ISOLATION AND CHARACTERIZATION OF SOME BACTERIA ANTAGONISTIC TO PLANT PATHOGENIC FUNGI

by

O e d j i j o n o

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for the degree of Master of Science (Microbiology),
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DECLARATION

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

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SUMMARY

Bacteria antagonistic to plant pathogenic fungi were isolated from various sources and tested for their ability to inhibit fungi or protect plants *in vitro*, in the glasshouse and in a field trial.

Five strains of bacterial antagonists were isolated from daisy roots (*Bacillus polymyxa* UT1), decomposing woodchips (*B. subtilis* UT2), clary sage roots (*Pseudomonas cepacia* UT3 and *P. putida* UT4) and from white clover roots (*Acinetobacter* sp. UT5). *In vitro*, the degree of inhibition of fungi by the five antagonists varied, with strain UT3 showing antagonism to all fungal pathogens (*Sclerotinia sclerotiorum*; *S. minor*; *Botrytis cinerea*; *Pythium ultimum*; *Fusarium solani*; *Phytophthora cinnamomi*; *P. cactorum*; *Gaeumannomyces graminis*; and *Penicillium echinulatum*) tested. The action of the antagonists depended on the media used. Isolates of UT1 and UT3 were inhibitory to the fungi on both PDA and KBM; the other strains were only inhibitory on PDA. Antifungal compounds produced by the antagonists were thought to be responsible for inhibiting the pathogens rather than ferric-siderophores, as addition of iron to the media did not affect the inhibitory activity of the antagonists.

The antagonists (strains UT1, UT2, UT3, UT4, and NIR-6 of *P. putida*) significantly reduced wheat take-all disease in an axenic sand assay with isolate UT3 (*P. cepacia*) showing the most inhibition to the pathogen. The antagonists maintained their viability and ability to protect the wheat roots after 3 weeks of the assay. In the glasshouse, the five antagonists (strains UT1 to UT5) reduced lettuce (*Lactuca sativa*) drop caused by either *Sclerotinia sclerotiorum* or *S. minor* compared with their controls after 10 weeks growth. In the field however, the antagonists did not significantly protect daisy

(*Olearia phlogopappa*) plants from the root rot disease caused by *Pythium* sp..

Metabolites produced by strains UT1 and UT3 were inhibitory to several fungi *in vitro*. Strain UT3 of *P. cepacia* produced two types of antibiotic designated A and B. Compound A (the main inhibitor) was inhibitory to both fungi and bacteria tested, while compound B (the minor inhibitor) was only inhibitory to the bacteria. The main compound had an R_f= 0.40 and the minor had an R_f= 0.87 on silica gel following development in a chloroform-methanol (7:3) solvent system. Both had very similar retention times (between 20 and 22 minutes) following separation by C_{18} reversed phase HPLC and elution with 0-100% methanol containing 0.1% trifluoroacetic acid. Both antibiotics were thermostable and resistant to acidic and alkaline treatments. Proton NMR indicated that the compound A may be an aromatic molecule but further characterization was not possible due to incomplete purification.

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1. INTRODUCTION

The use of pesticides for the prevention of plant diseases is known to lead to increased pathogen-resistance to them and to potentially damaging accumulation of the pesticides in the environment. Increased awareness of the hazards associated with pesticides has encouraged efforts to find effective alternatives. Control through biological means has attracted particular interest. Biological control is described as any means of controlling disease, or reducing the amount or the effect of pathogens, that relies on biological mechanisms or organisms other than man (Campbell, 1989). Traditionally, this method of control has been practiced by farmers through crop rotation or addition of organic manures.

Plants release nutrients which are readily utilized by microorganisms in the rhizosphere, as evidenced by the greater microbial populations and activities in these regions. Interactions between plants and microorganisms can cause both beneficial and detrimental effects on the plants. Biological control of soil-borne plant pathogens is achieved by inducing a beneficial association between introduced microorganism and the host to the detriment of the pathogens and/or by enhancing plant growth. Agents of biological control occur naturally in soil or in the plant rhizosphere. To be successful, biological control agents must establish and increase their populations in the root zone of target plants and maintain their effectiveness both *in vitro* and in natural habitats.

The aim of this study was to investigate bacteria antagonistic to soil-borne plant pathogens from various sources, and to test their efficacy against fungal pathogens *in vitro* and in the field. Attempts

were also made to characterise antimicrobial compounds produced by one of the most effective of the antagonists.

2. LITERATURE REVIEW

2.1 Progress in biological control of plant pathogens

After twenty-five years of progress in biological control (Cook, 1990), a number of biocontrol agents of plant pathogens are currently commercially available (Table 2.1.1), these being effective primarily against fungal pathogens.

Table 2.1.1 Commercial biological control agents of plant pathogens (Powell *et al.*, 1990).

Microorganism	Target	Country of registration
Bacteria		
Agrobacterium radiobacter	crown gall	USA, Australia, NZ
Bacillus subtilis	growth enhancement	USA
Pseudomonas fluorescens	bacterial blotch	Australia
P. fluorescens	seedling diseases	USA
Fungi		
Peniophora gigantea	*Fomes annosus*	UK
Pythium oligandrum	*Pythium* sp.	USSR
Trichoderma harzianum	damping off	Israel
T. viride	timber pathogens	Europe
Trichoderma sp.	root diseases	USSR
Fusarium oxysporum	*Fusarium oxysporum*	Japan

2.1.1 Bacteria as biological control agents

Bacteria representing 21 genera have been shown to have or are thought to have potential for biological control of plant pathogens (Weller, 1988; Tannii *et al.,* 1990). Some of them are also known as plant growth promoting rhizobacteria (PGPR), this term being coined for bacteria that colonized roots, promoted plant growth, and increased yields (Kloepper *et al.,* 1980a). The term "plant growth promotion" was used to describe the increased plant growth and crop yields that occurred following inoculation of seeds or roots with certain beneficial bacteria (Burr *et al.,* 1978; Suslow and Schroth, 1982), while the term "rhizobacteria" was used to describe bacteria with the ability to colonize roots aggressively (Schroth and Hancock, 1981).

Agrobacterium radiobacter

A. radiobacter strain K-84 was the first bacterial biocontrol agent used commercially to control crown gall caused by *Agrobacterium tumefaciens* (Kerr, 1972). Crown gall is distributed worldwide and caused serious economic losses before the introduction of the control agent. In Australia economic losses occur mainly in almonds, peach, roses and stone fruits (Kerr, 1980), and in Europe, North America, South Africa in apples, pears, grapes and stone fruits (Matthee *et al.,* 1977). A series of studies of the biological control of crown gall by *A. radiobacter* strain 84 on various plants showed spectacular results. For example, up to 60% of inoculated peach seeds were resistant to galls compared with 21% for uninoculated seeds (Kerr, 1972) and 100% resistance was demonstrated for peach seedlings following seed dipping and planting in infested soil, compared with 42% mortality of controls (Htay and Kerr, 1974). Similar results were also found for tomato (Htay and Kerr 1974; Moore, 1977), *Prunus* (Moore, 1977),

and other plants including *Rubus, Malus, Salix, Vitis, Libocedrus, Chrysanthemum, Crategus, Carya, Rosa, Pyrus* and *Humulus* (Moore and Warren, 1979).

Bacillus spp.

Bacillus spp. have received much attention as potential biological control agents of plant diseases since they produce endospores that are tolerant to heat and to dry conditions. One of the first reports was of a *Bacillus subtilis* strain A13 isolated from lysed mycelium of *Sclerotium rolfsii* which reduced damping-off and wire-stem of pepper seedlings caused by *Rhizoctonia solani* in soil mix (Broadbent *et al.,* 1971). The isolate was also found to stimulate growth of peppers and tomatoes in soil in the absence of the pathogen. Similar results using this isolate were reported by Merriman *et al.* (1974b), with seed treatment increasing grain yield and dry matter of cereals, although root disease caused by *Rhizoctonia* was not effectively controlled. The bacterium as seed treatment also increased marketable yields of carrots to 48% compared with 15% for controls. Strain A13 was inhibitory to several plant pathogens *in vitro* (e.g. *Phytophthora citrophthora, P. cinnamomi, P. nicotianae* var. *parasitica, Pytium ultimum, P. debaryanum, Fusarium oxysporum* f.sp. *lycopersici, Sclerotium rolfsii* and *Rhizoctonia solani*), and increased plant growth (e.g. germination, plant weight and grain yield) of cabbages, tomatoes, ornamental plants, wheat and sorghum in steamed and non-steamed soils or in the the field (Broadbent *et al.,* 1977). Yuen *et al.* (1985) reported that roots of carnation cuttings dipped in strain A13 suspensions reduced *F. oxysporum* f.sp. *dianthi*-wilt to 40% of that controls within 75 days in pot trials. Since 1983, *B. subtilis* strain A13 has been commercially available as a treatment for peanut

pathogens under the trade name of QUANTUM-4000 (Weller, 1988).

Merriman *et al.* (1974a) isolated antagonistic *Bacillus* spp. from pasture soil and from a continuous wheat soil that, *in vitro,* inhibited growth of *R. solani* and reduced post-emergence damping-off, stunting and root disease. An increase in dry weight of wheat was also recorded. Soaking of wheat grains in cell suspensions of four *Bacillus subtilis* isolates prior to sowing in pasteurized sand-clay mixture infested with *R. solani*, resulted in reduced root discolouration as well as increased grain yield and plant dry matter. However, no reduction of *Fusarium* infection of tubers was reported by Chambers and Millington (1974) when one of the above-mentioned isolates (strain 1-B80) was applied to cuttings of potato cultivars Kennebec and Sequoia prior to planting in soil infested with the pathogen. However fungal populations in a field soil experiment were significantly reduced by this strain for up to 140 days.

Bacillus spp. appear to be antagonistic to a wide range of fungal pathogens. Six isolates of *B. subtilis* (strains B-1, B-2, B-4, B-8, B-K, and B-O) isolated from sclerotia of *Sclerotium cepivorum* produced diffusible antibiotics which were antagonistic, *in vitro,* to the growth of *S. cepivorum.* Following seed treatment of onions grown in non-sterile soil in a controlled environment chamber, strains B-2, B-4 and B-8 were found to significantly reduce infections by *S. cepivorum* (Utkhede and Rahe, 1980). These antagonists also gave significant field-control of white rot in the onion varieties Festival and Autumn Spice. In a trial of 11 bacterial isolates, Utkhede and Rahe (1983) found a correlation ($P = 0.01$) between the width of inhibition zone of fungal growth on agar plates and protection against the fungi in pots using cultivar Autumn Spice. The isolate B-2 provided the best protection from onion white rot of the antagonists tested.

Although the antagonist B8 inhibited growth of *Phytophthora cactorum* on corn meal agar and in sterile field soil, under greenhouse conditions protection of apple seedlings was not significant, suggesting differences in susceptibility of the target pathogens (Utkhede and Gaunce, 1983).

Utkhede (1984) isolated 21 strains of *B. subtilis* from soil which lysed *Sclerotium cepivorum* mycelia *in vitro* and which also showed antagonism to *P. cactorum*. Six isolates significantly reduced crown rot infection of apple seedlings in sterile soil relative to controls. Utkhede and Sholberg (1986) further showed that the antagonists inhibited the growth of other fungal pathogens *in vitro* including *R. solani*, *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, and *Sclerotium cepivorum*. In field trials, most of the antagonists effectively prevented *Alternaria* rot and brown rot of harvested cherries.

Some strains of *B. subtilis* appear to be parasitic on fungal sclerotia. Coley-Smith and Dickinson (1971) found that sclerotia of *S. cepivorum* stimulated *B. subtilis* populations *in vitro*, the bacteria apparently using sclerotia as a carbon source in carbon-free media. Backhouse and Stewart (1989a) isolated two *B. subtilis* strains (B11 and B14) from soil or surface-sterilized sclerotia which were strongly antagonistic to *S. cepivorum*, *in vitro*. No sclerotia germinated when incubated in soil culture with the antagonists.

Tschen *et al.* (1989) examined the biological control of crysanthemum basal stem rot in cuttings caused by *R. solani*. Dipping the cut ends in a suspension of *B. subtilis* reduced the disease by 30% relative to controls in pot trials. Handelsman *et al.* (1990) isolated *B. cereus* (UW85) from healthy alfalfa roots which when applied as seed coatings reduced alfalfa seedling mortility to nil and increased seedling emergence by 30% in vermiculite infested with *Phytophthora*

megasperma f.sp. *medicaginis* compared with controls. A similar result was recorded in a small scale field trial. Sterile filtrates of these cultures were also effective in protecting seedlings from damping-off.

***Pseudomonas* spp.**

Pseudomonads are currently receiving much attention as biological control agents. Advantages of these bacteria are that they are common colonists of the root rhizosphere and are nutritionally versatile. Both antagonism to plant pathogens and promotion of plant growth have been noted for *Pseudomonas* spp..

Strains of *P. fluorescens* (TL-3) and *P. putida* (BK-1) isolated from the surface of potato tuber significantly increased the growth and yield of potatoes in greenhouse trials following dip-inoculation of seedpieces (Burr *et al.*, 1978). It was suggested that the yield increase was related to the suppression of various pathogens in the rhizosphere. *In vitro*, the pseudomonads exhibited antibiosis against *Erwinia carotovora* var. *carotovora*, and in field trials potato yields were increased up to 33% following application of the *Pseudomonas* spp. (especially strain TL-3) to seedpieces. Significant results were also reported by Kloepper *et al.* (1980a) in greenhouse assays. Geels and Schippers (1983b) evaluated 112 fluorescent *Pseudomonas* species isolated from potato tubers, for antagonistic activity against pathogenic fungi and bacteria. *In vitro*, 4% of these isolates were strongly antagonist, and Geels and Schippers (1983a) showed that of six of the strains tested, three (WCS 358, WCS 365, and WCS 374) produced siderophores while the other three (WCS 377, WCS307 and WCS 361) produced inhibitory substances other than siderophores. In a pot experiment, the former group suppressed the potato disease, but the latter isolates did not.

Pseudomonas fluorescens strain E6, isolated from celery roots, promoted radish growth in greenhouse and field trials (Kloepper *et al.*, 1980b). In glasshouse trials this strain increased potato growth by 86% compared with controls. This strain also stimulated growth of other plant species, such as sugar beet in field trials (Suslow and Schroth, 1982); and ornamental plants in pot trials (Yuen and Schroth, 1986b).

P. fluorescens strain Pf5 isolated from the rhizosphere of cotton seedlings successfully protected cotton seedlings against damping-off by both *R. solani* and *P. ultimum* (Howell and Stipanovic, 1979). This strain produced antibiotics, pyrrolnitrin and pyoluteorin, that were strongly inhibitory *in vitro* to *R. solani* and *Pythium ultimum*, respectively, and to the other cotton seedling pathogens, *Thielaviopsis basicola*, *Alternaria* sp. and *Verticillium dahliae* (Howell and Stipanovic, 1979; 1980). Strain Pf5 also showed its efficacy against the potato pathogen *Erwinia carotovora* in glasshouse trials (Xu and Gross, 1986b).

Kloepper *et al.* (1980b) isolated two strains (A1 and B10) of plant growth promoting fluorescent *Pseudomonas* spp. from the potato periderm that increased plant growth by 500% compared with controls in greenhouse trials when inoculated onto potato seedpieces. Promotions of seedling emergence and of potato yields by strain B10 was also reported by Xu and Gross (1986a, 1986b). The ability of strain B10 to inhibit the potato pathogen, *E. carotovora*, both *in vitro* and in the field was also shown. A reduction of *Fusarium* wilt in carnations by 40% up to 75 days after treatment following dipping of root cuttings in suspensions of strain B10 was reported by Yuen *et al.* (1985).

Suppression of take-all of wheat by *Pseudomonas fluorescens* has

been extensively investigated. Weller and Cook (1983) isolated two *P. fluorescens* strains (2-79 and 13-79) that inhibited *Gaeumannomyces graminis* var. *tritici* (Ggt) *in vitro,* and as soil treatments or seed coatings successfully suppressed take-all in greenhouse trials and in field-grown winter and spring wheat. A mutant of strain 2-79 (2-79RN_{10}), was highly inhibitory *in vitro* to Ggt both on potato dextrose agar (PDA) and King's B medium agar (KBM) (Weller *et al.,* 1988). They also showed that addition of 10-50μM $FeCl_3$ to agar medium resulted in the elimination of inhibition on KBM, suggesting that this strain produced iron-chelating compounds. Thomashow and Weller (1988) identified an antibiotic, phenazine-1-carboxylate, produced by strain 2-79 and active against the take-all fungus. *In vitro*, the antibiotic inhibited 4/22 fungi tested at 25μg ml^{-1} and 17/22 at 250μg ml^{-1} (Brisbane *et al.,* 1989). The combinations of strain 2-79 with fungicide metalaxyl or bitertanol when mixed in soil, resulted in no significant effect on the weight of wheat plants naturally infested with Ggt, although seedling emergence was more than doubled following the treatment. *P. fluorescens* strain 2-79 has been claimed to be a model successful bacterial biological control agent for take-all (Weller, 1988; Thomashow and Weller, 1990; Ryder *et al.,* 1990).

Other strains of *P. fluorescens* (R1a-80, R4a-80, R15b-80 and R1bc-80) isolated from soil-grown wheat which were even more suppressive of Ggt *in vitro* than strain 2-79, gave a reduction in seedling mortality but had no significant effect on plant height, number of heads or plant yield (Weller *et al.,* 1985). In a study of mutant strains of R1a-80 (plus four others) Poplawsky and Ellingboe (1989) showed that although *in vitro* antibiosis of two of these strains was greater than their parent strains, none was capable of inhibiting Ggt. This suggested that increasing antibiosis ~~could~~ does not necessarily ~~be~~ equated with an increased ability to suppress disease. Wong and Baker (1984) isolated

P. fluorescens from a soil suppressive to *Fusarium* wilt which gave significant protection to wheat grown in soil infested with Ggt in pot trials and to *Agrostis* turfgrass grown in soil infested with Ophiobolus patch (*G. graminis* var. *avenae*). The pseudomonads, as soil treatments, reduced disease and increased the dry weights of the plant tops. However whether the increased growth of plants was due to stimulation of growth or suppression of pathogens was uncertain.

P. fluorescens strain Pfcp isolated from cankered citrus leaf strongly inhibited *Sarocladium oryzae* (causing sheath rot of rice) or stimulated plant growth both *in vitro* and in field trials (Sakthivel and Gnanamanickam, 1987). The antagonist also gave protection to other plants following inoculation of roots. Other fluorescent and non-fluorescent pseudomonads isolated from rice roots were antagonistic *in vitro* to sheath-blight of rice caused by *R. solani* (Vasanta Devi *et al.,* 1989), with symtoms of disease being reduced by up to 74% in greenhouse trials and up to 72% in field experiments.

Additions of as little as 5% (v/v) of soil suppressive to black root rot (caused by *Thielaviopsis basicola*) to susceptible sites was sufficient to suppress the pathogen (Stutz *et al.,* 1986). Three of 15 isolates of *P. fluorescens* from this soil were half as effective against the pathogen as the undiluted suppressive soil, and one further isolate (strain CHAO) gave suppressiveness which was similar to the undiluted suppressive soil. By adding strain CHAO at 10^7 cfu/cm^3 of soil, black root rot of tobacco was reduced in 36 of 39 conducive soil samples. Keel *et al.* (1989) found that strain CHAO was inhibitory to *T. basicola* on tobacco grown in a vermiculite system, with root and shoot weights being increased compared with controls. A transposon-insertion siderophore-negative mutant of CHAO (CHA400) was also suppressive, indicating the antagonism to be primarily by a mechanism

other than Fe-chelation.

Elad and Chet (1987) isolated *P. putida* strains 805 and 310 and *P. cepacia* strains 808, 814 and Y11, from the rhizosphere of bean, cotton, radish, cucumber, and melons infested with *Pythium aphanidermatum* having abilities to suppress damping-off in cucumber by 17-67% in the glasshouse. The antagonists 805 and 808, either as a slurry or as coating on seeds grown in soil infested with *P. aphanidermatum*, reduced disease in cucumber by 62-67%, in melons by 35-65%, in tomatoes by 95-80%, in peppers by 55-50% or in beans by 75-65%, and significantly reduced pre-emergence damping-off. Plant growth promotion by isolates 805 and 808 of tomato, pepper, melon, bean, tobacco, cucumber and radish in the greenhouse was also noted by Elad *et al.* (1987).

Pseudomonas putida strain A12 isolated from the rhizosphere of flax roots, when applied as a soil mix in pot trials reduced wilt in flax caused by *Fusarium oxysporum* f.sp. *lini* to 10% compared with controls with 42.5% wilt at 30 days after treatment (Scher and Baker, 1982). A further strain of *P. putida* strain N-1 isolated from mycelial mats of *Fusarium* was reported to be inhibitory to the flax disease by Dupler and Baker (1984). In a subsequent trial, strain A12 reduced the disease in cucumber and radish grown in soil infested with *F. oxysporum* f.sp. *cucumerinum* and *F. oxysporum* f.sp. *conglutinans*, respectively. In contrast, Park *et al.* (1988) found that strain A12 did not suppress the disease in cucumber grown in soil infested with *F. oxysporum.* f.sp. *cucumerinum*, though the germination of chlamydospores of the pathogens was reduced in the rhizosphere of cucumber plants. There were reductions in the disease development, however, when the strain A12 was combined with other non-pathogenic *F. oxysporum* strains. Its mutants also significantly

reduced the disease of cucumber and the germination of *Fusarium*-chlamydospores compared with controls, either alone or in combination with the non-pathogenic isolates of *F. oxysporum*. The incidence of sugar beet damping-off caused by *Pythium ultimum* was reduced by both strains of *P. fluorescens-putida* ML5 and *P. putida* R20 when the antagonists were applied to sugar beet seeds (Osburn *et al.*, 1989).

Pseudomonas cepacia is recognized as a bacterial plant pathogen. However Kawamoto and Lorbeer (1976) reported that a particular strain, *P. cepacia* 64-22, showed ability to colonize onion roots and reduce onion seedling damping-off caused by *F. oxysporum* f.sp. *cepae* in pot trials. The suppression of radish damping-off, tomato *Fusarium*-wilt, and eggplant *Verticillium*-wilt following application of *P. cepacia* RB425 was reported by Homma *et al.* (1985). Some strains of *P. cepacia* were also reported to reduce infection of cucumber caused by *P. aphanidermatum* (Lumsden *et al.*, 1987) and inhibit *Botrytis cinerea* and *Penicillium expansum* causing blue mold and gray mold on apple and pear (Janisiewicz and Roitman, 1988). The production of particular antibiotics by *P. cepacia* and other antagonistic *Pseudomonas* spp. was thought to be involved in the antagonism.

A new species of *Pseudomonas, P. antimicrobica* that was strongly antagonistic to all of 33 fungal and six of eight bacterial pathogens tested, was described by Attafuah and Bradbury (1989). Surprisingly this antagonist was isolated from a mealybug (*Planococcoides njelensis* Laing). An antagonist-free extract exhibited activity against *Phytophthora palmivora* and *P. infestans* buffered at pH 3.4-8.0. In addition the zoospores and mycelia of these fungi did not grow when inoculated on agar into which the active substance had diffused.

Alcaligenes spp.

Yuen *et al.* (1985) first isolated *Alcaligenes* sp. strain MFA1 from the roots of carnations grown in soil that was inhibitory to *Fusarium*-wilt. A 40% reduction in severity of *Fusarium oxysporum* f.sp. *dianthi* wilt of carnation occurred after 75 days in pot trials when the roots of cuttings were initially dipped in suspensions of strain MFA1. In commercial greenhouses, the incidence of *Fusarium*-wilt in carnations was reduced by 13% 7 months after treatment with the same isolate. Yuen and Schroth (1986a) found that strain MFA1 was inhibitory to *F. oxysporum* f.sp. *dianthi* on KBM, but the inhibition did not occur on the iron-rich medium indicating siderophore production by the isolate. Addition of low concentrations of glucose and asparagine to soil inoculated with strain MFA1 resulted in an inhibition of germination of *F. oxysporum* spores, but there was no inhibition at higher concentrations. As a seed treatment, strain MFA1 significantly inhibited colonization of carnation seedling roots by *Fusarium oxysporum* f.sp. *dianthi* by between 24% and 37%.

Enterobacter spp.

The antagonistic activity of *Enterobacter* against pathogens was first reported by Nair and Fahy (1972). A strain of *E. aerogenes* isolated from peat protected a mushroom (*Agaricus bisporus*) from infection by *Pseudomonas tolaasii*, but the inhibitory ability of this bacterium failed *in vitro*. Protection of cucumber, pea and beet seedlings from rots caused by *Pythium* spp. was provided by *E. cloacae* (NRRLB-14095) with 75-92% of cucumber seedlings being healthy following seed-coating with the antagonist compared with 14% of untreated controls (Hadar *et al.*, 1983). Nelson *et al.* (1986) found a 40-62% increase in cucumber seedling emergence in soil naturally infested with *Pythium* spp. when the seeds were treated with

E. cloacae. The suppressive effect of a strain of *E. cloacae* (313) against *Rhizoctonia solani* was increased in combination with *Trichoderma hamatum* compared with treatment with the bacterial antagonist alone (Kwok *et al.*, 1987).

Erwinia spp.

Several workers have reported the use of *Erwinia* spp. as biocontrol agents against plant pathogens, with non-pathogenic *Erwinia* tending to have a similar niche with the pathogenic strains. Riggle and Klos (1970) reported that *E. herbicola* and pathogenic *E. amylovora* occurred together within fire blight cankers in orchards. *E. herbicola* isolated from fire blight cankers of pear showed a reduction in pear blossom infection *in vitro* when a suspension of the non-pathogenic strain was sprayed into the blossom a day prior to the *E. amylovora* infestation. Protection against pear and apple blights by *E. herbicola* was also reported by Beer and Rundle (1980), Beer (1981) and Beer and Rundle (1983).

E. herbicola was reported by Sneh *et al.* (1984) to provide significant protection to cucumber sedlings against *Fusarium*-wilt. Chlamydospore germination of this fungus was also inhibited by the antagonist. Wong and Hughes (1986) isolated a strain of *E. herbicola* from onion roots showing antagonism *in vitro* to *Sclerotium cepivorum* causing white rot of onion. Kempf and Wolf (1989) demonstrated significant correlation between *in vitro* inhibition of *Fusarium culmorum* by a strain of *E. herbicola* (B247) and suppression of wheat disease in pot tests. In addition, spraying this antibiotic-producing strain on wheat leaves before infesting *Puccinia recondita* f.sp. *tritici* spores resulted in a 76% disease reduction. In contrast, an antibiotic-deficient mutant (strain Tn247) was ineffective.

Bio-spraying of *E. herbicola* to reduce target pathogens is now showing increasing promise. Wilson *et al.* (1990) reported that *E. herbicola* strains WL40 and WL9 significantly reduced both blossom- and shoot-blight of pears. The latter strain gave over 80% control of blossom blight, equivalent to that provided by chemical agents while total control of shoot blight was achieved by this strain when applied at an antagonist : pathogen ratio of 10 : 1.

Chitinolytic bacteria

Chitinolytic bacteria have been reported to be antagonistic to fungal pathogens, particularly *Fusarium* sp.. Koths and Gunner (1967) demonstrated the biological control of *Fusarium* stem rot of carnations by the application of an *Arthrobacter* sp.. This bacterium was isolated from the carnation rhizosphere and lysed mycelia of *F. roseum, in vitro.* Protection of rooted carnation cuttings was shown by dipping the roots in bacterial suspension and planting in pots infested with *Fusarium roseum.*

Sneh (1981) isolated chitinolytic *Arthrobacter* sp. and *Serratia liquefaciens* that completely lysed hyphae of *F. oxysporum* f.sp. *dianthi in vitro* within 4-6 days. There was also a significant reduction in carnation disease-incidence when the roots of cuttings were dipped in a suspension of *Arthrobacter* sp. and grown in pots infested with the pathogen. The incidence of healthy treated plants was 92% compared with 38% healthy non-treated controls after 150 days growth. These results were also supported by field trials.

Reduction in the incidence of carnation wilt by *S. liquefaciens* and *Hafnia alvei* was reported by Sneh *et al.* (1985), with *S. liquefaciens* giving better protection than *H. alvei.* However, the protection occurred when the antagonists were applied to the cuttings before

rooting, but not when applied to the root systems of cuttings. There appeared to be considerable competition between the antagonist and the pathogen for initial establishment in the rhizosphere. Chanway *et al.* (1989) found that *Serratia* spp. enhanced plant growth of lentils. Strains of *S. proteamaculans* and *S. fonticola* increased the emergence, vigor, nodulation, C_2H_2 reduction and root fresh weight of lentils in field experiments. The former strain also significantly enhanced nodule numbers.

***Rhizobium* spp. and *Bradyrhizobium* sp.**

A reduction of *Fusarium*-root rot of soybean following application of nodulating *R. japonicum* was reported by Mew and Howard (1969) and a reduction of *Phytophthora megasperma* root rot both *in vitro* and in the glasshouse, following application of *Rhizobium japonicum*, was demonstrated by Tu (1978). Tu (1979) further reported that rhizobial inoculation of *P. megasperma*, *Pythium ultimum*, *Ascochyta imperfecta* and *Fusarium oxysporum* cultures reduced their sporulation by 47-75%. Non-septate fungi were reported to be more susceptible to rhizobial parasitism than septate fungi.

Tu (1980a) showed that increasing concentrations of *Rhizobium* decreased the severity of *Fusarium*-root rot and *vice versa*, with the greatest degree of protection being achieved when rhizobia were applied at the same time as the fungal inoculum. Chao (1990) tested various rhizobia against pathogenic and non-pathogenic fungi *in vitro*, and found that strains of *Rhizobium leguminosarum* biovar *phaseoli*, *R. meliloti*, and *Bradyrhizobium* sp. were antagonistic to *Trichoderma harzianum*, *T. koningii*, *Pythium ultimum*, *P. irregulare*, *Fusarium oxysporum* f.s. *pisi*, *F. solani* f.s. *phaseoli*, *F. moniliforme*, *F. roseum*, and *Rhizoctonia solani*. Culture filtrates

of a strain of *R. leguminosarum* biovar *phaseoli* showed the greatest inhibitory activity against all fungi tested. It was suggested that care should be taken when using a combination of *Rhizobium* and fungi as biocontrol agents.

Streptomyces spp.

Many streptomyces show inhibition of fungi on solid media, although about half of these fail to produce detectable inhibitory substances in liquid media containing the same nutrients (Schatz and Hazen, 1948). Of 20 *Streptomyces* cultures isolated by Hsu and Lockwood (1969), 18 produced zones of inhibition against *Glomerella cingulata* and *Mucor ramannianus*. Broadbent and Baker (1969) isolated *Streptomyces* spp. that were inhibitory to *Phytophthora, Fusarium, Sclerotium* and *Pythium* spp. in soil tubes and several were also inhibitory on agar medium. *Streptomyces* were prevalent in soils high in organic matter. Broadbent *et al.* (1971) isolated *Streptomyces* strains antagonistic *in vitro* to a similar spectrum of pathogenic fungi.

A strain of *Streptomyces griseus* strain 2-A24 isolated from both pasteurized and unpasteurized soils, reduced disease symptoms in pepper and wheat caused by *R. solani* (Merriman *et al.,* 1974a) with increases in grain yield by 30% over controls. Merriman *et al.* (1974b) found that though the root disease of cereals (barley, oats, wheat) and carrots caused by *R. solani* was not effectively controlled following seed treatment with the *Streptomyces* isolates, such treatment resulted in increased grain yield and dry matter production at one site and increased tiller number at two sites of a three-site trial. In addition, there was an increase in marketable yield of carrots by 15% over controls in the field. Chambers and Millington (1974) reported

the isolation of a *Streptomyces* strain able to significantly reduce *Fusarium*-infection and increase tuber yield of potatoes at 70 and 140 days after inoculation.

Antagonistic activity of *Streptomyces longisporus* against *Helminthosporium oryzae* and *Alternaria solani* was shown *in vitro,* in autoclaved soil and by its filtrate on agar (Chattopadhyay and Nandi, 1982). Disease reduction was also evident when infected potato seed pieces or rice grains were dipped in culture filtrates.

Rothrock and Gottlieb (1984) found that *Streptomyces hygroscopicus* var. *geldanus* (producing geldanamycin) protected peas from rhizoctonia root rot in previously sterilized soil if the bacterium was applied two or more days prior to fungal infestations, but it did not control the disease if applied at the same time as the pathogen. The prior application was apparently required by the antagonist to either become established or to produce antibiotic.

A *Streptomyces* sp. isolated from *Sphagnum* peat significantly reduced yield losses caused by *B. cinerea* in peat-grown lettuces following application as a spore suspension, but did not reduce those caused by *R. solani* (Tahvonen and Lahdenpera, 1990).

Pasteuria penetrans

Taxonomically, there has been a misidentification of the cladoceran- and nematode-parasitizing bacterium as *Bacillus penetrans* (and previously *Duboscqia penetrans*). It is now identified as belonging to the genus *Pasteuria* Metchnikoff (Sayre *et al.,* 1983; Sayre and Starr, 1985).

P. penetrans is able to prevent egg production by, and eventually to kill root-knot and meadow nematodes. Mankau (1975) found that "*B.*" *penetrans,* as a soil treatment, reduced the population of

Pratylenchus scribneri by 53.7% in soil or by 62.5% on bean roots. Tomato root-knot disease, caused by *Meloidogyne incognita,* was also significantly decreased in soil-grown tomatoes in the presence of this antagonist. Stirling (1984) reported that ground, air dried tomato roots, containing *Meloigogyne javanica* infected with "*B.*" *penetrans,* applied at rates of 212-600 mg/kg of field soil infested with root-knot-nematodes, significantly reduced the galling of tomato roots and nematode numbers compared with controls. A similar result was also obtained in pot trials with grapes. Applications of "*B.*" *penetrans* into soil infested with *M. incognita* reduced yield losses of tobacco by 23% or of winter vetch by 38% and in the following year by 24%, 35% and 55% for tobacco, soybeans and winter vetch, respectively (Brown *et al.,* 1985).

As a biocontrol agent, *Pasteuria penetrans* is able to parasitise important nematode pests of several crops and to persist in soil for long periods of time. It is resistant to desiccation and temperature extremes and is compatible with several pesticides including nematicides (Sayre, 1980; Mankau, 1980; Jatala, 1986). However the obligate nature of its parasitism, its lack of mobility and surface charge, its dependence on dispersal by water, animal and man and its inability to be cultured *in vitro* on standard bacteriological media presently limits its commercial utilization.

Other bacteria

Meshram and Jager (1983) showed *Azotobacter chroococcum* to be antagonistic to *Rhizoctonia solani in vitro* and this species has been widely reported to be a PGPR.

