BASIS FOR THE BIOCONTROL OF *PYTHIUM* BY FLUORESCENT PSEUDOMONADS

A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

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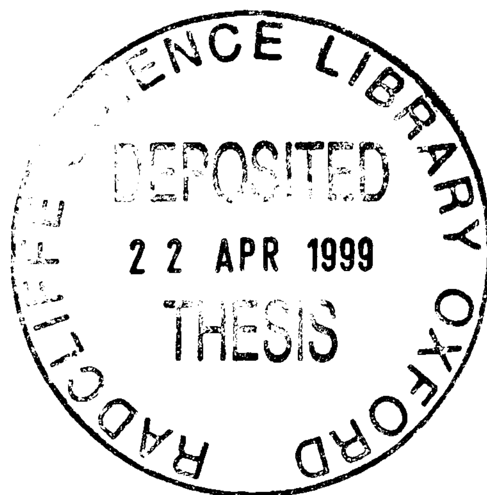

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Abstract

The aim of this thesis was to gain an understanding of the molecular and ecological basis for the biological control of *Pythium* by fluorescent pseudomonads. A fluorescent pseudomonad biocontrol agent (BCA), *Pseudomonas fluorescens* 54/96, identified as a potential candidate for commercial development, was analysed together with transposon induced mutants in a variety of assays for anti-fungal activity (Chapter 2). It was revealed that 54/96 had a fungistatic effect generated by a number of different mechanisms, which included nutrient competition and antibiosis. The synecology of this organism with *Pythium* was then compared to a similar organism (*P. fluorescens* SBW25) demonstrating a similar degree of anti-fungal activity (Chapter 3). The similarity of the population dynamics of these two strains prompted an examination of the genetic basis for the anti-fungal activity of the second strain, with the intention of comparing with 54/96 (Chapter 4). Again this revealed a multifactorial mode of action of SBW25 against *Pythium*. Whilst some mutants with reduced anti-fungal activity were deficient in growth on seed exudate others were unaffected, but the mechanisms appeared to be different to those utilized by 54/96. The comparison of strains was expanded to a larger collection of pseudomonad BCAs which were contrasted by a number of phenotypic and genotypic methods (Chapter 5). Various markers were identified which showed commonality within the different classes of BCA, the most useful of which was cyclopropanated fatty acids. These may prove to be a useful marker when screening for new pseudomonad BCAs. It was concluded that a greater understanding of the molecular, physiological and ecological basis of anti-fungal activity of bacterial will lead to the development of biocontrol strategies with improved efficacy.

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Abbreviations

°C	degrees centigrade
BCA	biological control agent
cfu	colony forming units
CI	Control index
cm, mm, µm	centimetre, millimetre, micrometre
CMM	cornmeal medium
d, h, min	days, hours, minutes
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid (disodium salt)
FA	fatty acid
FAME	fatty acid methyl ester
FDA	fluorescein diacetate
g, mg, ng	gram, milligram, nanogram
HCN	hydrogen cyanide
l, ml, µl	litre, millilitre, microlitre
LB, LBA	Luria-Bertani medium, + agar
M, mM	molar, millimolar
MGT	mean generation time
MSD	minimum significant difference
NSI	Nutritional similarity index
O.D.	optical density
PDA	potato dextrose agar
PDAA	potato dextrose agar supplemented with 320 mg l^{-1} aureomycin
PGPR	plant-growth promoting rhizobacteria
phl	2,4-diacetylphloroglucinol
phz	phenazine
plt	pyoluteorin
PSA-CFC	*Pseudomonas* selective agar with 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin and 50 mg l^{-1} cephaloridine
PSE	pea seed exudate
Pythium	*Pythium ultimum*
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SAR	systemic acquired resistance
SDDW	sterile deionized distilled water
Tris	tris (hydroxymethyl) aminomethane
TSBA	tryptic soy broth + agar
TWA	tap water agar
w/v	unit weight per unit volume
x*g*	times gravitational force

1. INTRODUCTION

1.1. Crop protection

As demand dictates that agricultural productivity should be increased, so agricultural practices that improve yield are constantly sought. Until recently the method by which this has been achieved is the application of agrochemicals. Fertilizers are applied to ensure sufficient nutrients are available for maximal plant growth and pesticides are applied to ensure crops are not damaged by either invertebrate or microbial pests. However, public awareness of the environmental implications of the use of large quantities of pesticides in agricultural practices is increasing. Not only is the development of resistance to the pesticides a real problem, but the financial cost to the consumer of application of existing products and the necessary development of new ones is exorbitant. Moreover, contamination caused by the extensive use of chemical pesticides that find their way into foodstuffs and water supplies has highlighted the need for environmentally friendly options (de Weger *et al.*, 1995; Asaka & Shoda, 1996) and thus alternative strategies for the control of plant diseases are being sought (Weller, 1988).

The use of microorganisms to prevent loss of crop yield caused by another is one such strategy that is being investigated. The first report of the use of microorganisms as biological control agents was in the 1920s and since then interest and information about the phenomenon have gradually increased (Campbell, 1989). However, with the increasing knowledge of microbial ecology and plant pathology in recent years the use of biological control has become a more realistic prospect. Indeed, there are now several commercially available biocontrol products. For example, in Australia the product 'NoGall' is an *Agrobacterium* strain antagonistic to crown gall disease of fruit and roses (Ryder, 1994), in the USA 'Dagger G' is a *Pseudomonas fluorescens* strain active against damping-off of cotton (Cook, 1993) and 'GlioGard' is the commercial name for *Gliocadium virens* active against seedling diseases of ornamentals (Cook, 1993).

However, despite these apparent successes, the use of biocontrol is still not as effective or reliable as the chemical counterparts (Weller, 1988). This is probably due to the inherent variability of applying a living organism to the environment. As environmental conditions fluctuate so the colonisation and *in planta* activity will be altered (Weller & Thomashaw, 1994). A full understanding of the ecology of an organism is therefore a prerequisite for its

release (Curl & Truelove, 1986; Campbell, 1989; Dowling *et al.*, 1994). This requires an insight into the evolutionary basis for the antagonistic phenomenon. Therefore the focus of this thesis has been to collect data pertaining to population dynamics and phenotypic and genetic analysis of fluorescent pseudomonads with the ability to suppress damping-off disease caused by the soil-borne fungal phytopathogen *Pythium*.

1.2. Soil-borne phytopathogens

Many micro- and macroscopic plant pathogens are found in cultivated soil. These range from insects and their larvae, to fungi, bacteria and viruses. Plant-microbe interactions have been responsible for catastrophic crop failures (Jackson & Taylor, 1996) and for this reason their study has received much attention with the aim of improving agricultural productivity (Jackson & Taylor, 1996).

There are 8000 different species of fungi from many genera that cause plant disease (Schafer, 1994). Some species, such as *Fusarium oxysporum* (Campbell, 1989), are particularly virulent and can completely decimate whole crop stands at any time during the season, whilst others, known as minor or opportunistic pathogens, are restricted in terms of their virulence or their narrow timing of infection.

1.2.1. Pythium

Pythium is a member of the Oomycetes, a lower fungi group. This group of fungi is capable of the production of spores both sexually and asexually. The sexually-produced spores, known as oospores, have a thick wall which ensures their long-term survival in soil (Johnson, 1988). Asexual spores, known as zoospores, are motile and released in batches from sporangia, which themselves are capable of remaining viable in soil for long periods (Stanghellini & Hancock, 1971b). However, in some oomycetes, sporangia do not release zoospores and germinate directly (Webster, 1970).

The economic effects caused by crop failure due to *Pythium* can be substantial and losses due to the pre- and post-emergence damping-off that it causes run in to millions of dollars annually (Cherif, Benhamou & Belanger, 1993). This group of phytopathogens is ubiquitous in cultivated soils (Hwang, Chakravarty & Prevost, 1993) and apparently shows no host specificity (Hendrix & Campbell, 1983) and causes a wide variety of symptoms. For example, *Pythium* spp. are capable of causing damping-off, foliar blight, crown rot, root rot and snow blight of turfgrasses (Abad, Shew & Lucas, 1994). However, despite this apparent dominance these fungi are considered as opportunistic pathogens (Campbell,

1989) in so much as disease is only apparent on young or weak plants (Weller, 1988). However, as well as their ability to cause disease in higher plants, *Pythium* species have also been shown to infect algae and other fungal species (Hendrix & Campbell, 1983).

Pythiaceous fungi are usually dormant in soil, existing either as oospores (Johnson, 1988; Francis & St. Clair, 1997) or sporangia (Stanghellini & Hancock, 1971b; Francis & St. Clair, 1997), reflecting the fact that they are inactive until a suitable opportunity arises. When a suitable opportunity does arise, for example, in close proximity to germinating seeds which exude nutrients, fungal germination and growth are extremely rapid (Hendrix & Campbell, 1983), in some cases infection occurs within a few hours of the seed being planted (Nelson & Craft, 1989). This quick response is, in part, the reason for their success. They are generally the first organisms to invade plant tissue but are soon displaced by more aggressive pathogens but by this time large quantities of resting structure have been produced (Hendrix & Campbell, 1983). Therefore, due to its long term persistence in soil, broad host range and rapid germination, growth and infection, *Pythium* disease is not easily controlled (Webster, 1970).

1.2.1.1. Existing methods of control

Pythium is a problem both in the field and glasshouse-grown crops (Paternotte & de Kreij, 1993; Harris *et al.*, 1994) even in hydroponic systems (Buysens, Höfte & Poppe, 1993) and must be controlled to obtain a good stand and yield (Hendrix & Campbell, 1983). At present, *Pythium* is controlled by a variety of methods which fall into two categories, agronomic and chemical (Orlandini & Signorini, 1993). Agronomic systems include the use of crop rotation, specialized soil tillage regimes and the use of resistant cultivars. There is evidence that dryer, warmer conditions are detrimental to disease development and therefore a significant reduction in damping-off can be achieved by planting when weather conditions are favourable (Hendrix & Campbell, 1983). Crop rotation is of little use for controlling *Pythium* because of its wide host range (Webster, 1970).

Cultivars and breeding lines that are resistant to pythiacious fungi exist in many plant species and genera (Hendrix & Campbell, 1983). Most have been discovered by chance but breeding programs are developing new lines that are either directly resistant to the fungi or are capable of growing in conditions that are unfavourable to the development of disease (Hendrix & Campbell, 1983). The main problem which such approaches is that many resistant cultivars are lower yielding than the commonly used equivalents and therefore a careful balance must be made when selecting cultivars.

Alternatively, chemical pesticides are applied by seed coating or fumigation. In glasshouses steam treatment or fumigation of potting media and pots in the most common method of eliminating *Pythium* (Harris *et al.*, 1994). However, problems still occur because of its persistence in dust in commercial glasshouses themselves and therefore fungicides are often applied to the potting media (Harris *et al.*, 1994). Some media is also composted before use and this has been shown to reduce the pathogen populations (Hendrix & Campbell, 1983).

There are a large variety of compounds are available for the chemical control of damping-off disease (Cohen & Coffey, 1986). However, it has only been since the 1970's that systemic fungicides with good activity against oomycetous fungi have been available (Cohen & Coffey, 1986). The problem with these systemic compounds is, that while they may be applied to the foliage of germinating seedlings at lower rates, they are often highly specific, and may not even control all species in one genera (Hendrix & Campbell, 1983). Another problem with the use of fungicides in general is the development of resistance in the pathogens (Hendrix & Campbell, 1983).

1.2.1.2. Alternatives to chemical control

Certain soils have been noted to impede fungal diseases and are known as disease-suppressive (Schroth & Hancock, 1982). In some cases the soil is naturally suppressive but in most suppressiveness is induced by continuous cropping with a susceptible cultivar (Cook *et al.*, 1995). It has been postulated that the induction may be due to changes in either the physical, chemical or biological properties of the soil, or indeed a combination of effects (Schroth & Hancock, 1982). A commonly held view in the field of biocontrol is that induced suppression is due to selection, by the plant, of microorganisms that are antagonistic towards pathogens (Cook *et al.*, 1995).

Systemic acquired resistance (SAR) is an induction of the plant's natural defence mechanisms (Hunt *et al.*, 1996; Ryals *et al.*, 1996). The induction may either be triggered by the pathogen itself or by some bacterial inoculants (Leeman *et al.*, 1995, 1996; van Veen, van Overbeek & van Elsas, 1997). The systemic nature of the resistance mechanism means that resistance to a root pathogen may be generated by the presence of the inducer on the leaf surface. There is speculation that biosurfactants produced by the bacteria may be responsible for this induction (Harling, pers. comm.) and there is mounting evidence that salycilic acid and lipopolysaccharide play a major role (Leeman *et al.*, 1995, 1996).

Other mechanisms by which *Pythium* diseases may be controlled are now coming to light. It is evident that an increased understanding of *Pythium* population genetics and biology may lead to more effective plant breeding and disease management strategies can be implemented (Francis & St. Clair, 1997). The application of microbial antagonists to seeds is another approach that has received attention (Campbell, 1989). However, seed treatment may not be feasible as there are a huge number of different plant species affected by damping-off (Harris *et al.*, 1994) and therefore any viable alternative to the fungicides used at present should function when applied direct to soil. This is particularly relevant as the time span when protection from *Pythium* is required spans only a few days during germination (Osburn *et al.*, 1989) and it is considered this pathogen should be simpler to control, once the appropriate systems have been developed, than others where plants are at risk throughout the entire growing season (Weller & Thomashaw, 1994).

1.3. Biological Control

Broadly speaking biological control is defined as the reduction of the effect of pathogens that relies on biological mechanisms other than man (Campbell, 1989). However, it is more accurately defined as the artificial introduction of antagonistic organisms that results in the control of a pathogen (Campbell, 1989). The interactions between two organisms that lead to the suppression of the density of one population is a natural phenomenon and essential to ecosystem function, but unfortunately the natural levels of control achieved are too low to be of use to man (Stotzky, 1997). These interactions have evolved over millennia and therefore attempts to redress the balance are bound to meet difficulty due to the buffering of the natural environment (van Veen, van Overbeek & van Elsas, 1997). Although the term 'biological control' was originally used to describe the regulation of insect pests by the introduction of predators, it is now more commonly associated with microorganisms (Campbell, 1989).

1.3.1. Microscopic organisms

One of the major areas in which biological methods look promising is the control of soil-borne fungal phytopathogens such as *Aphanomyces* (King & Parke, 1993), *Fusarium* (Couteaudier, 1992), *Gaeumannomyces* (Thomashow & Weller, 1988), *Pythium* (Howie & Suslow, 1991; Fukui *et al.*, 1994a) and *Rhizoctonia* (Hill *et al.*, 1994). Unfortunately, little is known about the ecology and epidemiology of these organisms and therefore the choice of an appropriate biocontrol agent is not a simple task. For example, the chemical stimulants which lead to the germination of dormant spores have not been fully characterized and the

sensitivities of these organisms to the various classes of antibiotics are not known. Therefore no information is available that will allow the choice of antagonist to be an educated one.

Much of the work published on the use of biocontrol agents (BCA) is based on isolates selected by screening large numbers of microbes for *in vitro* or *in planta* activity against fungi (Callan, Mathre & Miller, 1990; Handelsman *et al.*, 1990; King & Parke, 1993; Hill *et al.*, 1994) and this is still the preferred method (Campbell, 1989). Microorganisms from both the fungal and bacterial kingdoms have been selected from screening programs such as these. Whilst some particular genera appear to hold more promise for development into a commercially viable product that others (Campbell, 1989), this should not preclude the use of other organisms. However, it does appear that certain genera, namely the fungus *Trichoderma*, and the bacteria *Bacillus* and *Pseudomonas*, have been the most studied.

Trichoderma and other fungi have been used successfully for the control of many fungal pathogens, including those that cause damping-off (Lumsden *et al.*, 1990), both by mycoparasitism and antibiosis mechanisms (Campbell, 1989). Although commercial preparations are available, their use is not widespread (Campbell, 1989). Similarly, strains of *Bacillus subtilis* have also been made commercially available (Paulitz, 1992) but despite their apparent efficacy they are not widely used (Campbell, 1989). Pseudomonads, with their wide array of potential antimicrobial mechanisms have also shown promise for the control of damping-off. To this end, the search for pseudomonad BCAs has been extensive.

Some isolates such as *Pseudomonas cepacia* B37w were discovered by chance (Burkhead, Schisler & Slininger, 1994). One isolate used in this study, *P. fluorescens* 54/96 was selected from 7000 isolates from sugar beet rhizosphere because of its activity against *Pythium* in *in planta* assays (Fattori *et al.*, 1993). Another isolate used here, SBW25, was originally isolated as a candidate for field release purely on the basis of its prevalence in plant habitats (Bailey *et al.*, 1997).

1.4. The Pseudomonads

1.4.1. Description and Taxonomy

The family Pseuedomonadaceae are defined in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984) as strictly aerobic chemoorganotrophs. They are Gram-negative rods with motility provided by polar flagella. The type genus is *Pseudomonas*,

which has been divided up into a number of groups on the basis of ribosomal 16S RNA (rRNA) homology and DNA homology (Palleroni, 1984). The largest group, Group I, has been termed *Pseudomonas* sensu stricto (Brosch *et al.*, 1996). Recently, a more comprehensive analysis of the intrageneric relationships based on the sequencing of the 16S rRNA gene has been published (Moore *et al.*, 1996).

Whilst the majority of the pseudomonads appear to be poorly defined, the fluorescent species of rRNA group I (*Pseudomonas* sensu stricto) have been the focus of many studies. One of the reasons for this, may be the inclusion of both the human pathogen *P. aeruginosa*, and the plant pathogenic species, *P. syringae*. Amongst this group there are also many saprophytic species commonly isolated from water, soil and plant habitats (Moore *et al.*, 1996). For the purpose of this study, the term fluorescent pseudomonads will be used to identify the "*Pseudomonas fluorescens* intrageneric cluster" as defined by (Moore *et al.*, 1996), which includes the majority of species commonly isolated from plant-associated habitats.

This group of bacteria are amongst the most metabolically diverse, in terms of the ability to utilize a wide array of carbon sources, degrade xenobiotics and produce biologically active metabolites. For example, *Pseudomonas* spp. have been identified which produce anti-viral compounds (Lampis *et al.*, 1996), anti-fungal compounds (Rainey, Brodey & Johnstone, 1993) and anti-bacterial compounds (Whatling *et al.*, 1995). Their use for the removal of organic pollutants have also been highlighted (de Lorenzo, 1994). Given this wide array of functions it is probable that some will be of use to man. However, it has also been noted that microbes are generally selective in terms of their habitat preferences and therefore those required to function in a plant habitat should ideally be isolated from that habitat.

1.4.1.1. Plant-associated pseudomonads

Pseudomonads are virtually ubiquitous in plant associated habitats and are usually the most numerically dominant genus that can be cultured from these environments (Palleroni, 1992; Rainey, Bailey & Thompson, 1994; Ellis, Thompson & Bailey, 1998). To this end it is highly likely that they will possess features that enable them to compete well in such environments, probably at the expense of other microbes. Whilst the majority of these colonizers appear to be saprophytic with no obvious effects on the host plant (Beattie & Lindow, 1995), some are beneficial whilst others are pathogenic. It is also possible that individual genotypes may switch between these states, triggered by changes in environmental conditions (Beattie & Lindow, 1995).

1.4.1.2. Pseudomonads as biological control agents

Many of the so-called 'plant-growth promoting rhizobacteria' (PGPR) isolated are fluorescent pseudomonads (Weller & Thomashaw, 1994). Their presence in the rhizosphere of plants serves to improve the growth of that particular plant. The cause of improved plant health can be due to an bacterial-mediated increase in the supply of nutrients to the root, bacterial production of phytohormones or by the reduction in disease caused by soil-borne pathogens (Weller & Thomashaw, 1994). It is pseudomonads from this latter class that have been identified for manipulation for the purpose of biological control.

Pseudomonas species have been shown to cause fungistatic and fungicidal effects by a number of different methods. These include the production of antibiotics (Fravel, 1988), siderophores (Leong, 1986; Loper & Buyer, 1991) enzymes that degrade components of fungal cell walls (Fridlender, Inbar & Chet, 1993) and the metabolism of nutrients essential for fungal pathogenicity (Nelson, 1992). Some fluorescent pseudomonads can also stimulate the growth of mycorrhizal fungi and the formation of their symbioses with plants (Frey-Klett, Pierrat & Garbaye, 1997). Therefore, in order to develop an effective biocontrol strategy using organisms such as these it is essential to ascertain the mode of action and under what conditions it is most active.

