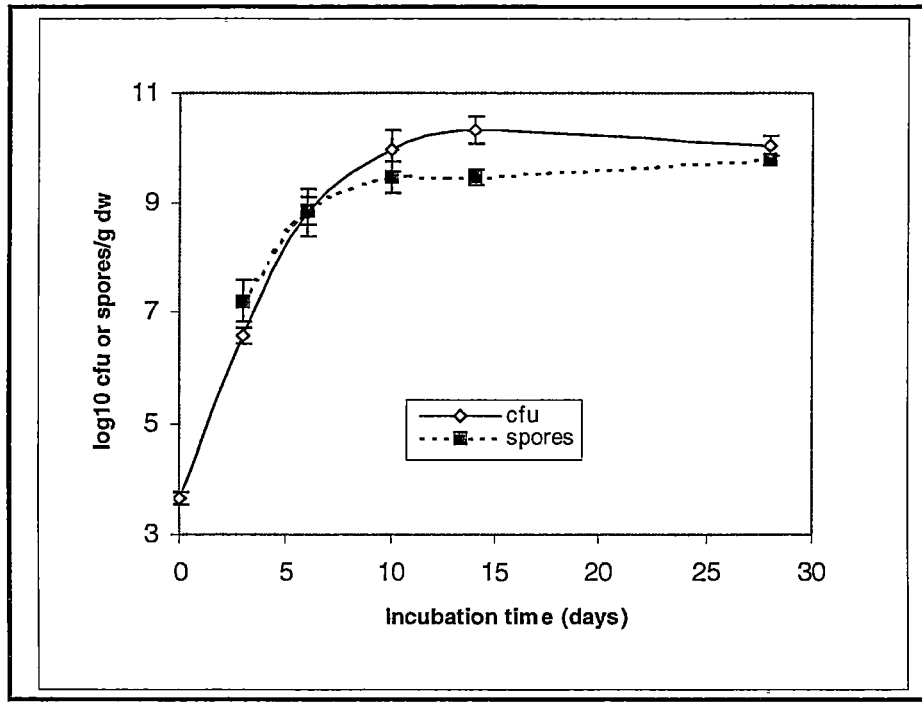


Figure 5-2: Growth of Td_{22} in raw WFW:millet seed (80:20 w/w) measured as cfu/g or spore density/g. Each value in the graph is an average of four replicate determinations ± standard error.

The nett biomass of Td_{22} in the various mixes after prolonged incubation (10 weeks), as determined from chitin contents (after deducting chitin content in controls lacking Td_{22}, this amounting to ~33.2 mg/g for the millet seed control or ~57.8 mg/g for compost control), indicated levels of approximately 50-60 mg/g mix except for mixes F and G, which contained about 35 mg/g mix. As might be expected, the fungal biomass (as determined by chitin content) was not positively correlated with spore density; mix E for example containing no spores still contained a significant amount of chitin (53.45 mg/g), attributable to hyphal content.

5.4.2 Growth of Td_{22} under non-sterile conditions

The growth and survival of Td_{22} in compost following different pre-treatments was followed for 28 days. The results are presented in Figure 5-3.

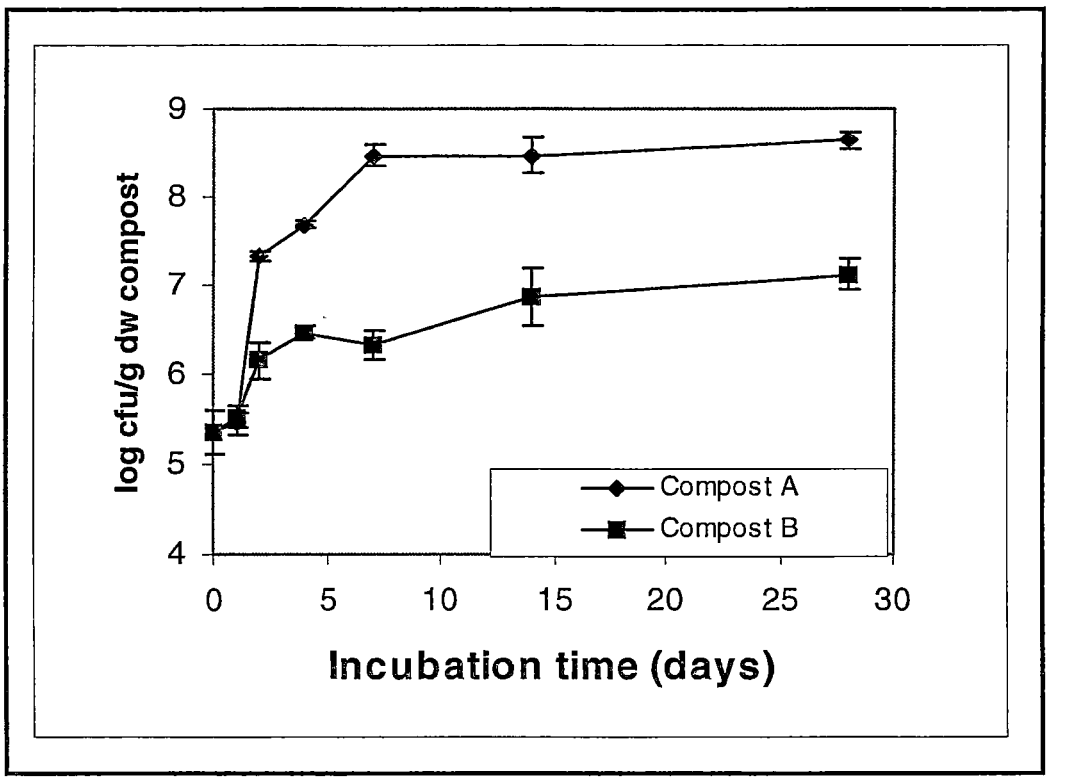


Figure 5-3: Population density of *Trichoderma* spp. (isolate Td_{22}) in three months old WFW compost, either briefly treated for 5 minutes at 121°C prior to inoculation (Compost A), or directly inoculated following re-wetting (Compost B). Both compost types were brought to approximately field capacity with 2% (w/v) Phostrogen® solution (~350 mL/L). Each value in the graph is an average of three replicate determinations ± standard error.

Significant increases in cfu of Td_{22} in steam-treated or untreated composts were observed in the first seven days, after which time the populations remained relatively constant at about 8.5 $\log_{10}$ cfu/g dw in compost A or about 7.0 $\log_{10}$ cfu/g dw in compost B. The growth in non-sterile, steam-treated or air-dried compost was equivalent in other respects to mix F (Figure 5-1). The identity of Td_{22} in these non-sterile conditions was confirmed by both morphological characteristics and isozyme profile. The pectolytic enzyme profiles produced by the fungal reference (Td_{22}) and by those fungi re-isolated from non-sterile compost are shown in Plate 5-1

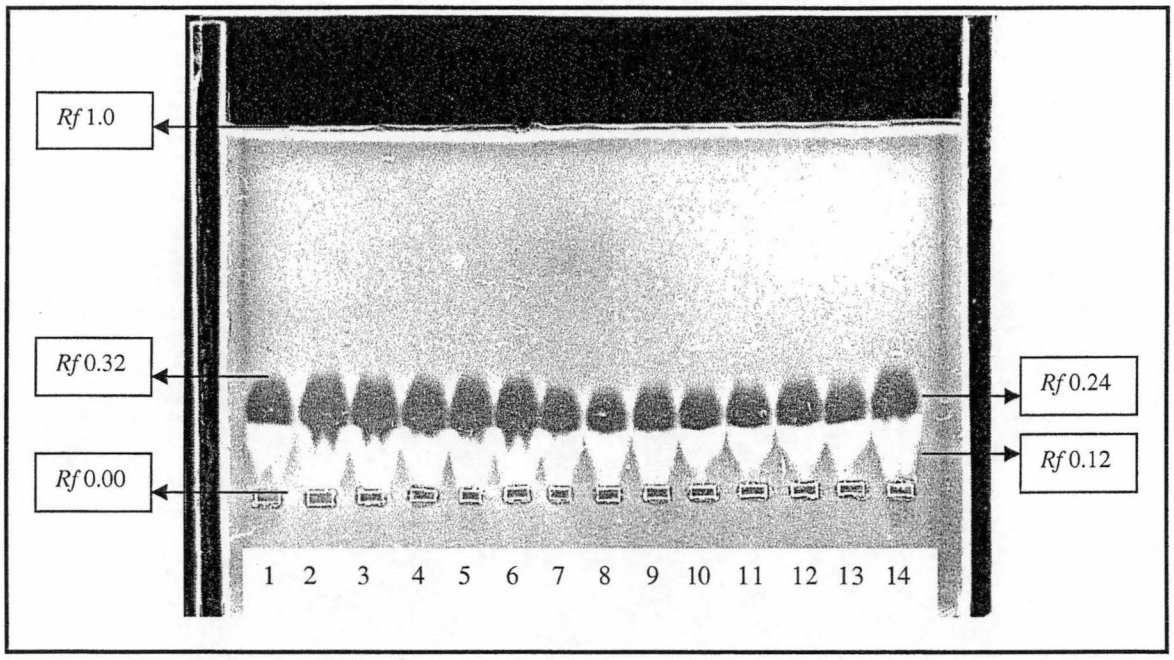


Plate 5-1: Contact print following polyacrylamide-gel electrophoresis to confirm survival of Td_{22} in three-month old WFW compost. Dark bands indicate polygalacturonase and light bands indicate pectinesterase. Wells 1, and 9 – 14 contained the control reference, Td_{22}, while wells 2 – 8 contained re-isolated fungi from compost B on day 7 of sample collection.

The populations of mesophilic bacteria in composts A (treated for five minutes at 121°C) and B (dried, untreated)(Figure 5-3) over 28 days are presented in Figure 5-4. The initial bacterial populations in the mixes depended on the method of preparation. The population of the mesophilic bacteria in compost A was approximately one order of magnitude lower than that in compost B after two weeks incubation. However after two weeks the growth rate in compost A exceeded that of compost B, to result in a population of 9.3 $\log_{10}$ cfu/g indigenous mesophilic bacteria after 28 days, in comparison with 8.5 $\log_{10}$ cfu/g in compost B (Figure 5-4).

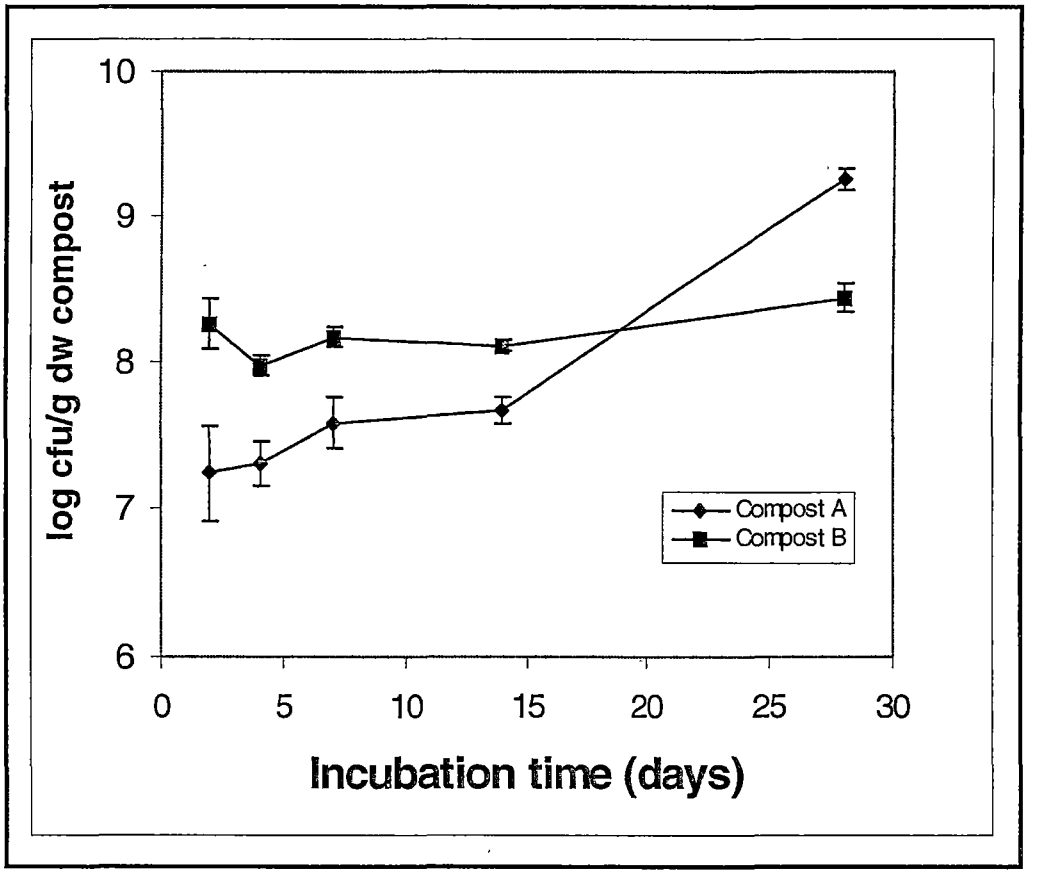


Figure 5-4: Population density of mesophilic bacteria in the compost after briefly steaming for 5 minutes at 121°C prior to inoculation (compost A), or directly inoculated after re-wetting (compost B). Each value in the graph is an average of triplicate determinations ± standard error.

5.5 Discussion

This investigation has demonstrated the potential use of WFW as a growth medium for the large-scale cultivation of a biological control fungus, *Trichoderma* sp. Td_{22}, which is known to be particularly effective against sclerotia-forming fungi, such as *Sclerotinia minor* and *Sclerotium cepivorum*. Td_{22} was found to grow well in nutrient-supplemented three-month old compost of WFW to reach cell densities of approximately 10^{10} cfu/g in 14 days. Three months of composting was used because in a preliminary study this time was required to eliminate toxicity for radish seedling germination and growth using a standard potting mixture as control (data not shown). Raw WFW and WFW-composts up to two months old were in contrast shown to be toxic in this regard. Likewise Jackson

(1998) reported that at least three months composting was required to eliminate most toxic compounds present in the raw eucalypt WFW to acceptable levels for potting media. According to Hoitink *et al.* (1996) decomposition of woody materials including toxic compounds by microorganisms occurs in all stages of composting, although the highest rate of degradation normally occurs in the second stage of the thermal composting where the temperature may reach 65°C or more. Work relevant to the degradation of toxic compounds in composts has been reviewed by Diaz *et al.* (1993) and Grebus *et al.* (1994).

Analysis of the three-month old WFW compost before nutrient amendment indicated a C:N ratio of ~40. Amendment of nutrient solution (NH_4NO_3-salts) into this compost, plus millet seed supplement was aimed at decreasing this ratio to ~30. Such amendment was seen to be suitable for the growth of Td_{22} as indicated in Figures 5-1 and 5-3. Barkdoll *et al.* (1991) has stated that an initial C:N ratio of ~50 or less in a compost matrix is ideal, although generally a C:N ratio of between 25-30 gives a faster rate of cellulosic decay (Diaz *et al.*, 1993). The achievement of very high cfu levels after 2-4 weeks (Figure 5-1) indicated that nutrient supplementation was sufficient to meet the Td_{22}-fungal requirements.

For the purpose of Td_{22} cultivation, composting the WFW was initially thought to be advantageous by removing toxic components. However it appears to be unnecessary as nutrient-amended, raw WFW-Td_{22} culture after 14 days gave comparable fungal cfu to that of the composted equivalent and subsequent assessment of this culture after 14 days incubation at the rate of 20% v/v amendment of potting mix showed it to have no residual toxicity to radish seed. This suggests that plant-toxic compounds of the WFW were degraded by Td_{22} during incubation.

That Td_{22} in the raw WFW medium appeared to be largely in the form of spores following 14-28 days incubation auguring well for its long-term survival (e.g. in commercial preparations) prior to application.

Assay of chitin content of growth medium provides a useful indicator of fungal biomass, because chitin is a specific component of fungal cell walls (Cousin, 1994 and Atlas, 1995). That the chitin content of the mix was not related to the number of spores (or cfu) after 10 weeks is curious, indicating either that the chitin content of spores is low, or more probably, that the cfu/biomass ratio of spores is much higher than that of fungal hyphae. Mix E for example, was not observed to contain spores, yet contained a significant amount of chitin (fungal hyphal biomass). From a production viewpoint, the

cfu produced in different mixes after 14 to 28 days is of more interest than fungal biomass (chitin content).

Air drying compost in the glasshouse for three weeks or brief steam-treatment (rather than sterilizing) (Nakasaki *et al.*, 1998) with a view to minimizing the indigenous microbiota prior to inoculation with Td_{22}, gave encouraging results. The cfu of this fungus cultured under non-sterile conditions were found to increase by two to three orders of magnitude, although they were always lower than those obtained under sterile conditions (Figure 5-3). The cfu were found to be higher in the briefly autoclaved compost (compost A) than those in the directly inoculated compost (compost B) following re-wetting. Application of heat treatment to the WFW compost (by briefly autoclaving at 121°C) may have changed its composition, but the most probable reason for the better growth of Td_{22} in this medium was the near-elimination of competition by mesophilic bacterial survivors in compost A relative to compost B. Similar results were also demonstrated by Nakasaki *et al.* (1998) who found a reduced growth rate of a strain of *Bacillus subtilis* following inoculation of this antagonist into non-sterile (as compared with sterile), but freshly-cut grass clipping compost. This reinforces the notion that competition between the indigenous microbiota and the inoculated antagonists is important in the directed establishment of such antagonists in a compost matrix.

The ability of Trichoderma spp. to produce cellulase and pectolytic enzymes as demonstrated in Figure 4-4 and Plate 5-1 has been reported previously by Metcalf (1997), Oksanen *et al.* (2000), Domingues *et al.* (2000), and Lee *et al.* (2000). These and other enzymes are important in the degradation of complex carbon sources contained in WFW and the ability to produce them is advantageous when in competition for available major energy sources.

Assay of isoenzyme profiles (e.g. pectolytic enzyme profiles as undertaken in the present study) of fungal isolates provided a very convenient tool to differentiate Td_{22} from other isolates of *Trichoderma* spp following re-isolation from non-sterile samples. This assay has been applied by many workers (e.g. Neate *et al.*, 1988; Leone, 1990; Metcalf and Wilson, 1999) to distinguish their fungal isolates from other related fungi based on isozyme *Rf*.

Millet seed amendment (at the rate of 20% w/w as applied in the present study) appeared to be important to induce the growth of Td_{22} in the raw or composted WFW (Figures 5-1 and 5-2) as it provided the fungus with relatively simple compounds such as starch and pectin. However, it was noticed that excessive amendment of this seed into the

WFW compost (mix B where 50% w/w millet seed was added and mix E where 100% millet seed was used) (Figure 5-1) tended to result in the late sporulation of this fungus coupled with lower cfu or chitin-biomass assays. Fungal sporulation normally occurs as a response to the environmental stress, such as nutrient depletion, which induce their 'intrinsic signals' to complete life cycle (Adams *et al.*, 1998). Perhaps the relatively low cfu assays observed in mix E (100% millet seed) after 28 days relative to say mix D with 20% millet (Table 5-1) simply reflects the different levels of sporulation of the two cultures at this time; the cfu being dominated by fungal hyphae in mix E and spores in mix D.

Metcalf (1997) reported the growth and maintenance of Td_{22} in 100% millet, however this study showed that the fungus could be grown to comparable or better cfu and spore numbers in compositions of e.g. 80% WFW and 20% millet seed. This provides considerable potential for the low-cost and large-scale production of this and possibly other fungal antagonists. Since this work was completed, the commercial potential using compost material has been realized by the provider of the Td_{22} culture.

5.6 Conclusions

Raw and three-month old compost of WFW were found to provide excellent substrates for the growth of Td_{22} to largely replace the relatively expensive millet seed used previously. The utility of WFW for this purpose is due in large part to the ability of Td_{22} to degrade complex polysaccharides, the millet then providing the fungus with a supplement of relatively simple compounds dominated by starch and pectin. The presence of indigenous microbiota in WFW compost was shown to be detrimental to the growth of Td_{22}, most probably due to their competition for nutrients. To achieve good growth rate and high cfu/g of Td_{22}, minimization of such indigenous microbiota by steam-treatment or air-drying was advantageous. Other methods aimed at the low-cost minimisation of the indigenous microbiota are addressed in Chapter 7.

Chapter 5A

A study on the growth response of bacterial antagonists in a mix of composted wood fibre waste and millet seed under sterile and non-sterile conditions

5A.1 Abstract

The potential use of composted WFW for the cultivation of bacterial antagonists of *S. minor* was examined with the result that a mix of millet seed (20% w/w) and WFW, suitably amended with nutrients, proved to be an ideal matrix for the growth of some of these bacteria. Densities in terms of cfu's ranged from 8.5 $\log_{10}$ cfu/g dw to 10.5 $\log_{10}$ cfu/g dw) under sterile conditions after 14 days incubation. Lower population densities of the antagonists were achieved under non-sterile conditions in the compost:millet mix of between 7.9-9.3 $\log_{10}$ cfu/g dry weight over the same period of time. However, when applied in a pot (glasshouse) trial to protect against *S. minor*, the millet seed appeared to stimulate the growth of this pathogen resulting in a high incidence of attack of lettuce plants after 2-3 weeks. Although the percentage of healthy seedlings increased following application of compost mix grown antagonists (at a rate of 5% v/v) when compared to the control treatment, these values were not statistically significant ($p>0.05$) in most cases.

5A.2 Introduction

Attempted manipulation of composting conditions or of mature compost for the cultivation of desired microorganisms has been slow to develop; particularly difficult is manipulation of non-sterile compost due to the high biological buffering provided by a diverse microbiota, combined with the problems posed by a microbial succession as temperatures change from mesophilic to thermophilic and back again, as occurs in the compost habitat. As described in chapter 5, one of the first successful demonstrations of the directed cultivation of bacteria in compost followed the inoculation of pasteurized grass clippings with a *Bacillus subtilis* strain known to be an agent of biological control

(Nakasaki *et al.*, 1998). The bacterium grew to concentrations of 10^8 cfu/g dw before sporulating, the spores surviving subsequent high temperature composting to inhibit *Rhizoctonia* large patch disease of grass. Compost that was not inoculated showed no disease suppressive effects. Other less successful reports of bacterial inoculation of mature composts, particularly for the suppression of *R. solani*, have been made by Phae *et al.*, (1990), Kok *et al.*, (1996), Hoitink *et al.*, (1997) and Rÿckeboer *et al.*, (1998).

A mix of composted or raw material of WFW and millet seed in the ratio of 80:20 (w/w) was previously reported to be most favorable for growth of *Trichoderma* sp. (Td_{22}) (Chapter 5), with peak cfu and biomass (chitin-content) being reached after 14 days incubation (Ramona and Line, 2002). The efficacy of the Td_{22} grown in this mix in protecting lettuces against *S. minor* attack in a series of glasshouse trials is described in Chapter 6.

The present study investigated the use of composted WFW and millet seed as a possible carrier or substrate for the cultivation of selected bacterial antagonists previously isolated from the rhizosphere of crop plants (Chapter 3).

5A.3 Materials and methods

5A.3.1 Bacterial antagonists

Several bacterial antagonists, such as *Pseudomonas corrugata*, *Bacillus polymyxa*, *B. megaterium*, *B. thuringiensis*, *B. mojavensis*, and *Lysobacter antibioticus*, isolated from various sources as described in Chapter 3, were investigated for growth response in the mix of composted WFW and millet seed (80:20 w/w). For long-term storage, these bacterial antagonists were cryo-preserved at -70°C in TSB medium supplemented with 30% glycerol as described in Section 3.3.1 (Chapter 3).

5A.3.2 Fungal antagonist (*S. minor*)

The origin, method of cultivation, and storage of *S. minor* has been previously described in Chapter 3.

5A.3.3 WFW compost production

The origin and the method of composting of raw WFW have been described in Chapter 5.

5A.3.4 Inoculum preparation

Selected bacterial antagonists were grown in a sterile trypticase soya broth (TSB) medium (Appendix 1B minus agar) following inoculation with loopfuls of 24-48 hour old TSA cultures. Flasks (500mL capacity) of inoculated medium (100mL) were incubated at 25°C under static conditions for 24-48 hours, until turbidity was achieved. Before use, the bacterial numbers were estimated by serial dilution plating (in triplicate) on TSA (Appendix 1B).

5A.3.5 Carrier preparation and inoculation under sterile conditions

Preparation of the composted WFW and millet seed (80:20 w/w) mix has been described in Chapter 5. Prior to sterilization, the pH of the nutrient-amended mix was adjusted to approximately neutral by addition of $CaCO_3$. On cooling, the mix was inoculated with suspensions of the antagonistic bacteria as described in Section 5A.3.4 at the rate of 10% (v/w) to give an initial density in the mix of between 5.9 $\log_{10}$ cfu/g dw and 8.0 $\log_{10}$ cfu/g dw. Flasks were incubated at 25°C with periodic assay for growth of the bacterial antagonists following dilution plating onto TSA (Appendix 1B).

5A.3.6 Carrier preparation and inoculation under non-sterile conditions

The preparation of the mix was the same as that described in Section 5A.3.5, however prior to inoculation the mix was pre-incubated at 60°C for one week (to simulate hot-composting conditions) with a view to minimize the density of indigenous mesophilic microbiota. On cooling, the mix was inoculated with suspensions of bacterial antagonists as described in section 5A.3.5. The inoculated mix was incubated at 25°C under static condition with periodic assay for growth of the bacterial antagonists following dilution plating onto TSA. The identity of the antagonists following re-isolation was confirmed on the basis of their colony morphologies. When necessary, determination of cellular morphology by Gram staining and of biochemical reactions, such as the ability to hydrolyse casein or starch, was also undertaken.

5A.3.7 Glasshouse trial

A glasshouse trial was conducted to investigate the efficacy of the bacterial antagonists grown under sterile conditions in a mix of composted WFW and millet seed (80:20 w/w) in suppressing challenge by *S. minor*. Soil (sampled from NW Tasmania)

was mixed with 5% (v/v) suppressive WFW mix, dispensed into pots of 1.5 L capacity, and inoculated with *S. minor* cultured on millet seeds, placed at 20mm below the surface of the mix at the rate of 2g millet-inoculum per pot. Pots inoculated with *S. minor* only and pots without *S. minor* inoculation but containing suppressive compost amendment served as controls. All pots were left under irrigated conditions in a shade house for one week prior to sowing. Ten lettuce seeds were sown per pot with four replicates per treatment. After sowing, the pots were maintained in the shade house for four weeks. The numbers of germinated seeds were recorded one week after sowing, prior to thinning to five seedlings per pot. Numbers of healthy seedlings were recorded at weekly intervals thereafter.

5A.3.8 Statistical analysis

Statistical analysis of data was conducted using the analysis of variance (ANOVA) as described in Chapter 4.

5A.4 Results

5A.4.1 Growth of bacterial antagonists in the mix of composted WFW and millet seed (80:20 w/w) under sterile conditions

The results of the bacterial growth response in the mix of composted WFW and millet seed under sterile conditions are presented in Figure 5A-1. All tested bacterial antagonists were found to grow well in the WFW mix with increases of between 2.5 and 4.4 orders of magnitude (depending on the bacterial species) in density generally occurring in the first 14 days. Following prolonged incubation (up to 56 days), the population density of these antagonists plateaued between 8.4 $\log_{10}$ cfu/g and 10.1 $\log_{10}$ cfu/g (Figure 5A-1).

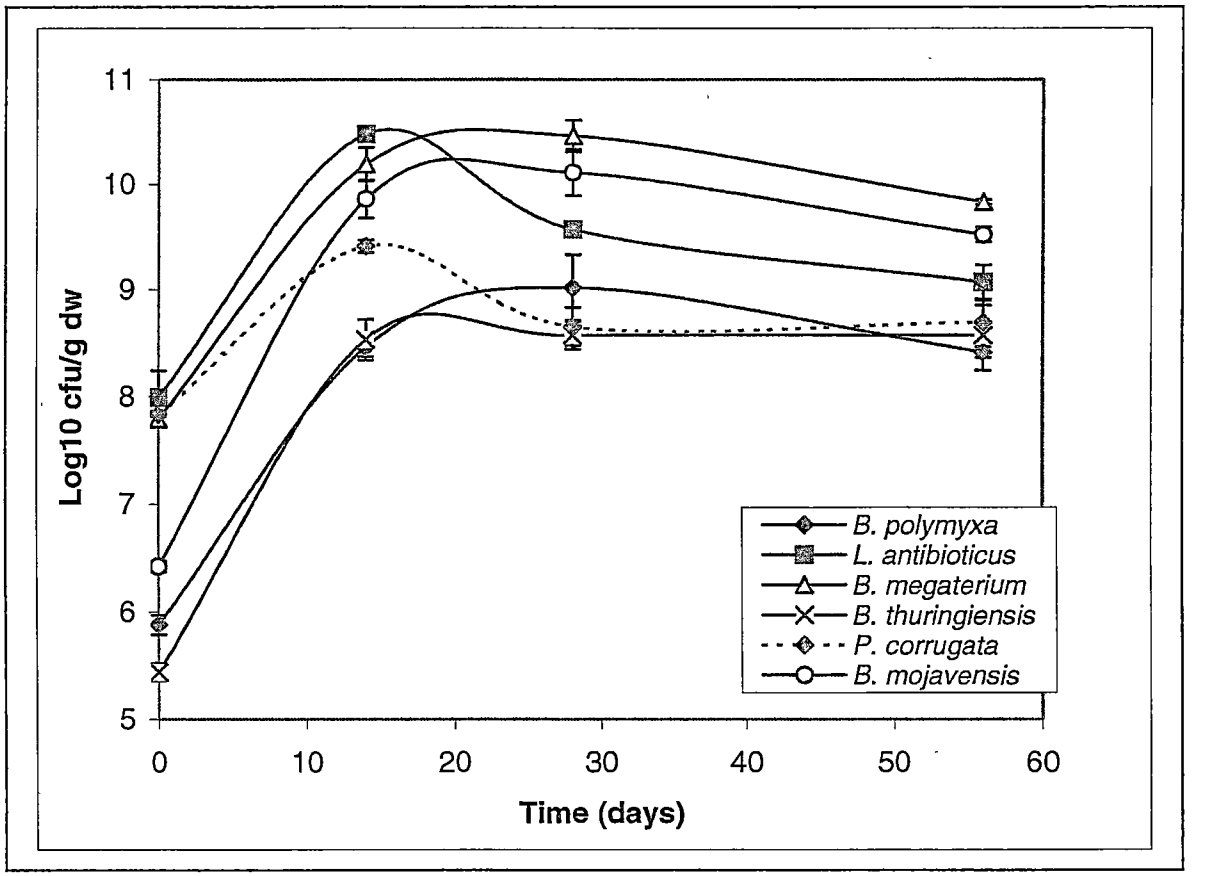


Figure 5A-1: Growth response of six bacterial antagonists in a mix of 80% composted WFW and 20% millet seed under sterile conditions. Each value shown is an average from four replicate mixes ± standard error.

5A.4.2 Growth of bacterial antagonists in the mix of composted WFW and millet seed (80:20 w/w) under non-sterile conditions

The growth of the bacterial antagonists in a mix of WFW and millet (80:20) under non-sterile conditions is shown in figure 5A-2.

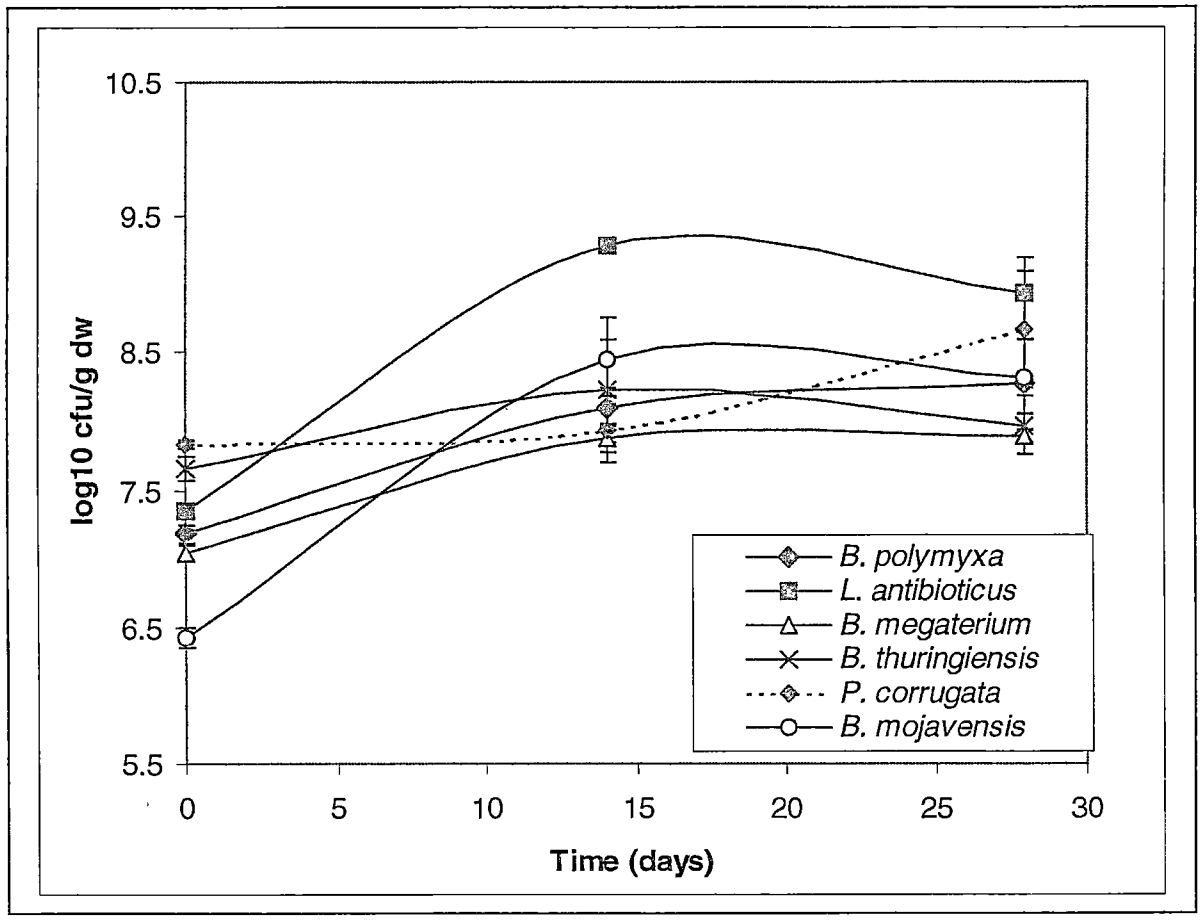


Figure 5A-2: Growth of six bacterial antagonists in a mix of 80% composted WFW and 20% millet seed under non-sterile conditions. Each value shown is an average from four replicate mixes ± standard error.

The growth rate of these bacterial antagonists appeared to be suppressed when grown under non-sterile conditions, indicated by a lower cfu, when compared to that recorded under sterile conditions (Figure 5A-2). In some cases, the cfu of the antagonist fell by more than one order of magnitude over the same period of incubation time. The *L. antibioticus* consistently showed the best growth response in this mix (both under sterile and non-sterile conditions), although its growth was somewhat suppressed when grown under non-sterile condition over the same period of time (Figure 5A-1 and 5A-2). All the tested antagonists however, reached a density of more than 7 $\log_{10}$ cfu/g dw in the mix under these conditions after 28 days incubation.

5A.4.3 Glasshouse trial

The efficacy of the suppressive mixes to protect lettuce seedlings/plants from attack by *S. minor* is shown in Table 5A-1.

Table 5A-1: Germination rate of lettuce seedlings in suppressive mixes, and the effectiveness of the mix-grown antagonists in protecting against *S. minor*. Each value is an average obtained from four replicate pots ± standard error.

Treatments*	Germination rate†	Healthy seedlings (%)¥	
		Week 2	Week 4
A0B0	92.5 ± 2.2	100 ± 0.0 a	100 ± 0.0 a
A0B1	90 ± 3.7	15 ± 5.0 b	5 ± 5.0 bc
A1B1	92.5 ± 2.2	20 ± 8.2 bc	15 ± 5.0 b
A2B1	92.5 ± 2.2	35 ± 5.0 c	15 ± 9.6 bc
A3B1	87.5 ± 2.2	35 ± 9.6 bc	10 ± 5.6 bc
A4B1	95 ± 2.6	25 ± 5.0 bc	0 ± 0.0 c
A5B1	92.5 ± 4.3	40 ± 8.2 c	20 ± 8.2 b
A6B1	87.5 ± 2.2	20 ± 8.2 bc	10 ± 5.8 bc

†The germination rate of lettuce seed was not statistically different at $p<0.05$ in all treatments.
*A0B0: nil control (neither pathogen nor antagonist were inoculated).
A0B1: pots inoculated with *S. minor* only (control treatment)
A1B1, A2B1, A3B1, A4B1, A5B1, and A6B1: Pots inoculated with both pathogen (*S. minor*) and *B. polymyxa, L. antibioticus, B. megaterium., B. thuringiensis, P. corrugata,* or *B. mojavensis*, respectively.
¥Values in the same column followed by the same letter are not significant statistically at $p<0.05$

The germination rate of the lettuce seeds in pots containing the antagonist, pathogen, or a combination of antagonist and pathogen-treated pots was high (ranging from 87.5 to 92.5%). Germination rates were not significantly different ($p>0.05$) when compared to the nil control (A0B0) showing 92.5% germination (Table 5A-1). Generally, the percentage of healthy seedlings in the pots treated with mix-grown antagonists was relatively higher than that in the control treatment pots (A0B1), although in most cases the results were not statistically significant ($p>0.05$) (Table 5A-1). *Lysobacter antibioticus* (A2B1) and *Pseudomonas corrugata* (A5B1) significantly protected the seedlings up to week two, with 35% and 40% healthy seedlings respectively, while only 15% survival was found in the control treatment (Table 5A-1). As more plants became infected with prolonged

incubation differences in the percentage of healthy plants between treatments and the control became statistically insignificant ($p>0.05$) (week 4, Table 5A-1). It would appear from this trial that the growth of the pathogen (*S. minor*) was stimulated in the soil by the millet seed of the mix, resulting in a more aggressive attack by the pathogen on the lettuce plants than would otherwise be the case.

5A.5 Discussion

The growth of the selected bacterial antagonists in three-month old WFW compost amended only with 0.5% w/w Phostrogen® was very poor (results not shown). A modification on this compost [amending it with 20% millet seed, re-wetting it with nutrient solution (NH_4NO_3-BMS) to approximately field capacity, and adjusting the pH to approximately neutral] resulted in excellent growth responses of the antagonists (Figure 5A-1). In some cases, the cfu-density reached by the bacterial antagonists under sterile conditions was comparable to that reached by the Td_{22} in the same mix (Chapter 5). This indicated that the mix of composted WFW and millet seed in the ratio of 80:20 (w/w) was also suitable for the cultivation of bacterial biological control agents. The presence of millet seed in the mix appeared to provide a ready source of available carbon and nitrogen, particularly in the initial stages of bacterial growth. WFW alone, which mostly consists of cellulose, remained largely undegraded by the inoculated antagonists, none of which was cellulolytic. The C:N ratio of raw WFW is approximately 218 (Jackson, 1998), however, following three months composting with appropriate nitrogenous amendment the C:N ratio was determined to be approximately 40, as described in section 5.5. Millet seed has been estimated to contain 1.7% w/w N, (Morrison, 1959). Hence its combination with WFW at the rate of 20% (w/w) would contribute further (if low) nitrogen supplementation for the growth of the bacterial antagonists.

As described in Chapter 4 the bacterial antagonists grew best at around neutral pH, this being achieved by addition of $CaCO_3$ to the otherwise acidic mix (pH 4.0-5.0) prior to inoculation.

The growth of bacterial antagonists under non-sterile conditions was somewhat reduced in terms of cfu/g mix when compared with that recorded under sterile conditions (Figure 5A-1 and 5A-2). A similar or greater effect of using non-sterile conditions was observed for the fungal agent Td_{22} (Chapter 5), with cfu/g mix falling by one to two orders of magnitude under non-sterile conditions. A similar reduction in cell numbers

under non-sterile conditions was also reported by Nakasaki *et al.* (1998) who reported the diminished growth of a *B. subtilis* strain in the presence of indigenous contaminants in a grass clipping compost substrate. Therefore, minimizing the density and/or diversity of indigenous microbiota prior to inoculation with antagonists is seen to be advantageous in achieving maximal numbers of inoculated antagonists.

Pre-treatment of the mix at 60°C for seven days (simulating hot-compost conditions) was found to reduce the diversity of the isolated indigenous bacteria, although the density of the total bacterial loading (cfu on TSA at 60°C) was not significantly reduced. This was not of concern, since the biota would be strongly dominated by thermophiles, which would be expected to compete poorly against the mesophilic biological control inoculants at temperatures of ~ 20°C.

As previously noted in section 5A.4.3, the millet seed used in the cultivation of the bacterial antagonists also appeared to stimulate the growth of the fungal pathogen (*S. minor*) in the pot trial. Apparently the complex carbohydrate-components of the millet were either beyond the metabolic capacity of the biological control bacteria to degrade, or (more likely) in excess of requirements over the period of antagonist-cultivation, leaving these components as substrates for the fungal pathogens. Therefore the use of millet seed as a nutrient supplement for the bacterial antagonists cultivation, to be subsequently used to control fungal pathogens in the field, cannot be recommended.

The finding that the use of millet/cellulose-medium (containing Td_{22}) as 10% or 20% inocula of potting media was antagonistic to the growth of *S. minor* is in qualified agreement (bearing in mind the above comment) with a report by Metcalf (1997) that *S. cepivorum* was suppressed by *Trichoderma koningii* in a composition of 100% millet seed. The use of other carbon sources rather than millet seeds, such as casein or starch for the cultivation of bacterial biological control agents is reported in Chapter 7.

5A.6 Conclusions

Modification of WFW compost, by amending with millet seed and adjusting the pH to neutral made it suitable as a growth medium for inoculated bacterial antagonists. However such amendment also appeared to provide an excellent nutrient source for the fungal pathogen (*S. minor*) on soil application. Therefore, the use of millet seed as a nutrient source for the bacterial antagonists is precluded. Although pre-treatment of the mix at 60°C for one week successfully eliminated most of the mesophilic bacteria, the

residual microbiota was still capable of some degree of suppression of the growth of inoculated bacterial antagonists relative to sterile counterparts following incubation at mesophilic temperatures. The possibility remains for the use of radical temperature shift (60°C to 25°C) to minimize the indigenous competition with desired inoculated biota on subsequent cultivation in bulk media.

Chapter 6

Assessment of composted wood fibre waste -grown Td_{22} for the protection of lettuce and pyrethrum from attack by *Sclerotinia minor*

6.1 Abstract

The effectiveness of Td_{22} grown in a mix of 20% millet seed and 80% WFW compost in protecting lettuce and pyrethrum plants from attack by *S. minor* was assessed. In pot trials, this suppressive mix was found to consistently protect these plants from *S. minor* attack. The degree of protection provided to lettuce plants was approximately proportional to the rate of suppressive mix amendment. Protection of 100% was recorded when soil was amended with Td_{22}-compost at the rate of 20% (v/v) four weeks after sowing. Mortality of controls at this time was 100%. Allowing a period of equilibration of the mix and pathogen in the glasshouse of at least four days prior to sowing significantly improved the biological control of *S. minor* by Td_{22}. To some degree, the effectiveness of Td_{22} may have been affected by the mode of application. Application of Td_{22} as a spore suspension or as spores in compost consistently and significantly provided protection to lettuce plants, while mycelial application was found to be less effective. The biological control fungus was re-isolated from root segments or soil samples at a frequency of between 80-90%, indicating it to have ecological competence in a competitive soil environment. Td_{22} was also found to be compatible with a commercially available foliar fungicide (Sumisclex®).