Filonow and Lockwood (1985) reported that several actinomycetes (*Actinoplanes missouriensis, A. utahensis, Amorphosporangium*

auranticolor, and *Micromonospora* sp.) as coatings on seeds, increased plant, root and shoot weights, and reduced root rot of soybeans grown in soil naturally infested with *Phytophthora megasperma.*

Kwok *et al.* (1987) reported that strains of *Flavobacterium balustinum* and *Xanthomonas maltophilia* isolated from radish or cucumber roots were antagonistic to soil borne plant pathogens. Both isolates suppressed *Rhizoctonia*-damping-off in hardwood tree bark compost media and the suppressive ability of *F. balustinum* was more effective in combination with a *Trichoderma hamatum* strain. Chen *et al.* (1987) found that both bacterial antagonists when added to a bark compost medium reduced disease severity of *Pythium* damping-off in cucumber.

Tannii *et al.* (1990) found several isolates of *Acinetobacter* sp. inhibitory to 19 potato pathogens tested *in vitro.* Two of these (M24-1 and M5-1) were reported to reduce scab severity by 48% and promote plant growth in two consecutive field crops.

2.1.2 Fungi as biological control agents

Peniophora gigantea (now ***Phlebia gigantea***)

A saprophytic basidiomycete, *P. gigantea*, was the first commercially available biocontrol agent, for the control of *Fomes annosus* (now *Heterobasidion annosum*) on pine trees (Risbeth, 1963). Inoculation of pine stumps with 1 x 10^4 oidia of the antagonist directly after felling significantly protected the wood from naturally infection of "*F. annosus*". As natural competitor of *H. annosum* on pines, *P. gigantea* has shown its efficacy for several years (Risbeth, 1975). However, its ability to protect tree species other than *Pinus radiata* was less effective. *Phlebia* competes with *Heterobasidion* for subtrates

with the production of antibiotics and also parasitizes *Heterobasidion* mycelia (Gindrat, 1979; Campbell, 1989). Commercial inocula of the antagonist are produced in dehydrated tablets and in fluid form. The former has a storage life of two months at 22°C and is prepared by dissolving in water, the latter has a shelf-life of 4 months at 20°C and is prepared by diluting in water plus dye.

Gliocladium spp.

A pathogen of *Rhizoctonia solani* Kuhn was first observed to be parasitized by *Gliocladium* on nutrient culture media by Weindling (1932) and production of a toxin (gliotoxin) by *G. fimbriatum* responsible for inhibition of the pathogen was reported by the same author (Weindling, 1937; 1941). Interest in the control of fungal plant pathogens by fungal antagonists has increased significantly since these initial reports (Papavizas, 1985). The antagonism is generally considered to be parasitic rather than by antibiotic production, although Howell (1987) showed (by the use of nonparasitic-mutants) that mycoparasitism was not the only mechanism of control. Lumsden and Locke (1989) thought that the control of pathogens was likely to be related to the production of specific metabolites. A considerable specificity in biological control was apparent, due to differences in susceptibility of strains of the same pathogen to a single biocontrol strain, in addition to specificity due to various strains of a biocontrol agent (Papavizas and Collins, 1990).

G. roseum. This fungus is found frequently in nature growing on other fungi. Shigo (1958) isolated *Gliocladium roseum* Bainier from oak-wilt trees which, *in vitro*, overgrew and killed *Ceratocystis fagacearum* as well as its mats in nature. Barnet and Lilly (1962) isolated several *G. roseum* strains from soil, from parasitized fungi

and from infected plants, that *in vitro* parasitized *C. fimbriata*, *Trichothecium roseum*, *Rhinotrichum macrosporum* and *Helminthosporium sativum*. The spores and vegetative cells of pathogens would die following contact with the parasite, indicating possible parasitism. However, Walker and Maude (1975) found that *G. roseum* produced substances which inhibited the growth of hyphae of *Botrytis allii*. In addition, they reported that the antagonist was often found to be a contaminant of isolates of *B. allii* taken from growing plants and onion bulbs and it also occurred on *Botrytis* sclerotia. Moody and Gindrat (1977) found a destructive mycoparasite of cucumber black root rot, *Phomopsis sclerotioides,* in a black highly organic soil. The pathogens were completely destroyed and disintegrated by *G. roseum* when washed mycelial mats of the pathogenic fungus were infested with conidia of the antagonist *in vitro*.

G. virens. Tu (1980b) isolated *G. virens* from decomposed sclerotia of *Sclerotinia sclerotiorum* infection of white bean. *In vitro*, the pathogen failed to form sclerotia when the antagonist and *S. sclerotiorum* were grown together on PDA plates. A distinct tropism of antagonist toward the host hyphae occurred when *G. virens* was inoculated after the fungus had formed sclerotia. McCredie and Sivasithamparam (1985) found that *G. virens* isolated by a baiting technique using *S. sclerotiorum*, failed to destroy the sclerotia in the soil at 22.5°C after 10 weeks. However, Phillips (1986) found a strain of *G. virens* from surface sterilized sclerotia of *S. sclerotiorum*, that *in vitro*, was inhibitory to mycelial growth of *S. sclerotiorum* when they were in contact. The incidence of rotted sclerotia in a glasshouse trial six weeks following antagonist addition was 76.6% compared with a control at 22.0%.

G. virens may also be an effective biocontrol agent of damping-off caused by *P. ultimum* and *R. solani*. Howell (1982) reported that addition of *G. virens* to cotton-seed planted in soil infested with either *R. solani* or *P. ultimum*, reduced pre-emergence damping-off by more than half, however this strain was not significant in suppressing post-emergence damping-off compared with controls. Lumsden and Locke (1989) tested twenty isolates of *G. virens* against *P. ultimum* and *R. solani* in zinnia, cotton and cabbage grown in soilless mix, finding three of them to effectively control damping-off of the three plants after 1 and 2 weeks of trial.

G. virens (G1-21) isolated from a mycelium of *Sclerotium rolfsii* was found to be a promising antagonist to *R. solani* infection in field trials. Application of conidia of the antagonist to seed potatoes infested with sclerotia of *R. solani* before planting, reduced sclerotial germination by 86% and disease incidence by 55% (Beagle-Ristaino and Papavizas, 1985). As well as reducing soil-borne levels of the pathogen, the population of the antagonist in the soil also increased over time. Lewis and Papavizas (1985) showed that as mycelial preparations, this strain of *G. virens* was able to protect beet seed from damping-off caused by *R. solani, in vitro*. The efficacy of the fungus in preventing damping-off of cotton sugar beet and radish seedlings was also shown in glasshouse trials.

The suppression of *S. rolfsii*-damping-off and bean blight by *G. virens* strains (strain G1-21 reported above and a further strain, G1-3) was reported by Papavizas and Lewis (1989), who also found them to be more effective in controlling *S. rolfsii* than strains of *Trichoderma harzianum*. One of these (G1-3) also reduced the pathogenicity of *S. rolfsii* sclerotia against snap beans after 32 days trial (Papavizas and Collins, 1990). Strains of *S. rolfsii* varied in their susceptibility to the activity of G1-3, with strains having larger

sclerotia being more resistant.

Other *Gliocladium* species have also been reported to be antagonistic against fungal pathogens. *G. catenulatum* isolated from sclerotia of *S. sclerotiorum* showed a hyperparasitic ability against *S. sclerotiorum* and *Fusarium* spp. (Huang, 1978). The antagonist was capable of killing vegetative hyphae, macroconidia and conidiophores of *Fusarium* spp. as well as the hyphae of *S. sclerotiorum*. The fungus killed the host cells by direct hyphal contact without penetration and without development of intracellular hyphae, causing the affected cells to collapse or disintegrate. Tschen *et al.* (1989) reported that *G. deliquescens* strain F-92 produced an antibiotic and inhibited growth of *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Rhizomucor miehei*, *Paecilomyces variotii*, and *Rhizoctonia solani in vitro*. A significant reduction in basal stem rot was evident when the basal stems of chrysanthemum cuttings coated with the antagonist were planted in sand infested with *R. solani*. Furthermore, they found that the crude extracts of F-92 was more efficient than the bacterial antagonist in preventing the infection of *R. solani* in the soil.

Trichoderma spp.

Trichoderma spp. are the most frequently reported of the fungi used as biological control agents. The ability of *Trichoderma* spp. to attack and destroy fungal pathogens on artificial media was first reported by Weindling (1932). Their involvement in controlling plant pathogens has been reviewed elsewhere (Papavizas, 1985; Adams, 1990), with six species (*T. hamatum, T. harzianum, T. viride, T. koningii, T. pseudokoningii,* and *T. polysporum*) known to act as biological control agents.

T. hamatum. Dennis and Webster (1971) tested 9 isolates of *T.*

hamatum that were active against *Fomes annosus* and further *in vitro* studies showed that 6 of these were antibiotic producers.

Reduction of strawberry fruit rots caused by *Botrytis cinerea* and *Mucor mucedo* following treatment with *T. hamatum* was reported by Tronsmo and Dennis (1977).

A strain of *T. hamatum* (85) isolated from soil naturally suppressive to *R. solani,* when tested on plates, covered, coiled and degraded the pathogen mycelia (Chet and Baker, 1981). Application of conidia of this fungus to pea or radish seeds protected them and their seedlings from *Pythium* spp. or *R. solani* damping-off *in vitro* (Harman *et al.,* 1980). A combination of this antagonist and *Chaetomium globosum* however was less effective on peas than *Trichoderma* alone. Similar suppression of both of the above pathogens with pea and radish as hosts was reported by Harman *et al.* (1981). They also showed that a combination of *T. hamatum* with *Rhizobium* as treatment of bean and pea seeds was not detrimental to nodulation and provided an added protective ability. Hubbard *et al.* (1983) found that the failure of *T. hamatum* (strain 85) to protect peas from seed rots caused by *Pythium* spp. in soils with low iron availability was due to antagonism of *T. hamatum* by fluorescent pseudomonads as a result of iron-immobilization.

Kuter *et al.* (1983) isolated *T. hamatum* strain 382 from hardwood bark compost, and the ability of this isolate to suppress *Rhizoctonia*-damping-off of radishes was subsequently demonstrated by Nelson *et al.* (1983) and Kwok *et al.* (1987). This strain gave better protection against the pathogen when it was combined with other bacterial antagonists.

Beagle-Ristaino and Papavizas (1985) reported that *T. hamatum* (Tri-4) applied as fermentor biomass (FB) reduced potato tuber-borne

sclerotial germination of *R. solani* by 83% in the glasshouse. In the field, the survival of the pathogen propagules was also significantly suppressed. A decrease in "population" of the pathogen was followed by an increase in the antagonist "population" by 1,000-fold within 4 weeks. As mycelial preparations, this antagonist reduced survival of *R. solani* in infested beet seed in soil and also provided protection of cotton, sugarbeet and radish from this fungus (Lewis and Papavizas, 1985).

T. harzianum. A strain of *T. harzianum* isolated from sclerotia of *Sclerotium rolfsii* was inhibitory to *S. rolfsii, Sclerotinia trifoliorum* and *B. cinerea in vitro* (Wells *et al.,* 1972). This isolate controlled the pathogen on blue lupins, tomatoes and peanuts in greenhouse trials. Under field conditions, reduction of disease of tomato transplants following application of the antagonist inoculum onto the soil surface occurred in one-third of the trials. Abd-El-Moity and Shatla (1981) found that complete darkness was an essential factor for mycoparasitism by *T. harzianum* against *Sclerotium cepivorum in vitro.* Sterile culture filtrate of this fungus obtained from a culture incubated in darkness inhibited the growth of the pathogen, but that from culture incubated in continuous light did not. Under greenhouse conditions, introduction of this antagonist into pasteurized soil infested with the pathogen significantly reduced the onion disease from 92% infection to 26-60% infection. In a field trial, the antagonist (as barley grain preparations) significantly controlled white rot disease of onion. Papavizas and Lewis (1989) reported four strains of *T. harzianum* which as conidial suspensions, suppressed damping-off of bean caused by *S. rolfsii* by 30-50% after 10 days and blight caused by *S. rolfsii* by 36-74% after 32 days trial. When a combination of the strains was used, suppression of the pathogen was only as effective as that attributable to single strains, suggesting that competition among strains

in mixtures reduced their combined effectiveness.

A protection of radish seedlings from damping-off caused by *R. solani* and an increase in seedling germination was achieved following application of a wheat bran inoculum of *T. harzianum* to five successive weekly plantings (Henis *et al.*, 1978). Control of damping-off of bean, tomato and eggplant seedlings by an isolate of *T. harzianum* was also reported by Hadar *et al.* (1979). Both reports noted that addition of pentachloronitrobenzene to the inoculum resulted in an additive effect on disease control and a synergistic effect on the decrease in population of pathogen propagules. Elad *et al.* (1980) reported an isolate of *T. harzianum*, as wheat bran preparations, significantly suppressed disease caused by *R. solani* in field trials with beans, cotton and tomatoes. This fungal antagonist was isolated from a soil naturally infested with both *R. solani* and *S. rolfsii*, and was inhibitory *in vitro* to both of these pathogens and to *Pythium aphanidermatum*. Disease incidence in carnations was reduced by 70% following treatment with a *Trichoderma* inoculum into field soil fumigated with methyl bromide (Elad *et al.*, 1981b). The best control was achieved when the antagonist was applied and established in the rooting mixture before transplanting in the field.

The ability of *T. harzianum* to control diseases caused by *R. solani* has been shown in a wide variety of plants. Elad *et al.* (1981a) reported the success of *T. harzianum* in suppressing black root rot in strawberry caused by *R. solani* in nurseries and in the field. A decrease of disease was also observed in antagonist-treated soil that was successively planted with bean seedlings. Bell *et al.* (1982) also showed significant antagonistic activities of *Trichoderma* isolates against fungal pathogens, especially *R. solani, in vitro*.

A strain of *T. harzianum* isolated by Chet and Baker (1981) from

a soil naturally suppressive to *R. solani* in Colombia and showing promise as a biological control agent of plant pathogens, was mutated to benomyl-tolerance, resulting in significantly increased plant growth of various floricultural and horticultural crops treated with benomyl (petunia, chrysanthemums, periwinkle, pepper, tomato, cucumber) (Chang *et al.,* 1986). This mutant aggressively colonized the rhizosphere compared with the wild type when applied as a seed treatment (Ahmad and Baker, 1987), a feature considered crucial for the effectiveness of a biological control agent. The root colonizing ability of this strain was found to be influenced by benomyl, soil pH and temperature. Ahmad and Baker (1988) further demonstrated a significant reduction in the incidence of pre-emergence damping-off of barley, cucumber, pea, radish, and tomato caused by *P. ultimum* following seed treatment with the rhizosphere-competent mutant. In contrast the wild type was less effective in controlling the pathogen. Reductions in pathogen population in rhizosphere soil and colonization of cucumber root tips by *Pythium* were also evident after treating with the mutant strain. Combination of this isolate with another antagonist of *Pythium nunn* to control *Pythium* damping-off in a range of soils resulted in no consistent control (Paulitz *et al.,* 1990). It was assumed that some physical, chemical, or biological factor had affected the host, the pathogen, or the antagonist.

T. harzianum was isolated abundantly from a compost that was suppressive to *Rhizoctonia* damping-off (Nelson *et al.,* 1983; Kuter *et al.,* 1983), and its addition to compost-planted radish seedlings significantly reduced the severity of the disease. The degree of suppression by this isolate was influenced by the age of the compost, although its suppressive ability was not altered following inoculation into pasteurized compost.

A strain of *T. harzianum* (WT-6) was reported to be effective as a biocontrol agent in the field against fruit rot of cucumber caused by *R. solani* (Lewis and Papavizas, 1980). Ultraviolet irradiation of this strain resulted in mutants with enhanced suppressive abilities against pathogens compared with the parent strain (Papavizas *et al.*, 1982). In pot trials these mutants were more effective than the wild isolate in controlling damping-off of peas caused by *P. ultimum*, damping-off of cotton and radish caused by *R. solani*, and white rot of onion caused by *S. cepivorum.*

Delivery methods of the antagonist into substrates influence the control of the pathogens, with mycelial (but not conidial) preparations of isolate WT-6 preventing damping-off of cotton, sugar beet and radish seedlings in the greenhouse (Lewis and Papavizas, 1985). In addition, as fermentor biomass preparations, this isolate significantly reduced sclerotial viability on potato tubers and in soil (Beagle-Ristaino and Papavizas, 1985).

Tschen *et al.* (1989) reported the protection of chrysanthemum from *Rhizoctonia*-basal stem rot following treatment with two strains of *T. harzianum* in sawdust composts. These isolates produced antibiotics *in vitro* and their crude extracts were inhibitory to *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Paecilomyces variotii* and *Rhizomucor miehei.* Control of *Rhizoctonia* disease in chrysanthemum by antagonists was shown to depend on the strain of the antagonists and the delivery systems used.

Inhibition of *Pythium* spp. by *T. harzianum* isolates has been reported by several workers. Chet and Baker (1981) showed successful control of *Pythium* infection of beans, and reductions of seed rot and damping-off caused by *Pythium* spp. in peas and cucumbers following conidial application of a strain T-12 were reported by Hadar *et al.* (1984). However, protection by this antagonist was not demonstrated

in a field trial, when low temperatures were suspected to be responsible for the failure. The antagonist grew optimally at 25-30°C while field temperatures fell within the range of 10-20°C. Vannacci and Harman (1987) found that isolate T-12 was also effective in reducing radish diseases caused by *Alternaria raphani* and *A. brassicicola* under greenhouse conditions. Ahmad and Baker (1987) reported that a mutant of strain T-12 (T-12B) tolerant to benomyl was more competent in colonizing cucumber roots than the wild type. This strain applied as a seed treatment was able to suppress pre-emergence damping-off of barley, cucumber, pea, radish and tomato induced by *P. ultimum* more effectively than the parent strain (Ahmad and Baker, 1988).

Sivan *et al.* (1984) isolated a strain of *T. harzianum* (T-315) from soil naturally infested with *P. aphanidermatum.* A culture filtrate of this species was inhibitory to the pathogen and as a wheat bran/peat preparation mixed with loamy sand, the antagonist significantly reduced the disease incidence in cucumbers, peas and tomatoes by 69, 81 and 85%, respectively. Similar results were achieved with conidial suspensions applied to seeds. T-135 was subsequently shown to suppress *R. solani* and *S. rolfsii* in beans (Sivan *et al.,* 1984).

Kelley and Rodriguez-Kabana (1976) reported a reduction in the growth of *Phytophthora cinnamomi* by *T. harzianum* (T-386) based on ß-glucosidase and phosphatase activities and further showed that the antagonist utilized the *Phytophthora* mycelium as well as nutrients released by *P. cinnamomi* as substrates. Glasshouse testing of isolate T-386 showed suppression of *P. cinnamomi* damping-off of pine seedlings in autoclaved soil under low moisture conditions, but it had no effect on the pathogen in wet condition or in non-autoclaved soil. It therefore was not considered an effective biocontrol agent for

P. cinnamomi (Kelley, 1976).

There have been several reports of the interactions between *T. harzianum* and *Gaeumannomyces graminis*. Maas and Kotze (1987) reported that two isolates (ATCC 20475 and PREM 47942) significantly inhibited mycelial growth of *G. graminis* var. *tritici in vitro* and reduced wheat take-all in soil artificially infested with this pathogen in the greenhouse, with strain PREM 47942 being the most effective in suppressing the disease. *T. harzianum* (strain 70) isolated from wheat rhizosphere protected wheat from take-all fungus in pot trials with sterilized soil, but did not in a non-sterilized soil (Dewan and Sivasithamparam, 1988b). Ghisalberti *et al.* (1990) showed an isolate of *T. harzianum* (strain 71) producing pyrones to be the most effective inhibitor of take-all of wheat.

T. koningii. Hadar *et al.* (1984) isolated a strain of *T. koningii* T8 (ATCC 56679) from soil which under controlled conditions, protected pea seed roots and seedlings from damping-off caused by *Pythium* spp., as well as the pre-emergence and post-emergence damping-off of cucumber. In soils naturally infested with *Pythium*, a strain of *T. koningii* (T8) was effective against seed rots of peas and snap beans. This antagonist also showed antagonism towards *Alternaria raphani* and *A. brassicicola* pathogens of radish plants (Vannacci and Harman, 1987).

Trichoderma spp. made up 71% of the fungi in soils found to be suppressive to *Gaeumannomyces graminis* var. *tritici* compared with a 34% incidence in conducive soils (Simon and Sivasithamparam, 1988b). Twenty three of 27 *Trichoderma* isolates from suppressive soils were *T. koningii*. When soils were inoculated with *T. koningii* the saprophytic growth of the pathogen was reduced in the inoculated soil, but not in the uninoculated soil (Simon and Sivasithamparam,

1988c). This antagonist produced antibiotics that inhibited the growth of only one of 13 bacteria tested from suppressive soils, yet inhibited 13 of the 20 bacterial isolates tested from conducive soils (Simon and Sivasithamparam, 1988a). Its antibiotic was identified as 6-*n*-pentyl-2H-pyran-2-one (pyrone), which also inhibited the growth of take-all fungus, and *Trichoderma* isolates including the producer strain. Simon (1989) found the antagonist reduced stellar discolouration of ryegrass (*Lolium rigidum* L.) caused by *G. graminis* by 95% in soil inoculated with the pathogen and antagonist two weeks before sowing under controlled environmental conditions, or by 52% if inoculated just before sowing. Reduction of take-all of wheat grown in pots containing field soil was also achieved, with application of antagonist two weeks prior to sowing giving best results.

Parasitic ability of *T. koningii* against *Sclerotinia sclerotiorum* both *in vitro* and in field trials was shown by Trutmann and Keane (1990). *In vitro* the antagonist parasitized the pathogen by coiling the host hyphae and growing within it. In the field, application of this isolate coated onto sclerotia resulted in significant reduction in the viability of sclerotia and an increased number of sclerotia infected with the antagonist in spring and summer. However, its application as a post-harvest soil treatment in winter did not protect the infection of bean stem by sclerotia. It appeared that *T. koningii* has potential as a control agent for *S. sclerotiorum* when applied in warmer conditions.

Trichoderma viride. A species of *T. viride* has long been known to produce the antibiotic gliotoxin in culture media and in soil (Wright, 1956) and the ability of this species to invade hyphae of various fungal pathogens was reviewed by Durrell (1968). A strain of *T. viride* successfully prevented silver leaf disease of plum trees (*Prunus mariana*) caused by *Stereum purpureum* as well as silver leaf

diseases of peach and nectarine trees (Dubos and Ricard, 1974).

Locke *et al.* (1985) reported the control of *Fusarium* wilt of chrysanthemums by *T. viride* (T-1) in the glasshouse. This strain and its mutant (T-1-R9), alone or in combination with *Aspergillus ochraceus*, suppressed the disease by more than 50% in vegetatively maintained plants. Protection provided by the mutant strain T-1-R9 was as effective as fungicide treatment. This isolate as fermentor biomass preparation also reduced stem canker and black scurf of potato caused by *Rhizoctonia solani* in the field (Beagle-Ristaino and Papavizas, 1985). In addition, sclerotial germination of the pathogen was reduced by 88% following treatment with T-1-R9 prior to planting in the glasshouse.

A *T. viride* isolate from the phylloplane of lettuce reduced ascospore germ-tube elongation of *Sclerotinia sclerotiorum* by 34% in leaf disc trials and reduced lettuce drop by 40% under controlled conditions (Mercier and Reeleder, 1987).

Trichoderma pseudokoningii. Dennis and Webster (1971) studied diffusible inhibitors produced by an isolate of *T. pseudokoningii*, and the parasitic ability of this fungus against fungal pathogen. Reduction of apple fruit rots caused by *Botrytis cinerea* was significant following application of this antagonist in the field (Tronsmo and Raa, 1977). Application of a strain of this fungus also resulted in significant reduction of strawberry fruit rots (Tronsmo and Dennis, 1977). This isolate produced non-volatile and volatile metabolites that were inhibitory to pathogenic fungi *in vitro*. The ability of the antagonist to control the pathogen, and produce antagonistic metabolites was temperature dependent.

Trichoderma polysporum. Tronsmo and Dennis (1977) found that volatile and non-volatile metabolites produced by an isolate of *T. polysporum* were inhibitory to *Botrytis cinerea* and *Mucor mucedo.*

Maas and Kotze (1987) reported that strain ATCC20475 significantly reduced wheat take-all in soil infested with *Gaeumannomyces graminis* var. *tritici* and increased plant growth in the glasshouse.

Fusarium spp.

It appears that not all *Fusarium* species are pathogenic to host plants. Sitepu and Wallace (1984) showed that *F. lateritium* inhibited spore germination and mycelial growth of *Sclerotinia sclerotiorum* on agar media. This antagonist protected 67% of lettuce seedlings against *S. sclerotiorum* compared with 100% mortality of control plants. Gupta and Agarwala (1990) reported that cauliflower-stalk rot caused by *S. sclerotiorum* was effectively controlled by a strain of *F. solani* in the glasshouse. Suppression of pathogenic *S. sclerotiorum* in beans by strains of *F. graminearum* and *F. heterosporum* was reported by Boland and Inglis (1989).

Vannacci and Harman (1987) found that a strain of *Fusarium* sp. isolated from cruciferous seeds reduced radish infections caused by *Alternaria raphani* in controlled environments. It also reduced the incidence of *A. brassicicola*-infected cabbage seedlings.

Sporidesmium sclerotivorum

A strain of *S. sclerotivorum* isolated from soil by Uecker *et al.* (1978) was strongly parasitic on *Sclerotinia* spp.. Adams and Ayers (1980) reported that this antagonist, in appropriate environmental conditions, completely destroyed sclerotia of *Sclerotinia minor* in field soil after 10 weeks trial. A significant decline in the numbers of sclerotia of *S. minor* and *Sclerotium cepivorum* by *S. sclerotivorum* in field trials was reported by Adams and Ayers (1981). They also found that the antagonist was able to control potato stem rot caused by

Sclerotinia sclerotiorum and onion white rot caused by *S. cepivorum.* Sclerotial viability of *S. minor* was significantly reduced (by 75-95%) following addition of the antagonist into soil (Adams and Ayers, 1982; Adams, 1989). Application of this antagonist to lettuce plants infested with the pathogen reduced disease incidence by 40-83% compared with the controls in four consecutive crops (Adams and Ayers, 1982).

Successful biological control of *Sclerotinia* spp. by *S. sclerotivorum* depends on soil populations of both the host and the mycoparasite. The extent of sclerotial decay was affected by the amount of antagonist added; *in vitro* about 5 macroconidia of the mycoparasite g^{-1} of soil were needed to get a successful infection of the host (Adams *et al.,* 1984). Application of the antagonist preparation at rates of 0.2, 2.0, or 20 kg/ha to lettuce plants infected with *S. minor* significantly reduced plant infections compared with the untreated controls in three consecutive crops in field trials (Adams and Fravel, 1990). In the third crop, 53, 68, and 72% disease control was obtained relative to the control at the 0.2, 2.0, and 20 kg/ha rates, respectively.

Sterile red fungus

Sterile red fungi are abundant on the roots of rye-grass (*Lolium rigidum*) and wheat (*Triticum aestivum*) (Waid, 1957; Dewan and Sivasithamparam, 1988a). When this fungus was associated with take-all fungus (*Gaeumannomyces graminis*) on the plant roots, the plant remained healthy. Application of the sterile red fungus into take-all fungus-infested soils growing wheat, rye-grass, barley, great brome, chick pea, lupins, medic, oats, peas, rape, and subterranean clover resulted in control of the disease and promotion of plant growth (Dewan and Sivasithamparam, 1988a; 1989a; 1989b). Success in controlling take-all of wheat following treatment with the sterile red

fungus in a field trial was also reported by Dewan and Sivasithamparam (1990). Exudate of the antagonist was shown to increase the growth of plants and was found to have antibiotic activity against *G. graminis* and *Phytophthora cinnamomi* (Gillespie-Sasse *et al.*, 1991).

Coniothyrium minitans

C. minitans was first isolated from sclerotia of *Sclerotinia sclerotiorum* by Campbell (1947). This species is known as the primary parasite of *Sclerotinia* spp.. Tribe (1957) reported that 85-99% of sclerotia of *Sclerotinia trifoliorum* were killed within 11 weeks following treatment with a sand-cornmeal culture of *C. minitans*. Application of this mycoparasite, as a pycnidial dust, into *S. trifoliorum*-infested soil resulted in inconsistent destruction or infection of sclerotia (Turner and Tribe, 1975).

Huang and Hoes (1976) observed destruction of hyphae and sclerotia of *S. sclerotiorum* by *C. minitans in vitro*. The mycoparasite penetrated the host causing destruction of cell walls. Turner and Tribe (1976) reported that this antagonist was also able to parasitise *S. minor*, *Botrytis cinerea*, *B. fabae* and *Sclerotium cepivorum*. Huang (1977) investigating hyperparasitism of *S. sclerotiorum* by *C. minitans* occurring naturally in sunflowers over the growing season, found that the antagonist parasitized and killed sclerotia produced on root surfaces (59% effective), inside the root (76% effective), and inside the stem (29% effective).

Pythium spp.

Most of genera of *Pythium* are known to be plant pathogens, but some of them have been found to be antagonistic to their relatives. Sneh *et al.* (1977) found a species of *Pythium* sp. parasitic upon *Phytophthora megasperma*, *P. cactorum*, *Pythium* sp., and

Aphanomyces euteiches in natural soil. Similarly, Lifshitz *et al.* (1984a) reported a strain of *Pythium* sp. which was mycoparasitic against pathogenic *Pythium* sp.. Addition of this antagonist to soil suppressed the pathogenic population and pre-emergence damping-off of cucumber in pot trials.

A strain of *Pythium oligandrum* showed its ability to parasitise a wide range of host fungi which were classified as highly susceptible, moderately susceptible, or resistant (Deacon, 1976; Deacon and Henry, 1978; Foley and Deacon, 1986). An isolate of this fungus coated onto cress seeds, sugar beet and carrot decreased damping-off of seedlings caused by either *P. ultimum* or *Phoma betae* in sand-culture or in naturally or artificially infested field soils (Al-Hamdani *et al.,* 1983; Lutchmeah and Cooke, 1985; Walther and Gindrat, 1987). Martin and Hancock (1987) found that *P. oligandrum* was a non-pathogenic fungus on 12 species of economic crop plants representing the families Leguminosae, Cucurbitaceae, Cruciferae, Compositae, and Chenopodiaceae. As seed treatments, this fungus was as effective as a fungicide in controlling pre-emergence damping-off of sugar beet caused by *P. ultimum* in the greenhouse. Campbell (1989) reported that oospore preparations of *P. oligandrum* were available commercially on a small scale. McQuilken *et al.* (1990a) reported successful controls of damping-off of cress (*Lepidium sativum*) and sugar beet (*Beta vulgaris*) caused by *Pythium* spp., *P. ultimum, Rhizoctonia solani,* and *Aphanomyces cochlioides* following treatment of the seeds with *P. oligandrum* in pot trials. In some cases the degree of control was found to be equivalent to fungicide treatment. McQuilken *et al.* (1990b) developed a liquid culture preparation of biocontrol agent *P. oligandrum* that appeared more beneficial than a solid preparation.

Since its discovery, *Pythium nunn* (strain N3) has been found to be a promising biocontrol agent for plant pathogenic fungi (Lifshitz *et al.,* 1984b). Application of this antagonist into soil conducive to *Pythium* sp. resulted in the suppression of pathogenic *Pythium* and an increased propagule population of *P. nunn* (Lifshitz *et al.,* 1984a). *In vitro*, *P. nunn* showed a strong mycoparasitism against several soil fungi (Lifshitz *et al.,* 1984c).

Paulitz and Baker (1987b) found that in sterilized-soil infested with *P. ultimum*, *P. nunn* (N3) reduced the incidence of damping-off in cucumbers. The suppression of the pathogen by antagonist was variable, depending on the delivery system, temperature, pH, matric potential, and organic amendments (Paulitz and Baker, 1987a). The ability of strain N3 to compete with *P. ultimum* on bean leaf fragments in naturally and artificially infested soils was shown by Paulitz and Baker (1988). Paulitz *et al.* (1990) combined *P. nunn* and *Trichoderma harzianum* T-95 (an aggressive root coloniser) in an attempt to control *Pythium* damping-off of cucumber. Inconsistent results were obtained for both fungal interactions and disease reduction depending on soil type.

Laing and Deacon (1990) claimed *P. oligandrum* to be the most aggressive mycoparasite of the *Pythium* antagonists tested based on its ability to reduce cellulolytic activities of the host fungi.

Verticillium biguttatum

Verticillium sp. is an obligate mycoparasite of *Rhizoctonia solani* on potato tubers. Jager *et al.* (1979); Boogert and Jager (1983); and Jager and Velvis (1983) conducted a series of trials using *V. biguttatum* as a biocontrol agent of potato tuber infections caused by *R. solani.* Subsequently it was shown that potato seed tubers

inoculated with *V. biguttatum* (strain M73) significantly reduced sclerotial infection (Jager and Velvis, 1985) and infection by pathogenic *R. solani* (Jager and Velvis, 1986) in the field. In a review on the efficacy of this antagonist, Boogert *et al.* (1990) concluded that it was unaffected in soils treated with fungicides. However, the natural development of *V. biguttatum* was completely dependent on *R. solani*, which in turn was dependent on potato plants for its survival.

Paecilomyces spp.

An isolate of *P. inflatus* from a compost suppressive to *Rhizoctonia* damping-off of radish (*Raphanus sativus* L.) effectively suppressed the disease in pot trials (Nelson *et al.*, 1983). Tschen *et al.* (1989) reported that two strains (CF302, CF407) of *P. marquandii* were hyperparasitic on *R. solani*, and both strains significantly controlled infection of *Rhizoctonia*-basal stem rot of chrysanthemum. *In vitro*, the antagonist produced antibiotics that were inhibitory to the pathogen. Seed treatments of sunflower (*Helianthus annuus* L.) and mung bean (*Vigna radiata* L.) with spore suspensions of *P. lilacinus* resulted in 80% and 84% disease reductions respectively, after 30 days growth in the field (Hussain *et al.*, 1990).

Penicillium spp.

Boosalis (1956) isolated *Penicillium vermiculatum* parasitic on *R. solani* from soil both *in vitro* and from field trials, yet this antagonist did not control *Rhizoctonia* damping-off or seedling root rot of peas in non-sterilized soil. Similarly Utkhede and Rahe (1980) isolated *Penicillium nigricans* (Bain) from sclerotia of *Sclerotium cepivorum* that was inhibitory to the pathogen *in vitro,* but provided no protection to onions from white rot disease in a field trial. Akem and Melouk (1989) reported that a strain of *Penicillium citrinum* when

applied as conidial suspensions, reduced the incidence of viable sclerotia of *Sclerotinia minor* by 74% in pasteurized soil and by 55% in non-pasteurized soil. A reduction of peach (*Prunus persica*) twig blight disease caused by *Monilinia laxa* following treatment with *Penicillium frequentans* in the glasshouse was reported by De Cal and Sagasta (1990). Application of this antagonist also gave significant disease reduction in field trials.