1.5. Improving the efficacy of *Pseudomonas* biological control agents

The major drawback to the use of biological control agents at present is their poor performance in the field when compared to the chemical equivalents (de Weger *et al.*, 1995). This is measured both in terms of the level of control achieved and the reproducibility of the control between applications. Thus to make biocontrol a commercially viable proposition this situation must be addressed.

In order to control a pathogen the biocontrol agent must reduce its reproduction or activity. Two factors dictate the interaction between two organisms: relative fitness and competitive ability (Kinkel, Newton & Leonard, 1995). Fitness, defined as the ability to grow and replicate in a fundamental niche, is a function of the individual organism and as such can not be altered. However, the competitive ability of a given organism is dependant on the presence of other organisms which share all or part of that niche. Thus these organisms effectively alter the niche of the original organism and the resultant realized niche will be

less favourable for that organism. A greater the overlap between the niches of two organisms will usually result in a greater difference between the ability of each to prosper in that given situation. Thus, it is this part of the equation that needs most study in the quest for a full understanding of biological control.

1.5.1. Ecological considerations

Competition in biological control systems can take on a number of forms and it must be remembered that it is probably a multi-faceted interaction of which only the net result is observed. The bias towards studying the mechanisms used by the beneficial organism to suppress the pathogen has led to a lack of knowledge about the mechanisms used by the plant pathogen to counter the antagonism and the information here reflects that bias. There are two approaches by which a biocontrol agent can suppress a plant pathogen: antibiosis and superior (high affinity) resource utilization. Antibiosis is a well documented phenomenon but its relevance in the natural environment has long been a point of some debate (Fravel, 1988). The ability of a potential biological control agent to utilize a resource more efficiently than the pathogen is the basis behind the second approach to suppression.

Inevitably, this is achieved by minimizing the niche available to the pathogen, where the niche is defined as all available resources including both physical space and nutrients (Campbell, 1989). Therefore this area covers both pre-emptive colonization and energy source uptake. Pre-emptive colonization is rather non-specific but involves the rapid growth of one organism which enables it to fully utilize the niche before another organism is able to do so. In terms of plant tissue colonization, a niche may be a specific site on the plant surface, such as a cleft between cells or an outer membrane attachment motif. Thus, a population is established which cannot be ousted by another organism as there will be insufficient resources to enable it to do so.

A germinating seed releases a large array of compounds into the surrounding soil. Some of these will stimulate a resurgence of growth from the quiescent state of fungal plant pathogens (Nelson, 1990, 1992). Competition for these stimulatory exudates has been suggested as a mechanism for biocontrol (Nelson, 1992). If the molecules are catabolized by another organism prior to their induction of germination then the incidence of disease will be reduced. Indeed, bacterial chemotaxis towards seed exudates has been demonstrated (Gamliel & Katan, 1992a) and implicated in the rapid establishment of populations in the rhizosphere of young plants (Gamliel & Katan, 1992b). In these cases the increase in the bacterial population (fluorescent pseudomonads) correlated with a reduction in total fungi numbers.

Any other essential resource in short supply in the environment will be competed for. One such resource is Ferric iron (Fe^{3+}). It is highly insoluble at normal physiological pH and therefore a wide variety of organisms, including higher plants, have evolved special systems for its sequestration (Loper & Buyer, 1991). The use of siderophores is one of the most common systems. Low molecular weight compounds are produced that chelate Fe^{3+} with high affinity and are then recognised by receptors on the cell surface which then transport the ion into the cell (Leong, 1986). The competition for this resource is at the level of the relative affinities different organisms have for Fe^{3+}. However, despite the large amount of work on siderophores, especially those produced by fluorescent pseudomonads, their role in biological control is minimal in some situations (Hamdan, Weller & Thomashow, 1991; Loper *et al.*, 1993) but more central in others (Loper & Buyer, 1991), being affected by soil, host plant, pathogen and individual bacterial strains.

There is a great deal of emphasis placed on the importance of rhizosphere colonization by the BCA in its ability to suppress disease (de Weger *et al.*, 1995; Latour *et al.*, 1996). Whilst this may not always be relevant in the control of *Pythium* damping-off, which occurs prior to the development of a root system, the principles that the BCA population must attain a sufficient size in the correct place (de Weger *et al.*, 1995) still apply. This is so that active compounds or other desired effects are released in adequate quantities to effect control. Therefore, any BCA for damping-off must be capable of surviving at effective densities in non-rhizosphere soil and show anti-fungal activity at the onset of seed germination.

1.5.2. Genetical considerations

Molecular genetics techniques have been applied to determine the role of various mechanisms in the activity of BCAs in the environment. For instance, by a process of transposon mutagenesis and subsequent complementation, phenazine-1-carboxylate was implicated in the control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow & Weller, 1988; Weller, 1988). Similar methods have been used to prove the involvement of pyrrolnitrin (Pfender, Kraus & Loper, 1993; Hill *et al.*, 1994), pyoluteorin (Kraus & Loper, 1995), 2,4-diacetylphloroglucinol (Fenton *et al.*, 1992; Keel *et al.*, 1992), other unknown antibiotics (Carruthers *et al.*, 1995), and cyanide (Voisard *et al.*, 1989) in biological control of fungal plant pathogens by pseudomonads.

Recent advances in recombinant DNA technology have also led to the possibility of improving bacterial biocontrol agents genetically (Burr & Caesar, 1999). For example, efforts have been made to engineer pseudomonads to overproduce various antibiotics such

as 2,4-diacetylphloroglucinol (Bosnall, Weller & Thomashow, 1997). These technologies have also provided the opportunity to genetically mark these organisms so that their persistence and activity in the target site can be accurately monitored (Bailey *et al.*, 1997). Such studies can provide vital information to allow efficacy to be precisely altered. There are several examples of how this has been achieved in pseudomonads. In one instance, the ice nucleation reporter gene (*inaZ*) was inserted into *P. fluorescens* Pf-5 downstream of an iron regulated siderophore promoter (Loper & Henkels, 1997). The results of this study may be extrapolated to illustrate the temporal and spatial nature of siderophore production by fluorescent pseudomonads in rhizosphere systems as well as indicating some of the physiochemical characteristics of soil that will effect their production.

More information is coming to light about the mechanisms controlling the production of antibiotics. The biosynthesis of some, such as 2,4-diacetylphloroglucinol in *P. fluorescens* F113 (Fenton *et al.*, 1997), is both cell density-dependant, and affected by the carbon source used for growth (Shanahan *et al.*, 1992). Some other antibiotic metabolites are under the control of stationary phase promoters (Sarniguet *et al.*, 1995). These examples demonstrate that the regulation of antibiotic production is highly complex, even in defined laboratory conditions. Thus, if the mechanisms controlling production are fully understood, then they may be manipulated to enhance the levels of antibiotics synthesized *in situ.*

Essentially, genetic manipulation of a given strain will attempt to simulate evolution (Stotzky, 1997) or to force evolution in a direction beneficial to man. Generally, the aim on manipulation of BCAs will be to enhance the levels and timing of *in situ* antibiotic production, and to improve their survival and stability in the fluctuating natural environment.

1.6. Project aims

The aim of this thesis has been to ascertain factors that may effect the efficacy of fluorescent pseudomonad biological control agents. In order to achieve this goal a variety of different approaches have been utilized. However, the initial aim was to develop a suite of reproducible assays that would serve as a basis for the analysis of the effect of different determinants in the ability to suppress damping-off in peas, for which the causal agent was *Pythium ultimum.*

With the aid of transposon mutagenesis technology it was intended to ascertain the mechanisms by which fluorescent pseudomonads suppress disease. Existing biocontrol-negative mutants were to be compared to the wild-type parent in an attempt to describe the

activity which had been curtailed by the insertional mutations. Thus both the phenotypic and genetic basis of anti-fungal activity would be characterized.

A study of the population dynamics of both the pseudomonads and the fungal pathogen should provide insight into the ecological interactions between the two populations. Such a study should reveal both the timing and nature of the antagonistic effect.

The final aim of this project was to compare a collection of pseudomonads to determine if any genotypic or phenotypic traits predispose pseudomonads to antifungal activity. It was hoped that a feature would be identified which would allow rapid screening of pseudomonad collections for potential BCAs in the future.

The data obtained from the combination of these approaches would provide a better insight into the interactions between pathogen and antagonist that lead to disease suppression and identify those areas in which it could be manipulated to improve the efficacy and reliability of biological control in the field.

2. DEVELOPMENT AND APPLICATION OF ASSAYS FOR DETERMINING THE FACTORS THAT AFFECT BIOCONTROL EFFICACY OF SELECTED *PSEUDOMONAS FLUORESCENS* STRAINS

2.1. Introduction

Field evaluations of biocontrol agents (BCAs) are prone to variable results, even when the same experiments are repeated in different growing seasons at the same location or at different locations at the same time (Hagedorn, Gould & Bardinelli, 1993). This inconsistency can not be accounted for either in terms of disease prevalence or environmental factors (Hagedorn, Gould & Bardinelli, 1993) and thus results are difficult to interpret.

It is this lack of consistency that probably accounts for the limitations to the widespread use of biological control for the reliable suppression of fungal phytopathogens (Weller, 1988; Hagedorn, Gould & Bardinelli, 1993). A greater understanding of factors which cause variation in biocontrol efficacy would allow predictions of the level of success achievable

under a given set of conditions. If these factors can be determined the application of biocontrol agents can be made more cost effective as they need only be applied when conditions are likely to be successful. An understanding of both the ecology and biology of the organisms must therefore be obtained as a prerequisite for BCA release to ensure that this approach is to work effectively (Dowling *et al.*, 1994), even as part of an integrated management system.

In order to experimentally evaluate the efficacy of potential biological control agents, reliable model systems are required. The need for rapid primary screening systems for identification of potential biocontrol bacteria has been highlighted (Kloepper, 1991; Renwick, Campbell & Coe, 1991; Stephens, Crowley & O'Connell, 1993), but their use for exploring the mechanisms and mode of action of biocontrol has been largely overlooked. Whilst small scale assays are used for screening for biocontrol negative mutants, their use for investigating basic ecology has seldom been investigated.

The design of *in vivo* assays needs careful attention to ensure that field conditions are simulated as accurately as possible, whilst the numbers of variables are reduced to provide a meaningful study. The components that must be included are the soil and plants, but elements such as temperature, light intensity, water content and the sizes and diversity of the microbial populations present must be accurately monitored to allow accurate replication. However, caution must be exercised when extrapolating data obtained using microcosms to predict field performance, as it has been shown that microcosms can assess differences between two closely related strains, but not predict actual microbial population sizes (Kinkel, Wilson & Lindow, 1996). Such detail is of particular importance if control efficacy is dose dependant.

The assessment of activity of BCAs on the different phases of the fungal growth and infection cycle can provide insight into the specific fungal processes interrupted by the BCA. These may include spore germination, hyphal extension and host infection. The means by which these are instigated, such as competition or antibiosis, may be revealed by logical analysis of the results, thus creating a framework for improvement of sustained activity and reproducibility of the biocontrol effect. The exploitation of *in vitro* assays to examine interactions between pathogen and antagonists holds great promise for ascertaining the mode of action of the BCA.

Determining the mode of action of a biocontrol agent is important for a number of reasons. Firstly, if the mode of action is known, the effectiveness of the biocontrol agent in field situations can be optimized (Curl & Truelove, 1986). For example, the timing and

frequency of inoculum application can be improved to enhance its antagonistic effects. Secondly, it will be possible to predict the situations where control will be successful and where it may fail, thereby avoiding fruitless application. Finally, if a BCA is to become a commercial product, it will have to pass strict regulatory testing procedures. For example, if the mode of action is one of simple competition for nutrients between antagonist and pathogen, then these procedures will be less stringent than if the BCA produces a whole array of secondary metabolites, each of which will have to be subjected to the same rigorous testing required for chemical pesticides (Lynch, 1992).

In this chapter, a suite of complementary assays have been developed to assist in resolving the mode of action of a proven pseudomonad BCA, *Pseudomonas fluorescens* 54/96 (Fattori *et al.*, 1993), and for confirmation of the activity of a second strain with possible biocontrol potential. These assays included both *in planta* assessment of disease suppression and *in vitro* studies to determine the effect of bacteria on the activity of the pathogen.

2.2. Materials and Methods

2.2.1. Pathogen / Host system

The pathogen host system used throughout this thesis was based on that used by Zeneca Agrochemicals (Murray, 1994). Esentially, the fungal pathogen, *Pythium ultimum*, was selected for its ubiquity and virulence against many plant species. The plant host used, the forage pea, *Psium satvium* var. 'Bohartyr' (Nickerson Seeds, Linconshire), was an untreated seed variety which was particularly susceptible to the strain of *Pythium* used here (Murray, 1994). Untreated seeds were used in all experiments.

2.2.2. Propagation and maintenance of Fungi

Pythium ultimum (Murray, 1994) was stored in the form of naturally infested soil. New batches were produced as required essentially as described (Murray, 1994). *Pythium* infested soil (6 g), the initial batch of which was obtained from Zeneca Agrochemicals (Jealotts Hill Research Station, Bracknell, UK), and wheatgerm (2 g) were mixed with 1 l Sterilized Mendip Loam (Minster Brand Products, Wimbourne, Dorset [Soil analysis: 47.5% sand, 29.3% silt, 23.2% clay; pH 6.65; water holding capacity 580 μl water per gram dry weight soil]). This mix was used to fill 20 x 15 cm seed trays which were planted with 25 untreated pea seeds (*Psium satvium* var. 'Bohartyr', Nickerson Seeds, Linconshire) and

watered. Seedlings were propagated at 15 °C with an 18 h photo-period and daily watering for 1 week. Watering was reduced during the second week. Fourteen days after sowing shoots were removed and the soil dried for 4 weeks at 15 °C, effectively killing the mycelial fragments and enriching for spores (Martin, 1992). The soil was then passed through a 2 mm gauge sieve and stored in sealed containers in the dark at 15 °C.

The pathogen was isolated from infested soil by plating diluted soil suspension onto Potato Dextrose Agar (PDA; Unipath, UK) supplemented with 320 mg l^{-1} aureomycin (Cyanamid, UK; PDAA; (Thompson *et al.*, 1993b)), or Tap water agar (TWA; 1.2% Agar No. 3 (Unipath, UK) in tap water, (Stanghellini & Hancock, 1970)). Plates were incubated at 20 °C for 18 h. *Pythium* was identified by its rapid growth rate (Stanghellini & Hancock, 1970).

Oospore suspensions were produced essentially as described by (Johnson, 1988). Briefly, 9 mm plugs from 3 day old cultures on PDAA were placed in 20 ml cornmeal medium (CMM; 10 g l^{-1} cornmeal heated to 60 °C and filtered before autoclaving) and incubated at 15 °C in the dark for 3-6 weeks. The oospores were separated from hyphae by mixing the culture with an equal volume of sterile deionised distilled water (SDDW) and blending in a Waring Blender. Hyphal mass was removed from the suspension by filtration through a 150 µm sieve and the remaining oospores washed twice by centrifugation at 4,000 x*g* for 10 minutes. Oospores were finally resuspended in SDDW and stored at 4 °C.

2.2.3. Storage of bacteria and preparation of bacterial suspensions

Two different *Pseudomonas fluorescens* strains were utilized in this study. The first, 54/96 (NCIMB 40186) was selected for possible commercialization (Fattori *et al.*, 1993) and its efficacy as a biocontrol agent for *Pythium* damping-off has been thoroughly investigated (Fattori *et al.*, 1993). Three mutants, produced by the introduction of transposon Tn*5-lac* (Kroos & Kaiser, 1984; O'Sullivan & O'Gara, 1988) into 54/96 (Stevenson, 1994) and labelled 54/96::Tn1569, 54/96::Tn1882 and 54/96::Tn3296, were assayed for inhibition of damping-off disease and growth of *Pythium in vitro*. The second, SBW25, was isolated from sugar beet (Bailey & Thompson, 1992; Bailey *et al.*, 1995).

Bacterial isolates were grown in 5 ml of Luria-Bertani broth (LB; Difco, UK) and shaken (150 rpm) at 28°C overnight. One millilitre of the resultant culture was added to 0.8 ml of 70% glycerol (v/v) and 0.85% sodium chloride (w/v) and stored at -70 °C. Isolates were revived by streaking the stored material on Tryptic Soy Broth (Difco, UK) solidified with 1.2% (w/v) agar (TSBA).

Inocula for all assays were produced by growing isolates in LB at 28 °C with shaking at 180 rpm for approximately 18 hours. Cells were then pelleted by centrifugation of the cultures at 5000 x*g* for 10 minutes and then washed twice in SDDW before resuspending in the original culture volume of SDDW. The resultant suspension contained approximately 10^9 cfu ml^{-1}.

2.2.4. In-vitro assays for determination of anti-fungal activity

2.2.4.1. Oospore Germination

2.2.4.1.1. Agar-based assay

All assays conducted on solid media utilized TWA. Bacterial suspensions (10^9 cfu ml^{-1}) were added to agar cooled to 42 °C at a rate of 1 ml suspension per 10 ml media. Various supplements (sucrose (6 mM), Fe^{3+} (1 mM) and Ca^{2+} (1 mM)) were also added to determine factors which may have effected the interaction. After drying overnight at 20 °C, plates were inoculated with 100 µl of *Pythium* oospore suspension (see above) and a single pea seed. Plates were scored for pathogen germination and growth after 4, 6 and 8 d incubation at room temperature. Developing pea roots were also examined for *Pythium* colonization under low power magnification and photographic records obtained. Germinating spores and *Pythium* colonies were also photographed under microscopic observation.

2.2.4.1.2. Vital staining and microscopy

Oospore suspensions, prepared as above, were mixed with fluorescent stains which allowed identification of active and non-active fluorescent cells (Söderström, 1977). The stains used were Calcofluor (CCF, Sigma, 100 mM in phosphate buffer (pH 7.0 -7.2) + 0.1% n-propyl gallate (Sigma) and fluorescein diacetate (FDA, Sigma 2 mg ml^{-1} in acetone). These two were mixed in the ratio of 5 µl FDA solution to 1 ml CCF solution immediately prior to use. Oospore suspensions (10 µl), were mixed with bacterial suspensions (10 µl) and incubated at room temperature for 2 h. This suspension was mixed with 3 µl stain, and observed under UV light at x500 magnification with a Vickers M41 Photoplan microscope with illumination from a Mercury Vapour lamp (Vickers Instruments, UK). All fungal structures present in the sample, dead or alive, stained blue with CCF as it binds to β-glucans in the cell walls, but only active fungi are stained green by FDA which is hydrolysed by esterases in the cytoplasm (Söderström, 1977).

2.2.4.2. Hyphal extension

The medium used for *in vitro* assays in the liquid phase was Pea seed exudate (PSE). This was produced by adding SDDW (50 ml) to pea seeds (50 g) and shaking at 18 °C for 1 h. The exudate was then filtered through Whatman No.1 paper before sterilizing by filtration through a 0.22 µm filter. PSE was stored for up to a week at 4 °C. PSE was diluted 1 in 10 in SDDW and triplicate 5 ml aliquots inoculated with either 100 µl of washed bacterial suspension prepared as above, 3 mm cores of 3 d old cultures of *Pythium* removed from PDAA or both. These PSE cultures were incubated at 28 °C with shaking (180 rpm) in 30 ml Universal tubes. After 4 d the diameter of the *Pythium* hyphal mass was measured. This method allowed comparisons of the activity of the different strains on hyphal growth.