Good survival of Td_{22} in the mix of 20% millet seed and 80% wood fiber waste was demonstrated up to 4.5 months at ambient temperature.

The cost per hectare to incorporate this suppressive mix at the rate of 20% (v/v) into a growing mixture medium for nursery lettuces was estimated to be AU$91.47. By contrast, control *S. minor* in the field (field application instead of nursery application) using chemical fungicides currently costs AU$181.80/hectare. In view of this, application of suppressive compost as the formulation described to control *S. minor* must compare poorly with that of chemical fungicides. If the millet seed can be replaced by less

expensive materials, such as barley combs or cracked barley (by products of beer manufacture), this or an equivalent suppressive mix could provide an environmentally compatible, economically viable alternative for disease control in farming practices.

6.2 Introduction

Sclerotinia spp cause diseases in a wide range of host plants (Singleton *et al.*, 1992). Lettuce drop, for example, caused by *Sclerotinia minor* and *S. sclerotiorum* has been reported in many lettuce-growing countries having cool and moist climates such as Australia, Canada, the Netherlands, New Zealand, United Kingdom, United States, and Venezuela. Both fungi may attack lettuce plants at any stage of their growth, particularly during maturity, and the disease incidence is often severe (Davis *et al*, 1997). Until recently, control of these fungi has relied on fungicides, but with the move away from chemical treatments attention has focussed on biological method of control, where cost competitiveness becomes important.

Due to difficulties in handling, storage, and delivery of liquid preparation of fungal biological control agents, solid or semi solid preparations are considered to be preferable (Hadar *et al*, 1979). Millet seed has previously been investigated as a medium to support the growth of a biological control fungus *Trichoderma koningii* (Metcalf, 1997). The price of the seed, however has made the large scale production of this agent impracticable. Therefore research on alternative media is warranted, with the specific aim of cost minimisation.

As previously described (Chapter 5), *Trichoderma* spp. Td_{22} active against *S. minor* and *S sclerotiorum* showed an excellent growth response in composted and raw WFW supplemented with 20% w/w millet seed, with spore numbers reaching ~10^{10} per gram dry weight compost after 14 days incubation (Ramona and Line, 2002).

In the present study, Td_{22} grown in the mixture described above was assessed for its ability to protect lettuce and pyrethrum seedlings/plants from attack by *S. minor* in glasshouse trials. Specific objectives of this study were to investigate:

1. The effect of the amount of compost:millet mix-grown Td_{22} amendment on the level of protection provided to lettuce seedlings/plants from *S. minor* attack.
2. The effect of pre-incubation time of the Td_{22} medium prior to sowing on the level of protection provided to lettuce seedlings/plants.

3. The effect of storage of the Td_{22} medium on the level of protection provided to lettuce seedlings/plants.
4. The relative effectiveness and compatibility of WFW compost:millet seed-grown Td_{22} with a fungicide (Sumisclex®) in protecting lettuce seedlings/plants against *S. minor* attack.
5. The ability of Td_{22} cultured in various other media to suppress disease in lettuce seedlings/plants caused by *S. minor*.
6. The ability of Td_{22} grown in compost:millet mix to protect pyrethrum plants from attack by *S. minor*.
7. The ability of Td_{22} to colonize lettuce and pyrethrum roots and soil.

6.3 Materials and Methods

6.3.1 Microorganisms

The origin, method of cultivation and storage of *Trichoderma* spp. (isolate Td_{22}) and the *S. minor* have been previously described in Chapter 5.

6.3.2 Lettuce seeds and soil

Lettuce seeds were purchased from Roberts Limited, Australia. A red laterite soil on which both lettuces and pyrethrum are grown was obtained from northwest Tasmania. The soil was sieved through a 5 mm mesh and steam pasteurized for 45 minutes at 60°C before use.

6.3.3 Compost production

WFW compost was produced according to the method described in Chapter 5. The three-month old compost was air dried in a glasshouse for several weeks and stored in vinyl bags.

6.3.4 Preparation of Td_{22} inoculum

The preparation of Td_{22} inoculum has been previously described in Chapter 5.

6.3.5 Preparation of *S. minor* inoculum

Dry millet seed (500g) was soaked in 400 mL of 0.2% (w/v) Phostrogen® (a NPK-minerals formulation produced by Phostrogen Ltd, Australia) solution in distilled water and autoclaved for 30 minutes at 121°C on each of two consecutive days. On cooling, several plugs of *S. minor* on potato dextrose agar (PDA) were aseptically inoculated into the seed medium and incubated at 25°C for four weeks by which time sclerotia had formed. Prior to use in the glasshouse trials, this *S. minor* inoculum was air dried at room temperature.

6.3.6 Production of suppressive compost

Dried WFW compost was mixed with millet seed in the ratio of 80:20 w/w (d.w.) and brought to field capacity (~ 1.5L/kg dry mix) with a solution containing (gL^{-1} distilled water): NH_4NO_3, 5.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$ 0.2; $CaCl_2.2H_2O$, 0.01; and $FeCl_3$, 0.01, and autoclaved for 30 minutes on each of two consecutive days. The resultant pH of the mix was 4.3 and water content (mean of triplicate measurements) was 56.7%. The mix was inoculated with 10% (v/dw mix) of Td_{22} described above to give an initial cell density of 5.03 $\log_{10}$ cfu/g dry mix and incubated at 25°C for two to four weeks, when heavy sporulation was evident.

6.3.7 Glasshouse experiments

6.3.7.1 Assessment of compost concentration on disease control

Lettuce seeds were sown in pots containing field soil amended with suppressive compost at levels of 2.5, 5.0, 10 and 20% v/v. The spore density of the antagonist (Td_{22}) in the compost was 1.62 x 10^9 spores/g ww. Lettuce pathogen (*S. minor*) grown on millet seed was evenly inoculated approximately 20 mm below the soil surface at rate of 2.0 g inoculum per pot. Soil without compost amendment, amended with pathogen only, or without pathogen inoculation served as controls. Five replicate pots per treatment, each containing five seeds were maintained for four to six weeks in a shade house. Pots were irrigated as required and the germinated seeds counted one week after sowing. Disease incidence on the lettuce seedlings was recorded from two weeks after sowing.

6.3.7.2 Assessment of pre-incubation on disease control

Pots of 1.5 L capacity were prepared with compost as described above (Section 6.3.7.1) and inoculated with dried *S. minor* on millet seeds, placed 20 mm below the surface of the mix at the rate of 2.0 g millet-inoculum per pot. Pots without *S. minor* inoculation or without suppressive compost amendment served as controls. All pots were placed in the shade house for at least four days prior to sowing. The time interval between the preparation and sowing time is referred to as pre incubation time by Steinmetz and Schőnbeck (1994). During this time, the pots were irrigated as required to keep the mix moist. Five, two-weeks old lettuce seedlings were sown per pot with five replicates per treatment. After sowing, the pots were maintained in the shade house for ten weeks. The number of healthy plants was recorded at weekly intervals.

6.3.7.3 Assessment of compost age on disease control

Td_{22}-inoculated compost of three different ages (15, 30, and 60 days old) was assessed for effectiveness in protecting lettuce seedlings/plants from attack by *S. minor* in the shade house. The experiment was conducted in 1.5 L pots using 5% v/v compositions of Td_{22}-inoculated compost. The inoculation of *S. minor* was the same as described in section 6.3.7.1. Ten seeds were sown per pot (to be later thinned to five seedlings per pot), with five replicates per treatment. The experiment was maintained for four weeks in the shade house, with the number of healthy seedlings/plants being recorded at weekly intervals, starting two weeks after sowing.

6.3.7.4 Assessment of the relative effectiveness of *Trichoderma*-inoculated compost with a fungicide

The effectiveness of Td_{22}-inoculated WFW compost (as a 5% constituent of the potting mixture) in protecting lettuce plants from attack by *S. minor* was compared with a fungicide (Sumisclex®) commonly used in lettuce farms. Sumisclex® at the recommended concentration of 10.5 mL/10 L, was applied as drench at the rate of 20 mL per pot four days before planting. *S. minor* application was as described previously (Section 6.3.7.1). Five, two-weeks old lettuce seedlings were sown in each pot with eight replicate pots per treatment. The pots were maintained in a shade house for eight weeks and the number of healthy seedlings/plants was recorded at weekly intervals, starting two weeks after sowing.

6.3.7.5 Evaluation of application methods

The effectiveness of three Td_{22} preparations for the protection of lettuce seedlings/plants from attack by *S. minor* was evaluated in a pot trial using field soil as the growth medium. The preparation comprised a Td_{22} spore suspension in saline, one week-old of fresh Td_{22} mycelial suspension in pectin broth medium, and Td_{22} grown in a mix of 80% WFW compost and 20% millet seed (dry weight basis). Five mL of either a spore suspension (7.7±0.07 $\log_{10}$ spores of Td_{22}/mL) or mycelial suspension (6.3±0.56 $\log_{10}$ cfu Td_{22}/mL) was mixed into surface layers of the potting medium; comparison being made with growth/challenge in pots containing 5% (v/v) Td_{22}-grown compost (2 weeks old) in field soil and with appropriate controls. The *S. minor* pathogen was inoculated as described in Section 6.3.7.1. Ten seeds (later thinned to five) were sown in each pot with five replicate pots per treatment. Pots were maintained in a shade house for six weeks with assessment of plant mortality at weekly intervals.

6.3.7.6 Root colonization studies and establishment of Td_{22} in the soil

The establishment of Td_{22} both in the soil (Td_{22}-amended pots) and on the root surface of plants was assessed at the end of the pot trial reported in Section 6.3.7.3. For this, lettuce plants from each treatment (including the nil control) were uprooted, rinsed in water, and segments cut from the top (approximately 0.5 cm) of each root sample (with 20 segments cut from each treatment, except the control treatment, where four root segments were plated from two surviving lettuce plants) and aseptically placed on pectin agar medium amended with 60 µg/mL tetracycline. Also at the termination of the trial the establishment of Td_{22} in the soil at different depths was assessed by randomly collecting 15 soil samples adhering to roots surfaces at depths of 2 cm, 5 cm, and 10 cm, and plating one drop of a $1/5^{th}$ dilution of these samples in sterile saline on pectin agar medium amended with 60 µg/mL tetracycline. All inoculated plates were incubated at 25°C for 4-7 days until fungal growth was observed. For purposes of morphological comparison, fungal growth on each plate was sub-cultured on the same medium as a Td_{22} culture after followed by incubation for one week when conidial development was observed. A plug of hyphae from each plate was also sub-cultured into pectin broth medium for pectic enzyme assay. The identity of Td_{22} isolates from soils and root segments was confirmed on the basis of morphologically appearance (Metcalf, 1997) and pectic enzyme profiles (Cruickshank and Pitt, 1987) (Appendix 3).

6.3.7.7 Effectiveness of Td_{22}-grown compost in protecting pyrethrum plants from attack by *S. minor*

A pot trial was undertaken using 0.5 L capacity pots containing field soil amended with 5% v/v compositions of a suppressive compost inoculated with Td_{22} 4.5 months previously. *S. minor* was inoculated as described in Section 6.3.7.1. Four, three-week old pyrethrum seedlings were sown per pot following pre-incubation of the soil mixture in a shade house for four days, with eight replicates per treatment. The experiment was maintained for seven weeks in the shade house, with the number of healthy seedlings/plants being recorded at weekly intervals, starting one week after sowing. The trials were destructively sampled at week eight. All surviving plant tops were harvested, dried at 65°C and subjected to dry weight determinations. The sclerotia of the pathogen (*S. minor*) in pots inoculated with either *S. minor* alone (A0B1) or with *S. minor* and Td_{22}-grown compost (A1B1) were retrieved according to the method specified by Metcalf (1997) using a 0.5 mm sieve. Twenty retrieved *S. minor* sclerotia were randomly sampled and plated onto pectin agar amended with 60 µg/mL tetracycline to assess the effectiveness of Td_{22} parasitization. The identity of Td_{22} colonies growing from *S. minor* sclerotia was confirmed on the basis of their morphological characteristics and pectolytic enzyme profiles (Cruickshank and Pitt, 1987). The ability of Td_{22} to colonize pyrethrum roots was assessed using the method described in Section 6.3.7.6.

6.3.7.8 Statistical analysis

The data was analysed using the MINITAB software for windows as described in Chapter 4.

6.4 Results

6.4.1 Effect of suppressive compost amendment on the protection of lettuce seedlings from attack by *S. minor*

The effect of Td_{22}-grown compost amendment on the germination rate of lettuce seed and on the protection of lettuce seedlings/plants from attack by *S. minor* is presented in Table 6-1.

Table 6-1: Germination rate of lettuce seeds and protection of lettuce seedlings/plants following varying Td_{22}-grown compost amendments of potting medium.

Treatments*	Percentage seed germination	Percentage of healthy seedlings/plants[r]	
		Week 3 after sowing	Week 4 after sowing
A0B0	92 ± 4.9 a	92 ± 4.9 ab	92 ± 4.9 ab
A0B1	96 ± 4.0 a	4 ± 4.0 d	0 ± 0.0 d
A2.5B0	96 ± 4.9 a	88 ± 4.9 b	88 ± 4.9 b
A2.5B1	92 ± 4.9 a	32 ± 8.0 c	32 ± 8.0 c
A5B0	96 ± 4.0 a	92 ± 4.9 ab	92 ± 4.9 ab
A5B1	100 ± 0.0 a	48 ± 4.9 c	44 ± 4.0 c
A10B0	96 ± 4.0 a	96 ± 4.0 ab	96 ± 4.0 ab
A10B1	100 ± 0.0 a	92 ± 4.9 ab	92 ± 4.9 ab
A20B0	88 ± 4.9 a	88 ± 4.9 b	88 ± 4.9 b
A20B1	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a

[r]Each value is an average of five replicates ± standard error. Values in the same column followed by the same letter(s) are not significantly different at $p<0.05$.
*A0B0: Neither pathogen nor antagonist was inoculated (nil control); A2.5B0, A5B0, A10B0, and A20B0: Pots amended with 2.5%, 5%, 10%, and 20% (v/v) compost-grown Td_{22}, respectively, in the absence of *S. minor*; A2.5B1, A5B1, A10B1, and A20B1: pots amended with 2.5%, 5%, 10%, and 20% (v/v) compost-grown Td_{22}, respectively in the presence of *S. minor*; A0B1: Pots inoculated with pathogen (*S. minor*) only (control treatment).
Td_{22} grown for two weeks in the millet seed:WFW compost mixture was used in this trial.

Between 88-100% of the lettuce seeds germinated in soil amended with 5-20% (v/v) compost-grown Td_{22} one week after sowing (Table 6-1). No inhibition of seed germination was evident at the higher application rate of Td_{22}-compost. No significant difference (at $p<0.05$) in the germination rate of the lettuce seeds was noted following compost-grown Td_{22} amendment of up to 20% (v/v), a result which is consistent with that reported in Chapter 5 of a high rate of radish seed germination in three-month old WFW compost.

As indicated in Table 6-1, the rate of amendment of the compost/fungus was proportional to disease control, ranging from 32% provided by 2.5% (v/v) amendment to 100% protection provided by 20% (v/v) amendment four weeks after sowing. This was

statistically significant at $p<0.05$ when compared to the control treatment (A0B1) where 100% mortality was observed at four weeks after sowing.

6.4.2 The effect pre-incubation of the biological control agent and pathogen on the disease control in lettuces

The protection provided to lettuces following pre-incubation of Td_{22} plus pathogen in the soil milieu prior to planting is presented in Table 6-2.

Table 6-2: Effect of pre-incubation on the efficacy of Td_{22}-grown compost to protect lettuce seedlings/plants from *S. minor* attack.

Treatments[r]	Percentage of healthy seedlings/plants*		
	Week 4	Week 7	Week 10
A0B0	100±0.0 a	100±0.0 a	100±0.0 ad
(A1B1)0	48±13.6 b	36±16.0 bc	36±16.0 bce
(A0B1)0	4±4.0 c	4±4.0 c	4±4.0 b
(A1B1)4	100±0.0 a	92±8.0 a	92±8.0 ad
(A0B1)4	48±13.6 b	48±13.6 b	48±13.6 ce
(A1B1)7	100±0.0 a	100±0.0 a	100±0.0 ad
(A0B1)7	92±4.9 a	92±4.9 a	44±11.7 ce
(A1B1)10	100±0.0 a	96±4.0 a	96±4.0 ad
(A0B1)10	92±4.9 a	92±4.9 a	52±8.0 ce
(A1B1)14	100±0.0 a	84±16.7 a	84±16.7 d
(A0B1)14	96±4.0 a	96±4.0 a	56±7.5 e

* Each value is an average of five replicates ± standard error. Values in the same column followed by the same letter(s) are not significantly different at $p<0.05$ according to lsd test following ANOVA.
[r]A0B0: Neither pathogen nor antagonist were inoculated (nil control); (A0B1)0, (A0B1)4, (A0B1)7, (A0B1)10, and (A0B1)14: pots amended with pathogen (*S. minor*) only (control treatment) and pre-incubated for 0, 4, 7, 10, and 14 days, respectively prior to sowing; (A1B1)0, (A1B1)4, (A1B1)7, (A1B1)10, and (A1B1)14: pots amended with 5% suppressive compost and pathogen (*S. minor*) and pre-incubated for 0, 4, 7, 10, and 14 days, respectively prior to sowing.

Pre-incubation prior to sowing significantly affected the efficacy of Td_{22}-grown compost ($p<0.05$). At four weeks after sowing and in the absence of a 'pre-incubation' prior to pathogen challenge, the Td_{22}-grown compost (suppressive compost) significantly

($p<0.05$) protected the lettuce plants from attack by *S. minor* [treatment (A1B1)0 relative to the control treatment (A0B1)0], a result consistent with its counterpart (treatment A0B1) reported in Table 6-1. However, the efficacy of the suppressive compost in (A0B1)0 treatments became non-significant statistically ($p>0.05$) compared to the control treatment [(A0B1)0] following prolonged incubation (Table 6-2). By contrast, in all cases of pre-incubation of the pathogen/antagonist in potting media for four or more days prior to planting, significant protection ($p<0.05$) was provided to the plants by the suppressive compost for the full period of the trial, up to 10 weeks after sowing.

6.4.3 Effect of suppressive compost storage on disease control

The effect of storage on the efficacy of the compost-grown Td_{22} to protect lettuce seedlings/plants from attack by *S. minor* is reported in Table 6-3.

Table 6-3: Effect of storage of compost-grown Td_{22} on the protection of lettuce against *S. minor* attack

Treatment*	% of healthy seedlings/plants†		
	2 weeks after sowing	3 weeks after sowing	4 weeks after sowing
A0B0	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
A0B1	44 ± 7.5 d	8 ± 4.9 c	8 ± 4.9 d
(A1B1)2 wks	84 ± 4.0 bc	52 ±14.9 b	44 ± 13.3 c
(A1B1)4 wks	88 ± 4.9 c	80 ± 6.3 b	80 ± 6.3 b
(A1B1)8 wks	96 ± 4.0 abc	64 ±11.7 b	56 ± 9.8 bc

*A0B0: Neither pathogen or antagonist was inoculated (nil control); (A1B1)2 wks, (A1B1)4 wks, and (A1B1)8 wks: pots were inoculated with 2g/pot pathogen culture and 5% (v/v) compost-grown Td_{22} after 2, 4, and 8 weeks of storage, respectively; A0B1: pots inoculated with pathogen (*S. minor*) alone (control treatment).

† Each values ± standard error is an average of five replicates. Values in the same column followed by the same letter(s) are not significant statistically at $P<0.05$, according to lsd test following ANOVA.

It was apparent from Table 6-3 that the Td_{22}-grown compost, as assessed at week two after planting, did not lose its efficacy to protect lettuce seedlings/plants after 8 weeks of storage at room temperature (approx. 20°C), the survival for which is not significant (at $p<0.05$) from that of its equivalent non-stored counterpart indicated in Table 6-1. Likewise, there was no significant *loss* of efficacy (following 2, 4 or 8 week's storage) of the compost-antagonist as determined at three and four weeks after planting.

6.4.4 Comparative effectiveness and compatibility of Td_{22}-inoculated compost with the fungicide Sumisclex®

The efficacy of Td_{22}-inoculated WFW compost:millet seed in protecting lettuces against *S. minor* was compared with the fungicide Sumisclex®. A combined application of this suppressive mix with this chemical fungicide was also included to assess their compatibility. The results are presented in Table 6.4.

Table 6-4: Comparative effectiveness and compatibility of Td_{22}-inoculated mix with Sumisclex®

Treatments[¥]	Percentage of healthy plants at week 7[¥]
A0B0	100 ± 0.0 a
A0B1	70 ± 10.7 b
A1B1	100 ± 0.0 a
A2B1	100 ± 0.0 a
A3B1	100 ± 0.0 a

[¥]A0B0: nil control (neither pathogen nor antagonist was inoculated); A0B1: control treatment (pots inoculated with pathogen only); A1B1, pots inoculated with suppressive mix and pathogen, A2B1, pots inoculated with Sumisclex® and pathogen, and A3B1, pots inoculated with a combination of suppressive mix, Sumisclex® and pathogen.
Each value is an average of 8 replicate pots. Values followed by the same letter are not significant statistically at $p<0.05$.

Although the level of pathogen attack after seven weeks was low, it was sufficient to demonstrate the effectiveness of the biological control agent and Sumisclex® ($P<0.05$). No comparison of these two treatments however was possible in this trial. The biological control fungus Td_{22} was re-isolated at a very high frequency on the root segments of lettuce (95% of the root segment samples) sampled from treatment A3B1 (pots inoculated with a combination of suppressive mix and Sumisclex® plus pathogen), indicating that this fungus was compatible with the fungicide at the concentration applied in this trial.

6.4.5 Effectiveness of Td_{22} in various formulations

The efficacy of Td_{22} applied in various preparations in protecting lettuce seedlings/plants from attack by *S. minor* in a pot trial is presented in Table 6-5.

Table 6-5: Efficacy of Td_{22} against *S. minor* applied in various formulations

Treatment***	Percentage healthy plants*			
	Week 3	Week 4	Week 5	Week 6
A0B0	100 ± 0.0 ab	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
(A1B1)spore†	92 ± 4.9 bcd	92 ± 4.9 a	92 ± 4.9 a	92 ± 4.9 a
(A1B1)mycelia**	88 ± 4.9 cd	84 ± 7.5 ab	84 ± 7.5 ab	84 ± 7.8 ab
(A1B1)compost•	100 ± 0.0 ab	96 ± 4.0 a	96 ± 4.0 a	96 ± 4.0 a
A0B1	84 ± 4.0 d	76 ± 4.0 b	68 ± 4.9 b	64 ± 7.5 b

* Each value is an average of 5 replicates ± standard error. Values in the same column followed by the same letter (s) are not statistically significant according to lsd test at $p<0.05$ following ANOVA.
**Mycelia of Td_{22} was produced by inoculating plugs of 3 day-old pectin agar-grown Td_{22} into the same medium minus agar. The flask was incubated for one week until sufficient mycelia were obtained. Some spores were also observed in this mycelial culture.
***A0B0: nil control; A1B1: pots inoculated with *S. minor* and suppressive compost; A0B1: pots inoculated with *S. minor* only (control treatment).
•Two week-old compost was applied in this trial.
†Spores of Td_{22} were harvested from 10 day-old cultures of Td_{22} grown on pectin agar and suspended in saline.

The effectiveness of Td_{22} in protecting against *S. minor* in this trial appeared to be affected by the mode of application, with the spore suspension and Td_{22}-grown compost application providing possibly better (but not significant at $p<0.05$) protection of lettuce seedlings than mycelial application (Table 6-5). When compared to treatments at different times, the efficacy of the suppressive mix applied at 5% (v/v in soil) consistently and significantly ($p<0.05$) protected lettuce seedlings/plants from attack by *S. minor* (see Table 6-1, 6-3, and 6-5).

In all trials where the soil was supplemented with compost-grown Td_{22} the growth rate of lettuce plants appeared to be significantly improved relative to controls as indicated in Plate 6-1.

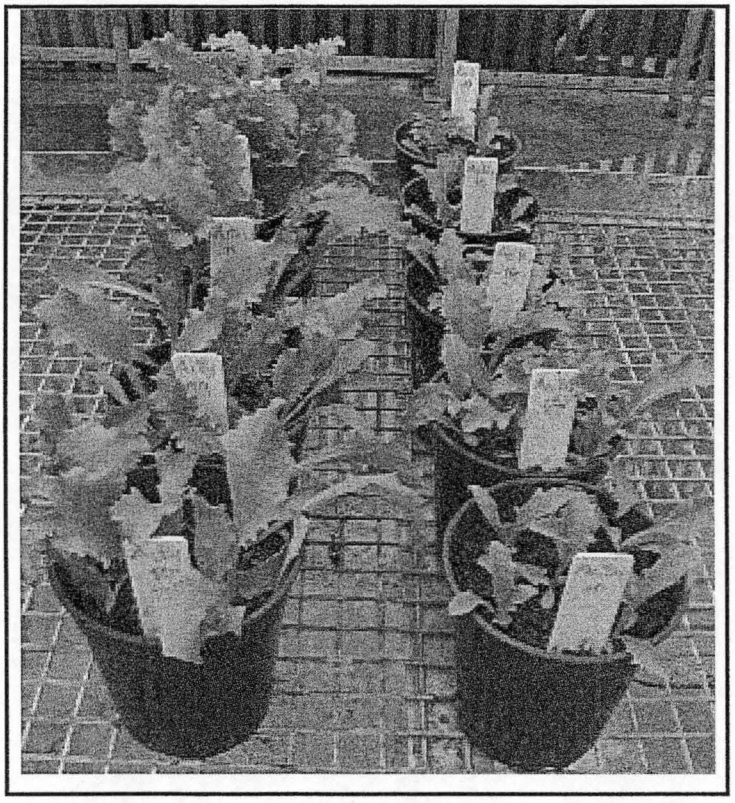


Plate 6-1: Growth improvement on lettuce plants as a result of compost-grown Td_{22} amendment. Pots on the left containing soil were amended with 5% v/v compost-grown Td_{22}, while the pots on the right were not (soil only).

6.4.6 Effectiveness of Td_{22} in colonising lettuce roots and soil

Td_{22} was reisolated from 85% to 90% of the root segments of lettuce plants inTd_{22}-inoculated pots, using pectin agar medium amended with 60µg/mL tetracycline (Table 6-6). A surprising finding was that 2/4 root segments from two surviving seedlings of pathogen-only (A0B1) control plants and from 4/20 root segments inoculated with neither antagonist nor pathogen (A0B0) were colonized by the Td_{22}. The presence of Td_{22} in these treatments was presumably attributable to cross-contamination from Td_{22}-inoculated pots, perhaps due to splashing during watering.

Table 6-6: Colonization of lettuce roots by Td_{22}

Treatment*	No. of root segments†	No. of samples colonised by Td_{22}	% segments colonised
(A1B1)2	20	17	85
(A1B1)4	20	17	85
(A1B1)8	20	18	90
A0B1	4	2	50
A0B0	20	4	20

*A0B0: Uninoculated pots (nil control); (A1B1)2, (A1B1)4, and (A1B1)8: pots inoculated with pathogen and compost-grown Td_{22} after 2, 4, and 8 weeks of storage, respectively; A0B1: pots inoculated with pathogen alone (control treatment).
†Root samples (reaching approximately 10 cm depth) were collected from randomly 10 survive plants on week 5 and shaken free of soil. Two root segments each of approximately 5.0 mm length from each plant were plated on tetracycline semi-selective medium for colonization determinations.
Pots were amended with 5% (v/v) compost-grown Td_{22} and 2g/pot of *S. minor* in millet seeds, where indicated.

After five weeks, soil samples (approximately 2g each) were taken from non-root regions and at different depths of the Td_{22}-inoculated pots to assess the distribution of this fungus. Td_{22} was re-isolated from 12/15 (80%) samples at 2cm depth, from 13/15 (87%) samples at 5cm depth, and from 10/15 (67%) samples at 10cm depth. It was also isolated from 2/15 (13%) samples taken from the surface to approximately 2 cm depth of uninoculated pots, suggesting that some degree of cross contamination had occurred as indicated above. The identity of Td_{22} isolated from root segments or soil samples based on morphological characteristics was confirmed in specific instances by pectolytic enzyme profiles.

6.4.7 Effectiveness of compost-grown Td_{22} in protecting pyrethrum plants from *S. minor* attack

The effectiveness of 4.5-month old compost-grown Td_{22} (unspent suppressive compost from the lettuce trials) to protect pyrethrum seedlings/plants from *S. minor* infection is indicated in Table 6-7.

Table 6-7: Effectiveness of Td_{22}-grown compost in protecting pyrethrum against *S. minor* attack.

Treatments*	Percentage of healthy seedlings/plants[r]			
	Week 1	Week 2	Week 4	Week 7
A0B0	100±0.0 a	100±0.0 a	100±0.0 a	100±0.0 a
A0B1	56±6.3 b	28.3±5.7 b	21.9±5.7 b	21.9±5.7 b
A1B0	100±0.0 a	100±0.0 a	100±0.0 a	100±0.0 a
A1B1	100±0.0 a	100±0.0 a	100±0.0 a	100±0.0 a

*A0B0: Uninoculated pots (nil control); A0B1: pots inoculated with pathogen only (control treatment), A1B0: posts inoculated with Td_{22}-grown compost only, and A1B1: pots inoculated with both *S. minor* and Td_{22}-grown compost.

[r]Each value is an average of 8 replicates pots with 4 pyrethrum seedlings/plants per pot. Values ± standard error followed by the same letter in the same column are not significant statistically at $p<0.05$, according to lsd test following ANOVA.

The effectiveness of suppressive compost in the total suppression of *S. minor* attack of pyrethrum seedlings/plants over a seven-week period, following its storage for 4.5 months, was demonstrated in this trial. This was in contrast to the *S. minor* control, which showed 78% mortality over this period.

The trial indicated both that the Td_{22} was not pathogenic to pyrethrum (plants grown with compost-grow Td_{22} in the absence of the pathogen showed 100% survival) and that it was versatile in its protective capacity [it was also known to protect onions (Metcalf, 1997) and lettuces from attack by *S. minor*].

Plate 6-2 shows pyrethrum plants in the pot trial at week 8, before harvesting for dry weight determination.

Dry weight determinations of the pyrethrum plants at week 8 are presented in Table 6-8. The average dry weight per plant in the *S. minor* control treatment (A0B1) was significantly lower ($p<0.05$) than that in Td_{22} amended treatments A1B0 and A1B1. However, the dry weight of the plant in this control treatment (A1B0) was not statistically significant ($p>0.05$) from that recorded in the nil-pathogen, nil-Td_{22} control (A0B0).

Plate 6-2: Pyrethrum plants in the pot trial at week 8 before harvesting for dry weight determinations. From left to right are pots inoculated with *S. minor* only (A0B1), pots co-inoculated with *S. minor* and compost-grown Td_{22} (A1B1), nil control (A0B0), and pots inoculated with compost-grown Td_{22} only (A1B0).

Table 6-8: The relative average dry weight of pyrethrum plants eight weeks after sowing.

Treatments*	Average dry weight per plant (g)**
A0B0	1.08 ± 0.1 ab
A0B1	0.7 ± 0.12 b
A1B1	1.13 ± 0.07 a
A1B0	1.10 ± 0.12 a

*A0B0: uninoculated control (nil control); A0B1: pots inoculated with *S. minor* only (pathogen control); A1B1: pots inoculated with both *S. minor* and compost-grown Td_{22}; A1B0: pots inoculated with compost-grown Td_{22} only.

**Each value is an average of 8 replicates ± standard error, except A0B1 (average of 5 replicates, with plants in other pots having died). Values followed by the same letter(s) are not significant statistically at $p<0.05$ using the lsd test following ANOVA.

At week eight, *S. minor* sclerotia in the control treatment pots (A0B1) and in the pots inoculated with both *S. minor* and compost-grown Td_{22} were retrieved using a 0.5mm mesh sieve. The relative sclerotial density per pot is presented in Table 6-9.

Table 6-9: Relative density of *S. minor* sclerotia after eight weeks (trial termination).

Treatments*	Number of *S. minor* sclerotia per pot**
A0B0	ND
A0B1	108 ± 11.74 a
A1B1	58.3 ± 8.58 b
A1B0	ND

*A0B0: uninoculated control (nil control), A0B1: pots inoculated with *S. minor* only (pathogen control), A1B1: pots inoculated with both *S. minor* and compost-grown Td_{22}, A1B0: pots inoculated with compost-grown Td_{22} only. All pots were inoculated equally with pathogen at the start of the trial.
** Each value is an average of 8 replicates ± standard error. Values followed by the same letter are not significant statistically using the lsd test following ANOVA.
ND: not determined

The density of *S. minor* sclerotia in the control treatment (A0B1) was significantly higher (almost double) that in pots where *S. minor* and compost grown Td_{22} were co-inoculated (A1B1) (Table 6-9), suggesting inhibition of growth or sclerotial development of the pathogen, or parasitism and death of the sclerotia. The latter possibility was supported by the finding that 92.5% of the sclerotia retrieved from the pathogen/Td_{22} treatment (A1B1) were parasitised by Td_{22}, even though 15.6% of those retrieved from the pathogen-only treatment (A0B1) were also found to be parasitised by the fungus (this probably attributable to splash contamination). The non-infected sclerotia retrieved in this trial were found to be capable of causing root-rot disease of lettuces.

As indicated for lettuces, Td_{22} was effective in colonizing roots of pyrethrum plants with 83% of the root segments collected from pots co-inoculated with *S. minor* and Td_{22}, and 80% of the segments collected from pots inoculated only with compost-grown Td_{22} being colonized by this antagonist. Td_{22} was also noticed at an average incidence of 42% from root segments of surviving plants in the *S. minor* control treatment (A0B1), and of 32.5% from root segments of the nil control (A0B0), this again being attributable to splash transport. The effectiveness of Td_{22} to colonize pyrethrum following pot inoculation was comparable to that found in lettuce plants (as described in Section 6.4.6).

6.4.8 Economic analysis of WFW compost-grown Td_{22} application relative to chemical fungicides

The relative costs associated with the use of suppressive compost (WFW compost-grown Td_{22}) and chemical fungicides to control *S. minor* in the field application are important considerations for growers.

The cost to produce WFW compost is estimated to be AU$9.26 per m^3 of raw material (Jackson, 1998) and the volume of the raw material is estimated to decrease by 45-50% during composting. Therefore, the cost of 1 m^3 of finished compost increases to between AU$16.84 and AU$18.5. Assuming 1 m^3 of wet WFW compost with approximately 40% moisture content weighs to 0.56 tonnes, the dry weight of this 1 m^3 compost will be 336 kg. The original formulation for the cultivation of Td_{22} provided by Dr D. Metcalf (who demonstrated the effectiveness of this strain to control root-rot in onion) was of 100% millet. This was reduced to 20% in the work reported here, amounting to 84 kg millet/m^3. The current retail price of the millet seed is ~AU$0.90 per kg; the total cost to produce 1 m^3 of medium for fungal antagonist, which consists of 80% WFW compost and 20% millet seed, will therefore be approximately AU$93.28. Assuming the cost of inoculation is AU$2.00 or ~US$1.00 as estimated by Granatstein (1998), the total cost of 1 m^3 of this suppressive compost (dw) will be approximately AU$95.28. This is comparable with the cost of producing 1 m^3 of growing medium consisting of 90% WFW compost and 10% perlite, reported at AU$92.15 (Jackson, 1998). It also compares well with the cost of other commercially available bark-based media produced for small-scale home or nursery use, of between AU$5 to AU$10 per 20 L bag or AU$250 to AU$500 per m^3 (Jackson, 1998), but is hardly economic for large-scale use by market gardeners at the anticipated application rate of 20-40% v/v.

It was apparent that most of the cost of producing suppressive compost production was attributable to the millet seed amendment, amounting to ~ 80% of the total expenditure. Assuming that 4 L growing medium is required to grow 100 seedlings, and 120,000 seedlings are needed per hectare (A. Houston, pers. comm.), 4.8 m^3 of growing medium is required per hectare, amounting to 0.96 - 1.92 m^3 suppressive compost (compost-grown Td_{22}) per hectare incorporated into the growing medium. Therefore, if treatment of the nursery bed soil was sufficient to control *S. minor* in the field (currently an unproven possibility), the cost per hectare to control *S. minor* using compost-grown biological control agents would be ~AU$91.47 to AU$182.94. By comparison, control *S.*

minor using an application of the fungicide Amistar® at the rate of 0.3 kg per hectare (the current price of this fungicide is AU$361/kg) plus Score® at the rate of 0.5 L/ha (the current price is AU$147/L) currently costs AU$181.80/ha (Geoffrey Cook, personal communication). It must be emphasised however that these treatments are for the field rather than the nursery bed soil, and in view of this, application of suppressive compost as the formulation described to control *S. minor* compares poorly in economic terms with that of chemical fungicides.

In view of these results, further work needs to be undertaken to examine other options for the cultivation of the Td_{22} antagonist, replacing expensive grain supplements entirely with waste materials such as barley combs or cracked barley (the by-products of beer manufacture) to reduce production costs.

6.5 Discussion

The effectiveness of *Trichoderma* sp. (Td_{22}) grown in a mix of millet seed and wood fibre waste compost (20:80 w/w) to protect against *S. minor* has been clearly demonstrated in a series of glasshouse trials in the present study. Application of compost-grown Td_{22} at rates up to 20% (v/v) was non-toxic (relative to controls) to lettuce seed in all treatments (Table 6-1).

The utilization of composted WFW and millet seed as described in the present study could be achieved in large scale at moderate cost while avoiding the problems of liquid cultures as outlined by and Hadar *et al* (1979). Application of compost-grown Td_{22} after allowing growth for two weeks or more appeared to be advantageous, because as described in Chapter 5 the growth of Td_{22} following at least two weeks incubation was mostly in the form of spores. This reduces concerns relating to viability in the field (if raising others relating to allergens), because fungal spores will be relatively more resistant than mycelia to environmental stress (Tronsmo, 1996).

A good disease control of the suppressive mix after 8 weeks of storage in the lettuce trial, or after 4.5 months of storage in the pyrethrum plant trial, was demonstrated (Table 6-3 and Table 6-7), and increasing the proportion of Td_{22}-compost from 5% to 10% or 20% was seen to be advantageous (Table 6-1). Similar results for fungal biological control agents in compost have been reported by Nelson and Hoitink (1982), Kuter *et al.* (1988) (who also reported a storage-related effect on the control of *Rhizoctonia* damping-off), and by Huang and Huang (2000). The survival of a strain of

Trichoderma koningii in a mixture of ryegrass seed and cornmeal for 30 weeks has also been reported by Warren *et al.* (2001).

Pre-incubation of pots inoculated with pathogen and biological control agent prior to planting seedlings, significantly affected the efficacy of the compost grown Td_{22} in protecting lettuce plants from attack by *S. minor* (Table 6-2). Pre-incubation for at least four days prior to planting appeared to be important to avoid early contact between the pathogen (*S. minor*) with its plant host (lettuce or pyrethrum in this study). In the absence of plant host, non-germinated sclerotia would remain dormant until the host was detected, allowing colonization of the growing root prior to antagonist attack and allowing the possibility of sclerotial parasitization before or after its germination. The latter possibility is supported by Table 6-2, where fewer plants became infected in the pots co-inoculated with *S. minor* and compost-grown Td_{22} if pre-incubation was prolonged, suggesting that the pathogen was parasitized during this period. The same effect of pre-incubation prior to sowing on the control of *Pythium ultimum* by *Trichoderma harzianum* and *Gliocladium roseum* has been reported by Steinmetz and Schönbeck (1993).

Notably, the efficacy of 5% v/v WFW compost-grown Td_{22} amendment for the control of *S. minor* infection of lettuces was comparable with Sumisclex® (at the recommended concentration of 10.5g/10 L) applied at 20 mL per pot, both treatments providing full protection (100% survival) to lettuce plants up to 7 weeks after planting (Table 6-4). No further conclusion can be drawn from the 100% protection provided by the combination of Sumisclex® and 5% WFW compost-grown Td_{22}, since the fungicide alone is seen to be fully effective in control of the pathogen. The presence of Td_{22} at a very high frequency (95% of the root segments sampled) on root segments of surviving plants sampled from this combined application indicated its compatibility with the Sumisclex®, opening the possibility for reduced dependence (lower application rates) on the chemical component. Biological control *Trichoderma* spp. have been reported to be compatible with a number of fungicides (e.g. McLean *et al.*, 2001 and http://www.bioworksbiologicalcontrol.com/rootshield/pdf/techsheets/CompatibilityChart.pdf).

It was found in this study that application of mycelial suspension of Td_{22} to protect lettuce from attack by *S. minor* resulted in levels of protection that were lower, but not significantly so, than those resulting from application of spore suspensions (Table 6-5).

It has been reported that organic amendment generally improves soil properties, such as bulk density, water holding capacity, and cation exchange capacity (Herrick and

Wander, 1997). Improvement in soil fertility following compost amendment has also been reported by Steffen *et al* (1995). The growth improvement of plants indicated in plates 6-1 and 6-2 following compost-grown Td_{22} amendment was perhaps partly due to this effect, although the availability of nutrient residues in compost would also help in this regard. Alternatively, the Td_{22} promoted the plant growth directly in the absence of a pathogen, although this possibility was not supported by the observation (Section 6.4.4) of no growth improvement in the pots of lettuces treated with spore suspension or mycelial suspension in the absence of compost. The findings in this study support the reports of Hoitink *et al.* (1991) and Maynard (1997) of an improved growth rate or yield of plants as a result of suppressive compost amendment.

It was perhaps significant that re-isolation of Td_{22} from root segments (Table 6-5) indicated that less than 100% of the segments were apparently colonized by the antagonist, reinforcing the probability that the increased incidence of disease noted for 5% compost-inoculum relative to 20% (Table 6-1) was attributable to a poorer colonization by the antagonist inoculum at the lower level of amendment.

The cost associated with the production of WFW compost-grown Td_{22} was still found to be high (~AU$95.28 per m^3). Since 1 tonne ww is approximately equal to 2 m^3, application of this product in the field to control plant pathogens at the rate of 89 tonnes per hectare as specified by US EPA (1997) and Granatstein (1998) is unrealistically expensive. Taking an alternative pathway, an application rate of 20% (v/v), 0-10 cm depth amounts to 125 tonnes/ha, which again is unrealistically high. Therefore, application of this suppressive compost can presently only be considered at the nursery level by amending the growing medium at the rate of 20 to 40% (v/v) so that root colonization by the antagonist (Td_{22}) is effective prior to field planting. The cost associated with the growing medium preparation will increase by ~AU$91.47 (20% amendment) to AU$182.94 (40% amendment) ha^{-1}, a cost comparable with that of chemical fungicide application in the field to control *S. minor* as discussed above, at approximately AU$181.80 ha^{-1} (Geoffrey Cook, personal communication).

Although the application costs of this suppressive compost/biological control agents are higher than those of chemical pesticides, they provides several long-term advantages (Nameth, 2001). These include:

1. In most cases they are safer to use than chemical-based fungicides.
2. Biological control helps reduce the loading of chemical-based fungicides.

3. Biological control helps reduce the risk of developing pathogen resistance to traditional chemicals.
4. In most cases, biological control is less phytotoxic.
5. In most cases, biological control results in lower restricted interval times than chemical-based pesticides.

To this list we can add, in the case of compost-grown biological control agents, the attributes provided by the compost of improved water holding capacity, improved tilth, improved nutrient status and improved biological buffering capacity. Depending on perspective, these considerations might outweigh the added expense associated with biological control. It seems probable that biological control will become increasingly competitive with chemical-based fungicides in farming practices, particularly if the costs of production of the biological control agents can be further reduced.