Epicoccum purpurascens

Mercier and Reeleder (1987) found that *E. purpurascens* reduced ascospore germ-tube elongation of *S. sclerotiorum* by 38.3% and lettuce infection by 46.7%. This antagonist also significantly reduced white mold incidence of snap bean (*Phaseolus vulgaris* L.) in both glasshouse and field trials (Zhou and Reeleder, 1989). Zhou and Reeleder (1990) exposed *E. purpurascens* grown on fungicide-amended media to ultraviolet light resulting in strains that were tolerant to fungicides and with enhanced efficacy against the pathogen.

Hyphochytrium catenoides

The parasitic ability of *Hyphochytrium catenoides* against oospores of pathogenic fungi in soil has been studied by Sneh *et al.* (1977), Ayers and Lumsden (1977), and Humble and Lockwood (1981). Application of this species to soil artificially or naturally infested with *Phytophthora megasperma* effectively reduced root rot of soybean in the greenhouse (Hsu and Lockwood, 1984). In field trials, however, *H. catenoides* provided no protection against the pathogen (Filonow and Lockwood, 1985).

Corticium sp.

A *Corticium* sp. was reported to significantly reduce seedling damping-off of beans, soybeans, and sugar-beet in *R. solani*-infested soils by Odvody *et al.* (1977). Disease incidence of fruit rot of cucumber caused by *R. solani* was reduced by 33% following treatment with the antagonist in the field (Lewis and Papavizas, 1980). Saprophytic activity of the pathogen was also reduced *in vitro* and in pot trials.

Other fungal antagonists

Gees and Coffey (1989) isolated *Myrothecium roridum* that was shown to be strongly inhibitory to *Phytophthora cinnamomi* (strain TW), *in vitro*. In the glasshouse, this isolate significantly controlled *P. cinnamomi* root rot of *Persea indica* in artificially and naturally-infested soils.

Klecan *et al.* (1990) reported a strain of *Tilletiopsis pallescens* parasitizing cultures of *Erysiphe graminis* f.sp. *hordei* (an obligate pathogen of barley aerial surfaces). Leaf segments of barley inoculated with both fungi were protected from powdery mildew disease.

Teratosperma oligocladium was reported by Adams and Ayers (1985) and Adams (1989) to be an aggressive mycopasite of *S. minor*. The antagonist was able to reduce the sclerotial viability by 94% in the soil within a 10-week period (Adams, 1989).

2.2 Mechanisms of pathogen suppression

2.2.1 Substrate competition

Substrate competition occurs when two or more organisms require the same nutrient, usually a carbon or nitrogen source. Those organisms having better uptake mechanisms or more effective extracellular enzymes will reduce nutrient availability to other organisms which eventually cannot compete and die (Campbell, 1989).

Inhibition of conidial germination of fungal pathogens by bacterial antagonists is partly or mainly due to nutrient competition (Brodie and Blackeman, 1975; Elad and Baker, 1985). Competition between *Pseudomonas* sp. and *Botrytis cinerea* on leaves was shown by detection of ^{14}C-glutamine uptake and metabolism. Brodie and Blackeman (1975) found that the *Pseudomonas* antagonist utilised a small proportion of the endogenous organic reserves of *Botrytis* conidia, but a major proportion was from exogenous substrates. Elad and Baker (1985) reported that a siderophore-negative *Pseudomonas* sp. remained inhibitory to clamydospore germination of *Fusarium oxysporum* in the rhizosphere of host plants, even when iron was added. Alabouvette (1990) reported that addition of glucose to provide energy for *Fusarium* resulted in a greater disease incidence in both suppressive and conducive soils, suggesting that competition for carbon by the antagonists was involved.

Nelson *et al.* (1986) found that certain sugars blocked the inhibition of *Pythium ultimum* by *Enterobacter cloacae*. In contrast to this Wisniewski *et al.* (1989) reported that attachment of the antagonist to hyphae and sporangiospores of *Rhizopus stolonifer* occurred following addition of glucose, indicating that bacterial attachment facilitates nutrient uptake by the bacterium.

Introduction of biocontrol agents into plant rhizospheres leads to reduced numbers of indigenous bateria (Weller, 1984; Elad and Chet, 1987), which may be a result of competition for root exudates in the rhizosphere, although other agencies of inhibition are also possible.

2.2.2 Siderophores

Most aerobic and facultative anaerobic microorganisms form extracellular, low molecular weight, high affinity iron (III) chelators (siderophores) to combat iron starvation (Leong, 1986; Neilands and Leong, 1986).

Kloepper *et al.* (1980a) were the first to show that iron (III)-binding siderophores produced by plant growth-promoting rhizobacteria (PGPR) of the genus *Pseudomonas* were involved in the biological control of potato pathogens. Meyer and Abdallah (1978) and Kloepper *et al.* (1980a) reported that pseudomonads produce several types of siderophore molecules that differ in their structure and ability to bind iron. It was found that siderophores produced by bacterial antagonists have higher iron stability constants than those siderophores produced by various pathogenic fusaria (Scher and Baker, 1982; Sneh *et al.,* 1984).

In the glasshouse, treatment of potatoes with PGPR strains A1, B10, and BK1 increased plant growth by 47, 63, and 74%, respectively. Inhibition of *Erwinia carotovora* and enterobactin-negative *Escherichia coli* by PGPR did not occur in high iron agar media suggesting that the inhibition was caused by iron deprivation induced by the siderophore activity (Kloepper *et al.,* 1980a). Growth promotion of potatoes following applications of PGPR was also reported to be due to iron deprivation (Geels and Schippers, 1983a; Xu and Gross, 1986b). Buyer and Leong (1986) proposed the mode of iron deprivation to be the inability of susceptible strains to utilize iron bound to siderophores

of the producing bacteria, since pathogenic strains which were able to utilise these siderophores were not inhibited.

Scher and Baker (1982) added iron chelators or *Pseudomonas putida* into conducive soil, with the result that the soil became suppressive to *Fusarium* wilt pathogens of flax, cucumber, and radish. Yuen and Schroth (1986a) reported that inhibition of *Fusarium oxysporum* by a strain of *Alcaligenes* sp. was mainly affected by the antagonist's siderophore, whereas siderophore-negative mutants did not suppress chlamydospore germination of the pathogen. Several reports also showed that induction of suppressiveness to clamydospore germination and *Fusarium* wilt pathogens could be achieved through iron competition (Vandenbergh *et al.,* 1983; Sneh *et al.,* 1984; Park, *et al.,* 1988).

Becker *et al.* (1985) reported that purified pseudobactin of isolate *P. fluorescens* (B10) inhibited the uptake of iron (III) by the roots of peas and maize and caused reduction of the synthesis of chlorophyll, interpreting this as competitive binding, as occurs with synthetic iron chelators.

The involvement of *Pseudomonas* spp. siderophores in plant growth promotion and biological control of *Pythium* spp. has been reported in wheat (Becker and Cook, 1988), and in cotton (Loper, 1988). The inhibition of *Pythium* was observed on media with low, but not high iron concentrations. *Pseudomonas* mutants lacking siderophores were not inhibitory to *Pythium* on agar media and did not promote plant growth in the soil, showing that production of siderophores was responsible for the suppression of pathogens and plant growth promotion. Similar results were reported by Wong and Baker (1984), and Weller *et al.* (1988), who showed that wheat take-all caused by *Gaeumannomyces graminis* was reduced following

treatment with siderophore-producing fluorescent pseudomonads, both *in vitro* and in the field.

An alternative mechanism of siderophore activity was proposed by Ahl *et al.* (1986) with regard to the inhibition of the fungal pathogen *Thielaviopsis basicola* by *P. fluorescens* (CHAO). It was suggested that, firstly siderophores make iron available in toxic concentrations to the fungus, and secondly the complexed iron-siderophores have by themselves an inhibitory effect on *T. basicola* like antibiotics. This contention was supported by Keel *et al.* (1989) who showed that suppression of the pathogen by strain CHAO was more effective in sufficient iron conditions, and iron competition was not a suppressive mechanism in this system.

The ability of siderophore-producing pseudomonads to suppress fungal pathogens in the soil is affected by soil pH. Alteration of the pH of soils conducive to *Fusarium* from 8.0 to 7.0 resulted in the soil becoming suppressive to the pathogen (Scher and Baker, 1980). However the further decrease of soil pH to 6.0 resulted in a significantly increased disease incidence over that at either pH 7.0 or 8.0. The ability of fluorescent pseudomonads to suppress *Fusarium* wilt of cucumber was enhanced by increasing the soil pH from pH 6.7 to pH 8.1, but not by decreasing it to pH 5.5 (Park *et al.,* 1988). Reduction of the growth of *Pythium aphanidermatum* was significantly greater in the presence of siderophores at pH 8.0 compared with that at pH 6.0 (Misaghi, *et al.,* 1988). The absence of siderophore-producing bacteria in acidic soils suggested that strategies for the development of suppressiveness could be the increasing of soil pH or the genetic manipulation of PGPR to greater tolerance of low pH (Baker, 1990).

Production of siderophores by fluorescent pseudomonads was influenced by temperature (Loper and Schroth, 1981); with strain B10

producing only one-tenth the amount of siderophore at 33°C than it produced at 28°C. Its siderophore production was inhibited at temperatures below 12°C which are commonly found in soil (Xu and Gross, 1986a).

2.2.3 Antibiotics

Antagonists producing antibiotics may play a major role in the biological control of plant pathogens, with serious attention only being given to them following a report by Hsu and Lockwood (1969) that an unidentified antibiotic produced by *Streptomyces* spp. was inhibitory to *Mucor ramannianus* and *Glomerella cingulata.*

Agrobacterium radiobacter

The antibiotic Agrocin 84, a substituted adenine nucleotide, produced by *Agrobacterium radiobacter* var. *radiobacter* strain 84, effectively protects against crown gall disease caused by *A. radiobacter* var. *tumefaciens.* A mutant of strain K84 (K84Agr$^-$) which was agrocin-negative was ineffective in controlling the gall compared with the parent strain in the field (Cooksey and Moore, 1982). This antibiotic is specifically toxic to agrobacteria carrying a nopaline or agrocinopine A type Ti plasmid (Kerr and Htay, 1974; Roberts and Kerr, 1974). Kerr and Htay (1974) found a significant reduction of the sensitive pathogen population in tomato wounds inoculated with strain 84 (at a 1:1 ratio of pathogen and antagonist), but the strain 84 was ineffective against agrocin-resistant pathogens. Kerr (1980) reviewed the evidence for the efficacy of agrocin 84 in the control of crown gall, concluding that only nonpathogenic agrobacteria producing agrocin 84 control crown gall, and only pathogenic agrobacteria sensitive to agrocin 84 can be controlled. Also genetic data showed that the production of agrocin 84 was coded by a plasmid.

Agrocin 84 is thought to inhibit DNA synthesis in sensitive strains (Das *et al.,* 1978; Murphy and Roberts, 1979), although Smith and Hindley (1978) demonstrated an impaired binding of pathogenic cells to host tissues, proposing that it was due to interference of the synthesis of the outer cell envelope of the bacteria.

Pseudomonas spp.

Pseudomonas fluorescens (strain Pf-5), produces two antibiotics: pyrrolnitrin (3-chloro-4-[2'-nitro-3'-chlorophenyl]-pyrrole) and pyoluteorin (4,5-dchloro-1 *H*-pyrrol-2-yl-2,6-dihydroxyphenyl ketone) (Howell and Stipanovic, 1979; 1980). The former antibiotic was strongly inhibitory to *R. solani* and other fungi associated with cotton seedling diseases except *Pythium ultimum*; the latter was toxic only to *P. ultimum*. Treating cotton seeds with 200 µg/ml pyrrolnitrin increased healthy seedlings by 13 to 70% above the controls. Similarly, inoculation of the seed with the antagonist planted into *R. solani*-infested soil increased the germination of healthy seedlings by 30 to 79% above the controls (Howell and Stipanovic, 1979). Treatment of cotton seed with a similar amount of pyoluteorin increased seedling survival in *P. ultimum*-infested soil by 33 to 65% (Howell and Stipanovic, 1980). Janisiewicz and Roitman (1988) reported that reduction of apple gray mold (caused by *Botrytis cinerea*) and pear blue mold (caused by *Penicillium expansum*) by *Pseudomonas cepacia* was due to the production of the antibiotic pyrrolnitrin. Both diseases were completely controlled by treating with the antibiotic at a concentration greater than 50 mg/l (Janisiewicz and Roitman, 1988). Similar results were also found by Janisiewicz *et al.* (1991). Homma *et al.* (1989) showed that pyrrolnitrin produced by a strain of *P. cepacia* was effective against several soil-borne plant

pathogens. This antagonist also produced two kinds of pseudane: 2-(2-heptenyl)-3-methyl-4-quinolinol and 2-(2-nonenyl)-3-methyl-4-quinolinol. Défago *et al.* (1990) identified some of the chemical suppressors of tobacco black root rot produced by *P. fluorescens* (CHAO) as pyoluteorin, 2,4-diacetylphloroglucinol, monoacetylphloroglucinol, and salicylic acid, although these were not the main inhibitors of the pathogen.

The antibiotic phenazine-1-carboxylic acid produced by *P. fluorescens* (strain 2-79) was reported to be responsible for the inhibition of wheat take-all (Gurusiddaiah *et al.,* 1986; Brisbane and Rovira, 1988; Brisbane *et al.,* 1989). The structure of this antibiotic was that of a monomer which was toxic to phytopathogens under acid conditions (Brisbane *et al.,* 1987; Brisbane and Rovira, 1988). Phenazine inhibited *Gaeumannomyces graminis in vitro* at a minimum concentration of 1 μg/ml, it was also inhibitory to several fungal pathogens and bacteria (Gurusiddaiah *et al.,* 1986). Mutants of strain 2-79 (Phz^-), deficient in production of this antibiotic lost their ability to control take-all in wheat seedlings (Thomashow and Weller, 1988). Production of phenazine on the wheat rhizosphere by strain 2-79 was evident both *in vitro* and *in vivo* (Brisbane and Rovira, 1988; Thomashow *et al.,* 1990). However, phenazine was not effective against plant pathogens in alkaline environments (Brisbane *et al.,* 1987; Brisbane and Rovira, 1988).

P. fluorescens strain HV37a produces three unidentified antibiotics inhibitory to *Pythium ultimum* (James and Gutterson, 1986). Production of these antibiotics was affected by glucose (at concentrations up to 2%) and at least five genes were involved (James and Gutterson, 1986; Gutterson *et al.,* 1986). Mutants deficient in glucose dehydrogenase were ineffective in inhibiting the fungus (James

and Gutterson, 1986).

Several *Pseudomonas* spp. isolated from rough lemon (*Citrus jambhiri* Lush) produce antibiotics inhibitory to the citrus pathogens (Gardner *et al.,* 1984). Unidentified antibiotics were also reported to be produced by fluorescent pseudomonads and played a role in suppressing potato soft rot caused by *Erwinia carotovora* (Xu and Gross, 1986b). A new pseudomonad species (*Pseudomonas antimicrobica*) was reported to release a broad range of water soluble, thermolabile antifungal and antibacterial substances by Attafuah and Bradbury (1989). A strain of *P. cepacia* which was suppressive to various corn fungal pathogens also produced an antifungal compound which was resistant to protease enzyme, heat, acid or alkali (Jayaswal *et al.,* 1990). The nature of this compound was different from previous compounds investigated from pseudomonads.

Bacillus spp.

Bacillus spp. have been reported as potential biocontrol agents for several fungal plant pathogens (Hodgson, 1970; Pusey and Wilson, 1984; Handelsman *et al.,* 1990). Antibiotic-producing *Bacillus subtilis* inhibited the stone fruit pathogen *Monilinia fructicola in vitro* and application of culture filtrates onto fungal-inoculated fruit gave similar protection to that provided by bacterial suspensions (Pusey and Wilson, 1984), with the same result being obtained against damping-off of alfalfa seedlings by sterile filtrates of *Bacillus cereus* (Handelsman *et al.,* 1990). Utkhede (1984) showed that strains of *B. subtilis* produce antibiotics inhibitory to mycelial growth of *Phytophthora cactorum* and Backhouse and Stewart (1989a) reported that toxic substances released by an isolate of *B. subtilis* were responsible for the loss of the viability of sclerotial *Sclerotium cepivorum.*

***Streptomyces* sp.**

The antibiotic geldanamycin produced by *Streptomyces hygroscopicus* var. *geldanus* was reported to cause suppression of rhizoctonia root rot of pea (Rothrock and Gottlieb, 1984). This antibiotic, at amounts equivalent to that produced *in vitro*, gave similar control to the live antagonist. In addition, geldanamycin could be recovered from soil inoculated with the bacteria.

Fungi

Toxic substances produced by fungal antagonist were known to be partly responsible for inhibiting some fungal pathogens. Identification of gliotoxin from *Trichoderma lignorum* (*Gliocladium fimbriatum*) (Weindling, 1941) stimulated further work on the biological control of plant pathogenic fungi. Brian and McGowan (1945) showed that *Trichoderma viride* produce a second fungistatic antibiotic, viridin, and Wright (1956) was able to show the production of gliotoxin by *T. viride* in wheat straw in soil. Aluko and Hering (1970) reported that the production of viridin and gliotoxin by *Gliocladium virens* against *Corticium solani* was of greater effectiveness than parasitic ability. Two further antibiotics, trichodermin and a peptide were found to be produced by *Trichoderma* spp. and by *G. virens* (Dennis and Webster, 1971). *In vitro*, the antibiotics were highly toxic to several plant pathogenic fungi. Howell (1982) also demonstrated that suppression of damping-off of cotton seedlings caused by *Pythium ultimum* by *G. virens* (strain GV-P) was due to antibiotic action. Howell and Stipanovic (1983) identified it to be this antibiotic as gliovirin (diketopiperazin) and reported highly toxic to the pathogen, but not to other fungi associated with cotton seedlings. A mutant of strain GV-P deficient for gliovirin was also deficient in antagonistic

ability against *P. ultimum*. Howell (1987) found that strains of *G. virens* produced gliotoxin that was inhibitory to *R. solani*, and gliotoxin was also shown to be the primary antibiotic responsible for inhibiting mycelial growth and sporangial germination of *P. ultimum* (Roberts and Lumsden, 1990).

Mercier and Reeleder (1987) reported that antifungal compounds produced by *Epicoccum purpurascens* were responsible for the disease reduction in lettuces due to *Sclerotinia sclerotiorum*. Brown *et al.* (1987) showed production of six antifungal compounds (epicorazines A and B; compounds X and Y, flavipin; and compound Z) by *E. purpurascens*. These compounds varied in their toxicity against different fungi, with *Pythium* spp. being more sensitive to the epicorazine fraction than to flavipin, *Phytophthora* spp. being more susceptible to flavipin, and *Fusarium oxysporum* being resistant to both.

2.2.4 Volatile substances

The role of volatile substances in disease suppression has been little investigated. Howell *et al.* (1988) showed that inhibition of *Pythium* damping-off *in vitro* by *Enterobacter cloacae* was due to ammonia released by the antagonist. This inhibitor in amounts up to 14 µl on agar medium was inhibitory to *P. ultimum* and *Rhizoctonia solani*, and the former was more sensitive than the latter. Production of ammonia by *E. cloacae* was reduced by addition of sugars (*D*-galactose, *D*-glucose, sucrose, and *ß*-methyl-*D*-glucoside).

The bacterium *Pseudomonas fluorescens* strain CHAO which was known to produce antibiotics and a siderophore, was found by Défago *et al.* (1990) to suppress some diseases mainly by synthesis of hydrogen cyanide. In contrast to the parent strain a mutant deficient in

HCN production was ineffective in controlling black root rot of tobacco or in increasing root hair formation. The production of HCN by strain CHAO was found to be induced by the presence of iron (III).

Strains of *Trichoderma harzianum* were found by Claydon *et al.* (1987) to produce two volatile metabolites identified as 6-n-pentyl-2H-pyran-2-one and 6-n-pentenyl-2H-one. These metabolites were fungistatic to a wide range of fungal pathogens and significantly reduced damping-off in lettuce seedlings by *R. solani in vitro.* The former of the metabolites was also found to be produced by a strain of *Trichoderma koningii* pathogenic to *Gaeumannomyces graminis* (Simon and Sivasithamparam, 1988a; Simon *et al.,* 1988). Production of volatile pyrones and the ability to suppress take-all fungus was variable among *Trichoderma* spp. (Ghisalberti *et al.,* 1990). A strain of *T. harzianum* suppressive to take-all of wheat produced two pyrones and other undetermined analogues, although two other isolates having similar efficacy did not produce any pyrones. Strains of *T. koningii* and *T. hamatum* which produced a simple pyrone compound also suppressed *G. graminis.*

2.2.5 Enzymes

Lytic action has a possible role in biological control of plant pathogens. Strains of *Bacillus* spp. and a strain of *Pseudomonas* sp. producing chitinase were shown to lyse mycelia of *Fusarium oxysporum, in vitro* (Mitchell and Alexander, 1961), with severity of vegetable root rot being reduced following addition of the bacteria, chitin, cellulose and ammonium chloride to *Fusarium*-infested soil. Monreal and Reese (1969) showed that chitinase was also produced by *Serratia marcescens* and *Enterobacter liquefaciens.* The former isolate's enzyme system is composed of an endochitinase, a chitobiase, and a factor (CH_1) required for the hydrolysis of crystalline chitin.

Morrissey *et al.* (1976) reported that chitinase released by a strain of *Arthrobacter* sp. was an essential factor responsible for lysing *Fusarium roseum.* Lim *et al.* (1991) also reported that extracellular enzymes of chitinase and laminarinase were responsible for the inhibition of *F. solani* by *Pseudomonas stutzeri* (YPL-1). These lytic enzymes inhibited the mycelial growth and lysed mycelia and germ tubes of the pathogen *in vitro.*

Several fungal antagonists (*Trichoderma hamatum, T. harzianum, Pythium nunn*) produce ß-1,3 glucanase, chitinase, and cellulase (Hadar *et al.,* 1979; Chet and Baker, 1981; Elad *et al.,* 1982; Elad *et al.,* 1983; Elad *et al.,* 1985; Sivan and Chet, 1989). These enzymes are capable of degrading cell walls of *Pythium* spp., *Rhizoctonia solani,* and *Sclerotium rolfsii.* Harman *et al.* (1981) showed that addition of fungal cell wall preparations and chitin increased the efficacy of *T. hamatum* in controlling the pathogen. Roberts and Lumsden (1990) showed that *Gliocladium virens* was able to produce the enzymes laminarinase, amylase, carboxymethylcellulase, chitinase, and protease. Although these enzymes were not primarily responsible for antagonism of *P. ultimum, in vitro,* they have the potential to degrade cell wall and membrane constituents of the pathogen. It was suggested that the action of the enzymes may facilitate the diffusion of the antibiotic gliotoxin to its binding site on the cytoplasmic membrane (Jones and Hancock, 1988; Roberts and Lumsden, 1990).

2.2.6 Parasitism

The literature on the biological control of pathogenic fungi by fungal parasitism has been reviewed by Schroth and Hancock (1981); Papavizas (1985); and Campbell (1989). Penetration of the host by the

mycoparasite is by over-growing and coiling, destruction of the hyphae and sclerotia, followed by the death of the cells. Reduction of disease caused by *Sclerotinia* spp., *Sclerotium* spp., *Rhizoctonia solani, Pythium* spp., *Botrytis cinerea, Fusarium* spp. and by other fungi, by the antagonists *Coniothyrium minitans, Sporidesmium sclerotivorum, Verticillium biguttatum, Gliocladium* spp., *Trichoderma* spp. and *Pythium oligandrum* has been reported by various authors (Elad *et al.,* 1980; Abd-El Moity *et al.,* 1982; Papavizas *et al.,* 1982; Hadar *et al.,* 1984; Lumsden and Locke, 1989; Papavizas and Lewis, 1989; Adams, 1989; Trutmann and Keane, 1990; McQuilken *et al.,* 1990; Adams and Fravel, 1990; Boogert *et al.,* 1990). The sclerotial viability of the pathogens was shown to be reduced and spores of the pathogens were also invaded following treatment with the antagonists in the soil (Smith, 1972; Ayers and Lumsden, 1977; Deacon and Henry, 1978; Ayers and Adams, 1979; Humble and Lockwood, 1981; Phillips, 1986; Velvis *et al.,* 1989; Papavizas and Collins, 1990). The following interactions between the host and the mycoparasite have been observed: penetration, mycoparasitic formation of appressorium-like structures or haustoria, and formation of intracellular hyphae on the host fungus (Huang and Hoes, 1976; Huang, 1978; Tu, 1980; Tu and Vaartaja, 1981; Lifshitz *et al.,* 1984a; Bullock *et al.,* 1986; Boogert *et al.,* 1989, Laing and Deacon, 1990, Trutmann and Keane, 1990).

2.2.7 Plant growth promoting factors

Plant growth promotion may be mediated by rhizobacteria or fungal antagonists which colonize the root aggressively and exclude deleterious fungi and bacteria (Schroth and Hancock, 1981; Kloepper and Schroth, 1981; Suslow and Schroth, 1982; Yuen and Schroth,

1986b; Chang *et al.,* 1986; Elad *et al.,* 1987; Van Peer and Schippers, 1989). Alternatively microbial agents of the rhizosphere may produce a range of growth promoting metabolites described below (Brown, 1972; Merriman *et al.,* 1974b; Broadbent *et al.,* 1977; Baker *et al.,* 1984; Windham *et al.,* 1986; Dewan and Sivasithamparam, 1988a; 1989a).

Reduction of pathogen populations and increased plant growth and yield of horticultural plants following applications of strains *B. subtilis* or *S. griseus* have been demonstrated in the glasshouse and in the field (Broadbent *et al.,* 1971; Chambers and Millington, 1974; Merriman *et al.,* 1974a; 1974b). Brown (1972) showed that rhizosphere bacteria produce plant hormones such as gibberellins and indolyl-3-acetic acid (IAA). Broadbent (1977) reported that *B. subtilis* strain A13 released gibberellin as well as inhibitory substances. Olsen and Misaghi (1984) found that increased growth of guayule (*Parthenium argentatum*) was due to nutrients and a growth regulator excreted by pseudomonads on the root surface. Chanway and Nelson (1990) detected IAA from the supernatant of a culture of *B. subtilis*, and this bacterium was shown to promote wheat growth in either sterile or non-sterile conditions. Selvadurai *et al.* (1991) also reported that IAA analogues produced by strains of *B. cereus* were responsible for the growth increase of wheat.

Windham *et al.* (1986) showed that *Trichoderma* spp. produce a growth-regulating factor that increases seed germination and plant growth of tomato and tobacco. A sterile red fungus (SRF) has been shown to produce antibiotic and plant growth promoting factors (Dewan and Sivasithamparam, 1989a; 1989b). This fungus promoted the growth of wheat and rye-grass and also protected the plants from take-all (Dewan and Sivasithamparam, 1988a; 1989b; Gillespie-Sasse, 1991). The antibiotic and growth promoting factors of SRF exudates

were reported to be water soluble and of low molecular weight (Gillespie-Sasse, 1991).

2.3 Root Colonization

2.3.1 Significance of root colonization

Success of biological control agents for the suppression of pathogens in the rhizosphere depends upon their ability to colonize the growing roots of host plants (Kloepper *et al.,* 1980b). Such bacteria, including PGPR, have been shown to promote plant growth, exclude deleterious rhizosphere microorganisms (DRMO) (Schroth and Hancock, 1981; 1982; Suslow and Schroth, 1982) or to produce antibiotics in the rhizosphere (Brisbane and Rovira, 1988; Thomashow *et al.,* 1990).

The growth stimulation or disease reduction of plants following treatment with *Pseudomonas* spp. has been correlated with the ability of the bacteria to colonize plant roots (Burr *et al.,* 1978; Kloepper *et al.,* 1980b; Suslow and Schroth, 1982; Schroth and Weinhold, 1986; Xu and Gross, 1986a). Introduction of PGPR to potato tubers resulted in colonization of the rhizosphere in numbers of about 10^5 cfu cm^{-1} root two weeks after plant emergence, reducing to 10^2 cfu cm^{-1} throughout the growing season (Kloepper *et al.,* 1980b). Similar results were reported for sugar-beet by Suslow and Schroth (1982). Higher populations of *P. fluorescence* on wheat roots, exceeding 10^7 cfu g^{-1} of root, and "aggressive colonization" of emerging roots of potato by fluorescent pseudomonads (antagonistic to *Erwinia carotovora*) following seed treatments was reported by Xu and Gross (1986a). Populations of the fluorescent pseudomonads on the roots ranged from 10^3 to 10^6 cfu g^{-1} roots (fresh weight) over the entire

season.

There are few report regarding colonization of host roots by fungal antagonists. Fungal biocontrol agents are known to colonize the pathogenic sclerotia or mycelia directly and are not generally restricted to the rhizosphere (Beagle-Ristaino and Papavizas, 1985). Chao *et al.* (1986) demonstrated that a strain of *Trichoderma harzianum* introduced as seed treatment only colonized pea roots when the plants were grown in sterile soil, indicating poor competitiveness against the normal rhizosphere flora. It was also shown that numbers of propagules of *T. harzianum* were naturally greater in non-rhizosphere soil than in rhizosphere soil. In contrast however, Dewan and Sivasithamparam (1989a) reported that the sterile red fungus (SRF) aggressively colonized the entire root system of wheat and rye-grass in sterilized and non-sterilized soil. In addition, the SRF was able to invade the root cortex. The significant colonization of root host by the SRF was correlated with its ability to inhibit take all.

2.3.2 Process of root colonization

Nutrient exudates of plant roots are generally stimulatory to the rhizosphere biota, leading to competition for occupancy of the habitat. Scher *et al.* (1985) reported that chemotaxis of strains of *P. fluorescens* toward seed exudates of soybean was the mechanism by which their seed and root colonization was achieved. Scher *at al.* (1988) concluded however that motility and chemotaxis were not correlated with root colonization of *Pseudomonas* spp. and *Serratia* spp.. According to James *et al.* (1985), rapid adherence of bacteria to root surfaces was only the first of several events in the colonization process. Howie *et al.* (1987) proposed that colonization of wheat roots by a strain of *P. fluorescens* (2-79) occurs in two phases. In phase 1,

the bacterium attach to the root surface and then are distributed along the elongating root tip, resulting in lower populations on the roots away from the seed. In phase 2, the bacteria proliferate and spread locally to the limit of the ecological niche. Phase 2 could occur concurrently with phase 1, but it continues after phase 1. Weller (1988) suggested that this process may apply to other biological control systems.

Van Peer *et al.* (1990) found that "agglutinin" of tomato roots significantly affected root colonization by *Pseudomonas* spp., but chemotaxis by *Pseudomonas* spp. was not stimulated by seed exudates. It has been hypothesized that binding of specific *Pseudomonas* strains by root agglutinin was an important initial phase of adherence of bacteria to the root surface (Anderson, 1983; James *et al.,* 1985; Van Peer *et al.,* 1990).

2.3.3 Factors effecting root colonization

Factors effecting root colonization include soil matric potential, bacterial characteristics, soil pH, soil temperature, soil type, indigenous microorganisms, and plant genotype (Weller, 1988).

Pseudomonas spp. are susceptible to low water activity with rapid loss of viability when exposed to desiccated soils (Chen and Alexander, 1973). Dupler and Baker (1984) reported that strains of *P. putida* were able to survive in dried soil if soil matric potential was decreased gradually (from -15 to -100 bars). The ability of *Pseudomonas* spp. to survive at low soil matric potential was due to an increase in internal osmotic tension of the cells (Chen and Alexander, 1973; Dupler and Baker, 1984). Soil matric potential at -0.3 to -1.4 bars was favourable for *P. fluorescens* (Parke *et al.,* 1986; Howie *et al.,* 1987) and at -0.1 to 0.33 bars for *Gliocladium roseum* and *Trichoderma* spp. (Stack *et al.,* 1987). Chao *et al.* (1986) and Davies and Whitbread

(1989) reported that water percolation played a role in the movement of introduced microorganisms in the rhizosphere.

Howie *et al.* (1987) found that at soil matric potential -4.0 bars, a mutant strain of *P. fluorescens* 2-79 could be recovered up to 3 cm below the initial point of inoculation, and extrapolation from the data indicated that colonization downward of roots could occur in soil to -7.0 bars. Only passive distribution (phase 1) and little or no subsequent multiplication (phase 2) occurred at this matric potential. It has been previously thought that the cells would be unable to maintain turgor potential for growth at matric potential below -4.0 bars.

Bacterial motility and osmosensitivity may play roles in root colonization. Loper *et al.* (1984) found that osmotolerance of *Pseudomonas* spp. was correlated with their ability to colonize the rhizosphere of potato. However, Howie *et al.* (1990) showed that osmotolerance did not necessarily assist rhizosphere colonization of cotton, since populations of an osmotolerant strain of *P. putida* and an osmosensitive strain of *P. fluorescens* were not significantly different at low soil matric potential.

Howie *et al.* (1987) and Scher *et al.* (1988) reported that motility was only of significance in the localized spread of rhizosphere bacteria. Liddell and Parke (1989) however claimed that motility in combination with percolating water was important in the downward spread of bacteria.

Reports of the effects of soil temperature on root colonization by introduced microorganisms are sparse and conflicting. *Pseudomonas* spp. were shown by Loper *et al.* (1984) to grow rapidly in the rhizosphere of potato at a temperature 24°C, although Xu and Gross (1986a) found that following seed inoculation, rhizosphere populations were higher at temperatures of 12-18°C. Davies and Whitbread (1989) reported that optimal temperatures for root colonization of radish by

fluorescent pseudomonads under laboratory and glasshouse conditions ranged from 18 - 22°C. These temperatures were considered to be similar to the ambient air temperatures, but are not representative of conditions prevailing in the field (Schroth and Becker, 1990).

Kloepper *et al.* (1980b) reported that colonization of potato roots by *Pseudomonas* spp. was higher in neutral-pH soils than in acidic soils. A similar result was reported by Park *et al.* (1988) who showed that in neutral to alkaline soils (pH 6.7-8.1) populations of bacterial biocontrol agents on cucumber roots were greater than those in acidic soil (pH 5.5).

Soil types can affect the introduced bacteria in colonizing plant roots. For example soils with low water-holding capacity are reported to severely depress bacterial colonization (Yuen and Schroth, 1986b) and populations of *Pseudomonas* spp. were found to be greater in sandy soil than in clay soil (Dupler and Baker, 1984; Bahme and Schroth, 1987). In the colonization of zinnia roots by *P. fluorescens* (E6), greater numbers were recorded in sandy loam than in sandy soil (Yuen and Schroth, 1986b). Similarly Bahme *et al.* (1988) showed that populations of *P. fluorescens* (A1-B) in the potato rhizosphere were at least initially greater in sandy loam soil compared with silty clay loam. Howie and Echandi (1983) however reported no difference in the rhizosphere colonization by pseudomonads of potatoes planted in loam or sand.