2.2.5. *In planta* Quantification of disease suppression

An assay for studying the factors involved in the biocontrol of *Pythium* was developed (Figure 2-1). Based on an existing procedure (Murray, 1994), it was adapted to allow large numbers of assays to be run simultaneously. Seed trays were replaced by 145 mm diameter petri dishes. Whatman No. 3 filter paper (5 sheets) was placed in the bottom of the dishes to maintain water content and covered with 60 g of uninfested or infested soil. The soil used in all assays was sterilized Mendip Loam (Minster Brand Products, Wimbourne, Dorset). When *Pythium* was included in the system, infested soil was mixed 1:10 (w/w) with the loam to give a final *Pythium* dose of approximately 10^4 cfu g^{-1}. Bacteria were added either by applying a suspension, prepared as described above, directly to the soil, resulting in a dose of approximately 10^8 cfu g^{-1} soil, or by soaking the seeds in the same suspension for 10 minutes as described previously (Thompson, Ellis & Bailey, 1995). Peas were pressed into the surface of the soil at approximately 1.5 cm intervals and the assays incubated in the dark at 21 °C, which has been shown to be optimal for disease development (Murray, 1994). All treatments were set up in triplicate. Seven days later the numbers of infected seedlings were recorded for all treatments and controls.

A variety of different factors were investigated for their effect on the efficacy of the biocontrol strain *Pseudomonas fluorescens* strains 54/96 and SBW25. For each of the following individual experiments triplicates for each treatment were set up at the same time to ensure that accurate comparisons were made.

2.2.5.1. Comparison of BCAs and application methods

The first assay was designed to determine the relative control abilities of 54/96 and SBW25 when applied as either a soil drench or by seed soaking as described above. The numbers of infected seeds were scored after 7 d.

2.2.5.2. Persistence of BCA and longevity of activity

Another assay was set up to test if the commercial biocontrol agent was more effective if it was applied to infested soil a number of days before planting pea seeds. *Pythium* and 54/96 were added to the soil as above and pea seeds were added to different plates 0, 2, 6, and 10 d later. The effectiveness of each treatment was assessed as normal at 7 d after planting in each case.

2.2.5.3. Comparison of mutant performance

The third assay was organized to compare the efficacy of the Tn*5* mutants of 54/96 (54/96::Tn1569, 54/96::Tn1882 and 54/96::Tn3296) with the parental strain. SBW25 was included in this assay to ascertain the reproducibility of its biocontrol efficacy over multiple runs by comparison with the results from the first assay. Each strain was applied as a soil drench and the numbers of infected seeds were recorded 7 d after planting.

2.2.5.4. Density dependent effects

The final assay examined the effect of both *Pythium* and 54/96 inoculation density on the incidence of disease and its control. The pathogen was applied to the soil at densities of 200%, 100%, 50%, 10%, 1% and 0.1% of the standard inoculum level, both on its own and in combination with the usual inoculum of the biocontrol agent. In addition, 54/96 was added to soil with the standard level of *Pythium* at densities of 200%, 50%, 10%, 1%, and 0.1% of the norm. Seven days after planting, disease severity was recorded in the regular manner and the numbers of *Pythium* and pseudomonads in the soil were determined for 4 of the treatments (Py200-Z, Py10-Z, Py1-Z and Py0.1-Z).

2.2.6. Data analysis and statistics

Hyphal extension assays were performed in triplicate and significant differences between colony diameters were determined by analysis of variance (ANOVA).

All seedling infection assays were performed in triplicate and a control index (CI) was calculated for each replicate assay of 25 seeds using Equation 2-1.

Equation 2-1:

$$CI = 1 - \left(\frac{(t-u)}{(i-u)} \right),$$

where $t =$ the number of infected seeds in the bacterial-treated assay, $u =$ the number of infected seeds in the *Pythium*-free assay and $i =$ the number of infected seeds in the *Pythium* infested assay. In each case u was a small number - infected seeds occuring due to the presence of other pathogens in the soil.

The CI data were arcsin transformed as appropriate for proportion data (Fry, 1993) and tested for homogeneity of variance using Bartlett's Chi-square test, Bartlett-Box F test, Cochran's C and Hartley's F statistics (Unistat® 4, Unistat Ltd., London). The data were then subjected to analysis of variance (ANOVA) to identify significant differences between treatments by the calculation of minimum significant differences (MSD, (Fry, 1993)).

2.3. Results

2.3.1. Production of oospores

Infested soil produced as described above, routinely yielded 10^5 *Pythium* propagules per gram of air dried soil. These counts were determined on both TWA and PDAA and no differences were seen between the two methods. All batches showed similar levels of pathogenicity in subsequent seedling trials when added at levels of 10^4 spores per gram of soil.

Microscopic examination of *in vitro* produced oospores revealed that the preparation was effectively free from other fungal structures. Some hyphal fragments were observed but these appeared to be lysed and therefore incapable of propagation. Plate counts revealed that suspensions contained approximately 10^4 *Pythium* propagules ml^{-1}.

2.3.2. Agar based assay

The rapid germination and growth of *Pythium* in the presence of the pea stimulus is accurately demonstrated in Figure 2-2. The subsequent illustration (Figure 2-3) demonstrates the prolific nature of the colonization of young pea roots by *Pythium* hyphae.

It was noted that spores 40 mm from the seed germinated up to 4 h later than those less than 5 mm from the seed.

The agar based assay, when used with purified oospores, proved to be a highly reliable method for determining the effect of different bacterial strains on oospore germination. Those isolates which did not arrest germination but did inhibit hyphal growth were identified using this method. Addition of bacteria to warmed (42 °C) agar did not appear to affect their antifungal activity. However, supplementation of the media with a carbon source (6 mM sucrose) or metal ions (1 mM $FeCl_3$ or 1 mM $CaCl_2$) affected both oospore germination and its inhibition by bacteria. Table 2-1 illustrates the performance of 54/96, its mutants and SBW25 in the oospore germination assays and the effect of supplementing the media with salts and a carbon source. The effect of the addition of the different bacterial inocula was varied. 54/96 was extremely effective in preventing oospore germination in this system for up to 10 days. Only one of the 54/96 mutants (54/96::Tn1569) retained this phenotype. The other mutants (54/96::Tn1882 and 54/96::Tn3296) did not prevent germination but reduced the rate of hyphal extension. SBW25 prevented oospore germination. The addition of 1 mM ferric chloride was inhibitory to *Pythium* oospore germination. In most cases the addition of sucrose reversed the effect caused by the presence of the bacteria. Oospore germination and hyphal growth were not inhibited, except by mutant 54/96::Tn1569 which reduced hyphal extension, and SBW25 which retained antifungal activity in the presence of 6 mM sucrose. In three cases (54/96, 54/96::Tn1569 and 54/96::Tn3296) the ability to arrest germination was lost in the presence of 1 mM$CaCl_2$ but the capacity to slow hyphal growth was retained. In the other two cases (54/96::Tn1882 and SBW25) both activities were lost in the presence of 1 mM $CaCl_2$.

2.3.3. Vital staining and microscopy

The use of fluorescein diacetate showed that *P. fluorescens* 54/96 had a biostatic effect on *Pythium* oospores (Figure 2-4). Oospores showed no visual signs of lysis or physical alteration in the presence of the bacterium. However, it was apparent that cellular metabolic activity was inhibited as the FDA was not cleaved to yield fluorescence. It could not be determined whether the lack of metabolic activity in the oospores was due to the production of specific anti-fungal compounds or competition for the low level of nutrients in the pea seed exudate. However, when the mixed suspensions of bacteria and fungi were plated on media containing the antibacterial agent, aureomycin, fungal growth was resumed, indicating that the effect was fungistatic and not fungicidal.

2.3.4. Hyphal extension

In liquid culture *Pythium* produced a single spherical mass of hyphae. This facilitated a method by which *Pythium* hyphal growth was quantified by simply measuring the diameter of the biomass. The effect of each bacterial strain on hyphal extension was readily determined. Hyphal growth was significantly inhibited by the presence of bacteria in the growth media (Figure 2-5). The Tn*5-lac* mutants of 54/96 inhibited the development by a significantly lesser degree than the wild-type (P=0.05). SBW25 limited hyphal growth to the same extent as the mutants of 54/96. This indicated that 54/96 inhibited the hyphal growth phase of *Pythium* by a mechanism inactivated by all three insertional mutations.

2.3.5. Quantification of disease suppression

The results from the pea seedling assays revealed that this system was both highly sensitive and reproducible. Seedlings infected by *Pythium* in the untreated assays were easily identified by prolific hyphal growth on the surface of the seed, which extended up to 15 mm into the surrounding soil. Effective control by the BCA was determined by scoring the number of germinating uninfected seeds. Addition of known biocontrol agents, such as 54/96, resulted in fewer numbers of seeds becoming infected.

2.3.5.1. Comparison of bacterial application methods

Figure 2-6 illustrates the effects on biocontrol efficacy of different methods of application. Soil drench proved to be more effective than seed soaking as a method for applying the bacterial inoculum. Indeed, when applied by soaking seeds, strain 54/96 shows no discernible reduction in seed infection.

2.3.5.2. Timing of introduction of biocontrol agent

The results of the second assay are shown in Figure 2-7. A trend towards a reduction in the incidence of disease was observed if *Pythium* infested soil was inoculated with 54/96 several days before planting the soil (not significant at 95% level). Application of the bacteria to soil 10 d before planting seeds increased the control index (*CI*) to 1.0 whereas in all other assays a *CI* of greater that 0.92 was not achieved when bacteria were applied at the time of planting.

2.3.5.3. Efficacy of Tn5 mutants

The different biological control activities of the 54/96 Tn5 mutants, 54/96::Tn1569, 54/96::Tn1882 and 54/96::Tn3296 were illustrated in the seed germination assay. The incidence of infection in assays treated with these strains compared to the parental strain were divergent (Figure 2-8). Mutants 54/96::Tn1882 and 54/96::Tn1569 showed no significant disease suppression when compared to the untreated control. However, control the activity of 54/96::Tn3296 was comparable to that of the wild type.

2.3.5.4. Effect of inoculum density on biocontrol efficacy

To determine the relative importance of BCA and pathogen densities further seed germination assays were established. Figure 2-9 shows the incidence of infection under various combinations of inoculum densities of the pathogen and biocontrol agent. Disease incidence increased with the increased population densities of the pathogen in soil. One hundred percent infection was achieved when the *Pythium* inoculum exceeded 4.8×10^3 cfu g^{-1} soil (50Py). Secondly, when the *Pythium* inoculum was 2.4×10^4 cfu g^{-1} soil (200Py) the normal dose of 54/96 (1.2×10^8 cfu g^{-1} soil) was less effective than under lower pathogen levels. Thirdly, when soil was inoculated with less than 8.5×10^5 cfu g^{-1} of 54/96 (0.1Z) control was less effective against the standard level of *Pythium* infestation of 1.3×10^4 cfu g^{-1} (Py). The numbers of *Pythium* and pseudomonads in the soil were determined at the end of the experiments. The numbers of pathogen were unaffected by the initial size of the pseudomonad population. However, irrespective of the initial inoculum level, the pseudomonad count fell to be a sustainable density of approximately 10^6 cfu g^{-1} soil in all cases.

2.4. Discussion

It was essential that all the assays developed in this study were reliable and reproducible. *In vitro* assays, whilst not necessarily effective for the identification of BCAs with commercial value (Kloepper, 1991), do allow direct assessment of anti-fungal activity under defined conditions. The identification of whether or not strains exhibit activity against resting structures or actively growing fungi reveals much about the potency or the intensity of the interaction. Logically, it would be more difficult to arrest the growth of an active organism than to prevent a non-active organism from developing (Howell, Beier & Stipanovic, 1988). If the mode of action of the BCA is to be fully understood, it is essential that a variety of assays that relate to the pathogen life cycle and its mode of infection are compared.

2.4.1. Evaluation of *in vitro* assays

The rapid germination and hyphal growth of *Pythium* was apparent from the microscopic observations of oospores in close proximity to pea seeds (Figure 2-2). The effect was reduced at greater distance from the seed. This correlated with the observations of the significance of seed exudation for the germination of fungal spores in soil (Stanghellini & Hancock, 1971a; Short & Lacy, 1976) and prompted the attempt to utilize pea seed exudates as a carbon source for assays of anti-fungal activity. If *Pythium* growth could be suppressed in the presence of PSE, then it follows that growth would be suppressed in field situations where plant exudates are known to enhance microbial populations (the rhizosphere effect, (Lynch, 1990)). In contrast, artificial media may contain compounds that affect *Pythium* in a different way to those it normally encounters and thus will not provide a true representation of natural conditions. In the agar assays exudates were presumed to diffuse from the seed placed on the surface and thus allowed temporal and spatial effects of the exudation to be observed. In liquid culture, the preparation of exudate from imbibing seeds demonstrated that, although undefined, it contains sufficient quantities of nutrients to sustain growth of both bacteria and fungi.

Despite claims that agar-based assays using seed exudate as the nutrient source can not be used to identify strains that inhibit fungi on the basis of nutrient competition (Stephens, Crowley & O'Connell, 1993), the results here provide no evidence to support such speculation. Indeed, it appears that bacterial strains out-compete the fungi for the limited nutrient supply, indicated by reduction in *Pythium* germination. Metabolic profiles (5.3.4, p1) have revealed that pseudomonads and *Pythium* can utilize a similar spectra of carbon sources, supporting the argument that nutrient competition may be important in the direct inhibitory effects observed. It is conceivable that one mode of action for 54/96 and SBW25 may be the reduction of damping-off disease by simply utilizing seed exudates before they reach the threshold concentration required for spore germination. For 54/96 the addition of sucrose negated this effect, indicating that the fungi may have been carbon limited or that production of an anti-fungal compound by 54/96 is inhibited by sucrose. The production of secondary metabolites only in the presence of certain carbon sources has previously been reported (Shanahan *et al.*, 1992). However, this was not the case for SBW25, establishing the presence of an alternative mechanism of fungal inhibition by this strain.

The presence of calcium ions reversed the effect of bacterial inhibition of oospore germination. Calcium has been shown to be an important factor in the germination and infection processes of many phytopathogenic fungi (Deacon & Donaldson, 1993; Warwar &

Dickman, 1996). Both 54/96 and SBW25 may be capable of assimilating calcium ions more efficiently than the fungi, thus depriving it of an essential resource, in much the same way as demonstrated for siderophores. Indeed, some bacterial siderophores have shown a high affinity for many different metal ions (Voisard *et al.*, 1994). However, there are many resources that could be limiting in such a system and therefore any theories developed about the mechanisms of biocontrol should not just concentrate on carbon and calcium.

Use of vital staining techniques revealed that bacterial treatment of oospores could render them metabolically inactive but this appeared to be reversible as they were not permanently damaged, either visually or in respect of their ability to resume growth when antibacterial agents were used. The nature of the interaction between pathogen and antagonist could not be determined, and may have been due to either the presence of some inhibitory compound (antibiosis) or competition. However, this definitive action against spores illustrates that the bacteria interact directly with the fungi.

All bacterial strains tested in the hyphal growth assay exhibited some repression of *Pythium*. It is probable that this effect was caused by competition for the relatively low concentrations of nutrients in the pea seed exudate. This may again be attributed to limitations caused by carbon, calcium or some other essential mineral. Nevertheless, 54/96 demonstrated significantly greater inhibition than any other strain, which suggested that it bore an additional mechanism not found in other strains. The fact that all three Tn*5* mutants of 54/96 were less inhibitory than the wild type implied that this mechanism was affected by all three mutations. It is possible therefore that all carry mutations in a single pathway for the production of an anti-fungal metabolite. Alternatively, each mutation could be in catabolic or assimilatory pathways for nutrients essential for hyphal extension of *Pythium*. Thus, compounds that were made unavailable by the wild type remain free in the media in the presence of the mutants.

2.4.2. Evaluation of *in planta* disease suppression assays

Any system for laboratory analysis of microbial interactions will, by design, have a reduced number of variables compared with the field situation. Each possible variable must be considered carefully when designing experiments to ensure that, whilst data interpretation is simplified, the information obtained can be used to predict results in the field. The composition of the indigenous microbial community and the interactions with the BCA will also contribute towards variation in the results between sites (Hagedorn, Gould & Bardinelli, 1993). Alternatively, the physiochemical characteristics of the soil and plant exudates may

play an important role in the production of anti-fungal metabolites by BCAs (Stephens, Crowley & O'Connell, 1993) and the pathogenicity of the fungal component.

Therefore, one of the major considerations will be the choice of plant growth medium. A commercially available, dried, sieved, sterilized loam was selected for these assays. It proved to be homogeneous and consistent in terms of water content, particle size and the relative absence of microflora. Sterilisation, whilst not allowing a true reflection of field conditions (Voisard *et al.*, 1994), would serve to remove variations caused by the presence of other organisms. Such soil-based compost is commonly used for the growth of glasshouse crops, one of the areas where *Pythium* damping-off and other diseases is a major economic problem (Paternotte & de Kreij, 1993; Harris *et al.*, 1994).

Another issue that requires careful attention is the method of addition of pathogen to the soil. It has been demonstrated that hyphae, oospores and sporangia show differing levels of pathogenicity (Murray, 1994) and that the method of production influences the response to germination stimuli (Nelson & Craft, 1989). The use of naturally infested soil would be the most relevant procedure, but due to the inherent variation in *Pythium* densities and virulence from environmental samples, it is not a practical approach. The addition of *Pythium* from a stock with known population densities was considered to be the best option and *in vivo* produced oospores were shown to be more pathogenic (Murray, 1994).

The method of applying the antagonists to the system also needs to be standardized. Biological control efficacy of *Pseudomonas* spp. was shown to be affected by the application method (Fridlender, Inbar & Chet, 1993; Hagedorn, Gould & Bardinelli, 1993) and survival in soil was affected by the physiological status of the bacteria upon addition (Vandenhove *et al.*, 1991). Whilst some researchers have found that *Pseudomonas fluorescens* populations in soil declined at a lower rate and stabilized at higher densities when introduced as a late exponential phase culture (Vandenhove *et al.*, 1991), the bacteria used for these assays were from stationary phase cultures. All strains were grown in LB and therefore were not iron starved upon their addition to soil, implying that if siderophores are one of the biocontrol mechanisms used by these bacteria, they are produced *in situ*. All bacterial cells were washed before use in any of the assays. All nutrient sources and extracellular metabolites had therefore been removed. This implied that any extracellular antifungal compounds must have been exported *in situ* and thus the bacteria were to some extent active in soil.

The disease suppression assays were set up to be optimal for *Pythium* induced damping-off. Use of a homogeneous batch of pathogen to mix into sterile soil provided a high

degree of reproducibility. Levels of pathogen used were far greater than those normally encountered in the field (approximately 10^2 propagules g^{-1} soil (Mazzola & Cook, 1991)), but the addition of bacteria in the numbers used here (approximately 10^8 cfu g^{-1} soil) was capable of controlling disease. Whilst good levels of disease control were achieved, it must be remembered that the soil had been sterilized and therefore the system had a low microbial diversity. Under these conditions biocontrol is more likely to be successful due to reduced competition from indigenous microbes (Marois & Coleman, 1995) but it is similar to the conditions experienced for glasshouse-grown crops.

The correction factor for the level of control (Equation 2-1), allowed direct comparisons to be made between different assays and assays run at different times. This was because both the underlying disease present in the soil and the variation in the virulence of the pathogen were taken into consideration.

2.4.3. Factors effecting disease suppression

The first assay was designed to test the most effective means of applying the bacterial inocula to the system. The most practical methods of applying bacteria inocula in the field are by seed coating or spraying directly onto the soil. *P. fluorescens* 54/96 was most effective when applied directly to the soil (Figure 2-6). This implied that pre-emptive colonization of the seed is not the most important factor for disease control but that initial interactions in the soil played a key role in the suppression.

The prevention of oospore germination was probably the key to the success of the soil drench. Other studies have noted that soil applications are more effective than seed applications for the control of damping-off, partly because greater numbers of bacteria can be added (Hagedorn, Gould & Bardinelli, 1993). This factor may play an important role in the different levels of control achieved here. If the fungi were allowed to germinate, hyphal growth towards the seed was rapid (Hendrix & Campbell, 1983) and so the pathogen was well established by the time the biocontrol agent and fungi were in close enough proximity for interactions between the two organisms to play a significant role.

The overall assumption of the importance of the soil interactions was further confirmed by the results of the second assay (Figure 2-7). It appeared that the incidence of disease may be reduced by increasing the interaction time between the pathogen and biocontrol agent before the addition of the *Pythium* germination stimulant (pea seeds). However, in order to prove this, many more replicates would need to be studied. If it did prove to be the case

this would imply that the biocontrol agent actively reduced the density of the virulent pathogen in the soil and did not just prevent its infection of the germinating seed.