6.6 Conclusions

Compost-grown Td_{22} consistently provided protection to lettuce or pyrethrum plants with the degree of protection related to the rate of its amendment. No toxicity was evident to lettuce seed germination at high rate (up to 20% v/v) application of the compost-grown *Trichoderma.* Pre-incubation of the suppressive compost-amended growing medium for at least four days prior lead to an improved biological control of *S. minor* by the Td_{22}. The efficacy of the Td_{22} in protecting against *S. minor* attack may have been affected to some degree by the mode of its application, with spore application appearing to be more consistent than mycelial suspension application.

Effective storage of the Td_{22} spores in a mixture of WFW compost and millet seed at the ratio of 80:20 without loss of efficacy against *S. minor* has been demonstrated for a period of 4.5 months. The ability of Td_{22} to protect lettuce and pyrethrum plants from attack by this pathogen, coupled with its known effective colonisation of onion roots (Metcalf, 1997) implies that it might have the capacity to protect a spectrum of plants from attack by *S. minor.* The Td_{22} culture has clearly maintained excellent ecological competence despite its extended sub-culture over several years in the laboratory, as demonstrated by its competitive colonisation of plant roots following inoculation.

The estimated cost per hectare to control *S. minor* using suppressive mix produced in this study was in the range of AU$91.47 to AU$182.94 depending on the rate of its amendment. Millet seed was identified as a target for replacement by less expensive

materials, such as waste barley combs or cracked barley (by-products of beer manufacture). Such replacement would significantly reduce production costs of the biological control product, possibly making it an attractive alternative to chemical treatments.

Chapter 7

Potential of self-heating compost for minimizing indigenous microbiota prior to inoculation with bacterial antagonists.

7.1 Abstract

The objective of this study was to investigate the potential of utilizing the self-heating capacity of compost to minimize the indigenous mesophilic microbiota prior to inoculation and cultivation of specific mesophilic antagonists at 25°C. It was demonstrated that all inoculated bacterial antagonists reached high densities in cooled, inoculated matured fish waste compost, with two antagonists (*Pseudomonas corrugata* and *Lysobacter antibioticus*) reaching cell densities of ~10^{10} cfu/g dw after 14 days incubation at 25°C. These two antagonists appeared to exclude most indigenous microbiota to become the dominant cultivable bacteria in these composts. In a glasshouse trial the *L. antibioticus* or *P. corrugata*-inoculated fish waste compost significantly ($p<0.05$) protected lettuce plants from attack by *S. minor* with the degree of protection ranging from 40 to 55%, relative to the control treatment. The efficacy of this suppressive fish waste compost in a field trial (applied at the rate of 12.1 tonnes ha^{-1}) was comparable to a commercially available biological control agent (Companion®) applied as drench. The percentages of healthy plants in field plots treated with *L. antibioticus* or *P. corrugata*-inoculated fish waste compost at 5 or 8 weeks after planting was ~15-18% greater than those in control treatments plots, but not significant statistically ($p>0.05$).

The estimated production cost of the suppressive fish waste compost was AU$44.52 or AU$51.52 per m^3 depending on the N sources used in its enrichment. The cost per hectare to incorporate this product into a growing medium at the rate of 20% v/v for nursery lettuces to control *S. minor* was estimated to be AU$42.74 - AU$49.46, approximately 50% being less expensive than WFW-millet seed-based suppressive compost. This however must still compare poorly with that of chemical fungicides application, currently cost AU$181.80/hectare (field application instead of nursery application) (Chapter 6).

7.2 Introduction

As described in Chapter 5 that the use of compost-inoculated antagonists to reduce disease incidence in valuable crops has been reported or reviewed by many workers, such as, Hoitink and Fahy (1986), Logsdon (1993), Nelson *et al.* (1994), Craft and Nelson (1996), De Cuester and Hoitink (1999), and Lievens *et al.* (2001). In addition, application of compost teas (e.g. Mcquilken *et al.*, 1994; Elad and Shtienberg, 1994) as an alternative method of disease control has also been reported, although results have been variable and the science is often lacking.

Until recently, much of the work on biological control has focused on the mechanisms by which the inoculated antagonists control the pathogens. Only a few (Phae *et al.*, 1990; Hoitink, 1990, and Nakasaki *et al.*, 1998) have been related to low-cost suppressive compost production.

Costs associated with sterilization remain a major obstacle to the low-cost mass production of microbially-manipulated suppressive compost. Autoclaving at this scale of production is not practicable, and fumigation has problems of penetration to depth and of subsequent residual toxicity. As previously reviewed, the method developed by Nakasaki *et al.* (1998) appeared to open the way to exploring methods for minimizing or eliminating the indigenous microbiota in the compost prior to inoculation with specific antagonists, without resorting to sterilization. A key finding of the work of Nakaski *et al.* (1998) was that inoculation of compost substrate when the diversity and density of the indigenous microbiota was at the lowest (freshly cut grass) resulted in the proliferation of the desired bacterium, which survived subsequent thermophilic temperatures as spores, which germinated to provide effective biological control following soil application. This method however, is only applicable to the production of suppressive compost with spore-forming bacterial antagonists, because the temperatures of the thermophilic phase (of about 80°C) are lethal to vegetative bacteria and fungi, including fungal spores (Granatstein, 1998; Diver, 1998).

In the present study, the low diversity of microbiota found in the late thermophilic stage of fish waste compost was selected as a target for microbial manipulation. The method combined several facets that favoured the growth of inoculated biota over the indigenous (mainly thermophilic) biota. The compost was rapidly cooled to ambient temperatures, it was enriched with nutrients not found in the compost and known to be utilized by the desired bacteria, and it was heavily inoculated with active cultures of these

bacteria, growing on the 'novel' energy sources added to the compost, and in sufficient numbers to give them a numerical dominance over the residual native mesophilic microbiota. The advantage of following this avenue was that it allowed the cultivation of both spore-forming and non spore-forming bacterial biological control agents in the compost.

The objective of this study was to investigate the potential of self-heating compost for minimizing or eliminating indigenous microbiota prior to inoculation with specific antagonists, with a view to developing a method for the large-scale production of low-cost compost which was consistently inhibitory to plant pathogens.

7.3 Materials and methods

7.3.1 Fish waste compost

Hot fish waste compost (maturity stage) was kindly provided by Hazell Brothers Ltd. For this experiment samples from a fish-wood waste compost at the late-thermophilic stage were taken from a large-scale pile at the depth of ~30 cm from the pile surface, where temperatures were between 52-55°C.

7.3.2 Bacterial antagonists

Five bacterial antagonists of plant pathogens (*Pseudomonas corrugata*, *Lysobacter antibioticus*, *Bacillus thuringiensis*, *B. pumilus, and B. mojavensis*) isolated from various sources as described in Chapter 3 were used in this study. The long-term storage and maintenance of these isolates has been described in Chapter 3.

7.3.3 Preliminary study on the ability of bacterial antagonists to utilize various C and N sources

The ability of the five antagonists of plant pathogens to utilize various C sources (starch and casein) and N sources (ammonium nitrate and urea) was investigated in a preliminary study. The tests for casein, starch, and urea utilization were conducted using methods described in Sections 3.3.5.1.8, 3.3.5.1.9, and 3.3.5.1.10 (Chapter 3), respectively. Following these tests, the antagonists were investigated for growth response in a liquid basic mineral salt (BMS) medium (Appendix 1S) supplemented with a combination of C and N sources described above. Depending on the ability of isolates to utilize the above C and N sources, one loopfull of each was inoculated into 50 mL of

BMS medium plus starch (0.5% w/v) and urea (0.1% w/v); BMS medium plus NH_4NO_3 (0.5% w/v) and milk as a source of casein (5% milk v/v); or BMS medium plus urea (0.1% w/v) and milk as source of casein (5% v/v). All inoculated flasks were incubated under static condition at 25°C for three days and the samples were subjected to cfu assay on TSA (Appendix 1B) using the dilution plating method. The presence in milk of a variety of carbon and energy sources other than casein is acknowledged, as indicated in Appendix 4.

7.3.4 Inoculum preparation

The bacterial antagonists to be used in the production of suppressive fish waste compost were grown in a BMS medium supplemented with: NH_4NO_3 and milk (for the *L. antibioticus* and *B. pumilus*), urea and starch (for the *B. thuringiensis*), or milk and urea (for the *P. corrugata* and *B. mojavensis*). The choice of substrates was based on the results obtained in Section 7.3.3. The concentration of those materials in the medium was as described above. Yeast extract was added to the BMS medium at the rate of 0.1% (w/v) in this preparation to enhance the cell density of the antagonists. The initial pH of the media prior to inoculation was between 7.1 - 7.2. The inoculated flasks were incubated under static condition at 25°C for three days when high cell densities were reached. Prior to use, the cell densities of these inocula were determined using serial dilution and plating on TSA.

7.3.5 Inoculation of fish waste compost and counting of antagonists

Fresh, hot, fish waste compost material was allowed to cool to room temperature (taking approximately one hour) and replicate 100g samples aseptically transferred to plastic bags. The samples (in triplicate) were then saturated for 15 minutes with inocula, previously diluted with the same fresh medium in a ratio of 1:4 (as in Section 7.3.4), drained, and incubated at 25°C for one month. The samples were assessed at weekly intervals for cfu on TSA and water content. The identities of the antagonists in these non-sterile composts were confirmed by comparing colony morphologies on TSA with those of the corresponding antagonists. If necessary, Gram staining or testing of biochemical reactions, such as their ability to utilize casein, urea, or starch was also undertaken. To investigate whether the inoculated *Bacillus* isolates sporulated throughout the experiment,

the dilutions were heated in a water bath at 80°C for 30 minutes, plated on TSA, and incubated at 25°C for two to seven days.

7.3.6 Glasshouse trial

The unspent suppressive fish waste compost (after cfu and water content determinations) as described in Section 7.3.5 was tested for its efficacy in protecting lettuce seedlings/plants from attack by *S. minor* in a shade-house experiment. The compost was mixed with soil to give a final concentration of 5% w/w (dry weight basis) (Nakasaki *et al.,* 1998), and dispensed into 0.5 L capacity pots. The pathogen (*S. minor*) (prepared as in Section 6.3.5, Chapter 6) was evenly spread approximately 20 mm from the mix surface at the rate of 1g/pot. Pots with no pathogen and suppressive compost amendment served as a nil control, while those inoculated with the pathogen only served as the control treatment. Following addition of the pathogen but prior to sowing, all pots were pre-incubated in the shade house for four days. Each treatment consisted of five replicate pots with four x two-week-old lettuce seedlings per pot. Healthy seedlings/plants were scored at weekly intervals up to four weeks after planting. The trial was terminated after eight weeks and the antagonists were re-isolated and enumerated from healthy plant roots following dilution plating on TSA.

7.3.7 Field trial

The efficacy of the suppressive fish waste compost in protecting lettuces against *S. minor* was assessed in a field trial at a lettuce farm (Houston's) at Cambridge (30 km from Hobart), in March 2002, where a severe *S. minor* attack or infection on lettuce plants has been previously observed. The suppressive fish waste compost modified by the directed growth of either *P. corrugata* or *L. antibioticus* (prepared as in Section 7.3.4), was applied at the rate of 12.1 tonnes/ha. Direct addition of cell suspensions (approximately 10^7 cfu/mL) of these antagonists and of *B. polymyxa* (a stock culture of the school of Agriculture, the University of Tasmania-Australia) was also assessed. A commercially available biological control agent (Companion®, known to contain a *B. subtilis* strain) and a combination of Companion with a chemical fungicide (Sumisclex®) were also included in the trial.

The suppressive fish waste compost was applied at one week before planting, while the other preparations were drenched to the plant roots just before planting. A

randomised block design was employed using 1.0 m^2 plots on raised beds with five replicate plots per treatment. Twelve x two-week-old lettuce seedlings were planted per plot. The percentage of healthy plants in each plot was assessed at five and eight weeks after planting. The treatments for this field trial are summarized in Table 7-1.

Table 7-1: Treatment list of the Cambridge field trial.

No.	Treatment	Rate	Application method
1	Untreated control	N/A	N/A
2	*Pseudomonas corrugata* in compost	12.1 tons/ha	Applied at one week before planting
3	*Lysobacter antibioticus* in compost	12.1 tons/ha	Applied at one week before planting
4	*B. polymyxa* suspension	10^7 cfu/mL (2L/plot[a])	Drenched just before planting
5	*P. corrugata* suspension	10^7 cfu/mL (2L/plot[a])	Drenched just before planting
6	*L. antibioticus* suspension	10^7 cfu/mL (2L/plot[a])	Drenched just before planting
7	Companion®	10.5mL/10L (2L/plot[a])	Drenched just before planting
8	Sumisclex® + Companion®	(50mL+ 10.5mL)/10L (2L/plot[a])	Drenched just before planting
9	Uninoculated fish waste compost.	12.1 tons/ha	Applied at one week before planting

[a]Equivalent to 20 tonnes drench/ha

7.3.8 Statistical analysis

Analysis of variance (ANOVA) of data obtained in this study was conducted using Minitab for windows software as described in Chapter 4.

7.4 Results

7.4.1 Preliminary investigations on the utilization of various C and N sources by microbial antagonists of plant pathogens.

A preliminary investigation was undertaken to determine which potential substrates could be utilized by the antagonists of *S. minor*. The results are presented in Table 7-2. *B. thuringiensis* was found to utilize both starch and casein as C sources and urea as an N source. All isolates metabolised casein as a C source, while three of the five isolates utilized urea as a N source.

Table 7-2: Utilization of starch, casein, or urea by bacterial antagonists.

Antagonists	Starch	Casein	Urea
Lysobacter antibioticus	-	+	-
Pseudomonas corrugata	-	+	+
Bacillus thuringiensis	+	+	+
B. pumilus	-	+	-
B. mojavensis	-	+	+

For isolates incapable of utilizing urea as an N source, BMS medium was amended with NH_4NO_3. The results are presented in Table 7-3.

Table 7-3: The relative density of the antagonists in various modified BMS medium.

Antagonists	Initial density Log_{10} cfu/mL	Density after 72 h (log_{10} cfu/mL)	Media
L. antibioticus	5.3 ± 0.01	9.2 ± 0.06	BMS+NH_4NO_3+milk
P. corrugata	5.3 ± 0.15	8.4 ± 0.04	BMS+urea+milk
B. thuringiensis	5.1 ± 005	7.3 ± 0.06	BMS+urea+starch
B. pumilus	5.7 ± 0.01	9.3 ± 0.02	BMS+NH_4NO_3+milk
B. mojavensis	4.8 ± 0.10	7.7 ± 0.01	BMS+urea+milk

¥Each value is an average of duplicate ± standard error.

As indicated in Table 7-3, the antagonists grew to high densities (10^7-10^9 cells/mL) in a liquid BMS medium amended with appropriate C and N sources after three days incubation at 25°C.

The growth rate of *B. thuringiensis* in BMS medium containing urea and milk or of *P. corrugata* in BMS medium containing NH_4NO_3 and milk was found to be lower than that shown in Table 7-3 (data not shown). Based on these results, the BMS medium amended with C and N sources as described in Table 7-3 was used in the preparation of inocula for the production of suppressive fish waste compost in further studies.

7.4.2 Growth responses of the bacterial antagonists in amended fish waste compost under non-sterile conditions

The growth of the antagonists and of the mesophilic indigenous bacteria in fish waste compost at 25°C are presented in Figure 7-1. All isolates grew well in this compost and reached densities of between 7.7 $\log_{10}$ cfu/g dw and 9.5 $\log_{10}$ cfu/g dw after one month of incubation (Figure 7-1A). Two antagonists (*P. corrugata* and *L. antibioticus*) showed the best growth response in the compost, reaching ~10^{10} cfu/g dw after 14 days incubation to the exclusion of most of the indigenous microbiota. By the end of the experiment they were present as apparent monocultures in the compost, since no background indigenous microbiota were observed on serial dilution TSA plates of compost samples. The population densities of the background of the indigenous microbiota in uninoculated compost ranged from 8.0 $\log_{10}$ cfu/g dw to 9.6 $\log_{10}$ cfu/g dw, tending to decrease from peaks reached after14 days as the incubation progressed (Figure 7-1B).

In vitro inhibition of some culturable indigenous mesophilic bacteria by *L. antibioticus* or *P. corrugata* on TSA, which probably caused their exclusion from the compost, is demonstrated in Plate7-1.

All but one isolate were inhibited by diffusible inhibitors produced by these antagonists. A cell free extract of *P. corrugata* was also demonstrated to be inhibitory *in vitro* to *S. minor* as well as to a an indigenous bacterium of fish waste compost (Plate 7-2). However the cell free extract of the *L. antibioticus* was not inhibitory to the same tested microorganisms, possibly due to their loss by evaporation on extraction.

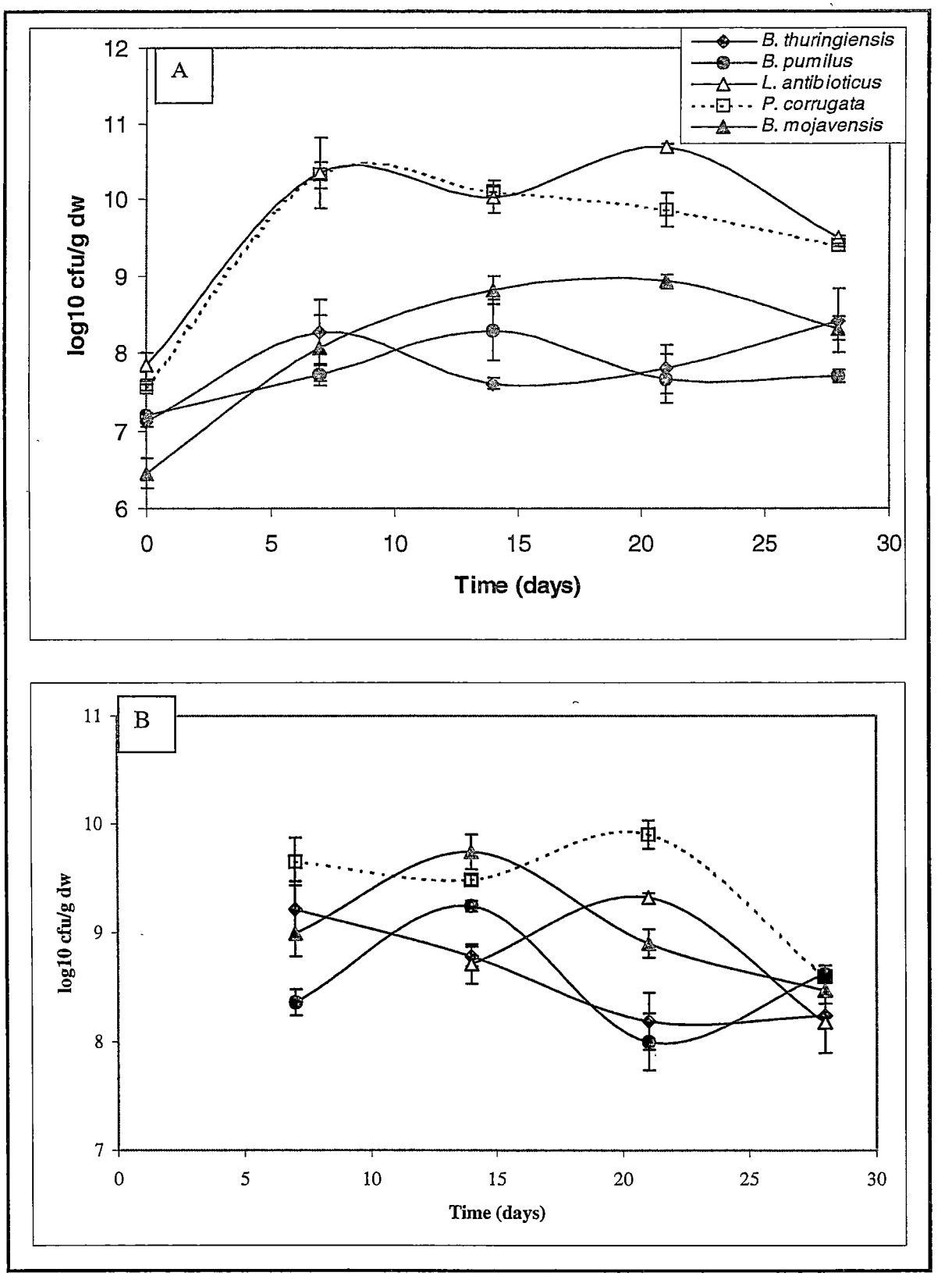


Figure 7-1: The growth response of the bacterial antagonist (A) and of the background indigenous mesophilic bacteria (B) in non-sterile fish waste composts. Each value is an average of triplicates ± standard error.

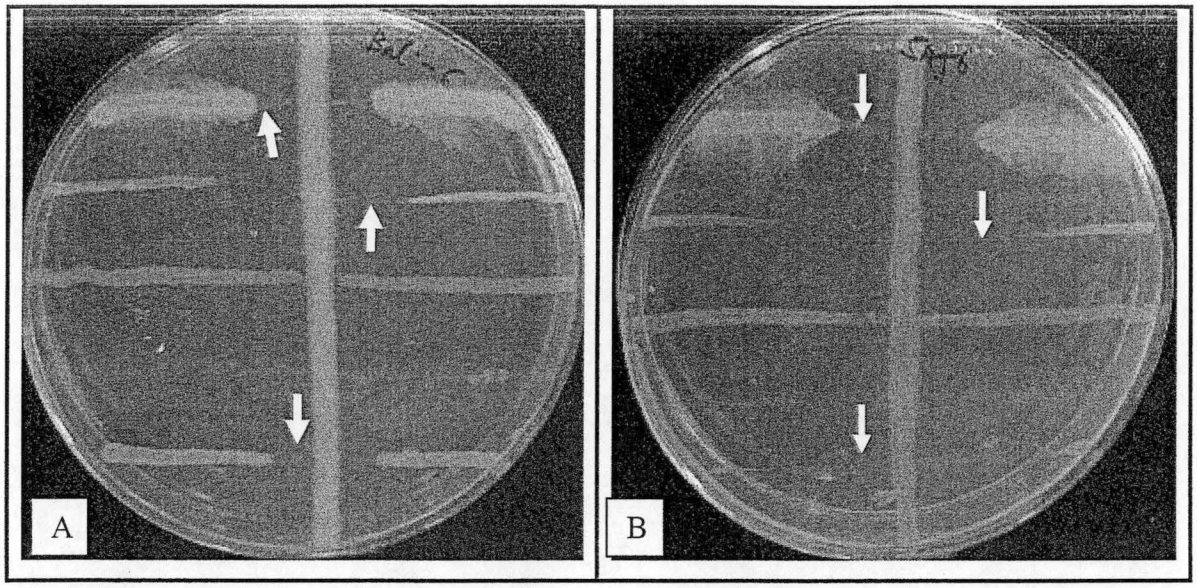


Plate 7-1: Antagonism of *L. antibioticus* (vertical streak left photo) or *P. corrugata* (vertical streak right photo) against some dominant cultivable aerobic bacterial isolates (horizontal streaks) from fish waste compost. The growth medium is TSA incubation was for seven days. Arrowheads show the growth inhibition of the culturable indigenous bacteria by the antagonists.

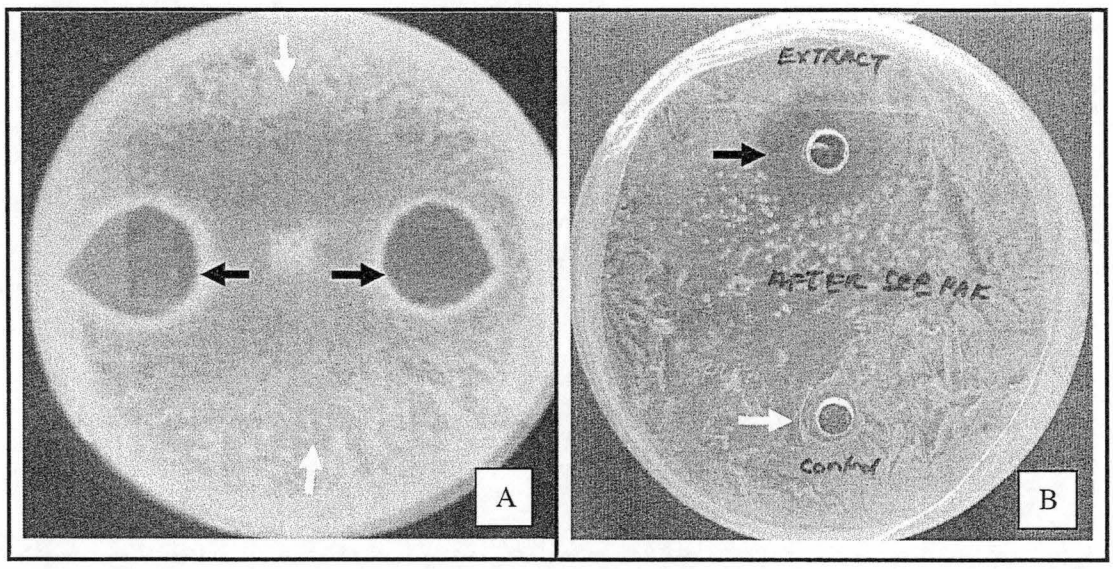


Plate 7-2: Inhibition on the growth of *S. minor* (A) and of a bacterium (B) isolated from fish waste compost, by the cell-free extract of *P. corrugata* on PDA and on TSA, respectively. Black arrowheads show inhibition zone of the fungus and of the bacterium following application of cell-free extract of *P. corrugata*. No inhibition was observed when solvent only (control) was deposited on either fungal or bacterial lawn (white arrow head).

All of the *Bacillus* spp. (*B. thuringiensis, B. pumilus*, and *B. mojavensis*) were found to sporulate in the fish waste compost with spore densities ranging from 95% to 99% of their total count (Figure 7-1) following 14-28 days incubation.

7.4.3 Glasshouse trial

The effectiveness of the suppressive fish waste compost in protecting lettuce seedlings/plants from attack by *Sclerotinia minor* at four weeks after sowing is shown in Figure 7-2.

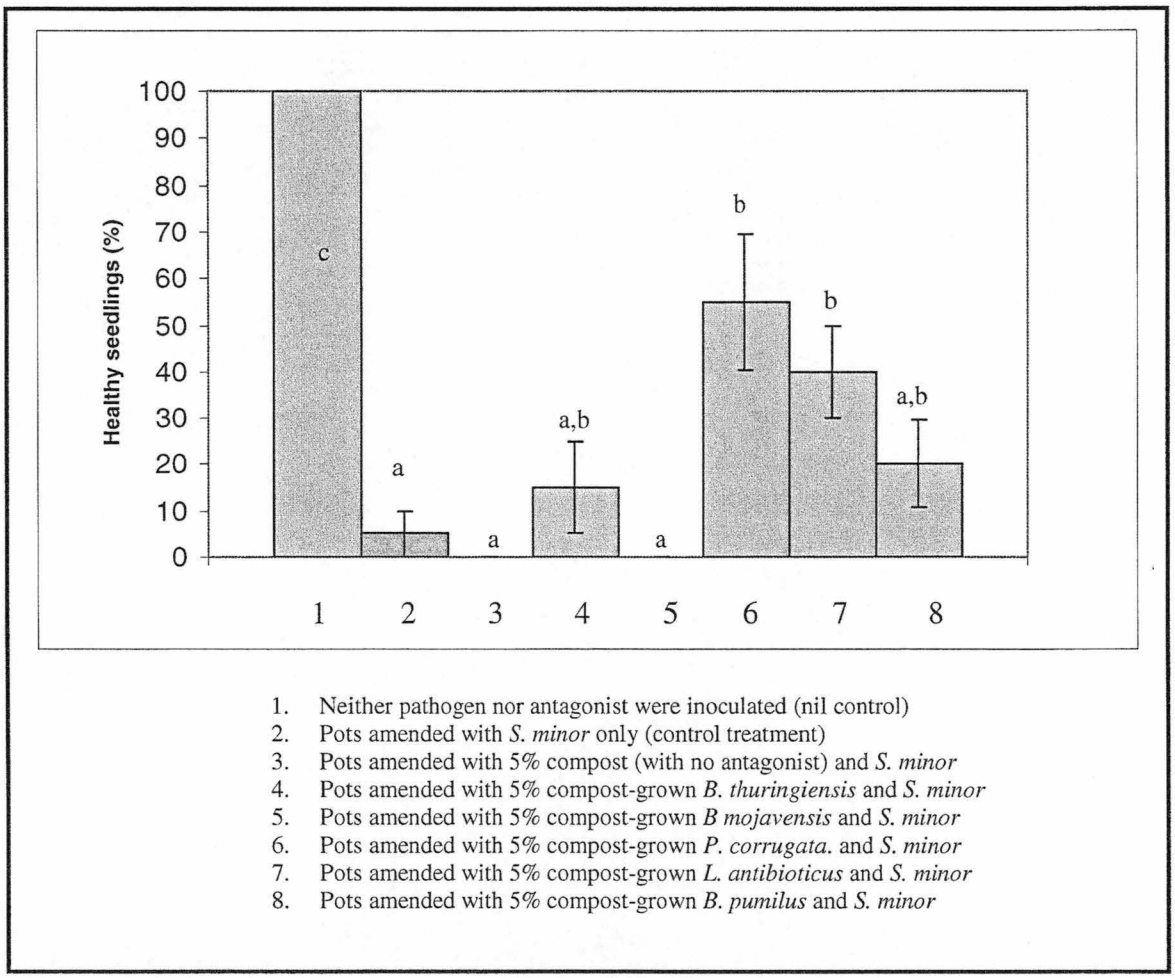


Figure 7-2: Protection of lettuce seedlings/plants from attack by *S. minor* by various bacterial biological control agents grown in fish waste compost four weeks after sowing. Each value ± standard error is an average of five replicate pots, each containing four seedlings/plants. Bars with the same letter(s) are not significantly different (p>0.05) according to the lsd test following ANOVA.

A significant ($p<0.05$ relative to controls) protection against fungal attack was provided by *L. antibioticus* and *P. corrugata* composts four weeks after sowing (Figure 7-2, Plate 7-3). Between 95% and 100% mortality was observed in control treatments after four weeks, compared with 45% and 60% mortality in pots amended with *P. corrugata* and *L. antibioticus* composts. The effectiveness of the compost-grown *P. corrugata* or *L. antibioticus* in protecting lettuce plants from *S. minor* attack, was better than that reported for these bacteria cultivated in composted WFW and millet seed of 15-20% (Chapter 5A), making fish-waste compost the preferred substrate for the cultivation of these bacteria, from the perspectives of both cost and effectiveness.

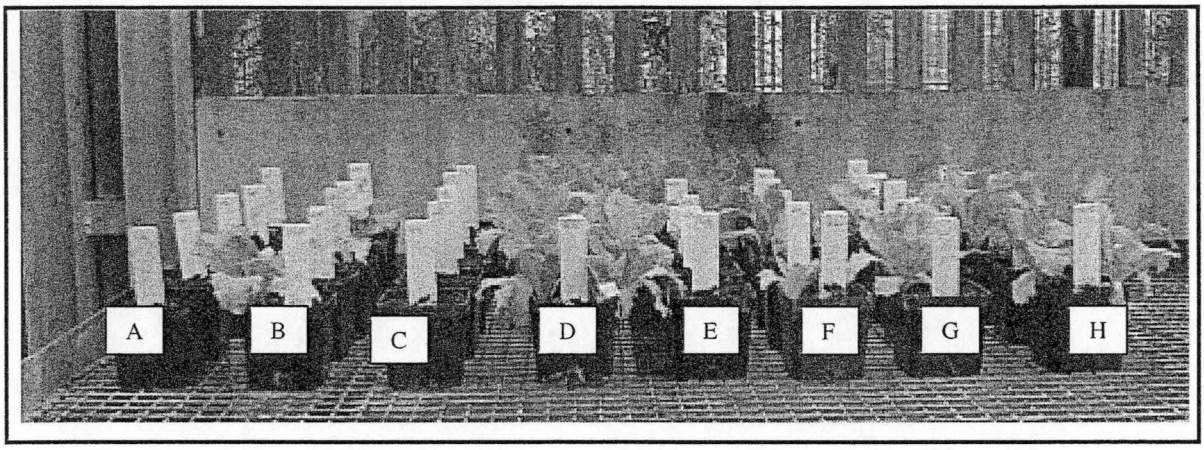


Plate 7-3: The relative protection of lettuce plants in a trial of bacterial-amended fish-waste compost (5% w/w) at seven weeks after planting. *S. minor* inoculum (1.0g/pot) was evenly spread at 20 mm from the mix surface four days before planting. Pots in rows from left to right are amended with: compost-grown *B. mojavensis* and *S. minor*; compost-grown *B. thuringiensis* and *S. minor*; compost control and *S. minor*; nil compost control and *S. minor* (nil control); *S. minor* only (control treatment); compost-grown *B. pumilus* and *S. minor*; compost-grown *L. antibioticus* and *S. minor*; and compost-grown *P. corrugata* and *S. minor*.

In a repeated pot trial (data not shown), addition of compost-grown *L. antibioticus* or *P. corrugata* consistently improved the survival percentage of healthy plants compared with the *S. minor* control (pots inoculated with *S. minor* only), but (in contrast to the above experiment) this protection was not significantly different to the protection provided by fish-waste compost in the absence of bacterial amendment. A combined

application of compost-grown *L. antibioticus* and *P. corrugata* at the same rate (2.5% w/w each) did not improve the resultant protection provided (data not shown).

Re-isolation of the antagonists from surviving plants eight weeks after planting showed significant numbers of some of the antagonists colonizing root segments (Figure 7-3), indicating that they possessed ecological competences in the rhizoplane of lettuce plants.

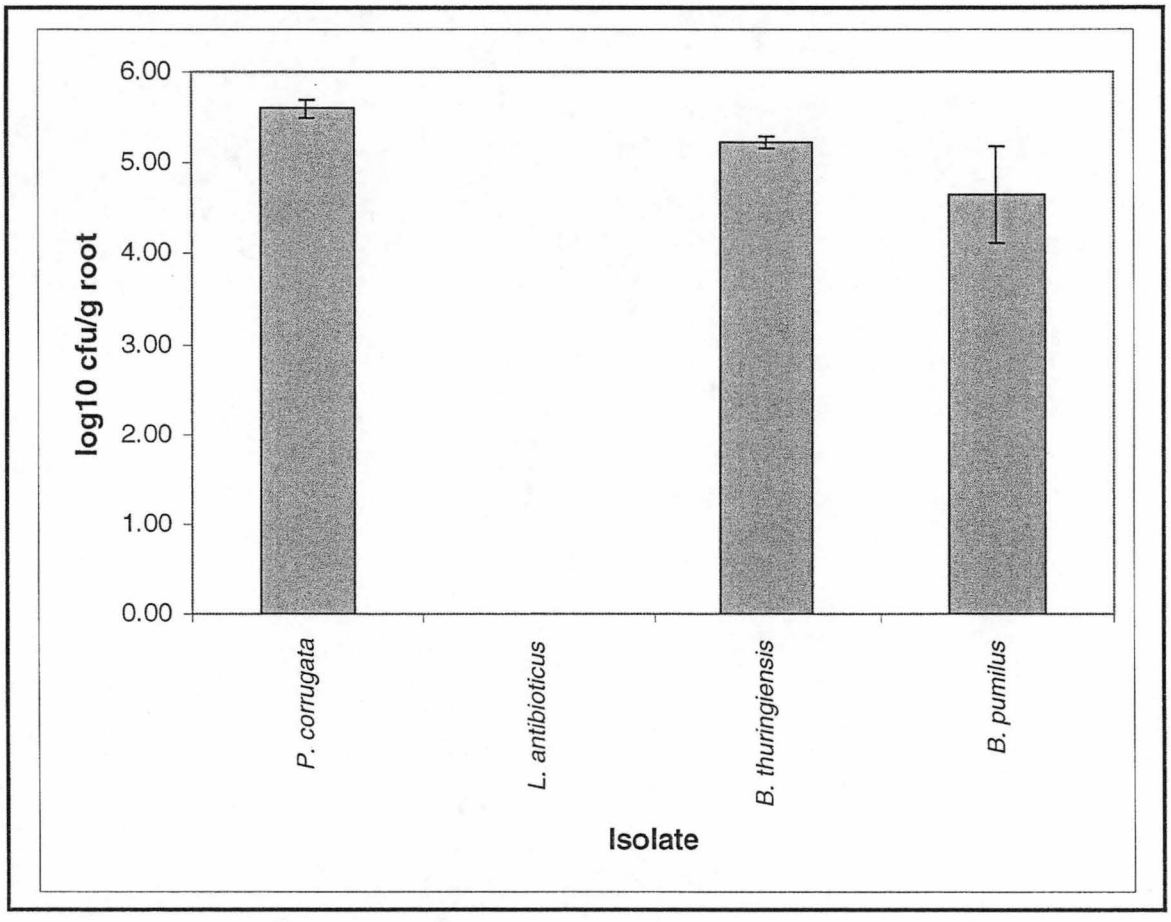


Figure 7-3: The relative abundance of some bacterial antagonists re-isolated from root segments of surviving lettuce plants eight weeks after planting. Each bar value is an average of three (*B. pumilus*) or five (*B. thuringiensis, P. corrugata*, and *L. antibioticus*) replicates ± standard error (depending on the number of surviving plants).

P. corrugata, *B. thuringiensis*, and *B. pumilus* appeared at densities on lettuce roots ranging from 4.7 – 5.6 $\log_{10}$ cfu/g root material at eight weeks after planting (Figure 7-2). No evidence of *L. antibioticus* survival was observed (Figure 7-2) at this time.

7.4.4 Assessment of the biological control of *S. minor* infection of lettuces in a field trial.

The relative survival of healthy plants in plots treated with various biological control agents was assessed at five and eight weeks after planting is presented in Table 7-4.

Table 7-4: The survival of healthy plants at five and eight weeks after planting.

Treatment	Percentage of healthy plants[¥]	
	Week 5	Week 8
Untreated control	75.0 ± 7.5 a	60.0 ± 11.3 ab
P. corrugata in compost	88.3 ± 4.3 ab	78.3 ± 2.0 b
L. antibioticus in compost	88.3 ± 4.3 ab	75.3 ± 8.3 abc
B. polymyxa suspension	79.7 ± 7.7 a	65.8 ± 10.6 ab
P. corrugata suspension	78.3 ± 6.8 a	68.3 ± 8.1 ab
L. antibioticus suspension	76.7 ± 3.1 a	70.0 ± 3.3 a
Companion®	83.3 ± 7.5 ab	68.3 ± 6.7 a
Sumisclex® + Companion®	98.3 ± 1.7 b	93.3 ± 3.1 c
Un-inoculated fish waste compost.	76.7 ± 3.1 a	60.0 ± 4.9 a

[¥]Each value is an average of five replicates ± standard error. Values in the same column followed by the same letter(s) are not significant statistically at $p<0.05$, according to lsd test following ANOVA.

From Table 7-4 it is apparent that only the treatment of Sumisclex® plus Companion® was significantly ($p<0.05$) better at protecting against *S. minor* infection than the control treatments at 5 and 8 weeks after sowing.

7.4.5 Economic analysis of suppressive fish waste compost application compared to chemical fungicides

The current cost of producing 1 m^3 of mature fish waste compost was estimated at AU$35.00 (Mike Pilcher, personal communication). Assuming 1 m^3 of mature fish waste compost with 40% moisture content weights ~0.5 tonnes and the same nutrient amendments are made as described above, then one m^3 fish waste compost for e.g. *P. corrugata* amendment will contain ~0.4 kg urea and ~19 L of milk. Assuming the costs of the amendment of mineral elements (e.g. crude of $FeCl_3$, K_2HPO_4, KH_2PO_4, $MgSO_4$, and $CaCl_2$) and milk waste product amounts to AU$4.83 (Simon Hills, Personal communication) and ~AU$2.53 (Reynolds, 2002), respectively, and the current price of the urea is AU$0.40 per kg (http://www.irri.org/Troprice/Ecomonics.htm), the total cost to produce 1 m^3 of enriched fish waste compost to be used for the cultivation of this antagonist will be ~AU$42.52. If the cost of inoculation is the same as that described in Chapter 6, the cost of 1 m^3 suppressive compost (fish waste compost-grown bacterial antagonist) rises to AU$44.52. This is less than half as expensive as that of WFW and millet-based suppressive compost production reported in Chapter 6.

If ammonium nitrate (NH_4NO_3) solution (0.5% w/v) is used to enrich the compost, the production cost will increase by ~AU$7.00 per m^3. This is because ~2 kg of NH_4NO_3 (35% N) per m^3 compost is needed. The current price of the NH_4NO_3 (in bulk) is AU$3.52 per kg (http://www.gilgames.com.au/offers/AmmoniumNitrate.html). This however, is still ~47% less expensive than WFW and millet seed-based suppressive compost production (Chapter 6). Application of modified suppressive fish waste compost at the nursery level (as described in Chapter 6) to control *S. minor* would reduce the cost by the same margins.

If antagonist suspensions (of equivalent density) in trypticase soya broth (TSB) at the rate of 10% (v/v) are used to inoculate the lettuce growing media, 480 L antagonist suspension will be required, as 4.8 m^3 growing media are needed to produce lettuce seedlings to be planted in 1 ha area (as described in Chapter 6). Assume 0.5% w/v TSB (normally used to grow bacteria *in vitro*) is used to prepare the antagonist suspension, 2.4 kg TSB will be needed. Therefore, the cost to apply the microbes as a TSB culture is several folds more expensive than the application of microbes grown in fish waste compost, as the current price of the TSB is AU$90/500g (Sigma®).

7.5 Discussion

A variety of factors contributed to the directed microbial growth of the desired bacterial antagonists in non-sterile compost;

i) The indigenous biota was subjected to temperature shock, with a rapid shift from thermophilic (58°C) to mesophilic (25°C) conditions, resulting in a low abundance of biota capable of competitive growth at the lower temperature. The cool-down phase of composting has been recognized as a prime target for inoculation, when competition by indigenous microbiota is lowest (Hoitink *et al.*, 1996; Granatstein, 1998; and Nakasaki *et al.*, 1998).

ii) The provision of low-cost C-sources not found in traditional compost, such as starch or casein, known to be utilized by the antagonists.

iii) The provision of low-cost nitrogenous amendments particularly urea to limit the growth of indigenous biota lacking urease enzymes.

iv) The provision of heavy inocula of actively growing antagonists, primed to utilize the 'novel' substrates that have been added to the compost mix.

These strategies were found to improve the establishment of the inoculated antagonists in the fish waste compost (Figure 7-1A), some of which reached high cell densities in near monoculture conditions after 14 days or more incubation. Enrichment of compost prior to inoculation with specific antagonists has also been reported by workers such as Hoitink (1990), Steinmetz and Schönbeck (1994), and Ramona and Line (2002). In most publications, a significant growth improvement of the inoculated antagonists was reported following this nutrient amendment into compost (or in agreement with the results reported in the present study). This indicates that nutrient amendment into compost prior to inoculation with specific antagonists is one of the most important factors that determine the successfulness of the antagonist establishment in such compost.

As discussed in Chapters 5 and 5A, minimizing the indigenous microbiota in compost prior to inoculation with potential antagonists is also crucial to achieving high numbers of the inoculum culture. A number of researchers have resorted to compost sterilization prior to inoculation (Hoitink *et al.*, 1997). This includes the use of gamma radiation by Phae and Shoda (1990) prior to inoculation with high density of antagonist inoculum, although they finally failed to grow their antagonists in that compost.

There have been a few reports (of mixed success) describing the cultivation of desired microorganisms in compost under non-sterile conditions as outlined in Chapter 2. Hoitink (1990) manufactured suppressive compost by inoculating 44-week old compost during the cool-down phase, although he admitted to limitations of this method, particularly as being impracticable for commercial operations. Ramamurty *et al.* (1996) reported that composting of eucalypt sawdust for 3-4 months at 25-45°C following inoculation with the mushroom fungus *Volvariella* resulted in an enhanced growth of wheat seedlings, although the cause of growth promotion was not determined. In a more recent study Nakasaki *et al.* (1998) achieved an outstanding method for a suppressive compost production using a *B. subtilis* strain as the biological control agent, but this method is only applicable for the cultivation of spore-forming bacterial biological control agents, the spores surviving the thermal phase of composting. In contrast, the method developed in the present study allowed the cultivation of both spore and non-spore forming bacterial antagonists to high densities and at moderate cost.

