Onion rhizobacteria as potential plant health promotants

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DECLARATIONS

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ABSTRACT

Sclerotium cepivorum Berk. (Berkeley, 1841), the causal agent of Allium White Root Rot (AWRR) disease poses a major threat to the sustainability of the Tasmanian onion industry, an export commodity which is worth $A40 million to the state's economy. Tasmanian research into alternatives to fungicides for the management of this disease has been primarily focussed on use of *Trichoderma* spp. as fungal antagonists and novel bioactive substances such as diallyl disulphides. In this study, antifungal bacteria from the onion rhizosphere were examined for their potential as plant growth promotion agents with the view to provide an additional component in the present integrated control strategy.

Bacteria isolated from root samples of healthy onions growing in an AWRR pathogen infested paddock were found to possess antifungal properties when co-cultured *in vitro* with the pathogen at 10°C (typical of cropping soil temperatures for onions in Tasmania). The five bacterial strains most active in *S. cepivorum* inhibition were identified as members of the Enterobacteriaceae. Molecular taxonomy studies indicated these were putatively novel species. These isolates were screened in glasshouse trials with one isolate, M1RB2, inducing production of significantly greater onion shoot biomass in dry warm conditions.

All bacteria trialled were suitable for formulation as a broadcast powder, and exhibited high tolerance to Benlate, Thiram and Folicur, the three fungicides most commonly used on Tasmanian onion crops. Studies on the impact of these fungicides on native microbial populations showed that bacterial numbers may be elevated by the application of a benlate-thiram seed dressing. These two traits are of particular significance if a commercial product was to be developed. ELISA surveys of the rhizosphere showed an increase in the proportion of M6SA1-like bacteria in pots to which the bacteria had been introduced but no significant change in total bacterial numbers.

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COMMON ABBREVIATIONS

ANOVA	analysis of variance
AWRR	Allium white root rot
BLAST	Basic Local Alignment Search Tool
CFU	colony forming unit
ddH2O	distilled, deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EMB	Eosin Methylene Blue Agar
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
PHYLIP	phylogenetic inference package
PROC GLM	general linear models procedure
rDNA	ribosomal deoxyribonucleic acid
rpm	rotations per minute
ssDNA	single strand deoxyribonucleic acid
UV	ultraviolet
TSA	trypticase soy agar
TSB	trypticase soy broth

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Introduction

The sustainability of the Tasmanian onion industry, an export commodity currently valued at $A40 million (Dean Metcalf pers comm), is presently under threat from several major soil-borne pathogens including *Sclerotium cepivorum* Berk., the causal agent of Allium White Root Rot (AWRR), and *Botrytis allii*. Research into soilborne disease management in Tasmania has focussed on further development of an integrated strategy to limit disease levels and spread of AWRR. The current integrated pest management (IPM) system uses disease-free sites where available, quarantine measures to prevent soil transfer between farms, planting outside the optimum disease development temperature period and fungicide treatments.

Public perception of the food safety problems related with synthetic chemical pesticides and their direct environmental impact have led to increased research into the area of alternatives such as the use of micro-organisms and organic soil amendments to promote plant health and suppress pathogenic organisms (Jacobsen and Backman, 1993). This philosophy is followed in Tasmanian research on onion white root rot with field applications of diallyl disulphides to provoke suicidal germination of sclerotia and inoculation with the fungal antagonist *Trichoderma harzianum* (Dennis, 1997). Extension of the current IPM strategy to include promotion of plant health as well as disease control was initiated in this project. This study examines the plant health promotion potential of rhizosphere bacteria isolated from AWRR infested red ferrosols on the state's North West Coast with the view to provide an agent or agents complementary to current control strategies.

Rhizobacteria have much potential as plant health promotants within the Tasmanian AWRR IPM program. Bacteria isolated from the rhizosphere have been studied and used as crop inoculants since the late 1800s with a U.S patent issued for *Rhizobium* as a legume inoculant in 1896 (Nobbe and Hiltner, 1896). Since those early beginnings, selected rhizobacteria have been commercialised in formulations such as wettable powders and seed coatings that are compatible with current agronomic practices (Fravel, et al., 1998). Strains of *Bacillus cereus*

that are tolerant to commercial fungicides have been released as the bacterium – fungicide treatment, Kodiak AT® (Gustafson Inc, Dallas Texas). Given the literature examples of rhizobacteria as plant health promotants and their compatibility with commercial cropping practices, this study aims to isolate onion rhizobacteria with plant growth promotion potential that could possibly be produced and trialled in formulations similar to those available commercially.

Literature Review:
Plant growth enhancement
by
free-living bacteria

INTRODUCTION

Environmental and food safety problems related with pesticides and the public perception of these problems have led to increased research into alternatives such as micro-organisms and organic soil amendments to promote plant health and suppress pathogenic organisms (Jacobsen and Backman, 1993). Rhizosphere bacteria have been examined for more than a century to improve agricultural production (Burris, 1988). This research has been driven by the 2-5% of the total bacteria isolated from the rhizosphere which invoke positive growth responses in plants (Schroth and Hancock, 1981). Hiltner (1904) first defined the rhizosphere as the plant exudate enriched zone around plant roots, which is colonised by microorganisms. Beneficial rhizobacteria may be divided into two major groups; symbionts such as *Rhizobium* and free-living rhizobacteria which can be found on, near or actually inside the plant root (Frommel, et al., 1991; Kloepper and Schroth, 1981a).

Symbiotic bacteria such as the nitrogen fixing genus *Rhizobium* have been the focus of much research with subsequent development of commercial inoculants such as for pasture and crop improvement. The second group is commonly known as plant growth promoting rhizobacteria (PGPR). PGPR may be defined as free-living root colonising bacteria that have apparently co-evolved with plants and are capable of increasing plant growth and yield (Kloepper, et al., 1980; Schroth and Hancock, 1981). The majority of reported PGPR strains are from Gram-negative genera such as *Pseudomonas* (Kloepper, 1993) and *Serratia* (Ordentlich, et al., 1987) although Gram-positive genera such as *Bacillus* and *Azotobacter* have also yielded effective plant growth promoters (Brown, 1974). Despite the spectra of potential PGPR isolated, few have reached full commercial development. Strains of *Agrobacterium radiobacter, Pseudomonas chloraphis, Ps. fluorescens, Burkholderia cepacia, Erwinia caratovora, Bacillus subtilis* have been registered for use as crop inoculants in several countries including the U.S., Australia, Mexico and Japan (Copping, 1998).

MECHANISMS OF PLANT GROWTH PROMOTION

Free-living bacteria may directly and indirectly promote plant growth by a variety of mechanisms (Baker, 1968). For the purposes of this review, these mechanisms are examined separately although an organism may itself express several of these mechanisms concurrently. For example, the siderophore producer *Pseudomonas* spp. BTP1 also induces resistance to *Pythium ultimum* in cucumbers (Ongena, et al., 1999). Fravel (1988) also observed that antibiosis is involved in most *in situ* antagonism but is often combined with other mechanisms.

Direct growth promotion

Direct impacts on plant growth by bacteria generally entail either providing the plant with a compound produced by the bacterium or facilitating the uptake of nutrients from the environment (Glick, 1995). Compounds produced by the bacteria that directly affect plant growth include:

- *phytohormones* such as IAA and zeatin which stimulate plant growth
- *siderophores* that convert iron to forms available to the plant
- *enzymes* to solubilise minerals and hydrolyse phytotoxins in the soil and
- *metabolites* to provoke plant resistance reponses against pathogens

(Brown, 1974; Glick, et al., 1998; Kempe and Sequeira, 1983; Neilands and Leong, 1986)

Plant growth regulators

One mechanism that may explain how PGPR affect plants is the bacterial production of plant growth regulators (PGR) such as IAA and cytokinins (Brown, 1974) and the deactivation of the phytohormone, ethylene (Glick, et al., 1998). Muller, et al. (1989) found that inoculating maize seedlings growing in nutrient solution with rhizosphere bacteria resulted in a synergistic increase in growth regulator concentration compared to sterile plant roots and bacterial

cultures. Regression analysis showed significant correlations between IAA and ABA concentration in the medium surrounding the roots and lengths of primary, secondary and adventitious roots and numbers of adventitious and secondary roots.

IAA

Many bacterial species including *Enterobacter cloacae, Pantoea agglomerans, Klebsiella aerogenes* and *Klebsiella oxytoca* and *Pseudomonas putida* have been shown to increase root hair growth by production of indole-3-acetic acid from tryptophan (Zimmer, et al., 1994). IAA is an auxin, a phytohormone which has vital functions in lateral root development and is present in growing shoots, fruits and leaves (Atwell, et al., 1999).

Pseudomonas putida GR12-2/aux1, an IAA overproducing mutant of the PGPR strain GR12-2, lost the ability to stimulate the elongation of the roots of canola seedlings under gnotobiotic conditions. The other possible mechanisms of PGPR available to the mutant such as growth rate, siderophore production, and 1-aminocyclopropane- 1-carboxylate (ACC) deaminase activity were identical to those of the wild-type strain indicating that the amount of IAA produced as well as actual production affects growth promotion (Xie, et al., 1996). IAA production by bacteria in the rhizosphere is probably regulated as liquid cultures of *Azospirillum* spp. can produce levels of IAA up to 10-3 M, a concentration which can inhibit root growth (Zimmer, et al., 1988).

Cytokinins

Young, et al. (1990)observed a relationship between production of cytokinin by pseudomonads and *Serratia* spp. and root elongation. Cytokinins promote cell division and have an active function in initiation of new shoot structures, dormancy release and retardation of senescence (Atwell, et al., 1999). *Pseudomonas* strains and *Azotobacter chroococcum* producing the cytokinin

compound, trans -zeatin and its derivatives increased growth in radish (Nieto and Frankenberger, 1989; Salomone, et al., 1997)

Ethylene

Plants produce ethylene in response to a variety of environmental stresses including fungal infection (Abeles, et al., 1992). This exogenous ethylene can contribute to the damage caused by the stress (Van Loon, 1984). Certain PGPR can reduce the levels of ethylene by a feedback loop involving IAA and the enzyme 1-aminocyclopropane -1carboxylic acid (ACC) deaminase (Glick, et al., 1998). The plant takes up the IAA which is produced by the bacteria; the IAA then stimulates the activity of an enzyme, ACC synthase, which converts S-adenosylmethionone to the ethylene precursor, ACC. The ACC exuded by the plant is hydrolysed by the bacterial ACC deaminase to ammonia and α-ketobutyrate. To maintain the gradient between the internal and external ACC levels, the plant must exude more ACC and thereby reduces the amount ACC available for ethylene production by the plant.

Burd, et al. (1998) found that *Klebsiella ascorbata*, a bacterium capable of surviving on ACC as a sole carbon source, reduced the ethylene damage caused in canola seedlings by exposure to high levels of nickel chloride. Removal of the ACC deaminase gene from *Enterobacter cloacae* UW4 diminished the ability of the bacterium to promote the elongation of canola roots under gnotobiotic conditions (Li, et al., 2000).

Increased nutrient availability to plants

PGPR have demonstrated the ability to increase the availability of several minerals including phosphorus, iron and nitrogen to treated plants (Bar-Ness, et al., 1992; Mahaffee and Kloepper, 1994; Rodriguez and Fraga, 1999). Nitrogen fixation by PGPR as a genuine growth promotion phenomenon has generally been discredited due to the limited amounts fixed by PGPR but it may play some subsidiary role to other mechanisms (Lynch, 1990; Mahaffee and Kloepper, 1994;

Shen, 1997). Hence, this review will concentrate on the role of bacteria in phosphorous and iron uptake.

Phosphorus

Many PGPR have the ability to solubilise inorganic and/or organic phosphorus from soils. These include various Enterobacteriaceae (Pompei, et al., 1993), *Pseudomonas* spp. (Richardson and Hadobas, 1997) and *Bacillus* spp. (Gaur and Ostwal, 1972). Bacteria can release unavailable phosphorus to plants by two main mechanisms: production of organic acids such as gluconic acid or 2-ketogluconic acid which solubilise mineral phosphates (Goldstein, 1986) and/or production of acid phosphatases and other enzymes which mineralise organic phosphorus (Rodriguez and Fraga, 1999). A strain of *Burkholderia cepacia* with the capacity to solubilise mineral phosphate and mineralise organic phosphate was shown to improve the yield of several crops including tomato, potato and coffee in field trials and is currently used as a commercial biofertilizer in Cuba (Rodriguez and Fraga, 1999).

Iron

Plants can obtain a supplementary iron source from microorganisms by utilising bacterial siderophores (Bar-Ness, et al., 1992; Crowley, et al., 1988). Siderophores are a group of low molecular weight compounds with high affinity for ferric iron which are produced extracellularly by bacteria in order to combat iron stress (Neilands and Leong, 1986). Bar-Ness, et al. (1992) reported that maize and cotton use the fluorescent siderophore ferrioxamine B as an iron source. Oat plants also possess a siderophore transport system suggesting PGPR play a significant role in plant iron nutrition (Crowley, et al., 1988).

Systemic acquired & induced systemic disease resistance

Induced resistance (IR) may be broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack (Kalbe, et al., 1996). IR may be

split into two categories, systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR and ISR are differentiated by the type of bacterium and by the signal-transduction pathways inducing the response. SAR is characterised by an initial attack by a pathogen that usually results in minor tissue necrosis whereas ISR is facilitated by benign rhizobacteria including avirulent strains of pathogenic species (Sticher, et al.,1997) This response was seen in potato seed pieces that developed resistance to *Pseudomonas solanaceum* after exposure to a non pathogenic strain (Kempe and Sequeira, 1983).

Based on the model developed using mutants of *Arabidopsis thaliana*, both ISR and SAR are regulated by the *npr1* gene but the signal activating the gene is different in each type (Pierterse, et al., 1998; Pierterse, et al., 1996). SAR is mediated by salicylic acid (SA) which triggers production of pathogenesis-related (PR) proteins. ISR does not involve SA or PR production but is instead regulated by jasmonic acid and ethylene (Van Loon, et al., 1998).

Indirect growth promotion

Kloepper and Schroth (1981a) contended that the promotive effect of introduced rhizobacteria was linked to reductions in the pathogen populations in the rhizosphere rather than direct promotive effects such as production of PGR. This hypothesis was supported by their experiments with PGPR treated radishes grown in field and autoclaved soils with the result that plant growth enhancement only occurred in the field soils.

Reductions in pathogen populations are mediated by several mechanisms. These include physical displacement of deleterious microorganisms by PGPR, production of antimicrobial compounds and promotion of other beneficial microorganisms.

Physical displacement of deleterious micro-organisms

Plant health and growth may be promoted by the exclusion of deleterious rhizosphere bacteria by PGPR. Cell and lateral root junctions are favoured by bacteria as colonisation sites due to the abundance of root exudates. Pre-emptive occupation of these sites by PGPR bacteria prevents colonisation by pathogenic species and subsequently promotes plant health (Suslow and Schroth, 1982).

Exclusion of pathogenic species by inoculating crops with closely related species has proved to be an effective use of PGPR. Successful disease control of the crown gall bacterium *Agrobacterium tumifaciens* has been achieved by applying treatments of *A. radiobacter* to fruit trees and vines (Htay and Kerr, 1974; Kerr, 1972; Kerr, 1980). The non-pathogenic *A.radiobacter* colonises potential infection sites on the host and prevents establishment of the pathogen. Similarly, an avirulent *Erwinia caratovora* applied Chinese cabbage prevents the establishment of soft rot causing *Erwinia* spp.. This strain has been registered in Japan as BioKeeper® (Copping, 1998).

Unrelated bacteria can also be effective excluders of deleterious microorganisms. Bacteria such as *Pseudomonas* spp. colonise the rhizosphere quickly and aggressively thereby out competing other bacteria for ecological niches (Latour, et al., 1996). Schippers et al. (1985, 1987) found that the introduction of fluorescent pseudomonads to potato crops decreased the population of deleterious microorganisms so that short term rotation potato crops had yields equivalent to those from long term rotations.

However, these colonisation effects can be transient. The antibiotic producer *P.fluorescens* CHA0 and its antibiotic overproducing derivative CHA0-Rif displaced part of the resident pseudomonad population on cucumber roots but this effect was only observable at 10 days after inoculation and not after 52 days (Natsch, et al., 1997). This has certain implications for the use of PGPR such as correct timing of application to ensure the bacterium is active at the time when the plant is most vulnerable.

Production of antimicrobial metabolites

Antibiotics

The literature contains abundant evidence that indicates antibiotic production does occur in the rhizosphere and plays a major role in the suppression of soil-borne pathogens (Fravel, 1988). Isolates of the genera *Agrobacterium, Burkholderia, Erwinia, Bacillus* and *Pseudomonas* have demonstrated the ability to produce low molecular weight (<300MW) antimicrobial compounds *in vitro* (Buchenauer, 1998). Agrocins, compounds produced by *Agrobacterium radiobacter* that control the crown gall pathogen *A. tumifaciens* have been comprehensively studied (Donner, et al., 1993; Jones and Kerr, 1989; Moore and Warren, 1979; Stockwell, et al., 1996). Likewise, the antibiotics produced by *Pseudomonas* spp. such as 2,4-diacetylphloroglucinol (Phl), phenanzine-1-carboxylic acid (PCA), pyoluteorin and pyrrolnitrin are well documented in the literature. Pyrrolnitrin is produced by only one strain of *Serratia marcescens* unlike the red antifungal pigment prodigiosin which is produced by nearly all strains (Grimont and Grimont, 1984). *Bacillus* spp. are known to produce bacilysin and fengymycin (Loeffler, et al., 1986).

Mutagenic studies are the main evidence for the role of antibiotics as indirect plant growth promotors by suppression of deleterious microorganisms. Antibiotic deficient mutants of antibiotic producing strains frequently lack the ability to control disease. Unlike its antibiotic-producing wild type, a phenanzine deficient strain of *Pseudomonas fluorescens* was unable to inhibit *Gaeumannomyces graminis* var. *tritici in vitro* and its ability to control take-all disease in wheat was impaired (Thomashow and Weller, 1988).

Chitinases

Many rhizobacteria have been reported as producing endochitinases i.e. *Enterobacter agglomerans* (Chernin, et al., 1995) *Bacillus polymyxa* (Mavingui and Heulin, 1994), *B. cereus* (Pleban, et al., 1997) and various *Serratia* spp. (Kalbe, et al., 1996; Kobayashi, et al., 1995). Production of chitinases may assist

rhizosphere bacteria in successful competition with fungi for chitin and thereby reducing fungal growth indirectly. Alternatively, chitinases may enable the bacteria to lyse living hyphae to use as a growth substrate, since chitin is an important constituent of most fungal cell walls (Deboer, et al., 1998).

Occurrence of summer patch disease of turfgrass caused by *Magnaporthe poae* has been reduced under controlled conditions by two chitinolytic bacteria, *Xanthomonas maltophila* and *Serratia marcesens* (Kobayashi, et al., 1995). *Enterobacter agglomerans* IC1270, a bacterium with strong *in vitro* chitinolytic activity, reduced the occurrence of disease caused by *Rhizoctonia solani* in cotton by 64-84% under glasshouse conditions. Two chitinase deficient mutants of this strain were unable to protect plants against the disease thereby confirming the role of chitinases in the suppression of *R. solani* (Chernin, et al., 1995).

Siderophores

Bioavailable iron is a limited resource in the rhizosphere: the solubility of ferric iron in aerated soils of neutral pH is about 10^{-18}M whereas most organisms require 10^{-6}M (Buchenauer, 1998). As mentioned previously in this review, bacteria produce iron chelators known as siderophores to sequester iron from the soil (Neilands and Leong, 1986). Schroth and Hancock (1981) suggested that siderophores of PGPR starve other microorganisms that have less efficient or reduced production of siderophores. Iron competition has been implicated in the control of *Fusarium oxysporum* wilt disease (Elad and Baker, 1985) and *Pythium ultimum* damping off of cotton (Costa and Loper, 1994).

Experiments with siderophore negative (Sid^-) mutants have shown the importance of siderophores in suppression of rhizosphere diseases. Sid^- mutants of fluorescent pseudomonad strains lacked the plant growth promotion capacity of the parental strains (Kloepper, et al., 1980). Similarly, biological control of *P. ultimum* damping off of cotton and growth responses in wheat were impaired in Sid^- mutants of *P fluorescens* spp. in comparison to the parent strains (Loper, 1988).