As expected, the incidence of disease was dependant on the number of pathogen propagules in the soil (Figure 2-9). However, the actual number required for significant disease occurrence was relatively low, but comparable to the numbers found in natural soil (approximately 300-400 propagules g^{-1} soil (Mazzola & Cook, 1991)). This demonstrated the aggressive nature of *Pythium* infection.

It appeared that at least 1000 pseudomonad cells for every *Pythium* propagule were required for effective biocontrol of the disease in soil. Given that there may be two possible mechanisms for biocontrol: competition for resources and antibiosis, large numbers of bacteria will be required to either (i) assimilate sufficient resources to inhibit fungal growth or (ii) produce enough antibiotic to raise its concentration to that which is deleterious to the pathogen. However, these mechanisms may only operate when there are large numbers of bacteria present, probably making the biocontrol activity a density dependant phenomenon. Biocontrol activity of other *Pseudomonas* BCAs has been observed to be affected by inoculum density either in a linear fashion or by the requirement for a minimum density (Kloepper, 1991). However, after 7 days the pseudomonad populations in the soil had fallen to approximately 10^6 cfu g^{-1} regardless of the initial inoculum level. This implied that there was a rapid die-off and perhaps the high initial inoculum was required to compensate for this.

Whilst ANOVA demonstrated that there are no significant differences (P=0.05) between the control indices of 54/96 or SBW25 from the different assays in which they were run, it is obvious that both isolates controlled *Pythium* infection less effectively in the assay illustrated in Figure 2-6. The average control indices (*CI*) achieved by both isolates in this assay were both less than 0.7 but in subsequent assays the *CI*s were greater than 0.85. This was probably due to the fact that the oospore inoculum was fresh and therefore at its most virulent for the first assay. Thereafter, storage of the inoculum reduced virulence to a lower but stable level. Variation within treatments of individual experiments was always small, but greater variation was seen between treatments replicated on different occasions. However, homogeneity of variance tests showed that comparisons between treatments were statistically reliable.

2.4.4. Conclusions

The anti-fungal activity of BCAs is often multi-factorial (Cook *et al.*, 1995), with no individual mechanism accounting for all disease suppression observed (Kraus & Loper, 1992). This appears to be the case for *P. fluorescens* 54/96, illustrated by the differing activities of the Tn*5* mutants. At first sight the results from the comparison of the mutants appeared to give some rather contradictory results. However, all three mutants showed different phenotypes and therefore the transposons were probably inserted into the genome at distinct loci. All three mutants showed some residual activity against hyphal growth but this was probably due to competition in the nutrient-poor media. The fact that all were significantly less effective implied that all three mutants had lost functions essential for inhibition of *Pythium* vegetative growth. Mutant 54/96::Tn1569 retained the ability to prevent oospore germination in the plate assay, whilst 54/96::Tn1882 and 54/96::Tn3296 showed a reduction in this capacity. However, despite the reduction in both functions, 54/96::Tn3296 showed no loss of biocontrol activity in the seed germination assay, whereas 54/96::Tn1569 and 54/96::Tn1882 did. These observations indicated that there were at least three different antifungal functions contributing to the biocontrol activity of 54/96.

Confirmation of the biocontrol activity of SBW25 was also provided by these assays. The systems developed here may therefore prove to be useful for screening fluorescent pseudomonads for the potential to control fungal phytopathogens. These assays also determined that SBW25 and 54/96 controlled *Pythium* by different mechanisms. SBW25 performed no better than the Tn*5* mutants of 54/96 in the reduction of hyphal growth and therefore was deficient in this mechanism. However, it performed as well as 54/96 in the disease suppression assays and prevented oospore germination, even in the presence of sucrose.

The results of the assays presented here indicate that simple manipulation of application techniques can be used to enhance the anti-fungal activity of BCAs. Whilst few of the assays appear to show any significant differences it is clear that the method of application is vital to biocontrol efficacy. Other trends are also apparent; incubation of BCA with the pathogen in soil prior to the addition of the oospore germination stimulus may improve the observed control, as does the addition of a large excess of BCA to the system. It may be the case that the bacteria become better adapted to soil conditions with time after their growth in artifical media. This implies that the most effective control will be achieved when application of the bacterial inoculum to soil occurs several days prior to planting - a variation in application method that has not previously been reported in the literature.

By using the simple assays as described here, it has been possible to develop hypotheses to explain the nature of the interaction between bacterial biocontrol agents and soil-borne fungal pathogens. Addition of bacteria to *Pythium* induced fungistasis in spores and a reduction of the hyphal growth rate. However, without molecular analysis of transposon mutants deficient in antifungal activity, it can not be confirmed whether the effect is due to resource competition, production of antifungal metabolites or a combination of factors. These methods have also been utilized, with great success, in the following chapters to demonstrate the activity of a variety of pseudomonads.

However, any one assay, used alone, would be of limited use for assessing the efficacy of biocontrol agents. When used together they provide an in-depth picture of the level of control achievable, and the nature of the interaction between the BCA and the fungal pathogen. Such an insight will be invaluable for determining the mode or modes of action of the BCA and can be used to determine the effects of mutations.

Table 2-1: Inhibition of Pythium oospore germination by fluorescent pseudomonads and factors which affect this process. These results were recorded 10 days after setting up the assays. Each symbol indicates the results from 1 replicated assay.

10 days	Additional factors			
Bacterial inoculum	None	1mM $FeIIICl_3$	1mM $CaCl_2$	6mM Sucrose
None	+ + + +[a]	- - - -[b]	+ + + +	+ + + +
54/96	- - - -	- - - -	± ± ± ±[c]	+ + + +
54/96::Tn1569	- - - -	+ - - -	± ± ± ±	± ± ± ±
54/96::Tn1882	± ± ± ±	- - - -	+ + + +	+ + + +
54/96::Tn3296	± ± ± -	- - - -	± ± ± ±	+ + + +
SBW25	- - - -	- - - -	+ + + +	± - - -

[a] Indicates prolific hyphal growth.
[b] Indicates that oospores showed no signs of germination.
[c] Indicates that whilst some oospores had germinated hyphal growth was very poor.

Table 2-2: Summary of the efficacy of 54/96 and three transposon insertion mutants in each of the assays described in sections 2.2.4 (p1) and 2.2.5 (p1) of this chapter.

Isolate	Seed germination	Hyphal growth	Oospore germination
54/96	++ [a]	++	++
54/96::Tn1569	+ [b]	+	++
54/96::Tn1882	- [c]	+	± [d]
54/96::Tn3296	++	+	±

[a] Strain shows maximum antifungal acivity in assay compared to wild type.
[b] Strain shows reduced antifungal acivity in assay compared to wild type.
[c] Strain shows greatly reduced antifungal acivity in assay compared to wild type.
[d] Strain shows no antifungal acivity in assay.

Figure 2-1: Schematic diagram illustrating the design of the disease suppression assay.

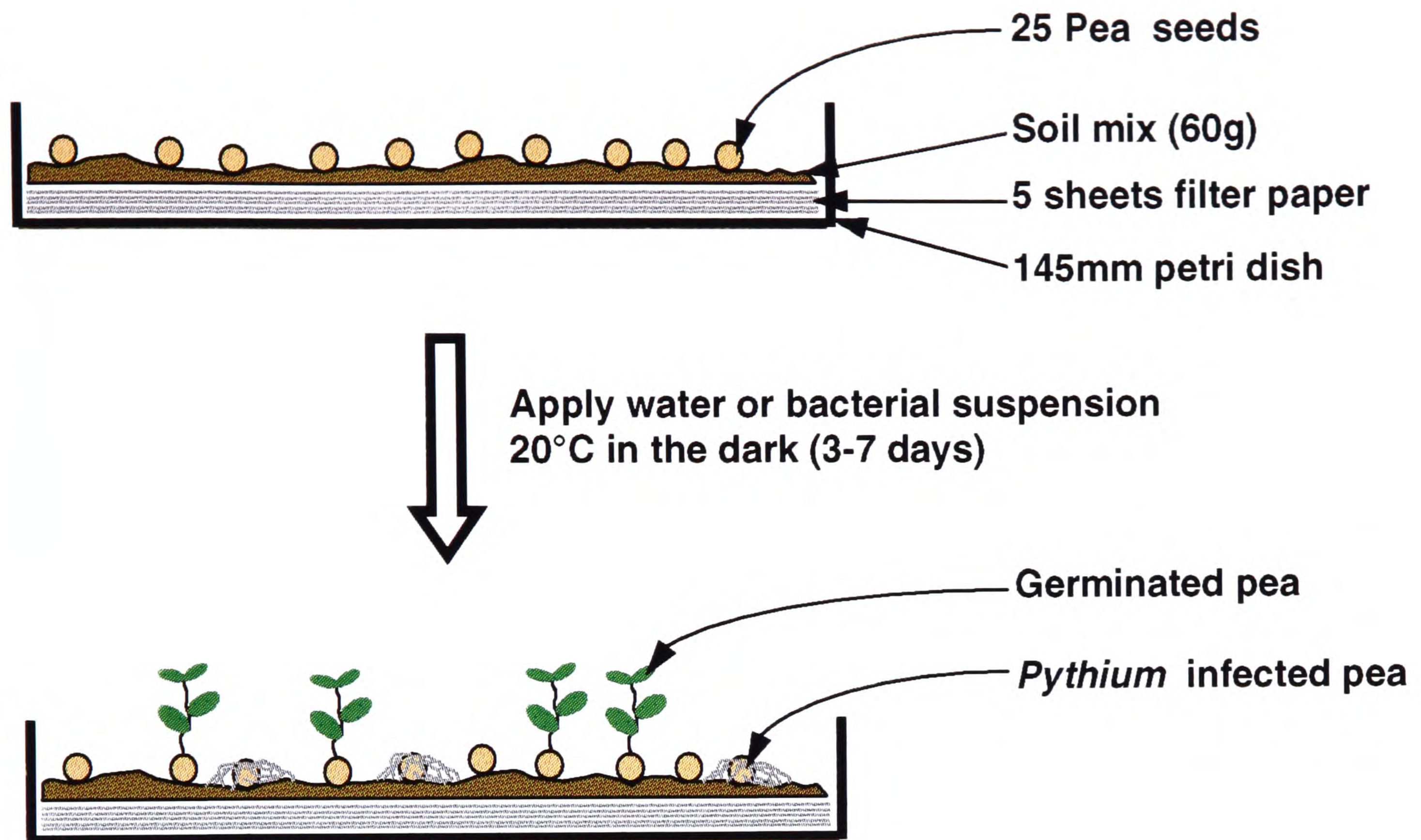


Figure 2-2: Development of a *Pythium* colony from a single propagule within 5mm of a germinating pea seed. The pictures were taken at 2, 4 and 6 hours after placing the pea (seen as a large dark object to the left of each picture) on the surface of the agar

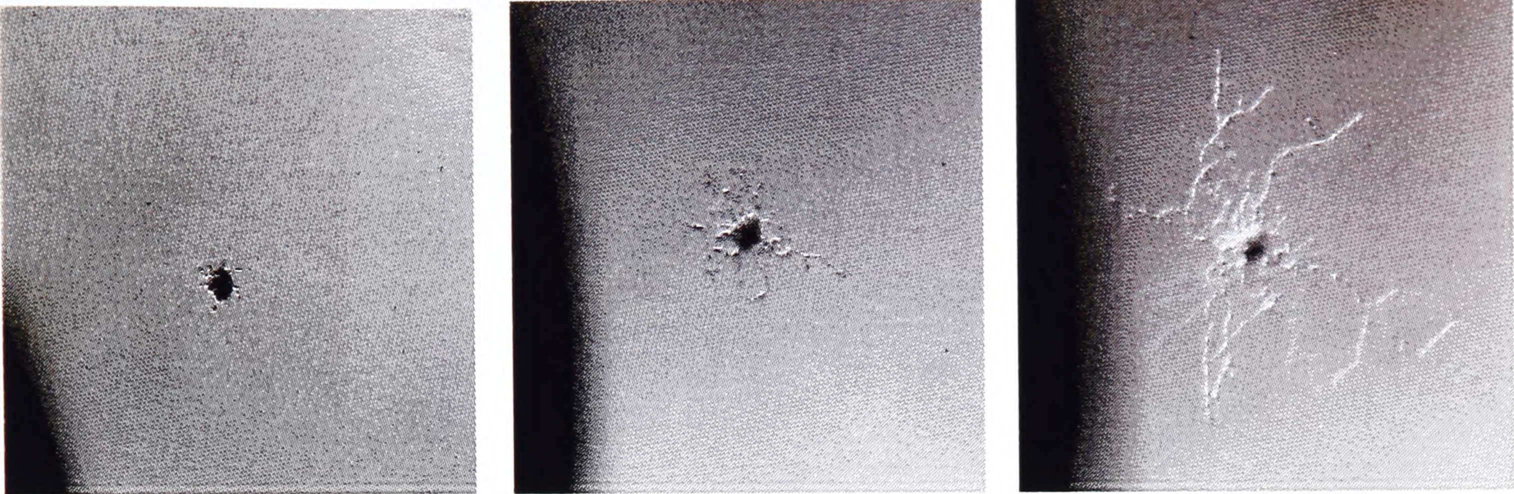


Figure 2-3: Prolific colonization and infection of a pea root tip. Hyphae can be seen as fine fibres and the shiny spherical objects are the *Pythium* fruiting bodies, sporangia.

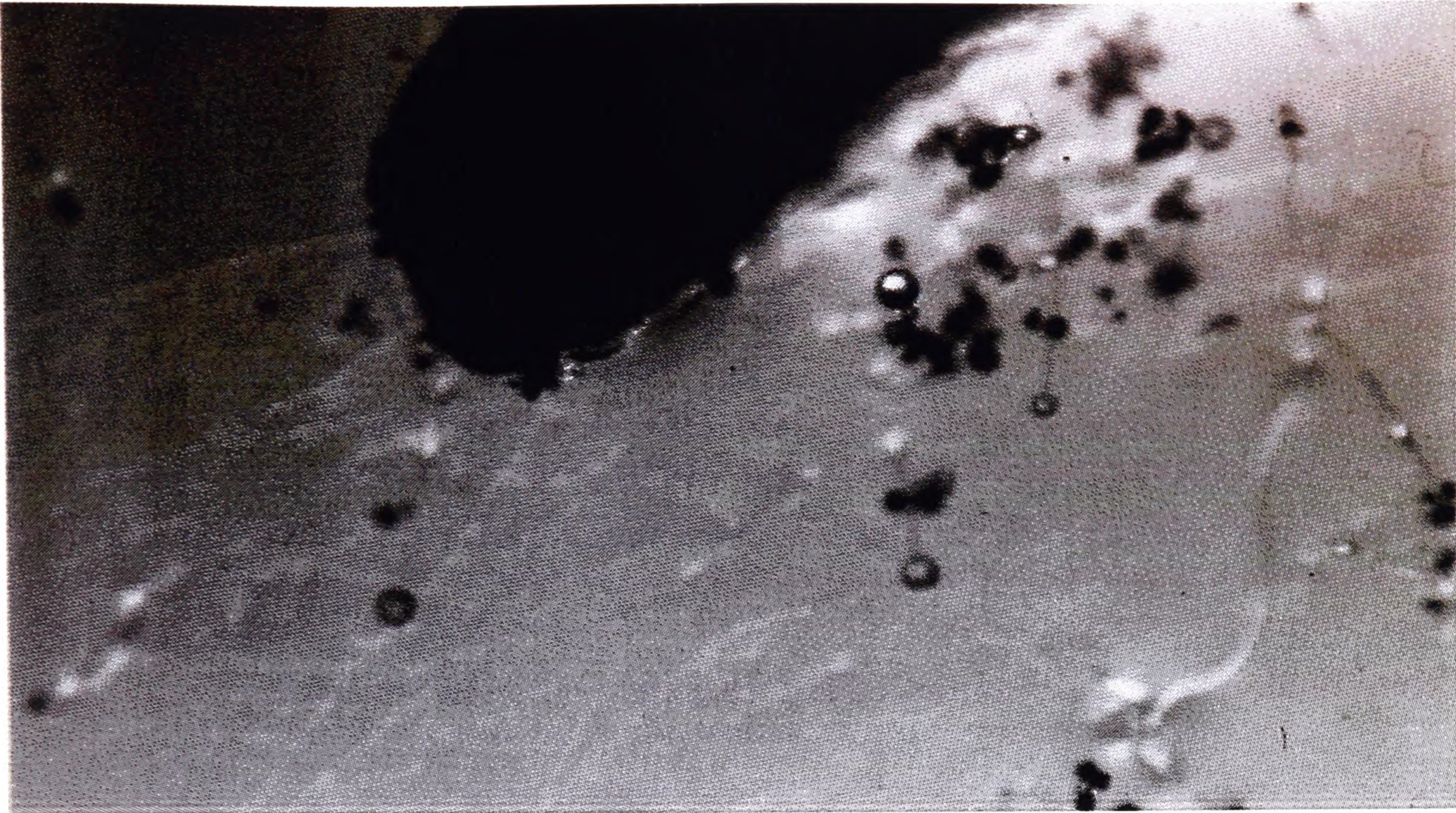
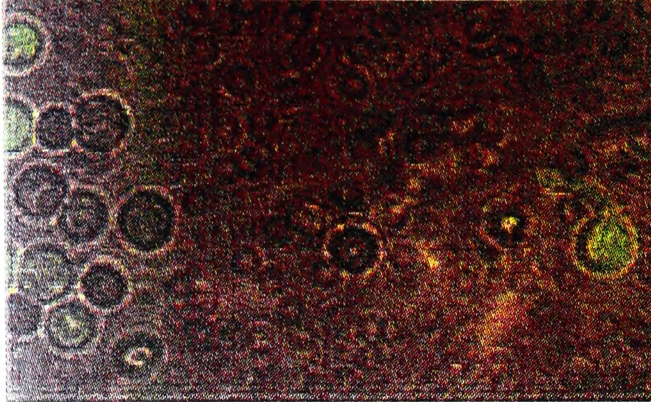


Figure 2-4: *Pythium* oospores stained with fluorescein diacetate and observed under UV illumination at x500 magnification. The image on the left illustrates germination oospores in pea seed exudate. The image on the right illutrates spores in pea seed exudate in the presence of 54/96.