The *in vitro* demonstration of the production of inhibitory compounds by *L. antibioticus* and *P. corrugata* (Plate 7-1) might explain their ability to exclude most indigenous microbiota and become the predominant biota in the compost (Figure 7-1). Inhibition of *S. minor* and a compost bacterium by a cell free extract of the *P. corrugata* (plate 7-2) indicated antibiosis to be the most likely mechanism involved. *P. corrugata* was also found to inhibit *S. minor in vitro* both on the $FeCl_3$-amended TSA (at the concentration of 100 μM and 1000 μM) and on TSA without $FeCl_3$ amendment (data not shown), indicating that siderophores did not play any role in this biological control mechanism. The isolation and screening of active compounds produced by *P. corrugata* is reported in Chapter 8. In the case of *L. antibioticus*, isolation of its active compound was unsuccessful. However, the viable cells of this antagonist was found to inhibit *S. minor* when challenged *in vitro* both on the $FeCl_3$-amended TSA (at the concentration of 100 μM and 1000 μM) and on TSA without $FeCl_3$ amendment, indicating that the mechanism of disease control by this antagonist was probably similar to that by the *P. corrugata.* The ability of *P. corrugata* and *Lysobacter* spp. to produce active compounds inhibitory to fungal or bacterial pathogens have also been reported by Chun (2000) and Hashizume *et al.* (2001), respectively. They used these antagonists to control plant pathogens, but the compounds involved in the biological control were not reported.

In a glasshouse trial, some antagonists found to be effective against *S. minor* in a non-replicated pot trial (Chapter 3), failed to produce positive results in this trial (Figure 7-2). *B. mojavensis* for example failed to protect against *S. minor* in this trial (bar 5 Figure 7-2), although its density in the fish waste compost was high (Figure 7-1). One possible reason for this inconsistent result is that this antagonist lost antibiotic production and competitiveness following subculture on nutrient medium (Weller, 1988), as described in Chapter 2. Also, since ~98% of the *B. mojavensis* population consisted of spores (dormant form) in the compost inoculum, a delay in their germination may have allowed sufficient time for the *S. minor* to become pathogenic.

The ability of some antagonists to colonise plant roots (being re-isolated from this source eight weeks after planting) indicated their potential as biological control agents. In the case of *L. antibioticus* where there was no evidence of root colonisation eight weeks after planting, the mechanism of protection is less clear. It is established that this bacterium produced antimicrobial compounds, which are normally only effective in the microenvironment of the root surface; hence an alternative to be considered is that the antagonist may have induced systemic acquired resistance to the plant.

Application of fish waste compost-grown *L. antibioticus* or *P. corrugata* at the rate of 12.1 tonnes/ha in the field trial gave encouraging results, with a relatively lower disease incidence when compared to the control treatment at five and eight weeks after planting. It was observed that the percentage of healthy plants in the plots treated with either fish waste compost-grown *L. antibioticus* or *P. corrugata* was 15 to 18% higher than that in the control treatment plots eight weeks after planting (Table 7-2), but not significantly different statistically ($P>0.05$). The amount of the suppressive compost applied in this field trial may have been too low to give a good disease control in view of the levels reported by the US environmental protection agency (US EPA, 1997) and Granatstein (1998) of between 89 and 178 tonnes/ha of composted solid waste used to combat *Rhizoctonia*-related disease. However application of the suppressive compost at these rates appears to be economically untenable relative to chemical fungicide application. Similar to the Td_{22}-grown WFW compost as described in Chapter 6, a niche for this product may be found at the nursery level where amendments up to 60% (v/v) in the growing medium are practicable. The establishment of protective biota in the root region of nursery plants could possibly provide residual protection over a critical period in the field.

7.6 Conclusions

It has been demonstrated that a combination of strategies has enabled a shift of balance away from indigenous compost microbiota towards microbiota of practicable utility to agriculturalists. Although previous researchers have achieved this in specific cases (particularly sporing *Bacillus* spp.), the current study has extended the range of desirable organisms that can be so cultivated, opening the way for wide-ranging developments. The production cost of suppressive fish waste compost was estimated to be AU$44.52 or AU$51.52 per m^3 depending on the N source used. Direct application of this product in the field to control plant pathogen (*S. minor*) at rates recommended by US EPA (1997) and Granatstein (1998) were economically impracticable, but they may have application at the nursery level, providing important residual biological control following transplantation to the field.

Chapter 7A

Assessment of the biological control of a foliar mildew disease in zucchini plants with suspensions of biological control agents

7A.1 Abstract

The objective of this study was to investigate the effectiveness of some biological control agents prepared as cell suspensions in trypticase soya broth (TSB) for the bacterial agents *Lysobacter antibioticus* and *Pseudomonas corrugata*, or in saline solution (0.85% w/v NaCl) for the fungus *Trichoderma* spp, isolate Td_{22} for controlling a foliar mildew disease (tentatively identified as downy mildew) in zucchini plants. If successful this would provide an advance on the traditional use of 'compost teas' for foliar disease control, since the primary compost would contain known antimicrobial biota rather than an unknown biota. It was found that between 22 and 83% protection ($p<005$) was provided by different antagonists following pathogen challenge relative to the pathogen control treatments after two weeks. However, the level of protection declined as the trial progressed, with between 46% and 60% of all leaves being infected regardless of treatment five weeks after the pathogen challenge. Application of Td_{22} (the best-performing antagonist) in combination with *L. antibioticus* or *P. corrugata* (or with both bacterial antagonists together) only reduced the nett effectiveness of the fungus.

The survival of the biological control agents on the surface of the leaves was very poor, although other reports have shown that survival need not be necessary to lasting control. The effect of these biota on the induction of systemic acquired resistance in target crops (such as grapes and poppies) has yet to be determined. If biological control treatments were to be implemented in commercial crops regular application may be required to maintain protection against this mildew. The best of these antagonists, Td_{22}, gave a level of protection which, according to literature reports, was equivalent to that provided by chemical treatments.

7A.2 Introduction

Downy mildew is a common foliar disease of plants, such as cucumber, grapes, cantaloupe, and all plants in the group of cucurbits. The disease is caused by fungi including *Plasmopara viticola* (Partridge, 1997; Kiefer *et al.*, 2002; Cohen *et al.*, 2003), *Peronospora trifoliorum* (Obert *et al.*, 2000; VanDyk, 2002), *P. viciae* (Pung and Cross, 2003), *P. destructor* (Gilles and Kennedy (2003), or *Pseudoperonospora cubensis* (Kuepper, 2000; Ishii, 2003). The pathogens attack all green parts of the plants, particularly the leaves. Downy mildew is characterized by pale–green, yellowish to brownish areas of irregular size and shape (oval to cylindrical) on the surface of infected leaves (Kuepper, 2000). The disease spreads rapidly via water splash or wind, and in some cases it may result in defoliation, stunted growth, or poor fruit development in the infected plants and severely infected plants may be killed (Kucharek, 1994).

Reported control of downy mildew disease has been provided by the use of crop rotation (Watson and Napier, 2001), chemical fungicides (Ries, 1996; Kuepper, 2000), irrigation management (Kuepper, 2002), or the use of resistant plant varieties (Watson and Napier, 2001; Kuepper, 2000). Kucharek (1994) and Kuepper (2000) have reported that the use of resistant plants in combination with fungicide application has been the favoured method of control in recent years. With the move away from the chemical fungicide application, the use of biological alternatives (e.g. 'compost teas') has received increased attention for the control of foliar diseases including downy mildew (Weltzein, 1991; Diver, 1998[a]). The efficacy of the 'compost teas' in controlling foliar pathogens is largely attributable to the presence of microbial antagonists (Brinton, 1995), although Diver (1998[a]) and Hoitink and Gardener (2003) have noted that its effectiveness and consistency in controlling foliar pathogens can be enhanced by inoculation with specific beneficial antagonists.

In the present study, the effectiveness of bacterial suspensions of *Lysobacter antibioticus* and *Pseudomonas corrugata* in TSB, and of a fungal spore suspension of Td_{22} in saline was assessed for the control of mildew, tentatively identified as downy mildew, in zucchini plants. The aim of this study is the development of 'compost teas' or their equivalents using microbial antagonists such as those described above as the active agents for the treatment of important Tasmanian commercial crops such as poppies and

grapes. The antagonists used in this study were found to grow well and reach high densities in the WFW or fish waste compost as reported in Chapter 5, 5A, and 7.

7A.3 Materials and methods

7A.3.1 Biological control agents

Two bacterial antagonists (*L. antibioticus* and *P. corrugata*) and the fungal antagonist Td_{22}, found to be effective to control *S. minor* in lettuce plants (Chapters 6 and 7), were investigated for effectiveness in controlling a foliar mildew disease (possibly downy mildew) in zucchini plants. The origin and maintenance of these antagonists has been described in Chapter 3.

7A.3.2 Foliar pathogen

The pathogen was obtained from infected leaves of grape, collected from the horticultural research centre (HRC), University of Tasmania, Australia. It was tentatively identified as downy mildew on the basis of characteristic leaf lesions, although according to one expert, zucchini plants should not be susceptible to downy mildew, as was found in this study. Confirmation of the disease is pending, however it will be referred to as downy mildew in subsequent text. Because the causative agents of downy mildew are obligate parasites, the infected leaves were collected immediately prior to the preparation of pathogen suspension in saline solution.

7A.3.3 Zucchini seeds

Zucchini seeds 'Blackjack' (Yates®) was purchased from Roberts Limited, Australia.

7A.3.4 Preparation of antagonist suspensions

The bacterial antagonists mentioned above were grown in 0.5% (w/v) trypticase soya broth (Appendix 1B minus agar) for 48 hours at 25°C under static condition. At this time the cell density of these antagonist was ~10^8 cells/mL. The spores of the Td_{22} were harvested from WFW compost-grown Td_{22} (unspent compost from lettuce or pyrethrum trials) by shaking this compost in saline solution (1:10 w/v) for ~10 minutes prior to use.

7A.3.5 Preparation of pathogen suspension

Infected grape leaves (~10 g) were added to 200 mL of sterile saline (0.85% NaCl) and shaken vigorously to release the pathogen.

7A.3.6 Pot trial

Zucchini seeds were sown in 1.5 L pots containing steam-sterilised standard potting mixture (Appendix 1A). After 14 days, the leaves of the seedlings were spray inoculated with antagonist suspensions prepared above (Section 7A.3.4). Combinations of *L. antibioticus* + *P. corrugata*, *L. antibioticus* + Td_{22}, *P. corrugata* + Td_{22}, and *L. antibioticus* + *P. corrugata* + Td_{22} (in equal mix) were also included in the trial. The pathogen suspension (Section 7A.3.4) was sprayed on plant leaves three days after the antagonist application. Each treatment consisted of five replicate pots with one plant (at approximately 10 cm height) in each pot. Plants sprayed with pathogen only or with saline solution only (pathogen and antagonist free) served as controls. Pots were maintained in a shade house for eight weeks with assessment of infection at two and five weeks after the pathogen introduction. To avoid cross contamination, the nil control pots (A0B0) were placed away from those sprayed with the pathogen. The disease severity index of the infected leaves was also rated on a scale of 0 to 5 (Nakasaki *et al.*, 1988), where 0 is asymptomatic, 1 = 1 to ≤ 20% leaf area being symptomatic, 2 = 21 to ≤ 40% leaf area being symptomatic, 3 = 41 to ≤ 60% leaf area being symptomatic, 4 = 61 to ≤ 80% leaf area being symptomatic, and 5 = 81 to 100% leaf area being symptomatic.

7A.3.7 Establishment of the biological control agents on the leaves

The trial was terminated after six weeks (six weeks after the pathogen introduction) for attempted re-isolation of the antagonists from randomly-selected uninfected leaves. To assess the establishment of bacterial antagonists, 10 g of leaves from each pot were added to 90 mL of saline solution and stomached for 3-5 minutes. Samples were assessed for cfu on TSA (Appendix 1B) following dilution plating and incubation at 25°C for 2-5 days. The identities of the bacterial antagonists were confirmed by comparing colony morphologies on TSA with those of the corresponding antagonists. If necessary, some biochemical tests as indicated in Section 7.3.5 were also undertaken. To assess Td_{22} establishment, 20 randomly-selected plugs (~3x3 mm) of leaves per treatment were aseptically removed, placed on pectin agar medium amended with 60

μg/mL tetracycline, and incubated at 25°C for 4-7 days until fungal growth was observed. Fungal growth on each plate was subsequently subcultured for morphological comparison with that of a Td_{22} stock culture grown on the same medium and incubated for one week until conidial development was observed.

7A.3.8 Statistical analysis

The data was analysed using the MINITAB software for windows as described in Chapter 4.

7A.4 Results

The effectiveness of the selected antagonists to protect zucchini's leaves from downy mildew infection (assessed as percentage of infected leaves and disease severity index) is presented in Table 7A-1.

Table 7A-1: Effectiveness of selected antagonists in protecting zucchini plants from downy mildew.

Treatments[¥]	Percentage of infected leaves (%) and disease severity index*	
	2 weeks after pathogen introduction	5 weeks after pathogen introduction
A0B0	3.33±3.33 d,e (0.20±0.20 c)	35.21±1.68 a (1.70±0.44 a)
A0B1	61.67±3.03 c (1.70±0.37 b)	59.85±3.92 b (2.00±0.35 a)
A1B1	34.29±5.76 a (1.00±0.00 a)	54.81±4.79 b (1.40±0.24 a)
A2B1	47.90±5.94 a,c (1.40±0.24 a)	46.67±2.04 b (1.90±0.29 a)
A3B1	10.24±5.50 e (0.50±0.26 c)	56.36±4.89 b (1.25±0.13 a)
A4B1	26.43±4.05 a,e (1.00±0.00 a)	52.88±5.16 b (1.10±0.10 a)
A5B1	26.67±8.99 a,e (1.00±0.00 a)	52.55±5.89 b (1.00±0.00 a)
A6B1	18.81±4.67 a,e (1.00±0.00 a)	47.92±2.50 b (1.13±0.11 a)
A7B1	37.86±4.74 a (1.50±0.32 a,b)	60.00±6.50 b (1.60±0.29 a)

* Percentage of infected leaves = (number of infected leaves/total leaves) x 100%.

*Each value is an average of five replicates ± standard error. Values with the same letter(s) in the same column are not significant statistically at $p<0.05$. Values in bracket indicate the rating of disease severity index of the infected leaves (scale details are given in Section 7A.3.6), rated according to Nakasaki *et al.* (1998).

¥A0B0, nil control (neither antagonist nor pathogen were inoculated), A0B1, control treatment (pathogen only was inoculated), A1B1, plants inoculated with *P. corrugata* and pathogen, A2B1, plants inoculated with *L. antibioticus* and pathogen, A3B1, plants inoculated with Td_{22} and pathogen, A4B1, plants inoculated with a combination of *L. antibioticus*, *P. corrugata* and pathogen, A5B1, plants inoculated with a combination of *L. antibioticus*, Td_{22} and pathogen, A6B1, plants inoculated with a combination of *P. corrugata*, Td_{22} and pathogen, A7B1, plants inoculated with a combination of all three antagonists and pathogen.

In all cases except A2B1 (plants inoculated with *L. antibioticus* and pathogen), the application of antagonists significantly ($p<0.05$) reduced the disease incidence when compared to the control treatment (A0B1) two weeks after pathogen introduction (Table 7A-1). The best disease control was seen for the fungus Td_{22}, (A3B1) at this time with 83% protection provided relative to the untreated-pathogen control. This however decreased to insignificant protection after five weeks. There appears to be some synergy (although n.s. at $p < 0.05$) between *L. antibioticus* and *P. corrugata* applied as a mixed culture (treatment A4B1) relative to separate applications of each (A1B1, A2B1), the combination giving 57% protection relative to the untreated-pathogen control. The co-inoculation of Td_{22} in any combination with the bacterial isolates only led to a reduction in effectivity, a result that might be attributed to the reduced concentration of the most effective biocidal agent added to leaf surfaces. The lack of observed synergy between the bacteria and fungus however may in part be attributable to antagonism between the antagonists, since *in vitro* dual culture showed both bacterial antagonists produced zones of inhibition against Td_{22} (Plate 7A-1).

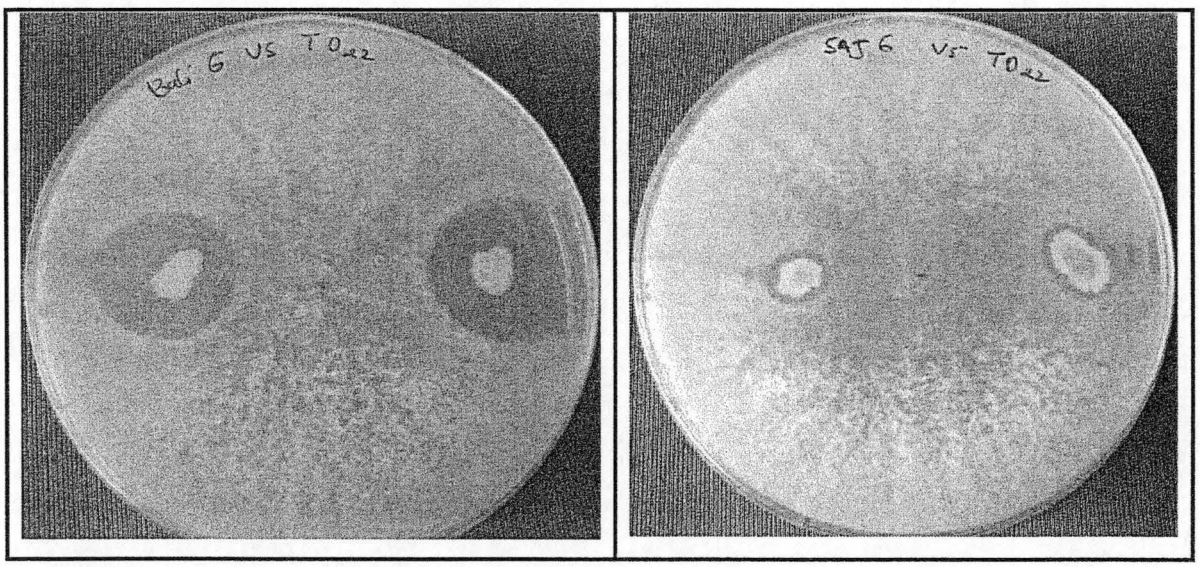


Plate 7A-1: ***In vitro*** **dual culture assay between *L. antibioticus* and Td_{22} (left) and between *P. corrugata* and Td_{22} (right) on TSA plate following incubation at 25°C for three days.**

The degree of protection provided by the antagonists against downy mildew became non significant statistically ($p>0.05$) against the control treatment after five weeks, when 60% of control plant leaves were infected.

The survival of the inoculated agents on the leaf surface was (as expected) poor. Td_{22} was re-isolated from two of 20 leaf samples taken after 5 weeks from treatment A5B1, (plants co-inoculated with *L. antibioticus* plus Td_{22} and pathogen), and from none of 20 leaves sampled from other treatments. Likewise, neither of the bacterial agents sprayed on the leaf surfaces was re-isolated after five weeks from any treatment.

7A.5 Discussion

Bacterial or fungal antagonist suspensions applied singly or in combination to control foliar diseases (e.g. downy mildew) in zucchini plants gave encouraging results with statistically significant reductions ($p<0.05$) in disease incidence relative to controls two weeks after the pathogen introduction (Table 7A-1). This may open the way to the manipulation of compost for the production of designer 'compost teas' to control foliar pathogens, or to the direct cultivation of one or more biological control agents for this purpose. All antagonists applied in the present study have been shown to grow well in compost (WFW compost or fish waste compost) (Chapter 5, 5A, and 7), and their mass production in such composts will be significantly less expensive than cultivation in synthetic media.

It was notable that the best result was obtained for the fungus Td_{22}, which had been grown in a mix of WFW compost and millet seed (80:20 w/w) and stored at ambient temperature (~20°C) for ~10 months before harvest and use in this experiment. The effectiveness of both fungal and bacterial antagonists could be expected to be improved by use of chelating agents and detergents in the formulation composition, as described in work reported subsequent to this experiment (van der Wolf and Birnbaum, 2003).

The demonstrated incompatibility of the bacterial and fungal antagonists has previously been noted in a similar study by Dandurand and Knudsen (1993). These workers reported that application of *Trichoderma harzianum* in combination with a biological control bacterium, *Pseudomonas fluorescens*, reduced the effectiveness of the fungus to control *Aphanomyces euteiches*, the causative agent of root rot on pea. In contrast, Raupach and Kloepper (1998) reported that application of mixed cultures of antagonists generally improved the disease control, suggesting that assessment of

compatibility of antagonists used in formulations is essential to improved disease control. The ability of Td_{22} to protect a range of plants from attack by a variety of fungi as demonstrated by this and other studies indicates that this fungus has a broad spectrum of disease control. The level of protection provided to zucchinis against downy mildew (83% after two weeks as shown in the present study) is comparable with that provided by chemical treatments, although regular re-application is field use. Notably, a similar conclusion has been reported in the case of fungicide treatment of downy mildew, with re-application at 10-14 day intervals recommended for reliable control (Ellis, 2001).

The development of mildew symptoms in nil-pathogen controls (A0B0) at five weeks (Table 7A-1) possibly resulted from natural infection (the disease was in a nearby vineyard) or from accidental human-mediated transfer. Such infection however was low in comparison with the inoculated plants and did not affect the outcome of this trial. The poor establishment of the biological control agents on the leaf surface was expected, and attributable to the combined effects of low water activity, high UV exposure and washing of leaf surfaces during overhead irrigation.

The mechanism of disease control by Td_{22} has not formed part of this investigation, but has been addressed at least for sclerotial infections of onions by Metcalf (2002).

7A.6 Conclusions

All but one (*L. antibioticus*) of the biological control agents assessed in the present study were found to be significantly ($p<0.05$) effective in protecting zucchini leaves from downy mildew for up to two weeks period in a glasshouse trial. This raises the potential use of one or more of these organisms for foliar disease control, especially if such control can be enhanced by use of surfactants/chelating agents. As currently assessed, protection beyond 14 days would require re-application of the biocontrol agents. The fungus Td_{22} was incompatible with both *L. antibioticus* and *P. corrugata*, while the combination of *L. antibioticus* and *P. corrugata* appeared to be compatible but without providing significant improvement in biocontrol relative to *P. corrugata* alone. The survival of these three antagonists on the leaf surface of zucchini plants was very poor.

Chapter 8

Preliminary screening and elucidation of active compounds produced by *Pseudomonas corrugata* (Strain SAJ6)

8.1 Abstract

Active compounds produced by *P. corrugata* (strain SAJ6) were examined with a view to determining the mechanism by which this antagonist controlled *S. minor* as described in Chapter 7. This bacterium was found to produce active compounds (molecular weights of 554 and 580) that were inhibitory to *S. minor*, a range of Gram-positive bacteria and some bacterial pathogens, such as *Pseudomonas aeruginosa* and *Listeria monocytogenes.* No inhibition was evident against *Candida albicans, Lysobacter antibioticus,* or *Staphylococcus aureus*. The role of siderophores in this antagonism was excluded from contention, since the inhibition of *S. minor* was not affected by the presence of high level of $FeCl_3$ in growth medium. It was therefore concluded that antibiosis was the most probable mechanism of disease control by this antagonist. Although the structure of the inhibitory compounds have not been elucidated, the data obtained in the present study provides information which could serve as a starting point for further study on their characterisation.

8.2 Introduction

A strain of *P. corrugata* isolated from the University of Tasmania farm in the course of this study showed strong *in vitro* antagonism to a range of fungal pathogens, including *S. minor* and *S. sclerotiorum.* This bacterium was also effective in controlling *S. minor* in glasshouse trials and in a field trial (Chapter 7). Strains of *P. corrugata* have been reported to cause pith necrosis disease in tomatoes and in a few cultivars of pepper (http://wsare.usu.edu/sare2000/071.htm). Its ability to produce active compounds inhibitory to a range of plant pathogens has also been reported by workers including

Emanuele *et al.* (1998) and Chun (2000). It has been used to control *Gaeumannomyces graminis* var. *tritici*, the causative agent of take-all disease in wheat (http://www.bspp.org.uk/icpp98/2.2/35.html), *Clavibacter michiganensis* subsp. *sepedonicus*, the causative agent of ring rot disease in potato stems, and *Helminthosporium solani*, the causative agent of silver scarf disease in potato (Chun, 2000).

Some characteristics of the active compounds produced by a strain of *P. corrugata* (SAJ6) were elucidated in the present study.

8.3 Materials and methods

8.3.1 *P. corrugata, S. minor*, and other microbes sensitive to *P. corrugata* active compounds

Sources of *P. corrugata* and *S. minor* have been described previously (Chapter 7). A bacterium sensitive to the compounds of *P. corrugata* was isolated from a commercial fish waste compost (Hazel Brothers Inc.); other isolates were obtained from the culture collection of the School of Agricultural Science, University of Tasmania.

8.3.2 Isolation of active inhibitory compounds

The isolation of active compounds from *P. corrugata* was undertaken using the method of Howell and Stipanovic (1979) with slight modification. Plates (20) of PDA were streak-inoculated with the antagonist, incubated at 25°C for 10 days, cut into ~1-cm squares and extracted with approximately the same volume of 80% aqueous acetone for one hour. The extracts were filtered through cheesecloth to remove debris and centrifuged at 9000 *g* (Beckman model J2-21) for 15 minutes to remove other particulates. Supernatants were partially evaporated under a reduced pressure at 40°C (Buchi rotavapor R) to remove the acetone, followed by addition of 5.0 g NaCl to each 100 ml aqueous concentrate and three extractions with approximately matching volumes of chloroform. Chloroform extracts were combined and evaporated to dryness at 40°C. The residues were then dissolved in 5.0 mL methanol, checked for activity against *S. minor* or a sensitive bacterium, and stored at –70°C until required.

8.3.3 Fractionation and preliminary elucidation of active compounds

Crude extracts obtained as described above (8.3.2) were fractionated using a reverse phase Sep_Pak® C_{18} cartridge (Waters). Active compounds were eluted from these cartridges using a range of solvents: methanol, isopropanol, dichloromethane, or 50%:50% hexane:isopropanol. These fractions were assessed for activity against a sensitive bacterium using the disc diffusion antibiotic susceptibility method of Kirby and Bauer (1966). Fractions showing antimicrobial activity were analysed further using high performance liquid chromatography (HPLC) with both UV-Vis and mass spectrometric (MS) detection. An HPLC (Waters alliance 2690) was coupled to a Waters 996 photo diode array detector, using a reverse phase C_{18} column (Waters Nova-Pak C_{18} 3.9 x 150 mm with Alltech Econosphere C_{18} 5 micron guard cartridge); the eluant from the diode array was passed on to a Finnigan LCQ mass spectrometer fitted with an atmospheric pressure chemical ionisation (APCI) source. The mass spectrometer conditions were as follows; capillary temperature was 180°C; APCI vaporizer temperature was 480°C; source voltage was 6.00 kV; source current was 5.00 μA; sheath gas flow was 60 mL min^{-1}; aux gas flow was 15 mL min^{-1}; capillary voltage was 5.5 V; and tube lens offset was 5.5 V. The elution gradients included a gradient of 50% solvent A (2% acetic acid in methanol):50% solvent B (0.1 M ammonium acetate) to 100% solvent A at 15 minutes then to 80% solvent A:20% solvent C (hexane) at 25 minutes and held at this for a further 10 minutes. The flow rate was at 0.8 mL min^{-1}. Re-equilibration was back to 100% methanol for 4 minutes then to starting conditions, which were held for 15 minutes prior to the next injection. For this analytical purpose, 20 μL of sample was injected. The diode array detector was monitored from 210nm to 450nm. Sample spectra were recorded once per second. The MS scan range was from *m/z* 150 to *m/z* 2000, with data-dependent MS/MS production scans alternating with normal scans. For data-dependent MS/MS scan, the isolation width was 3 daltons and the collision energy 30%. For semi preparative run, 40 μL sample was injected. Fractions collected were evaporated *in vacuo* at 40°C then dissolved in methanol for testing against the bacterium mentioned above. The MS data was analysed using Finnigan Navigator software.

8.3.4 Assay for antimicrobial activity

Antifungal activity of samples was assayed by depositing 20 µL of the sample (in methanol) onto the periphery of PDA plates (with appropriate methanol controls) followed by drying in a laminar flow cabinet. Plugs (1 cm^2) of 48 hour-old *S. minor* on PDA were placed in the centre of the plates, which were incubated at 25°C for 3-7 days. Antifungal activities were indicated by zones of inhibition around areas where the samples were deposited.

Antibacterial activity was determined using the method of Kirby and Bauer (1966). Alternatively, 15-20 µL methanol extracts (or methanol-only controls) were deposited into agar wells on a TSA plate previously seeded with a bacterial lawn. Antibacterial activity of the sample was indicated by clear zones around the filter paper disc or around wells where the active compounds were deposited. The HPLC fractions were also tested for bactericidal activity. Prior to assay, the fractions were evaporated *in vacuo* at 40°C, re-suspended in methanol, and assayed as above.

The crude methanol extract (after fractionation through a Sep_Pak® C_{18} cartridge) was also investigated for inhibition against several bacterial cultures, such as *Listeria monocytogenes, Lysobacter antibioticus, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Bacillus cereus. B. thuringiensis, B. mojavensis, B. pumilus, B. subtilis, B. plymyxa, B. megaterium,* and the yeast *Candida albicans*.

8.3.5 The effect of temperature on the stabilities of the active compounds

The effect of heat on the stability of the active compounds was examined by exposing the crude extract to room temperature for 5 days, 80°C for 10 minutes, or autoclaving it at 121°C for 5 minutes, according to the method described by Skerratt (2002). Following this, the samples were subjected to bioassay as described in Section 8.3.4.

8.4 Results

The presence of active compounds inhibitory to fungal or bacterial indicators was indicated by the formation of clear zones of inhibition surrounding filter paper discs, HPLC fractions, or spots of crude extract (Plate 8-1).

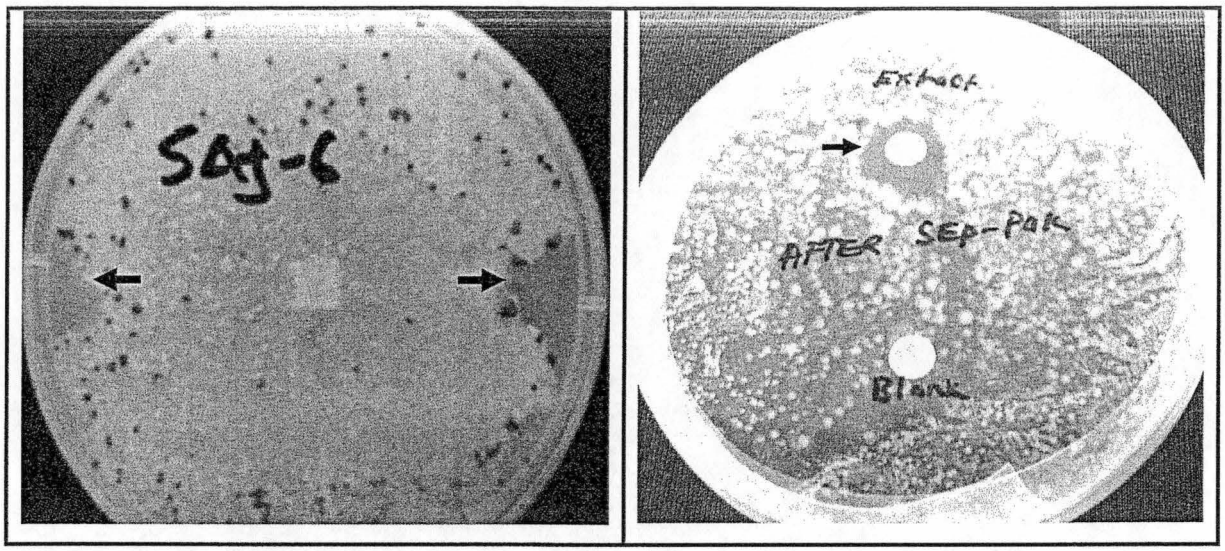


Plate 8-1: Inhibition zones produced by crude extract of *P. corrugata* against *S. minor* (Left Photo) and a sensitive bacterium isolated from fish waste compost (Right Photo). The blank is a filter paper disc treated with methanol only.

The crude extract of *P. corrucata* (following fractionation) was inhibitory to a range of bacterial species (Table 8-1). In general *Bacillus* spp. were more sensitive than other isolates to the crude extract of this antagonist (Table 8-1). The crude extract did not inhibit *C. albicans, L. antibioticus,* or *S. aureus.*

P. corrugata produces a yellow non-fluorescent pigment when grown on PDA, but not on other media, such as TSA or King's B. This pigment is soluble in water and particularly so in aqueous acetone (80% acetone in water). When shaken with chloroform following acetone evaporation, the pigment was retained in the water extract, indicating it to be a polar compound. The HPLC-UV analysis of the extract containing the pigment (at 400nm) showed a sharp peak eluting very early in a reversed-phase HPLC separation with some acetic acid in the aqueous phase (Figure 8-1A). This gave strong absorbance at 249nm and 397nm (the latter peak being equated with the yellow pigment) (Figure 8-1B).

The non-pigmented bioactive compounds were largely soluble in chloroform and methanol, indicating them to be relatively non-polar. Antibiotic activity of these compounds was stable after storing at room temperature for 5 days, autoclaving for 5 minutes, or heating in a water bath at 80°C for 10 minutes, indicating that they were not enzymes or other related proteins, which are normally inactivated at high temperatures.

The APCI HPLC total ion chromatogram of the active methanol extract showed obvious peaks that were absent from the PDA control methanol extract (Figure 8-2). The largest peak from the active extract eluted at approximately 14 minutes (Figure 8-2A). HPLC liquid secondary ion mass spectral analysis of this peak on a Kratos concept ISQ indicated a compound of MW 554 (strong ions at $[M+H]^+$ 555 and $[M+Na]^+$ 577). Two peaks (of MW 580 and 582) that appear closely related to the main peak compound were also observed.

Table 8-1: Antimicrobial spectrum of the crude extract of *P. corrugata*.

Indicator microorganisms	Qualitative inhibition*
Pseudomonas aeruginosa	+
Escherichia coli	+
Listeria monocytogenes	+
Staphylococcus aureus	-
Bacillus cereus	++
B. subtilis	++
B. polymyxa	+
B. mojavensis	++
B. pumilus	++
B. thuringiensis	++
B. megaterium	++
Lysobacter antibioticus	-
Candida albicans	-

*+ = inhibition zone ≤ 0.2 mm from the edge of the well to the edge of the bacterial lawn.
++ = inhibition zone> 0.2 mm from the edge of the well to the edge of the bacterial lawn.
- = no inhibition zone

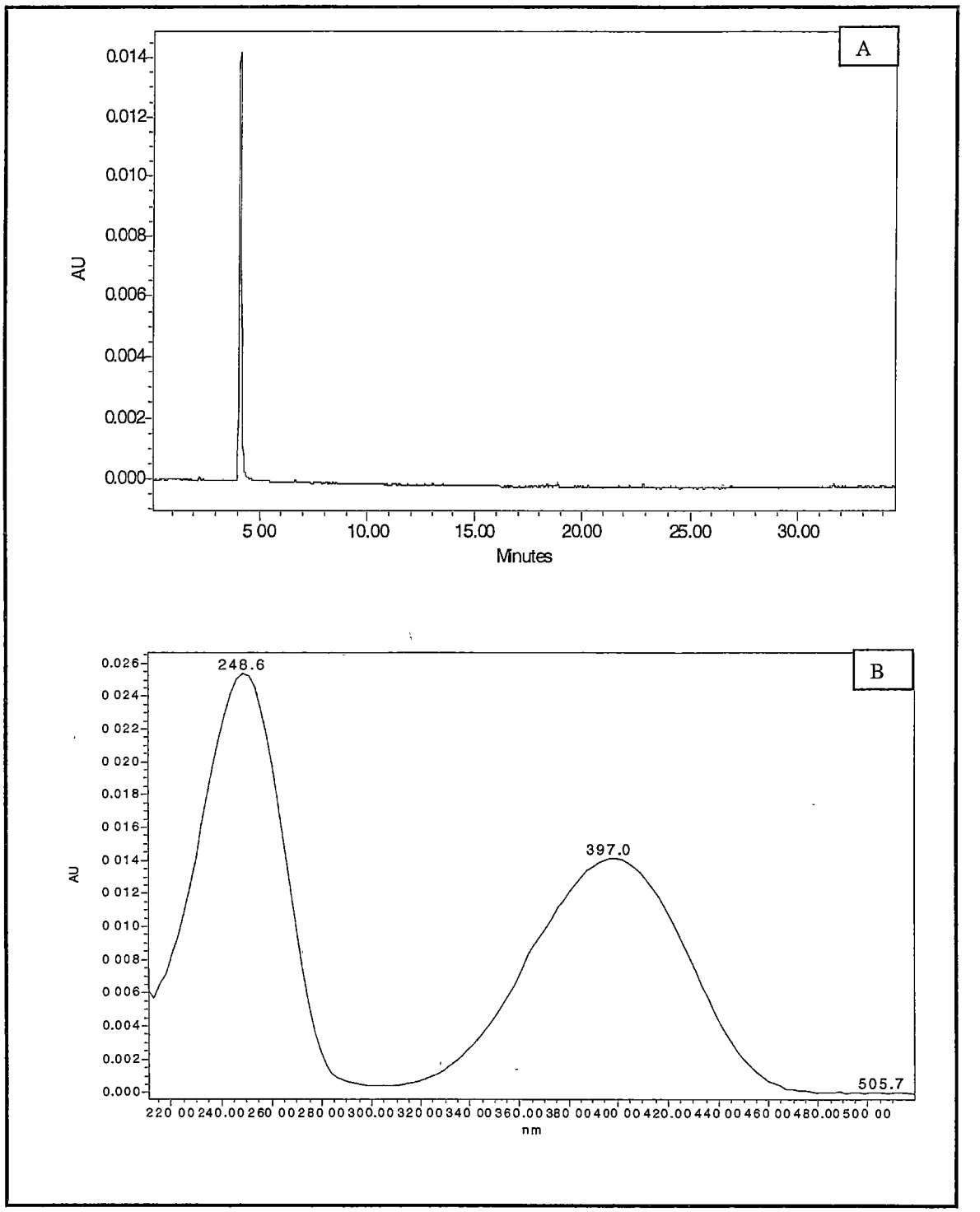


Figure 8-1: HPLC-UV analysis of the extract containing the non-fluorescent yellow pigment, produced by *P. corrugata* on PDA medium. (A) Yellow fraction UV chromatogram at 400nm. (B) UV spectrum of yellow compound. Details of the procedure are provided in Methods

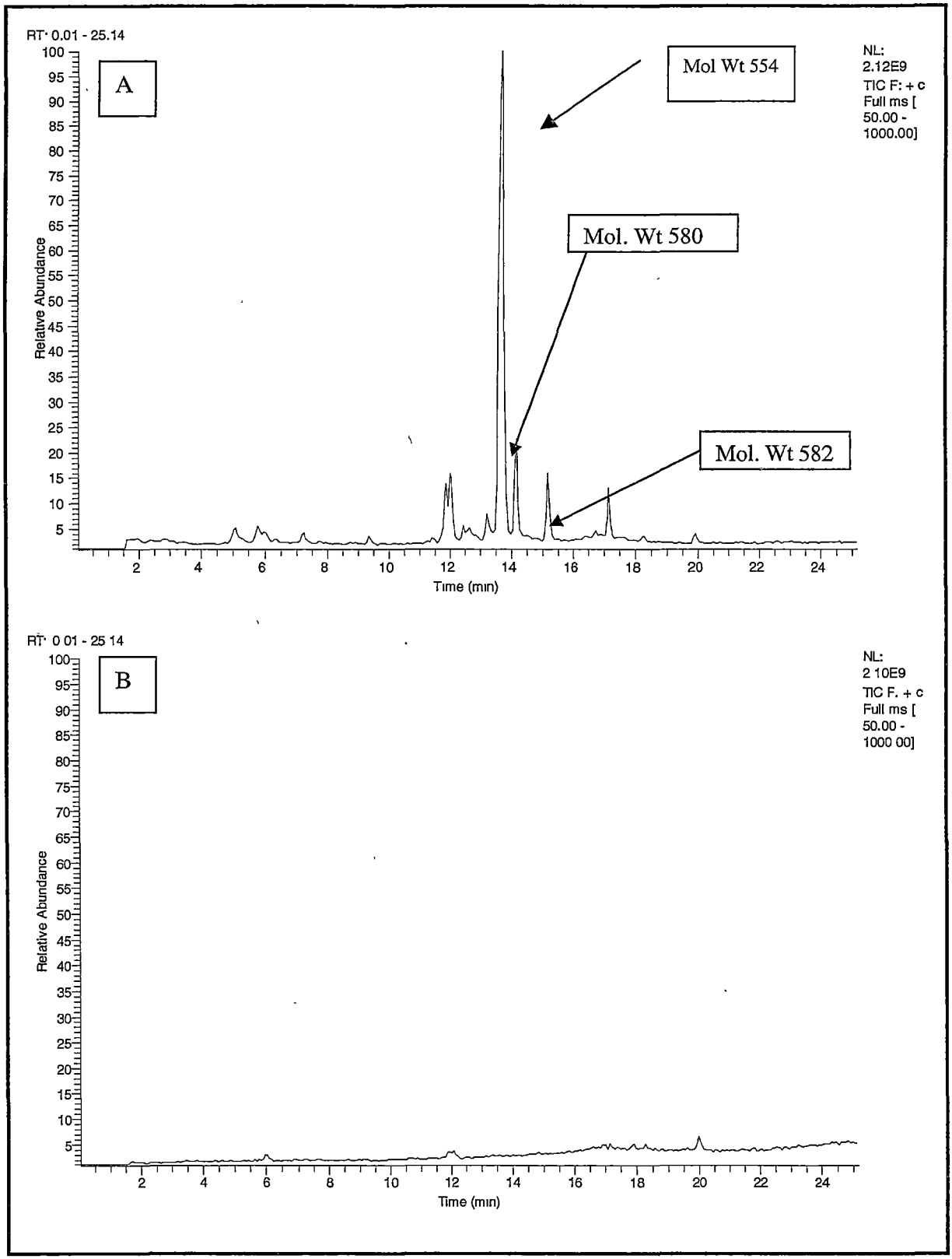


Figure 8-2: APCI HPLC MS total ion chromatogram of the active methanol extract (A) and PDA control methanol extract (B).

Two fractions from the total ion chromatograph (MW 554 and 580) were found to inhibit the indicator bacterium. No inhibition was observed for a fraction of MW 582.

The APCI mass spectrum of the main peak (MW 554) produced three distinct ions (Figure 8-3), two of those (*m/z* 409 and the $[M+H]^+$ ion at *m/z* 555) had the same retention time, indicating that they were probably from the same compound. The peak in the middle (arrow head, Figure 8-3) was apparently from a different compound, as it eluted at different retention time with the two peaks mentioned before. This compound almost co-eluted with the main peak.