The presence of pathogens apparently stimulates the growth of their antagonists as reported by Charigkapakorn and Sivasithamparam (1987) and Weller (1988) with regard to *Gaeumannomyces graminis* attack on wheat. These results suggest that plant lesions may provide an increased nutrient source for the growth of the antagonistic bacteria.

Different plant genotypes may be beneficial, neutral, or

detrimental to rhizosphere colonists (Gerhardson *et al.,* 1985; Åström and Gerhardson, 1988; Schroth and Becker, 1990), possibly as a result of differences in root exudates (Curl and Truelove, 1986). Åström and Gerhardson (1988) found that inoculation of wheat and pea genotypes with *Pseudomonas* spp. significantly affected the growth of plants in the glasshouse, yet populations of the introduced bacteria or the numbers of indigenous bacteria on the roots were not affected. It was concluded that plant response to the bacteria was under genetic control, a conclusion supported by subsequent research showing that in addition to the variation between plant species, the plants also showed an intraspecific variation in their reaction to the bacteria (Åström, 1991).

Mutualistic associations of biota can also contribute to the success of colonization by specific isolates. For example, *Curtobacterium* sp. aggressively colonized the roots of wheat and barley, but not maize. However, when the *Curtobacterium* sp. was co-inoculated with *Mycoplana* sp. into the rhizosphere of maize, the population of *Curtobacterium* was significantly stimulated and increased (Bennet and Lynch, 1981).

Weller (1986) found a significant difference among wheat cultivars with regard to their supporting root colonization by *P. fluorescens* 2-79. Also Chanway *et al.* (1988b) found that growth promotion in one wheat cultivar but not in another was observed following seed inoculation with *Bacillus* spp.. Specificity between *Bacillus polymyxa* and pasture plants was shown to occur in the yield of white clover and ryegrass (Chanway *et al.,* 1988a). Burr *et al.* (1978) and Howie and Echandi (1983) however found no differences in plant growth or in rhizosphere bacterial populations of cultivar potatoes treated with PGPR.

3. MATERIALS AND METHODS

3.1 Isolation of bacterial antagonists

Plants of daisy (*Olearia phlogopappa*) and clary sage (*Salvia sclarea*) were collected from areas of the University of Tasmania Farm with known histories of root rot diseases. White clover plants and woodchips were taken from the vicinity of the Department of Agricultural Science, University of Tasmania.

Plant roots were washed free of adhering soil under tap water and the excised roots surface sterilized with 1.8% calcium hypochlorite for 3-4 minutes, followed by four washings with sterile water. The woodchip sample was not surface sterilized. A weight of 5 g of each sample was added to 100 ml of sterile 0.85% saline in a plastic bag and stomachered for 5 minutes. Serial dilutions (0.1 ml) were spread onto Oxoid Potato Dextrose Agar (PDA) and King's B medium (KBM). After incubation for two days at 25°C, colonies appearing on 10^{-3} to 10^{-5} dilution plates were overlain with molten cooled PDA. After setting of the PDA these plates were spread with spore suspension of indicator pathogens. Screening was also conducted by spot inoculating bacterial candidates from serial dilution plates onto the periphery of PDA plates centrally inoculated with the pathogens. Zones of inhibition produced were noted and the bacteria responsible subcultured.

3.2 Fungal pathogens

The fungi *Sclerotinia minor*, *S. sclerotiorum* and *Botrytis cinerea* were supplied by Dr. J.A. Wong of the Department of Agriculture, Tasmania. *Pythium ultimum*, *Fusarium solani*,

Phytophthora cinnamomi, *P. cactorum* and *Penicillium echinulatum* were from the Department of Agricultural Science University of Tasmania culture collections. *Gaeumannomyces graminis* var. *tritici* strains C3 and C5 were supplied by Dr. Percy Wong (NSW Agriculture & Fisheries, Biological and Chemical Research Institute Rydalmere, NSW). Sclerotia of *Sclerotinia* spp. produced on sterile barley seeds were surface sterilized with 1.8% Ca-hypochlorite for 3-4 minutes and washed four times with sterile distilled water and then cultured onto PDA plates. Other fungal pathogens were cultured by cutting discs of agar containing the fungus and transferring this onto a fresh PDA plate. Incubation of cultures was at 25 °C.

Fungal inocula of *S. minor* and *S. sclerotiorum* for glasshouse infestations were prepared as follows: millet and barley seeds (150g) were moistened and sterilized by autoclaving at 121°C for 45 minutes in 1000ml flasks on two consecutive days. Ten to 14-day old cultures of the fungi on PDA were cut into one centimeter squares and these mixed into the flasks containing sterile seeds (millet for *S. minor* and barley for *S. sclerotiorum*). Cultures were incubated at 25°C for two months with shaking every week to promote homogenous growth.

3.3 Test for inhibition of fungal growth *in vitro*

A 1.5 cm diameter plug of fungal mycelia or a sclerotium of either *S. sclerotiorum* or *S. minor* from the PDA culture was placed on the centre of PDA plates and incubated at 25°C. After the fungal mycelial growth reached approximately 2 cm in diameter, either a loopful or 0.1 ml (10^8 cfu/ml) of each bacterial isolate was spot inoculated at 2 cm from the mycelial edge. The plates were incubated at 25°C for 7 to 14 days, and examined daily. In some studies, the PDA and KBM were

supplemented with filter sterilized ferric chloride at concentrations of 0, 5, 10, 20, 50, or 100 μM.

3.4 Effect of pH of the medium on the inhibition of fungi by antagonists

The pH of sterile, molten PDA was adjusted by adding either 1 M KOH or HCl solutions to obtain pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. Plates of these media were inoculated with either *S. minor* or *S. sclerotiorum* and assessed as discribed previously.

3.5 Culture and storage of bacteria and fungi

For routine examination, bacteria were subcultured on TSA (Appendix 1.1.1), KBM (appendix 1.1.3), or PDA (Oxoid). Slants of TSA or KBM were used for storage of the bacteria for up to three months at 4°C.

For long-term storage, cultures were maintained at -70°C and also freeze dried.

a) Cold storage at-70°C

Two to three day old TSA-plate cultures were flooded with sterile 20% glycerol in BBL Trypticase Soy Broth (TSB) and 1.0 ml of the emulsion transferred into sterile bijoux bottles containing plastic beads for storage at -70°C.

b) Freeze drying

Strains were suspended in freeze-dry suspension medium (FDSM) consisting of 50% sterile saline and 50% skim milk solution (20% powder w/v in water sterilized at 108°C for 30 minutes), and 200μl of

the suspensions were pippetted into ampoules. The ampoules were cooled at 4°C for 30 min, frozen under vacuum for a minimum of 5 hours, then sealed under vacuum, and stored at 3°C.

Long term maintenance of *S. minor, S. sclerotiorum* and *G. graminis* was by culturing on sterile millet, barley or wheat seeds, respectively in 250 ml flasks.

3.6 Identification of antagonists

Bacteria antagonistic to fungal pathogens were identified using Bergey's Manual of Systematic Bacteriology, volumes I (Krieg and Holt, 1984) and II (Sneath *et al.*, 1986). The following tests were performed.

3.6.1 Gram reaction and cellular morphology

Gram staining and examination of cellular morphology was performed on 48 hour cultures.

3.6.2 Flagellation and motility

Flagellar staining was performed using the Heimbrook *et al.* (1989) method. A loop of 24h-old culture from agar medium was mixed with a drop of water on a clean dry slide and then covered with a coverslip. After allowing 5 min for surface attachment of cells, two drops of stain (Appendix 1.2.7) were added to one edge of the coverglass. After a further 5-15 min, bacterial flagella was examined by light microscopy under oil immersion.

Determination of bacterial motility was performed using 6-12 h broth cultures and examination was by phase contrast microscopy.

3.6.3 Biochemical characteristics

The media and reagents used for all tests are described in Appendix 1.1 and 1.2 and with an incubation temperature of 25 °C, unless otherwise specified.

3.6.3.1 Oxidase

A loopfull of bacterial growth from TSA medium (Appendix 1.1.1) was placed on filter paper moistened with a few drops of 1% tetramethyl-*p*-phenylenediamine dihydrochloride solution. A positive test was indicated by the development of a violet colour within 30 seconds.

3.6.3.2 Catalase

Bacteria were grown on TSA medium for two days. Colonies were flooded with a few drops of 3% H_2O_2. A positive result was indicated by the evolution of oxygen.

3.6.3.3 Anaerobic growth

McCartney bottles containing 18 ml semisolid Hugh and Leifson medium (Appendix 1.1.20) were stab-inoculated and incubated for seven days. Anaerobic growth was shown by growth to the base of the deeps.

3.6.3.4 Poly-ß-hydroxy butyrate (PHB) accumulation

Plates of Palleroni and Doudoroff (1972) agar medium (Appendix 1.1.5) with NH_4Cl content at one-fifth normal concentration and supplemented with 0.5% *DL*-ß-hydroxybutyrate, were spot inoculated and incubated for five days. Bacterial colonies were then prepared for staining using Neisser's method [modified by Cruickshank *et al.* (1975)] as follows:

- stain with Neisser's methylene blue (Appendix 1.2.5) for 3 min.,
- wash off with dilute iodine solution (iodine solution of Kopeloff and Beerman's modification of Gram's method, diluted 1 in 10 with water) and leave for 1 min.,
- wash with water,
- counterstain with Jensen's neutral red solution (Appendix 1.2.6) for 3 min.,
- wash with water and dry.

Accumulation of PHB by bacteria was examined under light microscopy, with PHB granules staining deep blue and the remainder of the cell assuming a pink colour.

3.6.3.5 Arginine dihydrolase

Thornley's semisolid medium (Appendix 1.1.6) containing arginine was stab inoculated together with a control lacking arginine. The medium was then sealed with sterile melted paraffin and incubated for 7 days. A positive test was indicated by a change in the colour of the medium from yellow-orange to red (Smibert and Krieg, 1981).

3.6.3.6 Pyoverdin production

Strains were streak inoculated onto King's medium B (Appendix 1.1.3). Plates were examined at 1, 2, and 3 days incubation under ultraviolet light at 254 nm. A positive test was indicated by a fluorescent zone in the agar surrounding the growth.

3.6.3.7 Pyocyanin production

Plates of King's medium A (Appendix 1.1.2) were inoculated and incubated for 3-4 days. Positive results were indicated by a blue green zone in the agar surrounding the growth.

3.6.3.8 Lecithinase (Egg Yolk)

Plates of egg yolk agar (Appendix 1.1.9) were streak inoculated and incubated for 4 days. A positive result was indicated by an opaque zone around growth (Smibert and Krieg, 1981).

3.6.3.9 Nitrate reduction

Tubes of peptone water supplemented with KNO_3 (Appendix 1.1.7) were inoculated and incubated for 3 days. A drop of bacterial culture on a porcelain plate was mixed with a drop each of sulphanilic acid and α-naphthylamine solution (Appendix 1.2.3 and 1.2.2, respectively). The development of a red colour indicated that nitrate had been reduced to nitrite. If no red colour appeared, zinc dust was added. A red colour then indicated that nitrate had not been reduced. Lack of a red colour indicated that nitrite had been further reduced to a gaseous form (Smibert and Krieg, 1981).

3.6.3.10 Gelatin liquefaction

Plates of nutrient gelatin (Appendix 1.1.8) were stab inoculated, incubated for 7 days and examined daily. Before examination the plates were held at 20°C for 4 hours. A positive result was indicated by liquefaction around growth (Cruickshank *et al.*, 1975).

3.6.3.11 Levan formation from sucrose

Plates of nutrient agar (Appendix 1.1.4) containing 4% (w/v) sucrose were inoculated and incubated for 5-7 days. A positive result was indicated by a thick slimy growth of the colony (Skerman, 1969).

3.6.3.12 Voges-Proskauer reaction

Two tubes of MRVP broth (Appendix 1.1.12) were inoculated and

incubated for 2 days. One ml of culture was mixed with 0.6 ml of 5%-α naphthol and 0.2 ml of 40% (aq) KOH in a small tube and incubated for 15 minutes. A positive test was indicated by the development of a red colour. If negative, cultures were reincubated for a further 2 days and retested (Smibert and Krieg, 1981).

3.6.3.13 Hydrolysis of Tween 80

Strains were inoculated on TSA medium overlain with 1% Tween 80 agar and incubated overnight. Lipolytic activity was indicated by the opaque deposition of calcium soaps around growth.

3.6.3.14 Hydrolysis of casein

Strains were inoculated onto milk agar (Appendix 1.1.10) and incubated for 7 days. A positive result was indicated by a clear zone around the growth (Sneath, 1986).

3.6.3.15 Hydrolysis of starch

Plates of starch agar (Appendix 1.1.11) were streak inoculated and incubated for 3-7 days. After incubation, hydrolysis of starch was indicated by flooding with Gram's iodine and examining for clear zones around the growth (Sneath, 1986).

3.6.3.16 Utilization of citrate

Slants of Christensen citrate agar (Appendix 1.1.13) were inoculated and incubated for 14 days. Production of a red (alkaline) colour indicated utilization of organic acid (Smibert and Krieg, 1981).

3.6.3.17 Utilization of carbon sources

Sterile Palleroni and Duodoroff medium (Appendix 1.1.5) was supplemented with 0.5% of the following filter sterilized carbon

sources: *D*-glucose, *meso*-inositol, *L*-valine, trehalose, adonitol, levulinate, *D*-ribose, *L*-rhamnose, *m*-tartrate, tryptamine, *D*-xylose, *ß*-alanine, *D*-*L*-arginine, *D*-galactose, *L*-tryptophan, *D*-arabinose, *L*(+) arabinose, cellobiose, testosterone, geraniol, 2-ketogluconate, *p*-hydroxybenzoate, or histamine and 3 ml volumes distributed into bijoux bottles. These media were inoculated and incubated for one week. Utilization of the carbohydrates was shown by turbidity increase over inoculated controls containing no carbon source.

3.6.3.18 Acid from carbohydrates

Slants of medium for acid production from sugars (Appendix 1.1.19) contained 0.5% concentrations of the following carbohydrates: *D*-glucose, *L*-arabinose, *D*-xylose, and *D*-mannitol. These were inoculated by stabbing to the depth of the agar and incubated for 7-14 days. Positive results were indicated by a change in the colour of the medium from purple to yellow (Sneath, 1986).

3.6.3.19 Gas production

Bottles of glucose peptone broth (Appendix 1.1.17) containing Durham tubes were inoculated and incubated for 7 days. Gas production was indicated by gas production in the closed tube (Skerman, 1969).

3.6.3.20 Formation of indole

Bijoux bottles of 1% (w/v) Oxoid tryptone broth were inoculated and incubated for 14 days. To these cultures was added 0.5 ml of Kovacs reagent (Appendix 1.2.1). A positive result was indicated by development of a red colour in the alcohol layer (Sneath, 1986).

3.6.3.21 Formation of dihydroxyacetone

Plates of glycerol agar (Appendix 1.1.16) were streak inoculated and incubated for 10 days. The plates were flooded with copper sulfate solution and potassium sodium tartrate solution (Appendix 1.2.4). A positive result was indicated by a red halo around the growth (Sneath, 1986).

3.6.3.22 Deamination of phenylalanine

Duplicate slants of phenylalanine agar (Appendix 1.1.14) were inoculated and incubated for 7 days. To one of the slants was added 4-5 drops of 10% (w/v) ferric chloride solution. Formation of phenylpyruvic acid from phenylalanine was indicated by a green colour beneath the growth relative to the control (Sneath, 1986).

3.6.3.23 Degradation of tyrosine

Plates of tyrosine agar (Appendix 1.1.15) were streak inoculated and incubated for 7-14 days. A positive result was indicated by a clear zone around the growth (Sneath, 1986).

3.6.3.24 Growth at different temperatures

Strains were inoculated onto TSA plates and incubated for 3 days at 55°C, 5 days at 30°, 40°, and 50°C, and 21 days at 4°, 5°, and 10°C.

3.6.3.25 Growth in NaCl-media

McCartney's bottles of nutrient broth (Appendix 1.1.4) containing 0, 2, 5, 7, and 10% (w/v) NaCl were inoculated and incubated for 7 to 14 days. The ability of bacteria to grow in the various concentrations of NaCl was indicated by turbidity of the medium.

3.6.3.26 pH in V-P broth

Tubes of V-P broth (Appendix 1.1.18) were inoculated and incubated for 5-7 days. Culture pH following incubation was then measured.

3.6.3.27 Growth at pH 5.7

Slopes of Sabouraud dextrose agar (Oxoid) and tubes of Sabouraud dextrose broth (Oxoid) were inoculated with a loopful of nutrient-broth culture. Tubes of nutrient broths (pH 6.8) were also inoculated as controls. The cultures were incubated for 2 weeks or more prior to examination for growth (Sneath, 1986).

3.6.3.28 Resistance to antibiotics

Strains were spread inoculated onto TSA plates to which were added paper disks of appropriate antibiotics. The following antibiotics were tested: ampicillin, bacitracin, cephalothin, erythromycin, gentamycin, nalidixic acid, novobiocin, penicillin G, polymixin B, streptomycin, and tetracycline. Bacterial resistance to these antibiotics was assessed after 24h incubation.

For purposes of formulating selective media for the various bacterial antagonists, strains were grown on KBM supplemented with selected antibiotics at increased concentrations plus the fungicide pentachloronitrobenzene (PCNB) as given in Results.

3.7 Bioassay for wheat take-all disease

Axenic sand culture assays of root disease were performed according to the method of Brisbane and Rovira (1988). Antagonists used in this experiment were strain UT1, UT2, UT3, UT4 and a

Pseudomonas putida (strain NIR-6) supplied by Dr. P. Wong of the NSW Agriculture & Fisheries, Biological and Chemical Research Institute.

Large glass tubes (30 x 200 mm) containing 40 g of dry sand (coarse sand was sieved through a 2 mm mesh and washed with water to remove silt, clay and organic matter) and 8 ml of Hoagland's solution (Appendix 3) were plugged with cotton and autoclaved at 121 °C for one hour.

Plugs (11 mm diameter) of *G. graminis* strain C3 grown on PDA for 10 days were added to the sterile tubes plus 1.0 ml of suspensions of strains UT1, UT2, UT3, UT4, and NIR-6 at concentrations of between 2×10^8 - 8×10^8 cells ml^{-1}. Sterile water (1.0 ml) was added to controls. Wheat (*Triticum aestivum*) seeds of similar size were surface-sterilized with 1.8% Ca-hypochlorite, washed with sterile water four times and germinated on one-third strength TSA at 25°C. Three germinated seeds were then added to each of the tubes, covered with 10 g of dry sterile sand and incubated for 2 days at 25°C (by which time shoots had emerged). Subsequent incubation was at 17 $\pm$ 1°C in a controlled environment cabinet with a 10-hr photoperiod per day at a light intensity of 150 microeinsteins/m^2 $second^{-1}$. Following 3 weeks of growth, plants were removed from the tubes and the roots gently shaken to remove most of the sand. The wheat tops were cut from the roots at the base of the stem, dried in an oven at 70°C for 48 hours, cooled and weighed.

Roots with firmly adhering sand were weighed aseptically, then placed into McCartney's bottles containing 10 ml sterile saline and gently crushed by hand using a sterile glass rod. Subsequently 10-fold dilutions were made of the material and 0.1 ml aliquots of these spread onto TSA, PDA, KBM or selective media. Plates were incubated at 25°C and colonies counted on appropriate plates after seven days of

incubation. The results were expressed as numbers of colony-forming units (cfu) per g of root+adhering sand material.

3.8 Scanning electron microscopy (SEM) of bacterial roots

Wheat-root pieces (1 cm length) from seedlings in the tube assays inoculated with monoaxenic cultures of bacterial isolates (Experiment 3.7) were immersed in 3 volumes of cold glutaraldehyde [2.5% in 0.1 M Na cacodylate buffer (Appendix 2.1.3)] and fixed for 16-18 hrs at 4°C. The specimens were then washed three times for 30 minutes with 2 volumes of cold 0.1 M cacodylate buffer (Appendix 2.1.2). These fixed specimens were dehydrated in a graded ethanol series and infiltrated with acetone (Appendix 2.2). Samples in acetone were then critical point dried in CO_2 using a Polaron E-3000 critical point drying apparatus. The samples were examined under a Phillips 505 scanning electron microscopy, operated at 20 kV and photographed using Kodak Tmax 100 film.

3.9 Inoculation of lettuce seedlings with antagonists in glasshouse pots

Bacterial antagonists were grown on TSA at 25°C, for 2 days (strains UT1, UT2, UT3 and UT4) or for three days (UT5). Cells were then suspended in sterile, 0.85% saline and adjusted to 10^8 cells/ml based on optical density. Two week old lettuce seedlings (cultivar Pennlake) were removed from non-sterile potting mix and the roots washed in water before dipping them in the antagonist suspensions for one hour prior to planting of the seedlings in pots (15 cm diameter) containing 1.5 kg of potting mix. Potting mix comprised: Tasmanian

peat moss; coarse gravel; coarse washed sand; ground limestone; limil and "Osmocote" fertilizer (19 : 2.6 : 10 NPK) at 3 : 11 : 11 : 0.14 : 0.04 : 0.08 ratio (w/w). The pH of the mix was 6.2. One week after applying the antagonists, 2.0 g of fungal inoculum of either *S. minor* on millet or *S. sclerotiorum* on barley seeds was applied to pots by scratching into surface layers of the mix. Each treatment consisted of the bacterium alone, fungus alone, bacterium plus fungus (either *S. minor* or *S. sclerotiorum*), or neither bacterium nor fungus. All treatments comprised 10 pots and each pot was planted with one lettuce seedling. The plants were watered daily and Hoagland's nutrient solution (Appendix 3) was applied once a week. The experiment was maintained in the glasshouse for 12 weeks at temperatures ranging from 8°C (night) to 24°C (day) during June-September 1990.

After harvesting, reisolation of introduced bacteria from the root surface of lettuces was conducted as described previously using standard TSA and selective media.

3.10 Field assessment of antifungal activity of antagonistic isolates

Soil conducive* to root rot disease of daisy plants (*Olearia phlogopappa*) on a site at the University of Tasmania Farm was used for testing the ability of the bacterial antagonists to suppress the disease. The soil was sandy loam and had a pH of 5.2. During November 1990 to January 1991, the air temperatures ranged from 5°C to 40°C, and the mean soil temperature at a depth of 10 cm ranged from 14.6°C to 25°C.

Olearia cuttings of similar size were grown in the glasshouse for 13 weeks, and thinned. The plants were then removed from their containers, washed to free the roots from soil and the roots dipped in

* Previous disease outbreaks had been observed at this site

bacterial suspensions in water (10^9 cfu ml^{-1}) prepared as described previously (but with added protection against light exposure) for one hour. Plants were planted in a plot (7 x 7 m) of cultivated soil, 50 cm apart in a randomized complete block design with each block containing one plant of each treatment. Within each block (20 blocks), treatments were arranged at random based on tables of random permutations (Cochran and Cock, 1957). The plants were irrigated every five days and assessed after 9 weeks growth. For purpose of statistical analysis, the values 1 and 0 were given to healthy and diseased plants, respectively. Data were then transformed using the $\sqrt{Y + 1/2}$ tranformation (Y is the value given).

3.11 Isolation of fungi pathogenic to *Olearia* in the field

Plants that appeared to be dying were removed, and the roots washed in water. Root attack by the pathogen was evident by a black-rot, with isolation being achieved by cutting the infected root above and below the point of fungal infection, surface sterilizing with 1.80% Ca-hypochlorite for 3-4 minutes, washing 4 times in sterile water and placing root sections on water agar (WA) and PDA plates. The plates were incubated at 25°C for up to one week and fungal growth examined by light microscopy for purposes of identification.

3.12 Isolation and characterization of antimicrobial compounds produced by two of bacterial isolates

Two strains of the antagonists (UT1 and UT3) were chosen for their ability to produce antimicrobial substances (or antibiotics), because they were easy to cultivate, grew fast and showed a broad range of inhibition to the fungal pathogens (Table 4.1.1). The

antimicrobial compounds or antibiotics produced by antagonist strains UT1 and UT3 were isolated according to Howell and Stipanovic (1979), except that diethyl ether was used for the second extraction. The bacteria were grown on Oxoid PDA plates (20 plates) and incubated at 25°C for 7 days. Plate cultures were cut into 1 cm squares and extracted with 3 volumes of 80% acetone-water. The combined extracts were filtered through cheesecloth to remove pieces of agar. Other particulate matter was removed by centrifugation at 9,000x g for 15 minutes (Beckman, Model J2-21 Centrifuge). The supernatant was collected and partially evaporated *in vacuo* (Buchi Rotavapor-R) to remove the acetone. Five grams of NaCl was added for every 100 ml of the aqueous concentrate, which was then extracted three times with diethyl ether. The aqueous layer was retained for further examination. The ether extracts were combined and evaporated to dryness *in vacuo*. The resulting residue (crude extract) was dissolved in methanol (~20 ml) and stored at -20°C until required. Initial testing for antifungal activity was carried out by soaking filter paper discs in the sample, drying and then testing these against fungi on PDA plates. In addition, serial dilutions of dried crude extract were made by dissolving the crude extract in sterile water-methanol (1:1) at concentrations 0.01, 0.1, 1.0, 5.0, 10, 20, and 40 mg ml^{-1} and 0.1 ml of aliquots were tested against *S. sclerotiorum*, *S. minor*, and *G. graminis*.

The retention factor (R_f) of UT3 antimicrobial compounds present in the methanol soluble fraction of the crude extract was determined by using analytical thin-layer chromatography on silica gel (Merck 60 GF_{254}; 200x200; 0.2 mm) in various solvent systems. The solvent systems used were chloroform (100%), chloroform-acetone (9:1), chloroform-methanol (8:2; 7:3; and 6:4). The fluorescent and UV absorbing bands were detected under long (366 nm) and

shortwavelength (254 nm) ultraviolet light. The antimicrobial activity of the respective bands was determined directly on the TLC plates using bioautography. Following the determination of R_f values by this technique subsequent preparative chromatography was performed on silica gel plates (Merck-60 GF_{254}; 200 x 200 x 2.0 mm). Samples were streaked across the full width of plates and developed in the appropriate solvent. The running lane (16 cm) was then dried in a laminar flow cabinet. Bands having R_f values the same as those shown to contain active compounds were scraped into Erlenmeyer flasks and extracted with methanol-chloroform (1:1). In addition, all of the other bands were checked for possible antimicrobial activity not detected on the analytical TLC plates. The silica gel was removed by filtration through Whatman filter paper no. 42 and the solvent was evaporated *in vacuo*. Fractions obtained were then stored at 5°C.

High performance liquid chromatography (HPLC) was used to check the purity of separated fractions and was also evaluated as a means of separating the crude extract. A Waters gradient system was used which comprised: two Waters 6000 pumps, Waters U6K injector with a 2ml sample loop, Waters 490E 4 channel detector, and Waters Maxima data station providing system control and data analysis facilities.

Samples were dissolved in methanol and filtered (5µm) prior to injection. The mobile phase was a gradient of 0-100% (or a narrower range for preparative separations) methanol in water containing 0.1% trifluoroacetic acid (TFA).

Preparative separations of the crude extract were carried out on a 10x300mm Waters µ-Bondapak C_{18} reverse phase column at 3.5ml/min and analytical chromatograms were run using a Waters radial compression module fitted with a Novapak C_{18} column at a flow rate of

2ml/min. The detector was set at 220 and 330nm. Fractions collected were evaporated *in vacuo* at 40°C then dissolved in methanol for testing against fungi and bacteria.

Proton nuclear magnetic resonance spectra (NMR) were obtained on a Bruker AM300 spectrometer in deuterochloroform with tetramethylsilane as internal reference at δ 0ppm. Mass spectra were measured on a Vacuum General Micromass 7070F spectrometer using the direct insertion technique. A source temperature of 200°C and electron beam energy of 70eV was used.

3.12.1 Effect of heat, acid and alkali

The effect of heat, acid or alkali on the inhibitory activity of the UT3 compounds was determined as follows. The UT3 crude metabolites (15mg) were dissolved in 5ml aqueous methanol (1:1) and distributed into five small tubes. One tube was then immersed in boiling water for 10 minutes. To examine the stability at various pH's one drop of either HCl (bringing the pH to 2) or NaOH (bringing the pH to 9) was added and controls were provided by omitting these treatments. Fifty µl of these solutions was then spotted onto the periphery of PDA plates with centrally growing fungus (*S. minor, S. sclerotiorum,* or *G. graminis*) and the zones of inhibition were measured.

3.12.2 Solubility of the active compounds

Samples of the methanol soluble fraction of the crude extract were extracted with each of three organic solvents (ethyl acetate, chloroform, and acetonitrile) with sonication (Branson 1200 sonicator) and the organic layer was evaporated separately. The soluble and insoluble fractions were then redissolved in methanol and tested for

fungal inhibition.

3.12.3 Assays for antimicrobial activity

Bioautography of TLC plates was conducted according to the method of Homma *et al.* (1989). A small amount of the crude extract was spotted on the plate, developed in solvent, and dried under laminar flow. The TLC plate was then put into a plastic petri dish, sterilized with ultraviolet light for 15 minutes, and overlain with 10 ml of molten PDA or nutrient agar (NA). Either a spore suspension of *Ascochyta pisi* or a bacterial suspension of *Bacillus subtilis* was spread onto the PDA or NA. Both microorganisms were used routinely in the bioautography of TLC plates. The antimicrobial locality was observed as a clear zone after 5 to 24 hrs incubation at 37°C for the bacterium and 2-3 days at 25°C for the fungus.

Antifungal activity of samples was also checked by spotting 20-40 µl of the sample in methanol onto the periphery of PDA plates (one cm from the edge of the fungal growth) with centrally growing fungi (2-3 cm in diameter). The methanol was evaporated off under laminar flow and the plates were then incubated at 25°C for 2-7 days depending on the rate of fungal growth. The antifungal activity of the sample was indicated by zones of growth inhibition. Besides the species mentioned previously, the following fungi were also tested; *Phoma exigua exigua, Fusarium lateritum, Monilia fructicola, Rhizoctonia solani, Coniothyrium minitans, Trichoderma* sp., *Alternaria solani, Penicillium expansum, Colletotrichum gloeoshoriodes, Verticillium tenerum* and *Ascochyta pisi.*

To assay antibacterial activity, 20-40 µl of the sample or dilutions of sample in methanol was put into 3 ml of Oxoid nutrient broth in bijoux bottles and inoculated with 15 µl of 24 hr bacterial cultures.

Nutrient broth containing 20-40 µl methanol and inoculated with bacteria served as controls. The bottles were then incubated at 37°C for *Bacillus subtilis, Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Enterobacter cloacae,* and 25°C for *Bacillus cereus* var. *mycoides, B. thuringiensis, Erwinia* sp., *Pseudomonas aeruginosa,* and *P. syringae*. The inhibitory activity of the antibiotic was recorded after 5-48 hrs as the absence of turbidity due to bacterial growth. The fungi and bacteria were obtained from the Department of Agricultural Science culture collection, University of Tasmania.

4. RESULTS

4.1 Isolation of bacterial antagonists and *in vitro* antagonism against fungal pathogens

Bacteria associated with the roots of symptomless daisy and clary sage plants, healthy white clover and from decomposing woodchips were screened for *in vitro* antagonism to pathogenic fungi as described in Material and Methods. Of numerous isolates tested (>50), five exhibited antagonistic activity against one or more of the fungal pathogens tested on PDA (Table 4.1.1). These five isolates were from daisy (*Bacillus polymyxa* UT1), clary sage (*Pseudomonas cepacia* UT3 and *P. putida* UT4), white clover (*Acinetobacter* sp. UT5), and decomposing woodchips (*B. subtilis* UT2). Strains UT 1 and UT3 were antagonistic to fungi on both PDA and KBM, while other isolates although growing on both media were only antagonistic on PDA.

The fungal response to the antagonists varied with bacterial strain, UT3 showing antagonism to all 10 pathogens tested, contrasting with strains UT2 and UT5 which were antagonistic to only 6 or 7 of them. Isolate UT5 was particularly inhibitory to *S. minor,* though UT4 was the most consistently strong inhibitor. Isolate UT3 was the only one to show antagonism against *Pythium ultimum*, albeit slight, while strain UT2 always produced the smallest zones of fungal-inhibition. Isolates UT2 and UT5 were observed to lyse the fungal mycelia surrounding the zone of inhibition, with the mycelia becoming light brown instead of white. Interactions between bacterial antagonists and fungi (*S. sclerotiorum* and *S. minor*) are shown in Plates 4.1.1 - 4.1.2.

Table 4.1.1 Inhibition of fungal pathogens by bacterial isolates on PDA.

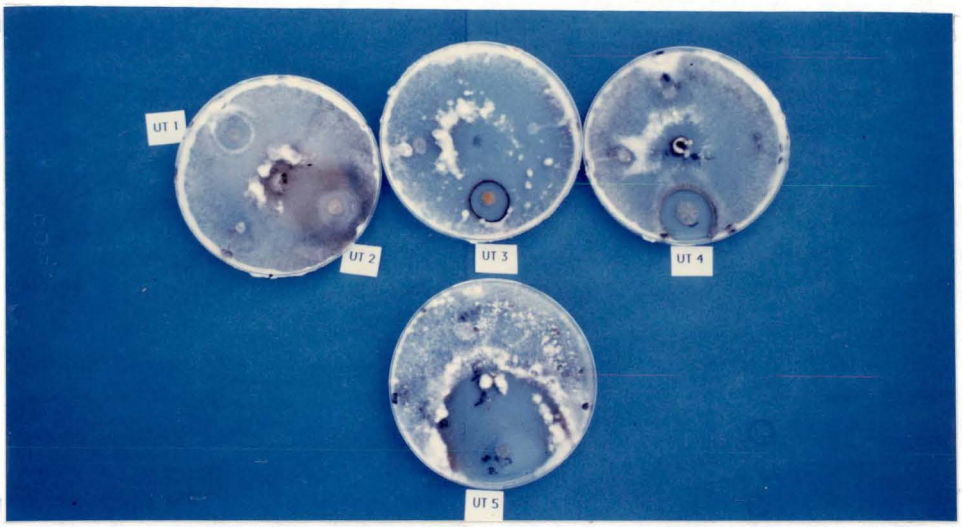
Pathogen	zones of inhibition[a] UT1	UT2	UT3	UT4	UT5
Sclerotinia minor	++	+	+	++	+++
S. sclerotiorum	+	+	++	++	+
Botrytis cinerea	+	+	+	++	+
Pythium ultimum	-	-	+	-	-
Fusarium solani	+	+	+	-	-
Phytophthora cinnamomi	+	-	+	++	-
P. cactorum	+	-	+	+	++
Gaeumannomyces graminis (C3)	++	+	++	+	+
G. graminis (C5)	++	+	++	++	++
*Penicillium echinulatum**	++	-	+	++	+

[a] Loopfuls of bacteria were spotted 5 mm from the edge of the plates and fungi inoculated in the centre. Inhibition zones were measured after 7 days incubation at 25°C: - = no zone of inhibition; + = zone of inhibition was less than 6 mm; ++ = zone of inhibition was 6-10 mm; +++ = zone of inhibition was more than 10 mm. * Sapstain Fungus

Additions of $FeCl_3$ into PDA or KBM agar resulted in no clear pattern of zone reduction with increasing iron concentration by any isolate, which would be expected if the inhibition was due to ferric chelation (Tables 4.1.2 and 4.1.3 and Plates 4.1.3-4.1.5). Tests of the effects of iron additions on the inhibition of growth of *G. graminis* (C3) by the antagonists were conducted on KBM, while PDA was used for *S. minor* and *S. sclerotiorum* since these fungi grew poorly on KBM. Only weak inhibition of UT2, UT4 and UT5 was apparent on KBM in the absence of $FeCl_3$ (Plates 4.1.3 bottom, 4.1.4 bottom and 4.1.5) and no effect on this inhibition was apparent following its addition.