Production of volatiles

Root pathogens may also be inhibited by hydrogen cyanide (HCN) and ammonia produced by rhizobacteria. Two roles for HCN have been suggested namely the induction of systemic resistance in the host plant and direct inhibition of deleterious microorganisms. The transfer of HCN synthesis genes from the PGPR bacterium *Ps. fluorescens* CHAO to the HCN negative non-PGPR strain P3 also conferred the ability to control *Thielaviopsis basicola* , the causal agent of black root rot of tobacco (Voisard, et al., 1989).

Enterobacter cloacae produces ammonia which is highly toxic to *Pythium ultimum* at low concentrations *in vitro*. Inhibition of bacterial ammonia production by addition of sugar to the culture medium prevented biocontrol activity (Howell, et al., 1988)

Assistance of other beneficial microorganisms

Free-living PGPR are known to improve the plant growth promotion effects of symbiotic bacteria. The synergy between the free-living *Bacillus* and the symbiotic nitrogen fixer *Rhizobium* promotes plant growth and development of legumes more than the isolated effects of *Rhizobium* (Halverson and Handelsman, 1991). *Bacillus polymyxa* stimulated population growth and nodulation by *Rhizobium etli* of *Phaseolus vulgaris* by stimulating root hair development in the host increasing the sites for nodulation (Petersen, et al., 1996).

Mycorrhizal helper bacteria (MHB) are a specialised subclass of PGPR. Certain ectomycorrhizal fungi such as *Laccaria bicolor* improve the growth of Douglas firs and *Pinus* spp. (Garbaye and Bowen, 1989). Co-inoculation of the fungus with a MHB such as *P. fluorescens* BBc6 improves the efficiency of the plant-fungus bond therefore indirectly promoting plant growth (Duponnois and Garbaye, 1991). This interaction has economic potential for the Douglas Fir nursery industry in France as the required dose of the relatively expensive

ectomycorrhizal inoculum are reduced when the bacterium is added (Frey-Klett, et al., 1999).

CONCLUSIONS

There exists a broad spectrum of rhizobacteria with demonstrated abilities to promote plant growth by a variety of mechanisms. With this premise in mind, the following body of work examines the potential of bacteria isolated from Tasmanian onion crops for plant growth and health promotion.

Chapter 1
Isolation & Screening
of
Rhizobacteria
with
Potential as Plant Health Promotants

INTRODUCTION

Introduction of beneficial bacteria to agricultural systems for the promotion of plant growth and health has become an area of increased interest since public backlash against the use of pesticides in agricultural production. The term "plant growth promoting rhizobacteria"(PGPR) may be defined as those root colonising bacteria that have apparently co-evolved with plants and are capable of increasing plant growth and yield (Kloepper, et al., 1980; Schroth and Hancock, 1981). The majority of reported PGPR strains are from Gram-negative genera such as *Pseudomonas* (Kloepper, 1993) and *Serratia* (Ordentlich, et al., 1987) although the Gram positive genera *Bacillus* and *Azotobacter* have also yielded effective plant growth promoters (Brown, 1974).

Bacterial strains selected initially for *in vitro* antibiosis as part of evaluating biological control activity frequently exhibit plant growth promotion activity in the absence of pathogens (Kloepper, 1993). Therefore, it was decided to isolate potential PGPR rhizobacteria which were antagonistic to the white rot fungus, *Sclerotium cepivorum,* a major pathogen of Tasmanian onion crops. As disease severity is highest when soil temperatures are between 11 and 15°C (Metcalf, 1997), bacteria were also selected for fast growth at winter soil temperatures (10°C). The final preliminary selection screen was designed to test each bacterium's ability to survive and multiply in the presence of Folicur®, Thiram® or Benlate®, three fungicides used to control *Botrytis* spp. and other lower fungi in Tasmanian commercial onion crops.

MATERIALS & METHODS

Origin & maintenance of bacterial isolates

Seventeen root samples were taken from bulbs of the onion cultivar Vecon Regular Cream Gold from a crop heavily affected by AWRR at Moriarty, Tasmania during February 1996. Portions of individual roots close to the bulb were aseptically removed from asymptomatic bulbs growing near plants obviously infected with *S. cepivorum* and harvested into sterile plastic snap lock bags. Sample selection was based on the premise stated by Henis and Chet (1975) "... saprophytic microorganisms inhabiting the surfaces of plant organs may serve as a biological buffer zone, preventing the pathogen from infecting its host."

Half of the total number of roots sampled from each bulb were plated directly on to trypticase soy agar (TSA) (Appendix I). The remaining portions of the samples were immersed in 10 ml of sterile 0.85% saline (w/v) then vortexed for 30 sec. A serial dilution to 10^{-2} in 9 ml of fresh sterile saline (Appendix I) was prepared then 0.1 ml of each sample was spread plated on TSA prior to incubation for seven days at 10°C.

Bacteria representing the dominant morphotypes on each plate were subcultured individually on TSA and incubated for 24 hours. All isolates were Gram stained (Lanyi, 1987) then the plates were stored for seven days at 4°C prior to antagonism tests. Bacterial isolates displaying inhibition of *S. cepivorum* were maintained a cryogenic preservation medium (Appendix I) at -80°C. Working cultures were recovered from cryogenic storage on to TSA every two months and stored at 4°C.

Origin & maintenance of *Sclerotium cepivorum* cultures

Sclerotium cepivorum SC4 cultures on onion agar were obtained from Dr. Dean Metcalf (Dept of Primary Industries, Water & Environment, New Town Tasmania). Cultures were recovered by transferring live mycelia from plate cultures to onion agar (Appendix I) or by forcing dormant sclerotia to germinate. Germination was initiated by crushing surface sterilised sclerotia (sclerotia soaked in 1.6% sodium hypochlorite for 1 min then for 10 min in 70% alcohol) with sterile forceps and plating the pieces on antibiotic amended TSA (Appendix I). Plates were then sealed with Parafilm and incubated at room temperature until germination occurred. Mycelia from the growing edge of the colony were transferred to TSA and incubated for 14 days before use in *in vitro* testing to prevent carryover of the antibiotics.

In vitro antagonism screening.

Isolates were initially screened for antagonistic activity against *Sclerotium cepivorum* by co-culturing four bacterial isolates against a 5mm square of a 14 day, 10°C culture of *S. cepivorum* SC4 on onion agar (Appendix I) (Plate 1). Duplicate sets of plates were incubated for 14 days at 10°C then examined for evidence of antifungal activity.

Plate 1: Example of four isolates co-cultured with S. cepivorum

Sixteen isolates displaying antifungal properties were then individually challenged against *S. cepivorum* on onion agar. A 4 cm line of a 24 hour, 25°C culture of each isolate was streaked 2 cm from a 5mm² block of 14 day old *S. cepivorum* mycelia (Plate 2). Three replicates of each plate were incubated for 14 days at 10°C. Plates were then examined macroscopically and microscopically for indication of different mechanisms of antagonism.

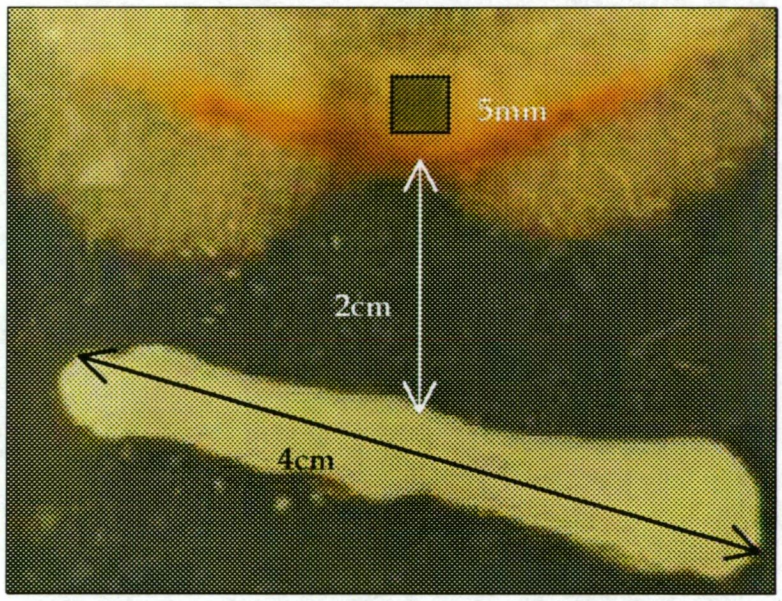


Plate 2: Individual isolate antagonism assay showing dimensions of plate layout

After fungal restriction measurements were made, a replicate of each plate was stored at 4°C and a second replicate incubated at 10°C. The 10°C plates were examined after 8 weeks and further developments of antagonism noted.

Quantification of antagonism by image analysis

Restriction of fungal growth by each isolate was analysed by scanning images of three replicate plates of fungal colonies with a OneScanner (Apple Computer Inc., California) using OFOTO® 2.0 software (Light Source Computer Images Inc. & Apple Computer Inc., California). A plate inoculated with a 5mm² of a 14 day

old culture of *Sclerotium cepivorum* was used as the control. The area of each image was converted into square centimetres by calibration to a known standard using NIH Image 1.58 (W. Rasband, National Institute of Health USA). The raw data was analysed for variance at the significance level $\alpha = 0.05$ using Microsoft Excel 7.0 software (Microsoft Corporation 1985-86).

Simple characterisation of antifungal activity

Five isolates displaying strong antifungal activity were examined for two modes of antifungal activity: production of iron chelating complexes (siderophores) and production of chitinase and pectinase. Production of siderophores was examined by reproducing the individual isolate assay as used for the quantification of antagonism but with onion agar amended with 10^{-4} M $FeCl_3$. Plates were visually assessed after 14 days at 10°C for evidence of impaired or enhanced suppression of fungal growth.

Pectinase activity was assayed on Paton's Pectate Gel medium (Appendix II) and chitinase activity on chitin agar (Appendix II). Isolates were incubated on two plates of each medium for 1 week at 25°C. Pectinase activity could be detected by deep liquefied pits in the medium (e.g. *Erwinia* spp.) or shallow non-liquefied pits (e.g. *Pseudomonas* spp.) (Sands, 1990). Chitin hydrolysis is indicated by clearing in the agar surrounding the bacterium (Smibert and Krieg, 1994).

Fungicide sensitivity

Sensitivity of each bacterial antagonist to fungicides routinely used on Tasmanian onion crops was assessed on TSA amended with Folicur® ($250gL^{-1}$ tebucanozole), Thiram® (800g/kg tetramethyl thiuram sulphide) or Benlate® (500 g/kg benomyl) at concentrations ranging from 0.001% to 10% (w/v or v/v) active ingredient (Appendix I). Fungicides were supplied by the Tasmanian Dept of Primary Industries, Water and Environment's New Town Laboratories. Four isolates were co-cultured on dual sets of each fungicide medium then examined for growth after 14 days incubation at 10°C.

RESULTS

Preliminary isolate screening

Forty-three of 122 bacterial isolates challenged *in vitro* against *Sclerotium cepivorum* were found to possess antifungal properties. Of these, 34 isolates originated from plates that were inoculated with samples of whole roots, with only nine isolates coming from the dilution series of the root samples. All isolates examined were Gram negative rods. Macro and microscopic study of the culture plates revealed several modes of *in vitro* antagonism as described and illustrated in Plates 3-4 and enumerated in Table 1.1. Type III antagonism (not illustrated) was assessed visually and was characterised by fast growth of the bacterium which competed for the space available for fungal growth.

Table 1.1: Summary of isolation results

ANTAGONISM TYPE	NUMBER OF ISOLATES
Type I Antagonism at a distance	30
Type II Localised lysis of hyphae	8
Type III : Space competition	6
No Antagonism	78

Plate 3a: Type I: Antagonism at a distance

Fungal growth has been restricted to a fixed distance from the bacterium M4SAX. Discolouration and collapse of the hyphae has occurred at the growing edge of the colony closest to the bacterium

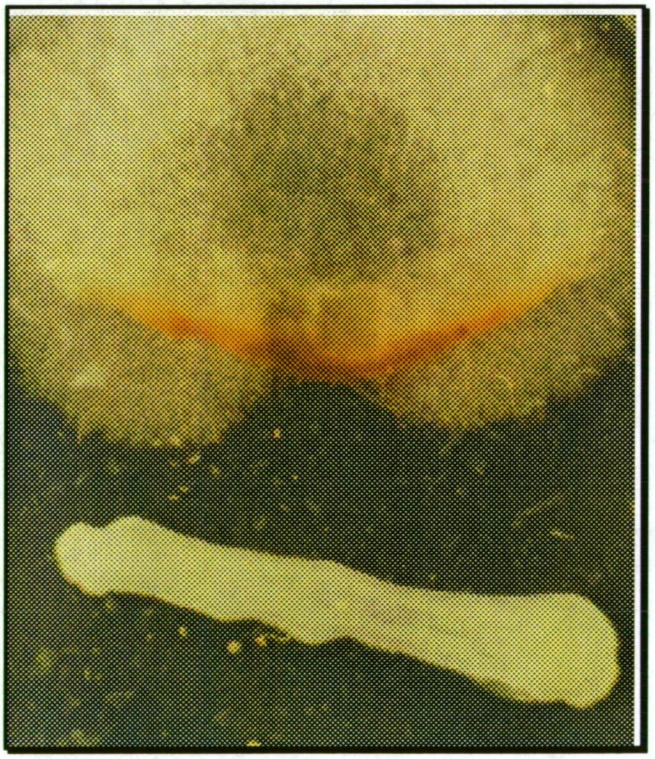


Plate 3b: Underside of plate showing antagonism at a distance

Unlike the case shown in Plate 1a, the fungus is not totally restricted and hyphae have overgrown the discoloured area

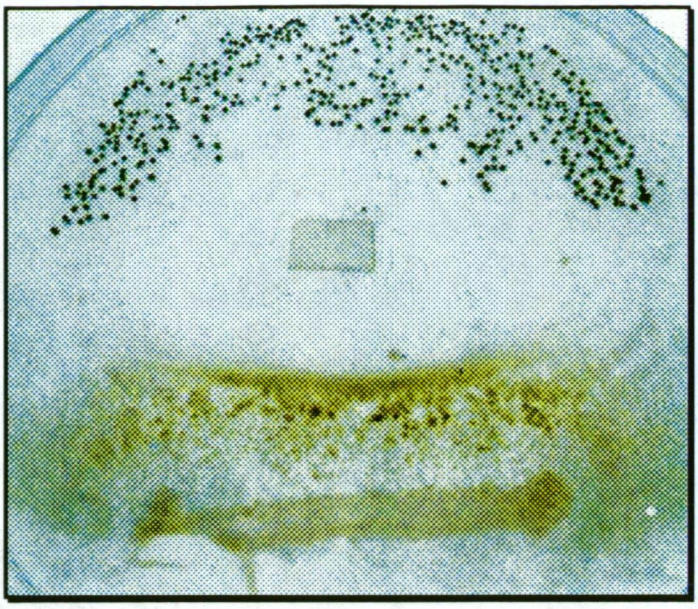


Plate 3c: Later stages of antagonism at a distance

Two months after the initial inoculation, it is seen that sclerotial maturation has been limited to the far side of the plate. Discoloured immature sclerotial initials may be seen at the edge of the bacterial colony

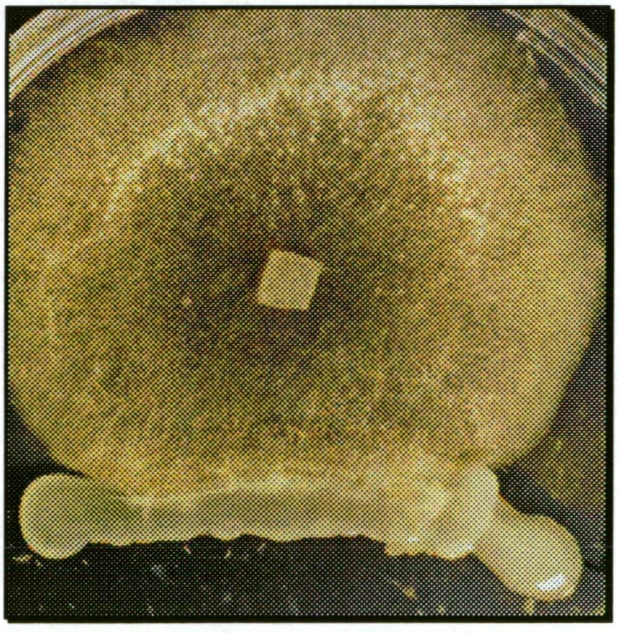


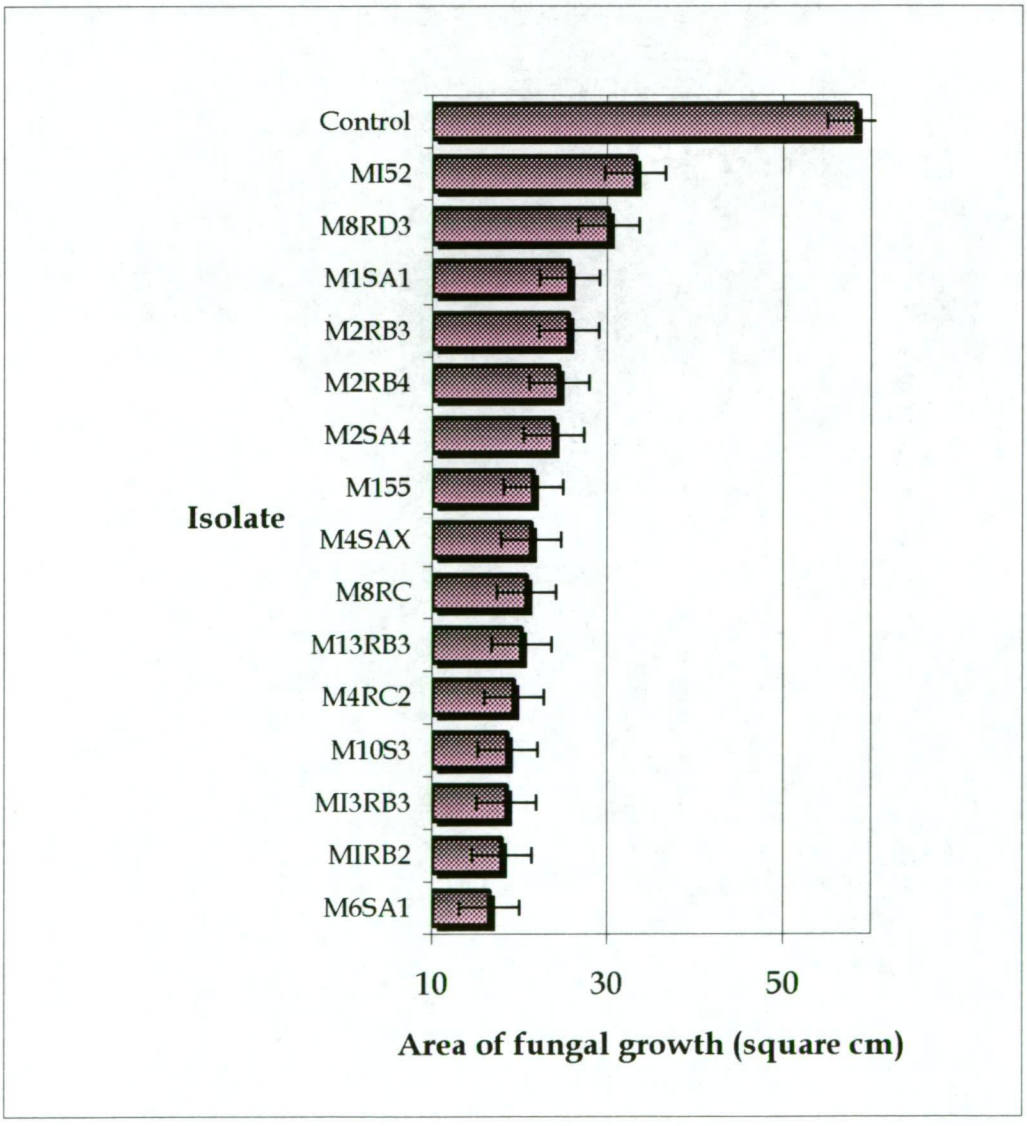
Plate 4: Type II: Localised lysis of hyphae

Advance of the fungal colony is restricted by the bacteria. Aerial hyphae tips at the colony margin lyse upon contact with the bacterium.