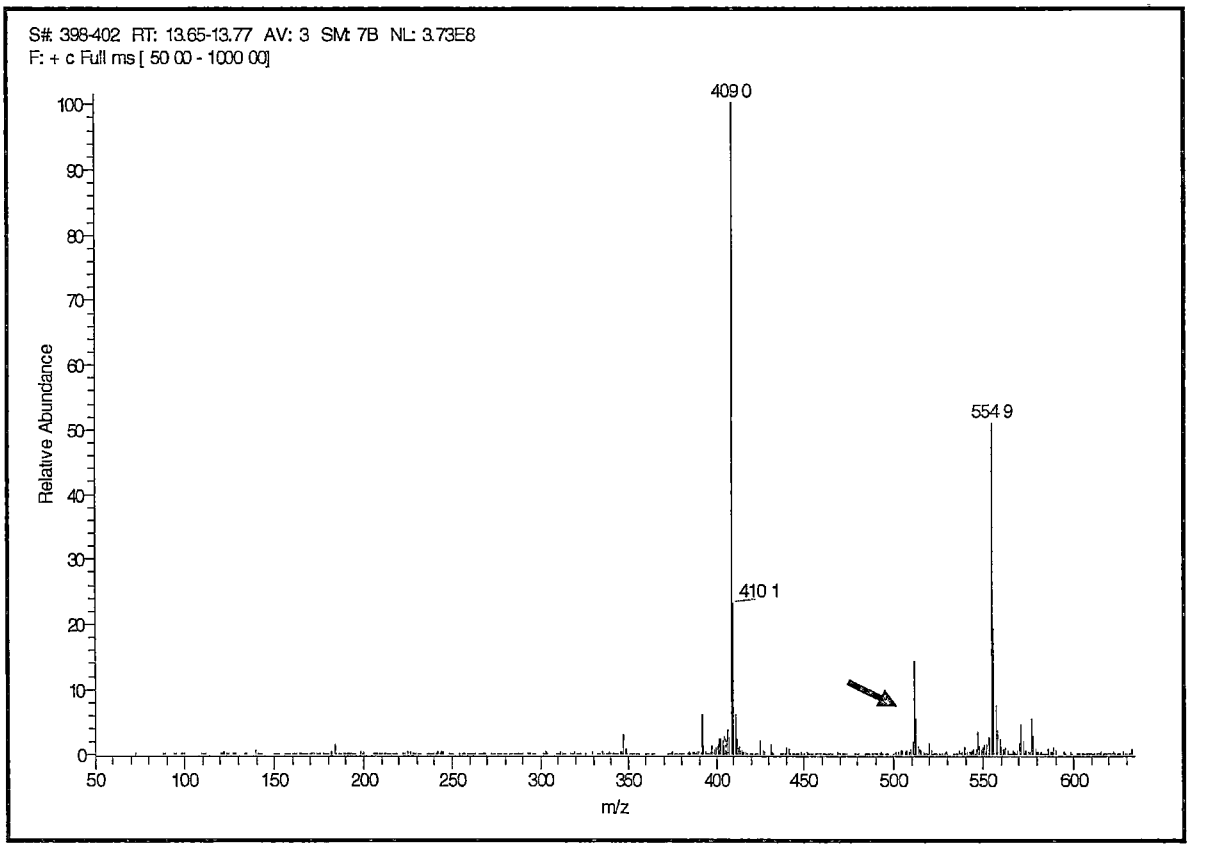


Figure 8-3: APCI mass spectrum of main peak (Mol wt. 554). The peak pointed by the arrow head is a different compound to that with the molecular weight of 554 as it eluted at a different retention time to the two other ions (*m/z* 409 and the $[M+H]^+$ ion at *m/z* 555).

Data-dependent MS/MS scans from the most intense ion in Figure 8-3 (m/z 409) is shown in Figure 8-4.

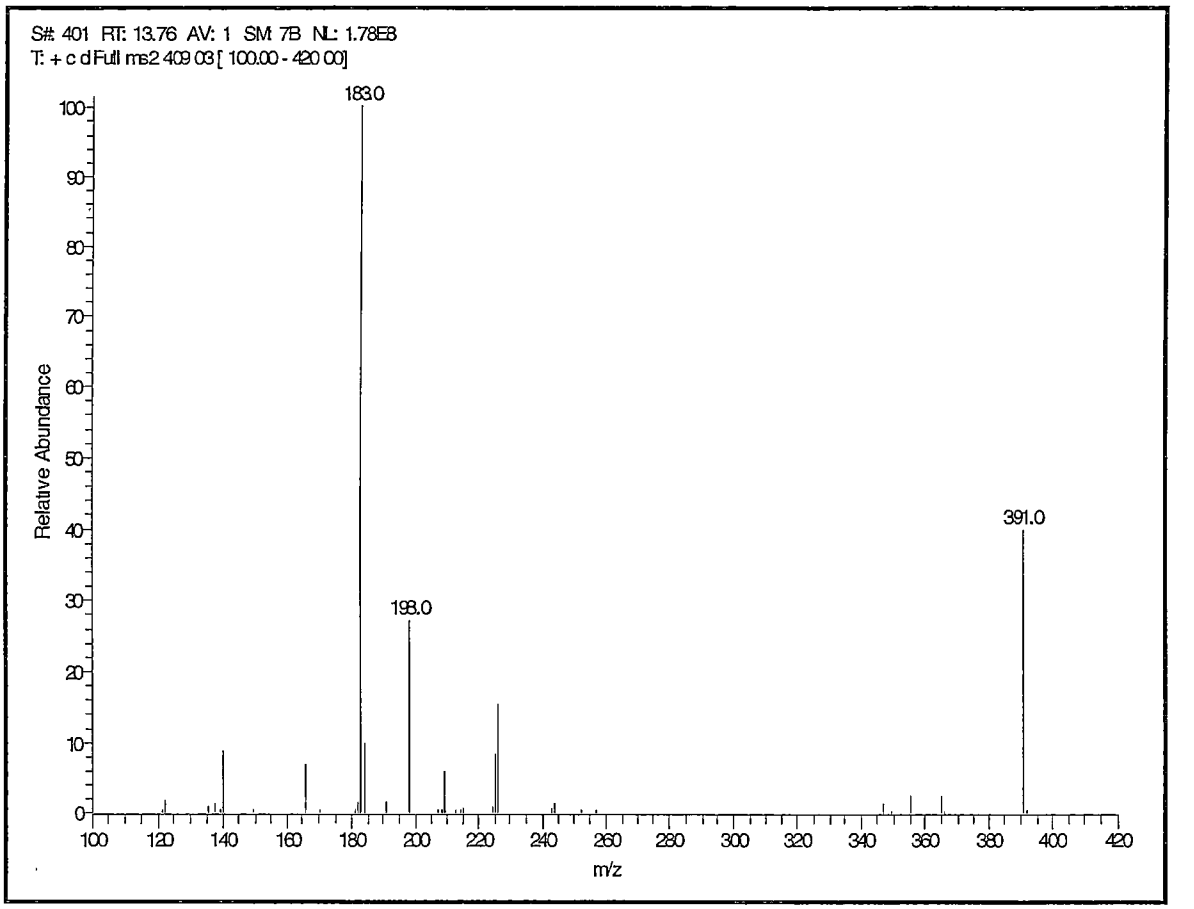


Figure 8-4: MS/MS product ions from the *m/z* 409 ion derived from the main molecular weight 554 compound.

A HPLC-UV trace at 254nm of the active methanol fraction showed the peaks of active compounds indicated in Figure 8-2 had the same retention times in this analysis (Figure 8.5A). The UV absorption spectrum of the most abundance peak (MW 554) is shown in Figure 8-5B. This compound absorbed UV strongly at 253.3nm, indicating an aromatic ring structure.

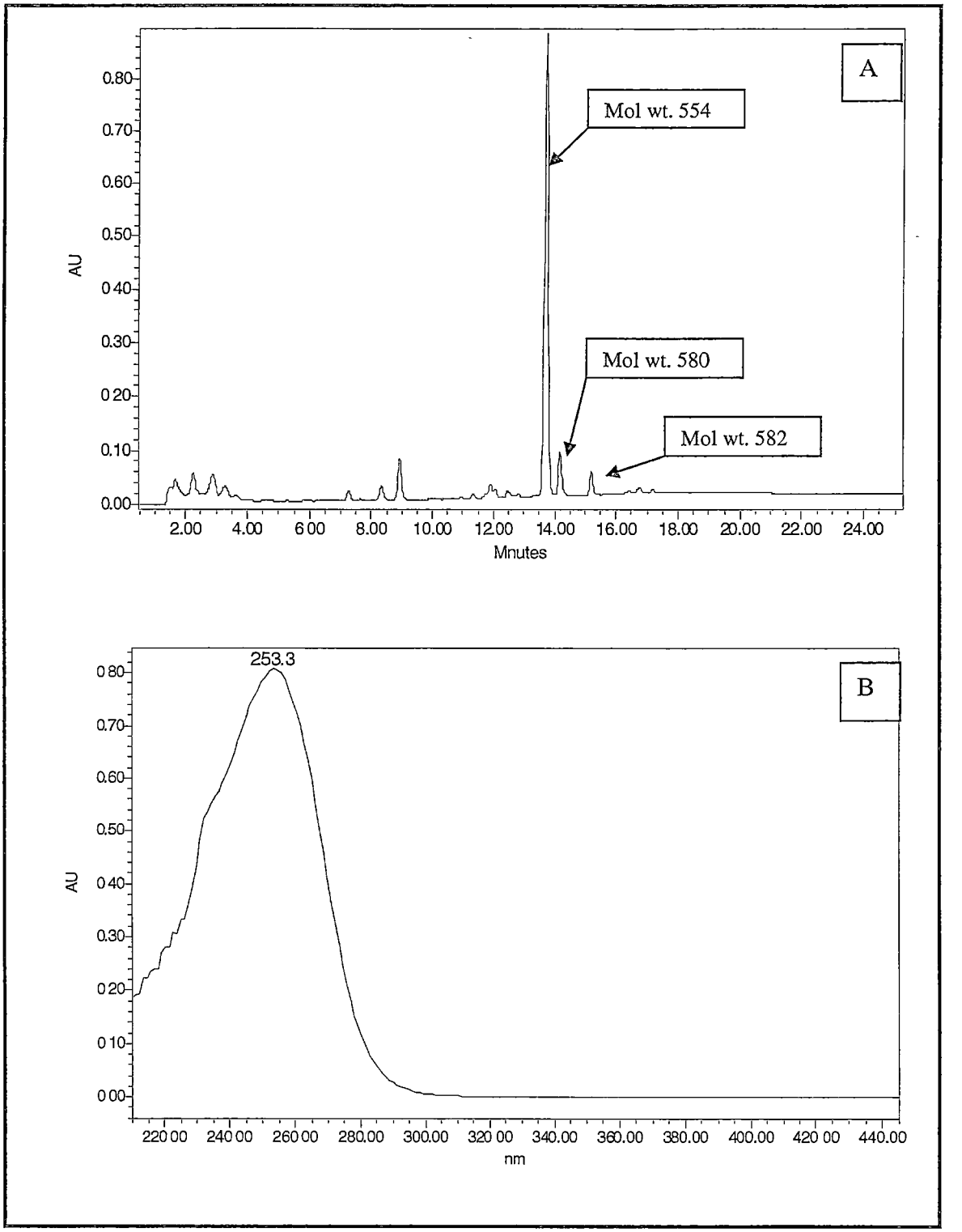


Figure 8-5: HPLC-UV trace at 254nm of active methanol fraction (A) and UV spectrum of the main peak (B).

8.5 Discussion

The present study demonstrated that *P. corrugata* strain SAJ6 produced active compounds inhibitory to fungal plant pathogens, such as *S. minor* and *S. sclerotiorum*. The crude extract of this antagonist was also found to be inhibitory to a range of bacterial pathogens, including *P. aeruginosa*, and *L. monocytogenes* and *Bacillus* spp. supporting the potential of these isolates for the control of a range of plant pathogens. The two compounds from strain SAJ6 having molecular weights of 554 and 580 were both inhibitory to an indicator bacterium isolated from fish waste compost. Such compounds produced by *P. corrugata* have previously been reported by Emanuele *et al.*, (1998) and Chun (2000), with the latter author recording inhibition against the bacterial pathogen *Clavibacter michiganensis* subsp. *spedonicus* and a fungal pathogen, *Helminthosporium solani*, the respective causative agents of ring rot and silver scarf disease in potatoes. The strain isolated by Chun (2000) has been patented in the USA (U.S. Patent 6,156,560) for its ability to protect against take-all and damping-off diseases in addition to those mentioned above. The unidentified active compounds have also been claimed to have medical application (http://www.irf.uro.uidaho.edu/TAFL/plantbiotech.htm).

There has been no report of active compounds produced by *P. corrugata* having molecular weights of 554 or 580, but since the molecular weights of the active compounds previously reported were not given, it is not known whether the compounds isolated in this study are different or new. The conclusion that the mode of action by strain SAJ6 was antibiosis is in agreement with the report by Emanuele *et al* (1998) who found that bioactive lipodepsipeptides were responsible for the growth inhibition of *B. megaterium*.

Pseudomonad species have been well documented for production of bioactive compounds (e.g. Coppoc, 1996; Bonsal *et al.*, 1997; Kerr, 2000), pyrrolnitrin or 3-chloro-4-[2'-nitro-chlorophenyl]-pyrrole (Howel and Stipanovic, 1979) phenazine-1,6-dicarboxylic acid (Kerr, 2000), 2,4-diacetylphloroglucinol and pyoluteorin (De La Fuente *et al.*, http://www.ag.auburn.edu/argentina/pdfmanuscripts/delafuente.pdf) being the most commonly reported. In terms of molecular weight, these compounds are far too small to match the active compounds produced by strain SAJ6. Furthermore, unlike pyoluteorin or pyrrolnitrin, the active compounds of SAJ6 (MW 554 and MW 580) do not contain any chlorine atoms, as they do not produce the very diagnostic chlorine 'isotope pattern' in their mass spectrum (Davies, personal communication). The compounds produced by

strain SAJ6 also appeared to be heat stable, indicating them to be also different from the generally heat labile compounds reported by Leary and Chun (1984) or Emanuele *et al.* (1998).

The compound isolated in the present study of MW 554 absorbed light primarily at the wavelength of 253.3nm (Figure 8-5B), remarkably close to that of an antibiotic-like compound from a strain of fluorescent pseudomonad of 254nm (Shanahan, *et al.*, 1992). However this compound was subsequently identified as 2,4-diacetylphloroglucinol (MW 210). Similarly, Rovera *et al.* (2000) reported a compound produced by a strain of *Pseudomonas* spp. that showed UV-visible absorption spectrum at the wavelength of 260nm characteristic of aromatic-ring compounds (Davies, pers, comm.; Rovera *et al.*, 2000).

The mass spectrum data did not allow any interpretation of the structure of the active compounds produced by the strain SAJ6 from first principles. In order to determine this structure NMR spectral analysis (^{1}HNMR and ^{13}CNMR analysis) is needed, requiring several mg of pure compound, a task which is beyond the scope of this study.

The strain SAJ6 was also found to produce non-fluorescent yellow pigment when grown on PDA but not on TSA or King's B medium. It was highly soluble in water, indicating a polar molecule. This pigment absorbed strongly at 249nm and 397nm and it was not inhibitory to a tested bacterium in an *in vitro* assay. The ability of *P. corrugata* to produce diffusible, non-fluorescent yellow pigment is very common, although it is not necessarily correlated with the production of active compounds (Krieg and Holt, 1984). This is in contrast to the orange pigment produced by an isolate identified as *P. aurantiaca*, which was claimed to be antimicrobial (Rovera *et al.*, 2000).

A compound different to that having a MW of 554 almost co-eluted with the main peak, as indicated in Figure 8-3. This was of some concern as the inhibition zone produced by *in vitro* assay of the MW 554 fraction could have been due to this compound. This needs to be elucidated by further study.

8.6 Conclusions

P. corrugata strain SAJ6 (effective against *S. minor* and *S. sclerotiorum*, both *in-vitro* and in pot trials), produces two active antimicrobial compounds inhibitory to *S. minor*, a range of Gram-positive bacteria, as well as some bacterial pathogens of animals, such as *Pseudomonas aeruginosa* and *Listeria monocytogenes* of medical importance.

The active compounds were chloroform or methanol soluble and found to have molecular weights of 554 and 580, which were too high to match any commonly produced by pseudomonad species, such as pyrrolnitrin, pyoluteorin, 3-chloro-4-[2'-nitro-chlorophenyl]-pyrrole, phenazine-1,6-dicarboxylic acid, or 2,4-diacetylphloroglucinol.

Chapter 9
General conclusions

The main objective of the study was to assess the effectiveness of antagonists in controlling plant pathogens and establish methods for their large-scale cultivation in low-cost materials. The use of WFW of paper mill origin and composted fish waste as media for the cultivation or carriage of antagonists of plant pathogens has been examined with a view to producing suppressive alternatives to chemical-based fungicides. These wastes are produced abundantly in Tasmania and elsewhere. In the present study, they were modified (enriched with ammonium nitrate-based mineral salt solution) to maximise the growth of the antagonists, this also having beneficial nutrient effect on field plants.

The study began with isolation and screening of agents that may have potential in the biological control of plant pathogens (Chapter 3). A total of 67 bacteria showing *in-vitro* antagonism to *Sclerotinia minor* or *S. sclerotiorum* were isolated in dual culture assays, the isolates originating particularly from the rhizosphere or rhizoplane of agricultural plants. In conformity with other reports, representatives of the genera *Pseudomonas* and *Bacillus* were the most frequently isolated inhibitory bacteria (Chapter 3), other antagonists belonging to the genera *Lysobacter, Acinetobacter, Flavobacterium, Alcaligenes, Chromobacterium, Moraxella, Proteus* and *Erwinia.* These cultures were further screened for protection of lettuces against *S. minor* attack in a non-replicated glasshouse trial, with five of the 21 isolates (those showing highest levels of inhibition *in-vitro*) providing 25-50% protection relative to controls over a six day period. This was in contrast to the *S. minor* controls, which showed 100% mortality in these time periods (Chapter 3).

A selected few of these isolates were subsequently assessed in a replicated glasshouse trial as described in Chapter 5A and 7, resulting in the identification of two isolates (*Lysobacter antibioticus* and *Pseudomonas corrugata*) as capable of consistently protecting lettuce seedlings/plants from *S. minor* attack. These two cultures were used

(together with a known biological control fungus provided from other research) in a later study of the low-cost cultivation of potential biological control agents in compost.

The effect of temperature and pH on the growth of selected antagonists, plus others supplied from elsewhere, was examined as described in Chapter 4, with a view to optimising these factors in the utilization of low-cost substrates for their cultivation. A temperature of 25°C and a pH range between 5.0 and 6.0 was most favourable for the growth of a biological control *Trichoderma* sp. (Td_{22}), while 25°C to 30°C pH coupled with a neutral pH combined was optimum for most of the bacterial isolates.

As a further measure to favour the growth of the selected agents in a compost matrix, an examination of various low-cost carbon and nitrogen sources that might be utilized by them was undertaken with a view to seeding the compost with one or more of these materials. The fungus Td_{22} was seen to utilize carboxy methyl cellulose (utilization of crystalline cellulose was probable, but unable to be confirmed in this work) while the selected bacterial antagonists were found to utilize starch, casein, urea, and NH_4NO_3 sources (Chapter 7). The ability of the selected bacterial antagonists to utilize starch or casein (as C sources) and urea (as an N source) made it possible to enrich the fish waste compost with a combination of those materials, favouring the inoculated biota relative to indigenous compost biota that either cannot utilize these substrates, or will take additional time (relative to pre-induced inocula) for the induction of degrading enzymes.

Following the determination of the optimal pH and temperature of the microbial antagonists as described above, the growth and survival of Td_{22} (Chapter 5) and of bacterial antagonists (Chapter 5A) in composted WFW both under sterile and non-sterile condition were assessed. The objective of this study was to investigate whether WFW compost could be used as a substrate or carrier for the selected biological control agents, with a view to the possible large-scale cultivation of these agents in this material. The advantage, if successful, would be that of low relative cost to mass-produce the suppressive agents. The WFW compost used for this purpose was produced according to the method of Jackson (1998). As the C:N ratio of the WFW was very high (~218 as reported by Jackson, 1998), a substantial amount of nitrogen supplementation was needed. Composting of the WFW for three months following amendment of urea, potassium nitrate, and super phosphate (to give an initial C:N:P:K ratio of 35:1:06:01)

resulted in a product with a C:N ratio of ~ 40 and without phytotoxicity to radish seed germination or growth.

The growth of Td_{22} was assessed in variants of WFW compost and millet seed mixes at pH values of 4-5 (Chapter 5); millet seed being the medium of choice in previous research (Metcalf, 1997). Td_{22} showed excellence growth responses in all variants of the mixes, the best being in a mix of 80% WFW compost and 20% millet seed where densities of ~10^{10} cfu/g were reached (Chapter 5). The inclusion of a component of millet seed in formulations for cultivation of Td_{22} was beneficial because it provided a ready supply of simple carbohydrates relative to the cellulosic WFW and because the protein-nitrogen content of the millet seed helped to bring the C:N ratio of the compost closer to the ideal ratio for microbial growth. The growth response of Td_{22} in the 80% WFW compost 20% millet seed opens the way for the large-scale and low cost cultivation of this and other potential fungal biological control agents. The 80:20 WFW:millet seed formulation adjusted to neutral pH levels was subsequently used to investigate the growth response of several bacterial antagonists (Chapter 5A), and for the cultivation of Td_{22} to assess its protective efficacy using lettuces challenged with *S. minor* (Chapter 6).

A costing of the process (Chapter 6) indicated an 80% savings could be made in the cultivation of Td_{22} as compared with its cultivation in 100% millet seed (Metcalf, 1997). However the use of millet for the cultivation of bacterial antagonists in the 80:20 WFW compost:millet seed mix might need reconsidering. Although high densities of the bacterial antagonists were achieved in this mix, pot trials indicated that the growth *S. minor* was stimulated in this mix (Chapter 5A), to subsequently result in a more severe infection than would otherwise occur. Starch and casein were investigated as alternative carbon sources to replace millet seed (Chapter 7) in fish waste compost media prior to antagonist inoculation. Both supplements, combined with appropriate sources of N, favoured the growth of the inoculated bacterial antagonists (known to utilize these supplements) in these media relative to non-utilizing native biota (Chapter 7).

Composting of WFW prior to use as a growth medium of Td_{22} was initially thought to be advantageous. However this was found to be unnecessary as the fungus showed excellent growth response and reached comparable cell densities in non-composted WFW following 14 days incubation (Chapter 5). The phytotoxicity of this

Td_{22}-cultivated raw WFW was absent when used at the rate of 20% (v/v) in soil mixture to germinate radish seeds, indicating the elimination of phytotoxic compounds by Td_{22} within the 14 days incubation period. This was highly advantageous because of time and labour savings resulting from the elimination of a composting step. Sieving the WFW raw material through a 1.0 cm mesh was useful in this study for maintaining consistency of the fungal and bacterial cultures (Chapter 5 and 5A). However for large scale culture the step could probably be eliminated.

That the antagonists grown under sterile conditions always achieved significantly higher densities than that grown under non-sterile conditions (Chapter 5 and 5A) was not surprising, indicating the competitive role of the indigenous biota in non-sterile material. This underscored the importance of minimising the impact of the native microbiota to achieve maximal growth of the inoculated organisms, as was undertaken in this study. Efforts to decrease the indigenous microbial loading prior to inoculation with the biological control agents including air-drying or briefly steam treating the compost prior to inoculation (Chapter 5A) were seen to improve the relative growth of the inoculated antagonists. However such methods are probably impracticable in large-scale operations and were superseded in this investigation by the use of other methods.

Solarization is an environmentally safe method that has been widely applied to eliminate harmful soil-borne organisms, but its effectiveness is highly dependent on the prevailing conditions of air temperature and solar insolation. It is particularly applicable and effective in tropical countries where daily air temperatures and radiative insolation are high throughout the year. Solarization of both raw and composted WFW during summer in Tasmania was also attempted, but the temperature of the material covered with black vinyl at a few cm from its surface peaked at less than 45°C (data not shown). This was insufficient to significantly reduce the indigenous mesophilic microbiota, resulting in a subsequent detrimental effect on the growth of the introduced biota. The practicality of solarization for this purpose at Tasmanian latitudes was concluded to be marginal.

Simulation of self-heating compost by exposing an enriched 'compost' (nutrient and millet-amended WFW compost) to 60°C for one week prior to inoculation with potential antagonists was assessed as described in Chapter 5A. This resulted in only minority populations of spore-forming *Bacillus* spp. and fungi surviving to subsequently

compete at 28°C against the inoculated biota. Their resultant growth of these inoculated antagonists was excellent, particularly of the bacteria *P. corrugata* and *L. antibioticus*, opening the way for the low-cost production of microbially-manipulated suppressive compost (Chapter 5A).

Following the above simulation, the potential of a commercially produced self-heating fish waste compost (55°C at a depth of 30 cm in cool-down phase) to reduce the diversity and population densities of indigenous compost microbiota capable of competing at mesophilic temperatures was evaluated (Chapter 7). As expected, diversity and population densities in the compost immediately after cool-down and which were capable of growth at 20-25°C were low, with some of the inoculated microorganisms becoming the dominant, or the only-cultivable biota, following further incubation at these temperatures.

In this study, two bacterial antagonists (*P. corrugata* and *L. antibioticus*) were seen to become the dominant cultivable biota in fish waste compost after 14 days incubation, to the exclusion of almost all indigenous compost microbiota (Chapter 7). This was partly attributed to their production of compounds inhibitory to other microbiota. Inhibition of *S. minor* or compost microbiota was also evident following exposure to cell-free extracts of *P. corrugata* (Chapter 7 and Chapter 8). Attempted isolation of the active compound(s) of *L. antibioticus* was unsuccessful, probably because of the volatility of these compounds, which tended to disappear during extraction. The possible role of siderophores in these two cases was eliminated from contention, since *in vitro* inhibition of *S. minor* and selected fish waste compost microbiota also occurred in the presence of high level of $FeCl_3$ in growth medium.

The molecular weight and some characteristics of the antimicrobial compounds produced by *P. corrugata* strain SAJ6 were elucidated (Chapter 8). Two active compounds having molecular weights of 554 and 580 showed inhibition of *S. minor* and of selected fish waste compost microbiota. These compounds were also found inhibitory to some human pathogens, including *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Chapter 8), implying that they may have some medical applications. Elucidation of the structure of these compounds was beyond the scope of this study

although the present report could serve as a starting point for their characterisation, possibly leading to synthetic production of new biocides or their analogues.

The efficacy of composted WFW-grown Td_{22} in inhibiting *S. minor* attack of lettuce and pyrethrum plants was investigated in pot trials (Chapter 6). The degree of protection of lettuce plants was positively correlated with the level of composted WFW-grown Td_{22} amendment, with an amendment rate of 20% v/v giving 100% protection at four weeks after sowing relative to100% mortality in of controls. No toxicity was evident at this rate of amendment. The efficacy of composted WFW-grown Td_{22} (applied at the rate of 5% v/v) for the protection of plants against *S. minor* was consistently significant in glasshouse trials (Chapter 6). Td_{22} also survived in the enriched WFW compost without losing its effectiveness against *S. minor* over a period of 4.5 months (a period subsequently extended to ten months in a subsequent trial), indicating that long-term storage of this fungus in solid low-cost waste materials is possible (Chapter 6). Td_{22} was also found to have the ability to colonise the growing roots of lettuce or pyrethrum plants, an important feature for the provision of protection by a biological control agent.

Pre-incubation of Td_{22} (grown in WFW compost:millet seed mixture) in a potting mix prior to sowing was generally found to improve the biological control of *S. minor* attack of lettuces (Chapter 6). Growth promotion of lettuce plants following application of composted WFW-grown Td_{22} was observed in a pot trials (Plate 6-1), but the cause appears to be related to the release of plant nutrients from the compost as it did not happen when Td_{22} was applied as a spore suspension or mycelial suspension (Chapter 6).

The efficacy of most of the bacterial biological control agents studied was of a lower order than that provided by Td_{22} (Chapter 7). Although these bacteria showed good disease controls in a non-replicated pot trial (Chapter 3), most failed to perform well in further pot-trial assay against *S. minor* (Chapter 7). Loss of biological control competence, particularly in bacteria, is reported to be common (Weller, 1988) and is attributable to a variety of causes as outlined previously.

A field trial of the use of fish waste compost-cultures (modified by the directed cultivation of *Lysobacter antibioticus* or *Pseudomonas corrugata*) gave encouraging results (Chapter 7). Application of suppressive composts at the rate of 12.1 tonnes per

hectare one week before planting improved the percentage of healthy plants at 5 and 8 weeks after planting, although this improvement was not statistically significant ($p>0.05$) when compared to the control treatment. The suppressive composts were however comparable in effectiveness to the protection provided by a commercially available biological control agent (Companion®) applied as drench at the rate of 2 L/m^2 (equivalent to 20 tonnes drench/ha). Possibly an increased rate of application of the compost-cultures would have provided significant protection to lettuce plants in this trial.

Costing of a WFW-millet based suppressive compost product (either inoculated with Td_{22} or bacterial antagonists) indicated production costs amounting to ~AU$95/m^3 (Chapter 6). The most expensive component of this compost was millet seed amendment, comprising ~ 80% of the total production cost. This lead to a search for alternatives to the millet, including by-products of beer manufacture costed at ~1/5th that of millet seed. The outcome was an effective alternative fish waste/malt-combings medium, at an estimated cost of less than half that of the WFW-millet-seed based compost (Chapter 7).

Based on the above estimation, and ignoring considerations of nutrient/soil benefits of compost amendment, direct application of these products in the field to control plant pathogens remains uncompetitive with chemical-based fungicides. In view of this, the use of microbially-manipulated compost as currently formulated may only be appropriate for use by organic growers or at the nursery level. The cost of such products applied at the nursery level was estimated to be several-fold less expensive than that of an antagonist suspension of equivalent cell density in 0.5% (w/v) TSB applied at the rate of 10% (v/v). The estimated cost of producing suppressive fish-waste compost or WFW medium at AU$42-95/m^3 compared well with that of commercially available bark-based potting media retailing in smaller quantities of 20L, at an equivalent of AU$250/m^3 in Tasmania.

In contrast to the use of suppressive compost, application of traditional alternatives such as bark-based materials (discussed above) have been reported to give variable results in the suppression of plant pathogens. Although chemical fungicide control of plant pathogens remains the most cost-effective agent for broad-field application, its effectiveness against many major pathogens is known to be decreasing,

leading to increasing application rates with associated increasing monetary and environmental cost. Therefore while the use of suppressive compost (as produced in the present study) may be presently restricted to small-scale use, its wider application can be predicted following selection of better biological control agents and the demonstration of their consistent effectiveness in low-cost compost matrices.

The ability of suspensions of antagonists of plant pathogens (the fungus Td_{22} and the bacteria *P. corrugata*, and *L. antibioticus*) to control mildew in zucchini plants was determined (Chapter 7A), the level of protection ranging from ~22% to 83% two weeks following pathogen challenge. The effectiveness rapidly declined as the trial progressed indicating that regular application of these antagonists would be required to provide reliable protection. The use of combined culture-applications did not improve the effectiveness of the better agents, and some antagonism between the antagonists was also observed. This work raises the possibility of developing 'compost teas' for the control of foliar diseases of agricultural crops having known and consistently high levels of effectiveness.

In conclusion, this study has successfully demonstrated the potential of the composted and non-composted WFW as well as fish waste compost as possible substrates or carriers for potential antagonists of plant pathogens. Enrichment of these materials with nutrient solutions (ammonium nitrate-BMS solution coupled with millet seed for the WFW enrichment, or ammonium nitrate or urea-BMS solution plus casein or starch for fish waste compost enrichment) was effective in improving the quality of these substrates or carriers for specific antagonists. The growth of the inoculated antagonists in the enriched media was significantly affected by the presence of indigenous mesophilic microbiota, with their reduction being crucial to achieving high populations of the desired microbiota. The use of commercial compost taken from the (still thermophilic) cool-down stage, coupled with other means for reducing competition by indigenous compost microbiota, provided a promising low-cost method for the large-scale production of biological control agents effective against plant pathogens.

This work builds on that of others, particularly Nakasaki *et al*, (1998) who achieved the cultivation of a biological control *Bacillus* spp. to high populations in freshly-cut grass clipping compost, their depending on the survival of spores of their

inoculated bacteria to survive the hot-phase. The developments that were utilized in this study, as previously described, greatly extend the range of desirable organisms that can be so cultivated and opens the way for wide-ranging applications of microbially-manipulated composts.

References

Abadias, M., Teixido, N., Usall, J., Benabarre, A., and Vinas, I. 2001. Viability, Efficacy, and storage stability of freeze-dried biocontrol agent Candida sake using different protective and rehydration media. Journal of Food Protection. 64(6): pp. 856-861.

Abdelzaher, H.M.A., Elnaghy, M.A., and Fadl-Allah, E.M. 1997. Isolation of *Pythium oligandrum* from Egyptian soil and its mycoparasitic effect on *Pythium ultimum* var. ultimum the damping-off organism of wheat.Mycopathologia. 139(2): pp. 97-106.

Adams, P.B., and Ayers, W.A. 1981. *Sporidesmium sclerotivorum*: distribution and function in natural biological control of sclerotial fungi. Phytopathology 71:90-93.

Adams, T.H., Wieser, J.K., and Yu, J.H. 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiology and Molecular Biology Reviews. 62 (1): pp. 35 ff.

Adejumo, T.O., Ikotun, T., and Florini, D.A. 1999. Biological control of *Protomycopsis phaseoli*, the causal agent of leaf smut of cowpea. Journal of Phytopathology. 147(6): pp.371-375.

Adikaram, N. and Abayasekara, C. 2003. Phytoalexins accumulation in induced resistance of four plants against anthracnose or gray mould. 8th International congress of plant pathology. Vol. 1: solving problems in the real world. Christchurch, New Zealand: pp. 42-43.

Agrios, G.N. 1997. Plant pathology, 4th Ed. Academic Press Inc., San Diego, USA.

Ahn, I.P., Park, K., and Kim, C.H. 2002. Rhizobacteria-induced resistance perturbs viral disease progress and triggers defense-related gene expression. Molecules and Cells. 13(2): pp. 302-308.

Aist, J.R. 1983. Structural responses as resistance mechanisms. In: Bailey, J.A. and Deveroll, B.J. (Eds.). The dynamic of host defence. Academic Press Sydney. Pp. 33-70.

Akem, C.N. and Melouk, H.A. 1989. Colonization of sclerotia of *Sclerotinia minor* by a potential biocontrol agent *Penicillium citrinum*. Rev. Plant Path. 68: pp. 127 (Abstract).

Akhtar, M. and Malik, A. 2000. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. Bioresource Technology. 74 (1): 35 – 47.

Alabouvette, C. 1998. Biotic interaction in the soil: An overview. ICPP98 Paper Number 2.7.1S. online. http://www.bspp.org.uk/icpp98/2.7/1.html.

Alexander, B.J.R. and Stewart, A. 2001. Glasshouse screening for biological control agents of *Phytophthora cactorum* on apple (*Malus domestica*). New Zealand Journal of Crop and Horticultural Science. 29(3): pp. 159-169.

Alraddad, A.M. 1995. Interaction of *Glomus mosseae* and *Paecilomyces lilacinus* on *Meloidogyne javanica* of tomato. Mycorrhiza. 5(3): 233-236.

Altschul, S.F., Madden, T., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search program. Nucleic Acids Research. 25. pp. 3389-3402.

Amer, G.A.M., Singh, D.V., Aggarwal, R., and Dureja, P. 1998. Microbial antagonism to *Neovossia indica*, causing Karnal bunt of wheat. Current Science. 75(12): pp. 1393-1396.

Amer, G.A. and Utkhede, R.S. 2000. Development of formulations of biological control agents for management of root rot of lettuce and cucumber. Canadian Journal of Microbiology. 46(9): pp. 809-816.

Andrews, J.H. 1985. Strategies for selecting antagonistic organisms from the phylloplane. In: Windels, C.E. and Lindow, S. (Eds.). Biological control in the phylloplane. American Phytopathological Society. St Paul. Minnesota. pp. 31 – 44.

Anon, 1999. Oligosaccharide elicitor signaling in plant-pathogen interaction. Online: http://www.glycoforum.gr.jp/science/word/saccharide/SA-A01E.html.

Anon, 2002. Cell defence in plants. Online: http://www.biology-online.org/1/12_cell_defense_3.htm.

Atlas, R.M. and Bartha, R. 1993. Microbial ecology: principles and applications. 3rd Ed. The Benjamin/Cummings Publishing Company, Inc. Redwood City.

Atlas, R.M. 1995. Principles of microbiology. 1st ed. Mosby-Year Book, Inc. St. Louis, Baltimore.

Avis, T.J., Hamelin, R.C., and Belanger, R.R. 2001. Approach to molecular characterization of fungal biocontrol agents: Some case studies. Canadian Journal of Pathology. 23: pp. 8-12.

Ayers, W.A., and Adams, P.B. 1979. Mycoparasitism of sclerotia of *Sclerotinia* and *Sclerotium* species by *Sporidesmium sclerotivorum.* Can. J. Microbiol. 25:17-23.

Backman, P.A., Brannen, P.M., and Mahaffee, W.F., 1994. Plant response and disease control following seed inoculation with Bacillus subtilis. In : Ryder, M.H., Stephens, P.M., and Bowen, G.D. (Eds.). Improving plant productivity with rhizosphere bacteria. CSIRO division of soils. Adelaide (South Australia). Pp. 3 – 8.

Backman, P.A., Wilson, M., and Murphy, J.F. 1997. Bacteria for biological control of plant diseases. In: Rechcigl, N.A. and Rechcigl, J.E. (Eds). Environmentally safe approaces to crop disease control.CRC Lewis Publishers, Boca Raton, FL: pp. 95-109.

Backhouse, D. and Stewart, A. 1989. Ultrastructure of antagonism of *Sclerotium cepivorum* by *Bacillus subtilis*. Journal of Phytopathology. 124: pp. 207-214.

Bacon, C.W. and Hinton, D.M. 2002. Endophytic and biological control potential of *Bacillus mojavensis* and related species. Biological Control. 23(3): pp. 274-284.

Bahme, J.B. and Schroth, M.N. 1987. Spatial-temporal colonization pattern of a rhizobacterium on underground organs of potatoes. Phytopathology. 77: pp. 1093-1100.

Baillieul, F., Genetet, I., Kopp, M., Saindrenan, P., Friting, B., and Kauffmann, S. 1995. A new elicitor of the hypersensitive response in tobacco: a fungal glycoprotein elicites cell death, expression of defense genes, production of salicylic cell, and induction of systemic aquired resistance. Plant Journal. 8: pp. 551-560.

Baker. K.F. and Cook, R.J. 1974. Biological control of plant pathogens. Freeman. San Fransisco. 433 pp.

Baker, K.F. 1987a. Evolving concepts of biological control of plant pathogens. Annual Review of Phytophatology. 25: pp. 67 – 85.

Baker, R. and Dunn, P.E. 1990. New direction in biological control : Alternative for suppressing agricultural pest and disease. Alan R. Liss, New York. 837 pp.

Baker, R. and Griffin, G.J. 1995. Molecular strategies for biological control of fungal plant pathogens. In: Reuveni, R. (ed.). Novel approaches to integrated pest management. Lewis, CRC Press, Boca Raton, 369 pp.

Barkdoll, A.W., Nordsedt, R.A., and Mithchell, D.J. 1991. Large scale utilization and composting of yard waste. Online. http://edis.ifas.ufl.edu/BODY_HE027.

Barnett, H.L. and Binder, F.L. 1973. The fungal host-parasite relationship. Annual Review in Phytopathology. 11 : pp. 273 – 292.

Bazzi, C., Alexandrova, M., Stefani, E., Anaclerio, F., and Burr, T.J. 1999. Biological control of *Agrobacterium vitis* using non-tumorigenic agrobacteria. Vitis. 38(1): pp. 31-35.

Becker, D.M., Kinkel, L.L., and Schottel, J.L. 1997. Evidence for interspecies communication and its potential role in pathogen suppression in a naturally occurring disease suppressive soil. Canadian Journal of Microbiology. 43(10): pp. 985-990.

Beever, R.E. 2003. Fungicide resistance: Botrytis cinerea versus dicarboximides as a case study. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 83.

Bélanger, R.R. and Benhamou, N. 2003. Defence responses associated with induced resistance. 8th International Congress of Plant Pathology. Christchurch, New Zealand: pp. 54.

Benhamou, N., Mazau, D., Grenier, J., and Esquerre-Tugaye, M.T. 1991. Time-course study of the accumulation of hydroxyproline-rich glycoproteins in root cells of susceptible and resistant tomato plants infected by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Planta. 184: pp. 196-208.

Benhamou, N. and Chet, I. 1993. Hyphal interactions between *Trichoderma harzianum* and *Rhizoctonia solani*: Ultra structure and cytochemistry of the antagonistic process. Phytopathology 83: 1062-1071.

Benhamou, N. and Brodeur, J. 2000. Evidence for antibiosis and induced host defense reactions in the interaction between *Verticillium lecanii* and *Penicillium digitatum*, the causal agent of green mold. Phytopathology. 90(9): pp. 932 – 943.

Benhamou, N; Gagne, S; Le Quere, D; Dehbi, L. 2000. Bacterial-mediated induced resistance in cucumber: Beneficial effect of the endophytic bacterium *Serratia plymuthica* on the protection against infection by *Pythium ultimum*. Pythopathology. 90(1): pp. 45-56.

Benhamou, N., Belanger, R.R., Rey, P., and Tirilly, Y. 2001. Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to *Fusarium* crown and root rot in tomato plants. Plant Physiology and Biochemistry. 39(7-8): pp. 681-696.

Berg, G., Kurze, S., Buchner, A., Wellington, E.M., and Smalla, K. 2000. Successful strategy for the selection of new strawberry-associated rhizobacteria antagonistic to *Verticillium* wilt. Canadian Journal of Microbiology. 46(12): pp. 1128 – 1137.

Berg, G., Marten, P., Minkwitz, A., and Bruckner, S. 2001. Efficient biological control of fungal plant diseases by *Streptomyces* sp DSMZ 12424. Journal of Plant Disseases and Protection. 108(1): pp. 1-10.

Bettini, P., Cosi, E., Pellegrini, M.G., Turbani, L., Vendramin, G.G., and Buiatti, M. 1998. Modification of competence for *in vitro* response to *Fusarium oxysporum* in tomato cells. III. PR-protein gene expression and ethylene evolution in tomato cell linestransgenic for phytohormon-related bacterial genes. Theoritical and Applied Genetics. 97: pp. 575-583.

Bigirimana, J. and Hofte, M. 2002. Induction of systemic resistance to *Colletotrichum lindemuthianum* in bean by a benzothiadiazol derivative and rhizobacteria. Phytoparasitica. 30(2): pp. 159-168.

Bol, J.F., Verbene, M., and Linthorst, H.J.M. 2003. Ethylene perception is required to generate the long distance SAR signal in tobacco. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 53-54.

Boland, G.J. and Inglis, G.D. 1989. Antagonism of white mold (*Sclerotinia sclerotiorum*) of bean by fungi from bean and rapessed flowers. Canadian Journal of Botany. 67: pp. 1775 – 1781.

Boller, T. 1991. Ethylene in pathogenesis and disease resistance. In: Matto, A.K. and Suttle, J.D. (Eds.). The plant hormone ethylene. CRS Press, Boca Raton, USA. pp. 293-314.

Bonsal, R.F., Weller, D.M., and Thomashow, L.S. 1997. Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas spp. in vitro* and in the rhizosphere of wheat. Applied and Environmental Microbiology. 63: pp. 951-955.

Bolwell, G.P. and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plants defence-a broad perspective. Physiological and Molecular Pathology. 51: pp. 347-366.

Brinton, W.F. 1995. The control of plant pathogenic fungi by use of compost teas. Biodynamics. January-February: pp. 12-15.

Broadbent, P., Baker, K.F., and Waterworth, Y. 1971. Bacteria and actinomycetes antagonistic to fungal root pathogens in Australian soil. Australian journal of biological science. 24: pp. 925 – 944.

Brock, T.D. and Madigan, M.T. 1991. Biology of microorganisms. 6th Eds. Prentice Hall, Englewood Cliffs. New Jersey.