Plate 4.1.1 Inhibition of *S. sclerotiorum* by antagonists on PDA after 2 weeks incubation at 25°C.

Plate 4.1.2 Inhibition of *S. minor* by antagonists on PDA after 2 weeks incubation at 25°C.


UT 1
UT 2
UT 3
UT 4
UT 5

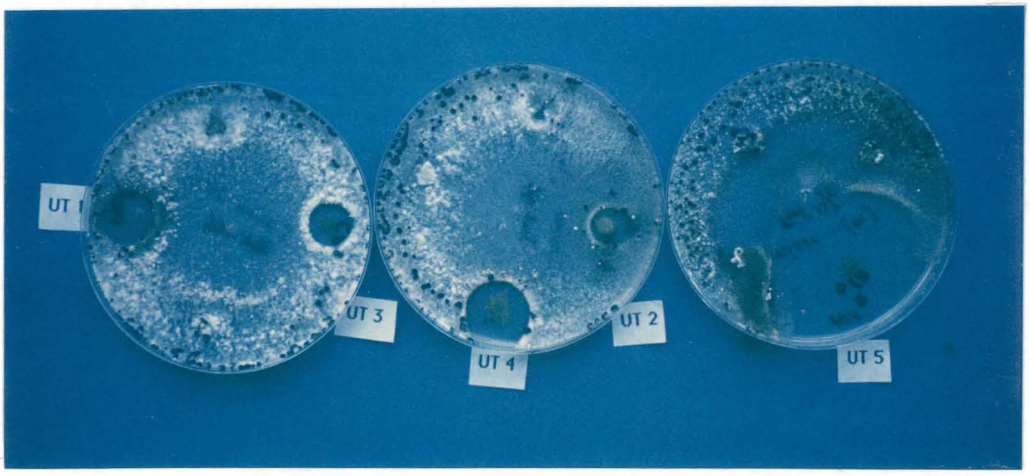

UT 1
UT 3
UT 4
UT 2
UT 5

Table 4.1.2 Mean inhibition zones (mm) of *Sclerotinia sclerotiorum* by bacterial antagonists on PDA amended with varying iron concentrations.[a]

Strain#	$FeCl_3$ concentrations (µM) 0.0	5	10	20	50	100
UT1	3.0 ab*	2.5 b	3.5 a	3.0 ab	1.0 c	2.5 b
UT2	1.5 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a
UT3	3.5 a	2.5 b	3.5 a	3.0 ab	2.5 bc	2.0 c
UT4	3.5 bc	4.5 a	4.0 ab	4.0 ab	3.5 bc	3.0 c
UT5	5.0 b	5.0 b	6.0 ab	7.0 a	6.5 a	6.5 a

* Within a row, values followed by the same letter do not differ significantly ($P < 0.05$) according to Duncan's Multiple Range test.

0.1 ml of 10^8cfu ml^{-1} suspensions were spotted on PDA.

Table 4.1.3 Mean inhibition zones (mm) of *Sclerotinia minor* by antagonists on PDA amended with varying iron concentrations.

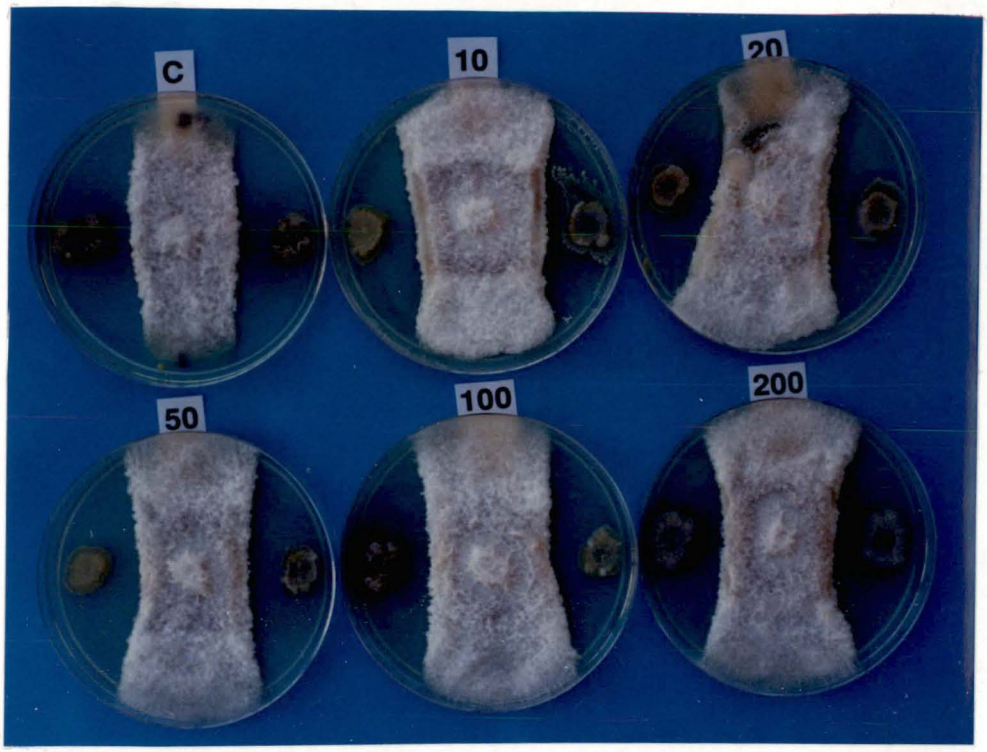
Strain#	$FeCl_3$ concentrations (µM) 0.0	5	10	20	50	100
UT1	5.0 a*	4.5 a	4.5 a	4.5 a	4.5 a	4.0 b
UT2	5.5 a	6.0 a	5.0 ab	4.5 b	5.5 a	4.5 b
UT3	3.5 a	2.0 b	3.0 a	3.5 a	1.5 b	2.0 b
UT4	10.5 a	10.5 a	9.5 ab	9.5ab	10.0 a	8.5 c
UT5	15.5 b	17.5 a	14.0 c	14.5bc	10.5d	14.0c

Symbols are as described in Table 4.1.2.

[a] A regression analysis may have been more appropriate here & in Tables 4.2.1 & 4.2.2

Plates 4.1.3 - 4.1.5 Effects of varying iron concentration on the antagonistic action of bacteria against *Gaeumannomyces graminis*. Iron concentrations in the KBM agar (μM) are shown on the plates (C: control/no $FeCl_3$).

Plate 4.1.3 Bactérial antagonists: strain UT1 (top), strain UT2 (bottom).


C
10
20
50
100
200

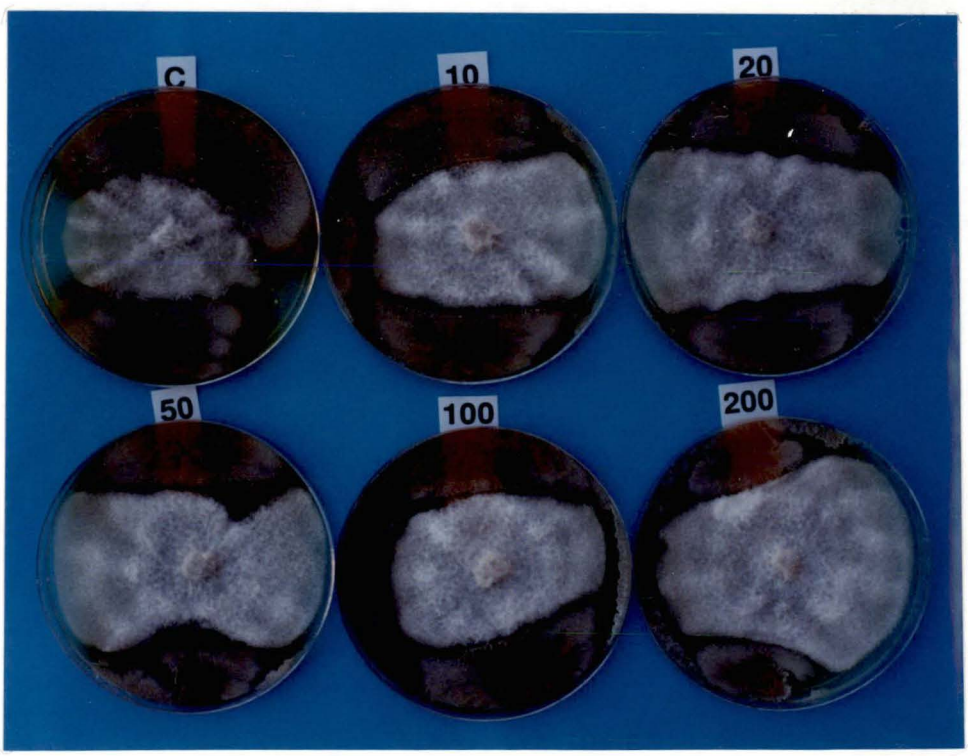
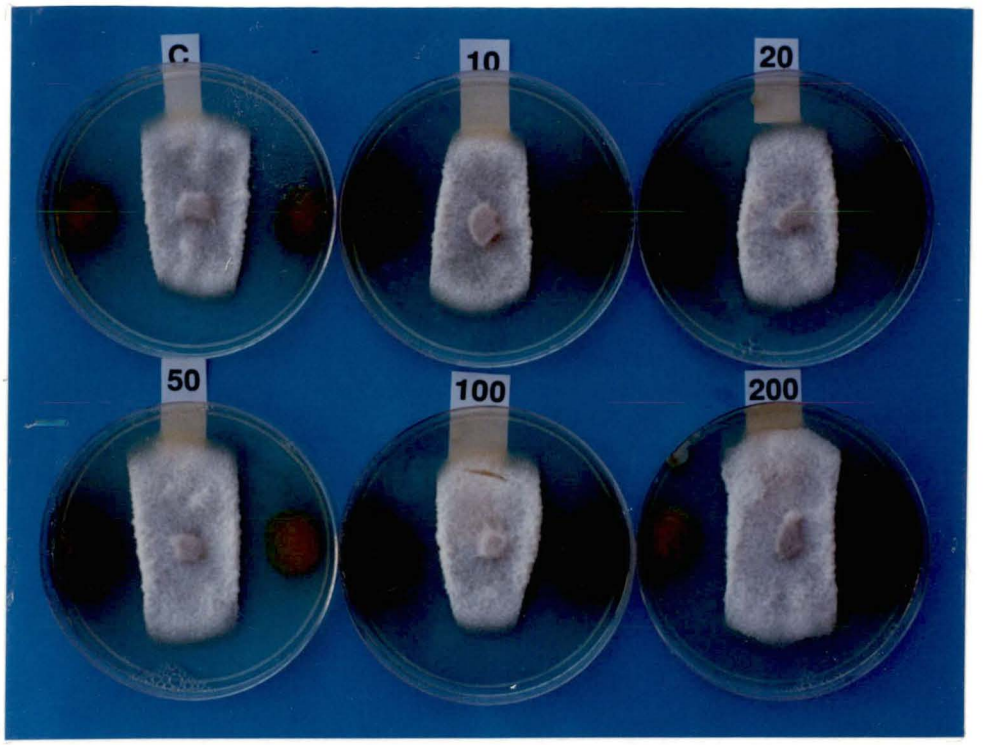

C
10
20
50
100
200

Plate 4.1.4 Bacterial antagonists: strain UT3 (top), strain UT4 (bottom).


C
10
20
50
100
200

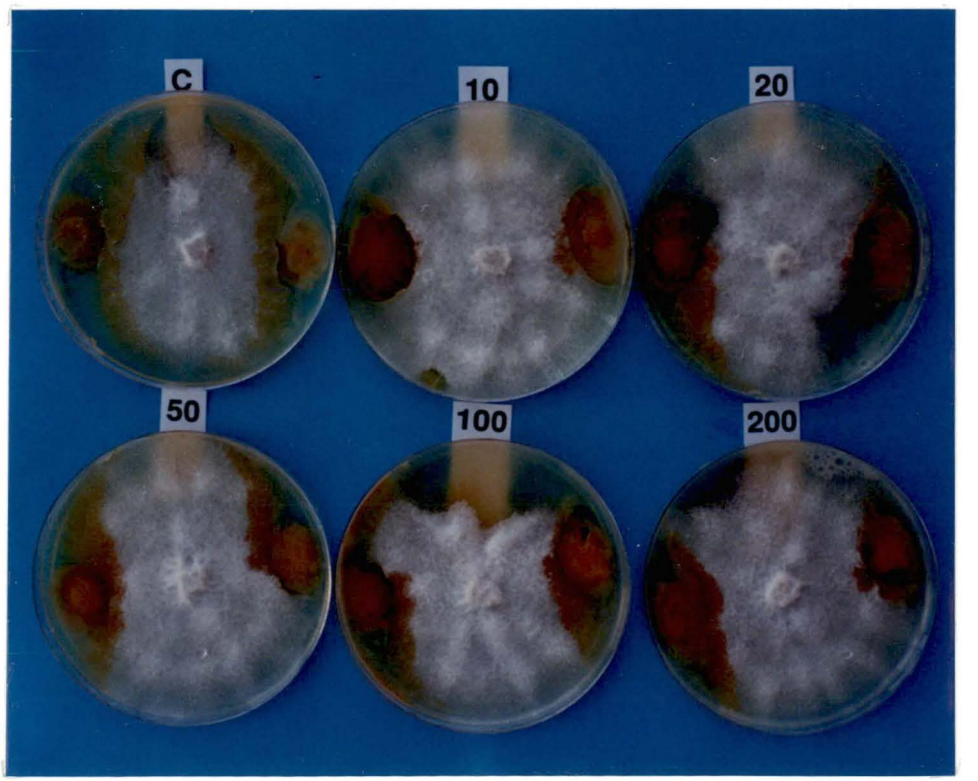
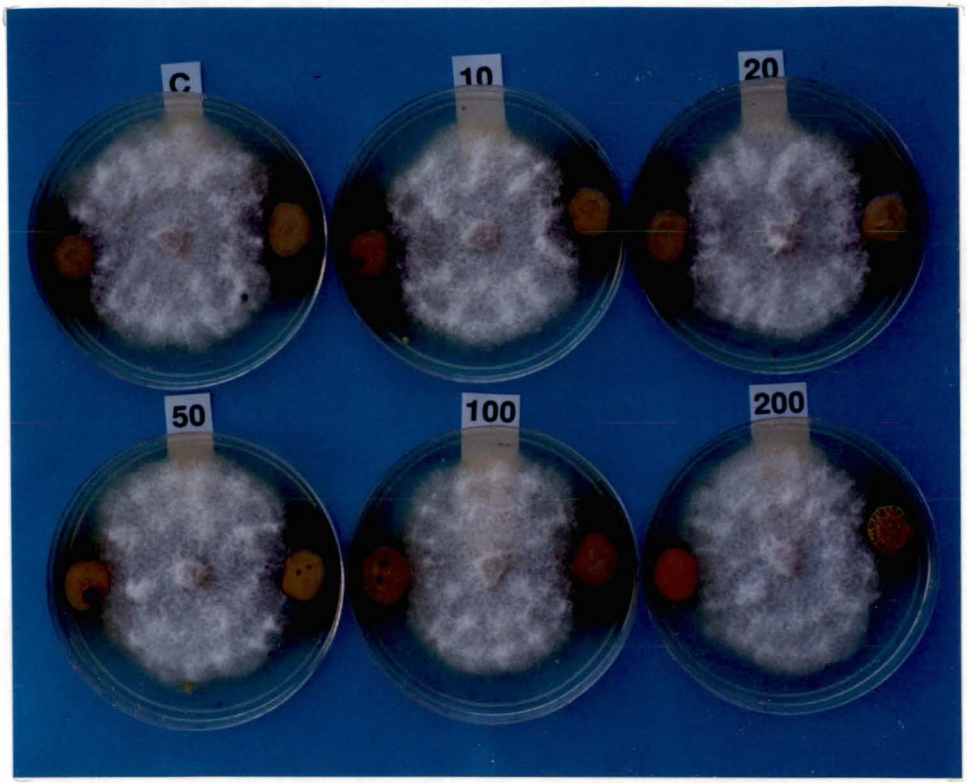

C
10
20
50
100
200

Plate 4.1.5 Bacterial antagonist strain UT5.


C
10
20
50
100
200

4.2 Effect of pH on zones of inhibition of *Sclerotinia* spp. by bacteria

Inhibition of *Sclerotinia* spp. by bacterial antagonists was significantly affected by medium pH (Tables 4.2.1 and 4.2.2), with zones of inhibition of the fungi appearing relatively constant over the range of pH 5.0 to 7.5. However acidic conditions inhibited growth of the bacteria and at pH 9.0 the fungi grew only poorly. This was reflected by the zones of inhibition of fungi at both extremes of pH.

Table 4.2.1 Effect of pH on inhibition of *S. sclerotiorum* by bacterial antagonists (zones of inhibition in mm)[1].

	pH										
Strains	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
UT1	-	2.5^{d} #	3.0^{c}	3.0^{c}	3.0^{c}	2.5^{d}	4.0^{b}	4.0^{b}	4.0^{b}	6.0^{a}	*
UT2	-	1.0^{c}	1.0^{c}	1.0^{c}	2.0^{ab}	1.0^{c}	1.5^{bc}	2.5^{a}	2.0^{ab}	2.0^{ab}	*
UT3	-	2.0^{e}	3.0^{d}	3.5^{cd}	3.5^{cd}	3.5^{cd}	4.0^{c}	4.0^{c}	7.0^{b}	8.5^{a}	*
UT4	-	5.0^{bc}	4.0^{cd}	3.0^{de}	3.0^{de}	3.5^{d}	4.5^{c}	4.5^{c}	5.5^{b}	7.5^{a}	*
UT5	-	6.5^{bc}	9.0^{a}	6.0^{c}	6.0^{c}	5.0^{d}	7.0^{b}	7.0^{b}	6.0^{c}	4.5^{d}	*

[1] Zones given are the mean obtained from 4 replicate plates, prepared and incubated as described in Table 4.1.2.

* Fungal growth was inhibited.

- Bacterial growth was inhibited.

Within a row, values followed with the same letter do not differ significantly ($P<0.01$) according to Duncan's Multiple Range test.

Table 4.2.2 Effect of pH on inhibition zones of *S. minor* by bacterial antagonists (zones of inhibition in mm)[1].

Strains	pH 4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0
UT1	-	$2.5^{d\#}$	4.5^{b}	4.0^{bc}	3.5^{c}	4.0^{bc}	3.0^{cd}	3.0^{cd}	3.0^{cd}	5.5^{a}	*
UT2	-	2.0^{c}	3.0^{b}	2.5^{bc}	2.5^{bc}	3.0^{b}	3.0^{b}	3.5^{ab}	3.5^{ab}	4.0^{a}	*
UT3	-	1.5^{de}	3.0^{d}	3.0^{d}	4.0^{c}	3.5^{cd}	3.0^{d}	5.0^{bc}	6.5^{a}	5.5^{b}	*
UT4	-	8.5^{bc}	9.0^{b}	9.0^{b}	8.5^{bc}	10.0^{a}	7.5^{e}	8.0^{cd}	8.0^{cd}	9.5^{ab}	*
UT5	-	14.0^{a}	15.0^{a}	15.0^{a}	10.5^{b}	11.0^{b}	6.0^{c}	6.0^{c}	5.0^{c}	10.0^{b}	*

[1] Symbols are as given in Table 4.2.1.

4.3 Identification of bacterial antagonists

Characteristics of the five antagonistic bacteria are given in Table 4.3.1. All isolates were able to grow on common media. On PDA, strains UT1, UT3 and UT4 formed thick slimy colonies, strain UT3 had yellow green colonies and strain UT4 formed yellow colonies and produced a yellow green diffusible pigment. On KBM agar, UT3 and UT4 colonies were yellow and non-fluorescent; colonies of strains UT1 and UT2 were similar to those formed on PDA; while isolate UT5 produced a diffusible dark brown pigment. Isolate UT3 also produced yellow slimy growth on TSA. Based on their characteristics, the five antagonistic isolates were identified as *Bacillus polymyxa* (UT1), *B. subtilis* (UT2), *Pseudomonas cepacia* (UT3), *P. putida** (UT4), and *Acinetobacter* sp. (UT5).

*Although atypical reactions were recorded for starch hydrolysis and utilization of various C-sources.

Table 4.3.1 Characteristics of bacterial antagonists.

Characteristics	Strains UT1	UT2	UT3	UT4	UT5
Cell diameter >1µm	-	-	-	-	-
Cell length (µm)	>2	>2	>1	>2	>2
Spore position	T	C			
Spore shape	E	E			
Sporangium swollen	+	-			
Gram stain	+	+	-	-	-
Cell morphology	rods	rods	rods	rods	rods
Motility	+	+	+	+	-
Flagella	P	P	one polar	1or2 polar	-
O/F reaction	F	O	O	O	O
Oxidase	-	+	+	+	-
Catalase	+	+	+	+	+
PHB accumulation			+	-	+
Arginine dihydrolase	-	-	-	+	-
Growth at (°C):					
4	+	-	-	+	-
10	+	-	-	+	-
30	+	+	+	+	+
41	+	+	-	-	-
50	-	+	-	-	-
55	-	-	-	-	-
Anaerobic growth	+	-			
Pyoverdin production	-	-	-	-	-
Pyocyanin production	-	-	+	-	-
Lecithinase (Egg Yolk)	-	+	+	+	+
Nitrate reduction	+	+	-	+	-

Characteristics	UT1	UT2	UT3	UT4	UT5
Denitrification	-	-	-	-	-
Levan formation from sucrose	+	-	-	-	-
Gelatin liquefaction	+	+	+	-	-
Hydrolysis of:					
Tween 80	-	-	+	+	+
Casein	+	+	+	+	-
Starch	+	+	+	+	+
Acid from:					
D- Glucose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Mannitol	+	+	-	+	+
D-Xylose	+	+	-	+	-
Gas from glucose	+	-			
Utilization of:					
Citrate	-	-			-
Levulinate	+	+	+	-	-
m-Tartrate	+	+	+	-	-
2-Ketogluconate	-	-	+	+	+
Glucose	+	+	+	+	+
Trehalose	+	+	+	+	+
D-Ribose	+	+	+	+	+
L-Rhamnose	+	+	+	+	+
D-Xylose	+	+	-	+	+
D-Galactose	+	+	+	+	+
D-Arabinose	-	-	-	+	+
L(+) Arabinose	+	+	+	+	+
Cellobiose	-	+	+	+	+
Meso-Inositol	-	+	+	+	-

Characteristics	UT1	UT2	UT3	UT4	UT5
Adonitol	-	-	+	+	+
Geraniol	-	-	-	-	-
L-Valine	-	-	+	+	+
ß-Alanine	-	-	+	+	+
DL-Arginine	-	+	+	+	-
L-Tryptophan	+	-	+	+	+
Testosterone	-	-	+	+	-
p-Hydroxybenzoate	-	+	+	-	-
Tryptamine	+	-	+	+	+
Histamine	-	+	+	+	+
Growth in:					
2% NaCl	+	+			
5% NaCl	-	+			
7% NaCl	-	+			
10% NaCl	-	+			
Voges Proskauer	+	+			
Deamination of phenylalanine	-	-			
Degradation of tyrosine	-	-	-	+	
pH in V-P broth:					
< 6	+	+			
> 7	-	-			
Growth in nutrient broth at pH:					
6.8,	+	+			
5.7	+	+			
Formation of:					
Indole	-	-			
Dihydroxyacetone	+	-			

Characteristics	UT1	UT2	UT3	UT4	UT5
Resistance to:					
Ampicillin 10 µg	-	-	+	+	+
Bacitracin 10 iu	+	-	+	+	-
Cephalothin 30 µg	+	-	+	+	+
Chloramphenicol 30 µg	-	-	-	+	-
Erythromycin 15 µg	-	-	+	+	-
Gentamicin 10 µg	-	-	-	-	-
Nalidixic acid 30 µg	-	-	-	-	-
Neomycin 30 µg	-	-	+	-	-
Novobiocin 30 µg	-	-	-	+	-
Penicilin G 10 iu	-	-	-	+	+
Polymixin B 300 iu	+	-	-	-	-
Streptomycin 10 µg	-	-	+	-	-
Tetracycline 30 µg	-	-	-	-	-

Symbols: T, terminal; C, central; E, ellipsoidal; P, peritrichous; O, oxidative; F, fermentative.

Antibiotic sensitivity testing of the isolates indicated potential selective agents for use in KBM: strains UT1, UT3 and UT4 tolerated bacitracin and pentachloronitrobenzene (PCNB) at concentrations up to 7.2 units ml^{-1} and 500 $\mu g\ ml^{-1}$, respectively; isolate UT1 also tolerated polymyxin B at concentrations up to 395 units ml^{-1}, while strains UT3 and UT4 tolerated ampicillin at concentrations up to 200 $\mu g\ ml^{-1}$. KBM containing these selective agents were identified as KB-PBP (for the isolation of UT1 and containing pentachloronitrobenzene 500 $\mu g\ ml^{-1}$, bacitracin 7.2 units ml^{-1}, and polymyxin B 395 units ml^{-1}) and KB-PAB (for the isolation of UT3 and UT4 and containing pentachloronitrobenzene 500 $\mu g\ ml^{-1}$, ampicillin 200 $\mu g\ ml^{-1}$, and bacitracin 7.2 units ml^{-1})

4.4 Suppression of wheat take-all by antagonists in axenic sand culture

The ability of the antagonists to inhibit *Gaeumannomyces graminis* var. *tritici* (C3) causing take-all disease was assessed in sand culture. The results after 3 weeks growth are shown in Table 4.4.1 and Plates 4.4.1 - 4.4.4. All antagonists significantly suppressed the wheat take-all disease ($P < 0.05$); strain UT3 being the most inhibitory to the pathogen, with dry weight of the plant tops being equivalent to the dry weight of controls treated with bacteria alone.

Applications of the isolates UT1, UT2 and NIR-6 to pathogen-treated plants resulted in mean disease ratings of from 4.7 to 4.9, with plants showing symptoms of lesions on all roots and often necrosis around the stem base (rating 5). Strain UT4 further reduced the effect of the pathogen, with roots of wheat seedlings showing lesions which coalesced around the base of the stem. Strain UT3 gave the lowest mean disease rating of 2.2 with plants showing typical lesions only on

three or more seminal roots.

Curiously there were significant reductions in the weight of wheat tops in plants with antagonists (only) added as compared with uninoculated controls. However, the seedlings looked healthy and no disease symtoms were found on the roots.

The numbers (log_{10}) of antagonists initially added to each tube were 8.60, 8.30, 8.30, 8.90 and 8.85 cfu (colony forming units) ml^{-1} for strains UT1, UT2, UT3, UT4 and NIR-6, respectively. At harvest, the numbers of cfu (in the same order) were 8.36, 7.48, 8.40, 9.0, and 8.70 cfu g^{-1} of root+sand (in associated with the fungus), and 8.55, 7.99, 8.66, 8.48, and 8.64 cfu g^{-1} of root+sand (bacteria alone). There were no marked differences in numbers (cfu/[g root+sand]) of any of the isolates at the harvest although clearly all must have proliferated to give numbers per g root+sand equivalent to numbers per ml of the initial inoculum.

In contrast to the above, scanning electron microscopy showed extensive colonization of the root surface by strains UT3 and UT4 (Plate 4.4.6) compared with apparently poor colonization by the *Bacillus* strains UT1 and UT2 and by strain NIR-6 (Plates 4.4.5 and 4.4.7). However, the latter results may possibly be due to a loose attachment of the bacteria to the root surface, resulting in their loss during the SEM fixation and dehydration processes.

Table 4.4.1 Suppression of take-all fungus *Gaeumannomyces graminis* var. *tritici* (C3) by antagonists on wheat seedlings in tube assays after 3 weeks growth.

Treatment	Dry weight of wheat tops (mg/plant)	Dw relative to bacterial control	Dw relative to pathogen-free control	Disease index 0 - 7
Pathogen-free control	26.8 ± 0.04 a©			0*
Ggt control	16.8 ± 0.1 e		0.63	6.8 a©
UT1	24.6 ± 1.9 b		0.92	0*
UT2	24.5 ± 5.1 b		0.92	0
UT3	22.5 ± 2.0 c		0.84	0
UT4	24.7 ± 3.9 b		0.92	0
NIR-6	23.5 ± 0.1 bc		0.88	0
UT1 + Ggt	21.5 ± 1.7 cd	0.87	0.80	4.8 b
UT2 + Ggt	22.2 ± 1.8 c	0.91	0.83	4.7 b
UT3 + Ggt	24.6 ± 1.8 b	1.09	0.92	2.2 c
UT4 + Ggt	22.3 ± 1.9 c	0.91	0.83	4.5 b
NIR-6 + Ggt	20.1 ± 0.1 d	0.86	0.75	4.9 b

© Within a column, values followed by the same letter do not differ significantly ($P < 0.05$) according to Duncan's Multiple Range test.

* Nil values were not included in the statistical analysis.

Dw: Dry weight. Four replicates/treatment were used

Ggt: *Gaeumannomyces graminis* var. *tritici*.

Root disease was assessed by the rating system of Wildermuth (1980):

Description of typical plants	*Disease Rating*
No visible sign of disease	0
Lesions on one or two seminal roots; no lesions on stem	1
Lesions on three or more seminal roots; no lesions on stem	2
Lesions on all roots and discrete lesions on the base of stem	3
Lesions on all roots and lesions coalesced around base of stem	4
As 4 but necrosis around stem base more severe	5
Seedling so severely diseased that only the first leaf produced; leaf still green	6
Seedling completely necrotic	7

Plates 4.4.1 - 4.4.4 Wheat seedling performances following treatments with *G. graminis* and bacterial antagonists after 3 weeks growth in axenic tube assay.

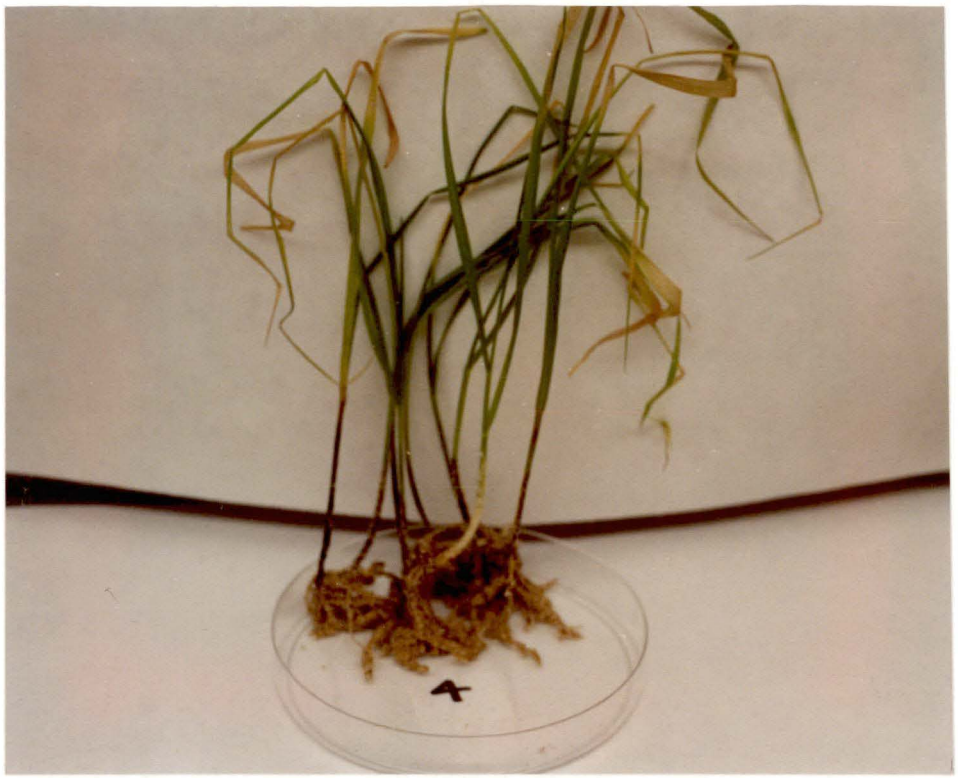
Plate 4.4.1 Experimental growth chamber (top), seedlings treated with Ggt alone (bottom left), seedlings treated with Ggt+strain UT1 (bottom right).

Plate 4.4.2 Seedlings treated with Ggt+strain UT2 (top), and seedlings treated with Ggt+strain UT3 (bottom).


2


3

Plate 4.4.3 Seedlings treated with Ggt+strain UT4 (top), and seedlings treated with Ggt+strain NIR-6 (bottom).


4


6

Plate 4.4.4 Pathogen-free controls.


K

Plates 4.4.5 - 4.4.7 Scanning electron microscopy of wheat-root surface colonization by bacterial antagonists following monoaxenix inoculation of seedlings in sterile sand.

Plate 4.4.5 Strain UT1 (top), and strain UT2 with some possible spores (bottom).