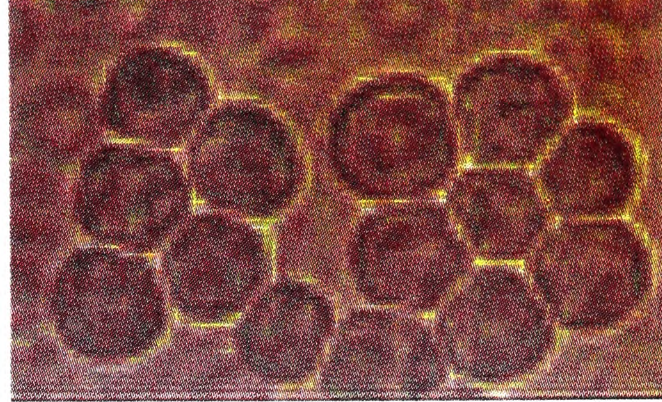


Figure 2-5: Effect of the addition of bacteria to the growth of *Pythium* hyphal mass in pea seed exudate liquid culture. Colony diameters were measured after 4 days incubation. Labels are as follows: 1569 = 54/96::Tn1569; 1882 = 54/96::Tn1882; 3296 = 54/96::Tn3296; Control = *Pythium* grown in the absence of bacteria; MSD = minimum significant difference

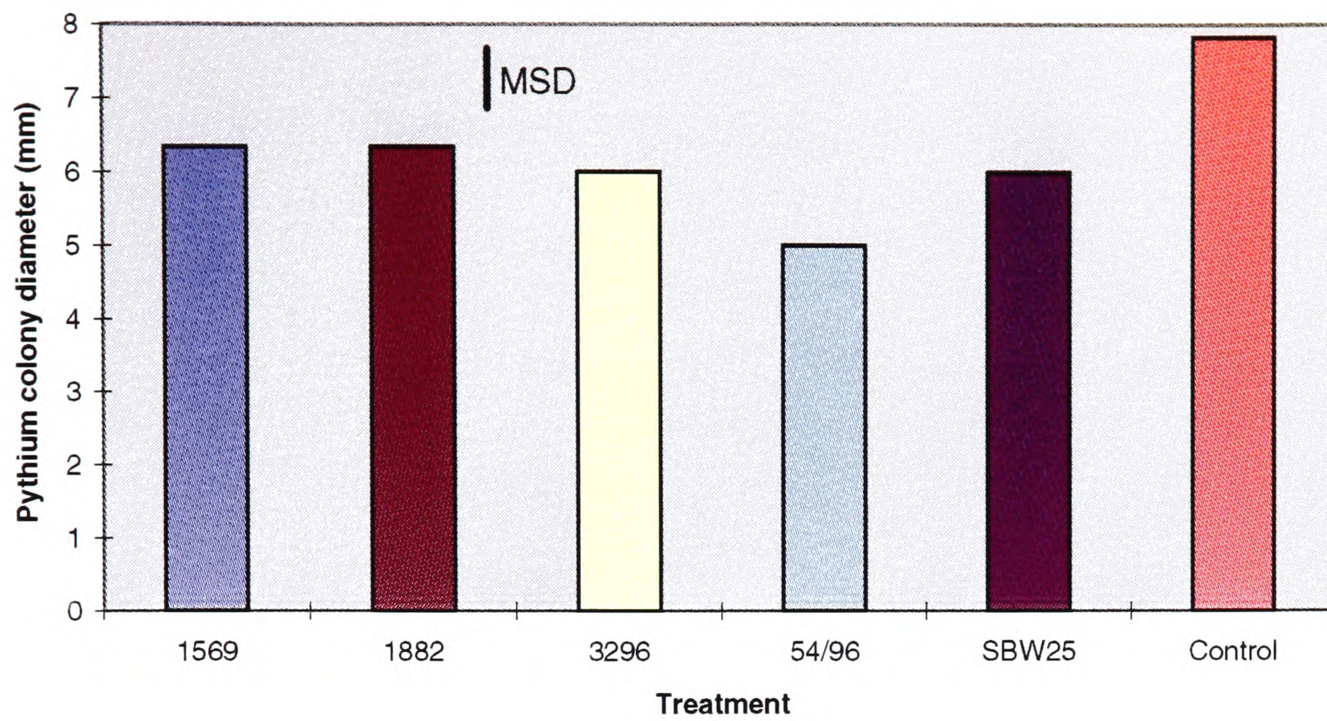


Figure 2-6: Effect of application method on the relative biocontrol efficacy of *Pseudomonas fluorescens* strains 54/96 and SBW25. Control Indices were calculated from numbers of infected seeds relative to the two controls. 30 ml of washed overnight cultures were applied for soil drench. Seeds were soaked for 10 minutes in 30 ml overnight culture. Key: infected = addition of *Pythium* but no bacterial inoculum; control = no bacteria or *Pythium* applied. The bar indicates Minimum Significant Difference.

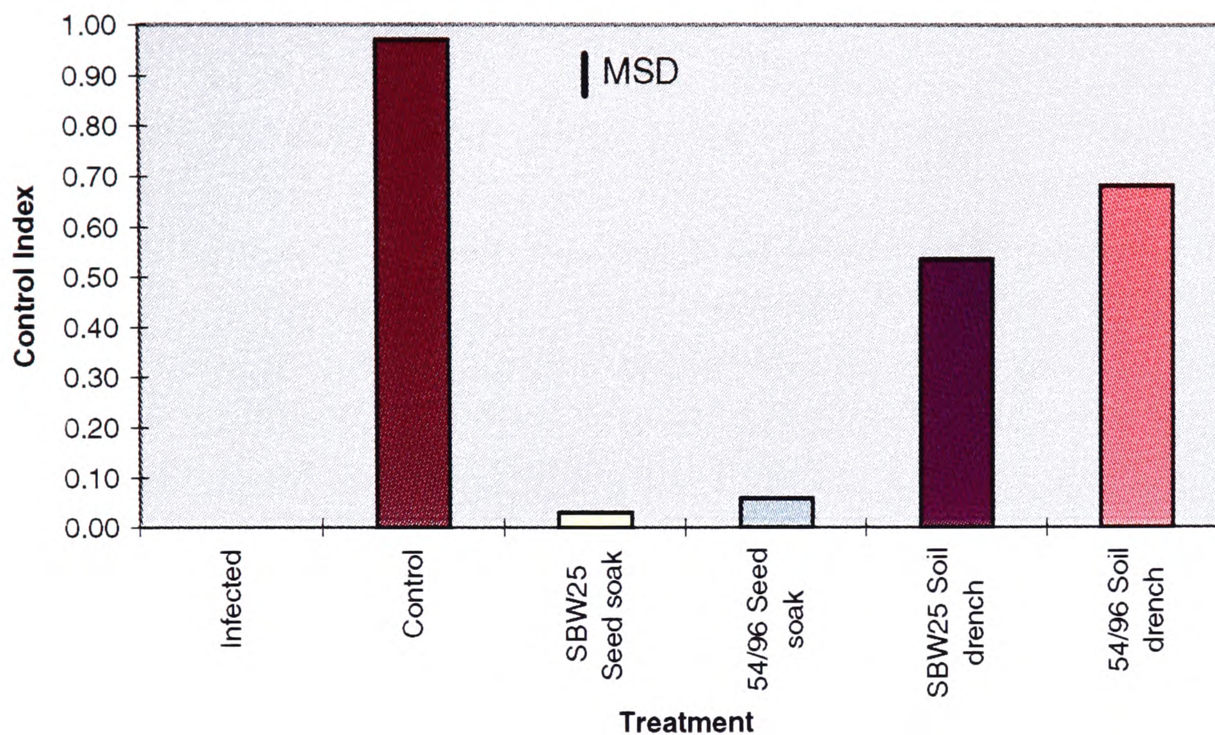


Figure 2-7: Effect of early application of *Pseudomonas fluorescens* 54/96 on the level of disease observed. Control Indices were calculated from numbers of infected seeds relative to the two controls. 30 ml of washed overnight cultures were applied for soil drench. Key: infected = addition of *Pythium* but no bacterial inoculum; control = no bacteria or *Pythium* applied. The bar indicates Minimum Significant Difference.

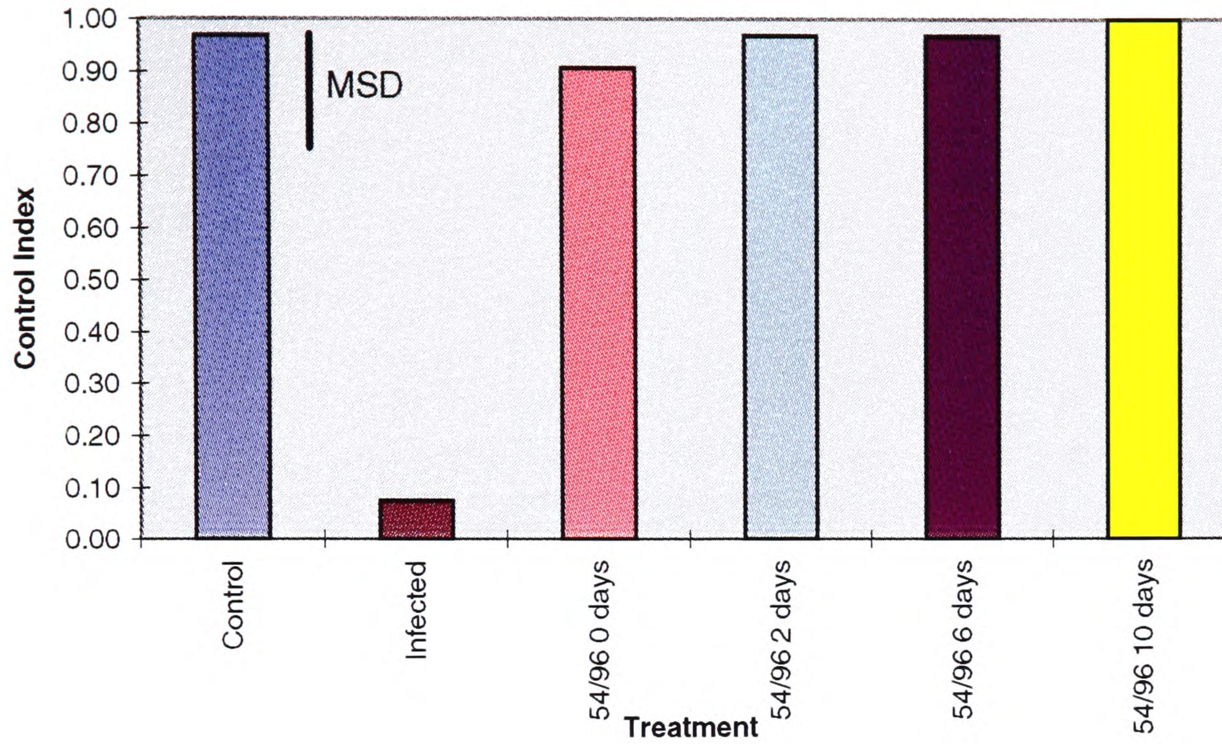


Figure 2-8: Relative efficacy of three Tn*5* insertion mutants and the parental strain of *Pseudomonas fluorescens* 54/96. Control Indices were calculated from numbers of infected seeds relative to the two controls. 30 ml of washed overnight cultures were applied for soil drench. Key: infected = addition of *Pythium* but no bacterial inoculum; control = no bacteria or *Pythium* applied. The bar indicates Minimum Significant Difference.

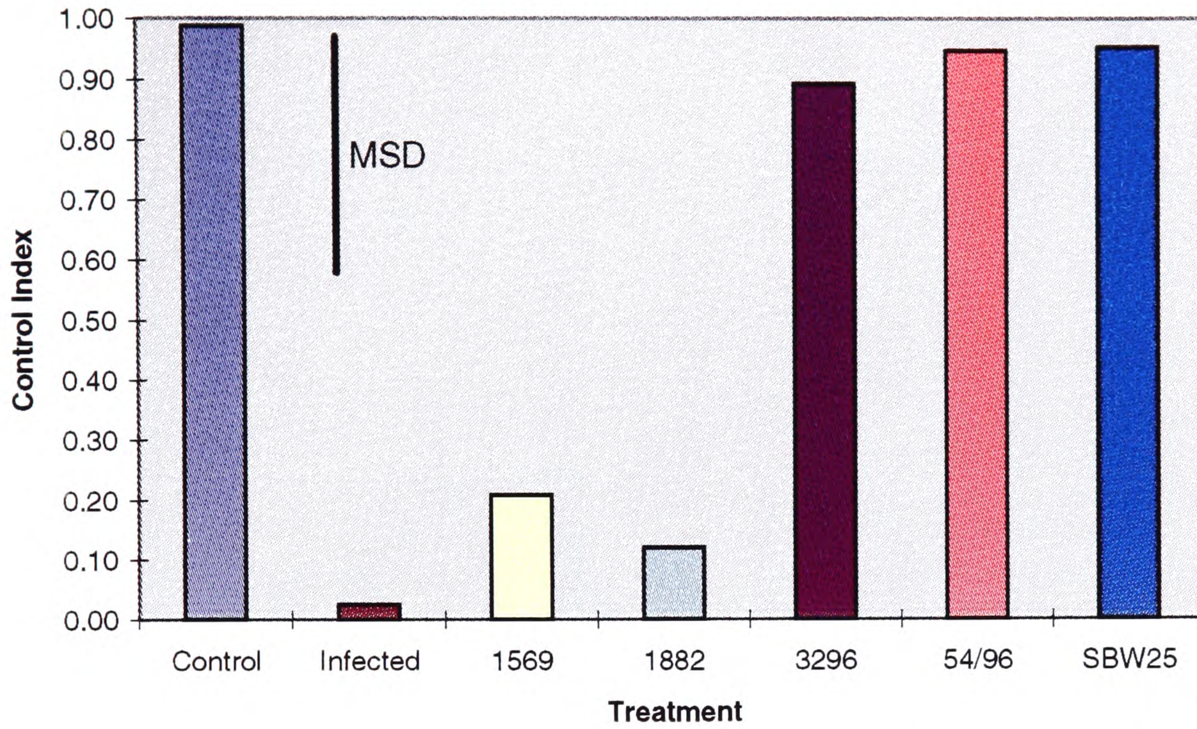
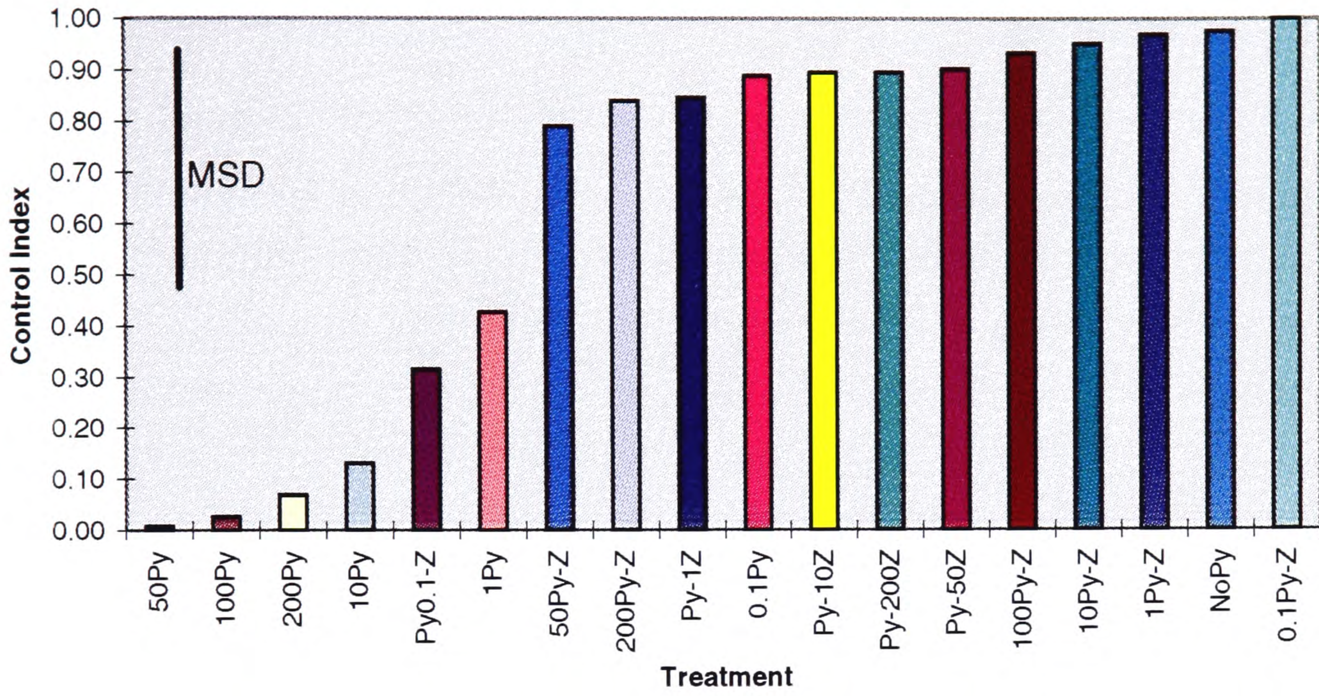


Figure 2-9: Effect of different relative doses of *Pythium* and *Pseudomonas fluorescens* 54/96 on the incidence of disease. Labels are coded as follows: The number preceding Py indicates the percentage of the normal *Pythium* inoculum density used (no number indicates normal) and the number preceding Z indicates the percentage of the usual inoculum density of 54/96 used (no number indicates normal). Control Indices were calculated from numbers of infected seeds relative to the two controls. The bar indicates Minimum Significant Difference



3. PSEUDOMONAD POPULATION DYNAMICS DURING DISEASE SUPPRESSION

3.1. Introduction

The genetic basis for the antagonism between pseudomonads and phytopathogens has been studied extensively (Pierson III & Pierson, 1996) but little work has focused on the ecology of the interactions. As the main drawback of this type of biocontrol has been the inconsistent results observed it seems imperative that the dynamics of pathogen and BCA populations need to be studied in greater depth. Biocontrol in macroorganism systems has been known to fail because of a lack of knowledge of the basic biology of the interacting organisms (Marois & Coleman, 1995). Understanding of how ecological concepts can be applied to microbiology may prove useful in the development of effective biocontrol strategies (Marois & Coleman, 1995). In microbial BCA applications, studies such as these have identified that disease suppression is dose dependant for some strains whereas others must be applied above a threshold level before any effects are observed (Osburn *et al.*, 1989; Harris *et al.*, 1994).

Whilst the perceived wisdom is that, in order to be an effective biocontrol agent, strains must be able to competitively colonize plant tissue (Harman & Lumsden, 1990; Bull, Weller & Thomashow, 1991; Cassinelli, Noris & Tolentino, 1993; Carroll *et al.*, 1995; Marois & Coleman, 1995), this seems to contradict evidence that the majority of anti-fungal compounds are secondary metabolites (Leisinger & Margraff, 1979), which by definition are produced whilst the bacteria are in the stationary phase (Sacherer, Defago & Haas, 1994). Although higher bacterial population sizes in wheat rhizosphere have been correlated with higher antibiotic concentrations (Thomashow *et al.*, 1990), it has not been determined whether phenazine is produced during the active growth phase, but its production is reduced when nutrients are plentiful (Mazzola *et al.*, 1992). Recently, it has been shown that phenazine production is under the control of a density dependant response regulator in some strains (Pierson III & Pierson, 1996), implying that cells must achieve a threshold density before phenazine production commences. However, some anti-fungal compounds are thought to be the by-product of catabolism. For example, ammonia is produced by actively growing *Enterobacter clocae* by deamination of plant derived amino acids and is inhibitory towards *Pythium* (Howell, Beier & Stipanovic, 1988). An in-depth study of the

comparative population dynamics of BCA and pathogen should go some way to resolving this inconsistency.

Recent studies have shown that biocontrol strains may exhibit low physiological activity in the rhizosphere (Marschner & Crowley, 1996; Marschner, Crowley & Higashi, 1997). Data from the quantification of root exudation (Lynch & Whipps, 1990) and microscopic examination of roots (Foster, 1986) indicate that this is highly likely. Therefore, it may be assumed that pseudomonads may be nutrient limited on roots thus exhibiting stationary phase responses . Logically, activity would be even lower than this in bulk soil due to the lower nutrient concentrations but may be increased in the spermosphere above the levels seen in the rhizosphere due to higher nutrient concentrations.

It is the apparent requirement that a bioprotectant against soil-borne phytopathogens should be rhizosphere competent that has meant that the search for BCAs has focused on the genus *Pseudomonas* (Kim, Weller & Cook, 1997). The degree of rhizosphere competence required will, of course, depend on the pathogen that is to be controlled and the mechanisms by which it causes disease. For example, wheat roots are vulnerable to infection by *Gaeumannomyces graminis* var. *tritici*, which causes take-all disease, throughout the growing season (Weller, 1984). Therefore the best adapted BCA would be capable of proliferation on wheat roots from the time of sowing until harvest. The identification of soil colonization as an important factor has only received limited promotion (Hagedorn, Gould & Bardinelli, 1993). It must be remembered that damping-off disease develops very soon after the introduction of seeds to infested soil (Osburn *et al.*, 1989) and therefore population dynamics should be studied during this period. For example, the timing of antibiotic production has been implicated in the efficacy of the suppression of *Pythium*-induced pre-emergence damping-off by *P. fluorescens* Pf-5 (Sarniguet *et al.*, 1995). It was suggested that control was poor because the level of antibiotic production was insufficient in the first three days after seed inoculation (Sarniguet *et al.*, 1995).

The spatial positioning of the bacteria may also play an important role in the control of disease. For example, Weller (Weller, 1984) states that bacteria which colonize the region of the root close to the tip would be more effective against take-all than those which only colonize a few centimetres of the root. In the case of damping-off, which occurs prior to germination, interactions in the soil may be more essential to disease control than those in the spermosphere. However, some workers studying the control of damping-off focus their attentions on seed colonization and neglect interactions in the soil (Fukui *et al.*, 1994b) and this is an area that needs to be addressed.