Bryk, H., Dyki, B., and Sobiczewski, P. 1998. Antagonistic effect of *Erwinia herbicola* on *in vitro* spore germination and germ tube elongation of *Botrytis cinerea* and *Penicillium expansum*. Biocontrol. 43 (1) : pp. 97 – 106.

Budge, S.P., Mcquilken, M.P., Fenlon, J.S., and Whipps, J.M. 1995. Use of *Coniothyrium minitans* and *Gliocladium virens* for biological control of *Sclerotinia sclerotiorum* in glasshouse lettuce. Biological Control. 5(4): pp. 513 – 522.

Budge, S.P. and Whipps, J.M. 2000. Potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using *Coniothyrium minitans* and reduced fungicide application. Pytopathology. 91(2): pp. 221 – 227.

Buell, C.R. 1999. Genes involve in plant-pathogen interactions. In: Agrawal, A.A., Tuzun, S., and Bent, E. (Eds.). Induced plant defenses against pathogens and herbivores. Biochemistry, Ecology, and Agriculture.APS Press. St. Paul, Minnesota.

Bull, C.T., Weller, D.M., and Thomashow, L.S. 1991. Relationship between root colonization and suppression of Gaeumannomyces graminis var tritici by Pseudomonas fluorescens strain 2-79. Phytopathology. 81: pp. 954-959.

Burla, M., Goverde, M., Schwinn, F.J., and Wiemken, A. 1996. Influence of biocontrol organisms on root pathogenic fungi and on plant symbiotic microorganisms *Rhizobium phaseoli* and *Glomus mosseae*. Journal of Plant Disease and Protection. 103 (2) : pp. 156 – 163.

Busam, G., Kessemayer, H.H., and Matern. 1997. Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiology. 115: pp. 1029-1038.

Caesar, A.J. and Burr, T,J. 1987. Growth promotion of apple seedlings and root-stock by specific strains of bacteria. Phytopathology. 77: pp. 1583-1588.

Calvente, V., Benuzzi, D., and de Tosetti M.I.S. 1999. Antagonistic action of siderophores from *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. International Biodeterioration & Biodegradation. 43(4): pp.167-172.

Campbell, R. 1986. The search for biological control agents against pathogens: a pragmatic approach. Biol. Agric.. Hort. 3: pp. 317 – 327.

Campbell, R., Renwick, A., and Coe, S.K.A.M. 1986. Antagonism and siderophores production by biocontrol agents, plant growth promoting organisms and the general rhizosphere population. In: Swinburne, T.R. Iron, siderophores and plant disease. NATO ASI Series A Live Sciences. Plenum Press, New York: pp. 179-189.

Campbell, R. 1994. Biological control of soil-borne diseases: Some present problems and different approaches. Crop Protection. 13(1): pp. 4 – 13.

Cartwright, D.K. and Benson, D.M. 1995 (a). Optimization of biological control of Rhizoctonia stem rot of *Poinsettia* by *Paecilomyces lilacinus* and *Pseudomonas cepacia*. Plant Disease. 79(3): pp. 301-308.

Cartwright, D.K. and Benson, D.M. 1995 (b). Biological control of *Rhizoctonia* stem rot of *Poinsettia* in polyfoam rooting cubes with *Pseudomonas cepacea* and *Paecilomyces lilacinus*. Biological Control. 5(2): pp. 237 – 244.

Chakraborty, S., Murra, G.M. Magare, P.A., Yonow, T., O'Brien, R.G., Croft, B.J., Barbetti, M.J., Sivasithamparam, K., Old, K.M., Dudzinski, M.J., Sutherst, R.W., Penrose, L.J., Archer, C., and Emmett, R.W. 1998. Potential impact of climate on plant diseases of economic significance to Australia. Australian Plant Pathology. 27(1): pp. 15-35.

Chanway, C.P., Turkington, R., and Holl, F.B. 1991. Ecological implications of specificity between plant and rhizosphere microorganisms. Advances in Ecological research. 21:pp. 121-169.

Chao, W.L. and Alexander, M. 1984. Mineral soil as carriers for *Rhizobium* inoculants. Applied and Environmental Microbiology. 47: pp. 94-97.

Cheah, L.H., Wilson, C.L., and Marshall, A.P. 1996. Rapid screening for antagonists against *Botrytis* storage rots using leaf and fruit tissue. Postharvest Biology and Technology. 8(3): pp. 223 – 228.

Chen, Y. And Inbar, Y. 1993. Chemical and spectroscopical analysis of organic matter transformations during composting in relation to compost maturity. In: Hoitink, H.A.J. and Keener, H.M. (Eds.). Science and Engineering of composting: design, environmental, microbiological and utilization aspects. Renaissance Publications, Worthington, OH: pp. 551-600.

Chen, J., Abawi, G.S., and Zuckerman, B.M. 1999. Suppression of *Meloidogyne hapla* and its damage to lettuce grown in a mineral soil amended with chitin and biocontrol organisms. Journal of Nematology. 31(4): pp. 719-725.

Chen, S.Y., Dickson,D.W., and Michell, D.J. 2000. Viability of *Heterodera glycines* exposed to fungal filtrates. Journal of Nematology. 32(2): pp. 190 – 197.

Chet, I., Harman, G.E., and Baker, R. 1981. *Trichoderma hamatum*: its hyphal interation with *Rhizoctonia solani* and *Pythium* spp.. Microb. Ecol. 7: pp. 29-38.

Chet, I., Inbar, J., and Hadar, Y. 1997. Fungal antagonists and mycoparasites. In: Lemke, P.A. and Esser, K. The mycota: A comprehensive treatise on fungi as experimental system for basic and applied research. Springer-Verlag Berlin Heidelberg: pp. 165-184.

Chun, W. 2000. Biological control with a plant pathogenic bacterium, *Pseudomonas corrugata*. SARE 2000 Conference Proceedings. Online: http://wsare.usu.edu/sare2000/071.htm

Chung, Y.R. and Hoitink, H.A.J. 1990. Interaction between thermophilic fungi and *Trichoderma hamatum* in suppression of *Rhizoctonia* damping-offin bark compost-amended container medium. Phytopathology. 80: pp. 73-77.

Clarkson, J.P. and Lucas, J.A. 1997. The role of antibiotic production by a strain of *Pseudomonas fluorescens* in the suppression of *Pseudocercosporella herpotrichoides*, the causal agent of eyespot disease of cereals. Journal of Applied Microbiology. 82(4): pp. 499-506.

Cohen, Y., Baider, A., Dong, H., Saidkarimov, A., and Ben-Daniel, B. 2003. Disease control with natural products. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 70-71.

Coley-Smith, J.R. 1979. Survival of plant pathogenic fungi in soil in the absent of host plants. In: Schippers, B. and Gam, W. (Ed,). Soil-borne plant pathogens. Accademic press. London: Pp. 39 – 57.

Collins, C.H. and Lyne, P.M. 1984. Microbiological methods. 5th Ed. Butterworths. London, Boston, Durban, Singapore, Sydney, Toronto, and Wellington.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press. St. Paul. Minesota.

Cook, R.J. 1990. Twenty-five years of progress towards biological control. In: Hornby, D., Cook, R.J., Henis, Y., Ko, W.H., Rovira, A.D., Schippers, B., and Scott, P.R. (Eds.). Biological control of soil-borne plant pathogens. C.A.B. International. Wallingford, U.K. pp. 1-14.

Cook, R.J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annual Review of Phytopathology. 31: pp. 53-80.

Cook, J., Bruckart, W.L., Coulson, J.R., Goettel, M.S., Humber, R.A., Lumsden, R.D., Maddox, J.V., Mcmanus, M.L., Moore, L., Meyer, S.F., Quimby, P.C., Stack, J.P., and Vaughn, J.L. 1996. Safety of microorganisms intended for pest and plant disease control – A framework for scientific evaluation. Biological control. 7(3): pp. 333 – 3351.

Coppoc, G.L. 1996. Peptide antibacterials. Online. http://www.vet.purdue.edu/depts/bms/courses/chmrx/polypep.htm.

Cousin, M.A. 1994. Chitin as a measure of mold contamination of agricultural commodities and foods. Journal of Food Protection. 59 (1): pp. 73-81.

Craft, C.M. and Nelson, E.B. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by Pythium graminicola. Applied and Environmetal Microbiology. 62: pp. 1550-1557.

Cruickshank, R., Duguid, J.P., Marmion, B.P., and Swain, R.H.A. 1975. Medical microbiology. 12th Ed. Churchill Livingstone. Edinburg, London and New york.

Cruickshank, R.H. and Pitt, J.I. 1987. Identification of species in *Penicillium* subgenus *Penicillium* by enzyme electrophoresis. Mycologia. 79: pp. 614 – 620.

Dandurand, L.M. and Knudsen, G.R. 1993. Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermosphere and rhizosphere of pea. Phytopathology. 83(3): pp. 265-270.

Dandurand, L.M., Mosher, R.D., and Knudsen, G.R. 2000. Combined effect of *Brasica napus* meal and *Ttrichoderma harzianum* on two soilborne plant pathogens. Canadian Journal of Microbiology. 46(11): pp. 1051-1057.

Danielsen, S. and Jensen, D.F. 1999. Fungal endophytes from stalks of tropical maize and grasses: isolation, identification, and screening for antagonism against *Fusarium verticilloides* in maize stalks. Biocontrol Science and Technology. 9(4): pp. 545-553.

Danielson, R.M. and Davey, C.B. 1973. Non nutritional factors affecting the growth of *Trichoderma* in culture. Soil Biology and Biochemistry. 5: pp. 495-504.

Datnoff, L.E., Nemec, S., and Pernezny, K. 1995. Biological control of *Fusarium* crown and root rot of tomato in Florida using *Trichoderma harzianum* and *Glomus intraradices*.Biological Control. 5: pp. 427-431.

Davis, R.M., Subbarao, K.V., Raid, R.N., and Kurtz, E.A. 1997. Compendium of lettuce diseases. APS Press, The American Phytopathological Society. Minnesota. USA: pp. 19 – 21.

DeBach, P. (Ed.). 1964. Biological control of insect pest and weeds. Reinhold. New York. 844 pp.

DeCal, A. and Sagasta, E.M. 1990. Biological control of peach twig blight (*Monilinia laxa*) with *Penicillium frequentans*. Plant Pathology. 39: pp. 612-618.

DeCeuster, H.J.M. and Pauwels, F.P.L. 1995. Soil disinfection in Belgian horticulture-Practice view. Acta Hortic. 382: pp. 37-50.

DeCeuster, T.J.J. and Hoitink, H.A.J. 1999. Prospect for composts and biocontrol agents as substitutes for methyl bromide in biological control of plant diseases. Compost Science and Utilization. 7(3): pp. 6-15.

Défago, G. 2003. Biocontrol in the rhizosphere. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 37-38.

De La Fuente, L., Bajsa, N., Bagnasco, P., Quagliotto, L., Thomashow, L., and Arias, A. Antibiotic production by biocontrol *Pseudomonas fluorescens* isolated from forage legume rhizosphere. Online. http://www.ag.auburn.edu/argentina/pdfmanuscripts/delafuente.pdf.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negretto, D., Gaffney, T., Gur-Rella, M., Kesmann, H., Ward, E., and Ryls, J. 1994. A central role of salicylic acid in plant disease resistance. Science. 226: pp. 1247-1250.

De Leij, F.A.A.M., Sutton, E.J., Whipps, J.M., Fenlon, J.S., and Lynch, J.M. 1995. Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Applied and Environmental Microbiology. 61: pp. 3443-3453.

del Rio, L. 2001. Biological control of *Sclerotinia sclerotiorum*. Online. http://www.ncsrp.com/whitemold/bioconwm.htm.

Dennis, C. and Webster, J. 1977. Antagonistic properties of species group of *Trichoderma*. Trans. Br. Mycol. Soc. 57: pp. 25-39.

Diaz, L.F., Savage, G.M., Eggerth, L.L., and Golueke, C.G. 1993. Composting and recycling municipal solid waste. Lewis Publishers. London.

Dijksterhuis, J., Sanders, M., Gorris, L.G.M., and Smid, E.J. 1999. Antibiosis plays a role in the contex of direct interaction during antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. Journal of applied microbiology. 86: pp. 13 – 21.

Dik, A.J., Koning, G., and Kohl, J. 1999. Evaluation of microbial antagonists for biological control of *Botrytis cinerea* stem infection in cucumber and tomato. European Journal of Plant Pathology. 105(2): pp. 115-122.

Dik, A.J. and Elad, Y. 1999. Comparison of antagonists of *Botrytis cinerea* in greenhouse-grown cucumber and tomato under different climatic conditions. European Journal of Plant Pathology. 105(2): pp. 123-137.

Diver, S. 1998. Disease suppressive potting mixes-Pest management technical note. ATTRA. Online: http://www.attra.org/attra-pub/dspotmix.html.

Diver, S. 1998[a]. Compost teas for plant disease control. Online: http://www.attra.org/attra-pub/comptea.html.

Dixon, R.A. and Lamb, C.J. 1990. Molecular communication in interactions between plants and microbial pathogens. Annual Review of Plant Physiology and Plant Molecular Biology. 41: pp. 339-367.

Domingues, F.C., Querioz, J.A., Cabral, J.M.S., and Fonseca, L.P. 2000. The influence of culture condition on mycelial structure and cellulase production by *Trichoderma reesei* Rut C-30. Enzyme and Microbial Technology. 26(5-6): pp. 394 – 401.

Dorrance A.E., Lipps, P.E., and Mills, M.R. *Rhizoctonia* damping-off and steam rot of soybeans. Ohio State University Extension Fact sheet. online article (http://ohioline.osu.edu/ac-fact/0025.html).

Drahos, D.J. 2001. Biology control: Background. Online. (http://www.bi-chem.com/green_releaf/professional/tech_info/tech_info_bio_ctrl.htm)

Dubos, B. and Ricard, J.L. 1974. Curative treatment of peach trees against silver leaf disease (Sterum purpureum) with *Trichoderma viride* preparation. Plant Disease Reporter. 58: pp. 147-150.

Duffy, B.K. and Weller, D.M. 1995. Use of *Gaeumannomyces graminis* var *graminis* alone and combination with fluorescent *Pseudomonas spp.* to suppress take-all of wheat. Plant Disease. 79: pp. 907-911.

Duffy, B.K., Simon , A., and Weller, D.M. 1996. Combination *Trichoderma koningii* and fluorescent Pseudomonads for contro of take-all on wheat. Phytophatology. 86: pp. 188-194.

Duffy, B.K., and Defago, G. 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of Tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology. 87 (12): pp. 1250 – 1257.

Duffy, B.K., and Defago, G. 2000. Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. Applied and Environmental Microbiology. 66(8): pp. 3142 – 3150.

Duijff, B.J., Pouhair, D., Olivain, C., Alabouvette, C., and Lemanceau, P. 1998. Implication of systemic induced resistance in the suppression of *Fusarium* wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. European journal of Plant pathology. 104(9): pp. 903-910.

Duponnois, R., Fargette, M., Fould, S., Thioulouse, J., Davies, K.G. 2000. Diversity of the bacterial hyperparasite *Pasteuria penetrans* in relation to root-knot nematodes (*Meloidogyne* spp.) control on *Acacia holosericea.* Nematology. 2: pp. 435-442.

Edwards, M.C., Fetch, T.G., Schwarz, P.B., and Steffenson, B.J. 2001. Effect of Barley yellow dwarf virus infection on yield and malting quality of barley. Plant Disease. 85(2): pp. 202-207.

Elad, Y., Chet, I., and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum.* Canadian J. Microbiol. 28: pp. 719 – 725.

Elad, Y. and Shtienberg, D. 1994. Effect of compost water extracts on Grey mould (*Botrytis cinerea.* Crop Protection. 13(2): pp. 109-114.

Elad, Y., Kirshner, B., and Sztejnberg, A. 1998. Management pof powdery mildew and gray mold of cucumber by *Trichoderma harzianum* T39 and *Ampelomyces quisqualis* AQ10. Biocontrol. 43(2): pp. 241 – 251.

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protection. 19(8-10): pp. 709-714.

Elhendawy, H.H., Zeid, I.M., and Mohammed, Z.K. 1998. The biological control of soft rot disease in melon caused by *Erwinia carotovora* subsp. *carotovora* using *Pseudomonas fluorescens*. Microbiological Research. 153 (1): pp. 55 – 60.

El-Katatny, M.H., Somitsch, W., Robra, K.H., El-Katatny, M.S., and Gubitz, G.M. 2000. Production of chitinase and β-1,3 glucanase by *Trichoderma harzianum* for control of phytopathogenic fungus *Sclerotium rolfsii*. Food Technology and Biotechnology. 38(3): pp. 173-180.

Ellis, M.A. 2001. Developing an effective fungicide spray program for wine grapes in Ohio. Online: http://viticulture.hort.iastate.edu/wsfeb01/ef.pdf.

Elson, M.K., Schisler, D.A., and Bothast, R.J. 1997. Selection of microorganisms for biological control of silver scurf *(Helminthosporium solani)* of potato tubers. Plant Disease. 81(6): pp. 647 – 652.

El-Tarabily, K.A., Sykes, M.L., Kurtboke, I.D., Hardy, G.E.S., Barbosa, A.M., and Dekker, R.F.H. 1996. Synergistic effects of a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytopthora cinnamomi* root rot of *Banksia grandis*. Canadian Journal of Botany. 74(4): pp. 618-624.

El-Tarabily, K.A., Soliman, M.H., Nassar, A.H., Al-Hassani, H.A., Sivasithamparam, K., McKenna, F., and Hardy, G.E.S. 2000. Biological control of *Sclerotinia minor* using a chitinolytic bacterium and actinomycetes. Plant Pathology. 49(5): pp. 573-583.

Emanuele, M.C., Scaloni, A., Lavermicocca, P., Jacobellis, N.S., Camoni, L., Di Giorgio, D., Pucci, P., Paci, M., Segre, S., and Ballio, A. 1998. Corpeptins, new bioactive lipodepsipeptides from cultures of *Pseudomonas corrugata*. FEBS letters. 433: pp. 317-320.

Falconi, C.J. and Mendgen, K. 1994. Epiphytic fungi on apple leaves and their value for control of postharvest pathogens Botrytis cinerea, *Monilinia fructigena* and *Penicillium expansum*. Journal of Plant Diseases and Protection. 101(1): pp. 38 – 47.

Fedi, S., Tola, E., Moenne-Loccoz, Y., Downing, D.N., Smith, L.M., and O'Gara, F. 1997. Evidence for signaling between the pathogenic fungus *Pythium ultimum* and *Pseudomonas fluorescens* F113: *P. ultimum* represses the expression of genes in *P. fluorescens* F113, resulting in altered ecological fitness. Applied and Environmental Microbiology. 63: pp. 4261-4266.

Földes, T. Bánhegyi, I., Herpai, Z., Varga, L., and Szigeti, J. 2000. Isolation of *Bacillus* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage microorganisms. Journal of Applied Microbiology. 89: pp. 840-846.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant disease. Annnual Review of Phytopathology. 26: pp. 75-91.

Fravel, D.R., Connick, W.J., and Lewis, J.A. 1998. Formulation of microorganisms to control plant diseases. In: Burges, H.D. (Ed.). formulation of microbial biopesticides. Cluwer Academic Publihers, Boston, Massachusetts: pp. 187-202.

Fravel, D. and Lazarovits, 2003. Commercialisation of biocontrol. 8th International Congress of Plant pathology. Solving problem the real world. Vol. 1-Invited papers. Christchurch, New Zealand: pp. 26-27.

Frietas, L.G., Ferraz, S., and Muchovej, J.J. 1995. Effectiveness of different isolate of *Paecilomyces lilacinus* and an isolate of *Cylindrocarpon destructans* on the control of *Meloidogyne javanica*. Nematropica. 25(2): pp. 109 – 115.

Frietas, L.G., Mitchell, D.J., and Dickson, D.W. 1997. Temperature effects on the attachment of *Pasteuria penetrans* endospores to *Meloidogyne arenaria* race 1. Journal of Nematology. 29 (4): pp. 547 – 555.

Gamard, P. and Deboer, S.H. 1995. Evaluation of antagonistic bacteria for suppression of bacterial ring rot of potato. European Journal of Plant Pathology. 101(5): pp. 519 – 525.

Gazoni, L., Cozzi, J., and Kobayashi, K. 1998. Survival of potential biocontrol bacteria in various formulations and their ability to reduce radish damping-off caused by *Rhizoctonia solani*. Journal of Plant Diseases and Protection. 105 (1): pp. 41 – 48.

Genetic Engineering newsletter. Online.
http://www.oeko.de/bereiche/gentech/newslet/documents/S-Gen-e5.pdf

Getha, K. and Vikineswary, S. 2002. Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f.sp *cubense* race 4: Indirect evidence for the role of antibiosis in the antagonistic process. Journal of Industrial Microbiology and Biotechnology. 28(6): pp. 303-310.

Gianessi, L.P. 1998. Changing the mindset regarding use of biocontrol product. Rutgers University Workshop on Alternative Paradigms for Commercializing Biological Control, New Brunswick, N.J. online. http://www.ncfap.org/RUTGRS01.HTML.

Giannakou, I.O., Gowen, S.R., and Davies, K.G. 2002. Aspects on the attachment of *Pasteuria penetrans* on root-knot nematodes. Russian Journal of Nematology. 10(1): pp. 25-31.

Gilles, T. and Kennedy, R. 2003. Development and testing of forecasting models for downy mildew (Peronospora destructor) on onions. 8th International Congress of Plant pathology. Vol 2. Christchurch, New Zealand: pp. 123.

Glazebrook. J. 1996. Plant defense responses. Online.
http://stein.cshl.org/atir/biology/protocols/cshl-course/8-defense.html

Goodman, R.N. and Novacky, A.J. 1994. The hypersensitive reaction in plants to pathogens. APS Press, St. Paul, MN: pp. 224.

Gorlach, K. 1994. Problems in the introduction of genetically engineered microorganism into the environment. Acta Microbiologica Polonica. 43: pp. 121-131.

Gorodecki, B. and Hadar, Y. 1990. Suppression of *Rhizoctonia solani* and *Sclerotium rolfsii* diseases in container media contaning composted separated cattle manure and composted grape marc. Crop Protection. 9: pp. 271-274.

Gowen, S.R., Tzortzakalis, E.A., Channer, A.G.D. 1998. Control of root-knot nematode *Meloidogynes javanica* by the parasite *Pasteuria penetrans* as influenced by the initial nematode population densities. Nematologica. 44 (4): pp. 369 – 379.

Gracia-Garza, J.A., Bailey, B.A., Paulitz, T.C., Lumsden, R.D., Releder, R.D., and Robert, D.P. 1997. Effect of sclerotia damage of *Sclerotinia sclerotiorum* on the mycoparasitic activity of *Trichoderma hamatum*. Biocontrol Science and Technology. 7(3): pp. 401-413.

Granatstein, D. 1998. Suppressing plant disease with compost. Online: http://www.goodfruit.com/archieve/May1-98/special3.html.

Grebus, M.E., Watson, M.E., and Hoitink, H.A.J. 1994. Biological, chemical, and physical properties of composted yard trimmings as indicators of maturity and plant disease suppression. Compost Science and Utilization. 2(1): pp. 55-71.

Grosch, R. and Grote, D. 1998. Suppression of *Phytophthoranicotianae* by application of *Bacillus subtilis* in closed soilless culture of tomato plants. Gartenbauwissenschaft. 63 (3): pp. 103 –109.

Gupta, S.K. and Agarwala, R.K. 1990. Biological control of *Sclerotinia* stalk rot of cauliflower. Rev. Plant. Path. 69: pp. 171 (Abstr).

Gupta, V., Willits, M.G., and Glazebrook, j. 2000. *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: Evidence for inhibition jasmonic acid signaling by SA. Mol. Plant-Microbe Interact. 13: pp. 503-511.

Hackenberg, C., Vrain, T.C., Sikora, R.A. 1999. Rhizosphere colonization pattern of *Agrobacterium radiobacter* strain G12A, an antagonistic rhizobacterium to the potato cyst nematode *Globodera pallida*. Microbiological Research. 154(1): pp. 57-61.

Hadar, Y., Chet, I., and Henis, Y. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harzianum*. Phytopathology. 69: pp. 64-68.

Hadar, Y., Harman, G.E., and Taylor, A.G. 1984. Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soil for biocontrol of seed rot caused by *Pythium* spp. Phytopathology. 74: 106-110.

Hadar, Y. and Gorodecki, B. 1991. Suppression of germination of sclerotia of *Sclerotium rolfsii* in compost. Soil Biology and Biochemistry. 23: pp. 303-306.

Hagedorn, C. And Hagedorn, S.A. 1998. Public perceptions of transgenics in biocontrol. AgBiotech News and Information. 10(7): pp. 217N-222N.

Halterman, D.A. and Martin, G.B. 1997. Signal recognition and transduction involved in plant disease resistance. Essay Biochem. 32: pp. 87-99.

Hammerschmidt, R. and Nicholson, R.L. 2000. A survey of plant defense responses to pathogens. In: Agrawal, A.A., tuzun, S., and Bent, E.(Eds.). Induced palnat defenses against pathogens and herbivores. APS Press. St. paul, Minnesota: pp. 55-71.

Hammond-Kosack, K.E. and Jones, J.D.G. 1996. Resistance gene-dependent plant defense responses. Plant Cells. 8: pp. 1773-1791.

Han, D.Y., Coplin, D.L., Bauer, W.D., and Hoitink, H.A.J. 2000. A rapid bioassay for screening rhizosphere microorganisms for their ability to induce systemic resistance. Phytopathology. 90(4): pp. 327-332.

Harada, H. and Ishikawa, H. 1997. Experimental pathogenicity of *Erwinia aphidicola* to pea aphid, *Acyrthosiphon pisum.* Journal of General and Applied Microbiology. 43 (6): pp. 363 – 367.

Hardy, G.E.St.J. and Sivasithamparam, K. 1991. Suppression of Phytophthora root rot by composted Eucalyptus bark mix. Australian journal of Botany. 39: pp. 153-159.

Haris, A.R. and Lumsden, R.D. 1997. Interaction of Gliocladium virens with Rhizoctonia solani and Pythium ultimum in non-sterile potting medium. Biocontrol Science and Technology. 7(1): pp. 37-47.

Harman, G.E., Chet, I., Baker, R. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. Phytopathology. 70: pp. 1167-1172.

Hashizum, H., Igarashi, M., Hattori, S., Hori, M., Hamada, M., and Takeuchi, T. 2001. Tripropeptins, novel antimicrobial agents produced by *Lysobacter* sp. I. Taxonomy, isolation and biological activities. Journal of Antibiotics. 54(12): pp. 1054-1059.

Hebbar, K.P., Davey, A.G., and Dart, P.J. 1992. Rhizobacteria of maize antagonistic to *Fusarium moniliforme.* a soil-borne fungal pathogen: isolation and identification. Soil Biology and Biochemistry. 24: pp. 979 – 987.

Heil, M. 2001. Induced systemic resistance (ISR) against pathogens-apromising field for ecological research. Perspectives in Plant Ecology Evolution and Systematics. 4(2): pp. 65-79.

Heimbrook, M.E., Wang, W.L.L., and Campbell, G. 1989. Staining bacterial flagella easily. Journal of Clinical Microbiology. 27: pp. 2612-2615.

Herrick, J.E. and Wander, M.M. 1997. Relationship between soil organic carbon and soil quality in cropped and rangeland soils: The importance of distribution, compossition, and soil bilogical activity. In: Lal, R. (Ed.). Soil process and the carbon cycle. Advance in soil science series. CRC Press. Boca Raton, F.L: pp. 405-425.

Hirt, H. 2002. Signal transduction of plant-microbe interaction. Online: http://emb1.bcc.univie.ac.at/PhD/Research/UNI/Hirt/hirttext.htm.

Hodges, C.F., Campbell, D.A., and Christians, N. 1993. Evaluation of *Streptomyces* for biocontrol of *Bipolaris sorokiniana* and *Sclerotinia homoeocarpa* on the phylloplane of *Poa pratensis*. Journal of Phytopathology. 139 (2): pp. 103 – 109.

Hoffmannhergarten, S., Gulati, M.K., and Sikora, R.A. 1998. Yield response and biological control of *Meloidogyne incognita* on lettuce and tomato with rhizobacteria. Journal of Plant Disease and Protection. 105 (4): pp. 349 – 358.

Hoitink, H.A.J. and Kuter, G.A. 1986. Effect of compost in growth media on soilborne pathogens. In: Chen, Y. and Avnimelech, Y. (Eds). The role of organic mater in modern agriculture. Martinus Nijhoff Publishers, Dordrecht, the Netherlands: pp. 289-306.

Hoitink, HA.J. and Fahy, P.C. 1986. Basis for the control of soilborne plant pathogens with compost. Annual Review of Phytopathology. 24: pp. 93-114.

Hoitink, H.A.J. 1990. Production of disease suppressive compost and container media, and microorganism culture for use therein. U.S. Patent 4,960,348. 13 Feb. 1990.

Hoitink, H. A. J., Inbar, Y., and Boehm, M.J. 1991. Compost can suppress soil-borne diseases in container media. American Nurseryman. September 15: pp. 91-94.

Hoitink, H.A.J., Boehm, M.J., and Hadar, Y. 1993. Mechanism of suppression of soilborne plant pathogens in compost-amended substrate. In: Hoitink, H.A.J. and Keener, H.M. (Eds.). Science and engineering of composting: Design, environmental, microbiological, and utilization aspects. Renaisance Publications, Worthtington, OH: pp. 601-621.

Hoitink, H.A.J., Maden, L.V., and Boehm, M.J. 1996. Relationships among organic mater decomposition level, microbial species diversity, and soilborne disease severity. In: Hall, R. (Editor). Principles and practice of managing soilborne plant pathogens. The American Phytophatology Sciety: pp. 237-249.

Hoitink, H.A.J., Stone, A.G., and Han, D.Y. 1997. Suppression of plant disease by compost. HortSci. 32: pp. 184-187.

Hoitink, H.A.J. and Gardener, B.M. 2003. Disease suppression through manipulation of microbial communities in compost and soil. 8th International congress of plant pathology. Vol. 1: solving problems of the real world. Christchurch, New Zealand: pp. 50-51.

Holland, R.J., Williams, K.L., and Khan, A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. Nematology. 1(part 2): pp. 131-139.

Holmes, K,A., Nayagam, S.D., and Craig, G.D. 1998. Factors affecting the control of *Pythium ultimum* damping-off of sugar beet by *Pythium oligandrum*. Plant Pathology. 47(4): pp. 516-522.

Holt, J.G. Krieg, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's manual of determinative bacteriology 9th Ed. Williams and Wilkins. Baltimore, Maryland. USA: pp. 93.

Hornby, D. 1983. Suppressive soil. Annual Review of Phytopathology. 21: pp. 65-85.

Howell, C.R. and Stipanovic, R.D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology. 69: pp. 480-482.

Howell, C.R. and Stipanovic, R.D. 1980. Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology. 70: pp. 712-715.

Howell, C.R. 1982. Effect of *Glyocladium virens* on *Phytium ultimum*, *Rhizoctonia solani*, and dumping-off of cotton seedling. Phytopathology. 72: pp. 496 – 498.

Howell, C.R. Stipanovic, R.D., and Lumsden, R.D. 1993. Antibiotic production by strains of *Glyocladium virens* and its relation to the biocontrol of cotton seedling diseases. Biocontrol Science & Technology. 3(4): pp.435-441.

Howell, C.R. Stipanovic, R.D. 1995. Mechanisms in the biocontrol of *Rhizoctonia solani*-induced cotton seedling disease by *Glyocladium virens* – antibiosis. Phytopathology. 85(4): pp. 469-472.

Hoynes, C.D., Lewis, J.A., Lumsden, R.D., Bean, G.A. 1999. Biological control agents in combination with fertilization or fumigation to reduce sclerotial viability of *Sclerotium rolfsii* and disease of snap beans in the greenhouse. Journal of Phytopathology. 147(3): pp. 175-182.

Huang, H.C. 1978. *Gliocladium catenuatum*: hyperparasite of *Sclerotinia sclerotiorum* and *Fusarium* species. Canadian Journal of Botany. 56: pp. 2243 – 2246.

Huang, Z., Thomashow, L.S., Mavrodi, D.V., Raaijmakers, J.M., Weller, D.M., and Cook, R.J. 1997. Transgenic strains for biocontrol of plant root disease. Pattent Application Filed 12 December 1997.

Huang, J.W. and Huang H.C. 2000. A formulated continer medium suppressive to *Rhizoctonia* damping-off of cabage. Bot. Bull. Acad. Sin.. 41: pp. 49-56.

Huang, H.C., Bremer, E., Hynes, R.K., and Erickson, R.S. 2000. Foliar application of fungal biocontrol agents for the control of white mold of dry bean caused by *Scleronia sclerotiorum*. Biological Control. 18(3): pp. 270 – 276.

Hultberg, M. Alsanius, B. Sundin, P. 2000. *In vivo* and *in vitro* interactions between *Pseudomonas fluorescens* and *Pythium ultimum* in the suppression of damping-off in tomato seedlings. Biological Control. 19(1): pp. 1-8.

Humphris, S.N., Wheatly, R.E., and Bruce, A. 2001. The effect of specific volatile organic compounds produced by *Trichoderma* spp. on the growth of wood decay basidiomycetes. Holzforschung. 55(3): pp. 233-237.

Hurlbert, R.E. 1999. Bacterial nutrition and environmental requirements. Microbiology (http://www.slic2.wsu.edu:82/hurlbert/micro1/pages/chap4.html).

Hutcheson, S.W. 1998. Current consepts of active defense in plants. Annual Review of Phytopathology. 36: pp. 59-90.

Ignatius, S.M.J., Chopra, R.K., and Muthukrishnan, S. 1994. Effects of fungal infection and wounding on the expression of chitinases and β-1,3-glucanases in near-isogenic line of barley. Physiologia Plantarum. 90: pp. 584-592.

Inbar, J. and Chet, I. 1992. Biomimic of fungal cell-cellrecognition by use of lectin-coated nylon fibres. Journal of Bacteriology. 174: pp. 1055 – 1059.

Inglis, G.D. and Kawchuk, L.M. 2002. Comparative degradation of oomycete, ascomycete, and basidiomycete cell walls by mycoparasitic and biocontrol fungi. Canadian Journal of Microbiology. 48(1): pp. 60-70.

Ishii, H. 2003. Status of strobilurin resistance and related research in Japan. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 82.

Jackson, M.J. and Line, M.A. 1997. Composting pulp and paper mill sludge-Effect of temperature and nutrient addition method. Compost Science and Utilization. 5(1): pp. 74 – 81.

Jackson, M.J. 1998. Compost-recycling of pulp amd paper mill sludge. Ph.D Thesis. School of Agricultural Science, The University of Tasmania. Australia.

James, T.D.W. and Sutton, J.C. 1996. Biological control of *Botrytis* leaf blight on onion by *Gliocladium roseum* applied as spray and fabric applicators. European Journal of Plant Pathology. 102(3): pp. 265 – 275.

Janisiewicz, W. J. 1988. Biocontrol of post harvest diseases of apple with antagonist mixtures. Phytopathology. 78: pp. 194-198.

Janisiewicz, W. J. 1996. Ecological diversity, niche overlap and coexistence of antagonists used in developing mixtures for biocontrol of postharvest diseases of apples. Phytopathology 86: pp. 473-479.

Jaworski, A., Kirschbaum, J., and Bruckner, H. 1999. Structures of trichovirins II, peptaibol antibiotics from the mold *Trichoderma viridae* NRRL 5243. Journal of Peptide Science. 5(8): pp. 341-351.

Jay, J.M. 1996. Modern food microbiology. 5th Ed. International Thomson Publishing. New York.

Jeffries, P. 1997. Mycoparasitism. In: Wicklow, D.T. and Söderström, B. (Eds.). The Mycota. A comprehensive treatise on fungi as experimental system for basic and applied research. Springer-Verlag, Berlin Heidelberg: pp. 149-164.

Jonathan, E.I., Barker, K.R., Abdel-Alim, F.F., Vrain, T.C., and Dickson, D.W. 2000.Biological control of *Meloidogyne incognita* on tomato and banana with rhizobacteria actinomycetes, and *Pasteuria penetrans*. Nematropica. 30(2): pp. 231-240.

Johnson, K.B. and DiLeone, J.A. 1999. Effect of antibiosis on antagonist dose-plant disease response relationships for the biological control of crown gall of tomato and cherry. Phytopathology. 89(10): pp. 974 - 980.

Jock, S., Volksch, B., Mansvelt, L., and Geider, K. 2002. Characterization of *Bacillus* strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora*. FEMS Microbiology Letters. 211(2): pp. 247-252.

Jones, C.R. and Samac, D.A. 1996. Biological control of fungi causing alfalfa seedling damping-off with a disease suppressive strain of *Streptomyces*. Biological control. 7 (2): pp. 196 – 204.

Jutsum, A.R., Heaney, S.P., Perrin, B.M., and Wege, P.J. 1998. Pesticide resistance: Assessment of risk and the development and implementation of effective management strategies. Pesticide Science. 54(4): pp. 435-446.

Kalbe, C., Marten, P., and Berg, G. 1996. Strain of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiological Research. 151 (4): pp. 433 – 439.

Kapulnik, Y. 1991. Plant growth promoting rhizobacteria. In: Waisel. Y., Eshel, A., Kafkali, U. (Eds.). Plant root, the hidden half. Marcel Dekker. New York: pp. 717 – 729.

Kearns, L.P. and Mahanty, H.K. 1998. Antibiotic production by *Erwinia herbicola* Eh 1087-Its role in inhibition of *Erwinia amylovora* and potential characterization of antibiotic biosynthesis genes. Applied and Environmental Microbiology. 64 (5): pp. 1837 – 1844.

Keen, N.T. 1990. Gen-for-gen complementarity in plant-pathogen interaction. Annu. Rev. Genet. 24: pp. 447-463.

Kerr, J.R. 2000. Phenazine pigments: antibiotics and virulence factors. Infectous Disease Review. 2(4): pp. 184-194.

Khmel, I.A., Sorokina, T.A., Lemanova, N.B., Lipasova, V.A., Metlitski, O.Z., Burdeinaya, T.V., and Chernin, L.S. 1998. Biological control of crown gall in grapevine and raspberry by two *Pseudomonas* spp. With a wide spectrum of antagonistic activity. Biocontrol Science and Technology. 8(1): pp. 45-57.

Khayami-Horani, H. and Ateyyat, M. 2002. Efficacy of Jordanian isolates of *Bacillus thuringiensis* against the citrus leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae). International Journal of Pest Management. 48(4): pp. 297-300.

Kiefer, B., Riemann, M., Buche, C., Kassemeyer, H.H., and Nick, P. 2002. The host guides morphogenesis and stomatal targeting in the grapevine pathogen *Plasmopara viticola*. Planta. 215(3): pp. 387-393.

Kirby W.M.M and Baur A.W. 1966. Antibiotic testing method by standardizing disc method. Am. J. Clin. Pathol.45: pp. 493-496.

Kloepper, J.W. and Schroth, M.N. 1978. Plant growth promoting rhizobacteria on radishes. In: Station de pathologie vegetale et phytobacteriologie, editors. Proceeding of the fourth international conference on plant pathologic bacteria, Vol. 2: INRA. Angers. Gilbert-Clarey, Tours: pp. 879-882.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. 1980 (a). Enhance plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature. 286: pp. 885 – 886.

Kloepper, J.W. and Schroth, M.N. 1981. Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. Phytopathology. 71(6): pp. 590 – 592.

Kloepper, J.W. 1991. Plant growth promoting rhizobacteria as biological control agents of soil-borne diseases. In: Bay-Petersen, J. (Ed.). The biological control of plant diseases. Taipei (Taiwan): Food and Fertilizer Technology: pp. 142-152.

Kloepper, J.W. 1993. Plant growth-promoting rhizobacteria as biological control agents. In: Meeting, Jr., F.B. Soil microbiaecology-application in agricultural and environmental management. Marcell Dekker, Inc. New York: pp. 255-274.

Kloepper, J.W. 1996. Host-specificity in microbe-microb interactions. Bioscience. 46 (6): pp. 406 – 409.

Kloepper, J.W., Rodriguez-Ubana, R., Zehnder, G.W., Murphy, J.F., Sikora, E., and Fernandez. 1999. Plant root-bacterial interactions in biological control of soil-borne diseases and potential extension to systemic and foliar diseases. Australian Plant Pathology. 28: pp. 21-26.

Knoester, M., Van Loon, L.C., Van den Heuvel, J., Hennig, J., Bol, J.F., and Linthorst, H.J.M. 1998. Ethylene-insensitive tobacco lacks non-host resistance against soil-borne fungi. Proc. Natl. Acad. Sci. USA. 95: pp. 1933-1937.

Knogge, W., van 't Slot, K.A.E., Gierlich, A., and Papavoine, T. 2003. Pathogen recognition and defence induction. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 64.

Kobayashi, D.Y., Guglielmoni, M., and Clarke, B.B. 1995. Isolation of the chitinolytic bacteria *Xantomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turf grass. Soil Biology and Biochemistry. 27 (11): pp. 1479 – 1487.

Koch, E., Kempf. H.J., and Hessenmuller, A. 1998. Characterization of the Biocontrol activity and evaluation of potential plant growth-promoting properties of selected rhizobacteria. Journal of Plant Diseases and Protection. 105 (6): pp. 567 – 580.

Kok, C,J., Hageman, P.E., Mass, P.W.T., Postma, j., Roozen, N.J.M., and Van Vuurde, J.W.L. 1996. Processed manureas carrier to introduce *Trichoderma harzianum*: population dynamics and biocontrol effect on *Rhizoctonia solani.* Biocontrol Science and Technology. 6: pp. 147-161.

Krause, M.S., Madden, L.V., and Hoitink, H.A.J. 2001. Effect of potting mix microbial carrying capacity on the biological control of *Rhizoctonia* damping-off of radish and *Rhizoctonia* crown and root rot of *Poinsettia.* Phytopathology. 91(11): pp. 1116-1123.

Krieg, N.R. and Holt, J.G. 1984. Bergey's manual of systematic bacteriology. Vol 1. Williams and Wilkins, Baltimore.

Krishnamurthy, K. and Gnanamanickam, S.S. 1998. Biological control of rice blast by *Pseudomonas fluorescens* strain PF7-14-Evaluation of a marker gene and formulations. Biological control. 13 (3): pp. 158 – 165.

Krishnamurthy, J., Samiyappan, R., Vidhyasekaran, P., Nakkeeran, S., Rajeswari, E., Raja, J.A.J., and Balasubramanian, P. 1999. Efficacy of *Trichoderma* chitinases against *Rhizoctonia solani*, the rice sheath blight pathogen. Journal of Biosciences. 24(2): pp. 207-213.

Kuć, J. 1995. Phytoalexin, stress metabolism and disease resistance in plants. Annual Review of Phytopathology. 33: pp. 275-297

Kucharek, T. 1994. Downy mildew of cucurbits. Online: http://edis.ifas.ufl.edu/BODY_VH006.

Kurze, S., Bahl, H., Dahl, R., and Berg, G. 2001. Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Disease. 85(5): pp. 529-534.

Kuepper, G. 2000. Downy mildew control in cucurbits. Online: http://www.attra.org/attra-pub/downymildew.html.

Kuter, G.A., Hoitink, H.A.J., and Chen, W. 1988. Effects of municipal sludge compost curing time on suppression for *Pythium* and *Rhizoctonia* diseases of ornamental plants. Plant Disease. 72: pp. 751-756.

Kuzina, L.V., Miller, E.D., Ge, B.X., and Miller, T.A. 2002. Transformation of *Enterobacter gergoviae* isolated from pink bollworm (Lepidoptera:Gelechiidae)gut with *Bacillus thuringiensis* toxin. Current Microbiology. 44: pp. 1-4.