10µm20.0kV 1.25E3 0004/00 UT 1

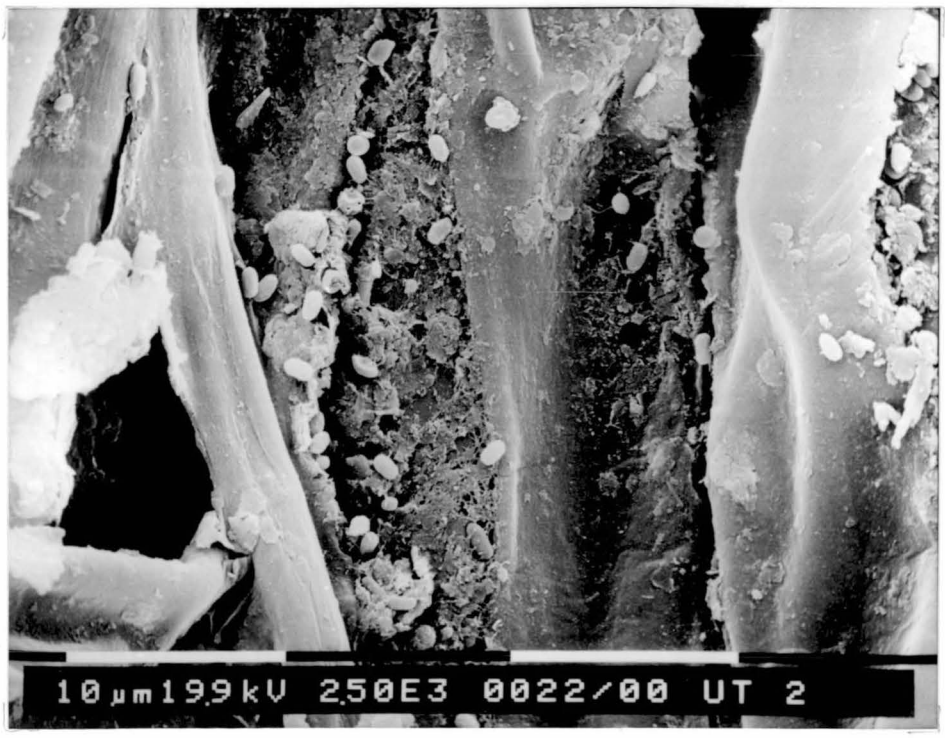
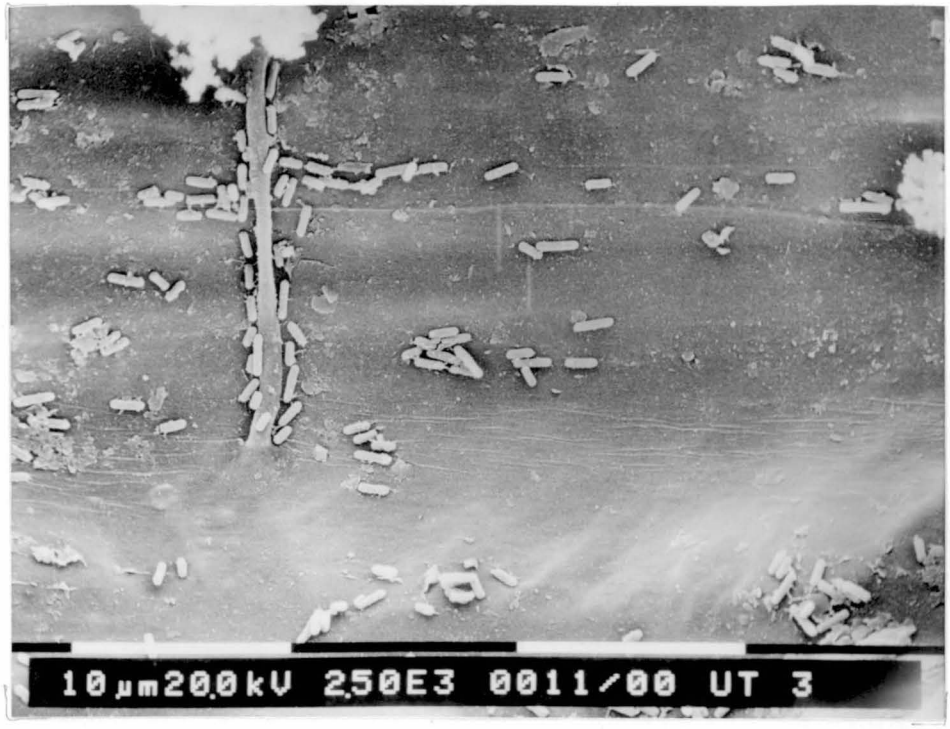

10µm19.9kV 2.50E3 0022/00 UT 2

Plate 4.4.6 Strain UT3 (top), and strain UT4 (bottom).


10µm20.0kV 2.50E3 0011/00 UT 3

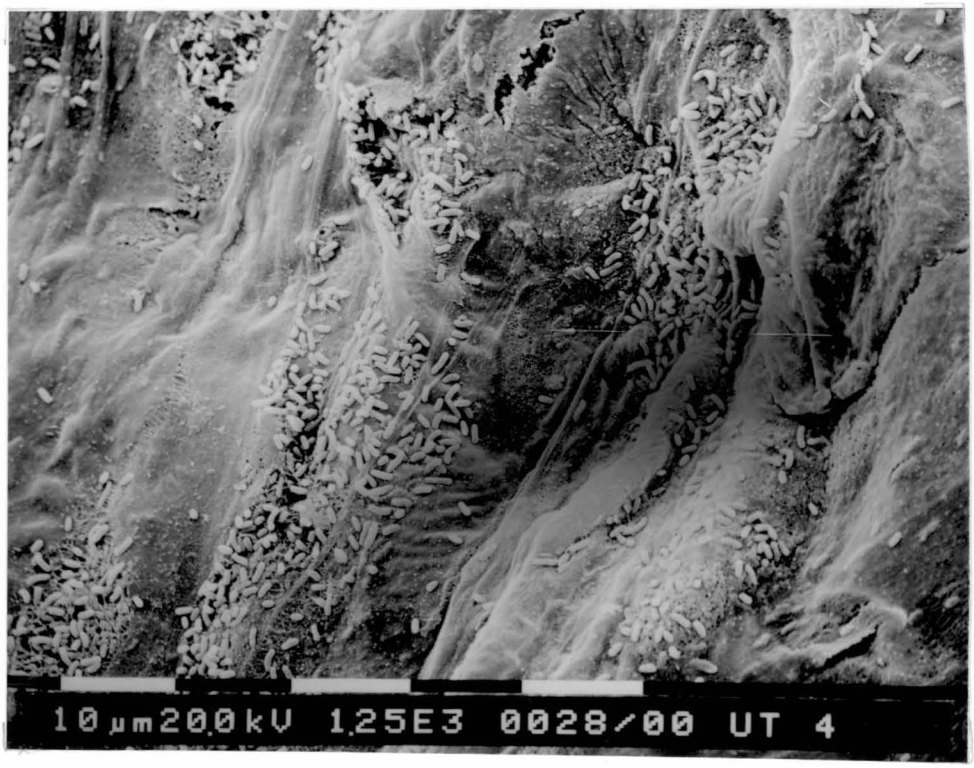
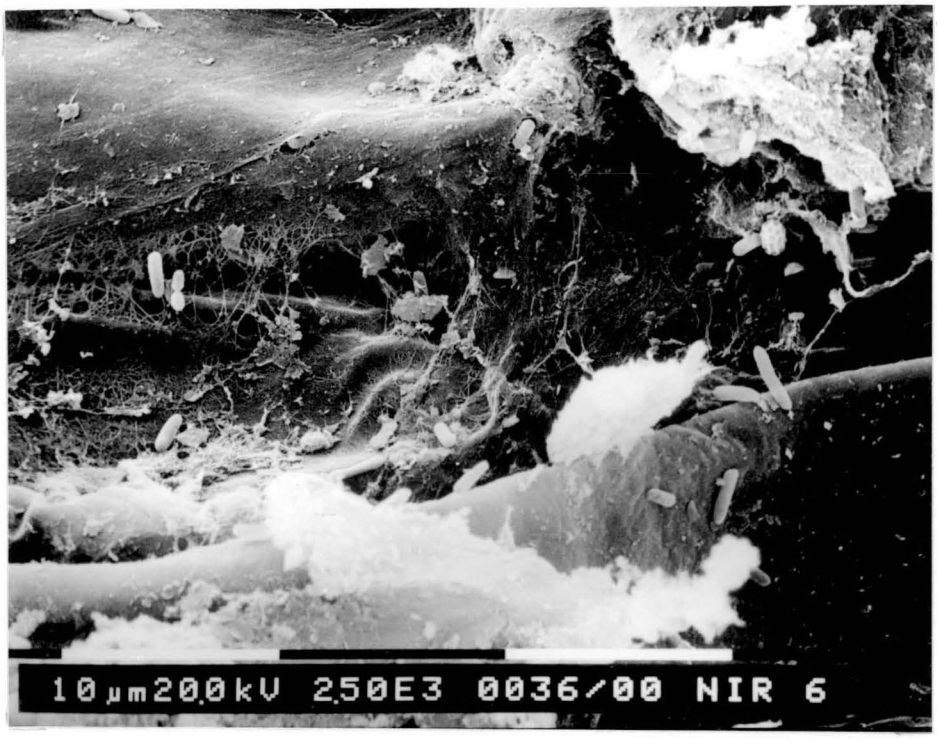

10µm20.0kV 1.25E3 0028/00 UT 4

Plate 4.4.7 Strain NIR-6.


10µm20.0kV 2.50E3 0036/00 NIR 6

4.5 Greenhouse pot assays

A greenhouse trial of the effectiveness of the various bacterial antagonists in protecting lettuce seedlings in potting mix infested with either *S. sclerotiorum* or *S. minor* was assessed after a 12 weeks period. Protection of lettuces from attack by *S. sclerotiorum* was demonstrated for all isolates (Figure 4.5.1). At 6-8 weeks into the trial only 20% of the control plants remained healthy compared with 80% of plants inoculated with the antagonists UT2 and UT5, or 60% of plants inoculated with UT1, UT3 and UT4. After 12 weeks growth, 10% of the control plants remained healthy compared with between 40 and 60% of the treated plants.

The antagonists UT1 and UT2 were less effective in controlling *S. minor* infection (Figure 4.5.2), although after 10 weeks growth 60% of plants inoculated with strains UT3, UT4, and UT5 remained healthy compared with 10% of the control plants.

Efforts to specifically reisolate the antagonists from the lettuce roots after the 12 weeks trial failed for strains UT1, UT2, UT3 and UT5*, however, strain UT4 could be reisolated from the lettuces infested with either *S. minor* or *S. sclerotiorum* in numbers of 2 x 10^3 and 4 x 10^3 cfu per gram roots, respectively.

*(These apparently being in low abundance)

Figure 4.5.1 Relative protection of lettuces provided by bacteria antagonistic to *Sclerotinia sclerotiorum* in pot trials[1].

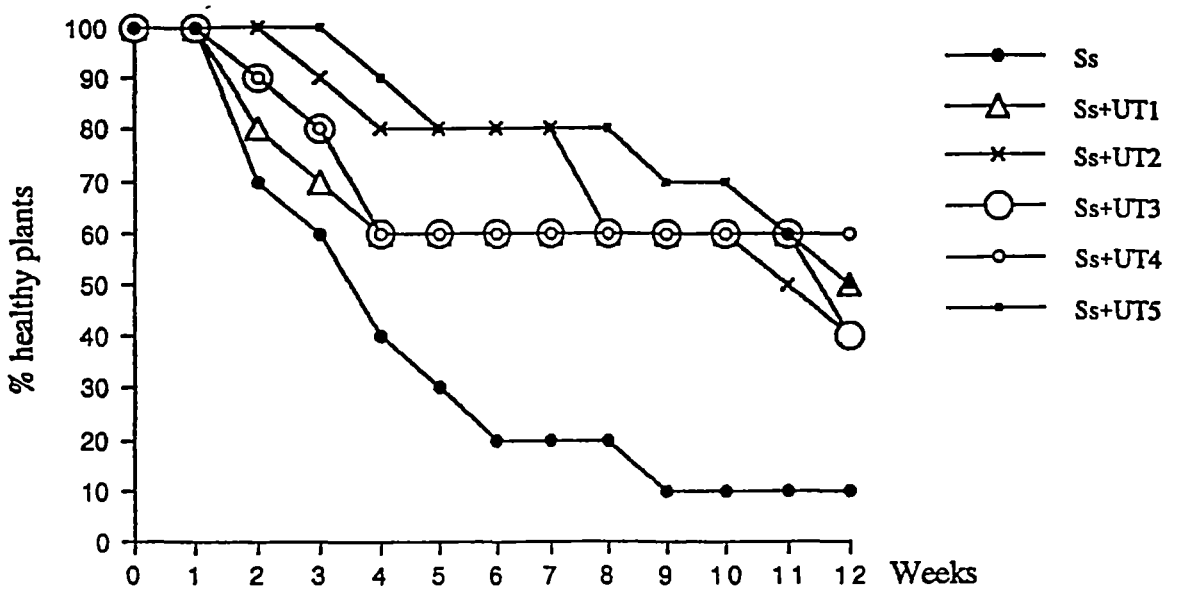
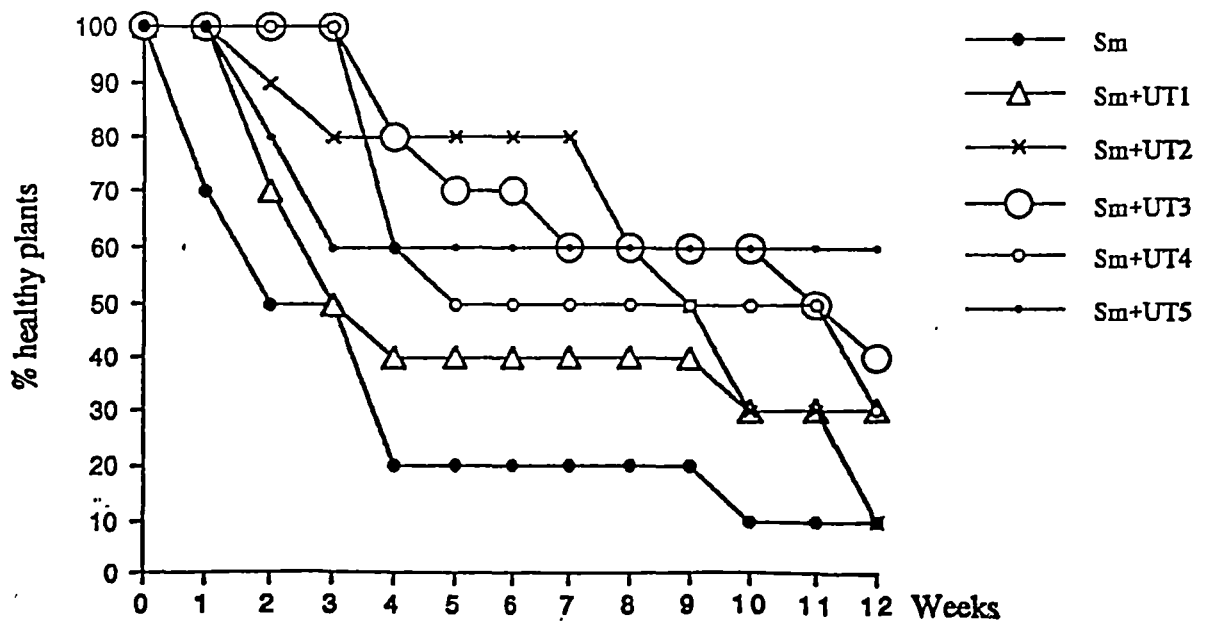


Figure 4.5.2 Relative protection of lettuces provided by bacteria antagonistic to *Sclerotinia minor* in pot trials[1].



[1] Each teatment is represented by 10 plants in separate pots.
Ss: *Sclerotinia sclerotiorum*, Sm: *Sclerotinia minor*.

4.6 Trial of the efficacy of antagonists in the field

A field trial of all isolates to assess their ability to protect daisy seedlings (*Olearia phlogopappa*) from root rot was established following their demonstrated effectiveness in protecting lettuces against *Sclerotinia* spp. in the glasshouse. The results are indicated in Table 4.6.1.

Differences between treatments were not statistically significant, although the number of healthy plants treated with isolates UT1, UT4, and NIR-6 were greater than the number of untreated controls.

Fungi associated with root rot of daisy were isolated from the roots of some of the plants and identified as *Pythium* sp. (aseptate hyphae and small, spherical sporangia). A representative isolate challenged with each of the five antagonists *in vitro* as described previously, was inhibited only by strain UT3, (as was the stock culture of *P. ultimum*) with zones of inhibition less than 3 mm.

Tabel 4.6.1 Field trial of the effectiveness of bacterial antagonists in protecting *Olearia* seedlings against root rot.

Treatments	numbers of healthy plants*	$\sqrt{Y+1/2}$ transformation
Control	10	0.97
UT1	13	1.02
UT2	11	0.99
UT3	11	0.99
UT4	12	1.02
UT5	10	0.97
NIR-6	12	1.02
SD@ of mean		0.26

* Out of 20 plants per treatment.

@ Standard deviation.

4.7 Antimicrobial compounds produced by isolates UT1 and UT3

The presence of antifungal substances produced by isolates UT1 and UT3 was indicated qualitatively by the production of zones of inhibition surrounding filter paper discs soaked in the concentrated samples of crude extract (UT1 in sterile water and UT3 in methanol). In subsequent quantitative tests, crude extracts of strains UT1 and UT3 were found to be inhibitory to *Sclerotinia* spp. and *Gaeumannomyces graminis* C3 at concentrations above 1000 μg ml^{-1} and 50 μg ml^{-1}, respectively (Plates 4.7.1 and 4.7.2). The highest activity was exhibited by the metabolites derived from strain UT3 (*P. cepacia*) which prompted further purification of the active compound produced by this strain.

The dry weight of UT3 crude extract varied from 13 to 160 mg/200 ml of PDA (10 plates) although the medium used, incubation, and extraction procedures were similar.

The crude extract of UT3 was yellow-orange in colour. The bioactive compounds were soluble in methanol, largely soluble in chloroform, slightly in ethyl acetate and insoluble in acetonitrile. The inhibitory activity of the crude extracts was not affected by heating at 100°C for 10 minutes, or by acid or alkali treatments (Table 4.7.1).

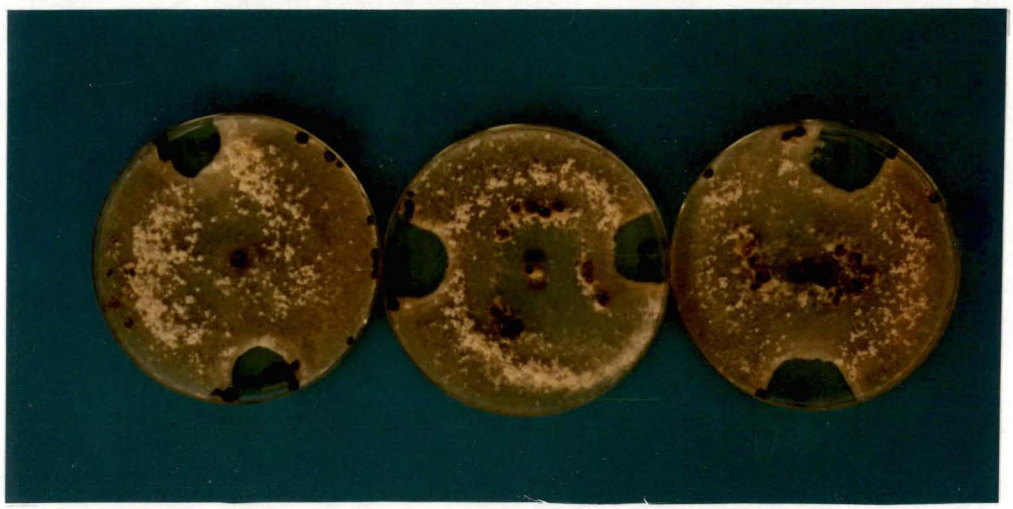
Table 4.7.1 Effects of heat, acid and alkali on the inhibitory activity of UT3 antifungal compounds.

Treatment	*S. sclerotiorum*	*S. minor*	*G. graminis* (C3)
pH 2	2.5*	3	4.5
Control	3	3	5
pH 9	3	3	5
Heating	3	3	5

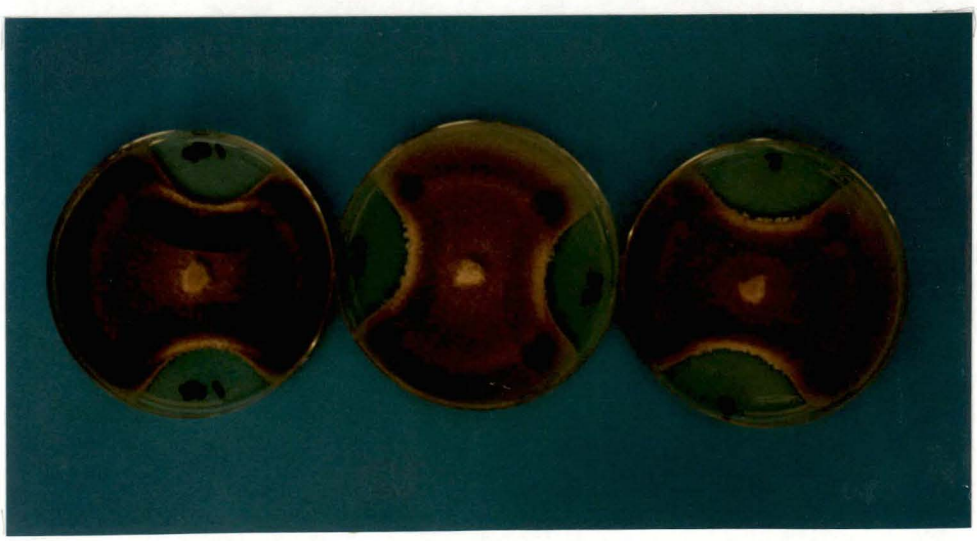
* Diameter of zones of inhibition (in mm) in agar.

Plate 4.7.1 Effect of UT3 crude extract on the growth of *S. minor* (1), *S. sclerotiorum* (2), and *G. graminis* (3) after 3 weeks incubation at 25°C. The concentrations of the extract were 100, 250, and 500 μg ml^{-1} (left to right).

(1)

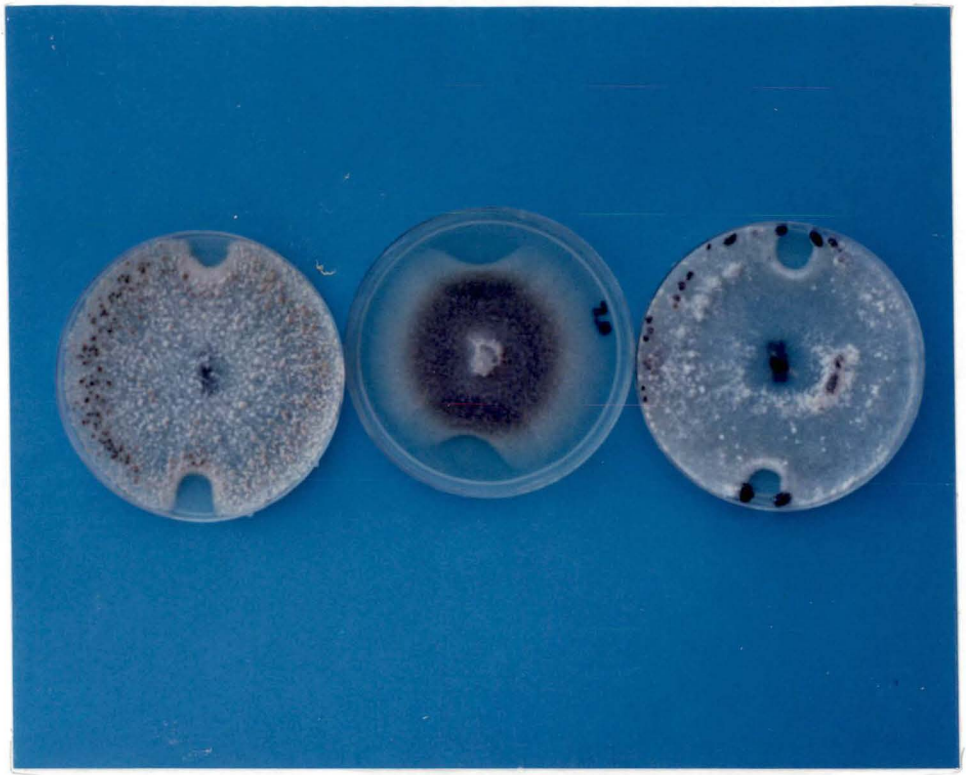


(2)



(3)

Plate 4.7.2 Effect of 50 μg ml^{-1} of UT3 crude extract on the growth of *S. minor*, *G. graminis*, and *S. sclerotiorum* (left to right) after 7 days incubation.



Thin layer chromatography of crude UT3 extract

Separation of the crude extract of *Pseudomonas cepacia* UT3 on thin-layer silica gel plates resulted in two zones of inhibition using a bacterial indicator (*Bacillus subtilis*) and one zone using a fungal indicator (*Ascochyta pisi*). This showed that two different antibiotics were produced by strain UT3. The lower R_f active compound was designated compound A and the higher R_f compound was designated compound B. Compound A was strongly inhibitory to both the bacterium and fungus tested while compound B was only active against the bacterium (Table 4.7.2). Plate 4.7.3 shows the active bands produced on TLC plates developed in different solvent systems, with *A. pisi* as the fungal indicator. Compound A was visible as a dark spot under illumination at 254 nm which weakly fluoresced under 366 nm ultraviolet light and compound B fluoresced strongly under illumination at 366 nm.

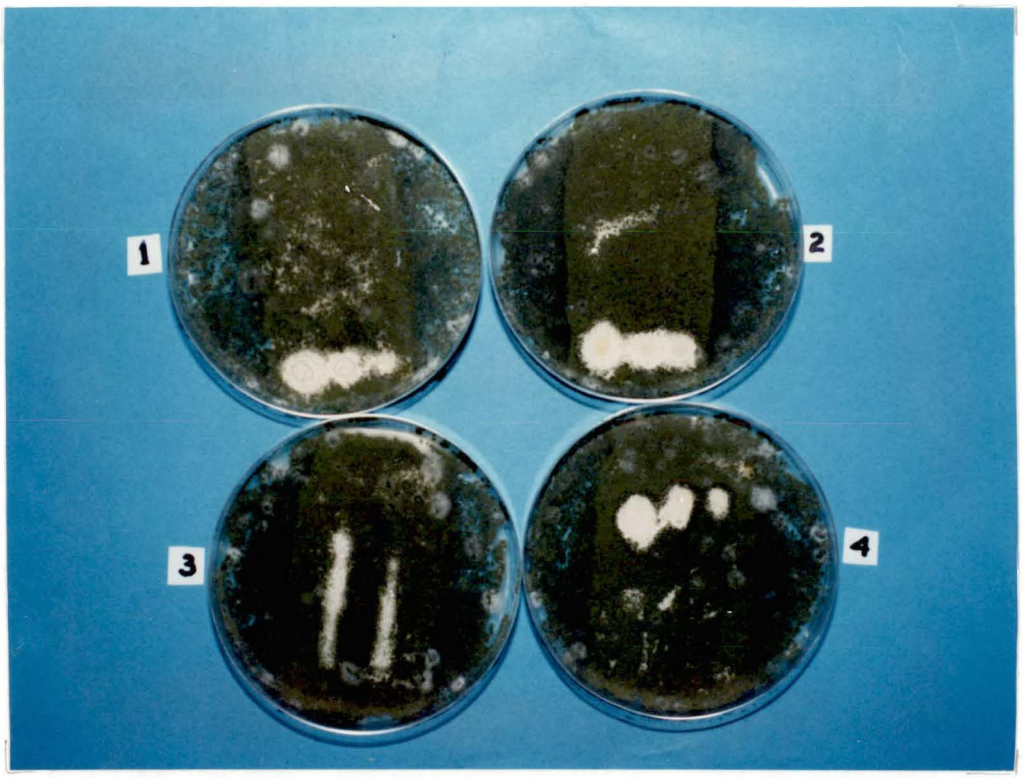
Table 4.7.2 Silica gel thin-layer chromatographic migration of the antibiotics produced by *P. cepacia* UT3.

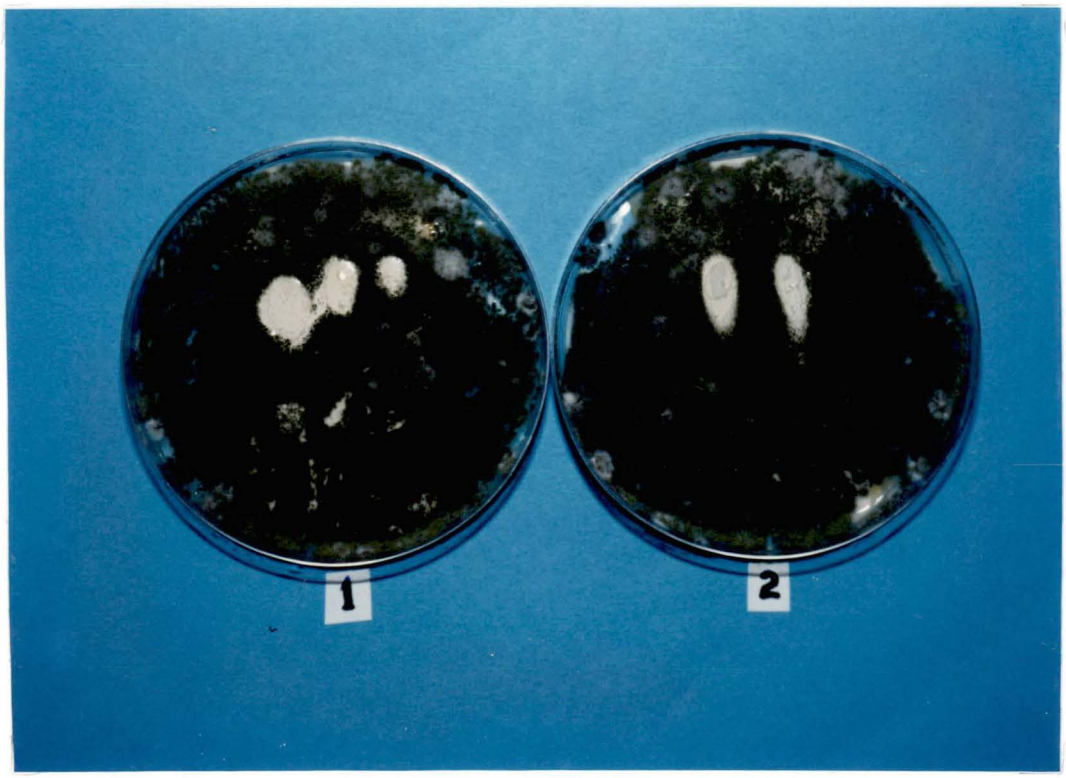
Solvent system	Compound	R_f value@
Chloroform (100%)	A&B	0
Chloroform-acetone (9:1)	A	0
	B	0.12
Chloroform-methanol (8:2)	A	0.28
	B	0.67
Chloroform-methanol (7:3)	A	0.40
	B	0.87
Chloroform-methanol (6:4)	A	0.67
	B	0.94

@ Based on bioautography of TLC plates.

Plate 4.7.3 Bioautography of TLC plates using *A. pisi* as a fungal indicator after 3 days incubation. The plates were developed in 100% chloroform (1), chloroform-acetone [(9:1) 2] chloroform-methanol [(7:3) 3] and chloroform-methanol [(6:4) 4]. Plates are aligned in these photographs with the TLC running bottom to top.

Plate 4.7.4 Bioautographycal comparison between UT3 crude extract (1) and HPLC-active fraction (2) developed in chloroform-methanol (6:4).


1
2
3
4


1
2

Tables 4.7.3 and 4.7.4 show that the antibiotics produced by strain UT3 of *P. cepacia* were qualitatively inhibitory to several fungi and bacteria tested. The take-all fungus and gram-positive bacteria were the most sensitive microorganisms to the compounds. It was also noted that at a similar amount of the antibiotics was bacteriostatic to *Escherichia coli* after 5 hrs incubation (based on optical density measured using a spectrophotometer).

Table 4.7.3 Zones of inhibition (mm) produced by antibiotics isolated from *P. cepacia* UT3 after one week incubation at 25°C.

Fungus	Crude extract	Partially Purified antibiotic fraction A4*
Gaeumannomyces graminis (C3)	6	6
Sclerotinia minor	3	3
S. sclerotiorum	3	3
Phoma exigua exigua	4	3
Fusarium lateritum	3	3
F. solani	0	0
Botrytis cinerea	4	4
Monilia fructicola	+	+
Pythium ultimum	+	+
Rhizoctonia solani	3	3
Coniothyrium minitans	0	0
Trichoderma sp.	0	0
Phytophthora cactorum	2	2
Alternaria solani	4	3
Penicillium expansum	2	2
Ascochyta pisi	4	5
Colletotrichum gloeoshoriodes	6	5
Verticillium tenerum	2	2

+: no zone of inhibition produced, but the antibiotic spot was not overgrown by the fungus. * see P115

Table 4.7.4 Antibacterial spectrum of the compounds produced by *P. cepacia* UT3

Bacterium	Crude extract	Purified antibiotic fraction A4
Bacillus subtilis	Inh	Inh
B. cereus var. *mycoides*	Inh	Inh
B. thuringiensis	Inh	Inh
Escherichia coli	Non*	Non*
Salmonella typhimurium	Non	Non
Staphylococcus aureus	Inh	Inh
Erwinia sp.	Non	Non
Enterobacter cloacae	Non	Non
Pseudomonas aeruginosa	Non	Non
P. syringae	Inh	Inh

Inh: the bacteria are inhibited after 48 h incubation at 25°C or 37°C; Non: not inhibitory after 48 h incubation.

*Bacteriostatic after 5 h incubation.

HPLC separation of the crude UT3 extract

A 26mg sample of the crude extract dissolved in methanol (150µl) was separated into 5 fractions (A1-A5) (Figure 4.7.1) by HPLC, using a linear 50-100% methanol gradient containing 0.1% TFA (pH 2) using the Waters µ-Bondapak column. It was found that some components tended to elute as very broad peaks in the absence of TFA. After evaporating the fractions, each fraction was dissolved in 1.5ml methanol for antimicrobial assay. It was found that inhibitory substances were only present in fraction A4.

It was observed that the aqueous fraction remaining after ether

partitioning of the crude extract was also qualitatively inhibitory to both bacteria and fungi. An aliquot of the aqueous fraction (1.5 ml from 70 ml total) was separated using a 0-100% linear methanol gradient containing 0.1% TFA (pH 2) using the Waters μ-Bondapak column (Figure 4.7.2). The six fractions (B1-B6) collected were evaporated to dryness; fractions B1-B4 were dissolved in water and fractions B5-B6 were dissolved in methanol-water (1:1) for antimicrobial assay. It was found that only fraction B6 was active.