The aims of the work in this chapter are therefore to study both the spatial and temporal aspects of the population dynamics of both pathogens and their antagonists with a view to ascertaining the site and timing of the interaction between the two organisms that leads to the suppression of disease.

3.2. Materials and Methods

3.2.1. Set up of assays

Assays were set up as described in 2.2.5 (p27). The strains used were 54/96, and SBW25. Each bacterial suspension was applied to uninfested or *Pythium*-infested soil in triplicate. Untreated controls with and without *Pythium* were also included. Twenty-five pea seeds (*Psium satvium* var. Bohartyr) were placed on the surface of the soil immediately after the application of the bacterial suspensions.

Two separate experiments were run, one to determine the population dynamics of BCA and pathogen on seeds, and the other to investigate interaction in the soil. Only the interactions with 54/96 were studied in the seed experiment, whereas both strains were used in the soil experiment. In all cases seeds were added to the system.

3.2.2. Sampling procedure

Single seeds were taken at random (irrespective of their level of infection) from each of the triplicate assays 0, 3, 5, and 7 d after planting and the microbial communities were extracted as follows. Individual seeds were placed in 30 ml glass Universal containers with sterile 10 ml quarter strength Ringer solution (QSR, Oxoid, UK) with 1 g sharp sand. This was shaken for 10 min with a Griffin wrist action shaker to remove cells from the spermosphere (and rhizosphere of older seedlings). This suspension was then subjected to 10-fold serial dilution. Aliquots (100 μl) were spread onto Potato Dextrose Agar supplemented with 320 mg l^{-1} aureomycin (Cyanamid, UK; PDAA; (Thompson *et al.*, 1993b)) and Pseudomonas agar base supplemented with 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin and 50 mg l^{-1} cephaloridine (PSA-CFC) plates for enumeration of *Pythium* propagules and colony forming units (cfu) of pseudomonads respectively. These plates were incubated at 20 °C for 48 h before counting.

Soil samples were taken at predetermined times (0, 6, 12, 24, 48, 72 and 168 hours) after planting seeds. Soil (1 g) was taken from each plate (3 bacterial treatments x 2 fungal treatments x 3 replicates = 18 samples) and added to 10 ml QSR. This was shaken for 10

min with a Griffin wrist action shaker to produce a homogeneous suspension. This mix was then treated as described above for seed sample suspensions.

3.2.3. Data analysis and statistics

All data were recorded on Microsoft® Excel and analysed using the Unistat® statistical package (Unistat Ltd., London). All count data (bacteria and fungi) were Log_{10} transformed prior to analysis and checked for homogeneity of variance using Bartlett's Chi-square test, Bartlett-Box F test, Cochran's C and Hartley's F statistics (Unistat® 4, Unistat Ltd., London). The data were then subjected to analysis of variance (ANOVA) to identify significant differences between treatments by the calculation of minimum significant differences (MSD, (Fry, 1993)) at P=0.05.

The number of generations per unit time (k) of each of the bacterial strains was calculated from the increase in numbers isolated within the first 6 h of their addition to soil or for the first three days of seed colonization using Equation 3-1.

Equation 3-1

$$k = \frac{(\log_2 N_t - \log_2 N_0)}{t},$$

where N_t is the population size at time t and N_0 is the population size at time zero. The mean doubling time is $1/k$. Doubling times were calculated for each replicate and differences between treatments were assessed using ANOVA and MSD.

3.3. Results

A low degree of variation was observed between replicates in the pseudomonad count data obtained. However, counts of *Pythium* propagules were more variable. Nonetheless, the two populations affected each other both on seeds and in the soil.

3.3.1. Microbial population dynamics on the seed

3.3.1.1. *Pythium*

The assay was designed to determine the interaction between the population sizes of the pathogen and the biocontrol agent on the seed and their relationship to the incidence of disease. Figure 3-1 illustrates that the rate and level of colonization of seeds by the

pathogen was not affected by the presence of the biocontrol agent. No significant differences between the numbers of *Pythium* isolated from seeds were detected, either in the presence or absence of the biocontrol agent, possible due to the high degree of variation seen between replicates. Numbers of *Pythium* isolated from the seed increased throughout the period of the study in both the presence and absence of the 54/96. However, in treatments where 54/96 was not applied, there was extensive hyphal growth on the surface of the seeds and in the surrounding soil. This was not observed in assays treated with 54/96.

3.3.1.2. Pseudomonads

The apparent mean doubling time for 54/96 colonising the pea seeds was approximately 3.6 h averaged over the first three days after planting and from triplicate measurements. The numbers of pseudomonads colonizing the seeds were dependant to some extent on the presence of the pathogen (Figure 3-2). Seeds were colonized to the same population density of pseudomonads when soil was inoculated with 54/96, regardless of the presence of *Pythium*. Yet, in uninoculated soil the presence of *Pythium* appeared to enhance the colonization of seeds by the indigenous pseudomonads. In contrast, pseudomonad colonization in the absence of both a bacterial and a fungal inoculum was poor.

3.3.2. Microbial population dynamics in the soil

3.3.2.1. *Pythium*

The numbers of *Pythium* isolated from soil remained at log 3.5 - 4 propagules g^{-1} for the duration of the 168 h study (Figure 3-3). Neither 54/96 nor SBW25 had any significant effect on the numbers of viable propagules.

3.3.2.2. Pseudomonads

Figure 3-4 and Figure 3-5 illustrate the numbers of pseudomonads isolated from soil after inoculation with 54/96 and SBW25 respectively. In both cases the bacteria appeared to multiply immediately after introduction to the soil. However, in two of the four conditions examined, population densities began falling between 6 and 12 hours after planting. For this reason mean generation times were calculated from data for the initial six hours of the experiment. The mean doubling times over the first 6 hours calculated for individual replicates varied between 3.5 and 63.4 h. Means, calculated from three replicates per

treatment, varied between 9.4 and 23.9 h but differences between the two strains were not significant due to the large variation observed.

It was observed that the basic trends were preserved for both bacterial strains (Figure 3-4 and Figure 3-5). In the absence of the *Pythium* inocula the populations of 54/96 and SBW25 initially increased in the first 6 h, following which they either stabilized (54/96, Figure 3-4) or increased at a reduced rate (SBW25, Figure 3-5) until 48 h after inoculation when populations declined steadily until the termination of the experiment (6.5-7 log units at 168 h). However, the presence of *Pythium* altered this pattern for both strains. That is, both bacteria populations responded in the same way to the presence of fungal propagules in the soil. Both 54/96 and SBW25 achieved higher densitiesafter 24 hours in soil when co-inoculated with *Pythium*. This was due to the fact that the initial increase continued for 12 h rather than 6 h as seen in the absence of the pathogen. However, 12 h after their addition, the populations of 54/96 and SBW25 decline rapidly. In the absence of *Pythium* this decline was delayed for a further 36 hours. There was no difference between the final densities reached by either strain in the presence or absence of *Pythium*.

3.4. Discussion

3.4.1. Interactions on the seed

In similar studies, one particular *Pseudomonas fluorescens* strain had a 5.2 h generation time on roots at 25 °C (Suslow & Schroth, 1982) and the population size of a strain identified as *P. fluorescens-putida* ML5 doubled in 3 h on sugar beet seeds (Osburn *et al.*, 1989) at 16 °C. This value was found to be dependant on the initial inoculum density; population increases were more rapid from smaller inocula (Fukui *et al.*, 1994b). A similar figure of 3.6 h was observed here for the colonization of pea seeds by 54/96 at 20 °C. However, unlike previous studies (Fukui *et al.*, 1994b), it was not possible to determine if the bacteria had gone through a lag phase before the resumption of exponential growth. If so, the generation times calculated here would be greater than the actual value. Therefore it can be concluded that 54/96 is an relatively efficient colonizer of the pea spermosphere.

Despite the variability which appeared to be inherent in the fungal count data (Figure 3-1 and Figure 3-3), important observations were made about the population dynamics of the pathogen. Colonization of seeds by *Pythium* did not appear to be affected by the biological control agents and the numbers that could be isolated increased exponentially, indicating that colonisation was an active process. This contrasts with previous studies (Osburn *et al.*,

1989) where the numbers of fungal propagules were reduced in the presence of BCA. However, there were several differences between the two studies. Different plants and bacterial strains were used but, perhaps most importantly, the methods for quantifying *Pythium* were different. In this study, pea seeds were vigorously abraded and the washings plated onto PDAA to generate a count of any fungal propagules adhering to the seed. Osburn *et al.* (Osburn *et al.*, 1989) surfaced sterilized sugar beet seeds and placed them directly onto water agar which enumerated those fungi which had successfully penetrated the seed coat. No distinction was made in the study presented here as to the ability of the fungi to infect seeds but the reduced incidence of disease in the bacterial treatments implied that many lost the ability to infect.

The variability seen in the fungal count data highlights the limitations of using plate counts for enumeration of fungi. A more effective means of ascertaining the effect of bacteria on fungi may be to use estimations of fungal biomass, such as determining ergosterol concentrations. These methods appear to show less variation than plate count procedures. The rate of hyphal growth could be calculated and therefore its impedance by the bacterial inocula could be quantified.

The apparently active colonization of seeds by *Pythium* poses a difficult question. The lack of visible hyphal growth around seeds in the presence of 54/96 implied that the fungi were inhibited. However, this does not explain how dormant oospores move to the seed from the soil without the discernible production of hyphae. Although *Pythium ultimum*, by definition, does not produce zoospores in culture (Webster, 1970), it is possible that they might be produced in soil which would account for the chemotactic movement towards the source of seed exudates. This would imply that sporangia as well as oospores are present in the infested soil. Alternatively, and probably more likely, an increase in the number of propagules that can be isolated may be attributed to the thin- / thick-walled duality of *Pythium* oospores (Lumsden & Ayres, 1975; Johnson & Arroyo, 1983; Johnson, 1988). Thick-walled oospores will only germinate after their conversion to the thin-walled form, a process that could take up to ten weeks in soil (Lumsden & Ayres, 1975). The increase in apparent numbers recorded *in vitro* on PDAA might therefore be attributed to an increase in the proportion of thin-walled spores but not in the overall number of oospores present.

Pea seeds were colonised by pseudomonads in the presence of *Pythium* even when no bacterial inocula had been applied. This demonstrated that the oospore-infested soil used as the inoculum contained pseudomonads that proliferated when the inoculum was applied. Their source may have either been the commercially prepared 'sterilised' soil itself where,

after a few weeks incubation indigenous strains were revived, or derived from the first batch of pea seeds grown to produce the oospore inoculum.

However, the presence of *Pythium* had no significant effect on the colonization of seeds by the introduced pseudomonads. Analysis of colony morphologies revealed that the only pseudomonads detected on the seed were derived from the inoculum. This implied that the indigenous pseudomonads were out-competed by 54/96.

3.4.2. Interactions in soil

The discovery that *Pythium* can be readily isolated on agar plates from soil incubated with high doses of BCA bacteria has lead to the hypothesis that the strains investigated in this study do not destroy the fungi, but simply retard growth or infection processes. By effectively removing the bacteria by plating onto media containing aureomycin, the fungal spores and hyphae are free from the constraint that the bacteria impose and grow rapidly. This is in agreement with the observations of others where some plant growth-promoting rhizobacteria (PGPR) bacteria have been described as biostatic as opposed to biocidal towards a number of plant pathogens (Suslow & Schroth, 1982). This assumption about the mode of action of 54/96 is supported by the observations of vital stained oospores (Chapter 2), which revealed that although essential metabolic activity was arrested by the presence of bacteria, there was no evidence of any physical damage. Therefore the effects would probably be reversible.

A possible impact of *Pythium* on the *Pseudomonas* sp. population densities in soil was observed soon after planting. The implication that the two populations interact at an early stage provides insight into the time frame in which the antagonistic effects leading to disease suppression were prevalent. The fact that the pseudomonads are naturally suppressed after an initial population increase in the presence of *Pythium* in soil allows speculation that the production of secondary metabolites may have been induced during this period.

The increase of *Pseudomonas* population densities in soil inoculated with fungi compared to non-fungal inoculated soils has been observed previously (Nemec, 1997). However, in contrast with those findings, other workers (Mazzola & Cook, 1991) have noted that the presence of *Pythium* spp. can inhibit root colonization by fluorescent pseudomonads. The nature of these interactions was strain specific (Mazzola & Cook, 1991). That is, colonization by different bacterial strains could be either enhanced or suppressed by the

same fungal species, and different fungal species had a variety of effects on the same bacterial strain.

For both bacterial strains examined here, the initial pseudomonad numbers in soil were higher, but not significantly so, in the presence of *Pythium*. One relevant observation made was that *Pythium* inoculated soil appeared to be more 'wettable' than the standard sterile soil, that is the bacterial suspensions were soaked up more quickly in fungi-infested soil. This indicates that the presence of fungi may have altered the physiochemical characteristics of the soil. This in turn may have an effect on the survival profiles of bacterial inocula.

3.4.3. Conclusions

As *Pythium* rapidly germinates in response to stimuli from seeds, any biological control agent must act rapidly following addition to soil. The ability to grow (replicate) is therefore of little importance but the mechanism by which the control is facilitated must be active or activated upon addition to soil. For control of seed and seedling diseases, such as damping-off, the bacteria should ideally be added in high numbers to overcome any threshold concentrations (Kloepper, 1991) and be in a state to respond to the fungal germination. The bacteria used in these experiments were prepared from stationary phase cultures and were added at relatively high doses compared to the studies of others. The washed preparations were made to deliberately lack nutrients and other factors at the time of inoculation, which indicated that the anti-fungal activity must be induced *in-situ*.

Whilst the pseudomonad biocontrol agents used do actively colonize germinating seeds, it appears that this was not a prerequisite for the control of damping-off disease. It would be logical to assume that the presence of the bacterium would reduce the colonization of the seeds by the fungi, as has been observed previously (Fukui *et al.*, 1994a, b) but *Pythium* propagules were isolated from pea seeds in equal numbers, irrespective of the presence of the bacteria. Conversely, bacterial colonization of seeds was unaffected by the presence of *Pythium*, indicating that there was little direct interaction between the two organisms on the seed surface. Whilst it has been stated that the ideal method for application of damping-off BCAs is directly onto the seeds (Parke, 1990), it would appear that the most important interactions between *Pythium* and pseudomonads are in the soil. However, this may be strain specific as it will probably depend on the mode of action of the BCA. For instance, if the bacterium acts by preventing spore germination it will be best placed in soil, whereas if it acts by preventing the infection process it would be best placed on the surface of the seed. It has been shown that the most effective method for the application of both 54/96

and SBW25 is by soil drenching as opposed to seed application (2.3.5.1, p31), the implication being that the most important activity of these strains in terms of biological control activity is their prevention of spore germination.

This study has revealed that the analysis of short-term population dynamics of introduced inocula can provide great insight into the activity and interactions which occur. In previous studies of the fate of bacterial inocula, samples were taken at greater time intervals (Doyle *et al.*, 1991; Doyle & Stotzky, 1993; Araujo *et al.*, 1994; van Veen, van Overbeek & van Elsas, 1997), and hence the immediate impact was missed. The rapid turnover of microbial cells will mean that the soil community should rapidly stabilize at an equilibrium after perturbation. Therefore it would appear that the changes in population densities will be most dramatic in the first few hours after the addition of the inocula. Thus the transient effects caused by the introduction of bacteria (Doyle & Stotzky, 1993) may be an artefact of the sampling strategy and the fact that the time scales used are not ecologically significant. It has been suggested that antibiotic production in the spermosphere and rhizosphere is at best transitory (Georgakopoulos *et al.*, 1994). The investigation of short term population dynamics of 54/96 and SBW25 supports this observation.

The effects of *Pythium* on pseudomonad numbers, although relatively short lived, were considered important as they ultimately led to the suppression of disease. The investigation of short term population dynamics described here has identified the time-frame whereby the interaction between fungi and bacteria results in the bacteria preventing the fungal infection of germinating seeds.

Figure 3-1: Numbers of *Pythium* propagules isolated from pea seeds after inoculation of the soil with *Pythium* oospores and bacterial suspensions. Each data point is the mean of three replicates. Key: Control = no bacterial of fungal inocula applied; *Pythium* = *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting; 54/96 = 54/96 inocula applied to soil at 10^8 cfu g^{-1} prior to planting; 54/96 + *Pythium* = 54/96 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting.

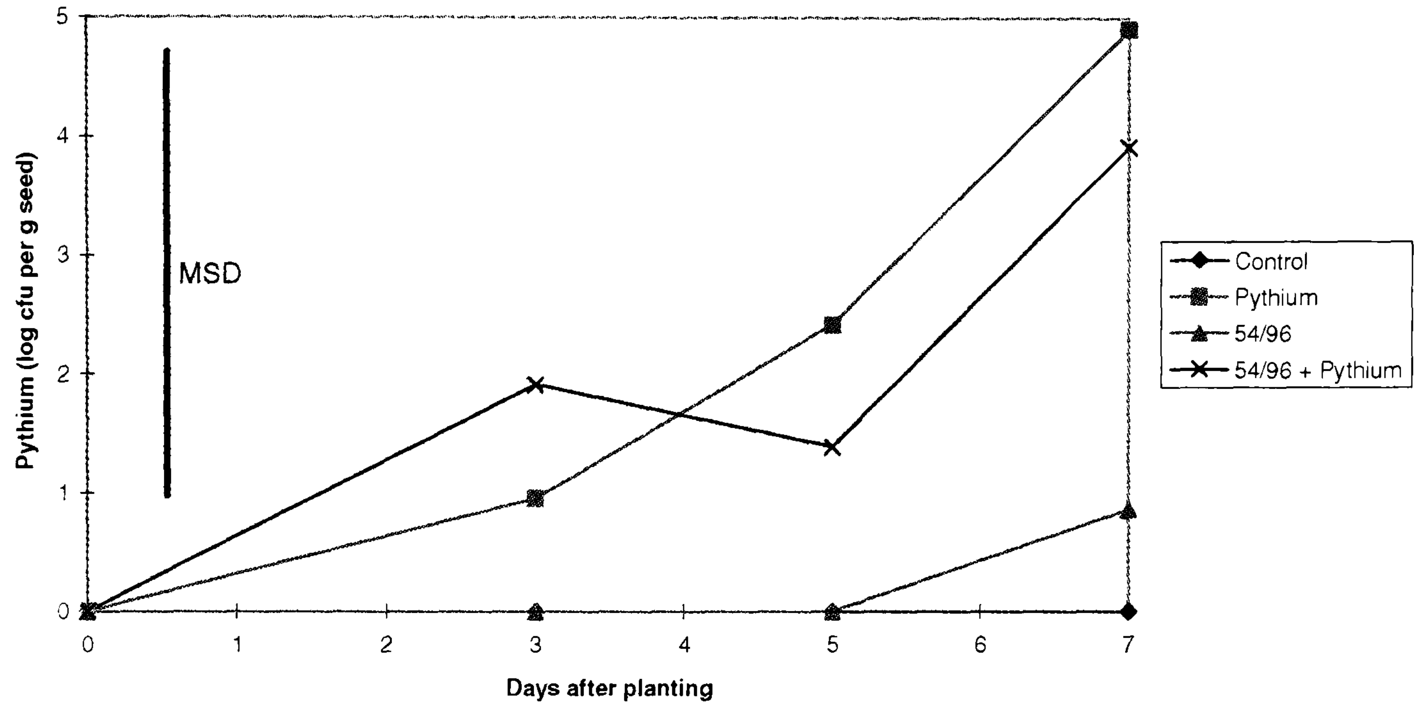


Figure 3-2: Numbers of *Pseudomonas* isolated from pea seeds after inoculation of the soil with *Pythium* oospores and bacterial suspensions. Each data point is the mean of three replicates. Key: Control = no bacterial of fungal inocula applied; *Pythium* = *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting; 54/96 = 54/96 inocula applied to soil at 10^8 cfu g^{-1} prior to planting: 54/96 + *Pythium* = 54/96 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-} prior to planting.