Quantification of antagonism by image analysis

All bacteria examined significantly restricted the growth of *S. cepivorum* after 14 days at 10°C in comparison to the unchallenged control. Results are shown graphically in Figure 1.1 with the averages and significance grouping given in Table 1.2

Figure 1.1: Average area of fungal growth after co-plating with individual bacterial isolates after 14 days at 10°C*



α= 0.05 for error bars

Table 1.2: Data for Figure 1

BACTERIUM	AVERAGE AREA OF FUNGAL GROWTH ON 3 PLATES (cm^2)	$LSD_{(0.05)} = 3.43\ cm^2$
M6SA1	16.63	a
MIRB2	18.05	ab
MI3RB3	18.57	abc
M10S3	18.71	abc
M4RC2	19.46	abc
M13RB2	20.31	bc
M8RC	20.82	bcd
M4SAX	21.38	bcde
M155	21.62	cde
M2SA4	23.95	def
M2RB4	24.58	ef
M1SA1	25.67	f
M8RD3	25.74	g
M2RB3	30.23	g
MI52	33.22	g
Control	58.47	h

Simple characterisation of antifungal activity

Siderophores

Supplementation of the onion agar with $FeCl_3$ had no observed effect on fungal growth in the presence of any bacterial antagonists.

Chitinolytic activity

All isolates examined possessed chitinolytic activity with 4-5 mm clearing zones around isolates M155, M1RB2 and MISA1 and 2-3 mm zones around M4SAX and M6SA1. None of the five isolates examined displayed pectinolytic activity as determined by pitting or liquefication of the pectin agar overlay.

Fungicide sensitivity of selected isolates

Of the 15 isolates screened for fungicide tolerance, only M1SA1, M2SA4 and M4SAX could not grow in the presence of high concentrations of Benlate. All isolates grew on Thiram and Folicur at all concentrations with the exception of M2SA4 which did not grow on 10 % Thiram. One isolate, M16RA, produced a deep orange-red pigment on the highest concentration of Thiram.

Visual assessments of the growth of all isolates are shown in Table 1.3 with growth assessed visually from poor (*) to good (***). Poor growth (*) was defined as individual colonies observed along the streak lines only with good growth (***) defined by merged colonies which had grown outside the boundaries of the original streak pattern.

Table 1.3: Fungicide sensitivity of selected bacterial isolates

FUNGICIDE	A.I. %	ISOLATE														
		M1RB2	M1SA1	M2RB3	M2RB4	M2SA4	M4RC2	M4SAX	M6SA1	M8RC	M8RD3	M10S3	M13RB2	M13RB3	M152	M155
Benlate	0.001	**	**	**	***	*	**	**	**	**	**	**	**	**	***	***
	0.01	**	**	**	***	*	**	**	**	**	**	**	**	**	***	***
	0.1	**	**	**	***	*	**	**	**	**	**	**	**	**	***	***
	1	*	-	**	***	*	**	-	**	**	**	**	**	**	*	***
	5	*	-	**	***	*	**	-	**	**	**	**	**	**	*	**
	10	*	-	**	**	-	*	-	**	*	**	*	**	**	*	*
Folicur	0.001	**	***	**	***	**	**	***	***	***	***	**	**	**	***	***
	0.01	**	***	**	***	**	**	***	***	***	***	**	**	**	***	***
	0.1	**	***	**	***	**	**	***	***	***	***	**	**	**	***	***
	1	**	***	**	***	**	**	***	***	***	***	**	***	**	***	***
	5	**	*	**	**	*	**	**	**	**	**	**	**	**	*	**
	10	**	*	**	**	*	**	*	**	**	*	**	**	**	*	*
Thiram	0.001	**	**	**	**	*	**	**	**	**	**	**	**	**	***	***
	0.01	**	**	**	**	*	**	**	**	**	**	**	**	**	***	***
	0.1	**	**	**	**	*	**	**	**	**	**	**	**	**	***	***
	1	**	**	**	**	*	*	**	**	**	**	**	**	**	**	**
	5	**	**	**	**	*	*	**	**	**	**	**	**	**	**	**
	10	**	**	**	**	-	*	**	**	**	**	**	**	**	**	**

*Key to visual scale - * = poor growth, ** =average growth, *** =good growth, - = no growth Poor growth (*) was defined as individual colonies observed along the streak lines only with good growth (***) defined by merged colonies which had grown outside the boundaries of the original streak pattern.*

DISCUSSION

One third of the bacteria isolated were antagonistic to *Sclerotium cepivorum*. Similar observations by Linderman, et al. (1994) led to the suggestion that this may be due to the host plant selectively enriching antagonists from the soil in response to external disease pressures. This conclusion was drawn from their observation that bacteria from onion rhizosphere soil, as a group, were more antagonistic against *Sclerotium cepivorum* than those from snapdragon rhizosphere soil. This proportion of antagonists on healthy bulbs near diseased plants may also support the premise on which selection of the samples was based i.e. "saprophytic microorganisms inhabiting the surfaces of plant organs may serve as a biological buffer zone, preventing the pathogen from infecting its host." (Henis and Chet, 1975)

The method of isolation appeared to have an effect on the number of antagonistic isolates recovered from each sample. Direct plating of the root samples on to agar resulted in recovery of 34 antagonists whereas the dilution series method only isolated nine. This suggests that the dilution washing may not have dislodged antagonists, which were firmly adhered to the root surface.

Preliminary identification of the bacterial strains chosen for further study (M1SA1. M1RB2, M155, M4SAX and M6SA1) fits the general description of the family Enterobacteriaceae i.e. facultatively anaerobic, Gram-negative, fermentative rods (Krieg and Holt, 1984). This family has been acknowledged as a significant component of the beneficial rhizosphere microbiota with isolates from the genera Enterobacter (Kempf and Wolf, 1989; Marchi and Utkhede, 1994), *Serratia* (Ordentlich, et al., 1987) *Erwinia* (Kalbe, et al., 1996) and *Kluyvera* (Burd, et al., 1998) reported as plant growth promoters and/or biological antagonists of fungi. However, the most aggressive antagonists isolated in this study do not fit the descriptions of these genera and appear to be novel members of the Enterobacteriaceae. This will be discussed further in Chapters 2 and 3.

The majority of the antagonistic isolates (29/42) restrained fungal growth at a distance. The zones of inhibition suggest that an antibiotic excreted by the bacterium was restricting fungal growth. This type of reaction between *Sclerotium cepivorum* and bacterial antagonists has been previously described by Ghaffar (1969) using linear measurements of inhibition and changes of fungal growth rates to compare the antagonists. The computer imaging used in this study may be a more suitable tool for comparison of different modes of antagonism as the fungal colony itself is quantified.

The number of antibiotic producing bacteria found suggests that these metabolites may be produced *in situ* as well as *in vitro*. There has been sufficient previous evidence in the literature to indicate that antibiotic production does occur in the rhizosphere and plays a major role in the suppression of soil-borne pathogens (Fravel, 1988). It can also be argued that some of the isolates recovered may produce antibiotics *in situ* but could not express these metabolites *in vitro* due to factors such as inappropriate media or absence of other stimuli.

Discolouration of the mycelium on the colony margin closest to the bacterium and lysis of hyphal tips has been previously reported by Backhouse and Stewart (1989). They postulated the mechanism of hyphal rupture was separate to that of growth inhibition and was a separate antibiotic or enzyme. This would seem likely in the light of the chitinolytic activity displayed by isolates in this study which do not produce antibiotics.

Prevention of maturation and pigmentation of the sclerotia of *Sclerotium cepivorum* has been reported for the fungal antagonists *Gliocladium virens* and *Trichoderma viride* but not by bacteria (Jackson, et al., 1991). This inhibition of maturation may possibly be linked to a bacterial metabolite that inhibits melanin synthesis thereby preventing development of the mature sclerotial rind. A sclerotium with an immature rind would be less likely to survive for prolonged

periods due to the role of melanin as an UV protectant and antibacterial agent (Bloomfield and Alexander, 1967).

The isolates chosen for further study, namely M6SA1, M1RB2, M4SAX, M1SA1 and M155, displayed chitinolytic activity. Not surprisingly, the bacterium M1SA1 which causes localised hyphal lysis produced the largest clearing zones in chitin amended agar. Other members of the Enterobacteriaceae antagonistic to fungi have been reported as producing endochitinases i.e. *E. agglomerans* (Chernin, et al., 1995) and *S. marcescens* (Kobayashi, et al., 1995). The potential combination of chitinases and antibiotics as an antifungal complex displayed by these isolates supports the theorem of Fravel (1988) who stated that antibiosis is involved in most *in situ* antagonism but is often combined with other mechanisms. This would be logical in light of the spectra of organisms present in the rhizosphere.

The absence of pectinases and siderophores was not surprising due to the environment from which the bacteria were isolated. Bacteria with the ability to hydrolyse pectin would not be of benefit to the host plant. The absence of detectable pectinolytic activity may also have been due to the synthetic media. In addition, the bacteria were isolated from a North Western Tasmanian red ferrosol which typically contains between 8 -13% free iron oxide at cropping depth (5-45 cm into the soil profile) (Baillie, 1995). This would suggest that massive production of siderophores would be required before any restriction of growth related to sequestering of iron would occur.

Tolerance of high levels of fungicides as displayed by 12 of the 15 isolates studied is not uncommon in bacteria isolated from intensive cropping situations. Such tolerance can be induced by prolonged or repeated exposure to fungicide (Abd-El Razik, et al., 1983). Degradation of the fungicides may also explain the isolates surviving exposure to high concentrations of agrochemicals. The bacteria were not tested for their ability to degrade fungicides although this may

be an important factor in potential commercial use. Bacteria isolated for antagonistic ability and fungicide tolerance such as those examined in this study may assist in the control of fungi already weakened by chemical controls and therefore increase general plant health.

Chapter 2

Morphological, Cultural & Biochemical Characterisation of Selected Rhizosphere Bacteria

INTRODUCTION

The preliminary studies outlined in Chapter One isolated several rhizobacteria with the capacity to suppress the *in vitro* growth of *Sclerotium cepivorum*. This lead to the next question: what species of bacteria are they? Elucidating the identity of a bacterium is useful as prior knowledge from literature can then be used to better understand the organism's ecology and suitability for use in a plant protection program. An example of this is that of *Serratia marcescens*, an effective biological control agent of summer patch disease of turf grass (Kobayashi, et al., 1995), but also an opportunistic human pathogen (Grimont and Grimont, 1984).

Morphological and cultural characteristics combined with biochemical assays are the primary keys to identifying a bacterium to the family level. These include Gram reaction, cell morphology, motility and the oxidative or fermentative metabolism of glucose (Smibert and Krieg, 1994). After these routine tests are performed, several methods may be used to narrow identification to genus and/or species level. The first methods used in this study involved the use of two commercial kits namely the API20E system for Enterobacteriaceae (BioMeriaux Vitek Australia Pty. Ltd, Castle Hill NSW) and Biolog GN microplates (Biolog Inc., Hayward, California). The API system uses a strip of 20 microtubules containing dehydrated substrates to which bacteria are added to produce an Analytical Profile Index (API). The pattern is then crossmatched to the manufacturer's database and a presumptive identification given. The Biolog system also uses dehydrated substrates but these are bound to a 96 well microplate along with a tetrazolium indicator. The colourless indicator turns purple in response to the oxidation of the substrate. The reaction pattern can then be manually entered into the manufacturer's Microlog program for identification or scanned with a microplate reader.

Both systems are quick with the possibility of identification within 24 to 48 hours. Unfortunately both systems are primarily intended for medical

applications and accordingly the manufacturer's databases and incubation requirements can be suboptimal for environmental specimens. Therefore a variety of tests in accordance with the schedule outlined in *Bergey's Manual of Bacterial Systematics* were also performed to confirm or extend the characterisation profiles given by the commercial kits.

MATERIALS AND METHODS

Five onion rhizosphere bacterium, namely M1SA1, M1RB2, M4SAX, M155 and M6SA1, were subjected to a battery of tests for identification according to *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984). All incubations were at 25°C under static dark conditions and duplicated unless otherwise stated.

Growth & Morphological Characteristics

Gram reaction & cell morphology

Macro and microscopic morphological characteristics and Gram reaction of bacterial cells were examined after 48 hours growth on trypticase soy agar (TSA) (Appendix I).

Motility

Drops of a 24-hour trypticase soy broth (TSB) (Appendix I) culture of each isolate were examined by phase contrast microscopy. One or more cells moving against the general bacterial stream or Brownian motion confirmed motility.

pH

Isolates were tested for growth in 9 ml of TSB buffered from pH 2.6 to pH 10 in diluent tubes. Media was buffered with phosphate at pH 6.0- 8.0 (Appendix I) and with citrate from pH 3.0 - pH 7.0 (Appendix I). An overlap of 1 pH unit (6.0

- 7.0) was used to determine the effect of the buffers on bacterial growth. Visible turbidity of the media after 7 days incubation was taken as an indication of growth.

Salinity

Nine ml of TSB amended with 0%, 1%, 4% and 8% NaCl in diluent tubes was inoculated with each isolate and incubated for 7 days. Growth was indicated by visible turbidity of the media or, in the case of the 8% NaCl broth, by streaking two loopfuls of culture on to TSA and incubating for 24 hours.

Differential/selective enteric media

Isolates were streaked on Eosin Methylene Blue Agar (EMB) (Oxoid Ltd., U.K.) and MacConkey agar (Appendix II) to determine growth and colony characteristics. Both media contain compounds that select for the growth of members of the Enterobacteriaceae and also indicators to differentiate between lactose and non-lactose fermenters. Lactose fermenters produce pink-red colonies on MacConkey agar and mucoid blue-black or metallic green colonies on EMB (Power and McCuen, 1988).

Biochemical Tests

Oxidation/fermentation of Carbohydrates

Oxidation and fermentation of the carbohydrates D-glucose, adonitol, arabinose, dextran, β(-) fructose, lactose, myo-inositol, α-L- rhamnose, maltose, mannose, raffinose, trehalose, α-D (+) melibiose, and sorbitol was determined using Hugh and Liefson media (Appendix II) amended with 1% of each individual carbohydrate. Cultures were stab inoculated into two tubes of each carbohydrate medium and examined after 1, 3 and 5 days for acid production. Fermentative metabolism of the carbohydrates was indicated by the medium

changing from blue to yellow in the base of the tube with oxidative metabolism indicated if there was a colour change in the top of the tube only.

Use of carbohydrates as sole carbon sources.

Each isolate was cultured in 3ml of basal mineral salts media (Appendix II) amended with 1% of the carbohydrates listed in the oxidation/fermentation of carbohydrates section. Cultures were examined after 14 days with visual turbidity of the media considered an indication of sole carbon source utilisation.

Casein hydrolysis

Strains were streaked on to skim milk agar (Appendix II) and incubated for seven days. Clearing of the agar around colonies indicated casein hydrolysis.

Catalase

Two drops of 3% H_2O_2 were added to a bacterial smear on a glass slide. Immediate formation of gas indicates the presence of catalase. No gas production indicates absence of catalase.

Citrate utilisation

Koser's citrate broth (Appendix II) was inoculated with each isolate and examined for turbidity after 24 hours. The test demonstrates the ability of a microorganism to utilise sodium citrate as a sole source of carbon with the ammonium ion as the sole nitrogen source.

Decarboxylation of arginine, lysine and ornithine

Three mls of Moeller Decarboxylase Broth (Appendix II) containing 1% arginine, lysine or ornithine was inoculated with each isolate. Cultures were examined

daily for four days with a violet/red-violet colour to the broth recorded as a positive for substrate decarboxylation.

Deoxyribonuclease activity

Isolates were streaked on to deoxyribonuclease agar (Oxoid Ltd., U.K.) and incubated for 24 hours. Plates were then flooded with 1N HCl and examined for zones of clearing around colonies. Polymerised DNA precipitates in the presence of HCl and renders the medium opaque. Deoxyribonucleases from the bacteria hydrolyse the DNA in the media to a form which does not precipitate with HCl resulting in clear zones around the bacterial colonies (Bridson, 1990).

Esculin hydrolysis

Esculin hydrolysis was tested using esculin iron agar (Appendix II). Esculin hydrolysers produce a dark brown/black complex in the medium surrounding the colonies (Power and McCuen, 1988).

Gelatin degradation

A 10 ml volume of gelatin testing medium (Appendix II) was stab inoculated with each isolate and incubated for 48 hours. Cultures were positive for gelatin degradation if the media remained liquid after 4 hours at 4°C.

Hydrogen sulphide production on Triple Sugar Iron agar (TSI)

Cultures were stab- and surface inoculated on a slant of TSI (Oxoid Ltd., U.K). After incubation for 7 days, slants were examined for acid and gas production from lactose, sucrose or glucose in the slant and the blackening at the base of the butt which indicates production of H_2S (Bridson, 1990).

Indole production

Two drops of Kovac's reagent were added to 24 hour peptone water (Appendix II) culture of each isolate. A light pink or cherry red colour change by the reagent indicates the presence of indole in the suspension and therefore tryptophanase activity. A negative result exhibits no colour change.

Levan production from sucrose

Plates of nutrient agar (Appendix II) amended with 5% sucrose were inoculated and incubated for 5-7 days. Levan production was indicated by formation of large mucoid colonies with a pearl-like lustre.

Malonate utilisation

Malonate broth (Appendix II) was inoculated with each bacterium. Malonate utilisers turn the broth blue whereas a negative result is yellow.

Methyl Red detection of glucose fermentation

Two drops of methyl red were added to a glucose-peptone broth (Appendix II) culture of each isolate. A red colour indicates acidification of the medium by fermentation of glucose. A yellow colour indicates a negative result.

Nitrate reduction

Bacto Nitrate broth (Difco Laboratories, Detroit, Michigan, USA) was inoculated with each strain. 0.1 ml of 0.8% sulphanilic acid (w/v in 5N acetic acid) and 0.1 ml 0.5% α-naphthylamine (w/v in 5N acetic acid) were added to 24 hour and 72 hour broth cultures. Nitrate reduction by the bacterium is indicated by a pink tone in the broth after addition of the reagents.

Oxidase

Filter paper was wetted with freshly prepared oxidase reagent (1% aqueous N,N,N,N'tetramethyl-paraphenylene diamine dihydrochloride). Colonies were transferred from TSA to the prepared filter paper with a glass pipette. The presence of cytochrome oxidase is indicated by formation of a purple product within five to ten seconds.

Phenylalanine utilisation

A few drops of 10% ferric chloride were poured on to a phenylalanine agar (Oxoid Ltd.,U.K.) slant which had been inoculated with each isolate and then incubated overnight. A green colour indicates deamination of phenylalanine to phenylpyruvic acid.

Starch hydrolysis

Plates of starch nutrient agar (Appendix II) were streaked with each isolate and incubated for 7 days. Clear zones around bacterial growth indicated starch hydrolysis after plates were flooded with Gram's iodine.

Tween hydrolysis

Plates of Tween 40 and Tween 80 agar (Appendix II) were streaked with patch inoculated with each isolate. If the isolate possessed lipase activity, an opaque halo of calcium soap crystals appeared around the individual colonies.

Urea hydrolysis

Plates of Christensen's urease agar (Appendix II) were heavily inoculated with each isolate and checked daily for 4 days for the purple-red colour change indicating production of ammonia from urea.

Voges-Proskauer reaction

Approximately 1ml of 40% NaOH and a knifepoint of creatine was added to a glucose-peptone broth (Appendix II) culture of each isolate. A light pink to cherry red colour indicates the conversion of carbohydrates to acetyl methyl carbinol via the butylene glycol metabolic pathway. No colour change denotes absence of this pathway (Bridson, 1990).

Commercial Identification Systems

Biolog

Biolog GN microplates (Biolog Inc., Hayward, California) were used to partially characterise carbon source utilisation of isolates displaying strong *in vitro* antagonism to *S. cepivorum*. Each bacterial isolate was grown on TSA for 24 hours at 25°C. Colonies were harvested from the plates using sterile cotton swabs and then suspended in sterile 0.85% NaCl. Suspensions were adjusted with a spectrophotometer to match the Biolog GN system turbidity standards at 590nm. Two plates were inoculated for each isolate and incubated as per the manufacturer's instructions. Colour changes in each well were scored visually at 4 hours and at 24 hours incubation at 25°C. Dark purple was scored as positive, light purple as "borderline' and clear as negative. Results were entered manually for analysis by the Microlog database version 3.1 (PC Format) (Biolog Inc., Hayward, California).