Kwok, O.C.H., Fahy, P.C., Hoitink, H.A.J., and Kuter, G.A. 1987. Interaction between bacteria and *Trichoderma hamatum* in suppression of *Rhizoctonia* damping-off in bark compost media. Phytopathology. 77: pp. 1206-1212.

Lam, E. 2003. Hypersensitive response and defence co-ordination. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 63.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell. 56: pp. 215-224.

Larkin,R.P. and Fravel, D.R. 1998. Efficacy of various fungal and bacterial biocontrol organisms for control of *Fusarium* wilt of tomato. Plant Disease. 82 (9): pp. 1022 – 1028.

Latour, X., Philippot, L., Corberand, T., Lemanceau, P. 1999. The establihsment of an introduced community of fluorescent pseudomonads in the soil and in the rhizosphere is affected by the soil type. FEMS Microbiology Ecology. 30: pp. 163-170.

Lazarovits, G., Conn, K.L., Abbasi, P.A., and Tenuta, M. 2003. Control of soil pathogens with organic amendment: Challenges and possibilities. 8th International congress of plant pathology. Vol. 1: Solving problems in the real world. Christchurch, New Zealand: pp. 51-52.

Leary, J.V. and Chun, W. 1984. Identification of a phytotoxin produced by *Pseudomonas corrugata*, causal agent of tomato pith necrosis. Phytopathology. 74: pp. 826.

Lederer, W., Lorenz, K.H., and Seemuller, E. 1992. Studies on antagonistic effects of Trichoderma isolates against Phytophthora cactorum. Journal of Phytophatology. 136: pp. 154-164.

Lee, I., Evans, B.R., and Woodward, J. 2000. The mechanism of cellulase action on cotton fibres: evidence from atomic force microscopy. Ultramicroscopy. 82 (1-4): pp. 213 – 221.

Lee, S., Sharma, Y., Lee, T.K., Chang, M., and Davis, K.R. 2001. Lignification induced by Pseudomonads harbourig avirulent genes on *Arabidopsis*. Molecules and Cells. 12(1): pp. 25-31.

Leeman, M., Vanpelt, J.A., Denouden, F.M., Heinsbroek, M., Bakker, P.A.H.M., and Schippers, B. 1995 (a). Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology. 85 (9) : pp. 1021 – 1027.

Leeman, M., Vanpelt, J.A., Denouden, F.M., Heinsbroek, M., Bakker, P.A.H.M., and Schippers, B. 1995 (b). Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to *Fusarium* wilt, using a novel bioassay. European Journal of Plant pathology. 101 (6): pp. 655 – 664.

Leeman M., Denouden, F.M., Vanpelt, J.A., Cornelissen, C., Matamalagarros, A., Bakker, P.A.H.M., and Schippers, B. 1996. Suppression of *Fusarium* wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root colonising fungi. European Journal of Plant Pathology. 102(1): pp. 21-31.

Lemanceau, P. and Alabouvette, C. 1991. Biological control of *Fusarium* diseases by fluorescent *Pseudomonas* and non pathogenic *Fusarium*. Crop Protection. 10: pp. 279-286.

Leone, G. 1990. *In vivo* and *in vitro* phosphate-dependent polygalacturonase production by different isolates of *Botrytis cinerea*. Mycol. Res. 94(8): pp. 1039-1045.

Leong, J. 1986. Siderophores: Their bicochemistry and possible role in the biocontrol of plant pathogens. Ann. Rev. Phytopathol. 24: pp. 187-209.

Lethbridge, G. 1989. An industrial view of microbial inoculants for crop plants. In: Campbell, R. and Macdonald, R.M. (Eds). Microbial inoculation of crop plants. IRL Press, Oxford: pp. 11 – 28.

Lewis, J.A. and Papavizas, G.C. 1980. Integrated control of *Rhizoctonia* fruit rot of cucumber. Phytopathology. 70: pp. 85 – 89.

Lewis, J.A., Robert, D.P., and Hollenbeck, M.D. 1991. Induction of cytoplasmic leakage from *Rhizoctonia solani* hyphae by *Gliocladium viren* and partial characterization of a leakage factor. Biocontrol Science and Technology. 1: pp. 21 – 29.

Lievens, B., Vaes, K., Coosemans, J., and Ryckeboer, J. 2001. Systemic resistance induced in cucumber against *Pythium* root rot by source separated household waste and yard trimmings composts. Compost Science and Utilization. 9(3): pp. 221-229.

Lifshitz, R., Sneh, B., and Baker, R. 1984. Soil suppressiveness to *Pythium ultimum* induced by antagonistic *Pythium* species. Phytopathology. 74: pp. 1054-1061.

Liu, L., Kloepper, J.W., and Tuzun, S. 1995 (a). Induction of systemic resistance in cucumber by PGPR-duration of protection and effect of host resistance on protection and root colonization. Phytopathology. 85 (10): pp. 1064 – 1068.

Liu, L., Kloepper, J.W., and Tuzun, S. 1995 (b). Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. Phytopathology. 85 (8): pp. 843 – 847.

Liu, D.Q., Anderson, N.A., and Kinkel, L.L. 1995 (c). Biological control of potato scab in the field with antagonistic *Streptomyces scabies*. Phytopathology. 85 (7): pp. 827– 831.

Liu, J.J. and Morrell, J.J. 1997. Effect of biocontrol inoculum growth conditions on subsequent chitinase and protease levels in wood exposed to biocontrol and strain fungi. Material und Organismen. 31 (4): pp. 265 – 279.

Locke, J.C., Marois, J.J., and Papavizas, G.C. 1985. Biological control of *Fusarium* wilt of geenhouse-grown chrysanthenum . Plant Disease. 69: pp. 167-169.

Logsdon, G. 1993. Using compost for plant disease control. Biocycle. 34(10): pp. 33-36.

Loper, J.E., Suslow, T.V., and Schroth, M.N. 1984. Lognormal distribution of bacterial populations in the rhizosphere. Phytopathology. 74: pp. 1454-1460.

Loper, J.E., Haack, C., and Schroth, M.N. 1985. Population dynamic of soil pseudomonands in rhizosphere of potato (*Solanum tuberosum* L). Applied and Environmental Microbiology. 49: pp. 416-422.

Lucas, J.A. 1998. Plant pathology and plant pathogens. 3rd Ed. Blackwell Science, Ltd. Osney Mead, Oxford. London.

Lund, S.T., Stall, R.E., and Klee, J.J. 1998. Ethylene regulates the susceptible response to pathogen infection in tomato. Plant Cell. 10: pp. 371-382.

Lusso, M.F.G. and Kuć.1995. Evidence of transcriptional regulation of b-1,3 glucanases as it relates to induced systemic resistance of tobacco blue mold. Molecular Plant-Microbe interactions. 8: pp. 473-475.

Maas, E.M.C. and Kotze, J.M. 1987.Trichoderma harzianum and *Trichoderma polysporum* as biocontrol agents of take-all of wheat in the greenhouse. Pytophylatica. 19: pp. 365-367.

Mabbayad, M.O. and Watson, A.K. 2000. Rejection of *Fusarium pallidoroseum* as a biological control agent of *Mimosa invisa* in upland rice. Biocontrol Science and Technology. 10(3): pp. 255 – 265.

Machala, M. and Vondracek, J. 1998. Estrogenic activity of xenobiotics. Veterinarni Medicina. 43 (10): pp. 311 – 317.

Madigan, M.T., Martinko, J.M., and Parker, J. 1997. Brock biology of microorganisms. Prentice-Hall International. USA.

Madrigal, C., Pascual, S., and Melgarejo, P. 1994. Biological control of peach twig blight (Monilinia laxa) with Epicoccum nigum. Plant Pathology. 43(3): pp. 554 – 561.

Madrigal, C. and Melgarejo, P. 1995. Morphological effects of *Epicocum nigrum* and its antibiotic flavipin on *Monilinia laxa.* Canadian journal of Botany. 73(3): pp. 425-431.

Makowski, R.M.D. and Mortenson, K. 1992. The first mycoherbicide in Canada: *Colletotrichum gloeosporioides* f.sp. *malvae* for round-leaved mallow control. In: Proceedings 1st International Weed Control Conference. Weed Science Society of Victoria, Inc., Melbourne. Vol 2: pp. 298-300.

Manocha, M.S., Chen, Y., and Rao, N. 1990. Involvement of cell surface sugars in recognition attachment and appressorium formation by a mycoparasite. Can. J. Microbiol. 36: pp. 771 – 778.

Margot, P. 2003. The challenge in chemical control of plant diseases. 8th. International Congress of Plant Pathology. Vol 1. Christchurch, New Zealand: pp. 71.

Mathivanan, N., Srinivasan, K., Chelliah, S. 2000. Biological control of soil-borne diseases of cotton, eggplant, okra and sunflower by *Trichoderma viride.* Journal of Plant Diseases and Protection. 107(3): pp. 235-244.

Mathre, D.E., Johnston, R.H., and Grey, W. 1998. Biological control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* under field conditions using a Phialophora sp. Biocontrol Science and Technology. 8: pp. 449-457.

Mathre, D.E., Cook, R.J., and Callan, N.W. 1999. From discovery to use: Transvering the world of commercializing biocontrol agents for plant disease control. Plant Disease. 83(11): pp. 972-983.

Maurhofer, M., Keel, C., Haas, D., and Defago, G. 1994. Pyoluteorin production by *Pseudomonas fluorescens* strain CHAO is invoved in the suppression of *Pythium* damping-off of cress but not of cucumber. European Journal of Plant Pathology. 100(3-4): pp. 221 - 232.

Maurhofer, M., Hase, C., Meuwly, P., Métraux, J.P., and Défago, G. 1994a. induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonizing *Pseudomonas fluorescens* strain CHAOL. Influence of the *gacA* gene and of pyoverdine production. Phytopathology. 84: pp. 139-146.

Mayer, J.M., Azelvandre, P., and Georges, C. 1992. Iron metabolism in *Pseudomonas*: Salicylic acid, a siderophore of *Pseudomonas fluorescens* CHAo. Biofactors. 4: pp. 23-27.

Maynard, A.A. 1997. Cummulative effect of annual additions of undecompost leaves and compost on the yield of eggplant and tomatoes. Compost Science and Utilization. 5: pp. 38-48.

Mazzola, M., Fujimoto, D.K., Thomashow, L.S., and Cook, R.J. 1995. Variation in sensitivity of *Gaeumannomyces graminis* to antibiotics produced by *Pseudomonas* spp. and effect on biological control of take-all of wheat. Applied and Environmental Microbiology. 61: pp. 2554-2559.

McClure, N.C., Ahmadi, A.R., and Clare, B.G. 1998. Construction of a range of derivatives of the biological control strain *Agrobacterium rhizogenes* K84: A study of factors involved in biological control of crown gall disease. Applied and Environmental Microbiology. 64(10): pp. 3977-3982.

Mccullagh, M., Utkhede, R., Menzies, J.G., Punja, Z.K., and Paulitz, T.C. 1996. Evaluation of plant growth promoting rhizobacteria for biological control of *Pythium* root rot of cucumbers grown in rockwool and effect on yield. European Journal of Plant

McKellar, M.E. and Nelson, E.B. 2003. Compost-induced suppression of Pythium damping-off is mediated by fatty-acid-metabolizing seed-colonizing microbial communities. Applied and Environmental Microbiology. 69(1): pp. 452-460.

Mclaren, D.L., Huang, H.C., and Rimmer, S.R. 1996. Control of apotheceal production of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* and *Tararomyces flavus*. Plant Disease. 80(12): pp. 1373 – 1378.

McLean, K.L and Stewart, A.2000. Application strategies for control of onion white rot by fungal antagonists. New Zealand Journal of Crop and Horticultural Science. 28(2): pp. 115 – 122.

McLean, K.L., Hunt, J., and Stewart, A. 2001. Compatibility of the biocontrol agent *Trichoderma harzianum* C52 with selected fungicides. 54th Conference proceedings (2001) of the New Zealand Plant Protection Society Incorporated: pp. 84-88. online: http://www.hortnet.co.nz

Mcquilken, M.P., Whipps, J.M., and Lynch, J.M. 1994. Effect of water extracts of a composted chicken manure-straw mixture on the plant pathogen *Botrytis cinerea*. World journal of Microbiology and Biothecnology. 10(1): pp. 20-26.

Mcquilken, M.P. and Whipps, J.M. 1995. Production, survival, and evaluation of solid-substrate inocula of *Coniothyrium minitans* against *Sclerotinia sclerotiorum.* European Journal of Plant Pathology. 101(1): pp. 101-110.

Meena, B., Radhajeyalakshmi, R., Marimuthu, T., Vidhyasekaran, P., and Velazhahan, R. 2002. Biological control of groundnut late leaf spot and rust by seed and foliar applications of a powder formulation of *Pseudomonas fluorescens.* Biocontrol Science and Technology. 12(2): pp. 195-204.

Merriman, P.R., Price, R.D., Kollmorgen, J.F., Piggott, T., and Ridge, E.H. 1974. Effect of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Australian Journal of Agricultural Research. 25: pp. 219-226.

Metcalf, D.A. 1997. Biological control of onion white root rot. Ph.D Thesis. School of Agricultural Science. The University of Tasmania-Australia.

Metcalf, D.A. and Wilson, C.R. 1999. Histology *of Sclerotium cepivorum* infection of onion roots and spatial relationships of pectinases in the infection process. Plant Pathology. 48: pp. 445-452.

Metcalf, D.A. and Wilson, C.R. 2001. The process of antagonism of *Sclerotium cepivorum* in white rot affected onion roots by *Trichoderma koningii.* Plant Pathology. 50(2): pp. 249-257.

Metcalf, D.A. 2002. Development of biological control agents, which control onion white root rot under commercial field conditions. Proceedings of Onions 2002 Conference. Yanco Australia 2002: pp. 63-68.

Misaghi, I.J. 1990. Screening bacteria for root colonizing ability by a rapid method. Soil Biology and Biochemistry. 22(8): pp. 1085 – 1088.

Mischke, S. 1998. Mycoparasitism of selected sclerotia-forming fungi by *Sporidesmium sclerotivorum.* Canadian Journal of Botany. 76(3): pp. 460-466.

Mishra, R.C., Singh, R., Singh, H.B., Dikshit, A. 2000. In situ efficacy of trichoderma harzianum as mycoparasite on *Sclerotium rolfsii* and *Rhizoctonia solani.* Tropical Agriculture. 77(3): pp. 205-206.

Mittal, N., Saxena, G., and Mukerji, K.G. 1995. Integrated control of root-knot disease in three crop plants using chitin and *Pecilomyces lilacinus.* Crop Protection. 14(8): pp. 647-651.

Miyamoto, J. and Klein, W. 1998. Environmental exposure, species differences and risk assesment. Pure and Applied Chemistry. 70 (9): pp. 1829 – 1845.

Mmochi, A.J. and Mberek, R.S. 1998. Trends in the types, amounts, and toxicity of pesticides used in Tanzania-Effort to control pesticide pollution in Zanzibar. Ambio. 27 (8): pp. 669 – 676.

Moenneloccoz, Y., Powell, J., Higgins, P., Mccarthy, J., and Ogara, F. 1998. An investigation of the impact of biocontrol *Pseudomonas fluorescens* F113 on the growth of sugarbeet and the performance of subsequent clover-rhizobium symbiosis. Applied Soil Ecology. 7 (3): pp. 225 – 237.

Morrison, F.B. 1959. Feeds and feeding: A handbook for the student and stockman. 22nd Ed. The Morrison Publishing Company, Clinton. Iowa.

Mousseaux, M.R., Dumroese, R.K., James, R.L., Wenny, D.L., Knudsen, G.R. 1998. Efficacy of *Trichoderma harzianum* as a biological control of *Fusarium oxysporum* in container-grown Douglas-fir seedlings. New Forests. 15(1): pp. 11-21.

Mpiga, P., Belanger, R.R., Paulitz, T.C., and Benhamou, N. 1997. Increased resistance to *Fusarium oxysporum, F. sp. radicislycopersici* in tomato plants treated with endaphytic bacterium *Pseudomonas fluorescens* strain 63-28. Physiological and Molecular Plant Pathology. 50 (5): pp. 301 – 320.

Mukherjee, P.K., Mukhopadhyay, A.N., Sarmah, D.K., and Shrestha, S.M. 1995. Comparative antagonistic properties of *Gliocladium virens* and *Trichoderma harzianum* on *Sclerotium rolfsii* and *Rhizoctonia solani*-Its relevance to understanding the mechanisms of biocontrol. Journal of Phytopathology. 143(5): pp. 275 – 279.

Nagtzaam, M.P.M., Bollen, G.J., and Termorshuizen, A.J. 1998. Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiment. Journal of Pythopathology. 146(4): pp. 165-173.

Nakasaki, K., Hiraoka, S., and Nagata, H. 1998 A new operation for producing disease-suppressive compost from grass clippings. Applied and environmental microbiology. 64 (10): pp. 4015 – 4020

Nameth, S.T. 2001. The nuts and bolts of bio-fungicides. OhioFloriculture. Online. http://floriculture.osu.edu/archive/aug01/bio-fung.html.

Nameth, S.T. 2002. Biocontrol for disease are here to stay. Online: http://bioworksbiocontrol.com/articles8.html.

Natsch, A., Keel, C., Pfirter, H.A., Haas, D., and Defago, G. 1994. Contribution of the global regulator gene *gacA* to persistence and desimination of *Pseudomonas fluorescens* biocontrol strain CHAO introduced into soil microcosms. Applied and Environmental Microbiology. 39(8): pp. 787 – 794.

Neate, S.M., Cruickshank, R.H., and Rovira, A.D. 1998. Pectic enzyme patterns of *Rhizoctonia solani* isolates from agricultural soils in south Australia. Trans. Br. Mycol. Soc. 90(1): pp. 37-42.

Neeno-Eckwall, E.C., Kinkel, L.L., and Schottel, J.L. 2001. Competition and antibiosis in the biological control of potato scab. Canadian Journal of Microbiology. 47(4): pp. 332-340.

Nelson, E.B. and Hoitink, H.A.J. 1983. Factors affecting suppression of *Rhizoctonia solani* in container media. Phytopathology. 72: pp. 275-279.

Nelson, E.B., Kuter, G.A., and Hoitink, H.A.J. 1994. Effect of fungal antagonists and compost age on suppression of *Rhizoctonia* damping-off in container media amended with composted hardwood bark. Phytopathology. 73: pp. 1457-1462.

Nelson, E.B. and Boehm, M.J. 2002. Microbial mechanics of compost-induced disease suppression. BioCycle. July, 2002.

Nicholson, R.L. and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. Annual Review of Phytopathology. 30: pp. 369-389.

Nielands, J.B. and Leong, S.A. 1986. Siderophores in relation to plant growth and disease. Ann. Rev. Physiol. 37: pp. 187-208.

Nielson, M.N., Sorensen, J., Fels, J., and Pedersen, H.C. 1998. Secondary metabolite and endochitinase-dependent antagonism toward plant-pathogenic microfungi of *Pseudomonas fluorescens* isolates from sugar beet rhizosphere. Applied and Environmental Microbiology. 64 (10): pp. 3563 – 3569.

Obert, D.E, Skinner, D.Z, and Stuteville, D.L. 2000. Association of AFLP markers with downy mildew resistance in autotetraploid alfalfa. Molecular Breeding. 6(3); pp. 287-294.

Odvody, G.N., Boosalis, M.G., Lewis, J.A., and Papavizas, G.C. 1977. Biological control of *Rhizoctonia solani*. Proc. Am. Phytopathol. Soc. 4: pp. 158 (Abstr).

Oedjijono. 1992. Isolation and characterization of some bacteria antagosnistic to plant pathogenic fungi. MSc. Thesis. Department of Agricultural Science, Tasmania University. Australia.

Oedjijono, Line M.A., and Dragar, C. 1993. Isolation of bacteria antagonistic to a range of plant pathogenic fungi. Soil Biol. Biochem.. 25 (2): pp. 247 – 250.

Oksanen, T., Pere, J., Paavilainen, L., and Viikari, L. 2000. Treatment and recycled kraft pulps with *Trichoderma reesei* hemicellulases and cellulases. Journal of Biotechnology. 78(1): pp. 1785 – 1790.

Olckers, T. 1999. Biological control of *Solanum mauritianum* Scopoli (Solanaceae) in South Africa: a review of candidate agents, progress and future prospects. African Entomology: pp. 65 – 73.

Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C., and Belanger, R.R. 2000. Systemic induction of phytoalexins in cucumber in response to treatments with fluorescent pseudomonads. 49(4): pp. 523-530.

Painuly, J.P. and Dev, S.M. 1998. Environmental dimensions of fertilizer and pesticide use, relevance to Indians agriculture. International Journal and Pollution. 10 (2): pp. 273 – 288.

Pajot, E., Le Corre, D., and Silue, D. 2001. Phytogard (R) and DL-beta-amino butyric acid (BABA) induce resistance to downy mildew (*Bremia lactucae*) in lettuce (*Lactuca sativa* L). European Journal of Plant Pathology. 107 (9): pp. 861-869.

Pal, K.K., Tilak, K.V.B.R., Saxena, A.K., Dey, R., and Singh, C.S. 2001. Suppression of maize root diseases caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* by plant growth promoting rhizobacteria. Microbuiological Research. 156 (3): pp. 209-223.

Pan, S.Q., Ye, X.S., and Kuć, J. 1992. Induction of chitinases in tobacco plants systemically protected against blue mold by *Perenospora tabacina* or tobacco mosaic virus. Phytopathology. 82: pp. 119-123.

Pandey, A., Palni, L.M.S., and Hebbar, K.P. 2001. Suppression of damping-off in maize seedlings by *Pseudomonas corrugata.* Microbiological Research. 156(2): pp. 191-194.

Parello, A., Monaco, C., and Cordo, C. 1997. Evaluation of *Trichoderma harzianum* and *Gliocladium roseum* in controlling leaf blotch of wheat (*Septoria tritici*) under *in vitro* and greenhouse conditions. Journal of Plant Disease and Protection. 104(6): pp. 588-598.

Parke, J.L. 1995. Biological control of plant pathogens. Midwest Biocontrol News. Online: http://www.entomology.wisc.edu/mbcn/fea303.html.

Parke, J.L. 2000. Burkholderia cepacia: Friend or foe?. APS net Education Centre. Online: http://www.apsnet.org/education/feature/BurkholderiaCepacia/Top.html.

Partridge, J.E. 1997. Downy mildew of grapes. Online: http://plantpath.unl.edu/peartree/homer/disease.skp/hort/grape/GrDwnMil.html

Pascual, S., Melgarejo, P., and Magan, N. 2000. Accumulation of compatible solutes in *Penicillium frequentans* grown at reduced water activity and biocontrol of *Monilinia laxa*. Biocontrol Science and Technology. 10(1): pp. 71-80.

Paul, B. 1999a. *Pythium periplocum*, an aggressive mycoparasite of Botrytis cinerea causing the gray mould disease of grape-vine. FEMS Microbiology Letters. 181(2): pp. 277-280.

Paul, B. 1999b. Suppression of Botrytis cinerea causing the grey mould disease of grape-vine by an aggressive mycoparasite, *Pythium radiosum*. FEMS Microbiology Letters. 176(1): pp. 25-30.

Paulitz, T.C. and Baker, R. 1987. Biological control of Pythium damping-off of cucumbers with *Pythium nunn*: Population dynamics and disease suppression. Phytopathology. 77: pp. 335-340.

Paulitz, T.C. and Linderman, R.G. 1989. Interaction between pseudomonads and VA mycorrhizal fungi. New Phytol.103: pp. 37-45.

Paulitz, T.C., Ahmad, J.S., and Baker, R. 1990. Integation of *Pythium nunn* and *Trichoderma harzianum* isolate T-95 for the biological control of *Pythium* damping-off of cucumber. Plant Soil. 121: pp. 243-250.

Paxton, J.D. 1981. Phytoalexin-a working redefinition. Phytopathology. Z101: pp. 106-109.

Peng, H.X., Sivasithamparam, K., and Turner, D.W. 1999. Chlamydospore germination and *Fusarium* wilt of banana plantlets in suppressive and conducive soils are affected by physical and chemical factors. Soil Biology and Biochemistry. 31(10): pp. 1363-1374.

Penyalver, R. and Lopez, M.M. 1999. Cocolonization of the rhizosphere by pathogenic *Agrobacterium* strains and nonpathogenic strains K84 and K1026, used for crown gall biocontrol. Applied & Environmental Microbiology. 65(5): pp. 1936-1940.

Penyalver, R., Oger, P., Lopez, M.M., and Farrand, S.K. 2001. Iron-binding compounds from Agrobacterium spp.: Biological control strain *Agrobacterium rhizogenes* K84 produces a hydroxamate siderophore. Applied and Environmental Microbiology. 67(2): pp. 654-664.

Perrin-Ganier, C. 1998. Soil and environment : The fate of pesticides and organic pollutants. Ocl-Oleagineux Corps Gras Lipides. 5 (5): pp. 341 – 343.

Phae, C.G., Saski, M., Shoda, M., and Kubota, H. 1990. Characteristics of *Bacillus subtilis* isolated from compost suppressing phytopathogenic microorganisms. Soil Sci. Plant Nutr. 36: pp. 575-586.

Phae, C.G. and Shoda, M. 1990. Expression of the suppressive effect of Bacillus subtilis on phytopathogens in inoculated compost. J. Ferment. Bioeng. 6: pp. 409-414.

Pierson, E.A. and Weller, D.M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology. 84: pp. 940-947.

Pieterse, C.M.J., Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A., and Van loon, L.C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell. 8: pp. 1225-1237.

Pieterse, C.M.J., Van Pelt, J.A., Ton, J., Parchmann, S., Mueller, M.J., Buchala, A.J., Metraux, J.P., and Van Loon, L.C. 2000. Rhizobacteria-mediatedinduced systemic resistance (ISR)in Arabidopsis requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiol. Mol. Plant Pathol. 57: pp. 123-134.

Pieterse, C.M.J., Verhagen, B.W.M., Ton, J., Van Wees, S.C.M., Van Pelt, J.A., and Van Loon, L.C. 2003. Induction of systemic resistance by root-zone bacteria: from perception to expression of resistance. 8th International Congress of Plant pathology. Christchurch, New Zealand: pp. 53.

Pleban, S., Chernin, L., and Chet, I. 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Letters in Applied Microbiology. 25 (4): pp. 284 – 288.

Poovala, V.S., Kanji, V.K., Tachikawa, H., and Salahudeen, A.K. 1998. Role of oxidant stress and antioxidant protection in acephate-induced renal tubular cytotoxicity. Toxicological Sciences. 46(2): pp. 403-409.

Powell, K.A.,Faull, J.L., and Renwick, A. 1990. The commercial and regulatory challenge. In: Hornby, D. (Ed.). Biological control of soil-borne plant pathogens. C.A.B. International, Wallingford, Oxon, UK. Pp. 445-463.

Pung, H. and Cross, S. 2003. The control of downy mildew disease, caused by *Peronospora viciae*, on processing peas in Australia. 8th International Congress of Plant Pathology. Vol. 2. Christchurch, New Zealand: pp. 71.

Raaijmakers, J. M., Van der Sluis, I., Koster, M., Baker, P.A.H.M. Weisbeek, P,J., and Schippers, B. 1995[b]. Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. Canadian journal of Microbiology. 41: pp. 126-135.

Raaijmakers, J. M., and Weller, D.M. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. Mol. Plant-Microbe Interact. 11:144-152.

Raaijmaker, J.M. and Weller, D.M. 2001. Exploiting genetic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: Characterization of superior root-colonizing P-fluorescens strain Q8r1-96. Applied and Environmental Microbiology. 67(6): pp. 2545-2554.

Rajappan, K., Vidhyasekaran, P., Sethuraman, K., and Baskaran, T.L. 2002. Development of powder and capsule formulations of Pseudomonas fluorescens strain Pf-1 for control of banana wilt. Journal of Plant Disease and Protection. 109(1):pp. 80-87.

Ramamurthy, V., Sharma, R.K., Yadaz, K.R., Kaur, J., Vrat, D., and Kothari, R.M. 1996. *Volvariella*-treated *Eucalyptus* sawdust stimulates wheat and onion growth. Biodegradation. 7: pp. 121 – 127.

Ramona, Y. and Line, M.A. 2002. Potential for the large scale production of a biocontrol fungus in raw and composted paper mill waste. Compost Science and Utilization. 10(1): pp. 57-62.

Raupach, G.S. and Kloepper, J.W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology. 88 (11): pp. 1158 – 1164.

Raupach, G.S. and Kloepper, J.W. 1998. Mixture of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology. 88(11): pp. 1158-1164.

Rebuffat, S., Goulard, C., Hlimi, S., and Bodo, B. 2000. Two unprecedented natural Aib-peptides with the (Xaa-Yaa-Aib-Pro) motif and unsual C-terminus: Structure, membrane-modifying and antibacterial properties of pseudokinins KL III and KL VI from the fungus Trichoderma pseudokoningii. Journal of Peptide Science. 6(10): pp. 519-533.

Renwick, A., Campbell, R., and Coe, S. 1991. Assessment of *in vivo* screening system for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Pathology. 40: pp. 524 – 532.

Reuveni, M., Agapov, P., and Reuveni, R. 1994. Induced systemic protection to powdery mildew in cucumber by phosphate and potasium fertilizers: Effects of inoculum concentration and post-inoculation treatment. Canadian Journal of Plant Pathology. 17: pp. 247-251.

Reuveni, M., Oppenheim, D., and Reuveni, R. 1998. Integrated control of powdery mildew on apple trees by folliar spray of mono-potasium phosphate fertilizer and sterol inhibiting fungicides. Crop Protection. 17: pp. 563-568.

Reynolds, J. 2002. Pasteurizing waste milk on dairies and calf ranches. Online: http://www.wildwestspeakers.com/food_animal_9.htm.

Rhodes, D.J., Logan. C., and Gross, D.C. 1987. Selection of *Pseudomonas spp.* inhibitory to potato seed tuber decay. Phytopathology. 76: pp. 1078.

Ridout, C.J., Coley-Smith, J.R., and Lynch, J.M. 1988. Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Microb. Technol. 10: pp. 180 – 187.

Ride, J.P. 1978. The role of cell wall alterations in resistance to fungi. Annals of Applied Biology. 89: pp. 302-306.

Ridder, E.R., Nokes, S.E., and Knutson, B.L. 1999. Optimization of solid-state fermentation parameters for the production of xylanase by *Trichoderma longibranchiatum* on wheat bran in forced aeration system. Transaction of the ASAE. 42(6): pp. 1785 – 1790.

Ries, S.M. 1996. Downy mildew of grape. Online: http://www.aces.uiuc.edu/ipm/fruits/rpds/705/705.html.

Rissler, J. 1991. Biotechnology and pest control: Quick fix vs. Sustainable control. Online. http://www.psrast.org/herbic.htm.

Ristaino, J.B. and Thomas, W. 1997. Agriculture, methyl bromide, and ozon hole. Plant Disease. 81: pp. 964-977.

Rovera, M., Cabrera, S., Correa, N., and Rosas, S. 2000. Characterization of antifungal agents produced by *Pseudomonas aurantiaca* for use as biocontrol inoculant. In: Pedrosa, F.O., Hungria, M., Yates, M.G., and Newton W.E. (Eds.). Nitrogen Fixation: From molecules to crop productivity. Kluwer Academic Publisher. Pp. 603.

Rÿckeboer, J.K., Deprins, K., and Coosemans, J. 1998. Compost onderdrunkt de kiemplanted-schimmels *Pythium ultimum* en *Rhizoctonia solani*: Veredele compost doet better. Vlacovaria. 3: pp. 20-26.

Safiyazov, J.S., Mannanov, R.N., and Sattarova, R.K. 1995. The use of bacterial antagonists for the control of cotton diseases. Field Crop Research. 43(1): pp. 51-54.

Samac, D.A. and Kinkel, L.L. 2001. Suppression of the root-lesion nematode (*Pratylenchus penetrans*) in alfalfa (*Medicago sativa*) by *Streptomyces* spp. Plant and Soil. 235(1): pp. 35-44.

Schäfer, W. 1994. Molecular mechanisms of fungal pathogenicity in plants. Annual Review on Phytopathology. 32: pp. 461-477.

Scher, F.M., Kloepper, J.W., Singleton, C., Zaleska, I., and Laliberte, M. 1988. Colonization of soybean roots by *Pseudomonas* and *Serratia* species: Relationship to bacterial motility, chemotaxis, and generation time. Phytopathology. 78: pp. 1055 – 1059.

Schippers, B. 1988. Biological control of pathogens with rhizobacteria. Philosophical Transactions of the Royal Society of London. 3(18): pp. 283-292.

Schippers, B. 1992. Prospect for management of natural suppressiveness to control soil-borne pathogen. In: Tjamos, E.C., Papavizas, G.C., and Cook, R.J. (Eds). Biological control of plant diseases, progress and challenge for the future. Plenum Publishing Corp. New York.

Schippers, B., Scheffer, R.J., Lugtenberg, B.J.J., and Weizbeek, P.J. 1995. Biocoating of seeds with plant growth promoting rhizobacteriato improve plant establishment. Outlook on Agriculture. 24: pp. 179-185.

Schisler, D.A. and Slininger, P.J. 1994. Selection and performance of bacterial strains for biologically controlling fusarium dry rot of potatoes incited by Gibberella pulicaris. Plant Disease. 78(3): pp. 251 – 255.

Schisler, D.A., Slininger, P.J., and Bothast, R.J. 1997. Effects of antagonist cell concentration and two-strain mixtures on biological control of Fusarium dry rot of potatoes. Phytopathology. 87: pp. 177-183.

Schisler, D.A., Slininger, P.J., Kleinkopf, G., Bothast, R.J., Ostrowski, R.C. 2000. Biological control of fusarium dry rot of potato tubers under commercial storage conditions. American Journal of Potato Research. 77 (1): pp. 29-40.

Schmidlisacherer, P., Keel, C., and Defago., G. 1997. The global regulator gaga of *Pseudomonas fluorescens* CHAO is required for suppression of root diseases in dicotyledons but not in gramineae. Plant Pathology. 46(1): pp. 80-90.

Schneider, R.W. 1982. Suppressive soil and plant disease. American Phytopathological Society. St. Paul. Minnesota. 88 pp

Schneider, S. and Ullrich, W.R. 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. Physiological and Molecular Plant Pathology. 45: pp. 291-304.

Schoeman, M.W., Webber, J.F., and Dickinson, D.J. 1999. The development of ideas in biological control applied to forest products. International biodeterioration and biodegradation. 43 (3): pp. 109-123.

Schroth, M.N. and Hancock, J.G. 1981. Selected topics in biological control. Ann. Rev. Microbiol. 35: pp. 453 – 476.

Schroth, M.N. and Hancock, J.G. 1982. Disease-suppressive soil and root colonising bacteria. Science. 216: pp. 1376 – 1381.

Schroth, M.N., Loper, J.E., and Hildebrand, D.C. 1983. Bacteria as bioconteol agents of plant disease. Current Perspective in Microbial Ecology. Proceeding of the 3rd International Symphosium on Microbial Ecology. Michigan State University: pp. 362-373.

Schroth, M.N. and Becker. 1990. Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In : Hornby (Ed.). Biological control of soil-borne plant pathogens. C.A.B. International. Wallingford. U.K. pp 389 – 414.

Sezen, K., Yaman, M., and Demirbag, Z. 2001. Insecticidal potential of *Serratia marcescens* Bn10. Biologia. 56(3): pp. 333-336.

Shanahan, P., O'Sulivan, D.J., Simpson, P., Glennon, J.D., and O'Gara, F. 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influenceing its production. Applied and Environmental microbiology. 58(1): pp. 353-358.

Shimizu, B., Fujimori, A., Miyagawa, H., Ueno, T., Watanabe, K., and Ogawa, K. 2000. Resistance against *Fusarium* wilt induced by non pathogenic *Fusarium* in *Ipomoea tricolor*. Journal of Pesticide Science. 25(4): pp. 365-372.

Siddiqui, Z.A., and Mahmood, I. 1999. Role of bacteria in the management of plant parasitic nematodes. A Review. Bioresource Technology. 69 (2): pp. 167 – 179.

Siddiqui, I.A., Qureshi, S.A., Sultana, V., Ehteshamul-Haque, S., and Ghaffar, A. 2000. Biological control of root rot-root knot disease complex of tomato. Plant and Soil. 227(1-2): pp. 163 – 169.

Simmonds, N.W. 1991. Genetics of horizontal resistance to diseases of crops. Biological Reviews. 66: pp. 189-241.

Singh, P.P., Shin, Y.C., Park, C.S., and Chung, Y.R. 1999. Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Pythopathology. 89(1): pp. 92-97.

Singleton, L.L., Mihail, J.D., and Rush, C.M.1992. Method for research on soilborne phytopathogenic fungi. APS Press. St. Paul, Minnesota.

Sitepu, D. and Wallace, H.R. 1984. Biological control of *Sclerotinia sclerotiorum* in lettuce by *Fusarium lateritium*. Aust. J. Exp. Agric. Anim. Husb. 24: pp. 272 – 276.

Sivakumar, D., Wijeratman, R.S.W., Wijesundera, R.L.C., Marikar, F.M.T., and Abeyesekere, M. 2000. Antagonistic effect of *Trichoderma harzianum* on postharvest pathogens of rambutan (*Nephelium lappaceum*). Phytoparasitica. 28(3): pp. 240-247.

Skerratt, J.H. 2002. Bacterial and algal interactions in a Tasmanian estuary. Ph.D thesis. School of Agricultural Science, The University of Tasmania-Australia.

Smibert, R.M. and Krieg, N.R. 1981. General characteristics. In: Gerhardt, P. and Murray, R.G.E. (Eds). Manual of methods for general bacteriology. American Society for Microbiology. Washington. : pp. 409 – 444.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E., and Hot, J.G. 1986. Bergey's manual of systematic bacteriology. Williams and Wilkins. Baltimore, Hongkong, London, Munich, Philadeplhia, Sydney, and Tokyo.

Sneh, B. 1981. Use of rhizosphere chitinolitic bacteria for biological control of *Fusarium oxysporum* f. sp. *dianthi* in carnations. Journal of Phytopathology. 100: 251-256.

Soesanto, L. 2000. Ecology and biological control of Verticillium dahliae. Abstract of Ph.D Thesis. Online: http://www.wau.nl/ppi/tesis/Abstract_Loekas.htm

Sorensen, J. 1997. The rhizosphere as habitat for soil microorganisms. In: Van Elsas *et al.*(Ed.). Modern soil microbiology. Marcel Dekker, Inc. New York: pp. 21 – 42.

Soytong, K., Kanokmedhakul, S., Kukongviriyapa, V., and Isobe, M. 2001. Application of *Chaetomium* species (Ketomium((R))) as a new broad spectrum biological fungicide for plant disease control: A review article. Fungal Diversity (7): pp. 1-15.

Srivastava, A.K., Singh, T., Jana, T.K., Arora, D.K. 2001. Induced resistance and control of charcoal rot in *Cicer arietinum* (chickpea) by *Pseudomonas fluorescens*. Canadian Journal of Botany. 79(7): pp. 787-795.

Steffen, M.S., Dann, J.K., Harper, S.J., Fleischer., Mkhize, S.S., Grenoble, D.W., MacNab, A.A., Fager, K., and Ruso, J.M. 1995. Evaluation of the initial season for implementation of tomato production system. Journal of American Society of Horticultural Science. 120: pp. 148-156.

Stein, B.D., Klomparens, K., and Hammerschmidt, R. 1993. Histochemistry and ultrastructure of the induced resistance response of cucumber plants to *Colletotrichum lagenarium*. Journal of Phytopathology. 137: pp. 177-188.

Steinmetz, J. and Schönbeck, F. 1994. Conifer bark as growth medium and carrier for *Trichoderma harzianum* and *Gliocladium roseum* to control *Pythium ultimum* on pea. Journal of Plant Disease and Protection. 101(2): pp. 200-211.

Stewart, A. 2003. Strategies for biological control of sclerotial pathogens. 8th International congress of plant pathology. Vol. 1: Solving problem in the real world. Christchurch, New Zealand: pp. 26.

Stockwell, V.O., Johnson, K.B., and Loper, J.E. 1998. Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. Phytopathology. 88 (6): pp. 506 – 513.

Su, X.Q., Zou, F.H., Guo, Q., Huang, J., and Chen, T.X. 2001. A report on a mosquito-killing fungus, *Pythium carolinianum*. Fungal Diversity. 7: pp. 129-133.

Sunaina, V., Kishore, V., Shekhawat, G.S., and Kumar, M. 1997. Control of bacterial wilt of potatoes in naturally infested soil by bacterial antagonists. Journal of Plant Diseases and protection. 104 (4): pp. 362 – 369.

Suslow, T.V. 1980. Growth and yield enhancement of sugar beets by pelleting seed with specific *Pseudomonas* spp. (Abst.). Plant Pathology News. 12(9): pp. 40.

Sztejnberg, A., Galper, S., Mazar, S., and Lisker, N. 1989. *Ampelomyces quisqualis* for biological and integrated control of powdery mildews in Israel. Journal of Phytopathology. 124: pp. 285 – 295.

Talavera, M., Mizukubo, T., Ito, K., and Aiba, S. 2002. Effect of spore inoculum and agricultural practices on the vertical distribution of the biocontrol plant-growth-promoting bacterium *Pasteuria penetrans* and growth of *Meloidogyne incognita*-infected tomato. Biology and Fertility of Soils. 35(6): pp. 435-440.

Tang, W.H., Wang, W., Wang, Y., Zhang, L.Q., and Wang, Y. 2003. Systemic aquired resistance and its mechanism in melons. 8th International Congress of Plant Pathology. Vol.1. Christchurch, New Zealand: pp. 42.

Tariq, V.N. and Devlin, P.L. 1996 Sensitivity of fungi to Nikkomycin Z. Fungal Genetic and Biology. 20(1): pp. 4 – 11.

Tatagiba, J.D.S., Maffia, L.A., Barreto, R.W., Alfenas, A.C., and Sutton, J.C. 1998. Biological control of *Botrytis cinerea* in reidue and flowers of rose (*Rosa hybrida*). Phytoparasitica. 26(1): pp. 8 – 19.

Templeton, G.E. 1982. Biological herbicides: Discovery, development, deployment. Weed Science. 30: pp. 430-433.

Thomashow, L.S., and Weller, D.M. 1998. Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var *tritici.* Journal of Bacteriology. 170 : pp. 3499 – 3508.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. 1998. Separate jasmonate-dependent and salicylate-dependent defense response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA. 95: pp. 15107-15111.

Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.A. 2000. Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonante. Plant. Phys. Biochem. 38: pp. 421-427.

Thomson, I.P., Liley A.K., Ellis, R,J., Bramwell, P.A., and Bailey, M.J. 1995. Survival, colonization, and dispersal of genetically modified *Pseudomonas fluorescens* SBW25 in the phytosphere of the field gron sugar beet. Nature Biotechnology. 13: pp. 1493-1497.

Thrane, C., Nielsen, T.H., Nielsen, M.N., Sorensen, J., Olsson, S. 2000. Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on *Pythium ultimum* in sugar beet rhizosphere. FEMS Microbiology Ecology. 33(2): pp. 139-146.

Travis, J., Rytter, J., and Hickey, K.2002. Fruit pathology: Downy mildew of grapes. Online: http://www.cas.psu.edu/docs/CASDEPT/PLANT/fpath/Grape/DownyM.html.

Trejoestrada, S.R., Paszczynski, A., and Crawford, D.L. 1998. Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. Journal of Industrial Microbiology and Biotechnology. 21 (1-2): pp. 81 – 90.

Tronsmo, A. and Dennis, C. 1977. The use of Trichoderma species to control strawberry fruit rots. Neth. J. Pl. Path. 83: pp. 449-455.

Tronsmo, A. and Raa, J. 1977. Antagonistic action of *Trichoderma pseudokoningii* against the apple pathogen Botrytis cinerea. Journal of Phytopathology. 89: pp. 216-220.