The analytical chromatograms of the crude extract and the aqueous solution (Figure 4.7.3) both showed peaks of similar retention time eluting between 20 and 25 minutes. This region appears to contain all of the antimicrobial components in both the organic extract and the aqueous fraction. Because it was likely that the same compounds were present in both fractions, it seemed that the active compounds had not been completely extracted. The pH of the aqueous solution was measured and found to be 5.5, very close to that of the PDA medium used to culture the bacterium. The solution was basified to pH 10 with 5% sodium hydroxide and extracted 3 times with ether. This extract showed no antimicrobial activity. The aqueous layer was then acidified to pH 2 with 5% hydrochloric acid and extracted with ether. This extract showed qualitatively similar antimicrobial activity to the initial crude extract. Analytical HPLC showed a similar composition to that of the initial extract (Figure 4.7.4) and bioautographs of thin layer chromatograms showed zones of inhibition at similar R_f values. The aqueous layer after neutralisation was devoid of activity after this treatment. This experiment indicated that the active compounds were moderately acidic, being completely extracted at pH 2 but not at pH 5.5.

When fraction A4 (from HPLC of the crude extract) was analysed by thin-layer chromatography as described above, it showed two* active zones having similar R_f values as the active substances in the crude extract (Table 4.7.2). Plate 4.7.4 shows active bands from the crude

* These probably indicate the breakdown of a larger compound.

extract and from fraction A4 on bioautography of TLC plates with *A. pisi* as fungal indicator. Clearly these two compounds having very different R_f values on TLC, eluted at similar retention times on reversed phase HPLC.

Thin-layer chromatographic separation of the crude UT3 extract

Although the resolution of individual components on reversed phase HPLC was very good it seemed that thin-layer chromatography on silica gel might provide a better preliminary separation of the active substances. Larger samples could also be handled more easily. The crude extract (including the extract after acidification) amounted to 397 mg and this was chromatographed on 4 plates (20x20cm, 0.25mm) using 30% methanol-chloroform as developing solvent. Four fractions were collected;

Fraction C1	R_f 0.86-1.00	54.9 mg
Fraction C2	R_f 0.63-0.86	74.0 mg
Fraction C3	R_f 0.40-0.63	104.6 mg
Fraction C4	R_f 0.11-0.40	160.4 mg

As expected from preliminary bioautography, fraction C3 contained the active components and the higher R_f active compound (Compound B) was apparently absent. This might be due to decomposition of this compound during storage or during TLC separation. Analytical HPLC showed that fraction C3 was very similar to fraction A4 from the preparative HPLC separation. TLC analysis combined with bioautography showed however that considerable purification could be achieved by changing the developing solvent to one containing ethyl acetate. Chromatography on a single plate (20x20cm, 0.20mm) using 30% ethyl acetate and 30% methanol in chloroform provided two main fractions;

Fraction C3/1 R_f 0.69-0.77 10.6 mg

Fraction C3/2 R_f 0.57-0.69 16.2 mg

The antimicrobial activity was present entirely in fraction C3/1 and at this stage further purification was not attempted. These two fractions were therefore further characterised by chromatographic and spectroscopic analysis.

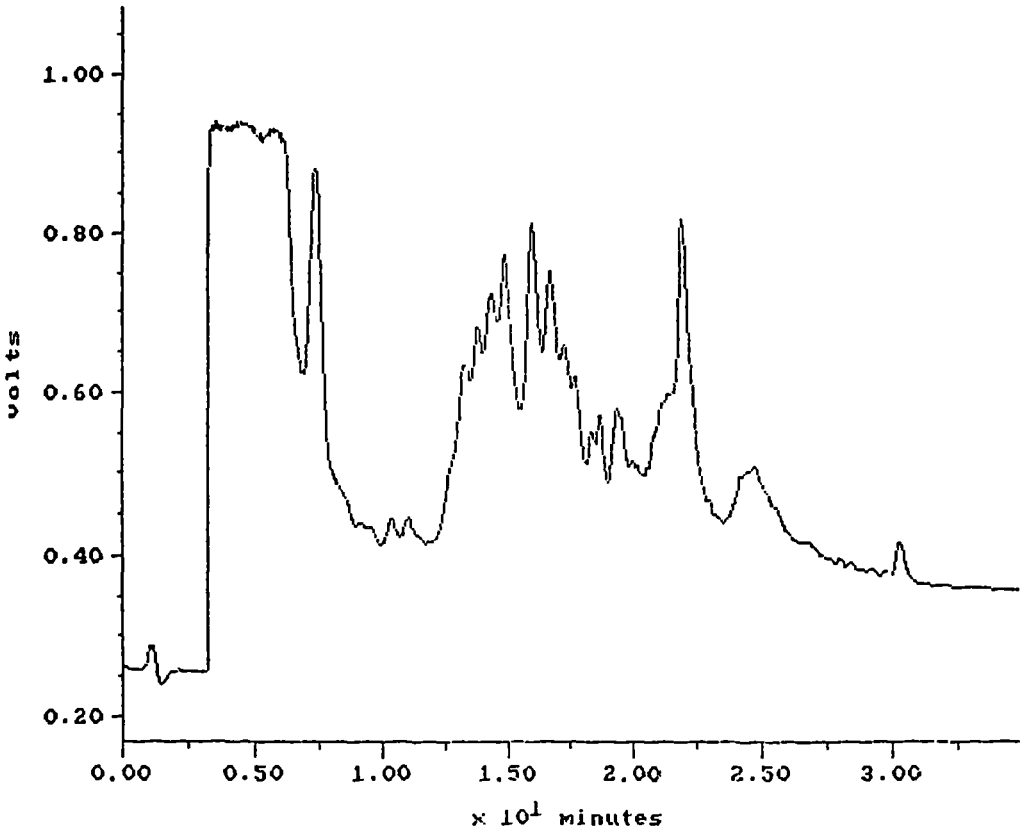
TLC analysis showed one spot under 254nm irradiation for fraction C3/1 and two for C3/2. These corresponded to components present in the crude extract. Neither of the samples contained components reacting with the anthrone spray reagent for sugars, however several ninhydrin positive components were present in the crude extract which were also present in fractions C3/1 and C3/2. These spots were all of lower R_f than the UV active component of C3/1 detected on irradiation at 254 nm. Bioautography showed that the antimicrobial compound present in C3/1 corresponded with the UV active component and not with any of the lower R_f-spots. Spraying with 5% sulphuric acid in ethanol and heating at 100°C revealed that both C3/1 and C3/2 contained several other components of low R_f which were not visible under UV nor stained with ninhydrin. Analytical HPLC chromatogram of fraction C3/1 and C3/2 are shown in Figure 4.7.5. The fraction showing the antimicrobial activity (C3/1) contained components eluting between 20 and 22 minutes, identical to the components in fraction A4 from the preparative HPLC separation which showed bioactivity. The inactive fraction (C3/2) did not contain these components.

Proton NMR spectra of fractions C3/1 and C3/2 (Figure 4.7.6) showed that they were somewhat similar, exhibiting a dominant polymethylene envelope at ~1.2 ppm and terminal methyl resonances at ~0.8 ppm. Olefinic protons resonating between 5.0 and 5.3 ppm were evident as were protons attached to carbons bearing oxygen showing

broad resonances between 3.4 and 4.4 ppm. This suggested the presence of fatty acid glycerides in both fractions but fraction C3/1 also exhibited resonances at 7.05 and 7.65ppm (broad doublets). These resonances are likely to be an AX system of aromatic protons perhaps attributable to the bioactive component.

The mass spectrum of C3/1 confirmed the presence of fatty acid glycerides. Characteristic peaks at m/e 98, 112, 116 and 129 were observed. Peaks due to molecular ions were also present at m/e 534, 548 and 562. These were confirmed by chemical ionisation using ammonia as reagent gas. Accurate mass measurement indicated that the ion at m/e 548 had molecular formula $C_{35}H_{64}O_4$ corresponding to a diglyceride of palmitic acid and palmitoleic acid (or a double bond isomer). The other ions differing by 14 mass units were probably due to diglycerides incorporating C_{15} and C_{17} fatty acids in place of palmitic acid. These acids are often found in bacterial lipids. As to the nature of the bioactive component present, no information could readily be obtained from the mass spectral investigation of these impure fractions.

Figure 4.7.1 Preparative HPLC separation of UT3 compounds from the organic fraction detected at 220nm (top) and 330nm (bottom). Flow rate: 3.5 ml min^{-1}, gradient: 50% - 100% methanol containing 0.1% TFA in 20 minutes.


1.00
0.80
0.60
0.40
0.20
volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

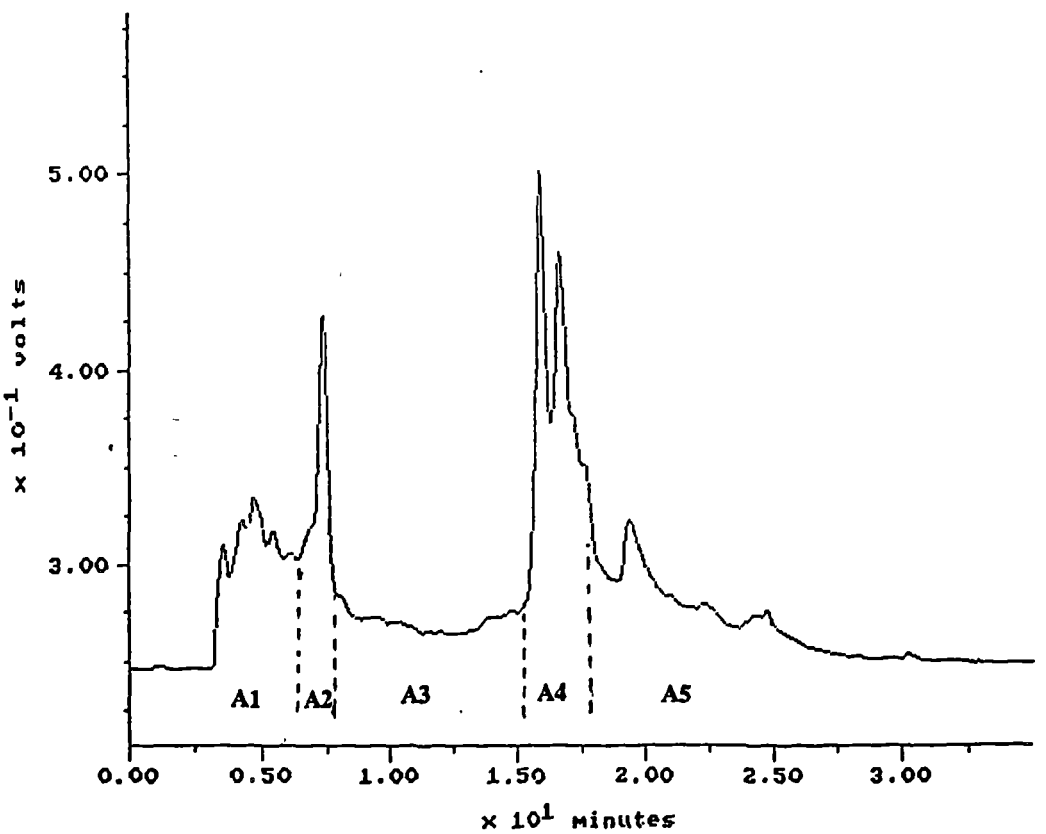
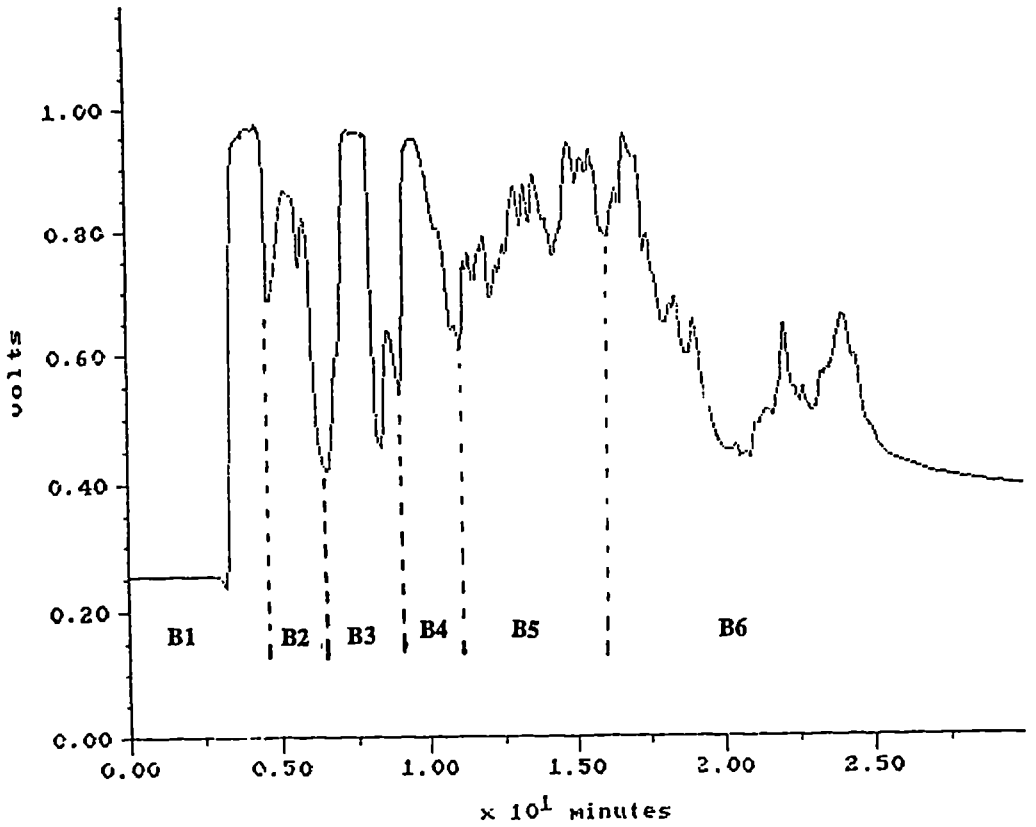

5.00
4.00
3.00
x 10⁻¹ volts
A1
A2
A3
A4
A5
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

Figure 4.7.2 Preparative HPLC separation of UT3 compounds from the aqueous fraction detected at 220nm (top) and 330nm (bottom). Flow rate: 3.5 ml min^{-1}, gradient: 0% methanol isocratic 5 min and 0 - 100% methanol containing 0.1% TFA in 15 minutes.


1.00
0.80
0.60
0.40
0.20
0.00
volts
B1
B2
B3
B4
B5
B6
0.00
0.50
1.00
1.50
2.00
2.50
x 10¹ minutes

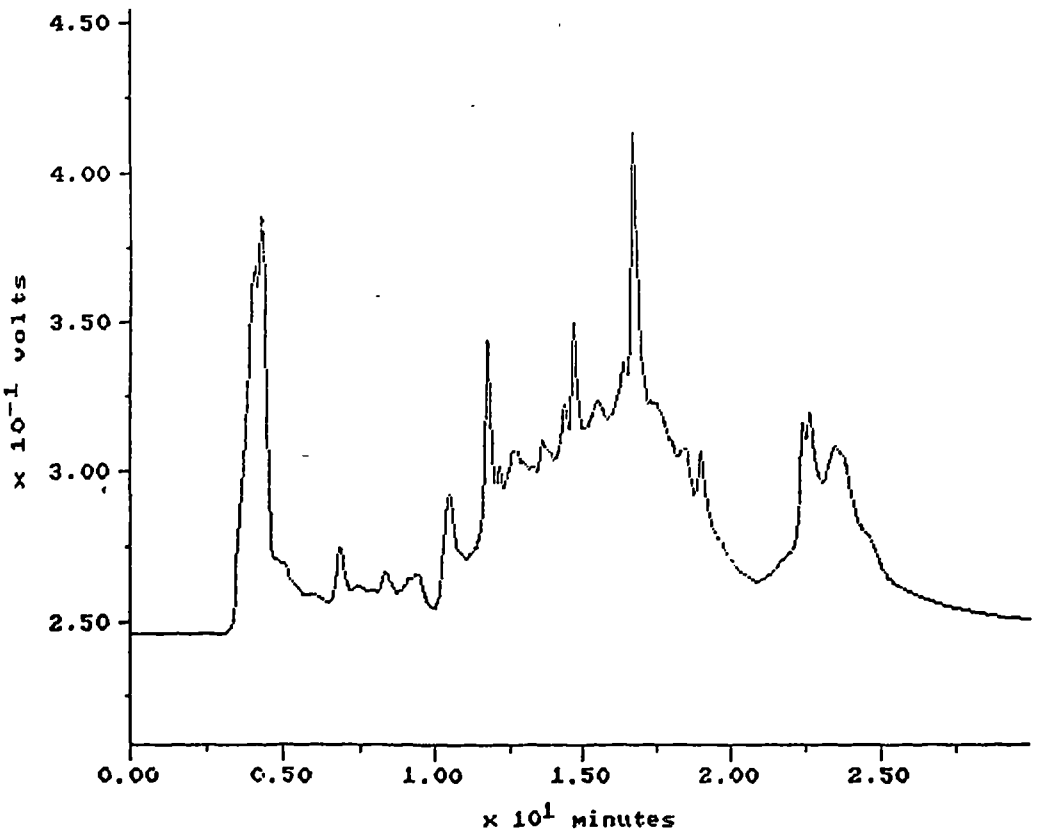
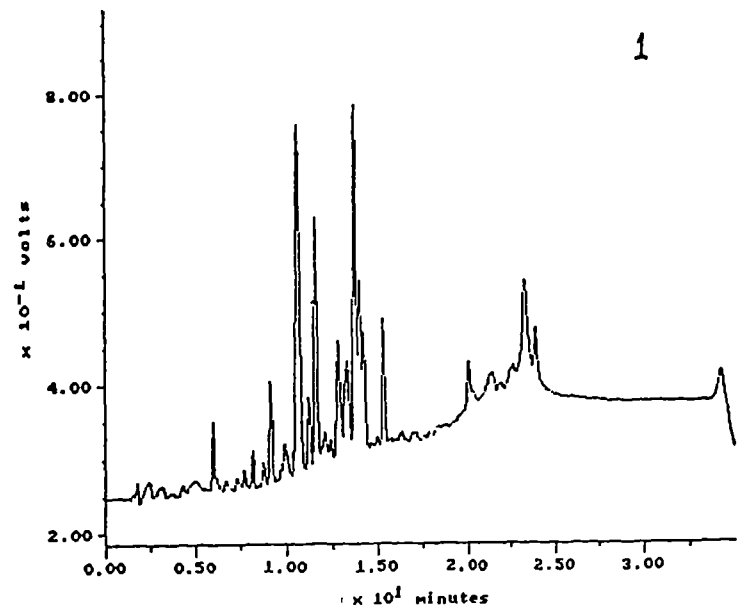
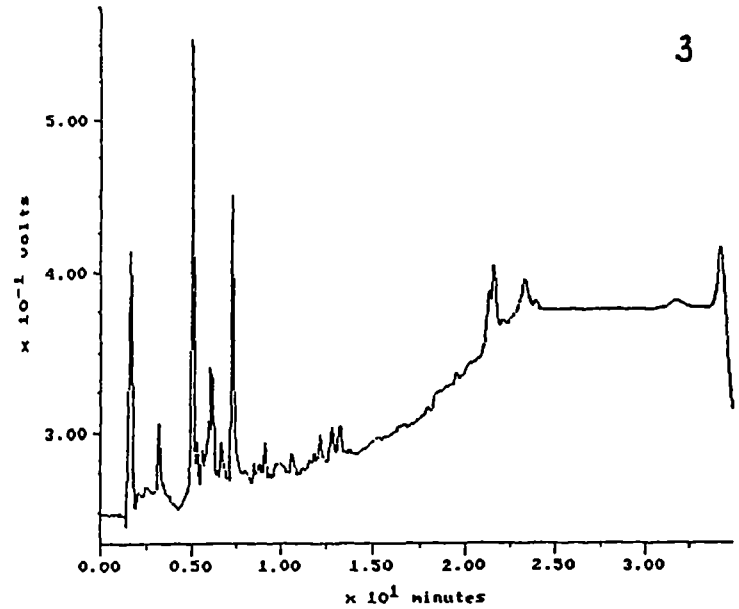
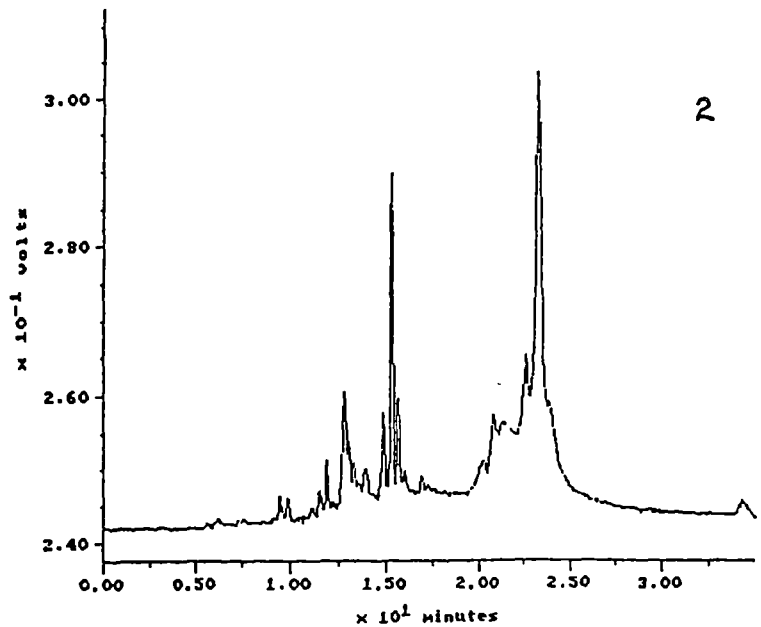

4.50
4.00
3.50
3.00
2.50
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
x 10¹ minutes

Figure 4.7.3 Analytical HPLC of the organic fraction detected at 220nm (1) and 330nm (2) and the aqueous fraction at 220nm (3) and 330nm (4).


1
8.00
6.00
4.00
2.00
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes


3
5.00
4.00
3.00
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes


2
3.00
2.80
2.60
2.40
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

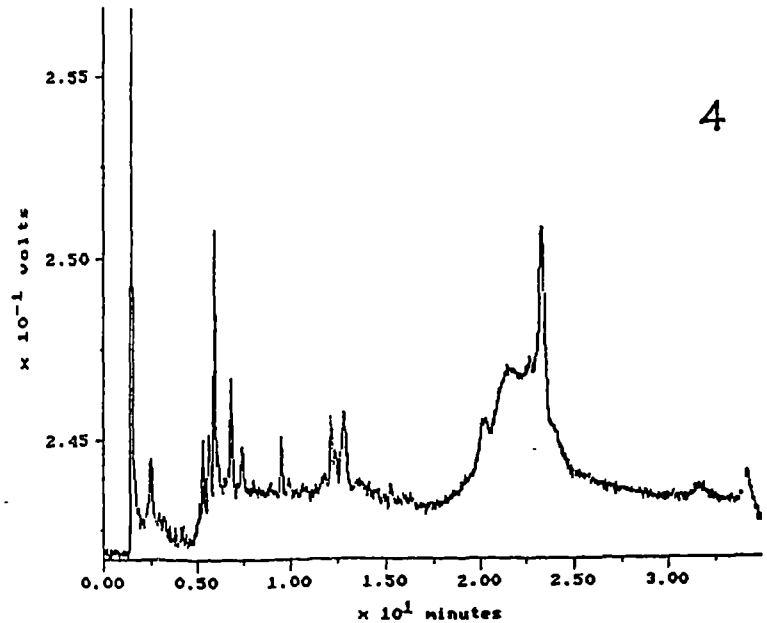
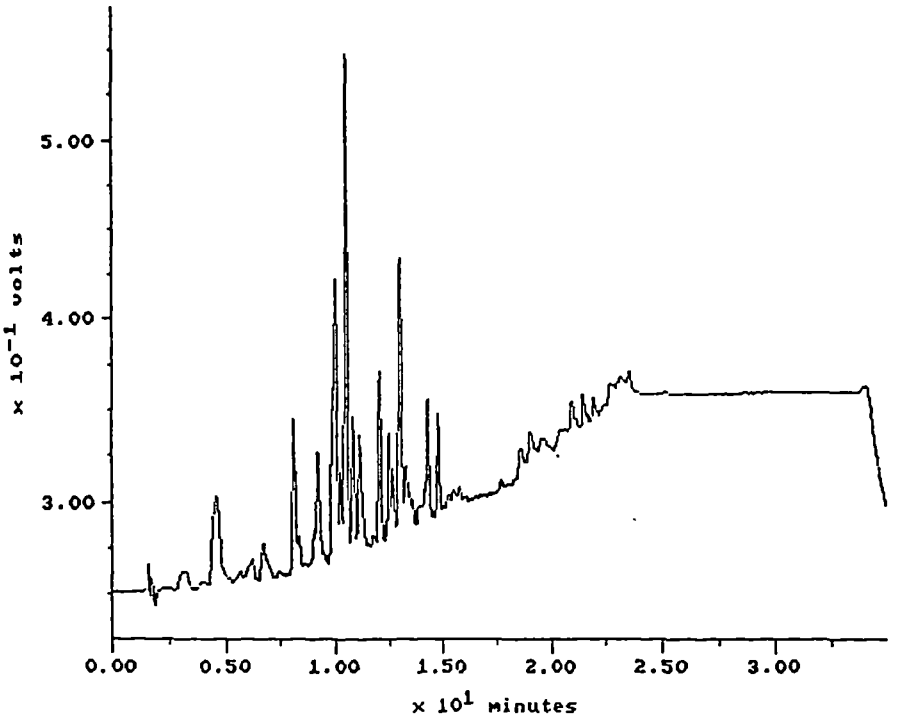

4
2.55
2.50
2.45
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

Figure 4.7.4 Analytical HPLC of the aqueous-acidic fraction detected at 220nm (top) and 330nm (bottom).


5.00
4.00
3.00
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

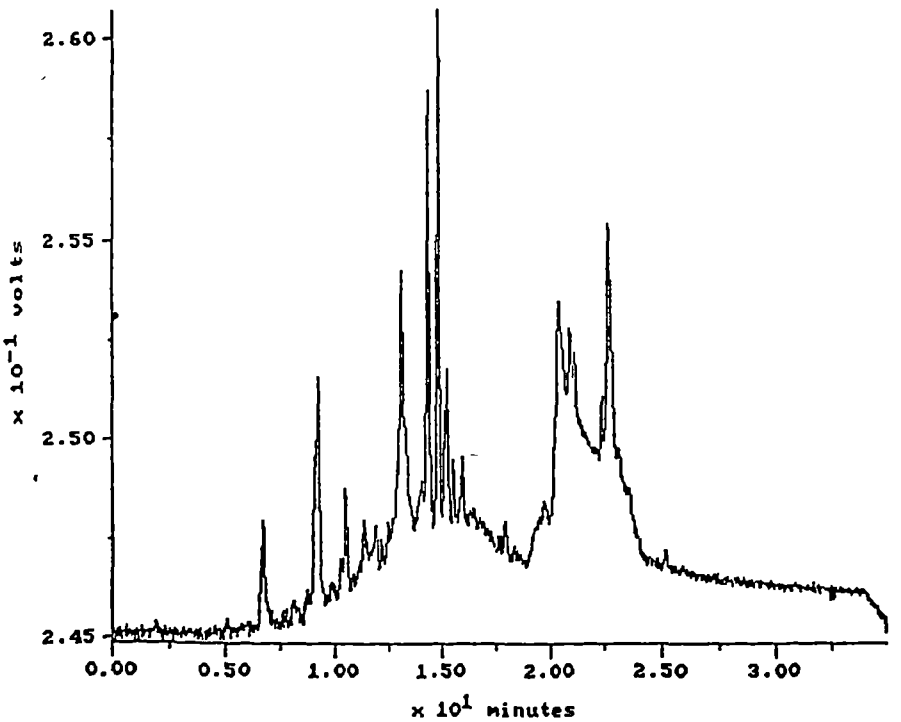
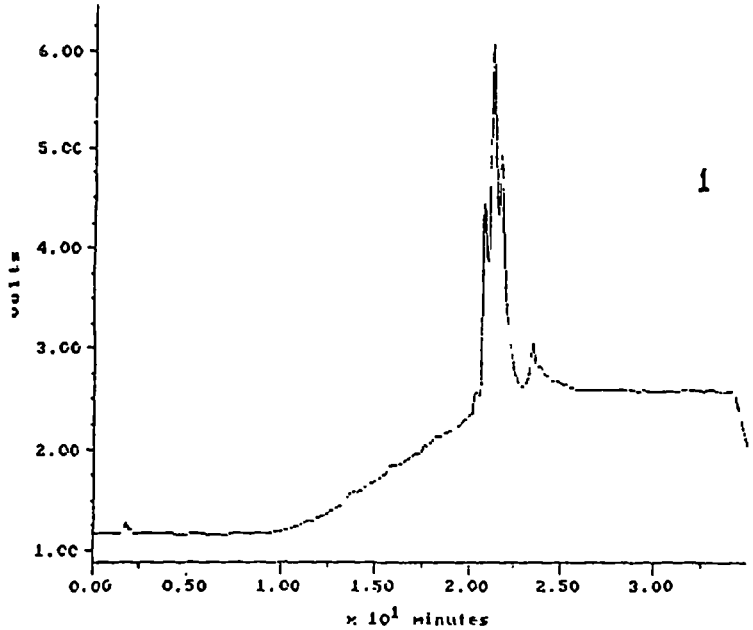
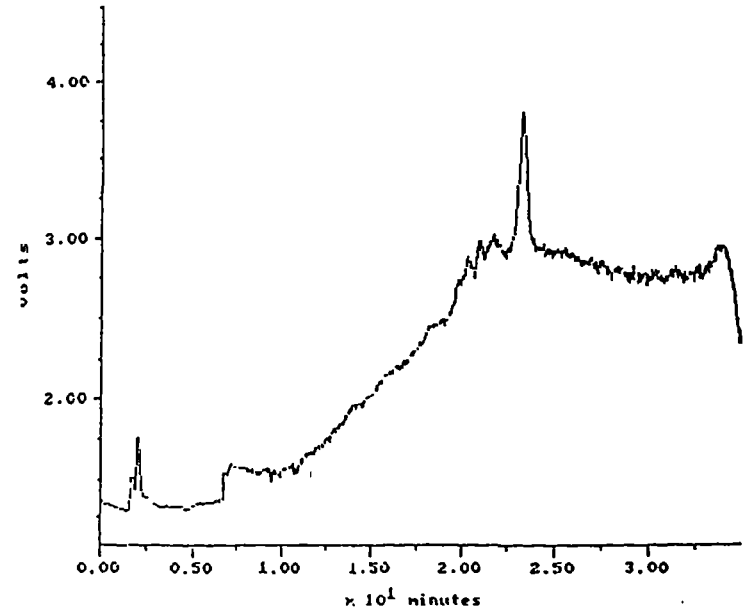
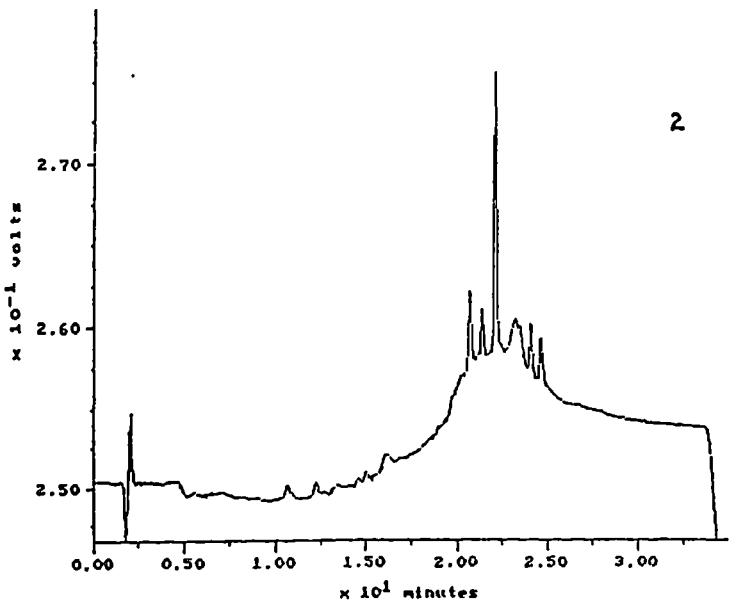

2.60
2.55
2.50
2.45
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

Figure 4.7.5 Analytical HPLC of fraction C3/1 detected at 220nm (1) and 330nm (2) and fraction C3/2 at 220nm (3) and 330nm (4).