API 20E

API 20E (BioMeriaux Vitek Australia Pty. Ltd, Castle Hill NSW) strips designed for identification of Enterobacteriaceae were prepared as per manufacturer's instructions and incubated for 24 hours at 37°C. The standard IMViC tests (Indole, Methyl Red, Voges-Proskauer and Citrate tests) were also performed in the appropriate broth cultures (Appendix II) at 25°C as environmental isolates of the Enterobacteriaceae may give false negatives at 37°C (Grimont and Grimont,

1984). Extra tests for motility, nitrate reduction, growth on MacConkey agar (Oxoid Ltd.,U.K.) and oxidation or fermentation of glucose (Hugh and Liefson Test) were performed in the appropriate media by the methods described earlier in this chapter to provide a more definitive API number. Identifications using the API number were made by BioMeriaux Vitek Australia Pty. Ltd, Castle Hill, NSW.

RESULTS

All isolates were Gram negative motile rods capable of growth at pH 4.0 to pH 8.0. Isolate M1RB2 was additionally capable of growth at pH 3.6. On TSA, all five isolates appeared round smooth shiny colonies 1-4mm in diameter. Colonies of the isolates M1RB2 and M6SA1 on EMB were a metallic green signifying lactose fermentation whereas the other three isolates were purple non-fermentors. All isolates grew on MacConkey agar with M1RB2 appearing brown, M1SA1 and M6SA1 appearing light purple and the remaining isolates appearing pink-purple. A summary of the entire range of biochemical tests performed may be found in Table 2.1.

Carbon source utilisation tests

All five isolates could utilise the 16 carbohydrates tested as sole carbon sources. Utilisation of salicilin by all isolates produced a yellow by product. The results of the oxidation and fermentation tests are given in Table 2.2.

Table 2.1: Summary of taxonomic test results

IDENTIFICATION TEST		ISOLATE				
		M1RB2	M1SA1	M155	M4SAX	M6SA1
Motility		+	+	+	+	+
Indole		-	-	-	-	+
Methyl Red		+	+	+	+	+
Voges Proskauer		-	-	-	-	-
Citrate		+	+	+	+	+
H_2S on TSI		-	-	-	-	-
Christensen's Urea		+	+	+	+	+
Phenylalanine deaminase		-	-	-	-	-
Decarboxylation of:	Lysine	-	-	-	-	+
	Ornithine	-	+	-	-	+
	Arginine	-	-	-	-	-
Gelatin		+	+	+	+	+
Malonate utilisation		+	+	+	+	+
Glucose, acid & gas		+	+	+	+	+
Esculin hydrolysis		+	+	+	+	+
Levan production		+	+	+	+	+
Tween hydrolysis:	80	-	+	+	+	+
	40	-	+	+	+	+
Deoxyribonuclease		-	-	-	-	-
Nitrate -nitrite		+	+	+	+	+
Oxidase		-	-	-	-	-
Caseinolysis		+	+	+	+	+

+ = positive as determined by the relevant method section

Table 2.2: Oxidation/fermentation of carbohydrates

CARBOHYDRATE	ISOLATE				
	M1RB2	M1SA1	M155	M4SAX	M6SA1
Adonitol	-	o	-	-	-
Arabinose	+	+	+	+	+
Dulcitol	-	-	-	-	o
Fructose	+	+	+	+	+
Inositol	-	o	o	o	-
Lactose	o	o	-	o	o
Maltose	+	+	+	+	+
Mannitol	+	+	+	+	+
Mannose	+	+	+	+	+
Melibiose	+	+	+	+	+
Raffinose	+	+	+	+	+
Rhamnose	-	o	-	-	-
Salicilin	+	+	+	+	+
Sorbitol	+	o	+	+	+
Sucrose	+	+	+	+	+
Trehalose	+	o	+	+	+
Xylose	+	o	+	+	+

- = Growth but no acid production, o = oxidation, + = fermentation

Commercial identification kits

M4SAX and M1RB2 share the same API of 120-676-365 and were typed as *Serratia* spp. M1SA1 and M1RB2 were both profiled as API 320-676-365 that was unacceptable to the manufacturer's database. Likewise, M6SA1 exhibited an unacceptable profile of API 724 677 365.

The Biolog similarity index is rated as significant if above 0.500 with indices below 0.300 considered too low for confidence. Biolog strongly identified MIRB2 as *Serratia plymuthica* (0.862). Less conclusive but still significant results were given for M6SA1 as *Pseudomonas corrugata* (0.529) and M4SAX as *Klebsiella terrigena* (0.509). M155 did not return a significant result but the closest match was *Enterobacter aerogenes* (0.417). Biolog did not identify MISAI due to the closest species, *Klebsiella pneumoniae B* having a similarity index of 0.099. The results of both commercial identification systems are summarised in Table 2.3.

Table 2.3: Summary of commercial identification kit results

	IDENTIFICATION KIT	
Isolate	Biolog *	API**
M1SA1	*Klebsiella pneumoniae* B sim 0.099	Unacceptable profile
M1RB2	*Serratia plymuthica* sim 0.862	*Serratia ficaria* (4 tests against) *Serratia plymuthica* (3 tests against)
M6SA1	*Pseudomonas corrugata* sim 0.529	Unacceptable profile
M4SAX	*Klebsiella terrigena* sim 0.509	*Serratia ficaria* (4 tests against) *Serratia plymuthica* (3 tests against)
M155	*Enterobacter aerogenes* 0.417	Unacceptable profile

** Similarity data calculated by the Microlog program. Closest match given*
*** Tests against = the number of test deviating from the type organism*

INTERPRETATION & DISCUSSION.

All five isolates fit the general description of the family Enterobacteriaceae Rahn. i.e. oxidase negative, glucose-fermentative Gram negative rods (Krieg and Holt, 1984). The family Enterobacteriaceae are an important component of the rhizosphere with several genera including *Enterobacter, Erwinia* and *Serratia* having been isolated by studies screening for antagonists of fungal plant pathogens (Kalbe, et al., 1996). Jackson, et al (1991) also retrieved Enterobacteriaceae antagonistic to *S. cepivorum* from infected onions and rhizosphere soils.

The typing of M6SA1 as a *Pseudomonas* species by Biolog is contradicted by the phenotypic tests that place it within the Enterobacteriaceae. Discrepancies between Biolog and manual testing may be due to insufficient growth after 24 hours at 37°C and thereby a failure to activate the redox chemistry. The primary key for the bacterium as an Enterobacteriaceae is its ability to ferment glucose. This automatically excludes it from being in the Pseudomonaceae as this family oxidises glucose (Krieg and Holt, 1984). Neither this bacterium nor M155 could be conclusively identified by either commercial identification system or by crossmatching with the Enterobacteriaceae in *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984).

M4SAX was identified as a possible *Klebsiella* by Biolog and was unable to be identified by API. The Biolog identification is unlikely to be accurate as *Klebsiella* spp. are by definition non-motile and M1SA1 is motile. Similarly, M1SA1 is methyl red positive and Voges Proskauer (VP) negative whereas *Klebsiella* spp are generally methyl red negative and VP positive.

M1RB2 and M4SAX were both identified as *Serratia* species by API with M1RB2 also being identified as such by Biolog. *Serratia* are commonly found in association with plants (Krieg and Holt, 1984) and some isolates have been found to possess antifungal effects against *Fusarium* (Sneh, et al., 1984), *Verticillium,*

Rhizoctonia and *Sclerotinia* (Kalbe, et al., 1996). However, the nutritive patterns of M1RB2 and M4SAX are not typical of the *Serratia* species listed in Bergey's manual in their methyl red reaction, malonate utilisation and growth on Christensen's urease agar.

The inability to definitively characterise environmental isolates such as the five investigated in this chapter to species is not an uncommon problem in bacteriology. Bacteria, unlike fungi, are often difficult to classify since few bacteria from natural environments have been adequately characterised. Traditionally, commercial identification and taxonomy is heavily biased towards species and strains of medical importance such as *Salmonella* and *Escherichia coli.* Further more, bacterial taxonomy is an area of intense debate regarding actual classifications and groupings within families. Standard sets of biochemical tests such as those delineated by Bergey's manual generally lead to a generic designation and less frequently to species classification. As stated by Campbell (1989) " Almost invariably the diagnosis reads ' it is close to species x, but is atypical in that....' " This has certainly been the case for these five isolates which leads to the next part of this study: the use of 16s DNA sequencing to elucidate their identities.

Chapter 3
16S rDNA Sequencing
of
Selected Rhizosphere Bacteria

INTRODUCTION

Attempts to sequence the 16sDNA gene of the five isolates characterised in Chapter Two were decided upon as a continuance of the identification process. Nucleic acid sequencing methods are a powerful tool in elucidating the identity of and relationships between, bacterial species. Highly conserved genes such as those encoding 16s ribosomal RNA are sequenced with the small amount of variation in the sequence used to establish phylogenetic relationships (White, et al., 1990). The sequence data itself can be compared to databases such as GENBANK at the United States National Centre for Biotechnology Information to determine whether it is an identified species.

One of the first steps in sequencing is the production of sufficient quantities of DNA by the polymerase chain reaction (PCR). PCR is the rapid amplification of DNA using oligonucleotide primers and thermostable DNA polymerases (Saiki, et al., 1988). The template DNA is lysed by the polymerase at high temperature to form single strands (ssDNA). Primers then anneal to the ssDNA and nucleotides are added to the template until a double stranded replicate of the original DNA is produced. The PCR products can then be directly sequenced by a dideoxynucleotide (ddNTP) chain-termination method also known as Sanger sequencing (Sanger, et al., 1977). Amplification of the PCR product is begun with appropriate primers and polymerase in a reaction mix containing a controlled ratio of labelled ddNTPs and the corresponding dNTPs. Nucleotides are added to the ssDNA until a ddNTP is randomly incorporated into the sequence at which point chain growth is terminated. The series of labelled strands produced may then be separated by gel electrophoresis with the resultant fragment pattern giving the DNA sequence of the target gene (Ludwig, 1991).

METHODS AND MATERIALS

Extraction of Bacterial DNA

DNA was extracted from the isolates of interest using a method modified from (Marmur, 1961). Bacterial cultures were grown on TSA plates at 25°C to late logarithmic phase. Cells were harvested from plates using clean microscope slides and then suspended in 4 ml saline EDTA (Appendix IIIa)

Cell walls were lysed by addition of 0.5 ml of lysozyme solution (100mg/ml in saline EDTA) to the bacterial suspension prior to incubation at 60°C for 30 min. 0.2 ml sodium dodecyl sulphate (SDS) (Appendix IIIa) and 0.1 ml of proteinase K (Appendix IIIa) were then added before a further 15 min incubation at 60°C. Another 0.3 ml of SDS was added and incubated for 15 min at 60°C. 1.325 ml of sodium perchlorate (Appendix IIIa) was added to the solution which was then transferred to a wrist action shaker and shaken for 10 min. One volume of chloroform-isoamyl alcohol (Appendix IIIa) was added before an additional 30 min shaking.

The resultant suspension was centrifuged at 4000 rpm. for 20 min then the aqueous phase was harvested with a wide bore Pasteur pipette to avoid shearing of the DNA. The chloroform-isoamyl alcohol extraction and centrifuge step was then repeated. Two volumes of chilled ethanol were added and the precipitate collected by spooling it around a rounded glass hook. The spooled material was airdried then resuspended in approximately 5 ml of ddH2O.

Agarose gel electrophoresis

Presence and purity of DNA and PCR extension products was determined by running a 5 µl sample mixed with 2 µl 6x loading buffer (Appendix IIIa) through a 1% agarose gel (Appendix IIIa). Samples were run for 40 min at 80 volts in a Horizon ® 58 Horizontal Gel Electrophoresis Apparatus (Gibco BRL) filled with

1X TAE buffer as an electrical conduit. The molecular ladder marker SPP-1 (Bresatec) was run alongside samples as a comparison for band weight. DNA bands present in the gels were visualised under short wave ultraviolet light from a UV transluminator (Ultra Lum. Inc.).

Purification of DNA

After the purity of the DNA was tested by gel electrophoresis, two chloroform-isoamyl alcohol extractions (as outlined in the DNA extraction procedure) were performed. The DNA was then precipitated with a 1:10 volume of 3M sodium acetate and one volume of isopropanol.

Samples were salt extracted to remove polysaccharides (Fang, et al., 1992). Each sample was diluted with 1 volume of 4M NaCl and the DNA precipitated with two volumes of absolute ethanol. After 30 min on ice, the mixture was centrifuged at 14000 rpm for 30 min at 4°C. The supernatant was carefully removed with a pipette and the pellet washed twice with 70% ethanol. The pellet was then air-dried and resuspended in 1 ml ddH20.

DNA from isolates M4SAX and M6SA1 proved recalcitrant to sequencing and both were re-extracted with polyethylene glycol (PEG). One volume of 13% PEG in 3M NaCl was added to each sample which was then mixed thoroughly and chilled for 30 min. The mixture was then centrifuged at 14000 rpm for 30 min at 4°C and the supernatant carefully removed with a pipette. The DNA pellet was washed twice with 70% ethanol, then air-dried and resuspended in ddH20.

Determination of DNA concentration

The concentration of DNA in solutions (DNA_{conc}) was determined either directly using a Hoefer TKO 100 minifluorometer with calf thymus DNA as a standard or indirectly by measuring absorbance at 260 nm using a GeneQuant RNA/DNA calculator (Pharmacia). Readings with GeneQuant were converted to μg/μl by the following equation (Dobson, 1993)

$$DNA_{conc}\,(mg/ml = \mu g/\mu l) = (A_{260} \times 50\mu g/ml \times D)/1000\ \mu g/ml$$

where D = dilution factor and A_{260} is absorbance at 260 nm.

Polymerase chain amplification of 16S rDNA

The polymerase chain reaction (PCR) (Saiki, et al., 1988) was used to selectively amplify 16S rDNA from the isolates M155, M1RB2, M1SA1, M4SAXand M6SA1 using the primers 1492 (reverse) (Lane, 1991) and A (forward) (Edwards, et al., 1989). The optimal PCR reaction mix was as follows

10x Reaction buffer IV (Advanced Biotechnologies)	10	μl
dNTP's (100 μM)	8	μl
$MgCl_2$ (25 mM)	8	μl
Taq polymerase (Advanced Biotechnologies)	0.25	μl
Dimethyl sulphoxide	5	μl
ddH_2O	90.75	μl
A primer (10 pmol/μl)	5	μl
1492 primer (10 pmol/μl)	5	μl
ds DNA (100-400 μg/μl)	1	μl

The isolates M4SAX and M6SA1 failed to produce high quality product from the above method so the Advantage™ KlenTaq Polymerase Mix kit (Clontech Laboratories, California) was used in the following reaction cocktail.

10x KlenTaq reaction buffer	10	µl
dNTP's (100µM)	8	µl
KlenTaq polymerase	2	µl
ddH_2O	69	µl
A primer (10 pmol/µl)	5	µl
1492 primer (10 pmol/µl)	5	µl
ds DNA (100-400 µg/µl)	1	µl

The PCR reactions were performed in a Corbett Research FTS 960 Thermal Sequencer using the following temperature regime:

Cycle 1 (x1)	94°C for 3 min (denaturation) 50°C for 2 min (annealing) 72°C for 6 min (extension).
Cycle 2 (x 36)	94°C for 1 min (denaturation) 50°C for 2 min (annealing) 72°C for 6 min (extension).

The final extension step after the 36 repetitions of Cycle 2 were completed was 72°C for 6 min followed by cooling to 4°C. Products were then stored at 4°C before purification.

Purification of PCR products

Presence of PCR products in the completed reaction was confirmed by gel electrophoresis and the bands excised in the same manner described earlier for the original DNA. All PCR products were purified using Qiagen QIAquick™ Gel Extraction kits in accordance with the manufacturer's instructions (Qiagen Inc, GmBH).

Sequencing

Sequence reactions for M1RB2, M155 and M1SA1were rendered by the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer) as per the manufacturer's instructions. The overlapping primers A (Edwards, et al., 1989), 1492 (Lane, 1991) 26 (Edwards, et al., 1989), 20 and 785 (Embley, et al., 1988) were used to give duplicate results for each sequence region.

The same primer set was used in attempts to sequence the isolates M4SAX and M6SA1 with the ABI PRISM dRhodamine Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and the Thermosequenase Dye Terminator Version 2.0 Kit (Amersham Pharmacia Biotech). All sequence reactions were prepared as per the manufacturer's instructions.

Sequencing reactions were carried out in a Corbett Research FTS 960 Thermal Sequencer with the following temperature regime: 5 min at 96°C followed by 36 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. At the completion of the 36th cycle, the products were held and stored at 4°C.

Purification of extension products

The extension products were transferred to a 1.5 ml centrifuge tube containing 2µl of 3M sodium acetate (pH 4.6) and 50 µl of chilled 95% ethanol. The tubes were incubated on ice for 10 min then centrifuged at 14 000 rpm for 30 min in a table top centrifuge held at 4°C. The supernatant was then carefully removed and discarded. The pellet was rinsed with 250µl 70% ethanol and spun for 5 min at 14 000 rpm at 4°C. Residual ethanol was removed with a micropipette and the pellet was dried in a tabletop rotary vacuum drier (500 mb pressure).

Automated sequencing and alignment

All purified extension products were sequenced on an Applied Biosystems A377 Automated Sequencer by CSIRO Hobart Marine Laboratories, Salamanca Place, Hobart.

Phylogenetic analysis

All sequences obtained were compared to the GenBank nucleotide library at the United States National Centre for Biotechnology Information using the Basic Local Alignment Search Tool (BLAST) (Altschul, et al., 1990) via the Centre's web site (http://www.ncbi.nlm.nih.gov). The sequences of M155, M1RB2 and M1SA1 were manually aligned against the 16S rDNA sequences of the most closest related bacterial species (as determined by BLAST) using an appropriately distant species, *Aeromonas hydrophila,* as an outgroup. Related species sequences were retrieved from the Ribosomal Database Project or from the Genbank database. GenBank accession numbers for all sequences used are shown in Appendix III b followed by the aligned sequence series.

Evolutionary distances between the aligned sequences were determined by PHYLIP v 3572 (Felenstein, 1991) using the maximum likelihood algorithm and DNAdist. The phylogenetic tree shown in Figure 3.1 was generated with PHYLIP v 3572 by the Neighbours joining method and DRAWGRAM using the evolutionary distance data given in Table A, Appendix IIIb.

RESULTS & DISCUSSION

Sequences were produced from isolates M155, M1SA1 and M1RB2. Poor quality sequences with insufficient data to attempt a Genbank search were obtained from M6SA1 and M4SAX. These bacteria may possess DNA with strong secondary structure that is resistant to the Sanger sequencing methodology as used in this study. Hillis, et al. (1996) recommends the use of Maxam-Gilbert chemical sequencing in such cases which, by use of harsher lysing methods, is able to break down more complex DNA configurations. Unfortunately, such methods are outside the scope of this study. Problems may also be inherent in the sequencing protocols chosen for these particular bacterium i.e. suboptimal reaction conditions and choice of primer sets.

BLAST searches on the sequenced bacteria, M155, M1SA1 and M1RB2 showed these bacteria to be members of the Enterobacteriaceae. This is consistent with the traditional and kit identification result of Chapter 2 but the nearest relatives as determined from the GenBank database are somewhat different to those identified by BIOLOG or API. GENBANK has its flaws as an identification tool for environmental bacteria as, like BIOLOG and API, it is dominated by sequences of commercial or medical importance (Hillis, et al., 1996). However, the 348,150 sequences available for comparison at the time of searching makes GENBANK significantly more powerful than the API or BIOLOG databases.

The phylogenetic tree generated by PHYLIP (Figure 3.1) shows M155 lying within a subgroup containing *Rahnella* spp. and *Yersinia* spp. with M1SA1 and M1RB2 forming a distinct cluster separated from *Aranicola proteolyticus* and *Serratia ficaria.* Given the separation of these bacteria from other members of the Enterobacteriaceae as determined by this molecular study, it is not surprising that the attempt to identify them by commercial kits or *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984) was unsuccessful. It would appear, given the results of the molecular and traditional taxonomic studies, that M155, M1SA1 and M1RB2 are members of novel species and perhaps genera. Further

investigation with techniques such as DNA-DNA hybridisation studies (J. Bowman, pers. comm.) would be required to conclusively prove their uniqueness within the Enterobacteriaceae and for the naming of these potentially new species.