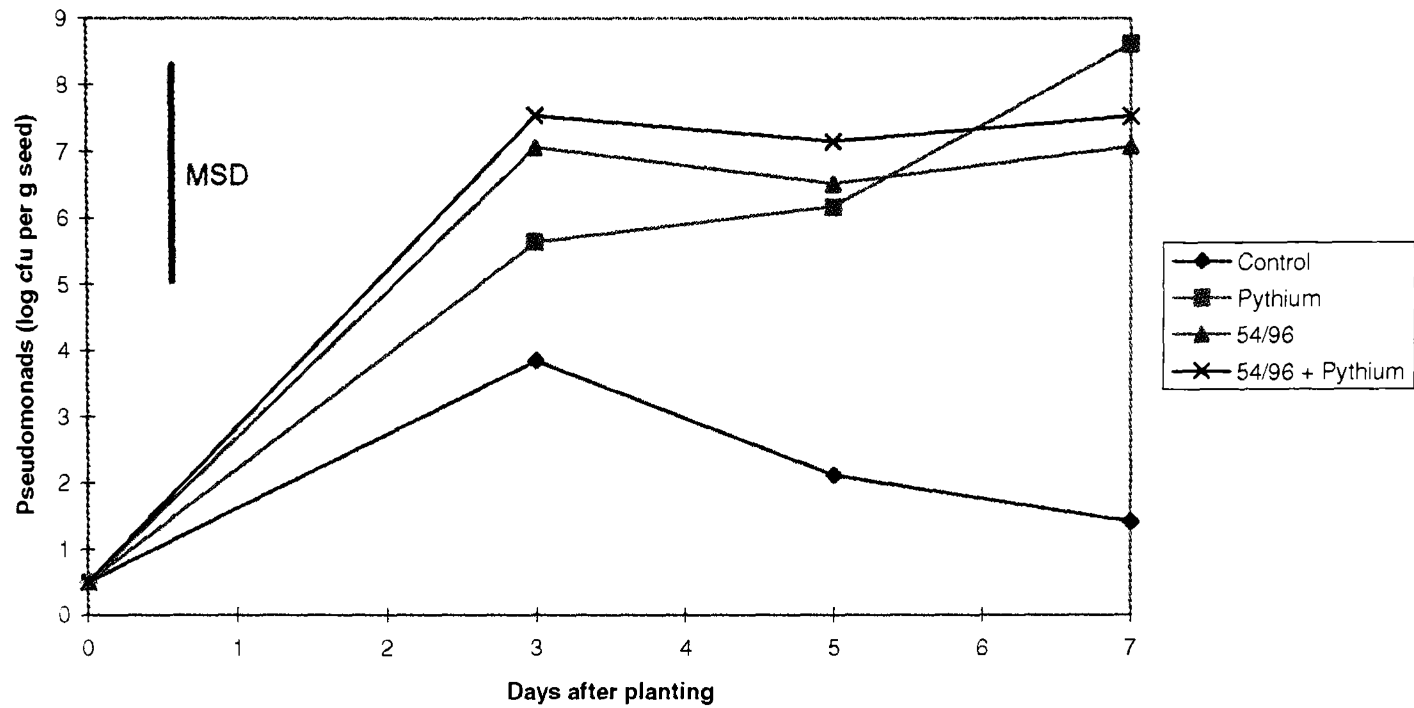


Figure 3-3: Numbers of *Pythium* propagules isolated from soil after inoculation with *Pythium* oospores and bacterial suspensions. Each data point is the mean of three replicates. Key; *Pythium* = *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting; 54/96 + *Pythium* = 54/96 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting; SBW25 + *Pythium* = SBW25 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting.

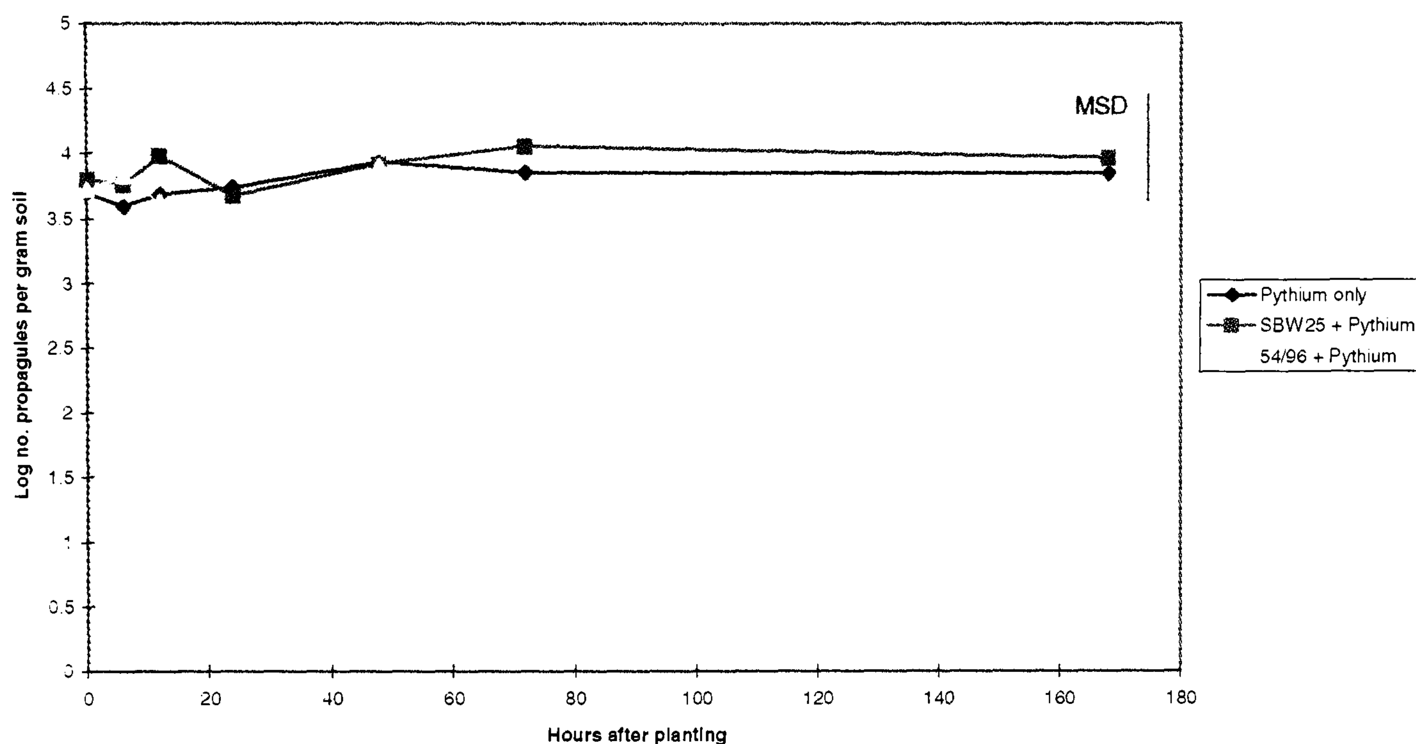


Figure 3-4: Comparison of numbers of *Pseudomonas fluorescens* 54/96 isolated from soil after inoculation either with or without *Pythium* oospores. Each data point is the mean of three replicates. Key; 54/96 = 54/96 applied to soil at 10^8 cfu g^{-1} prior to planting; 54/96 + *Pythium* = 54/96 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting.

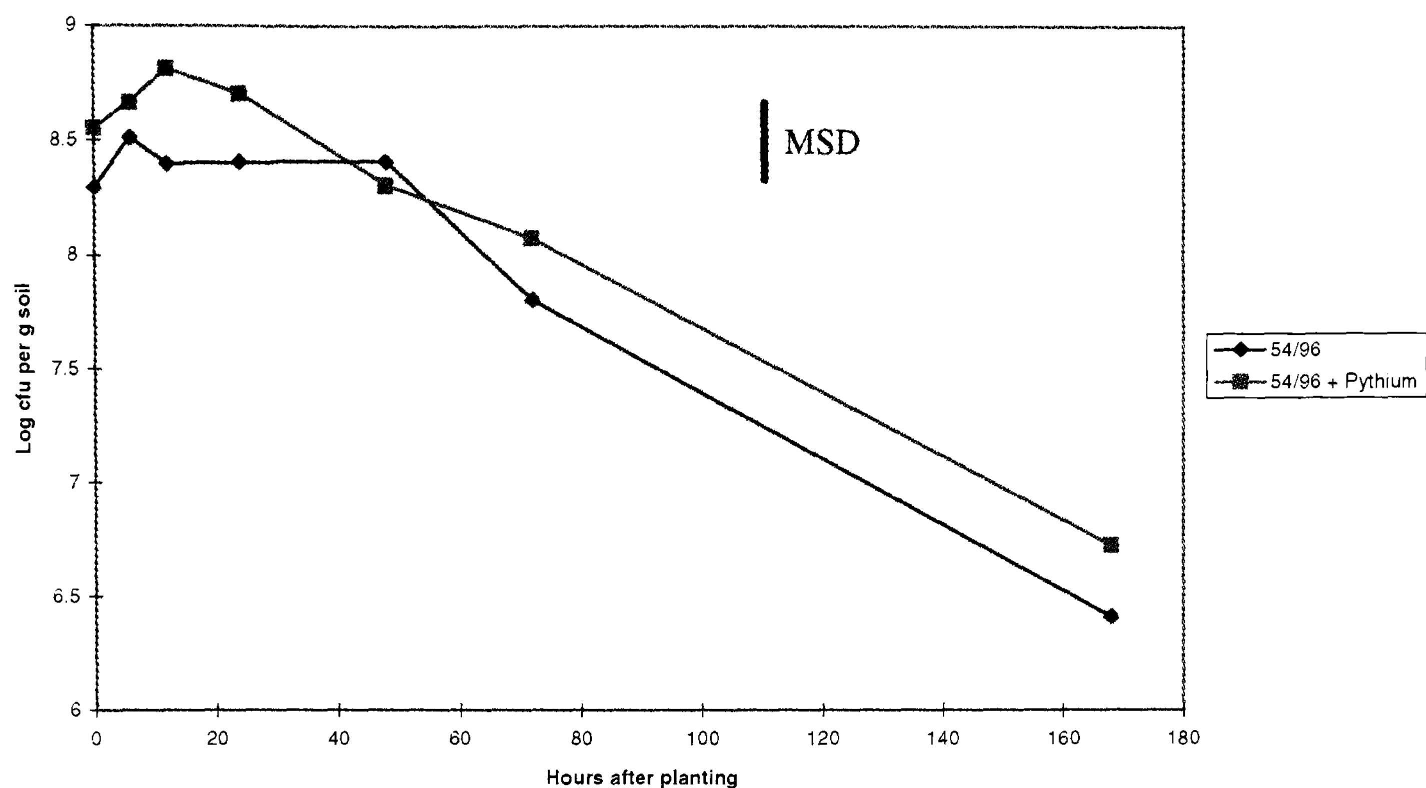
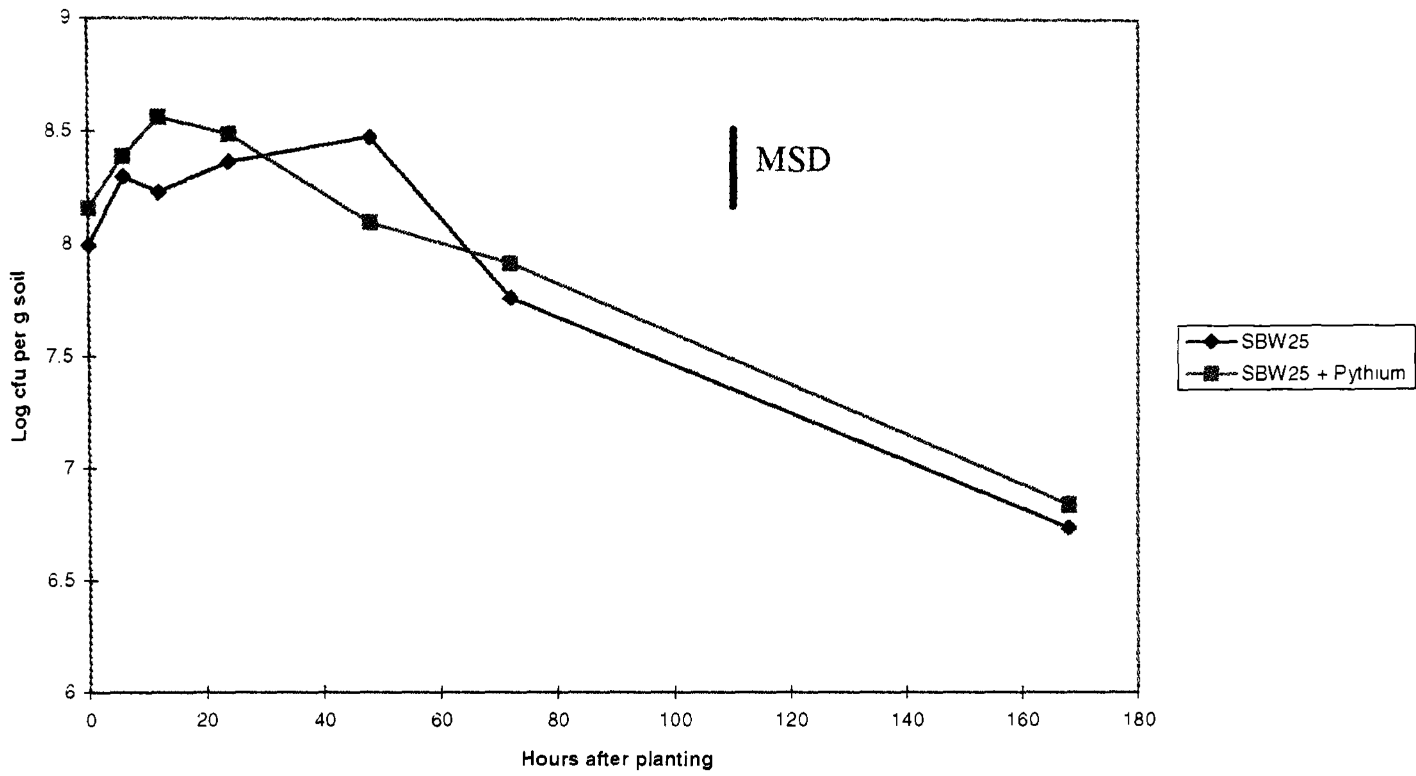


Figure 3-5: Comparison of numbers of *Pseudomonas fluorescens* SBW25 isolated from soil after inoculation either with or without *Pythium* oospores. Each data point is the mean of three replicates. Key;SBW25 = *SBW25* applied to soil at 10^8 cfu g^{-1} prior to planting; SBW25 + *Pythium* = SBW25 inocula applied to soil at 10^8 cfu g^{-1} and *Pythium* applied to soil at 10^4 propagules g^{-1} prior to planting



4. MOLECULAR CHARACTERIZATION OF THE BIOLOGICAL CONTROL ACTIVITY OF *PSEUDOMONAS FLUORESCENS* SBW25

4.1. Introduction

Many fluorescent pseudomonads have been identified as putative candidates for the biological control of fungal phytopathogens (Chapter 5). The majority of these have been isolated randomly from soil samples by screening for anti-fungal activity *in situ* or in microcosms. However the major limit to the commercial development of bacterial control agents is their inconsistent performance in the natural environment (Weller, 1988). One of the main causes of this appears to be the unpredictable survival and activity of the introduced organisms (van Veen, van Overbeek & van Elsas, 1997).

The organism described here, *Pseudomonas fluorescens* SBW25 was isolated from the phytosphere of sugar beet (Bailey *et al.*, 1995) and was selected for chromosomal marking in order to track the fate of a genetically modified microorganism in the environment. Therefore, the autecology of a genetically modified derivative, SBW25EeZY6KX, has been thoroughly investigated in wheat and sugar beet crops both in glasshouse (Ellis, Thompson & Bailey, 1995; Thompson, Ellis & Bailey, 1995) and field (De Leij *et al.*, 1995; Thompson *et al.*, 1995b; Bailey *et al.*, 1997) situations. In the controlled environment of the glasshouse the GMM consistently attained levels of at least 60% of the total culturable bacteria in some plant tissues (Thompson, Ellis & Bailey, 1995). However, when released into the field situation colonization was more variable both between individual plants and between different growing seasons (Thompson *et al.*, 1995b). It was shown that the organism actively grew and colonized emerging plant tissue (Thompson *et al.*, 1995b), and that it persisted in plant-associated habitats in the field for at least 250 days after inoculation (De Leij *et al.*, 1995; Thompson *et al.*, 1995b). It has been noted that this organism can also survive in fallow soil for at least 100 days, but its recoverability is adversely effected by desiccation (Ellis, unpublished observations).

In addition to an extensive knowledge of the ecology of this organism, a great deal of work has concentrated on its taxonomic position (Bailey *et al.*, 1997), chemotaxonomy (Thompson *et al.*, 1993a), phytopathogenicity (Bailey *et al.*, 1997), outer membrane

proteins (de Mot *et al.*, 1994), genomic organization (Rainey & Bailey, 1996) genetic stability in the environment (Denning *et al.*, 1997), plasmid biology (Lilley & Bailey, 1997a, b), phenotypic and genotypic plasticity (Morell, 1997) and identification of factors that affect its ability to colonize plant tissue (Rainey, 1997; Turnbull *et al.*, 1997). To this end it is a well characterized organism and thus any further studies will serve to enhance this position and build a clearer picture of plant-associated pseudomonads.

As its autecology has been studied under a variety of conditions, use of SBW25 as a biocontrol agent should be more predictable than other fluorescent pseudomonads, for which little is known about their population dynamics in the natural environment (Bailey *et al.*, 1997). The well-documented persistence of SBW25 in both rhizosphere and fallow soil, will also facilitate its use against soil-borne phytopathogens. Indeed, it has been shown to colonize plant tissue as well as the proven biocontrol strain, *P. fluorescens* F113 (Lugtenberg *et al.*, 1997) It has been speculated that pseudomonad populations consist of a multitude of locally adapted genotypes (Bailey *et al.*, 1997). However, as SBW25 is detectable under most conditions it is probably more versatile than other genotypes and therefore it should perform well in environmental field release. Intolerance of environmental fluctuations has also been quoted as a reason for the inconsistence of biocontrol applications (Duffy, Simon & Weller, 1986), but SBW25 has shown that it is capable of survival throughout the year in the extremes of British weather, thus providing further weight to the justification for its use here. As bacteria will not perform a function when it compromises their ecological fitness (de Lorenzo, 1994) it is important that strains with proven fitness are used for environmental release.

SBW25 was identified as a putative BCA during routine screening of a number of other fluorescent pseudomonads (see chapters 2 and 5 for details). However, further analysis revealed that it did not produce any of the characterized anti-fungal metabolites such as phenazines, phloroglucinols, or hydrogen cyanide (Table 5-4, p111). It has been shown in this thesis that cyanide production appears to bear the greatest influence on antifungal activity in a significant proportion of pseudomonads (5.3.6, p102). The other key factor, a relatively high proportional content of cyclopropane fatty acid, was also absent. However, SBW25 did fall within a genetic cluster that protected pea seedlings from *Pythium* and produced a diffusable antifungal agent on agar (5.3.3, p100).

It was therefore decided to ascertain the novel mode of action of this organism. In order to determine whether this strain could be used as a biological control strain an understanding

of the genetic basis of the antifungal activity is required. Whilst the potential has been noted, until its genetic basis is proven its use is speculative.

Transposon mutagenesis is now a routine procedure for use in many Gram-negative bacteria (de Lorenzo & Timmis, 1994), including *Pseudomonas*, and therefore has been used to identify the genetic basis for many phenotypic traits in this genus, such as antibiosis (Carruthers, Conner & Mahanty, 1994; Rajendran *et al.*, 1994; Kraus & Loper, 1995; Whatling *et al.*, 1995), adhesion (DeFlaun *et al.*, 1994) and motility (Winstanley *et al.*, 1994). With the development of the 'mini-transposons', resultant genomic insertions have become more stable and the cloning and identification of genetic loci and affected genes more reliable (de Lorenzo *et al.*, 1990; Herrero, de Lorenzo & Timmis, 1990; de Lorenzo, 1994; de Lorenzo & Timmis, 1994). This is because the gene encoding the transposase function is situated outside the transposon itself (that is, it acts *in trans*) and is not transmitted to the recipient genome (de Lorenzo & Timmis, 1994). This means that once inserted the transposon cannot be excised and inserted elsewhere in the genome. This factor, combined with the existence of restriction maps of these transposons (de Lorenzo *et al.*, 1990), prompted the use of the mini-Tn*5 xylE* system in this study. This variant carries a constituitively expressed kanamycin resistance gene for the isolation of transformants on selective media and a promoterless *xylE* gene, which can function as a promoter probe (de Lorenzo, 1994; de Lorenzo & Timmis, 1994). When inserted into the correct orientation in an actively expressed gene, the transposon mutant will exhibit catechol 2,3-dioxygenase activity. This means that as well as the isolation of genes inactivated by insertional mutation, this system can be used to identify promoters and genes associated with the expression of desirable traits in the environment (de Lorenzo, 1994). For example, genes expressed constituitively, on artifical media or under environmental conditions can be differentiated.