1
6.00
5.00
4.00
3.00
2.00
1.00
volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes


3
4.00
3.00
2.00
volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes


2
2.70
2.60
2.50
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

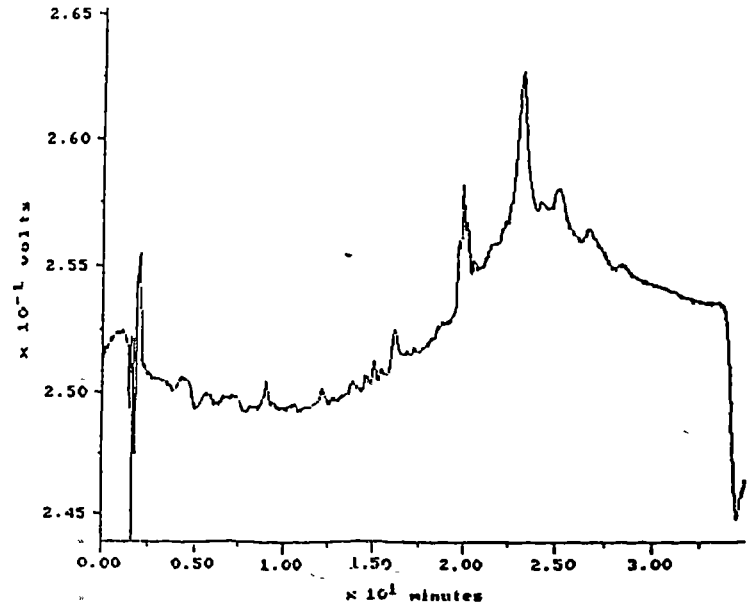
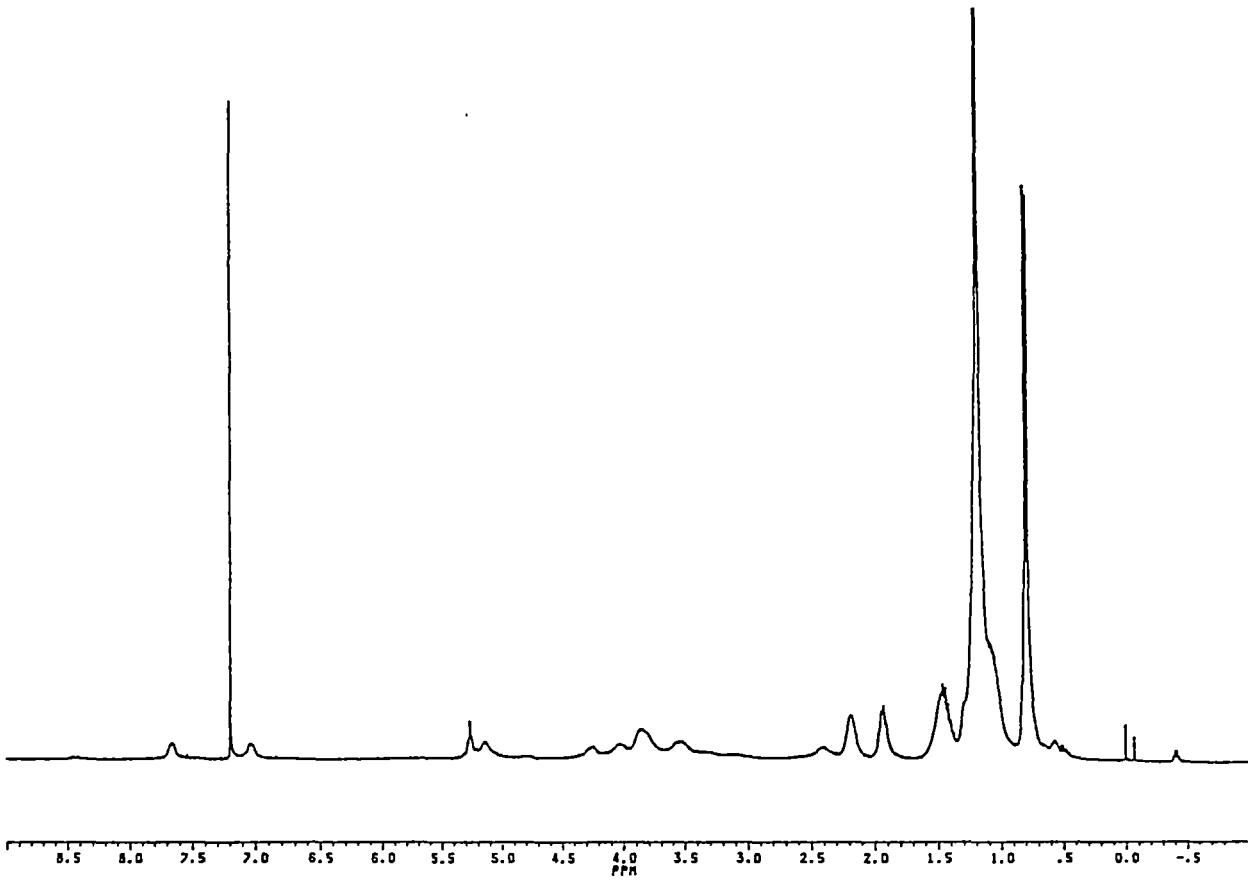
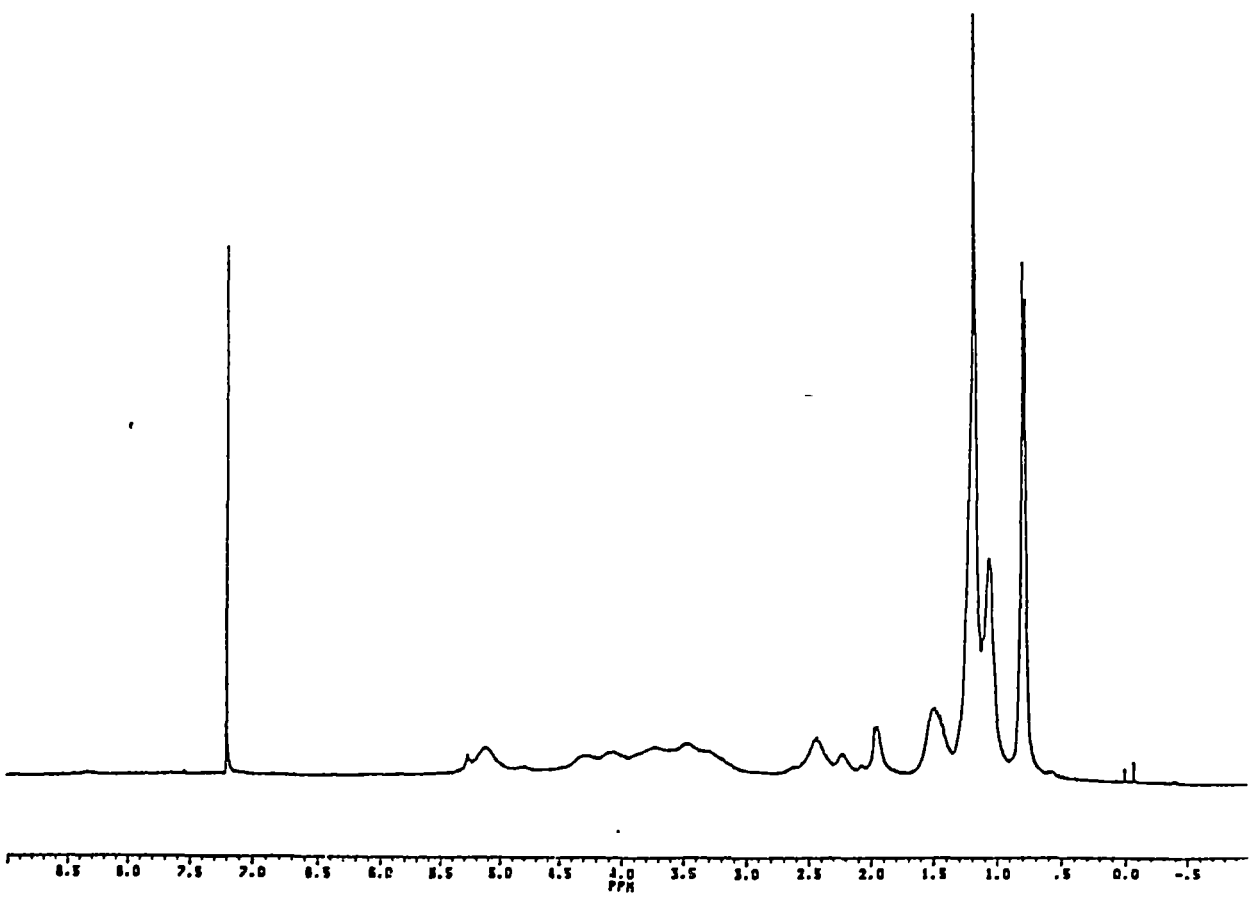

4
2.65
2.60
2.55
2.50
2.45
x 10⁻¹ volts
0.00
0.50
1.00
1.50
2.00
2.50
3.00
x 10¹ minutes

Figure 4.7.6 Proton magnetic resonance spectrum of fraction C3/1 (top) and fraction C3/2 (bottom) from *P. cepacia* UT3.


8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0 -.5
PPM


8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 .5 0.0 -.5
PPM

5. DISCUSSION

Isolation of bacterial antagonists and *in vitro* antagonism against fungal pathogens

Isolation and assay of bacterial antagonists was undertaken on PDA, since this medium is iron-rich and low in phosphate, providing a favourable nutritional environment for bacterial secondary metabolism and antibiotic production (Xu and Gross, 1986b). Five strains of bacteria antagonistic to fungal pathogens were isolated from various sources. *Bacillus polymyxa* strain UT1 was isolated from roots of symptomless daisy (*Olearia phlogopappa*), a Tasmanian native species showing some promises for the production of an essential oil as food flavouring. Strains UT3 of *Pseudomonas cepacia* and UT4 of *P. putida* were isolated from symptomless clary sage (*Salvia sclarea*), another essential oil plant containing linalool and linalyl acetate, and sclareol, of significance to the perfume industry. Both plants had a root rot disease causing more than 50% mortality during 1989/1990 plantings at the University of Tasmania Farm, Cambridge (C. Read, pers. comm.). *B. subtilis* strain UT2 and *Acinetobacter* sp. strain UT5 were isolated from decomposing woodchips and white clover (*Trifolium repens*), respectively. Decomposing woodchips was chosen as a promising source-material for antagonists of fungi causing sap-staining of moist, cut timber. However evidence for such antagonism by strain UT2 was lacking (Table 4.1.1). While there are numerous reports of the potential utilization of *Pseudomonas* and to lesser degree *Bacillus* species for biological control of plant pathogens, there appear to be few reports of an *Acinetobacter* sp. in this regard.

The bacterial antagonists were initially screened against *Sclerotinia minor*, *S. sclerotiorum*, and *Botrytis cinerea*, with strains *P. cepacia*, *B. polymyxa* and *P. putida* exhibiting a broad spectrum of antagonism against the fungi tested with the notable exception of *Pythium ultimum* (Table 4.1.1). In addition to the production of inhibition zones, isolates *B. subtilis* (UT2) and *Acinetobacter* sp. (UT5) appeared to lyse the fungal mycelia after prolonged (2-3 weeks) incubation. Mycelia of *S. minor* and *S. sclerotiorum* from the edge of the inhibition (after 3 weeks interaction) failed to grow on fresh PDA following subculture. Backhouse and Stewart (1989b) found a similar result in the interaction between *B. subtilis* and *Sclerotium cepivorum*. They suggested that antibiotics of the antagonist acted on the fungal metabolism, since it was shown that fungal mitochondria became swollen, hyphae were more vacuolated, and death of cells followed the rupture of hyphal walls and leakage of cytoplasm.

Weller *et al.* (1988) assumed that bacterial inhibition of *G. graminis* var. *tritici* on PDA (high iron medium) was due mainly to the production of antibiotics, whereas inhibition on KBM (low iron medium) was due to the production of a siderophore if such inhibition was reduced by addition of $FeCl_3$. In the present study, none of the antagonistic bacteria were significantly affected in their antagonism by the addition of $FeCl_3$, leading to the conclusion that the inhibition was due to the production of antibiotics. Differences were noted regarding antibiotic production by the isolates on different media (Tables 4.1.2-4.1.3 and Plates 4.1.3-4.1.5). Xu and Gross (1986b) grouped *Pseudomonas* spp. on their antibiosis on PDA and KBM agar, as strains producing inhibitory zones only on KBM (group 1), only on PDA (group 2), on both PDA and KBM (Group 3), or on neither PDA nor KBM (group 4). According to this scheme, strain UT3 (*P. cepacia*) belongs to antibiosis group 3 and isolate UT4 (*P. putida*) to

antibiosis group 2. It is accepted that selection for antagonism to fungi *in vitro* is necessarily followed by pot and field assay.

Effect of medium pH on the inhibition of *Sclerotinia* spp. by antagonists

Extremes of medium pH are critical factors for the growth of fungi and bacteria. *Sclerotinia* spp. grow optimally in acidic conditions with their growth rate decreased as medium pH increases (Willetts and Wong, 1980), whereas the growth of bacteria was optimal at neutral pH and slightly tolerant to alkaline conditions. These traits of the microorganisms were reflected in the zones of inhibition of fungi by the antagonists at both extremes of pH. Since the bacteria and fungi were both growing on the same medium, the diminished inhibitory activity at lower pH values was indicative of sub-optimal growth conditions for the bacteria rather than diminished activity of the antibiotic. This was confirmed by the similar inhibition zones produced by crude extracts of one of the isolates (UT3) treated with acid and alkali (Table 4.7.1).

Description of bacterial antagonists

***Bacillus* spp..** Both strains of UT1 and UT2 were Gram-positive sporing rods, < 1 μm cell width, > 2 μm cell length, motile with peritrichous flagella. Spores were ovoid, being terminal in UT1 and central in UT2. The ability of strain UT1 to grow anaerobically, produce levan from sucrose, liquefy gelatin and produce gas from glucose confirmed it to be *B. polymyxa*. Characteristics leading to the identification of strain UT2 as *B. subtilis* were its obligately aerobic

growth, production of nitrite from nitrate, production of acid from a variety of sugars, tolerance to >7% salinity, and its positive Voges-Proskauer reaction. Lack of ability to fix nitrogen anaerobically by strain UT1 was typical of *B. polymyxa*, while the lack of citrate utilization by strain UT2 was typical of *B. subtilis*. Strain UT2 was sensitive to all 13 antibiotics tested (Table 4.3.1), whereas *Bacillus polymyxa* (UT1) showed resistance to bacitracin, cephalothin and polymixin. This isolate, but not UT2, was also able to grow on KBM agar-supplemented with 7.2 units ml^{-1} of bacitracin and 395 units ml^{-1} of polymixin B.

***Pseudomonas* spp..** Pseudomonads are characterized as aerobic Gram-negative rods, being motile with one or more polar flagella, and catalase-positive (Palleroni, 1984). Characteristics leading to the identification of strain UT3 as *P. cepacia* were: the accumulation of poly-β-hydroxybutyrate as a carbon reserve material, the inability to hydrolyse arginine or to denitrify and the ability to produce pyocyanin pigment on King's A medium. Characteristics of strain UT4 leading to the identification as *Pseudomonas putida* were its inability to accumulate poly-β-hydroxybutyrate or to hydrolyse gelatin, its inability to denitrify, its growth at 41°C, and particularly its failure to hydrolyse gelatin (Stanier *et al.*, 1966). Aberrant characteristics for this species were the production of lecithinase and the lack of phenazine pigment production. Both strains UT3 and UT4 were resistant to most of the antibiotics tested (Table 4.3.1) and were capable of growing in a medium supplemented with 200 $\mu g\ ml^{-1}$ of ampicillin and 7.2 units ml^{-1} of bacitracin.

***Acinetobacter* sp..** Strain UT5 was characterized as being aerobic, Gram-negative non-motile rods (but displaying twitching motility), catalase positive, and oxidase negative, characteristics typical

of the genus *Acinetobacter* (Juni, 1986). Characteristics supporting this identification were its inability to reduce nitrite from nitrate and to hydrolyse gelatin. The strain was resistant to the ß-lactam antibiotics tested (ampicillin, cephalothin, and penicillin G).

Characteristics of the *Bacillus* and *Pseudomonas* isolates which may contribute to their promise as biological control agents are the ability of *Bacillus* spp. to sporulate, enabling them to survive in the soil under adverse conditions and the aggressive root colonization shown by the *Pseudomonas* isolates (Plate 4.4.6).

Suppression of wheat take-all by antagonists

The testing of bacterial colonization of root surfaces or antagonism to pathogens using tube assay is a convenient stepping stone to greenhouse or field trials (Weller *et al.,* 1985; Chao *et al.,* 1986; Brisbane and Rovira, 1988; Maplestone and Campbell, 1989; Keel *et al.,* 1989; Handelsman *et al.,* 1990).

Significant protection against wheat take-all was provided by all isolates used in the present study, with strain UT3 (*P. cepacia*) providing significantly better protection than other isolates (Table 4.5.1). Strains UT4 and NIR-6 (both *P. putida*) were less effective than strain UT3 but more effective than the remaining strains. Further work is therefore necessary to assess the efficacy of the antagonists against take-all fungus in the glasshouse and in the field, especially since tube assays do not always correlate with field results (Weller, 1985). Isolate NIR-6 (*Pseudomonas putida*) which was included in the present studies has been reported to inhibit *G. graminis* both *in vitro* and in the field (P. Wong pers. comm.). Wong and Baker (1984) reported that strain NIR-6 significantly protected wheat and *Agrostis* turfgrass

from take-all in pot trials and this isolate was also reported to inhibit *Fusarium oxysporum in vitro* as well as suppress *Fusarium*-wilts of pea, cucumber, radish, and bean in the glasshouse (Elad and Baker, 1985; Park *et al.*, 1988).

The introduced bacteria in the absence of the pathogen did not increase the dry weight of wheat seedling tops compared with untreated controls, indicating that plant growth promotion was not involved. A significant increase in the dry weight of the plants was however observed in the sand-tube assay by strain UT3 in association with the pathogen compared with the control treatment of isolate UT3 alone. ~~was not supported by the glasshouse pot trial.~~

The ability of the antagonists to colonize wheat roots was assessed in sand-culture by recovering the antagonists from the roots+adhering sand and by examining of the roots by SEM. From the SEM examination, strains UT3 and UT4 appeared to be more aggressive than the *Bacillus* or strain NIR-6 in colonizing the root surfaces, although the numbers of the various antagonists reisolated from root-sand material by dilution plating did not differ significantly. These numbers, however, may not reflect the populations of the antagonists observed on the rhizoplane by SEM. Brisbane and Rovira (1988) also found less effective colonization of root surfaces of wheat by *Bacillus* in comparison with *Pseudomonas*. Bahme and Schroth (1987) in a study of *Pseudomonas fluorescens* populations on potato roots found that 41% were associated with rhizosphere soil, 54% were loosely adhering to the root surface, and 5% were tightly attached or internal to the root surface. This showed that most root-associated bacteria are easily removed during SEM preparations.

Bowen and Rovira (1976) reported that in the initial phase of root colonization by antagonists, the bacteria offer little protection against pathogens that can infect rapidly, and root colonization by introduced

Pseudomonas spp. was shown to follow the growth of take-all fungus on wheat roots by Weller *et al.* (1988). Weller *et al.* (1988) also reported that the establishment of introduced bacteria along roots and in lesions was necessary for pathogen suppression to occur.

Effectiveness of antagonists against *Sclerotinia* root rot

No work has been reported on the biological control of *Sclerotinia minor* or *S. sclerotiorum* by bacteria, yet in Australia, sclerotinia rot on vegetables causes in the order of 64% of the loss attributable to pathogens (Wong *et al.*, 1980). Lettuce drop caused by *S. minor* and *S. sclerotiorum* (especially *S. minor*) was until recently of major importance in Tasmania (Dr. J. A.-L. Wong, pers. comm.). The present study showed that application of bacterial antagonists (strains UT1, UT2, UT3, UT4, and UT5) to lettuce roots resulted (after 10 weeks growth) in 60-70% survival of plants challenged with *S. sclerotiorum* and a 30-60% of survival of plants challenged with *S. minor* compared with 10% survival of control plants in both cases. Two of the antagonists (isolates UT3 and UT5) showed consistent suppression of *S. sclerotiorum* and *S. minor.*

The diameter of zones of inhibition of both *Sclerotinia* species by the antagonists *in vitro* did not correlate well with the protection of lettuce against the disease in pot trials. The exception was strain UT5 which provided the best control both *in vitro* and in the glasshouse. *S. minor* was more susceptible to antagonism by the bacteria than *S. sclerotiorum in vitro*, but in pot trials, the lettuce drop caused by *S. minor* was the more severe. Adams and Fravel (1990) found that application of *Sporidesmium sclerotivorum* into *S. minor*-infested soil reduced lettuce drop by between 53 and 72% over five successive

crops in the field, although such protection cannot be compared with that provided by isolates in the present study.

The protection of lettuces from sclerotinia rot by the antagonists appeared to be maintained over weeks 4 to 9 (Figures 4.4.1 and 4.4.2), indicating that the introduced bacteria successfully established themselves on the root surface. It was noted that in the course of the trial, the outer leaves of lettuces frequently made contact with the potting-mix substrate, thus providing an additional, uncontrolled avenue for pathogen invasion. The significance of this factor on the incidence of infection is not known.

At harvest, only strain UT4 of *Pseudomonas putida* was recovered from the roots of lettuces. This failure to isolate other inoculated strains might be due to a loss of viability (outcompeted) or to a change in their resistance to antibiotics used in reisolation though the latter possibility seems improbable.

Application of antagonists to protect *Olearia* root rot in the field

The field trial of the antagonists, against root rot of *Olearia phlogopappa* was unsuccessful. The fungal pathogen of the plant was identified as *Pythium* sp., and only one of the bacterial antagonist (UT3) was effective against it. This was however not isolated from *Olearia*, with the only antagonist originating from this plant showing no effectiveness *in vitro* against *Pythium*. Another possible reason for the failure of this trial was water stress resulting in an unfavourable environment for root colonization by inoculated strains. Irrigation of plants was conducted every five days, which appeared to be inadequate because plant wilting was observed with recovery after irrigation.

Selection of antagonistic bacteria from environments (and plants) similar to those in which they will be applied is a logical procedure for successful control (Weller, 1983; 1988; Handelsman *et al.,* 1990). Examples of this principle are provided by the reports of *P. fluorescens* strains Pf-5 (Howell and Stipanovic, 1979; 1980) and 2-79 (Weller *et al.,* 1983; Weller, 1985; 1988; Thomashow and Weller, 1990), which were isolated from the rhizosphere of cotton and wheat respectively, and which successfully controlled the pathogens associated with them.

Production of antimicrobial compounds by strain UT3

The mode of antagonism by strains UT1-UT5 was demonstrated to be (at least in part) the production of antimicrobial substances. The antimicrobial compounds of one of these antagonists (*Pseudomonas cepacia* UT3), were investigated further.

The partially purified antibiotic from strain UT3 (separated by either thin-layer chromatography or HPLC) inhibited the growth of several fungal pathogens and bacteria. Other workers have also shown that antibiotics produced by *Pseudomonas* spp. were inhibitory to a wide range of fungal plant pathogens and bacteria (Fuller *et al.,* 1971; Leisinger and Margraff, 1979; Howell and Stipanovic, 1979; 1980; Gurusiddaiah *et al.,* 1986; Janisiewicz and Roitman, 1988; Homma *et al.,* 1989; Jayaswal *et al.,* 1990). The UT3 antibiotics were mainly active against the gram-positive bacteria tested, having little effect on the gram-negative bacteria. A similar action was also reported for "pseudomonic acid" produced by a strain of *P. fluorescens* by Fuller *et al.* (1971). Pyrrolnitrin and phenazine antibiotics produced by strains of *P. cepacia* (Janisiewicz and Roitman, 1988; Homma *et al.,* 1989) and *P. fluorescens* 2.79 (Gurusiddaiah *et al.,* 1986)

respectively have been shown to be active against fungal plant pathogens and moderately inhibitory to several Gram-positive and - negative bacteria.

The active compounds of strain UT3 were not affected by mild heat, acid or alkali, indicating that their activity was not sensitive to these conditions. Similar stability was also shown by an unidentified antifungal compound produced by *P. cepacia* RJ2 (Jayaswal *et al.*, 1990) and by pyrrolnitrin over a pH range 3.4 - 10 (Janisiewicz *et al.*, 1991). The antimicrobial substances of UT3 appeared to be quite polar, since on TLC they did not move in 100% chloroform but migrated only following addition of methanol to the solvent system. Pyrrolnitrin in contrast has an R_f= 0.48 in chloroform (Howell and Stipanovic, 1979) and an unidentified antifungal compound produced by *P. cepacia* RJ2 reported by Jayaswal *et al.* (1990) also migrated to R_f 0.57 in chloroform. Therefore the antibiotics produced by *P. cepacia* UT3 were different to these.

Conclusions

The purpose of the study was to identify and characterize bacterial biological control agents of fungal pathogens. The five bacteria obtained were identified as *Bacillus polymyxa* (UT1), *B. subtilis* (UT2), *Pseudomonas cepacia* (UT3), *P. putida* (UT4), and *Acinetobacter* sp. (UT5). Isolation of *Bacillus* spp. and *Pseudomonas* spp. which were inhibitory to several fungal pathogens supported previous workers' findings that these beneficial bacteria are naturally associated with plants, and produce mutual interactions between them.

The ability of the antagonists (strains UT1-UT5) to reduce lettuce

drop in the glasshouse and to significantly protect wheat from *G. graminis* in a tube assay, indicated these bacteria to be promising biological control agents for sclerotinia rot and take-all fungus.

Partial isolation of antibiotic substances from strain UT3 of *P. cepacia* showed them to be uneffected by mild heat, acid or alkali; they were soluble in methanol and chloroform. Two inhibitory compounds were present; compound A had an R_f= 0.40 and compound B an R_f= 0.87 in a chloroform-methanol (7:3) solvent system, but neither compound migrated in 100% chloroform. They both had a retention time between 20 and 22 minutes on C18 reversed phase HPLC eluting with a linear gradient of 0-100% methanol containing 0.1% TFA in 25 minutes at a flow rate of 2ml min^{-1}. Proton NMR data indicated that compound A may be an aromatic compound, but mass spectral analysis did not provide any further information due to the large proportion of diglycerides in the partially purified sample.

Further purification and characterisation of the UT3 antibiotics was not attempted and further work will be required to elucidate their structures and evaluate their full antibiotic potency.

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APPENDICES

1 Growth media and reagents

1.1 Growth media

All media were sterilized for 15 minutes at 121°C unless otherwise stated.

1.1.1 Tryptone soya agar (TSA)

Tryptone soya powder without dextrose (BBL)	0.5%
Yeast extract (Oxoid)	0.1%
D-Glucose	0.2%
Agar	1.5%

1.1.2 King, Ward and Raney's (1954) medium A

Bacto-peptone (Difco)	20 g
Glycerol	10 mL
$MgCl_2$ (anhydrous)	1.4 g
K_2SO_4 (anhydrous)	10 g
Agar	15 g
Distilled water	1 L

Dissolve the ingredients by heating at 100°C and adjust pH to 7.2 before sterilizing.

1.1.3 King, Ward, and Raney's (1954) medium B

Proteose peptone (Oxoid)	20 g
Glycerol	10 mL
K_2HPO_4 (anhydrous)	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g

Distilled water	1 L

Dissolve the ingredients by heating at 100 °C, and adjust pH to 7.2 before sterilizing.

1.1.4 Nutrient agar

Beef extract (Oxoid)	3 g
Peptone (Oxoid)	5 g
Agar	15 g
Distilled water	1 L

Combine the ingredients and adjust the pH to 6.8. **Nutrient broth** is made excluding agar.

1.1.5 Palleroni and Doudoroff (1972) base medium

(g/litre of 0.033 M Na-K phosphate buffer [pH 6.8]):

NH_4Cl	1.0
$MgSO_4.7H_2O$	0.5
Ferric ammonium citrate	0.05
$CaCl_2$	0.005

Add the first two ingredients to the buffer and sterilize. The Fe-ammonium citrate and $CaCl_2$ are added aseptically from a single stock solution that has been sterilized by filtration.

1.1.6 Thornley semisolid arginine medium (Smibert and Krieg, 1981)

Peptone (Oxoid)	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Phenol red	0.01 g
L-Arginine.HCl	10.0 g
Agar	3.0 g

Distilled water	1 L

Combine the ingredients and adjust pH to 7.2.

1.1.7 Nitrate reduction medium (Cruickshank *et al.*, 1975)

KNO_3	0.2 g
Peptone (Oxoid)	5.0 g
Distilled water	1 L

Combine the ingredients and pour 5 ml into tubes.

1.1.8 Nutrient gelatin (Cruickshank *et al.*, 1975)

Nutrient broth (see 1.1.4)	1 L
Gelatin	120 g

Add the gelatin to the broth and sterilize. After sterilizing, pour the medium into sterile plastic plates and maintain at temperature below 22°C.

1.1.9 Egg yolk agar (Smibert and Krieg, 1981)

Peptone (Oxoid)	20.0 g
Na_2HPO_4	2.5 g
NaCl	1.0 g
$MgSO_4$, 0.5% (wt/vol) solution	0.1 mL
Glucose	1.0 g
Agar	12.5 g
Distilled water	500 mL

Combine materials and adjust pH to 7.3 to 7.4. After sterilizing, cool the medium to 60°C. Surface sterilize an egg with alcohol and after drying separate the yolk from the white. Add the yolk to the melted agar and mix to homogenicity. Dispense the medium into plates and

allow to solidify.

1.1.10 Milk agar (Sneath, 1986)

Skim milk powder	5 g
Agar	1 g

Dissolve each ingredient in 50 ml of distilled water and sterilized separately. After cooling to 50°C, they are mixed and poured into plates.

1.1.11 Starch agar

Trypticase soy broth without dextrose (BBL)	27.5 g
Agar	15.0 g
Starch (w/v)	0.2 %
Distilled water	1 L

Sterilize by autoclaving and pour into plates.

1.1.12 Methyl red-Voges-Proskauer (MRVP) broth (Smibert and Krieg, 1981)

Peptone (Oxoid)	7.0 g
NaCl	5.0 g
Glucose	5.0 g
Distilled water	1 L

Combine ingredients and adjust pH to 7.6. After sterilizing, dispense 5 ml of the broth into tubes.

1.1.13 Christensen citrate agar (Smibert and Krieg, 1981)

Na-citrate	3.0 g
Glucose	0.2 g
Yeast extract (Oxoid)	0.5 g

Cysteine hydrochloride	0.1 g
Ferric ammonium citrate	0.4 g
KH_2PO_4	1.0 g
NaCl	5.0 g
Na-thiosulfate	0.08 g
Phenol red	0.012 g
Agar	15.0 g
Distilled water	1 L

Combine ingredients and adjust pH to 6.7. After sterilizing, pour 5 ml of the medium into McCartney's bottles in slanted position.

1.1.14 **Phenylalanine agar** (Sneath, 1986)

Yeast extract (Oxoid)	3 g
DL-phenylalanine	2 g
Na_2HPO_4	1 g
NaCl	5 g
Agar	12 g
Distilled water	1 L

Combine ingredients and adjust pH to 7.3. After sterilizing, pour the medium into McCartney's bottles at slanted position.

1.1.15 **Tyrosine agar** (Sneath, 1986)

L-tyrosine	0.5 g
Distilled water	10 mL
Nutrient agar (see 1.1.4)	100 mL

Sterilize tyrosine in water and nutrient agar separately and then mix aseptically. After cooling (50°C), pour the medium into sterile plastic plates.

1.1.16 Glycerol agar (Sneath, 1986)

Nutrient agar (see 1.1.4)	100 mL
Yeast extract (Oxoid)	1 g
Glycerol	2 mL

Sterilize by autoclaving and pour into plates.

1.1.17 Glucose petone broth (Skerman, 1969)

Peptone (w/v) (Oxoid)	5%
Glucose (w/v)	5%

Both in distilled water.

Adjust pH to 7.3 and distribute into McCartney's bottles filled with Durham tubes.

1.1.18 Voges-Proskauer broth (Sneath, 1986)

Proteose peptone (Oxoid)	7 g
Glucose	5 g
NaCl	5 g
Distilled water	1 L

Distribute the mixture in 5-ml amounts in test tubes.

1.1.19 Medium for acid production from sugars (Sneath, 1986)

$(NH_4)_2HPO_4$	1 g
KCl	0.2 g
$MgSO_4$	0.2 g
Yeast extract (Oxoid)	0.2 g
Agar	15 g
Distilled water	1 L
Bromocresol purple, 0.04% (w/v)	15 mL

Adjust the pH of basal medium to 7.0 before adding 15 ml of

bromocresol purple. Sterilize the medium and 10% (w/v) carbohydrate solutions separately. Add aseptically sufficient carbohydrate solution to tubes of sterile basal medium to give a final concentration of 0.5% (w/v).

1.1.20 Hugh and Leifson medium (Smibert and Krieg, 1981)

Peptone (Oxoid)	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3.0 g
Distilled water	1 L

Combine ingredients and adjust pH to 7.1. After sterilizing, cool to 50°C and filter sterilized of 10% glucose is added to give a final concentration of 1%. Dispense the mix into McCartney's bottles.

1.2 Reagents

1.2.1 Kovacs reagent (Smibert and Krieg, 1981)

p-Dimethylaminobenzaldehyde	3.0 g
Iso amyl alcohol	75mL
HCL, concentrated	25mL

1.2.2 Alpha-naphthylamine solution (Smibert and Krieg, 1981)

N,N-Dimethyl-1-naphthylamine dihydrochloride	0.6 g
Acetic acid, 5 N solution	100 mL

Dissolve by gentle heating.

1.2.3 Sulphanilic acid solution (Smibert and Krieg, 1981)

Sulfanilic acid	0.8 g
Acetic acid, 5 N solution	100 mL

Dissolved by gentle heating.

1.2.4 Dihydroxyacetone reagent (Sneath, 1986)

Solution (a):

Copper sulfate (hydrous)	34.66 g
Distilled water	500 mL

Solution (b):

Potasium sodium tartrate	173 g
Sodium hydroxide	50 g
Distilled water	500 mL

Solutions (a) and (b) are stored in a refrigerator and mixed in a 1:1 ratio immediately before use.

1.2.5 Neisser's methylene blue stain (Cruickshank *et. al.*, 1975)

Methylene blue	1 g
Ethyl alcohol (95%)	50 mL
Glacial acetic acid	50 mL
Distilled water	1 L.

1.2.6 Neutral red solution of Jensen's modification of Gram's method (Cruickshank *et al.*, 1975)

Neutral red	1 g
Acetic acid 1%	2 mL
Distilled water	1 L

1.2.7 Flagellar stain solution (Heimbrook *et al.*, 1989)

Solution 1:

5% phenol (aq.)	10 mL
Tannic acid	2 g
K Al $(SO_4)_2$ $12H_2O$ (saturated aq.)	10 mL

Solution 2:

12 g crystal violet in 100 ml of 95% ethanol.

Mix 2 ml solution 2 with 20 ml solution 1, filter through paper, keep in a syringae fitted with 0.22 μ filter (needle stuck in rubber). Before using, keep the stain in the fridge.

2 Scanning electron microscopy (SEM) preparations

2.1 Fixatives and cacodylate buffers

2.1.1 Stock solution : 0.2 M cacodylate buffer

$Na(CH_3)_2AsO_2.3H_2O$	21.4 g
Distilled water to make	500 mL

Dissolve in 100 ml of distilled water and adjust pH to the desired level with 0.2 M HCl. Then add distilled water to make 500 ml and store at 4°C. Check pH and readjust if necessary before use.

2.1.2 0.1 M cacodylate buffer

0.2 M cacodylate buffer	100 mL
Distilled water	100 mL

Store at 4°C and readjust pH before use.

2.1.3 Cacodylate buffered glutaraldehyde (2.5%)

0.2 M cacodylate buffer	50 mL
25% glutaraldehyde	10 mL
Distilled water to make	100 mL

The final concentration of fixative is 2.5% in 0.1 M buffer. Stable for 4 months when stored at 4°C.

2. 2 Dehydration of wheat root specimens

Specimens were generally dehydrated in a graded ethanol series (with 20 minutes in each) in 25%, 50%, 60%, 70%, 80%, 90% and 100% ethanol (after the second change of 70% ethanol specimens could be stored for up to 2 months at room temperature prior to completion of the dehydration). Dehydration was followed by two changes each of 15 minutes in a 1:1 mixture of absolute ethanol/acetone, then two changes of 10 and 15 minutes in absolute acetone. Samples in acetone were then critical point dried.

3 Hoagland's nutrient composition (Jones, 1983)

Stock solution	to use, mL/L
1 M KH_2PO_4	1.0
1 M KNO_3	5.0
1 M $Ca(NO_3)_2.4H_2O$	5.0
1 M $MgSO_4.7H_2O$	2.0
0.5% Fe-ammonium citrate	1.0

Micronutrient stock solution	gr/L
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$Na_2MoO_4.H_2O$	0.02

The micronutrient is used at 1 ml/l nutrient solution.