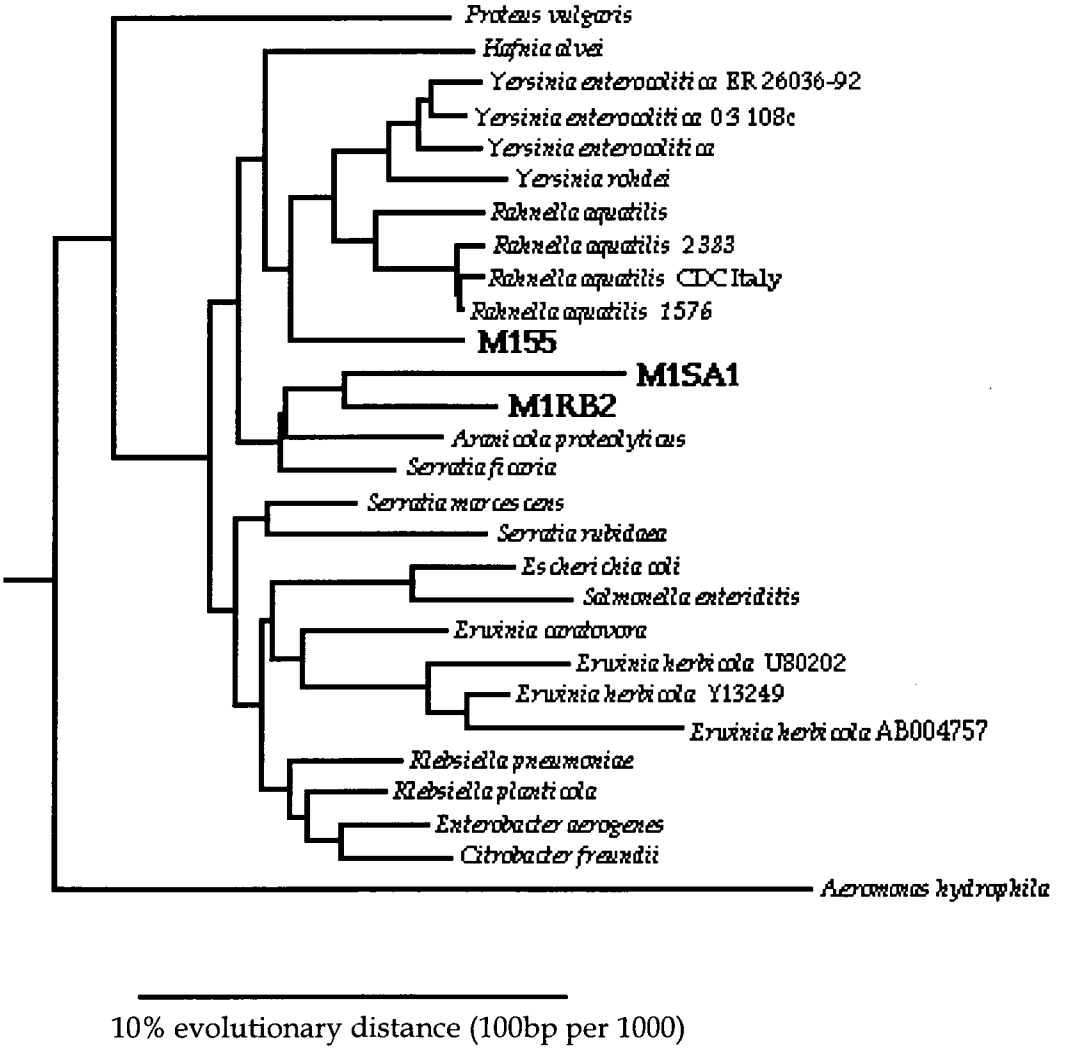


Figure 3.1: Phylogenetic relationships between selected rhizosphere bacterium and other selected eubacteria

Chapter 4

Preliminary Studies:

the shelf life & application of bacterial formulations, interactions with Sclerotium cepivorum

&

preparation of M6SA1 antibodies

INTRODUCTION

The isolation of an organism with suitable *in vitro* characteristics for potential plant health promotion is often the easiest part of the development process. Five organisms described in the preceding chapters were selected for further study based on their *in vitro* characteristics. These characteristics include fast growth at 10°C, differing modes of suppressing fungal growth and tolerance of high levels of commercial fungicides. The following chapter establishes the preliminary issues related to the interaction of the bacteria with the host plant including delivery of the bacteria to the rhizosphere, the predicted colonisation ability of one particular isolate and development of tools for recovering isolates from the rhizophere during soil-plant system studies. The effects of two bacteria on the host plant *in vitro* with and without pathogen pressures from *S. cepivorum* are also investigated.

Formulations of PGPR additives affect many aspects of the success of these introduced organisms in the rhizosphere, including proliferation and survival of the organism and effectiveness of plant health promotion. Factors relating to product storage and use such as safety of handling, ease of preparation and application and economics of commercial use are also affected by formulations. Reviews by Fravel, et al. (1998), Lumsden and Lewis (1989) and Paau (1998) discuss these issues in further detail. The choice of formulation depends on the target site, the bacterium and host and the possibility of integrating the formulation into current pest management and cropping practices (Fravel, et al., 1998). Four formulation types were chosen in this study: a simple seed dressing, seed dressing with a xanthan gum additive as an adherent, a drench consisting of the bacterium suspended in phosphate buffer and a powder formulation consisting of bacteria in a xanthan gum-talcum powder matrix. Samples of the powder and the two seed dressings were taken to determine the relative decline in bacterial populations over nine months at 4°C. The drench was not included in this study as it was felt that that a drench would not have a particularly long or practical shelf life. *In vitro* testing of the effect of the various formulations on

seed germination were also performed in sand culture under controlled conditions.

The results of the *in vitro* trials led to a further examination of M1RB2 and it's interaction with the host and the pathogen of choice, *S. cepivorum*, in an axenic sand culture system. This bacterium was also used to determine the theoretical number of sites available for bacterial colonisation on the plant root by an adsorption assay modified from (Zamudio and Bastarrachea, 1994). Root colonisation is the process by which introduced bacterium survive and multiply in the spermosphere, attach to the root surface and colonise the developing root system (Mahaffee and Backman, 1993). This is an important factor in plant health promotion because of the fundamental requirement of the bacteria to be present for the benefit of the host.

The final part of this chapter focuses on the initial development of a serological assay to detect the test bacterium in the root system of plants from the 1997 shadehouse trial (Chapter 5). A polyclonal antibody (PAB) was developed for use in indirect enzyme linked immunological assays (ELISA). Antibacterial PABs have several advantages over other tracking systems such as genetic modification and antibiotic resistance. These include

- the avoidance of culturing for quantification and detection which can prove to be unreliable (Leff and Leff, 1996)
- genetic purity of the test organisms especially as introduced genes may not operate under certain environmental stresses such as anaerobiosis (Lindow, 1995) and
- the ability to detect non viable cells (Chantler and McIllmurray, 1987).

The main disadvantage of PABs is their lack of specificity and the inconsistency in quality across batches. Monoclonal antibodies are superior to PABs in these respects but their production is more difficult thereby making their use in routine screening of environmental bacterium less common (Chantler and McIllmurray, 1987).

METHODS AND MATERIALS

Formulations

Five bacteria were trialled for their longevity and effect on onion seedling germination when incorporated into four different formulations: a simple seed dressing with no adjuvants, seed dressing with xanthan gum as an adherent, a drench consisting of the bacterium suspended in phosphate buffer and a powder formulation consisting of bacteria in a xanthan gum-talcum powder matrix. All materials used in the bacterial treatments except seed and talcum powder were autoclaved at 121° for 15 minutes before use.

Drench

Bacterial growth from TSA was harvested into sterile phosphate buffer pH 7.0 (Cruickshank, et al., 1975) after 24 hours at 25°C. The bacterial suspension was then adjusted to 30% transmission (approx $1x10^{11}$ CFU ml^{-1}) at 540nm using a Bausch and Lomb spectrophotometer. This same suspension was also used in seed coating and powder production.

Seed dressings

One gram of onion seed cv Vecon Regular Creamgold was added to bottles containing 10 ml of bacterial suspension (30% transmission at 540nm,approx $1x10^{11}$ CFU ml^{-1}). The bottles were then shaken at 100 rpm for an hour before the seeds were removed and placed in open sterile petri dishes for overnight drying in the laminar flow cabinet. The xanthan gum seed dressing followed the same general method but 5 ml of a 2% (w/v) solution of xanthan gum was mixed into the bacterial suspension prior to seed treatment.

Powder (after Kloepper & Schroth 1981b)

Equal volumes of bacterial suspension and 20% xanthan gum solution (w/v) were combined in sterile foil trays then a volume of talcum powder (Johnson & Johnson) equivalent to 5 times the combined volume of the bacteria- xanthan gum mixture was added. Each tray was dried over night in a laminar flow cabinet. The powder mixtures were ground with a flame-alcohol sterilized mortar and pestle to produce a finer grained powder.

Controls

All controls used the appropriate volume of phosphate buffer instead of the bacterial suspension. Subsamples of each formulation were aseptically transferred to sterile containers. These were stored in a dark refrigerator at 4°C until required for longevity studies.

Longevity studies

One gram samples of powder and 10 seeds of each dressing type were taken at the time of production then at subsequent 3 month intervals. The samples were emulsified in 9 ml 0.85% (w/v) sterile saline and the appropriate saline dilution series made. Then 100µl of the dilution series was plated on to TSA (Appendix I) with counts performed visually after 24 hours at 25°C.

Effect of bacteria & formulation on germination

Two bacteria, M1RB2 and M6SA1 were used in a controlled environment study to determine their effect on seedling germination. Each bacterium was applied in all four of the formulations with five replicates of each treatment. All seeds used were surfaced sterilised by a 1 min soak in 70% ethanol followed by 10 min in 1.6% NaOCl then three washes in sterile distilled H_2O.

Each experimental unit consisted of a 70 mm^3 Nalgene tissue culture container filled with washed beach sand until it weighed 400 g. The sand had previously been passed through a 0.5-1 mm nested sieve and rinsed twice with tap water before being soaked in modified Long and Ashton nutrient solution (Meidner, 1984) (Appendix I) and air-dried for two days. The filled jars were then autoclaved for 20 min at 121°C and left to cool.

Each container was planted with 6 onion seeds from the same batch as the pot trial and then amended with 7.5 ml of drench or 0.5 g powder. The jars were then transferred to a growth cabinet set at 10°C with an 18 hour daylength. Daylight was approximated with a mixture of 640 watts of fluorescent lighting and 400 W of incandescent lighting. Emergence was recorded after 42 days. The raw germination data was transformed to a log count variable for normality then analysed for variance by Bruce McCorkell of the Tasmanian Department of Primary Industries, Water and Environment at New Town, Hobart

Adsorption assay for colonisation potential

An estimate of the theoretical number of sites available for bacterial colonisation on the plant root was made by an adsorption assay modified from Zamudio and Bastarrachia (1994). Onion seedlings were grown in axenic sand culture for 28 days on a laboratory bench at ambient temperature. The plants were then removed from the soil and the roots washed three times in sterile water.

Two seedlings were dipped for 60 seconds in 2 ml of one of a range of concentrations of M1RB2 ($1x10^4$- $1x10^8$ cfu/ml in sterile 0.9% saline). Excess bacterial suspension was removed from each seedling by transferring them in 9 ml of 0.9% (w/v) sterile saline and vortexing for 10 sec. After a further two washes, the root sample was weighed then ground in a sterilised mortar with 9 ml of sterile 0.9% saline. Appropriate serial dilutions of each sample were made then a 100 µl sample of each was spread plated on to two TSA plates (Appendix

I). Counts were made after 24 hours at 25°C with numbers being rounded to the closest whole exponential number i.e $1x10^4$ or $1x10^5$.

Interaction of M1RB2 with *Sclerotium cepivorum*

Two 28 day old seedlings were dipped in a $1x10^7$ suspension of M1RB2. Another two seedlings were dipped in sterile saline as a control. Each seedling was transferred to a Petri plate filled with sterile damp sand. A 5 mm^2 plaque of a 14 day,10°C culture of *S.cepivorum* was placed 15 mm away from the seedling. The plates were then sealed with Parafilm and incubated at 10°C with constant light. Observations of the seedling and fungal growth were made after 14 days.

Preparation and purification of anti-M6SA1 antibodies

Preparation of bacteria for the immunisation series

Isolate M6SA1 was prepared as an immunological agent using the method of Gerencser (1979). Colonies of M6SA1 were harvested after 24-48 hours growth on TSA and emulsified in 2-3 ml of sterile phosphate buffer (pH 7.2) (Appendix I). The suspension was aseptically transferred to a sterile 50 ml tube and the cells were collected by centrifugation at 4000 rpm for 30 min.

The resultant pellet was washed 3 times with buffer to remove any antigens present in the original medium. Washes involved centrifuging the suspension at 4000 rpm for 30-60 min, removal of the supernatant and addition of fresh buffer. Washed cells were resuspended in buffer and 5% formaldehyde (v/w) added prior to incubation for one hour at room temperature. Cells were resuspended to a concentration of $1x10^{10}$ (approx 25% trans at 540 nm) and formaldehyde added to a final concentration of 0.25% (w/v).

Aliquots of the bacterial suspension were mixed with an equal volume of the appropriate Freund's complete or incomplete adjuvant (Sigma Aldrich Chemical Co.). The two liquids were mixed by aspiration with a 19 gauge syringe until a

droplet of the mixture remained cohesive when added to water. Individual doses were stored at -80°C and thawed a few hours prior to injection.

Preparation of antibacterial antibodies

Two New Zealand White crossbred rabbits were immunised with formaldehyde-killed cells of bacterium M6SA1. On day one, the rabbits were injected subcutaneously with 0.5ml of the bacterium combined with an equal volume of Freund's complete adjuvant (Sigma Chem Co.). This was followed on day 26 with 0.5 ml of bacterium-incomplete Freund's adjuvant (Sigma Chem Co.) mixture administered by intramuscular injection to the thigh. On day 44, approximately 10 ml of blood was taken from the marginal ear vein of each rabbit and collected into sterile silicon coated tubes.

Purification of χ globulins by ammonium sulphate precipitation & anion exchange chromatography.

Blood samples from each rabbit were left at room temperature for 30 min to clot then the serum was decanted into clean sterile centrifuge tubes. The serum was centrifuged three times for 5 min at 2000 rpm with the resultant pellet discarded each time to remove any remaining cellular material.

A 1.0 ml aliquot of serum was diluted with 9 ml water and then 10 ml of saturated ammonium sulphate (80 g per 100 ml distilled water) was added dropwise whilst stirring. Stirring was continued for a further 30-60 mins at room temperature. The precipitate was collected by centrifugation at 8000 g for 10 min then resuspended in 2.0 ml of 1/2x phosphate buffered saline (PBS) (Appendix IV).

The crude antibody/protein mixture was dialysed against 500 ml of 1/2x PBS at 4°C for 1 hour then transferred to fresh PBS and dialysed for a further 3 hours. The transfer was repeated and dialysis continued overnight. The dialysis

procedure was considered complete when a 2.0 ml sample from the dialysis buffer failed to form a precipitate with 1.0 ml of 2% barium chloride.

The dialysed antibody/proteins were filtered through a 6 ml column of CM Affi-gel Blue (Biorad) pre-equilibrated as per the manufacturer's instructions. The globulins were eluted with bed volumes of 1/2x PBS and adjusted to an optical density of 1.4 at 280 nm (approx 1.0 mg/ml). Sodium azide at 0.02% (w/v) was added as a preservative before storage in silicone coated bottles at 4°C.

Affinity with M6SA1 & cross-reaction with other Enterobactericeae

The purified antibodies were tested for specificity to M6SA1 by indirect double antibody sandwich ELISA. *Pseudomonas fragi* NCBM 8542 provided by Karina Neumeyer (Dept of Agricultural Science, University of Tasmania) was used as a negative control. Appropriate bacterial dilutions (1/10-1/1000 of one plate of a 24 hour, 25°C bacterial culture on TSA emulsified in 50 ml of buffer) were prepared in carbonate coating buffer (Appendix IV) and 100 µl added to each test well via an eight channel multipipetter. Buffer wells were inoculated with 200µl of carbonate buffer then the plates were incubated either at 4 hours at 37°C or overnight at 4°C.

After incubation, wells were washed twice by soaking with 200µl washing buffer (Appendix IV) for 10 min then rinsed thoroughly with tap water. The antibacterial rabbit antibody was diluted in extraction buffer (Appendix IV) to a range of concentrations (1/100 - 1/2000) and 100 µl added to each well. Plates were then incubated for 4 hours at 37°C or overnight at 4°C.

Plates were washed as described previously then 100 µl of goat or sheep antirabbit alkaline phosphatase conjugated antibody (Sigma Chem Co.) diluted in extraction buffer (1/1000 for goat or 1/2000 for sheep) was added to each well before further incubation for 4 hours at 37°C or overnight at 4°C.

After the completion of the conjugated antibody incubation, plates were washed and 100µl of substrate solution (Appendix IV) added to each well. Plates were incubated at room temperature until a distinct yellow colour developed. The extent of colour development in each well was analysed with Genesis v 1.79 software (Life Sciences UK Ltd) via a 405 nm filter in a Labsystems Multiskan RC plate reader.

Cross reaction of the antibodies with four identified members of the Enterobacteriaceae provided by Dr. Christian Garland (Aquahealth, University of Tasmania)(*Citrobacter freundii* ACM 2917, *Enterobacter aerogenes* ATTC 13048, UQM 1976 and *Escherichia coli* ATCC 25922, UQM 1901) and four unidentified Enterobacteriaceae isolates from onion roots was tested and analysed by the same method.

RESULTS

Longevity studies

Tables 4.1a–c show a general decline in bacterial CFU per sample unit over the sampling period. The control series of the powder and the drench had no detectable colonies at the 10^2 level. The $CFUg^{-1}$ counts of the two seed dressings were adjusted to allow for the original levels of bacterium present on the seeds using the control as a benchmark. All five bacteria tested had a CFU count higher than 50% of their original populations after seven months storage as a powder formulation. Only two bacteria had performed similarly when formulated as plain seed dressings with four performing well in the xanthan gum seed dressing.

M4SAX and M6SA1 generally had higher population percentages than the other three bacteria in any given formulation. In particular, M4SAX declined slowly in the xanthan gum formulation. M155 did not appear to survive well in seed dressing formulations and did not compare well to the other bacteria after four

months storage as a powder. These results were not compared statistically due to the data being generated from an unreplicated sample. The lack of replication was due to the small amount of sample available from the original batch of inoculants.

Tables 4.1a-c Bacterial counts detectable from formulation samples at given time intervals in storage at 4°C

a. Xanthan gum seed dressing (sampled from 10 seeds)

BACTERIUM	MONTH (% OF ORIGINAL CFU COUNT)		
	October	February	May
M155	100	0*	0*
M1RB2	100	62	49
M1SA1	100	66	54
M4SAX	100	92	88
M6SA1	100	66	64

b. Seed dressing (sampled from 10 seeds)

BACTERIUM	MONTH (% OF ORIGINAL CFU COUNT)		
	October	February	May
M155	100	55	0*
M1RB2	100	0*	0*
M1SA1	100	0*	0*
M4SAX	100	81	53
M6SA1	100	74	59

c. Powder (sampled from 1 g)

BACTERIUM	MONTH (% OF ORIGINAL CFU COUNT)		
	October	February	May
M155	100	63	56
M1RB2	100	74	61
M1SA1	100	71	57
M4SAX	100	87	69
M6SA1	100	81	74

indicates the bacterium was not detected at 10^2 on TSA

Effect of the bacterium on the host plant

Interaction with plant and pathogen

After 14 days, the uninoculated controls were infected with *S. cepivorum* whereas the seedlings treated with bacteria were not infected. However the plants inoculated with M1RB2 were withered and chlorotic.

Germination testing

M1RB2 had a significant effect on the mean germination rate in comparison to the control but this was not significantly different to M6SA1 ($\alpha = 0.05$) (Table 4.2). However, many of the seedlings treated with M1RB2 exhibited chlorosis and necrosis after successful germination and emergence.

Table 4.2: Effect of bacterium on onion seedling germination regardless of formulation

BACTERIUM	GERMINATION MEAN *(LOG COUNT)*
M1RB2	0.867 a
M6SA1	0.730 ab
Control	0.473 b

**Means followed by the same letter are not significantly different (pr.<0.05)*

Drench increased the germination mean above that of any other formulation across bacterium and control treatments ($\alpha = 0.05$) (Table 4.3). Powder had a lesser effect but was still significantly greater than the two seed dressing types.