Tronsmo, A. 1996. *Trichoderma harzianum* in biological control of fungal diseases. In: Hall, R. (ed.). Principles and practice of managing soilborne plant pathogens. APS Press, St. Paul, Minnesota: pp.213-236.

Troxler, J., Berling, C.H., Moenneloccoz, Y., Keel, Y., and Defago, G. 1997. Interaction between the biocontrol agent *Pseudomonas fluorescens* CHAO and *Thielaviopsis basicola* in tobacco roots observed by immunofluorescence microscopy. Plant Pathology. 46(1): pp. 62-71.

Tsahouridou, P.C. and Thanassoulopoulos, C.C. 2001. Proliferation of *Trichoderma koningii* in the tomato rhizosphere and the suppression of damping-off by *Sclerotium rolfsii*. Soil Biology and Biochemistry. 34(6): pp. 767-776.

Turhan, G. 1993. Mycoparasitism of *Alternaria alternata* by an additional 8 fungi indicating the existence of further unknown candidates for biological control. Journal of Phytopathology. 138(4): pp. 283 – 292.

Turner, G.J. and Tribe, H.T. 1975. Preliminary field plot trial on biological control of Sclerotinia sclerotiorum by *Coniothyrium minitans*. Plant Pathology. 24: pp. 109 - 113.

Turner, G.J. and Tribe, H.T. 1976. On *Conithyrium minitans* and its parasitism of *Sclerotinia species*. Trans. Br. Mycol. Soc. 66: pp. 97 – 105.

Tuzun, S., Gay, P.A., Lawrence, C.B., Robertson, T.L., and Sayler, R.J. 1997. Biotecnological applications of inheritable and inducible resistance to disease in plants. In: Gresshoff, P.M. (Ed.). Tecnology transfer of plant biotechnology. CRC Press Inc., New York: pp. 25-40.

Tuzun, S. and Bent, E. 2000. The role of multigenic and microbially-induced resistance in plants. In: Agrawal, A.A., Tuzun, S., and Bent, E. (Eds.). Induced plant defenses against pathogens and herbivores. APS Press. St. Paul, Minnesota: pp. 95-115.

US EPA. 1997. Inovative uses of compost disease control for plants and animals. Online: http://www.epa.gov/epaoswer/non-hw/compost/disease.txt.

Utkhede, R.S. and Rahe, J.E. 1980. Biological control of onion white rot. Soil Biology and Biochemistry. 12: pp. 101-104.

Utkhede, R.S.. 1996. Potential and problem of developing bacterial biocontrol agents. Canadian Journal of Plant Pathology. 18 (4): pp. 455 – 462.

Utkhede, R.S. and Smith, E.M. 1997. Effectiveness of dry formulations of *Enterobacter aglomerans* for control of crown and root rot of apple trees. Canadian Journal of Plant Pathology. 19 (4): pp 397 – 401.

Van der Wolf, J.M. and Birnbaum, Y. 2003. Compound of natural origin for seed treatment. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 323.

van Dijk, K. and Nelson, E.B. 1998. Inactivation of seed exudate stimulants of *Phytium ultimum* sporangium germination by biocontrol strains of *Enterobacter cloacae* and other seed-associated bacteria. Soil Biology and Biochemistry. 30 (2): pp. 183 – 192.

van Dijk, K. and Nelson, E.B. 2000. Fatty acid competition as a mechanism by which Enterobacter cloacae suppresses *Pythium ultimum* sporangium germination and damping-off. Applied and Environmental Microbiology. 66(12): pp. 5340-5347.

Van Bruggen, A.H.C. and Termorshuizen, A.J. 2003. Integrated approaches to root disease management in organic farming systems. 8th International Congress of Plant Pathology. Vol. 1. Christchurch, New Zealand: pp. 49.

VanDyk, J. 2002. Spring alfalfa diseases in 2002. Online: http://www.ipm.iastate.edu/ipm/icm/2002/5-13-2002/springdis.html.

Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytophatology. 36: pp. 453-485.

Van Loon, L.C. 1999, Improve biological control of *Fusarium* wilt by combinations of antagonistic microorganisms in the rhizosphere of crop plants. STW. Online. http://www.stw.nl/projecten/U/ubi3227.html.

Vanninen, I., and Hokkanen, H. 1997. Efficacy of entomopathogenic fungi and nematodes against *Argyresthia conjugella* (Lep. Yponomeutidae). Entomophaga. 42(3): pp. 377-385.

Vavrac, J., Michalicova, A., and Ondrej, M. 1997. Suppression of *Rhizoctonia solani* damping-off disease of cucumbers in autoclaved soils amended with beneficial microorganisms. Biologia. 52(3): pp. 365-371.

Vidhyasekaran[a], P., Rabindran, R., Muthamilan, M., Nayar, K., Rajappan, K., Subramanian, N., and Vasumathi, K. 1997. Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. Plant Pathology. 46: pp. 291 – 297.

Vidhyasekaran[b], P., Sethuraman, K., Rajappan, K., and Vasumathi, K. 1997. Powder formulations of *Pseudomonas flourescens* to control pigeonpea wilt. Biological Control. 8: pp. 166 – 171.

Visca, P., ciervo, A., Sanfilippo, V., and Orsi, N. 1993. Iron-regulated salicylate synthesis by *Pseudomonas* spp. J. Gen. Microbiol. 139: pp. 1995-2001.

Wall, L.G. 2000. Consequences of an overview on PGPR workshop in Argentina: The field should be wider. 5th International PGPR Workshop, Villa Carlos Paz, 29 October-3 November 2000, Argentina.

Walsh, U.F., Morrisey, J.P., and O'Gara, F. 2001. *Pseudomonas* for biocontrol of phytopathogens:from functional genomics to commercial exploitation. Current Opinion in Biotechnology. 12: pp. 289-295.

Walther, D. and Gindrat, D. 1987. Biological control of Phoma and *Pythium* damping-off of sugar beet with *Pythium oligandrum*. Journal of Phytopathology. 119: pp. 167-174.

Warren, R.A., Yang, H., and Ryder, M.H.2001. Survival of formulations of *Trichoderma koningii* isolate 7A and other new biological control agents when stored at different temperatures. In: Potter, I.J. (Ed.). Proceedings of the 2nd Australasian Soilborne Diseases Symposium: pp. 156.

Watanabe, K., Abe, K., and Sato, M. 2000. Biological control of an insect pest by gut-colonizing *Enterobacter cloacae* transformed with ice nucleation gene. Journal of Applied Microbiology. 88(1): pp.90-97.

Watson, A. and Napier, T. 2001. Downy mildew control in lettuce. Online: http://www.agric.nsw.gov.au/reader/10722.

Wegener, C., Bartling, S., Olsen, O., Weber, J., and von Wertstein, D. 1996. Pectate lyase in transgenic potatoes confers pre-activation of defense against *Erwinia carotovora*. Physiological and Molecular Plant pathology. 49: pp. 359-376.

Weibelzahlfulton, E., Dickson, D.W., and Whitty, E.B. 1996. Suppression of *Meloidogyne incognata* and *M. javanica* by *Pasteuria penetrans* in field soil. Journal of Nematology. 28 (1): pp. 43 – 49.

Wegener, C., Bartling, S., Olsen, O., Weber, J., and von Wertstein, D. 1996. Pectate lyase in transgenic potatoes confers pre-activation of defense against *Erwinia carotovora*. Physiological and Molecular Plant pathology. 49: pp. 359-376.

Weller, D.M. 1984. Distribution of a take-all suppressive strain of *Pseudomonas fluorescens* on seminal root of winter wheat. Applied and Environmental Microbiology. 48: pp. 897-899.

Weller, D.M. 1985. Application of fluorescent pseudomonads to control root disease. In: Parker, C.A., Rovira, A.D., Moore, K.J., Wong, P.T.W., Kollmorgen, J.F. (Eds.). Ecology and management of soil-borne plant pathogens. American Phytopathol. Soc.: pp. 137-140.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26 : pp. 379 – 407.

Weller, D.M. and Thomashow, L.S. 1999. Premier bacteria suppress wheat take-all. Agricultural Research.30: pp. 10-12.

Weller, D.M. and Thomashow, L.S. 2003. Take-all decline: a model for biocontrol of soilborne pathogens. 8th International congress of plant pathology. Vol. 1: Solving problems in the real world. Christchurch, New Zealand: pp. 48-49.

Wells, H.D., Bell, D.K., and Jaworski, C,A. 1972. Efficacy of *Trichoderma harzianum* as a biocontrol for *Sclerotium rolfsii*. Phytopathology. 62: pp. 442-447.

Weltzein, H.C. 1991. Biocontrol of foliar fungal diseases with compost extracts. In: Andrews, J.H. and Hirano, S.S. (eds.). Microbial ecology of leaves. Springer-Verlag, New York: pp. 430-450.

Wharton, P. 2001. Host defense. Sorgum anthracnose disease. Online: http://www.sorghumanthracnose.org/hostdef.html

Whipps, J.M. 1991. Effect of mycoparasites on sclerotia forming fungi. In: Beemster, A.B.R., Bolen, G.J., Berlagh, M., Ruissen, M.A., Schippers, B., and Tempel, A. (Eds.). Biotic interactions and soil-borne diseases. Elsvier, Amsterdam: pp. 129.

Whipps, J.M., Mcquilken, M.P., and Budge, S.P. 1993. Use of fungal antagonists for biocontrol of damping-off and *Sclerotinia* diseases. Pesticide Science. 37(4): pp. 309 – 313.

Whipps, J.M. 1997. Ecological consideration involved in commercial development of biological control agents for soil-borne diseases. In: Van Elsas *et al.*(Eds.). Modern soil microbiology. Marcel Dekker, Inc. New York. Pp. 525 – 546.

Whitehead, N.A. and Salmond, G.P.C. 2000. Quorum sensing and the role diffusible signalling molecules in plant-microbe interaction. In: Stacey, G.S. and Keen, N. (Eds.). Plant-microbe interaction. American Pathological Society. St. Paul, Minnesota, USA: pp. 43-92.

Whiteman, S.A. and Stewart, A. 1998. Suppression of *Botrytis cinerea* sporulation on irradiated grape leaf tissue by the antagonistic bacterium *Serratia liquefaciens*. New Zealand Journal of Crop and Horticultural Science. 26 (4): pp. 325 – 330.

Wiendling, R. 1932. *Trichoderma lignorum* as a parasite of other fungi. Phytopathology. 22: pp. 837 - 845.

Wiendling, R. 1937. Isolation of toxic substances from the culture filtrates of *Trichoderma* and *Gliocladium*. Phytopathology. 27: pp. 1175 – 1177.

Wiendling, R. 1941. Experimental consideration of the mold toxins of *Gliocladium* and *Trichoderma*. Phytopathology. 31: pp. 991 – 1003.

Wittig, H.P.P., Johnson, K.B., and Pscheidt, J.W. 1997. Effects of Epiphytic fungi on brown rot blossom blight and latent infections in cherry. Plant Disease. 81(4): pp. 383 – 387.

Wulff, E.G., Mguni, C.M., Mortensen, C.N., Keswani, C.L., and Hockenhull, J. 2002. Biological control of black rot (*Xanthomonas campestris* pv. *campestris*) of brassicas with an antagonistic strain of *Bacillus subtilis* in Zimbabwe. European Journal of Plant Pathology. 108(4): pp. 317-325.

Yamada, M. 2001. Methods of control of injury associated with continuous vegetable cropping in Japan - Crop rotation and several cultural practices. JARQ-Japan Agricultural Research Quarterly. 35(1): pp. 39-45.

Yan, Z.N., Reddy, M.S., Ryu, C.M., McInroy, J.A., Wilson, M., and Kloepper, J.W. 2002. Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. Phytopathology. 92(12): pp. 1329-1333.

Ye, X.S., Pan, S.Q., and Kuć, J. 1990. Association of pathogenesis-related proteins and activities of peroxides, β-1,3 glucanase and chitinase with systemic induced resistanceto blue mould tobacco but not to systemic tobacco mosaic virus. Physiological and Molecular Plant Pathology. 36: pp. 523-531.

Yuan, W.M. and Crawford, D.L. 1995. Characterization of *Streptomyces lydicus* WYEC 108 as a potential biocontrol agent against fungal root and seed rots. Applied and Environmental Microbiology. 61 (8): pp. 3119 – 3128.

Yuen, G.Y., Schroth, M.N., and McCain, A.H. 1985. Reduction of *Fusarium* wilt of carnation with suppressive soil and antagonistic bacteria. Plant Disease. 69: pp. 1071-1075.

Yuen, G.Y. and Schroth, M.N. 1986. Inhibition of *Fusarium oxysporum* f.sp. *dianthi* by iron competition with an *Alcaligenes* sp.. Phytopathology. 76: pp. 171-176.

Zhou, T., Reeleder, R.D., and Sparace, S.A. 1996. Influence of nutrients on growth of *Epicocum nigrum.* Canadian journal of Microbiology. 42(7): pp. 647-654.

Zriba, N., Sherwood, J.E., and Mathre, D.E. 1999. Characterization and effectiveness of *Phialophora* spp. isolated from a Montana take-all suppressive soil in controlling take-all disease of wheat. Canadian Journal of Plant pathology. 21(2): pp. 110-118.

Appendix 1

In vitro media, planting media, and reagents

A. Standard potting mix

Each 50 L of potting mix media contains 35 L pine bark, 10 L sand, 5 L peat moss, 25 g $FeSO_4$, 90 g limil, and 300 g Osmocote slow-release fertilizer. All the components except the Osmocote were mixed in a concrete mixer and steam sterilized at 71°C for 45 minutes. On cooling the Osmocote fertilizer was added to the mix.

B. Trypticase soya agar (TSA)

TSA contains /L (distilled water); 3 g Trypticase soya broth (Oxoid), 1g yeast extract (Oxoid) and 15 g Davis agar. All components were dispersed in distilled water, autoclaved for 15 minutes at 121°C and on cooling, dispensed into sterile petri plates.

C. Potato dextrose agar (PDA)

This medium containing 39 g powdered PDA (Oxoid) was dispersed in 1 L distilled water. The medium was autoclaved for 15 minutes at 121°C and on cooling, dispensed into sterile petri plates.

D. Pectin Agar

Pectin agar medium contains /L distilled water; 0.9 g $NH_4H_2PO_4$, 2 g $(NH_4)_2HPO_4$, 0.1 g $MgSO_4.7H_2O$, 0.5 g KCl, 10 g pectin, and 30 g Davis agar. All components were dispersed in distilled water and the pH was adjusted to 4.5 prior to sterilization at 121°C for 15 minutes and dispensing into sterile petri plates. An antibiotic, such as tetracycline or streptomycin was added as necessary.

E. Flagella stain

This stain is composed of two solutions; solution A contains 10 mL of 5% phenol, 2 g tannic acid, and 10 mL of saturated $K.Al(SO_4)_2.12H_2O$; solution B contains 12 g crystal violet in 100ml of 95% ethanol. To make 22ml of this stain, 2 mL of solution B and 20

mL of solution A were mixed, filtered through Whatman filter paper and stored at 4°C in a syringe.

F. Oxidative/Fermentative medium

This medium contains /L distilled water; 2 g peptone, 5g NaCl, 0.3g KH_2PO_4, 3mL of 1% bromothymol blue, and 3 g Davis agar. All components were dissolved in distilled water and sterilized at 121°C for 15 minutes. On cooling (at ~ 50°C), a 10% filter-sterilized glucose solution was added to give a final concentration of 1% in the medium, prior to dispensing into sterile capped tubes.

G. Starch agar medium

This medium was prepared by overlaying sterile TSA in petri plates with sterile medium comprising 0.2% starch, 1.5% agar.

H. Urease medium

This medium contains g/L distilled water; 1 g peptone, 5 g NaCl, 2 g KH_2PO_4, 1 g glucose, and 20 g Davis agar. All components were dissolved in distilled water and the pH was adjusted to 6.8 prior to sterilization at 115°C for 20 minutes. On cooling, 100 mL of 20% filter sterilized urea were added and the medium dispensed into sterile petri plates.

I. Nutrient agar

Each 23 g of nutrient agar powder (Oxoid), contains 3 g beef extract, 5g peptone, and 15 g agar. This quantity was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes prior to cooling and dispensing into sterile petri plates.

J. Saline-EDTA buffer

This buffer consisted of 0.1M NaCl and 0.1M di-sodium EDTA, pH 8.1.

K. Binding buffer

This buffer was made of 6M sodium perchlorate, 50mM Tris-HCl, 10mM di-sodium EDTA and the pH adjusted to 8.0.

L. Washing buffer

This buffer was made of 20mM Tris-HCl, 2 mM di-sodium EDTA, and 0.8M NaCl in 50% v/v absolute ethanol. The pH was adjusted to 7.6.

M. PCR mastermix

This reagent contains PCR buffer, $MgCl_2$, deoxynucleotides dATP, dCTP, dGTP, and dTTP), and taq polymerase.

N. Primer 10F and Primer 1492R

Primer 10F is 5'-AGTTGATCCTGGCTCAG-3' and primer 1492R is 5'-TACGGYTACCTTGTTACGACTT-3'.

O. TAE buffer

This buffer contains 40mM Tris, 40mM glacial acetic acid, 1mM disodium EDTA, with the pH adjusted to 8.0.

P. Loading dye

The loading dye contains 0.25% bromophenol blue and 0.25% xylene cyclanol FF in 40% sucrose.

Q. Nutrient broth

Nutrient broth contains /L distilled water; 1.0 g Lab-Lemco powder, 2.0 g yeast extract (Oxoid), 5.0 g peptone, and 5.0 g NaCl. All components were dissolved in distilled water, dispensed in 10 mL aliquots into 30 mL McCartney bottles and autoclaved for 15 minutes at 121°C.

R. Casein agar

This medium was prepared by overlaying sterilized TSA (Appendix 1B) with autoclave-sterilized medium containing 1.5% agar and 3% milk. The milk was steam-sterilized for 5 minutes before mixing with autoclaved 1.5% agar when the temperature of the agar medium was ~ 50°C.

S. Basal mineral salt (BMS) medium

BMS medium contains /L distilled water; 2.0 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.01 g $FeCl_3$, and 0.5 KH_2PO_4. All components were dissolved, dispensed into 100 mL bottles and autoclaved for 15 minutes at 121°C.

T. Nutrient gelatin medium

Gelatin was added to molten nutrient agar to give 5% w/v and autoclaved at 121°C for 15 minutes prior to dispensing into sterile petri plates.

U. Citrate medium

Koser's citrate medium contains /L distilled water; 3.0 g sodium citrate, 1.5 g $NH_4NaHPO_4.4H_2O$, 1.0 g KH_2PO_4, and 0.2 g $MgSO_4.7H_2O$. All components were dissolved, dispensed into Bijoux bottles, and autoclaved at 121°C for 15 minutes.

V. Phostrogen® Food and Tonic (Phostrogen Australia Pty)

N:P: K ratio of the mixture is given as 15:4.4:12.5.

Nitrogen present as:

Potassium nitrate	4.4%
Monoammoniumphosphate	2.0%
Ammonium sulphate	8.4%

Total water soluble phosphorus (P) present as:

Monoammoniumphosphate	4.4%

Total water soluble potassium (K) present as:

Potassium nitrate	12.5%

Magnesium (Mg) present as:

Magnesium sulphate	1.5%

Manganese (Mn) present as:

Manganese sulphate	0.03%

Iron (Fe) present as:

EDTA chelate	0.26%

Sulphur (S) present as:

Mixed sulphates	12%

Appendix 2

Estimation of the doubling times and the specific growth rate of the bacterial antagonists

The doubling time and the specific growth rate of the bacterial antagonists were estimated from the log phase of their growth curve (Stanbury and Whitaker, 1984). The bacterial growth at this phase can be mathematically described as follows:

$$dx/dt = \mu x \quad (1)$$, where:

x is the bacterial cell concentration

t is time

μ is specific growth rate

If the equation (1) is rearranged and integrated, it will result in:

$$\int_{x_1}^{x_2} dx/x = \mu \int_{t_1}^{t2} dt \quad (2).$$

This equal to:

$$\ln x_2 - \ln x_1 = \mu (t_2 - t_1) \text{ or } \ln x_2/x_1 = \mu (t_2 - t_1) \quad (3)$$

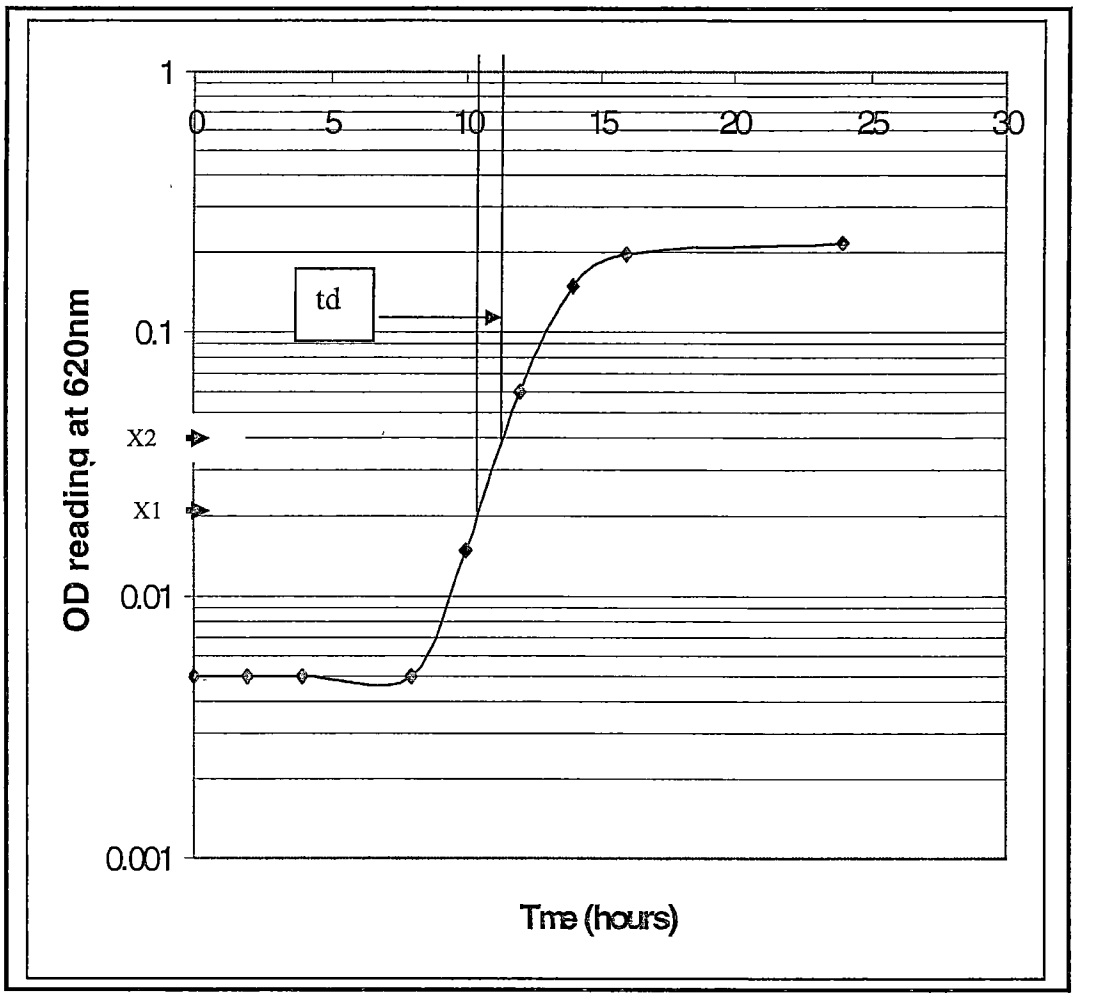
Consider $x_2 = 2\, x_1$ and $(t_2 - t_1) = td$, the equation (3) will become:

$$\ln 2 = \mu . td \quad (4)$$

The specific growth rate (μ) can then be estimated using the following equation:

$$\mu = \ln 2/td \quad (5)$$

The value of the td (doubling time) is estimated from the log phase of bacterial growth curve. The following curve is an example of doubling time (td) estimation from growth curve of *B. thuringiensis* grown at 30°C in half strength nutrient broth.



In the curve above, the value of x_2 is chosen 0.04 (within the exponential growth), which is twice as much as x_1 (0.02). The estimated doubling time (td) of this bacterium is approximately 1.07 hour.

Appendix 3

Iso-enzyme profile determination

Sample preparation

A cube (5 x 5 mm) was cut aseptically from a 5 day-old growth of the fungus Td_{22} on pectin agar medium (appendix 1D) and inoculated into 2 ml of pectinase medium (Appendix 1D minus agar) in 5 mL bijoux bottles followed by incubation at 25°C for 10 days under static condition.

Polyacrylamide gel preparation

Polyacrylamide gel was prepared by dissolving 0.1 g of citrus pectin, 10 g of acrylamide, 0.25g *N,N'*methylenebisacrylamide, and 0.1 mL *N,N,N'N'*tetramethylenediamide in 100 mL of gel buffer, comprising (g/L distilled water); Tris(hydroxymethyl)aminomethane, 4.598; and citric acid monohydrate, 0.525. The pH of the buffer was 8.7. Just prior to pouring, 0.1 g ammonium persulphate crystals were dissolved in the mixture. Gel moulds were held obliquely during initial filling to avoid air bubbles from being trapped in the gel moulds. The mixture was sufficient for three gels, which normally polymerize in about 10 minutes. Prior to electrophoresis, the upper perspex plates were removed leaving the gels supported on glass plates.

Gel electrophoresis for pectolytic and amylolytic enzymes detection

Ten micro liters of ten-day-old fungal cultures in pectinase medium in bijou bottles were loaded into wells of polyacrylamide gel. The electrophoresis was carried out at 2-4°C. The glass plate containing polyacrylamide gel was placed on copper plate that had been coated with several drops of kerosene to allow uniform heat conduction from the gel. The copper plate was suspended approximately 7 cm above the tank floor and each tank contained 500 mL of borate buffer pH 8.7, which consisted of (g/L distilled water); boric acid, 7.22 and sodium tetraborate decahydrate, 15.75. A platinum wire electrode was immersed across each tank adjacent to the central partition. Hospital lint wicks was used to facilitate electrical contact and applied to the gel ends. A small spot of 0.05% bromophenol blue in gel buffer was applied to the cathodic end of the gel immediately

prior to the application of 12 mA of constant current at an initial potential difference of 60 V per gel.

1.2.4 Gel staining and result recording

After electrophoresis, the polyacrylamide gel was soaked in 0.1 M malic acid for 1 hour at room temperature then stained overnight with 0.01% (w/v) ruthenium red at 4°C. To increase the contrast of the bands for the characterization of pectolytic enzyme, gels were immersed for about two minutes in 0.1% (w/v) ammonium persulphate solution.

A permanent record of results was prepared by direct photographic printing onto high contrast photographic paper in which the gels functioned as the negative (Cruickshank and Pitt, 1987).

Appendix 4

Milk composition

Water	**87.5%**
Lactose	**4.9%**
Protein	**3.2%**
Casein	2.9%
Lactalbumin	0.52%
Lactoglobulin	0.20%
Fat	**3.7%**
Minerals	**0.72%**
Calcium	0.12%
Phosphorus	1.01%
Chlorine	0.11%

Source: http://davidm.umecit.maine.edu/avs346/Lec4Milk_compostion.htm

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Potential for the Large-Scale Production Of a Biocontrol Fungus In Raw and Composted Paper Mill Waste

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The growth and survival of a *Trichoderma spp* (Td_{22}) antagonistic to *Sclerotinia minor* Jagger and *Sclerotium cepivorum* Berk was studied in raw wood fibre waste (WFW) of paper mill origin and in mature compost of this material In nutrient-amended, sterilized WFW or WFW compost (both supplemented with 20% w/w millet seed), the biocontrol fungus reached densities in the order of 10^{10} colony forming units (cfu)/g after 14 days incubation Lower population densities of Td_{22} were achieved under non-sterile conditions in the compost.millet mix of between 10^7-10^9 cfu/g after 28 days, depending on pretreatment. Viable spore density of Td_{22} in raw WFW amended with nutrients and 20% w/w millet seed reached approximately 10^{10} cells/g after 14 days incubation. This study indicates that cellulosic paper mill waste could provide an abundant low-cost growth medium for the large-scale culture of this or other biocontrol fungi.

Introduction

Problems of storage of microbial antagonists to plant pathogens have been largely overcome with the demonstrated maintenance of bacterial antagonists in matrices such as peat and vermiculite for 5 months or more (e.g. Vidhyasekaran *et al.* 1997; Gazoni *et al.* 1998). However the large-scale growth of these antagonists can be problematic because of the high cost of growth media, particularly where a large inoculum of biocontrol agent is needed. Cost of the cultivation medium is presently a critical factor in any assessment of merit relative to chemical treatments, a problem sometimes exacerbated by the perceived need for proprietary media formulations.

The use of compost to suppress plant diseases has been extensively examined, with recent reviews of the topic by e.g. Craft & Nelson (1996) and Hoitink, *et al.* (1997). Use of compost as a growth-medium for microbial antagonists rather than a storage material or an agent of non-specific inhibition, is however, very recent. An indication of the potential for manipulating the microbiota of compost to advantage was provided by Ramamurthy *et al.* (1996), who demonstrated that composting of eucalypt sawdust inoculated with the mushroom fungus *Volvariella* resulted in a product which enhanced the growth of wheat seedlings. Likewise Nakasaki *et al.* (1998) successfully cultivated a biocontrol *B. subtilis* to high population levels in composted grass clippings, with subsequent demonstration of the effectiveness of the modified compost against *Rhizoctonia* large patch disease of turf grass.

A crucial factor for the large-scale production of microbial antagonists in compost or other growth media, is their cultivation and survival in cell densities sufficient for end-use effectiveness, with Kodiak® (containing *Bacillus subtilis* effective against *Rhizoctonia spp.* and *Fusarium spp.* in cotton) being one of the few such products marketed in the USA (Brannen and Kenedy 1997).

Td_{22} (Metcalf, 1997a) is a *Trichoderma sp.* isolate known to give excellent field control of *Sclerotinia minor* Jagger and *Sclerotium cepivorum* Berk, the causative agents of lettuce drop and white-rot of onions respectively. Td_{22} is normally cultivated on auto-

claved moist millet seeds or on citrus pectin agar. Although its efficacy in field experiments has been amply demonstrated, its large-scale cultivation for agricultural application has been limited by cost considerations.

In the present study, raw and composted WFW was investigated for possible use as a base medium for Td_{22} cultivation and carriage. Cellulosic waste was considered worthy of examination in view of its attributes of good water-holding/aeration capacity and its potential utilization as a source of energy and carbon by Td_{22}.

Materials and Methods

Fletcher Challenge Mill WFW

Norske Skog Paper Mills Limited, Tasmania, landfills approximately 33,000 tonnes of WFW p.a. The material is of mixed eucalypt and *Pinus radiata* origin, comprising holocellulose as its primary constituent, with very low levels of metal contaminants and being deficient (from a recycling perspective) in N and P. In concentrated form it is phytotoxic to seeds, although the potential for its recycling to the field following composting was demonstrated by Jackson and Line (1997).

A compost was produced from this material by amending mill waste with single-superphosphate, urea and KNO_3 to give a C.N:P:K ratio of 35·1.0.6:0.1. This mix was composted at ambient temperatures for three months, at which time no residual toxicity was apparent to radish seed germination and growth.

Growth of Td_{22} Inoculum

Td_{22} inoculum was grown in sterile citrus pectin broth medium (pH 4.5) comprising (gL^{-1} distilled water): $NH_4H_2PO_4$, 0.9; $(NH_4)_2HPO_4$, 2.0, $MgSO_4.7H_2O$, 0.1, KCl, 0.5, citrus pectin, 10.0 (Metcalf, 1997b). Flasks (500mL) of inoculated medium (100 mL) were shaken for 7 days in a water bath at 25°C. Before use the potency of this inoculum, measured in cfu on pectin agar (the above medium solidified with 1.5% w/v agar), was determined by serial dilution plating (in triplicate).

Assessment of Growth of Td_{22} Under Sterile Conditions

Growth of Td_{22} was assessed in raw WFW, WFW composted for 3 months, millet seed, as well as mixes of WFW plus millet seed and composted WFW amended with either potato starch or Phostrogen® (a NPK-minerals formulation produced by Phostrogen Ltd, Australia). Unless stated otherwise these materials (100g in 750mL flasks, 3 replicates/treatment) were brought to field capacity (amounting to approximately 1.5L/kg for the 80:20 WFW/millet mix) with a solution containing (gL^{-1} distilled water): NH_4NO_3, 5.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; $FeCl_3$, 0.01, and autoclaved at 121°C for 30 minutes on each of two consecutive days. All flasks were inoculated with 10mL of Td_{22} suspension described above to give an initial density of between 3.7–5.5 $\log_{10}$ cfu/g dry mix. Inoculated mixes were incubated at 25°C for 4 weeks with periodic assay of growth of Td_{22} following dilution plating onto Oxoid Potato Dextrose Agar (PDA).

The relative biomass of Td_{22} under sterile conditions was assessed using the assay for chitin described by Chen and Johnson, 1983. Microscopic estimation of spore numbers in the various mixes was made using a haemocytometer. All estimates of cfu/g, spores/g or chitin biomass/g are given on a dry weight basis (100°C to constant weight).

Assessment of the Growth of Td_{22} Under Nonsterile Conditions

Air-dried WFW compost (dried to ambient moisture content under glasshouse conditions) was brought to field capacity with 2% w/v Phostrogen® solution and 100g quantities placed into 750mL flasks. The compost was then either autoclaved at 121°C for 5 minutes prior to inoculation, or directly inoculated with Td_{22} suspension described above to give 5.4 $\log_{10}$ cfu/g compost. Flasks were incubated at 25°C for 4 weeks with periodic assay of cfu on PDA for Td_{22} and on Trypticase Soy Agar (TSA) for bacteria [TSA comprising (gL^{-1} distilled water): Oxoid Trypticase Soy Broth Powder, 3.0; Oxoid yeast extract, 1.0; Davis agar, 15.0]. The identity of representative Td_{22} colonies was confirmed by isoenzyme analysis of pectic enzymes as described by Cruickshank and Pitt, 1987.

Glasshouse Assay of Effectiveness of WFW-Grown Td_{22}

Effectiveness of the 14-day culture of Td_{22} in an 80:20 w/w WFW compost:millet seed mix in protecting against *Sclerotinia minor* challenge was assessed in a pot-trial. Treatments of 10% and 20% v/v culture in soil were used with appropriate controls. Each treatment comprised five replicate pots. *S. minor* inoculum was mixed 20mm beneath the soil surface prior to adding 5 lettuce seeds per pot. Plants were assessed for health at weekly intervals for 4 weeks.

Statistical Analysis

Analysis of variance (ANOVA) of data obtained from this study was carried out using Minitab software for Windows. The significance of differences between means ($p < 0.05$ in all cases where significance is reported) was tested using the least significant difference (LSD) test following ANOVA.

Results

Growth of Td_{22} in Various Mixes under Sterile Conditions

Td_{22} grew well in all mixes tested except in 100% millet seeds, in which cfu fell after 14 days of incubation (Figure 1). The best growth response was observed in either raw or composted WFW supplemented with nutrients and 20% (w/w) millet seed, plateauing at 14 days incubation (at 10.3 and 9.9 $\log_{10}$ cfu/g respectively). Both results were significantly higher than those for a nutrient supplemented compost control after 14 or 28 days. Likewise, nutrient-supplemented WFW compost containing 50% w/w millet seed or 1% w/w starch did not improve the growth (cfu) of Td_{22} over the unamended control. Replacing the mineral nutrient supplements to WFW compost with 2% w/v Phostrogen® resulted in a very similar growth response (cfu) by Td_{22} (mix A cf. Mix F).

Haemocytometer assay of spore numbers in the raw 80%WFW:20% millet seed mix (showing the best growth of Td_{22}) indicated these to be of the same order of magnitude as the cfu determined from dilution plate counts (Figure 2). Total estimated spore numbers in other mixes were significantly lower than the cfu.

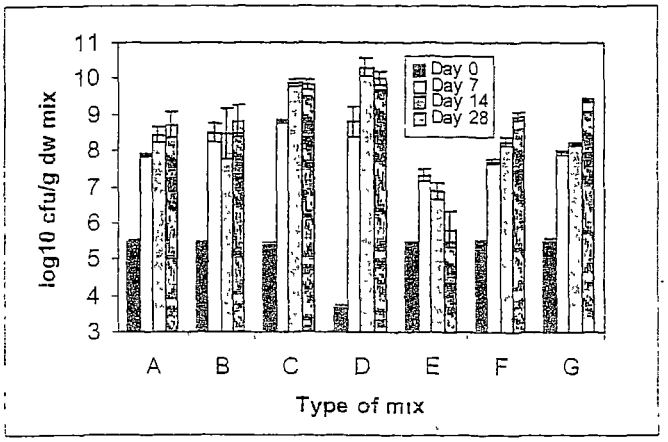

Figure 1 Growth of Td_{22} in various mixes under sterile conditions Each value is an average of 3 replicates ± standard error Initial and final pH levels (respectively) are indicated in brackets
Mix A· WFW compost (4.6-5 4)
Mix B 50% WFW compost + 50% millet seed w/w (4 8-7 5)
Mix C 80% WFW compost + 20% millet seed w/w (5 3-5 5)
Mix D. 80% Raw WFW + 20% millet seed w/w (5.0-5 5)
Mix E Millet seed (5 6-7 8)
Mix F WFW compost re-wetted with 2% w/v Phostrogen® solution (4 9-5 6)
Mix G WFW compost amended with 1% w/w potato starch (4 7-5 4).

Biomass of Td_{22} in the various mixes after prolonged incubation (10 weeks) as determined from chitin contents, indicated levels of approximately 50-60 mg/g mix except for mixes F and G, which contained about 35 mg/g mix.

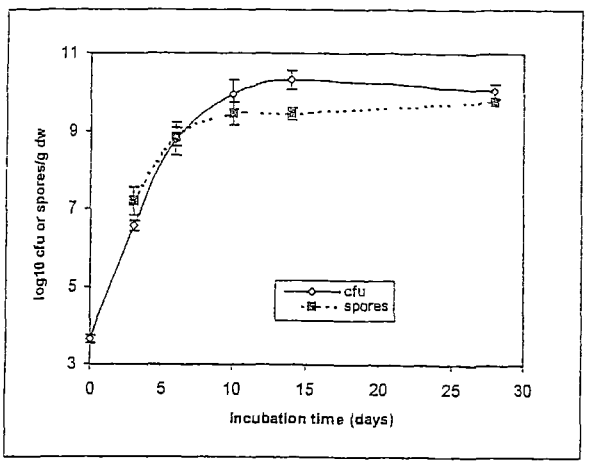

Figure 2. Growth of Td_{22} in raw WFW·millet (80 20% w/w) mix measured as cfu/g or spore density/g Each value is an average of 4 replicates ± standard error

Growth of Td_{22} in Nonsterile Compost

The growth of Td_{22} was followed for 28 days in non-sterile, steam-treated or air-dried composts, equivalent in other respects to mix F (Figure 1), after which time 8.6 $\log_{10}$ cfu/g and 7.1 $\log_{10}$ cfu/g were recorded in the respective mixes. The identity of

Td_{22} in the air-dried compost was confirmed by both morphological characteristics and isoenzyme profile. Over the same period the populations of mesophilic bacteria in the mixes reached 9.3 and 8.5 $\log_{10}$ cfu/g respectively.

Glasshouse Assay of Effectiveness of WFW-Grown Td_{22}

No toxicity to the germination (100%) or growth of lettuce seedling was observed in mixes of 10% or 20% v/v of composted WFW-millet culture mixed with soil. Challenge with *Sclerotinia minor* resulted in 100% mortality in soil-only pots after 4 weeks, compared with 8% mortality in the 10% v/v WFW-culture:soil mix and 0% mortality in the 20% v/v WFW-culture:soil mix.

Discussion

This investigation has demonstrated the potential use of WFW as a growth medium for the large-scale cultivation of spores of a biocontrol fungus, Td_{22}, known to be particularly effective against *Sclerotinia minor* and *Sclerotium cepivorum*, with cfu reaching 10^{10}/g culture in 14 days. Composting the WFW was initially thought to be advantageous to avoid deleterious effects on plant seeds (the original material was toxic to radish seedlings) and to provide nutrient balance. However it appears to be unnecessary for the purpose described; nutrient-amended, raw WFW-Td_{22} culture after 14 days gave comparable cfu to that of the composted equivalent, with no toxicity to radish seed germination evident at a high rate of application (20% v/v). After 14 days incubation the biocontrol agent in the raw WFW medium appeared to be largely converted to spores, auguring well for long-term survival in cultivation medium prior to application.

Except for mixes F and G, similar chitin contents were found in all mixes after 10 weeks incubation indicating similar hyphal abundance at this time, with presumably similar potential for spore production. From a production potential however the cfu produced in the different mixes after 14 to 28 days is of greater interest.

Air drying compost in the glasshouse for 3 weeks or brief steam-treatment (rather than sterilizing) (Nakasaki *et al.* 1998) with a view to minimizing the indigenous biota prior to inoculation with Td_{22} gave encouraging results, with two to three orders of magnitude increase in cfu Possibly better alternatives such as solarisation (McLean *et al* 2001) known to be effective for eliminating pathogens in glasshouse soil have yet to be examined.

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References

Brannen, P.M. and D.S. Kennedy. 1997. Kodiak® -A successful biological control product for suppression of soil-borne plant pathogens of cotton. *Journal of Industrial Microbiology and Biotechnology*, 19:169-171.

Chen, G.C. and B.R. Johnson. 1983 Improved colorimetric determination of cell wall chitin in wood decay fungi. *Applied and Environmental Microbiology*, 46:13-16.

Craft, C.M. and E.B. Nelson. 1996. Microbial properties of composts that suppress damping-off and root rot of creeping bentgrass caused by *Pythium graminicola. Applied and Environmental Microbiology,* 62:1550-1557.

Cruickshank, R.H. and J.I. Pitt. 1987. Identification of species in *Penicillium* subgenus *Penicillium* by enzyme electrophoresis. *Mycologia,* 79:614-620.

Gasoni, L., J. Cozzi and K. Kobayashi. 1998. Survival of potential biocontrol bacteria in various formulations and their ability to reduce radish damping-off caused by *Rhizoctonia solani. Journal of Plant Disease and Protection,* 105.41-48.

Hoitink, H.A.J., W. Zhang, D.Y. Han and W A. Dick. 1997. Making compost to suppress plant disease *BioCycle,* April, 1997:40-42.

Jackson, M.J and M.A. Line. 1997. Composting pulp and paper mill sludge-effect of temperature and nutrient addition method. *Compost Science and Utilization,* 5.74-81.

McLean, K.L., J. Swaminathan and A. Stewart 2001. Increasing soil temperatures to reduce sclerotial viability of *Sclerotinium cepivorum* in New Zealand soils. *Soil Biology & Biochemistry,* 33:137-143.

Metcalf D.A. 1997a. Development of a biological control agent for Allium white root rot in Tasmania. Proceedings of the Second International Symposium on Edible Alliaceae (Adelaide, Australia, November 1997) p40.

Metcalf, D.A. 1997b Biological control of onion white rot. PhD thesis, School of Agricultural Science, University of Tasmania, Australia.

Nakasaki, K., S. Hiraoka and H. Nagata. 1998. A new operation for producing disease-suppressive compost from grass clippings. *Applied and Environmental Microbiology,* 64:4015-4020.

Ramamurthy, V., R.K. Sharma, K.R. Yadaz, J.Kaur, D Vrat and R.M. Kothari. 1996 *Volvariella*-treated *Eucalyptus* sawdust stimulates wheat and onion growth. *Biodegradation,* 7:121-127.

Vidhyasekaran, P., R. Rabindran, M. Muthamilan, K. Nayar, K. Rajapan, N. Subramanian and K. Vasumathi. 1997. Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. *Plant Pathology,* 46:291-297