Table 4.3: Effect of formulation on onion seed germination regardless of bacterium

FORMULATION	MEAN
Drench	1.599 a
Powder	0.673 b
Xanthan Gum	0.350 c
Seed dressing	0.139 c

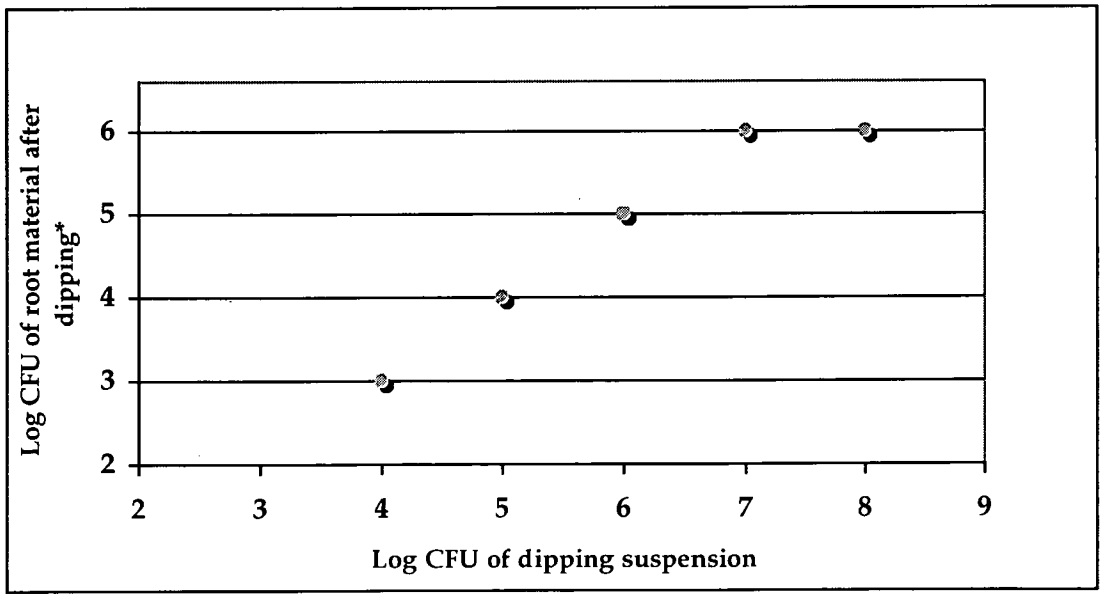
**Means followed by the same letter are not significantly different (pr.<0.05)*

Establishment and recovery of the bacterium in the rhizosphere

Adsorption assay for colonisation potential

The number of bacteria adhering to the roots was approximately one log count less than the suspension applied until a threshold of 10^6 was reached (Figure 4.1). This limit was assumed to be the maximum number of adhesion sites available on the sterile root.

Figure 4.1: M1RB2 adhering to aseptically grown onion roots (log CFU g^{-1})



** data from two replicates with identical log values*

Preparation of immunological tracking techniques using M6SA1

The antibodies displayed high affinity for the bacterium with colour development within 10 minutes. Colour development within the wells inoculated with the negative control, *Pseudomonas fragi*, was equivalent to readings from the uninoculated wells when the conjugated antibody was diluted lower than 1/100 (0.070-0.085 for *P. fragi*, 0.053-0.083 for the blank wells).

The antibody cross-reacted with all the Enterobacteriaceae tested. The known bacterial strains had less affinity at low concentrations of the antibody than M6SA1 or the other onion root strains (0.769-1.372 for the known Enterobacteriaceae, 2.575-3.319 for the onion rhizobacteria and 2.613- 3.126 for M6SA1). The onion root strains showed similar affinity to M6SA1 but may be closely related so cross reaction was not unexpected. Further testing of all bacteria with lower dilutions of goat antirabbit IgG (1/2000-1/20 000) had colour development for M6SA1 at 1/2000 after 60 mins. The 1/5000 dilution showed some reaction after twenty-four hours.

DISCUSSION

These preliminary studies suggest that four of the bacteria studied can survive for seven months at 4°C when formulated as seed coatings or as a powder. The fifth bacterium, M155, does not survive well as a seed coating but is more stable as a powder. Based on the single point longevity observations, the bacterial populations decline at a slower rate in a xanthan gum-talcum powder formulation. This may be linked to the apparent promotive effect of the xanthan gum when added to the seed coating. Xanthan gum is known to play a key role in the survival of the *Xanthomonas* spp. which produce it. Kloepper and Schroth (1981b) postulated that this polysaccharide coats and protects rhizobacteria from environmental stresses such as dessication.

The talcum powder formulation also had a stimulatory effect on plant growth when taken as a discrete factor in the axenic germination trials. The gum matrix may have sequestered moisture or nutrients for the growing seed. There was no significant interaction between the formulations and the bacterium.

The two bacteria tested, M1RB2 and M6SA1 gave the seeds some advantage in germination ability under axenic conditions when compared to the control. Emergence enhancement activity by PGPR strains has been reported previously but the actual mechanisms are unclear (Kloepper, 1993; Mahaffee and Kloepper, 1994). This increase in early vigour may help the young plant to combat emergence disease such as damping off caused by *Phytophthora* and *Pythium* spp.

M1RB2 protected the seedling against infection by *Sclerotium cepivorum* in the sterile sand plate set experiment. However, this bacterium may also be pathogenic or produce phytotoxic substances under sterile conditions as withering and chlorosis of plants treated with M1RB2 was observed in both the pathogen and germination experiments. Maurhofer, et al. (1992) reported that a overproduction of the antibiotic from a *Pseudomonas fluorescens* strain with the ability to suppress *Pythium ultimum* on cucumber, cress and sweet corn causes damage to the host. Since M1RB2 was shown to have the highest level of antibiotic production in *in vitro* testing, a similar effect may be taking place. This may not be a problem in the field due to the adsorption of excess antibiotic on soil particles or less aggressive colonisation by this bacterium under non-sterile conditions.

The adsorption assay showed that the number of bacteria adhering to the root plateaus at approximately 10^6 regardless of the concentration of bacteria in the dipping solution. Zamudio and Basterrachea (1994) also observed this binding saturation effect and suggested that a specific number of binding sites may exist on either the plant or the bacterial cells. Adhesion of the bacterium to the root is

an early stage in the process of root colonisation which may in turn influence how well the bacterium can promote plant health.

The polyclonal antibodies raised against M6SA1 showed high affinity for the bacterium and some cross-reaction with other enterobacterial strains. Such a cross-reaction could be expected as the family Enterobacteriaceae are defined in part by a common antigen (Krieg and Holt, 1984). Increased antibody specificity to M6SA1 could be obtained by cross adsorbtion with other common soil enterobacteriaceae such as Serratia spp. However, use of the goat antirabbit IgG diluted to 1/2000 during the ELISA procedure appeared to separate M6SA1 from related strains for the purposes of the rhizosphere sample assays outlined in Chapter Five.

Chapter 5
Glasshouse Trials
&
Rhizosphere Ecology Studies
of
Selected Bacterial Strains

INTRODUCTION

As mentioned in the introduction to Chapter Four, formulations can affect the success of introduced organisms and determine the effectiveness of plant health promotion. The powder formulation developed in Chapter Four performed well under axenic conditions but may not have the same performance rating in non-sterile soil. To test this theory, the axenic trial conducted in Chapter Four was reproduced in a glasshouse trial under simulated Tasmanian winter conditions during the summer of 1996.

Based on the results of the 1996 and the *in vitro* trials, the isolates M6SA1 and M1RB2 were assessed for plant growth promotion in a shadehouse pot trial conducted in the winter and spring of 1997. The two bacteria were applied as powder formulations to two different soils that were infested with *S. cepivorum*. This was to determine whether these bacteria could be effective in non-native conditions with a disease pressure present. This trial also examined the *in situ* compatibility of the bacteria with commercial seed dressings.

The rhizosphere population of the 1997 pot trials was examined to assess the impact of introducing the PGPR agents to the soil. Root samples were assayed for their aerobic bacterial population composition using one general and two selective media. As Pseudomonaceae are common to the rhizosphere and both introduced bacteria are members of the Enterobacteriaceae, King's B agar,which is semi-selective/differential for fluorescent pseudomonads, and Eosin Methylene Blue agar (EMBA),which selects for Enterobacteriaceae, were chosen for population assays (Bridson, 1990).

Long term survival and proliferation of introduced plant growth promotants is important for season-long benefits to the host plant. The ELISA detection system outlined in Chapter 3 was used to determine whether M6SA1-like bacteria were present after a growing season and whether they were present in higher proportions in the augmented pots when compared to the controls.

METHODS & MATERIALS

1996: Effect of five bacterial strains applied as four formulations on plant growth

Pot preparation

A red ferrosol known to be infested with *S. cepivorum* at a rate of approximately 200 sclerotia per kg was collected from Addison's farm at Moriarty, North West Tasmania, air dried and passed through a 4 mm sieve. A 1:1 (w/w) mixture of field and laboratory produced sclerotia (as described below) was added at a rate of 0.1g sclerotia/kg soil. Rigid plastic pots with a 10 cm diameter were filled with 400 grams of the amended soil then sown with four onion seeds (cv. Vecon Regular Creamgold). This seed had not been treated with fungicide and was supplied by Dr. J.J.D Dennis of Field Fresh Tasmania Pty. Ltd. Seeds were covered with 1 cm of soil after the appropriate treatment was added.

Sources of Sclerotium cepivorum

Laboratory batch culture

Mycelia from cultures of *S.cepivorum* SC4 were transferred to 500 ml flasks containing autoclaved onion scales (15 min at 121°C). After three weeks incubation at room temperature, sclerotia were harvested from the flasks by wet sieving. The damp sclerotia were air dried in a laminar flow cabinet before storage in a darkened area. Sclerotia were also produced on onion agar plates (Appendix I) and collected by brushing the agar surface.

Field collection

Field sclerotia were harvested from infected onions from several sites in North West Tasmania including Addison's Farm at Moriarty and the production line of

Perfecta Produce. These were recovered by scraping the sclerotial masses off infected bulbs or by wet sieving of rotted onion tissues.

Bacterial treatments

Each bacterium was formulated by the four methods trialled and described in Chapter 4 namely as a powder, a drench and as a seed dressing with and without xanthan gum as an adjuvant. Powder was applied at a rate of 1 g/pot at sowing depth and the drench was applied to the soil surface at a rate of 20 ml/per pot. Treated seeds were added directly to soil. Uninoculated seed and unamended powder were used as control treatments.

Experimental design & data collection

Twenty replicates of each treatment were arrayed in a completely randomised layout within an air-conditioned glasshouse maintained at 10°C (+/- 5°C). This gave a total of 400 pots (5 bacteria x 4 formulations x 20 replicates). The pots were watered daily. Emergence was measured at five weeks and 18 weeks. Plants were harvested after 24 weeks and the wet weight of the shoot system was recorded. The aerial parts were dried for 14 days in a 70°C oven before dry matter weights were recorded.

Analytical methods

Germination results were analysed for variance with two-way replication using Microsoft Excel (Microsoft Corp 1983-1997). Prior to analysis, the raw data was transformed using arcsine square root to equilibrate the weighting of all observations (Steel and Torrie, 1980).

Harvest results were analysed by Dr. David Ratkowsky of the University of Tasmania's School of Agricultural Science using the SAS General Linear Models Procedure. The data sets were analysed with an unbalanced model using only data from the emerged plants (N = 444) and as a balanced model with zero

values for the unemerged plants (N=480). Tests generating least significance criteria for each variable were analysed at the $\alpha = 0.05$ probability level.

1997: Effect of two powder-formulated bacterial strains and an optional fungicide seed dressing on plant growth

Pot preparation

The two soils used for this trial were North West Tasmanian red ferrosols known to be infested with *S.cepivorum* but differing in organic matter content and sclerotial loading. The soil collected from Addison's farm at Moriarty has a long history of onion cropping and a relatively low organic matter content with approximately 200 sclerotia per kg whereas the soil collected from Perfecta Produce has a relatively high organic matter content with approximately 50 sclerotia per kg (D. A. Metcalf, pers. comm.). The soil was air-dried before use in pots.

Each experimental unit consisted of a black plastic bag filled with 5 kg of natural untreated soil with an overlying 4 kg layer of soil which had been amended with 1 g of a 1:1 (w/w) mixture of field and laboratory produced sclerotia. Twenty onion seeds cv Vecon Regular Cream Gold were planted in each bag with the appropriate fungicide.

Bacterial & fungicide treatments

Each bacterium was formulated as a powder by the method described in Chapter Four. Powder was applied at a rate of 6 g/pot at a sowing depth of 1 cm. Seeds with fungicide had a commercial standard benomyl-thiram coating consisting of 2g of Benomyl and 5 g of Thiram per kg of seed. Untreated seed and unamended powder were used as control treatments.

Experimental design & sampling

Ten replicates of each treatment were placed in a shadehouse in a blocked randomised layout. This gave a total of 240 pots (3 bacteria x 2 formulations x 2 fungicide treatments x 2 soils x 10 replicates). The pots were watered for 15 min each day from an overhead sprinkling system. Plants were harvested after 24 weeks and the total fresh biomass of the shoot system produced by each pot recorded. Root samples were taken at 24 weeks for the rhizosphere population study.

Analytical methods

Biomass results were analysed by Dr. David Ratkowsky of the University of Tasmania's School of Agricultural Science using the SAS General Linear Models Procedure. T Tests generating least significance criteria for each variable were analysed at the $\alpha = 0.05$ probability level.

Survey of rhizosphere population composition using selective media

Root samples were taken from two pots of each treatment. Each sample was rinsed with 9 ml of sterile saline to remove excess soil. The root material was then shaken to remove excess solution and then transferred to a petri dish for weighing. After weights were recorded, the samples were transferred to a fresh 9 ml volume of sterile saline, vortexed for 30 sec and then tenfold dilution series made. 50 ul of each sample was plated using a Spiral Biotech Autoplate 4000 spiral plater (Spiral Biotech Inc. Bethesda USA) on TSA, King's B and EMB to survey for total non- fastidious aerobic microorganisms, fluorescent pseudomonads and enterobacteriaceae respectively. Plates were then incubated at 25°C for 24 hours. Colony forming unit (CFU/g) counts were done and the resultant data analysed for variance by Bruce McCorkell of the Dept of Primary Industries, Water and Environment, New Town Tasmania. The raw data was normalised by a square root transformation prior to analysis.

ELISA Detection of M6SA1

Bacteria representing the dominant morphotypes on the plates generated from the pots from the control and M6SA1 series were tested for their reaction to the antibody raised against M6SA1. Each sample was tested in duplicate using the ELISA protocol outlined in Chapter 4 with a modified washing step following the criscross plate layout designed by Darby Munro (Dept of Primary Industries, Water and Environment, New Town, Tasmania) which minimises the effect of edge well bias.

Results for each bacterium were analysed using Microsoft Excel (Microsoft Corp. 1983-1997) with a presumptive positive identification as M6SA1 or a closely related bacterium determined by the relative magnitude of the absorbance peak when related to a set of controls. The controls consisted of M6SA1 in neat (a plate of a 24 hour, 25°C culture on TSA emulsified in 20 ml of carbonate buffer), 1/10 and 1/100 concentrations as a positive, *Klebsiella oxytoca* provided by Lyndal Mellefont, University of Tasmania, as a negative and an uninoculated well as an indication of background absorbance.

RESULTS

1996: Effect of five bacterial strains applied as four formulations on plant growth

Effects of experimental design and environment

There was very little indication of infection with *S. cepivorum* of any of the seedlings during the duration of the trial. This may have been a result of record maximum summer temperatures in Hobart during January 1997 that overcame the glasshouse cooling system for a period of three weeks approximately 12 weeks after sowing. These external temperatures forced temperatures within the glasshouse above the 10°C (+/-5°C) stated in the experimental design to approximately 45°C with a corresponding cracking and drying of the soil in pots.

Effects of bacterial formulation on plant emergence

The ANOVA (Appendix V) shows that only formulation had a significant effect on seeding emergence on either of observation dates. Powder treatments, when analysed across bacterial treatments, had the highest emergence scores on both dates although they were not significantly different from the drench after 18 weeks. The seed dressing compared favourably with the drench at five weeks but had a decline in germination percentage after 18 weeks. The xanthan gum seed dressing had the lowest emergence rate on both dates.

Table 5.1: Effect of formulation on germination at 5 & 18 weeks

TREATMENT	GERMINATION RATE AT 5 WEEKS	GERMINATION RATE AT 18 WEEKS
Powder	63.8 a	69.8 a
Drench	57.0 b	67.1 a
Seed Dressing	57.9 b	56.6 b
Xanthan Gum	45.7 c	52.7 b

** Values followed by the same letter are not significantly different from the values in the same column.*
α = 0.05 5 weeks MSE = 29.30853477 DF= 15, LSD = 3.3305, Tcrit = 2.131
18 weeks MSE = 39.33184 DF= 15, LSD =3.858, Tcrit = 2.131

Treatment effects on shoot biomass at harvest

Appendix V gives the summary of the PROC GLM analysis for this trial allowing for Type I and Type III errors for the balanced (n=480) and the unbalanced (n=444) models

Balanced model

Isolate M6SA1 significantly increased (α = 0.05) fresh shoot biomass production in comparison to all other bacteria except M4SAX (Table 5.2) when analysed by the balanced model. A continuum of statistical differences is then seen with

M4SAX not significantly different from M155 but different from the three lowest ranking bacteria. M155, M1SA1 or M1RB2 did not significantly differ from the control.

The dry weight data had only two distinct significance categories with M1RB2 producing a dry mean shoot yield almost double that of the mean of the closest bacterium, M6SA1 (Table 5.3). This result contrasts with the wet weight assessment of this bacterium where it did not vary from the control treatment.

Table 5.2: Effect of bacterium on mean wet weight

BACTERIUM	MEAN WET WEIGHT * (g)
M6SA1	1.6731 a
M4SAX	1.6265 a,b
M155	1.3412 b,c
CONTROL	1.2286 c
M1SA1	1.2001 c
M1RB2	1.1425 c

**Values followed by the same letter are not significantly different from the values in the same column*
α= 0.05 df= 456 MSE= 0.947895 T_{crit} =1.97 LSD = 0.3025 n=480

Table 5.3: Effect of bacterium on mean dry weight

BACTERIUM	MEAN DRY WEIGHT * (g)
M1RB2	0.57040 a
M6SA1	0.29325 b
M4SAX	0.26887 b
M155	0.24336 b
Control	0.23312 b
M1SA1	0.21238 b

** Values followed by the same letter are not significantly different from the values in the same column*
α= 0.05 df= 456 MSE= 0.085445 T_{crit} =1.97 LSD= 0.0908, n=480

Powder formulations produced the greatest biomass in both wet and dry weight assessments (Tables 5.4 & 5.5). The drench treatment wet weights are not significantly different from the powder treatments but were approximately half those of the powder treatment when dried. The means of the two seed treatments were not significantly different from each other in either wet or dried

weight assessments but were significantly less than the drench and powder means in both cases.

Table 5.4: Effect of application method on wet weight

APPLICATION METHOD	WET WEIGHT MEAN* (g)
Powder	1.7633 a
Drench	1.5774 a
Xanthan Gum	1.0749 b
Seed dressing	1.0591 b

** Values followed by the same letter are not significantly different from the values in the same column.*
α= 0.05 df= 456 MSE= 0.947895 T_{crit} = 1.97 LSD= 0.247

Table 5.5: Effect of application method on dry weight

APPLICATION METHOD	DRY WEIGHT MEAN* (g)
Powder	0.54906a
Drench	0.27639b
Seed dressing	0.19496c
Xanthan Gum	0.19385c

** Values followed by the same letter are not significantly different from the values in the same column.*
α= 0.05 df= 456 MSE= 0.085445 T_{crit} = 1.97 LSD= 0.0742 n=480

Unbalanced model

Application of isolate M1RB2 more than doubled dry matter production in comparison to the other treatments (Table 5.6) when analysed by the unbalanced model. However, there was no significant difference between the wet weights of the different bacterial treatments in this scenario. Shoot biomass production was significantly higher in the drench and powder treatments than in either of the two seed dressing types based on wet weight data. (Table 5.6)

Table 5.6: Effect of bacteria on wet weight & dry weight

BACTERIUM	MEAN WET WEIGHT * (g)	MEAN DRY WEIGHT* (g)
M1RB2	1.873 a	0.844 a
M4SAX	2.295 a	0.382 b
M6SA1	2.209 a	0.391 b

Control	2.008 a	0.405 b
M155	1.981 a	0.328 b
M1SA1	1.922 a	0.374 b

* Values followed by the same letter are not significantly different from the values in the same column.
$\alpha = 0.05$ *Wet Weight MSE = 1.905814 DF= 420, LSD = 0.4462,* T_{crit} *=1.97*
Dry Weight MSE = 0.159415, DF = 420, LSD =0.1291, T_{crit} *=1.97*

Table 5.7: Effect of application method on wet weight

APPLICATION METHOD	MEAN* (g)
Drench	2.3502a
Powder	2.2911 a
Seed dressing	1.812 b
Xanthan Gum	1.6734 b

* Values followed by the same letter are not significantly different from the values in the same column.
$\alpha = 0.05$ *MSE = 1.905814 DF= 420, LSD = 0.4462,Tcrit =1.97*

The dry weight data showed that powder treated plants are once more almost twice the weight of shoots from the other three formulations (Table 5.8). The drench treated plants were significantly heavier than those treated with the xanthan gum dressing but not from those treated with a plain bacterial dressing.

Table 5.8: Effect of application on dry weight

APPLICATION METHOD	MEAN* (g)
Powder	0.74323 a
Drench	0.40587 b
Seed dressing	0.32824 b,c
Xanthan Gum	0.29848 c

** Values followed by the same letter are not significantly different from the values in the same column.*
$\alpha = 0.05$ *MSE = 0.159415 DF= 420, LSD = 0.1055, Tcrit =1.97*

1997

Effects of the experimental design and environment

There was no visible development of AWRR despite temperatures during the trial period being conducive to disease development ie. average air temperatures

for Hobart during winter are between 3.1°C and 13.9°C (http://www.bom.gov.au/climate/averages). There was no significant effects related to the experimental layout ie. the blocked trial design (Appendix V). This suggests that pots within the trial blocks were not subjected to significant environmental variation based on their location.

Treatment effects on shoot biomass at harvest

The summary of the PROC GLM analysis for this trial allowing for Type I and Type III errors is given in Appendix V. There were no interactions between the treatment variables (block, bacteria, soil type or fungicide interaction).

However, there was a definite effect of soil type (P = 0.001) with the average total shoot biomass produced from Perfecta produce soil (relatively high organic matter, low sclerotial loading) being over twice that of with soil from the intensively cropped Addison's farm site (Table 5.9). Neither of the two bacteria nor the fungicide seed dressing had significant effects on plant growth compared to the relevant control (Table 5.10 & 5.11).

Table 5.9: Effect of soil type on pot biomass production

SOIL TYPE	MEAN* (g)
Perfecta Produce	44.022 a
Addison's farm	20.417 b

** Values followed by the same letter are not significantly different from the values in the same column*
α=0.05, df=99 MSE = 891.9631, Tcrit =1.98 LSD =10.819

Table 5.10: Effect of bacterium on pot biomass production

BACTERIUM	MEAN* (g)
M1RB2	35.112 a
M6SA1	33.623 a
Control	27.924 a

** Values followed by the same letter are not significantly different from the values in the same column*
α=0.05, df =99 MSE = 891.9631, Tcrit =1.98 LSD =13.251

Table 5.11: Effect of fungicide on pot biomass production

FUNGICIDE	MEAN* (g)
Treated seed	33.381 a
Untreated seed	31.059 a

** Values followed by the same letter are not significantly different from the values in the same column*
α=0.05, df =99 MSE = 891.9631, Tcrit =1.98 LSD =13.251

Survey of rhizosphere population composition using selective media

The ANOVA (Appendix V) shows that fungicide and media type had significant impacts on the number of colony forming units generated from the root samples when analysed regardless of other factors. Roots from the seed treated with fungicide had a higher average bacterial count across all media (Table 5.12) than those from untreated seed.

The selective media had counts that were significantly different from each other (Table 5.13). TSA had the highest count as expected being the least selective medium. The number of colonies on the King's B plates was over twice the number of colonies on the EMB plates (presumed to mostly represent enterobacterial species).

Table 5.12: Effect of fungicide on mean CFUg-1 of bacteria

FUNGICIDE	MEAN*
Yes	1908 a
No	1316 b

** Values followed by the same letter are not significantly different from the values in the same column*
α=0.05, df =1 MSE = 4.387E+05, LSD = 443.0

Table 5.13: Effect of media on mean CFUg^{-1} of bacteria

MEDIA	MEAN
TSA	2357 a
KB	1774 b
EMB	705 c

** Values followed by the same letter are not significantly different from the values in the same column*
α=0.05, df =1 MSE = 4.387E+05, LSD=542.5

ELISA detection of M6SA1

Of the 80 bacteria tested from each treatment series, seven bacteria from the M6SA1 pots were presumptively identified as M6SA1 as their ELISA reaction was equivalent to or higher than that of the 0.18 and 0.127 recorded for the positive controls. Of the same number tested from the control series, only one bacterium registered as positive with a reading above 0.119 & 0.126 as recorded for the controls.

DISCUSSION

Two important issues of the experimental design and analysis must be taken into account when the results of these two trials are examined. Firstly, the lack of disease induction by infection with *S. cepivorum* and, secondly, the handling of zero values. Variation in disease pressure is a common drawback of biological screening programs (Lewis and Papavizas, 1991). It is almost impossible to regulate the amount of pathogen inoculum that will survive in the field under natural conditions or in growth room trials. This was exemplified in both pot

trials where inexplicable failures of disease development occurred despite environmental conditions being favourable for disease development during the majority of the duration of both trials. The failure of the air conditioner approximately 12 weeks after sowing and the subsequent drying out of the soil may have contributed to lack of infection by *S. cepivorum* in several ways. Firstly, high temperatures may have resulted in the thermal death of mature sclerotia with actively growing mycelia prior to infection thereby effectively removing the majority of potential infection events. Secondly, the temperatures may have inhibited plant growth and root development thereby preventing the normal course of disease development in which the infection travels from the root to the root stem and plate at which point plant death occurs. (Crowe and Hall, 1980). A third possible reason for failure of disease development may also explain the lack of infection of the winter trial: the sclerotia present may have remained dormant during the period of the trial and not germinated even when conditions were conducive.

Absence of AWRR in the pot trials also precludes any definite statement about the potential biological control activity of these bacteria as this result neither supports nor disproves their status as biological control agents. Further trials with conditions more suitable for white rot disease would be required to see whether the *in vitro* biological control potential of these isolates is also an *in situ* quality. The impact of sublethal infections which can decrease bulb weight and pre-emergence seedling mortality (Booer, 1945) could have been lessened but these are difficult to quantitively measure and assess. The possible impact of this type of infection on the bacteria's promotive capabilities could be assessed in future experiments comparing growth in soils infested with AWRR to growth in soils from disease free sites.

Pearce (1965) noted the implications of growth/no growth data in biological trials particularly the difference between a missing plot and an observation of zero. These issues were particularly relevant for the 1996 trial where each plant was recorded as a separate data unit and there were many pots with missing

plants or no growth. It was therefore decided to analyse the data in two ways i.e. as an unbalanced model using only data from the emerged plants (N=444) and as a balanced model with zero values for the unemerged plants (N=480). The first model could more accurately reflect a commercial cropping situation as missing plants play no part in tonnages per hectare but the second model is more statistically correct. The discussion of these data sets therefore should be considered in light of these statistical considerations.

M6SA1 and M1RB2 appeared superior to the other isolates in their potential as plant growth promotants in the 1996 trial. In terms of dry biomass production, M1RB2 had a significantly greater mean shoot weight (α=0.05) than any other bacterial isolate under both statistical models. However, this was not reflected in fresh weight assessments under the balanced model. M6SA1 had a significant positive effect (α=0.05) on fresh shoot biomass production when analysed by the balanced model. The significant promotion of seedling emergence by both bacteria seen in the gnotobiotic assay (Chapter 4) was not observed in the pot trial. However, M1RB2 and M6SA1 showed some of the highest emergence rates when trends in the raw data were examined.

The promotive effect demonstrated in the 1996 was not reproduced in the 1997 trial as no significant effect of bacterial treatment on pot biomass was observed. Further trials involving M6SA1 and M1RB2 could be conducted to determine whether this was an isolated incident or a true indication of lack of plant growth promotion under winter conditions.

The effect of formulation on plant biomass production was carried through from the *in vitro* work to the glasshouse trials. Powder, additive irrespective of bacterial amendment, had the consistently highest emergence scores in the 1996 trials and the highest mean of any application in terms of biomass whether fresh or dry. Paau (1998) commented that the wettability of this type of formulation and the gum matrix for retaining moisture may act as a seed primer.

Soil type also had a major effect on plant growth. The average total shoot biomass produced from pots filled with soil from plots at Perfecta produce was over twice that of those filled with soil from the intensively cropped Addison's farm site. This effect could be expected purely because of the better structural and nutritional qualities of soil from the Perfecta site.

It must be noted that all plant growth promotion activity was assessed purely on shoot weights prior to full bulb production in pots. A field trial harvested at commercial cropping stage would show whether the bacteria enhance bulb yield and thereby emphasise their suitability for commercial development.

Changes in the population dynamics of the rhizosphere by exclusion or suppression of deleterious or inert microorganisms is one mechanism by which introduced PGPR enhance plant growth (Kloepper and Schroth, 1981a). The rhizosphere population of the 1997 pot trials was examined by traditional selective media techniques and by immunological methods to determine whether such a shift had occurred. Neither M6SA1 nor M1RB2 had a significant impact on the composition of the microbial populations surveyed on selective media. Molina, et al. (2000) also found that addition of *Pseudomonas putida* strain KT2442 had no significant effect on the numbers of several soil bacteria including those of total fluorescent pseudomonads. Kloepper (1993) argued that introduced bacteria represent taxonomic groups identical to the native populations and therefore reduce the relevance of standard isolation and differentiation techniques to this type of study.

Kloepper's hypothesis can be supported in part by the ELISA results. A shift in microbial population can be seen as levels of M6SA1-like colonies were much higher in the pots to which the bacterium had been introduced than in the control pots. This effect was not observable in the agar plate surveys. However, the small sample size must be considered as this result would be more

statistically robust if a larger sample size were taken. It must also be taken into account that native bacteria may have reacted to the antibodies.

The benlate-thiram seed dressing had a significant impact on the number of bacterial colony forming units generated from the root samples. Roots from the seed treated with fungicide had a higher average bacterial count than those from untreated seed. Harden, et al. (1993) found that biomass was increased in the presence of fungicides but the proportion of bacteria able to mineralise glucose was unaffected. Smith, et al. (2000) found that microbial communities displayed a mixed response to the application of benomyl with bacterial counts either reduced or unaffected.

Improvements could be made in the methods of sampling in further examinations of bacterial populations in this type of trial. The sampling methods used in this study did not allow for the detection of variation in bacterial numbers along the root. Bacteria are generally found in higher numbers 1-3 cm from the root apices and lateral root junctions as these areas are a prime source of many root exudates (Brown, 1975). The distribution and numbers of M6SA1 along the root could be studied in more depth by whole root scanning microscopy using the antibody developed in this study as a immunofluorescent tag for the bacteria on the root surface.

Conclusions

Bacteria isolated from healthy onion roots were capable of suppressing the growth of *Sclerotium cepivorum in vitro* by production of antifungal metabolites, chitinases and by competitive exclusion. These bacteria also showed characteristics that would be important for a commercially viable product such as fungicide tolerance, activity at 10°C and longevity in a powder formulation. The isolate MIRB2 was shown to protect onion seedlings from infection under sterile conditions but demonstrated some phytotoxicity.

All bacteria chosen for further study were members of the Enterobacteriaceae, a family defined as oxidase negative, glucose fermenting Gram-negative rods. Neither of the two commercial kits used nor the traditional agar based protocols outlined in Bergey's Manual could provide a conclusive identification of the bacteria to species. 16sDNA sequencing and phylogenetic analysis was achieved for only three of the five species. The grouping of the bacteria within the phylogenetic tree and the failure of the four identification systems used to elucidate the bacterial identities suggest that the five isolates are members of a novel species grouped within the Enterobacteriaceae. Further studies such as DNA hybridisation and G+C analysis would be necessary to confirm this conclusion (Dr. J. Bowman, pers. comm).

Powder formulations generally had the best shelf life of any formulation of the individual bacteria. Unamended powder alone had significant effects on seedling emergence and plant growth in gnotobiotic and soil conditions. Further work on the powder formulation would include isolation of the plant growth promotive factor in this formulation ie the xanthan gum or the talcum powder and optimisation of the formula.

M6SA1 and M1RB2 promoted onion shoot growth under glasshouse conditions over the 1996-97 Tasmanian summer and slightly increased seedling emergence in the glasshouse and in a growth cabinet. Significant growth promotion was not observed in shadehouse pot trials over the 1997 Tasmanian winter. Trials to

investigate biological control potential for these isolates in conditions more favourable for AWRR development and their effect on whole bulb packout would significantly further this study.

Rhizosphere population dynamics were altered by the addition of a benlate-thiram dressing to the seed. ELISA analysis of the rhizosphere showed an increase in the amount of colonies displaying antibody reactions similar to M6SA1 could be seen in the treatments to which this bacterium had been added although the total CFU per gram of the total population was not significantly affected.

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Appendix I:

General Media

AGARS & BROTHS

All media was autoclaved at 121°C for 15 min unless otherwise indicated.

Trypticase soy agar

Trypticase Soy Broth medium	3	g
Yeast Extract	1	g
Agar	15	g
Distilled Water to final volume	1	l

Trypticase soy broth

Trypticase Soy Broth medium	3	g
Yeast Extract	1	g
Distilled Water to final volume	1	l

An appropriate buffer was substituted for water if a buffered medium was required.

Cryogenic preservation media

Trypticase Soy Broth medium	3	g
Yeast Extract	1	g
Glycerol	300	g
Distilled Water to final volume	1	l

The medium was autoclaved in 10 ml aliquots then transferred to sterile plastic cryogenic tubes.

Antibiotic amended TSA for sclerotial revival.

Penicillin G	50	mg
Polymyxin B sulphate	50	mg
Streptomycin sulphate	50	mg

The antibiotics were added to 1L of cool (40°C) sterile TSA and mixed thoroughly. All antibiotics were sourced from Sigma Chemical Co.

Fungicide amended TSA

	ACTIVE INGREDIENT	CONCENTRATION (% W/V OR V/V)					
FUNGICIDE		0.001	0.01	0.1	1	5	10
Benlate	500g/kg benomyl	0.005 g	0.05 g	0.5 g	5 g	25 g	50 g
Folicur	250g/l tebucanazole	0.01 g	0.1g	1 g	10 g	50 g	100 g
Thiram	800g/kg a i.	3 µl	30 µl	300 µl	3 ml	15 ml	31 ml

Fungicides were added to cooled (approximately 35°C) agar prior to pouring plates to prevent thermodegradation of the active ingredients. Powder formulated fungicides were sterilised prior to use by dissolving them in ethanol before addition to TSA.

Onion agar

Macerated onion bulb scales	50	g
Agar	15	g
Distilled Water to final volume	1	l

This medium was autoclaved at 110°C for 20 min with very loose lids on the bottles in which it was autoclaved.

BUFFERS & DILUENTS

Phosphate buffer (Cruickshank, et al., 1975)

Stock solutions

A.: 0.2 M monobasic sodium phosphate (31.2 g $NaH_2PO_4.2H_2O$ in 1000ml)

B : 0.2 ml dibasic sodium phosphate (71.7 g of $Na_2HPO_4.12H_2O$ in 1000ml)

pH	*x*	*y*
6.0	87.7	12.3
6.6	73.5	26.5
7.0	39.0	61.0
7.6	13.0	87.0
8.0	5.3	94.7

x ml of A was added to *y* ml of B to give the desired pH . The solution was then diluted to a total of 200 ml and the pH checked.

Citrate-phosphate buffer (Cruickshank, et al., 1975)

Stock solutions

A: 0.1M citric acid (19 g in 1000 ml)

B: 0.2M dibasic sodium phosphate (71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml)

pH	x	y
2.6	44.6	5.4
3.0	39.8	10.2
3.4	35.9	14.1
4.0	30.7	19.3
4.4	27.8	22.2
5.0	24.3	25.7
5.4	22.2	27.8
6.0	17.9	32.1
6.4	15.4	34.6
7.0	6.4	43.6

x ml of A was added to *y* ml of B to give the desired pH. The solution was then diluted to a total of 100 ml and the pH checked.

Long & Ashton's nutrient solution (Meidner, 1984)

	Stock solution (g/100 ml)	ml stock/L medium
KNO_3	5.06	8
$Ca(NO_3)_2 .4H_2O$	11.033	8
$MgSO_4.7H2O$	4.60	8
$NaH_2PO_4.2H_2O$	5.20	4
FeK-EDTA	0.330	5
H_3BO_4	0.310	1
$MnSO_4.H_2O$	0.223	1
$ZnSO_4 .7H_2O$	0.029	1
$CuSO_4.5H_2O$	0.025	1
NaCl	0.585	1
$Na_2MoO_4.2H_2O$	0.012	1
CoSO4.7H2O	0.00056	1

Appendix II

Diagnostic & Selective Media

All media was autoclaved at 121°C for 15 mins unless stated otherwise

Basal Mineral Salts (BMS) medium

Macronutrients

KH_2PO_4	1.36	g
Na_2HPO_4	1.42	g
KNO_3	0.5	g
$(NH_4)_2SO_4$	2.38	g
$MgSO_4.7H2O$	0.05	g
$CaCl_2$	0.01	g
Agar	15	g
1.0 ml Micronutrient Solution	1	ml
Distilled water to final volume	1	l

Micronutrient Solution

H_2BO_4	290	mg
$MnSO_4.H_2O$	150	mg
$(NH_4)_2Fe(SO_4)_2.6H2O$	350	mg
$CuSO_4.5H2O$	4	mg
$ZnCl_2$	2	mg
$CoCl_2.H_2O$	4	mg
$Na_2MoO_4.2H_2O$	2.5	mg
Distilled water to final volume	100	ml

Chitin agar

Basal Salts

K_2HPO_4	1.0	g
$MgSO_4.7H_2O$	0.5	g
NaCl	0.5	g
NH_4Cl	1.0	g
$CaCl_2.2H_2O$	0.1	g
$FePO_4.2H_2O$	0.001	g
Agar	15	g
Distilled Water to final volume	1	l

Add colloidal chitin to 500 ml Basal Salts Medium until the media becomes opalescent. Pour half plates of BSM and then overlay with chitin-amended agar.

Colloidal Chitin Preparation

Dissolve about 20 g of chitin in 100 ml conc. HCL until a pourable paste is made. Leave at room temperature for 2-3 hours then pour the slurry into 2-3 litres of water and allow chitin to settle out. Wash chitin with distilled water and centrifuge at 3000 rpm for 10 min. Repeat wash and centrifuge step six times to ensure chitin has been neutralised. Autoclave at 121°C for 15 min.

Christensen's urease agar (Bridson, 1990)

Urea agar base (Oxoid)	2.4	g
Distilled water	95	ml

Autoclave agar base media as usual then cool to 50°C. Prepare a sterile 40% urea solution by passing the solution through a 0.45 μm filter. Add 5 ml of the urea solution to the basal media, mix well and pour plates.

Decarboxylase broth

Moeller Decarboxylase Base (Difco)	10.5	g
L-amino acid	10	g
Distilled water to final volume	1	L

Dispense 3 ml into 5 ml Bijoux bottles prior to autoclaving

Esculin iron agar (Power and McCuen, 1988)

Esculin	1.0	g
Ferric ammonium citrate	0.5	g
Agar	15	g
Distilled water to final volume	1	L

Gelatine testing medium (Smibert and Krieg, 1994)

Trypticase Soy Broth	3	g
Yeast Extract	1	g
Gelatine	120	g
Distilled water to final volume	1	L

Dispense 10 ml volumes of media into 30 ml McCartney bottles and autoclave.

Glucose-phosphate peptone water (Cruickshank, et al., 1975)

Peptone	5	g
K_2HP0_4	5	g
Water	11	ml
10% Glucose solution	50	ml

The peptone-phosphate solution was pH adjusted to 7.6 then autoclaved and cooled prior to addition of the filter sterilised glucose

Hugh & Liefson test media

10% carbohydrate solution	1.25	ml
Peptone	0.25	g
NaCl	0.625	g
K_2HPO_4	0.3	g
Agar	3	g
1% Bromothymol Blue	0.4	ml
Distilled water	125	ml

All ingredients except the carbohydrate were combined, adjusted to pH 7.2 and autoclaved. The carbohydrate solution was passed through a 0.45µm filter prior to addition to the autoclaved media. The media was then dispensed into sterile tubes.

King's medium B (Atlas, 1993)

Proteose peptone No. 3	20	g
Glycerol	10	ml
K_2HPO_4	1.5	g
$MgSO_4.7H_2O$	1.5	g
Agar	15	g
Distilled Water to final volume	1	l

Koser's citrate (pH 6.8) (Cruickshank, et al., 1975)

NaCl	5.0	g
$MgSO_4$	0.2	g
$NH_4H_2PO_4$	1.0	g
KH_2PO_4	5.0	g
Distilled water	11	ml

Dispense 3 ml into 5 ml Bijoux bottles prior to autoclaving

Nitrate test solutions (Lanyi, 1987)

Solution A

Sulphanilic acid	8	g
5N acetic acid		
glacial acetic acid	300	ml
distilled water	700	ml

Dissolve by gentle heat and store at 4°C

Solution B

α-naphthylamine	5	g
5N acetic acid	1	L

Nutrient agar

Lab lemco powder	1	g
Yeast extract	2	g
Bacteriological peptone	5	g
Sodium chloride	5	g
Agar	15	g

Paton's pectate gel medium (modified) (Stead, 1990)

Basal Media

Peptone	5	g
Lab-lemco	5	g
Sodium lactate	7	ml
Agar	12	g
distilled water to final volume	1	L

Adjust to pH 7.2 and autoclave at 121°C for 15 min. Pour as basic plates

Pectate overlayer

Sodium polypectate	2	g
distilled water	100	ml
Na_2 EDTA	0.01	g
Bromothymol blue	trace	

Heat distilled water to 80°C and gradually stir in the polypectate with constant stirring and heat. Add the EDTA and stir at 80°C until the pectate has dissolved. After adding the bromothymol blue, adjust pH to 7.2 and dispense into 5 ml volumes. Autoclave at 115°C for 4 min.

Pour 5 ml of the cold overlayer to a plate containing the solid base. Allow to set for four hours and dry for 12 hours before use.

Skim milk agar

Pancreatic digest of casein	1.5	g
Yeast extract	2.5	g
Glucose	1.0	g
Agar	15	g
Distilled water to final volume	1100	ml

Skim milk solution

Skim milk powder	4	g
Distilled water to final volume	100	ml

Autoclave agar and milk solutions separately. Combine when cool and then plate.

Malonate broth, Ewing modified (Power and McCuen, 1988)

Yeast extract	1.0	g
K_2HPO_4	0.6	g
KH2PO4	0.4	g
$(NH_4)_2SO_4$	2.0	g
NaCl	2.0	g
Sodium malonate	3.0	g
Dextrose	0.25	g
Bromothymol Blue	0.025	g
Distilled water to final volume	1	L

Dispense into 10 ml diluent tubes and autoclave at 121°C for 15mins.

Positive reaction is blue, negative is yellow

Tween agar (Smibert and Krieg, 1994)

Trypticase Soy Broth	3	g
Yeast Extract	1	g
$CaCl2.H_2O$	1	g
Agar	15	g
Distilled Water to final volume	1	l

Sterilise Tween 40, 60 or 80 separately to the basal medium and cool to 45-50°C. Add sterile Tween to the molten agar to give a final concentration of 1% (v/v). Shake until Tween is completely dissolved and pour plates.

Appendix IIIa

Reagents for DNA extraction and purification

DNA EXTRACTION REAGENTS

Saline EDTA

NaCl	8.75	g
Disodium ethylene diamine tetraacetic acid (EDTA)	37.2	g
Distilled water to final volume	1.0	L

Mix and adjust pH to 8.0 with NaOH.

Sodium dodecyl sulphate

Sodium dodecyl sulphate	2.0	g
Distilled water to final volume	1.0	L

Mix and adjust to pH 7.0 with NaOH.

Proteinase K

Proteinase K	10	mg
Tris-EDTA	1.0	ml

Incubate solution at 37°C for 2 hours then heat at 80°C for 2 min. Divide into 0.2 ml aliquots and freeze at -20°C.

Sodium perchlorate

Sodium perchlorate	70.2	g
Distilled water to final volume	100	ml

Store at room temperature.

Chloroform-isoamyl alcohol

Chloroform	240	ml
Isoamyl alcohol	10	ml

Store at 4°C.

Tris EDTA

Tris (hydroxymethyl) methylamine	0.121	g
Disodium EDTA	0.074	g
Distilled water to final volume	1.0	L

Adjust to pH 7.0 with NaOH. Refrigerate.

3M Sodium acetate

Sodium acetate	246	g
Distilled water to final volume	1.0	L

Store at room temperature.

GEL REAGENTS

1 % Agarose gel

Agarose	1	g
Ethidium bromide (10 mg/ml stock)	5	µl
1x TAE buffer to final volume	100	ml

6X loading buffer

Bromophenol blue	0.125	g
Sucrose	20	g
Distilled water	50	ml

1x TAE

Tris base (Tris hydroxymethyl) amino methane	4.84	g
Glacial acetic acid	1.14	ml
0.5M EDTA (pH 8.0)	2	ml
Distilled water to final volume	1	L

0.5M EDTA

EDTA	74.84	g
Distilled water	400	ml

Adjust to pH 8.0 with NaOH

Appendix IIIb

Accession numbers & sequences for Chapter 3

ACCESSION NUMBERS FOR FIGURE 3.1

Aeromonas hydrophila	X60404
Aranicola proteolyticus	U93263
Citrobacter freundii	M59291
Enterobacter aerogenes JCM1235	AB004750
Erwinia caratovora	M59149
Erwinia herbicola	AB004757
Erwinia herbicola	U80202
Erwinia herbicola	Y13249
Escherichia coli	JO1695
Hafnia alvei	Z83203
Klebsiella planticola	X93216
Klebsiella pneumoniae	X87276
Proteus vulgaris	D63398
Rahnella aquatilis	X79938
Rahnella aquatilis CDC 21234	U88435
Rahnella aquatilis CDC 1-576	U88434
Rahnella aquatilis CDC Italy 2383	U88439
Salmonella enteritidis SE22	U90318
Serratia ficaria	AB004745
Serratia marcescens	M59160
Serratia rubidaea JCM1240)	AB004751
Yersinia enterocolitica ER-26036-92	Z49829
Yersinia enterocolitica (strain O:3 108 c)	Z75316
Yersinia enterocolitica	M59292
Yersinia rohdei	X752

TABLE A: PHYLIP CALCULATIONS OF EVOLUTIONARY DISTANCE USED TO CALCULATE FIG. 3.1

Aeromonas hydrophila	0																														
Escherichia coli	0.1107	0																													
Erwinia carotovora	0.1107	0.0465	0																												
Erwinia herbicola 2	0.1226	0.0537	0.0404	0																											
Erwinia herbicola 3	0.1054	0.0442	0.0298	0.0225	0																										
Erwinia herbicola	0.1309	0.0577	0.0527	0.0373	0.0247	0																									
Hafnei alvei	0.1123	0.0634	0.0432	0.0606	0.0577	0.0655	0																								
Proteus vulgaris	0.1108	0.072	0.0631	0.0757	0.0642	0.0823	0.0654	0																							
Serratia marcescens	0.1153	0.0407	0.036	0.049	0.0529	0.0621	0.043	0.0618	0																						
Yersinia enterocolitica	0.0987	0.053	0.0439	0.0597	0.0473	0.0719	0.0393	0.0651	0.0433	0																					
Rahnella aquatilis	0.0921	0.0526	0.0407	0.0654	0.0526	0.0727	0.0441	0.0614	0.0393	0.0227	0																				
Klebsiella pneumoniae	0.1196	0.0398	0.0316	0.0477	0.0441	0.0602	0.0437	0.0593	0.0227	0.0481	0.0483	0																			
Klebsiella planticola	0.1215	0.0405	0.0299	0.038	0.0377	0.0518	0.0341	0.0618	0.0209	0.0394	0.0446	0.0213	0																		
M15A1	0.136	0.0781	0.0579	0.0718	0.0674	0.0882	0.0573	0.0852	0.0551	0.0628	0.0593	0.0587	0.557	0																	
M1RB2	0.116	0.0608	0.048	0.0601	0.0566	0.0771	0.0526	0.0772	0.0418	0.047	0.0491	0.0458	0.045	0.0415	0																
M155	0.106	0.0599	0.0451	0.0638	0.0544	0.0669	0.0372	0.0652	0.0403	0.032	0.0351	0.0453	0.0399	0.0561	0.0353	0															
Enterobacter aerogenes	0.1288	0.0479	0.0363	0.0429	0.0433	0.0622	0.0389	0.0693	0.0295	0.0458	0.0507	0.0206	0.0134	0.0577	0.0503	0.0434	0														
Citrobacter freundii	0.1223	0.0349	0.0334	0.0382	0.0378	0.053	0.0458	0.0707	0.0329	0.0524	0.0552	0.0279	0.0267	0.0653	0.056	0.0517	0.0188	0													
Salmonella enteriditis	0.1141	0.0511	0.0391	0.0539	0.0539	0.0626	0.065	0.0777	0.0431	0.0617	0.0629	0.04	0.0421	0.0807	0.0705	0.071	0.0503	0.0343	0												
Serratia ficaria	0.1141	0.0511	0.0391	0.0539	0.0626	0.1242	0.0632	0.022	0.0448	0.0435	0.0371	0.041	0.0602	0.0544	0.055	0.0463	0.0449	0.0553	0.035	0											
Serratia rubidaea	0.1206	0.0542	0.0481	0.0487	0.0517	0.0682	0.0597	0.0625	0.03	0.0562	0.0519	0.0386	0.041	0.0602	0.0544	0.055	0.0463	0.0449	0.0553	0.035	0										
Yersinia enterocolitica 3	0.0987	0.0527	0.0446	0.0577	0.0444	0.0698	0.0384	0.063	0.0398	0.0083	0.0254	0.0467	0.0373	0.0635	0.0483	0.0323	0.0433	0.0492	0.0624	0.0431	0.0492	0									
Yersinia rohdei	0.104	0.06	0.0476	0.0599	0.0558	0.0753	0.0481	0.0696	0.0413	0.0266	0.0274	0.0496	0.0439	0.0669	0.0552	0.0344	0.0495	0.0538	0.0693	0.0438	0.047	0.0201	0								
Yersinia enterocolitica 2	0.1003	0.0574	0.0467	0.0602	0.0514	0.071	0.0404	0.0631	0.042	0.0111	0.027	0.0494	0.0457	0.0628	0.0471	0.0224	0.0515	0.0532	0.0704	0.0397	0.0504	0.012	0.0162	0							
Rahnella aquatilis 2383	0.1038	0.0595	..0414	0.0615	0.0537	0.0722	0.0425	0.0641	0.0411	0.0359	0.02	0.0526	0.0453	0.0501	0.0438	0.0457	0.0489	0.0531	0.0682	0.0411	0.0457	0.0313	0.0313	0.0356	0						
Aranicola proteolyticus	0.1086	0.0566	0.0427	0.0567	0.051	0.0627	0.0431	0.0618	0.0376	0.041	0.0398	0.0435	0.0399	0.0535	0.0301	0.0332	0.0464	0.049	0.0614	0.0262	0.0515	0.0395	0.0459	0.0383	0.0299	0					
R. aquatilis CDC Italy	0.0988	0.0585	0.0447	0.0635	0.0557	0.0726	0.0436	0.0629	0.0425	0.0359	0.022	0.0513	0.0418	0.051	0.0466	0.0475	0.0477	0.053	0.066	0.0407	0.0461	0.0293	0.0307	0.0375	0.0049	0.0301	0				
Rahnella aquatilis 1576	0.0997	0.0574	0.0405	0.0615	0.0538	0.0698	0.0416	0.0604	0.0403	0.0344	0.0188	0.0493	0.0425	0.0497	0.0427	0.044	0.0478	0.0511	0.0651	0.04	0.0446	0.0295	0.028	0.0341	0.0028	0.0271	0.0027	0			

ALIGNED SEQUENCES FOR PHYLIP

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Appendix IV

ELISA Reagents

Carbonate coating buffer (pH 9.6)

Na_2CO_3	1.59	g
$NaHCO_3$	2.93	g
Distilled water to final volume	1	l

Adjust pH if necessary, then filter sterilise and store aseptically at 4°C. Sodium azide (0.1g/L) may be added as a preservative.

Phosphate buffered saline (PBS) (pH 7.4)

PBS 10x stock	100	ml
Distilled water to final volume	1	l

Adjust pH if necessary, then filter sterilise and store aseptically at 4°C. Sodium azide (0.1 g/l) of may be added as a preservative.

PBS 10x stock (pH 6.7)

NaCl	80.0	g
KH_2PO_4	2.0	g
$Na_2HPO_4.2H_2O$	14.4	g
KCl	2.0	g
Distilled water to final volume	1	l

Check pH after mixing, filter sterilise and store aseptically at room temperature.

Washing buffer

PBS 10x Stock	100.0	ml
Distilled water	900.0	ml
Tween 20	0.5	ml
Skim milk powder	1.0	g

Extraction buffer

PBS 10x Stock	100.0	ml
Polyvinyl pyrrolidine (PVP 40 000)	20	g
Albumin	2	g
Sodium azide	0.1	g
Distilled water to final volume	1	l

Dissolve by vigorously stirring without heating and store at 4°C

Substrate buffer

Diethanolamine $[CH_2(OH)CH_2]_2NH$	97.0	ml
Distilled water	800	ml

Add 5N HCl until pH falls to 9.8 then adjust volume to 1 L with distilled water. Filter sterilise and store aseptically at 4°C.

Substrate solution

Dissolve one 5 mg tablet of p-nitro phenol phosphate (PNPP) (Sigma Aldrich) in 10 ml of substrate buffer.

Appendix V

Statistical analysis summaries

of the

1996 & 1997 pot trials

ANOVA FOR SEED EMERGENCE 1996

VARIABLE (5 WEEKS)	SS	DF	MS	F	P-VALUE	F CRIT
Bacteria	86.88862	5	17.37772332	0.592923647	0.70581492	2.273023
Treatments	743.7001	3	247.900021	8.458287763	0.00158199	2.489788
Error	439.628	15	29.30853477			
Total	1270.217	23				

VARIABLE (18 WEEKS)	SS	DF	MS	F	P-VALUE	F CRIT
Bacteria	124.6928	5	24.93856	0.634055	0.677046	2.273023
Treatment	883.2367	3	294.4122	7.485342	0.002724	2.489788
Error	589.9775	15	39.33184			
Total	1597.907	23				

PROC GLM SUMMARY 1996: UNBALANCED MODEL

No. of observations in data set=480
All dependent variables are consistent in respect to the presence or absence of missing values. However only 444 observations can be used in this data set.

VARIABLE (WET WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F
Model	23	114.87595675	4.99460682	2.62	**0.0001**
Error	420	800.44175592	1.90581370		
Corrected Total	119	915.31771267			

R-SQUARE	C.V.	ROOT MSE	WET WEIGHT MEAN
0.1225504	67.31974	1.3805121	2.0567943

VARIABLE (WET WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F
Type I error					
Bacteria	5	10.40130569	2.0826114	1.09	0.3644
Application	13	37.81760692	12.60586897	6.61	**0.0002**
Bacteria. Application	15	66.65704413	4.44380294	2.33	**0.0033**
Type III error					
Bacteria	5	11.78653243	2.35730649	1.24	0.2908
Application	13	39.26354080	13.08784693	6.87	0.0002
Bacteria. Application	15	66.65704413	4.44380294	2.33	**0.0033**

VARIABLE (DRY WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F
Model	23	71.93693754	3.12769294	19.62	**0.0001**
Error	420	66.95442878	0.15941531		
Corrected Total	443	138.89136632			

R-SQUARE	C.V.	ROOT MSE	DRY WEIGHT MEAN
0..517397	88.34395	0.3992684	0.45194771

VARIABLE (DRY WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F
Type I error					
Bacteria	5	13.45747826	2.69149565	16.88	**0.0001**
Application	3	14.42346767	4.80782256	30.16	**0.0001**
Bacteria. Application	15	44.05599161	2.9370611	18.42	**0.0001**
Type III error					
Bacteria	5	12.25461519	2.45092304	15.37	**0.0001**
Application	3	15.18448763	5.06149588	31.75	**0.0001**
Bacteria.Application	15	44.05599161	2.9306611	18.42	**0.0001**

PROC GLM SUMMARY 1996: BALANCED MODEL

Number of observations in data set = 480

VARIABLE (WET WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Model	23	99.90409000	4.34365609	4.58	**0.0001**
Error	456	432.24020812	0.94789519		
Corrected Total	479	532.14429812			

R-SQUARE	C.V.	ROOT MSE	WET WEIGHT MEAN
0.187739	71.13378	0.9735990	1.36868750

VARIABLE (WET WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Type I & III Sum of Squares					
Bacteria	5	20.72865031	4.14573006	4.37	**0.0007**
Application	13	45.76833760	15.25611253	16.09	**0.0001**
Bacteria. Application	15	33.40710208	2.22714014	2.35	**0.0030**

VARIABLE (DRY WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Model	23	39.69059523	1.72567805	20.20	**0.0001**
Error	456	38.96292579	0.08544501		
Corrected Total	479	78.65352101			

R-SQUARE	C.V.	ROOT MSE	DRY WEIGHT MEAN
0.504626	96.29287	0.2923097	0.30356326

VARIABLE (DRY WEIGHT)	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Type I&III error					
Bacteria	5	7.15319377	1.43063875	16.74	**0.0001**
Application	13	10.18047280	3.39349093	39.72	**0.0001**
Bacteria. Application	15	11.35692866	1.49046191	17.44	**0.0001**

*Values below 0.05 are significant

PROC GLM SUMMARY 1997

VARIABLE	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Model	20	29267.74137000	1463.38706850	1.64	0.0577
Error	99	88304.34202667			
Corrected Total	119	117572.08339667			

R-SQUARE	C.V.	ROOT MSE	TOTALWT MEAN
0.248934	92.69368	29.86575046	32.21983333

VARIABLE	DF	SUM OF SQUARES	MEAN SQUARE	F	PR>F*
Block Number	9	3913.17121333	434.79680148	0.49	0.8799
Bacteria	2	1151.59346167	575.79673083	0.65	0.5266
Soil Type	1	16715.88075000	16715.880750	18.74	**0.0001**
Bacteria.Soil Type	2	2619.11323500	1309.55661750	1.47	0.2353
Fungicide	1	161.65765333	161.65765333	0.18	0.6712
Bacteria.Fungicide	2	2142.64000167	1071.32000083	1.20	0.3052
Soil.Type.Fungicide	1	107.76865333	107.76865333	0.12	0.7289
Bacteria.Soil Type.Fungicide	2	2455.91640167	1227.95820083	1.38	0.2572

*Values below 0.05 are significant

ANOVA OF RHIZOSPHERE POPULATION COMPOSITION ON SELECTIVE MEDIA (CFU G^{-1} OF ROOT SAMPLE)

VARIABLE	DF	SUM OF SQUARES	MEAN SQUARE	V.R	F PR*
Soil	1	1.114E+06	1.114E+06	1.29	0.263
Fungicide	1	6.301E+06	6.301E+06	7.30	**0.010**
Media	2	3.369E+07	1.684E+07	19.51	**<0.001**
Bacterium	2	3.203E+06	1.602E+06	1.86	0.107
Soil.Fungicide	1	1.415E+06	1.415E+06	1.64	0.208
Soil.Media	2	2.277E+05	1.138E+05	0.13	0.877
Fungicide.Media	2	1.754E+06	8.771E+05	1.02	0.371
Soil.Bacterium	2	2.999E+05	1.499E+05	0.17	0.841
Fungicide .Bacterium	2	1.581E+06	7.904E+05	0.92	0.409
Media.Bacterium	4	4.020E+06	1.005E+06	1.16	0.341
Soil.Fungicide.Media	2	1.373E+05	6.866E+04	0.08	0.924
Soil.Fungicide. Bacterium	2	2.168E+06	1.084E+06	1.26	0.296
Soil.Media.Bacterium	4	3.615E+06	9.037E+05	1.05	0.396
Fungicide.Media. Bacterium	4	7.479E+06	1.870E+06	2.17	0.091
Residual	39	3.367E+07	8.633E+05		
Total	71	1.011E+08			

*Values below 0.05 are significant