4.2. Materials and Methods

4.2.1. Bacteria, fungi and plants

Table 4-1 lists bacteria, fungi, plants and plasmids used in this study. *E. coli* strains were grown routinely in Luria-Bertani (LB) broth. *Pseudomonas* isolates were cultured on Pseudomonas Agar Base supplemented with 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin, and 50 mg l^{-1} cephaloridine (PSA-CFC; Unipath, UK). Fungal isolates were grown on Potato Dextrose Agar (PDA; Unipath, UK) with 320 mg l^{-1} aureomycin (Cyanamid, UK). Other

antibiotics were used, when appropriate, at the following concentrations (mg l^{-1}): ampicillin (A, 50), kanamycin (K, 50), tetracycline (T, 10).

4.2.2. Transposon mutagenesis

In order to introduce the transposon into the pseudomonad it was mated with an *E. coli* strain bearing a plasmid carrying the transposon (de Lorenzo & Timmis, 1994). Overnight cultures of *P. fluorescens* SBW25 and *E. coli* S17/1 λpir (pUTmini-Tn*5xylE*) (de Lorenzo & Timmis, 1994) were grown overnight in 10 ml LB and LB-AK respectively. Cells were washed three times in LB and resuspended in 10 ml LB. Each culture was then incubated at room temperature for 30 min. Three hundred microlitres of S17/1 λpir (pUTmini-Tn*5xylE*) culture was added to 700 µl of SBW25 culture, the cells pelleted together and resuspended in 50 µl LB. This suspension was spotted onto a 13 mm diameter 0.2 µm pore size nitrocellulose disc on the surface of a TSBA plate and incubated at 28 °C for 6 h. The disc was then removed and vortexed in 3 ml of tenth strength LB. The suspension was diluted and plated onto PSA, PSA-K and LB-AK for recipients, transconjugants and donors respectively. PSA plates were incubated at 28 °C and LB plates at 37 °C.

4.2.3. Selection of mutants

Kanamycin resistant SBW25::mini-Tn*5xylE* colonies were picked from original isolation plates onto grids on PSA-K and replicated onto a second PSA-K plate which had previously been spread with 100 µl of *Pythium* oospore suspension (Johnson, 1988). All plates were incubated at 25 °C for 3 d. During this time, *Pythium* hyphae developed around and over the colonies which had a reduced biological control activity.

Individual mini-Tn*5xylE* mutants were assayed for 2,3-catechol dioxygenase activity. Strains were grown overnight on Tryptic Soy Broth Agar (TSBA) at 28 °C. Cells were transferred to filter papers which were then spotted with 10 µl of a 1% (w/v) solution of catechol. The development of a yellow colour within a few minutes was indicative of catechol 2,3-dioxygenase activity. This denoted the fact that the transposon had been inserted in the correct orientation into a gene which was expressed under laboratory conditions.

4.2.4. Comparison of FAME profiles

Initially, phenotypic differences were assessed by fatty acid methyl ester (FAME) analysis. Mutants were streaked on TSBA in triplicate and incubated at 28 °C for 24 h. Cells (50 mg) were harvested and the FAMEs extracted as described previously (Thompson *et al.*, 1993a). Essentially, the bacterial cells were saponified in NaOH in aqueous methanol, methylated in hydrochloric acid in aqueous methanol and the FAMEs were extracted with a hexane / methyl-tert butyl ether solvent mix. FAME profiles were obtained using Hewlett-Packard 5890 series II gas chromatograph with a 25 m (length) x 0.2 mm (internal diameter) x 0.33 µm (film thickness) phenyl methyl silicone capillary column. The analysis was performed under the control of the Microbial Identification System software (Microbial ID Inc., Newark, DE, USA). The mean FAME profile from the replicates was used to construct a database entry. A dendrogram based on the Euclidian distance between pairs of mutants and clustered using the unweighted pair group method with arithmetic averages (UPGMA) was produced. Significant differences in the proportions of individual fatty acids were determined by analysis of variance (Fry, 1993).

4.2.5. Relative growth rates in an 'ecological' substrate

The growth rates of four of the mutants showing different phenotypes with different changes in biocontrol activity were compared to that of wild type SBW25 in a medium consisting of exudate from pea seeds. Pea seed exudate (PSE) was produced by adding SDDW (50 ml) to pea seeds (50 g) and shaking at 20 °C for 1 h. The exudate was then filtered through Whatman No.1 paper before sterilizing by filtration though a 0.22 µm filter. PSE was stored for up to one week at 4 °C. Each strain was grown overnight in LB (10 ml) at 28 °C with agitation. The cells were washed three times with an equal volume of distilled water before resuspension in the same. These suspensions (10 µl) were each used to inoculate triplicate aliquots of PSE (300 µl) in honeycomb well plates. These plates were placed in an automated microplate reader (Bioscreen C, Life Sciences Ltd., UK) and incubated at 28 °C with agitation. The optical density at 600 nm wavelength (OD_{600}) was recorded every 15 min over a 24 h period. The average OD_{600} was determined for each time point and a graph was plotted. Differences in the growth profiles were determined visually.

4.2.6. In planta screening of biological control activity

A simple assay for quantifying the biological control of *Pythium* was developed and described in 2.2.5 (p27). The soil used in all assays was sterilized Mendip Loam (Minster

Brand Products, Wimbourne, Dorset) and *Pythium* oospores added to a final dose of approximately 10^4 cfu g^{-1}. Bacteria were added to the system by applying a suspension directly to the soil. Peas were pressed into the surface of the soil and the assays incubated at 20 °C in the dark. All treatments were set up in triplicate. Seven days later the numbers of infected seedlings were recorded. Differences in the ability to reduce damping-off disease were determined by analysis of variance (Fry, 1993).

4.2.7. Isolation of genetic loci involved in antifungal activity

The generalized strategy adopted for the cloning of wild type genomic fragments from SBW25 is illustrated in Figure 4-1. The experimental details for each step are described below. Unless otherwise indicated, all procedures are referenced from (Sambrook, Fritsch & Maniatis, 1989).

4.2.7.1. Generation of SBW25 cosmid library

Genomic DNA was isolated from an overnight culture of SBW25 by the CTAB method as described previously (Ellis, Thompson & Bailey, 1998). Approximately 300 µg of DNA was partially digested with 1 Unit of the restriction enzyme *Sau*3A in a volume of 3 ml at 37 °C for 1 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with an equal volume of isopropanol. DNA was resuspended in 2 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Size-fractionation was achieved using a 10-40% (w/v) sucrose gradient in 1 M NaCl, 20 mM Tris-HCl, 10 mM EDTA and 0.2% (w/v) N-laurylsarcosine as described elsewhere (Ivens & Little, 1995). Sucrose solutions were made up at the following concentrations: 10%, 15%, 20%, 25%, 30%, 35% and 40%. Starting with the highest concentration 5 ml of each was sequentially pipetted into 38 ml centrifuge tubes to create a sucrose step gradient which was then allowed to stand overnight at room temperature. Each 35 ml gradient was then loaded with 1 ml of partially digested DNA. Gradients were centrifuged at 26,000 xg for 17 h at 10 °C. Tubes were carefully removed from the centrifuge rotor and pierced at the base with a hypodermic needle. Fractions (~1 ml) were collected in microcentrifuge tubes and the DNA (10 µl of every third fraction) sized by electophoresis on a 0.4% agarose gel. A range of fractions with DNA between approximately 15-25 kb was selected and precipitated with 2 volumes of ethanol plus 2 volumes of 70% ethanol at -20 °C overnight. After revisualization on agarose gel (0.4%) a single fraction containing fragments of approximately 20-24 kb was then selected and reprecipitated with 2 volumes of ethanol.

The cosmid vector, pIJ3200 (Liu *et al.*, 1990) was digested with *Bam*HI and dephosphorylated with alkaline phosphotase (Boehringer, UK). Vector and size fractionated genomic DNA were mixed at a 1 to 1 molar ratio and ligated together for 18 h at 4 °C with 4 units T4 DNA ligase (Gibco BRL, UK). The resultant recombinant cosmids were packaged into lambda heads and tails using a commercial packaging kit (Promega, UK) following the manufacturers instructions. The packaging mix was diluted and each aliquot was mixed with 6 volumes of an overnight culture of *E. coli* XL1-Blue MR (Stratagene, UK) and incubated at 37 °C for 30 min. Prewarmed LB (12 ml) was then added and incubated for a further 45 min. The culture was then pelleted and resuspended in 500 µl LB before spreading on selective plates (LB-T plus 1.5% agar).

Colonies were picked into individual wells of microlitre plated containing 200 µl LB-T. These plates were grown at 37 °C overnight. A small aliquot (50 µl) was applied to positively charged nylon membrane (Boehringer) using dot blot apparatus under vacuum. Cells were lysed and DNA was fixed to membranes by soaking in 0.5 M NaOH for 4 min. Cell debris was removed by vigorous washing in 5 x SSC. Membranes were allowed to dry and baked at 80 °C for 2 h. The cosmid bank was stored by adding 80 µl of 70% (v/v) glycerol, 0.85% (w/v) NaCl to the remaining culture volume and freezing at -70 °C.

4.2.7.2. Cloning of genomic regions flanking transposon insertions

Genomic DNA was extracted from each of the mutants using the CTAB method described above. The DNA was digested with *Pst*I. This restriction enzyme cleaves within the transposon but leaves the kanamycin resistance gene intact (de Lorenzo *et al.*, 1990). The digested DNA was ligated into dephosphorylated *Pst*I-digested pBluescript® II SK(+) at 4 °C overnight. The resultant mix of plasmid clones were electroporated into competent XL1-Blue® MRF' cells. After 3 h incubation in SOC at 37 °C the cultures were plated out onto LB-TAK for selection of plasmids carrying the kanamycin resistance gene from the transposon insertion.

Plasmids were extracted by the alkaline lysis procedure (Sambrook, Fritsch & Maniatis, 1989) and digested with *Pst*I and *Not*I to cleave the kanamycin resistance gene from the flanking genomic region. DNA fragments were separated by agarose gel electrophoresis and purified from the gels as follows using the Qiaex II DNA extraction kit (Qiagen® Ltd, UK). The ethidium bromide complexed DNA fragments were excised form the gels under UV illumination and solubilized. DNA was then absorbed to silica particles and the buffer removed. The bound DNA was then washed before elution from the particles in TE buffer.

4.2.7.3. Identification of homologous cosmids.

The cosmid bank membranes were probed with the *PstI-NotI* fragments prepared from the cloned flanking regions described above (4.2.7.2,.p83). Fragments were labelled and hybridization was performed as described in the ECL protocol (Amersham, UK). Cosmids bearing DNA homologous to the probes were identified by their binding of labelled DNA. Membranes were stripped by incubation at 37 °C in a large volume (>500 ml) of 0.2 M NaOH, O.1% (w/v) SDS before probing with another fragment (Boehringer Mannheim).

4.2.7.4. Cosmid complementation of mutants.

Homologous cosmids were transferred into SBW25 transposon insertion mutants by a tri-parental mating procedure (Simon, Priefer & Pühler, 1983). Overnight cultures of SBW25 transposon insertion mutants, *E. coli* XL1-Blue MR containing the cosmids of interest and *E. coli* HB101 (pRK2013) were grown overnight in 10 ml LB-K, LB-T and LB-K respectively. Cells were washed three times in LB and resuspended in 10 ml LB. Each culture was then incubated at room temperature for 30 min. Three hundred microlitres of XL1-Blue MR (pIJxxx) culture was added to 700 µl of mutant SBW25::mini-Tn*5 xylE* culture and 400 µl of HB101 (pRK2013) culture, the cells pelleted together and resuspended in 50 µl LB. This suspension was spotted onto a 13 mm diameter 0.2 µm pore size nitrocellulose disc on the surface of a TSBA plate and incubated at 28 °C for 6 h. The disc was then removed and vortexed in 3 ml of tenth strength LB. The suspension was diluted and plated onto PSA-K, LB-CFC-KT and LB-T for recipients, transconjugants and donors respectively. PSA and LB-CFC-KT plates were incubated at 28 °C and LB plates at 37 °C.

4.2.7.5. Subcloning from homologous cosmids

Cosmids homologous to the genomic regions flanking the transposon insertions were extracted from the host XL1-Blue MR cells using the alkaline lysis procedure (Sambrook, Fritsch & Maniatis, 1989). Essentially, cells from 3 ml of overnight culture were pelleted and resuspended in 100 µl 50 mM glucose, 25 mM Tris (pH8) and 10 mM EDTA. After a five minute incubation at room temperature 200 µl of 0.2 M NaOH, 0.1% (w/v) SDS were added and the samples incubated on ice for 5 min. To this was added 150 µl of 5 M sodium acetate (pH 4.8) and the cell debris pelleted by centrifugation. The supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Plasmid DNA was precipitated by the addition of 2 volumes of chilled ethanol and

incubation at -70 °C for 15 min. The DNA was pelleted by centrifugation, air dried and resuspended in 50 µl TE.

Aliquots of the purified plasmids were digested with *Eco*RI, *Pst*I and a combination of both enzymes at 37 °C overnight. Digested plasmids were electrophoresed on a 0.8% agarose gel at 60 V for 3 h. DNA was visualized by staining with ethidium bromide and under UV illumination. DNA was transferred overnight to positively charged nylon membrane (Boehringer Mannheim, UK) using 0.4 M NaOH as the transfer buffer. After transfer, DNA was fixed to the membrane under UV for 3 min followed by baking at 80 °C for 2 h. These membranes were then probed with the transposon flanking regions as for the probing of the cosmid library. The smallest single fragment was then identified and used as the target fragment for subcloning.

The plasmid pBluescript®IISK+ was digested with the enzyme corresponding to those at the ends of the target fragment and then dephosphorylated. Both the plasmid and cosmid digests were phenol/chloroform/IAA (25:24:1) extracted, ethanol precipitated and dried before resuspending in TE. The DNAs were then mixed at an equimolar ratio in ligation buffer in the presence of 10 units of T4 DNA ligase in a total volume of 10 µl. The mix was incubated at 4 °C overnight.

4.2.7.6. Production of electroporation competent cells.

E. coli XL1-Blue MRF' were grown overnight in 10 ml LB-T at 37 °C. This was used to seed a further 400 ml LB. This was incubated at 37 °C until the optical density of the culture at 600 nm (OD_{600}) was 0.3. This took approximately 3-4 h. The cells were pelleted by centrifugation at 5000 x g in a Beckman centrifuge. The pellet was resuspended and repelleted in decreasing volumes of cooled 10% (v/v) glycerol. The steps were 1 x original volume, $\frac{1}{2}$ volume, $\frac{1}{10}$ volume, $\frac{1}{50}$ volume, $\frac{1}{100}$ volume and finally $\frac{1}{200}$ volume. This final suspension was divided into 50 µl aliquots and stored frozen at -70 °C.

4.2.7.7. Electroporation

Ligation reactions (2 µl) were mixed with 50 µl aliquots of competent cells and placed on ice for 5 min in electroporation cuvettes (Biorad, UK). Cells were pulsed at 2.5 kV, 25 µF, 200 Ω (GenePulser, Biorad, UK). Prewarmed enriched broth (SOC: 2% bacto tryptone, 0.5% bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was immediately added and incubated at 37°C for 2 hours. A ten-fold serial

dilution was performed on the resultant culture and plated onto LB containing the appropriate antibiotics and X-Gal to identify those plasmids carrying inserts. These plates were incubated at 37 °C overnight.

4.2.7.8. Identification of plasmids containing subcloned fragments

Colonies bearing plasmids with inserts were distinguished from those without by their lack of blue coloration. Approximately 20 were selected and the plasmids extracted, restricted and electrophoresed to ascertain those which contained inserts of the size corresponding to the hybridizing fragment from the cosmid digest (4.2.7.5). Confirmation was achieved by probing these digest with the relevant transposon-flanking fragment (4.2.7.2).

4.3. Results

4.3.1. Transposon mutagenesis and screening of mutants.

Six hundred and twenty-seven transposon mutants were screened; the assay developed for this study for screening for loss of activity against *Pythium* proved to be effective (Figure 4-2). Of these, 12 of the insertion mutants (2%) had been overgrown by the *Pythium* on the inoculated plates. These mutants were selected for further study, together with 7 others that showed altered phenotypes of interest. These are listed in Table 4-2. Of these, 9 demonstrated catechol 2,3-dioxygenase activity after cultivation on TSBA (Table 4-2). That approximately 50% of the insertions resulted in *xylE* expression indicated that transposon insertions were random and in active genes.

The 19 transposon mutants selected from the primary screen were all assessed for their *in planta* activity against *Pythium*. Twelve mutants controlled damping off to the same extent as the wild type, 6 showed reduced antifungal activity whilst 1 mutant (F1, which produced a diffusable brown pigment) demonstrated a slight but non-significant ($P < 0.05$) improvement over the wild type SBW25 (Figure 4-3). Four mutants, A7 (reduced BCA activity), B73 (reduced BCA activity), D7 (reduced BCA activity) and F1 (enhanced BCA activity), were selected for further genetic analysis.

4.3.2. FAME analysis of mutants

Several of the transposon insertion mutants had significantly different FAME profiles. The magnitude of these differences are illustrated in a dendrogram (Figure 4-4). Cluster A labelled on this figure identifies those variants which would be described as the same strain

according to MIS-MIDI literature (Sasser, 1990), which states that 2 euclids is the maximum deviation within a strain group, 6 euclids for a species group and 10 euclids for a genus group. The mutants not within this cluster therefore would not automatically be assigned to the same strain and one, B26, would be classified as a different species (Sasser, 1990).

4.3.3. Relative growth rates of selected mutants

The four selected mutants demonstrated different growth profiles in pea seed exudate (Figure 4-5). D7 showed the greatest deviation from the wild type growth profile as it multiplied at a much slower rate but sustained this throughout the 24 h period. In comparison, wild type SBW25 reached its maximum population density after 9 h. Mutant B73 also multiplied at a slower rate in this substrate and achieved a lower maximal cell density than the wild type, but within the same time scale as wild type SBW25. Two of the transposon mutants (A7 and F1) appeared to multiply no differently to the wild type in this media. However, despite the differences observed, there were no differences between the strains in the optical densities observed at 24 h.

4.3.4. Generation of cosmid library

Using the equation of Sambrook *et al.* (Sambrook, Fritsch & Maniatis, 1989), it was estimated that the 1056 cosmid bearing colonies picked into microtitre plates would represent approximately 97% of the SBW25 genome, given its 6.6 Mb size (Bailey *et al.*, 1995) and an average cosmid insert size of 22 kb.

4.3.5. Identification of cosmids homologous to genomic regions bearing transposon insertions and subcloning of minimal fragments

Each fragment, excised from mutant genomes' immediately adjacent to the inserted transposon, hybridized with at least 3 recombinant cosmids in the bank. Each of these was purified from the corresponding glycerol stock and digested with *Eco*RI, *Pst*I and both enzymes.

Restriction and hybridization analysis of these cosmids identified shorter DNA fragments within the genomic insert with homology to the transposon flanking region from each mutant. Subcloning of these fragments generated plasmids containing relatively short DNA fragments. The fragment sizes obtained for each of the four mutants are as follows: A7 -

1.6 kb, B73 - 6 kb, D7 - 1.7 kb, F1 - 1.7 kb. These fragments were of a suitable size and in a suitable vector (pBluescript®II SK(+)) for sequence analysis.

4.3.6. Genetic complementation of mutants

One cosmid was selected for each mutant and transferred to that mutant by tri-parental mating at an average frequency of 10^{-4} transconjugants per recipient as determined by their resistance to both kanamycin and tetracycline. However, cosmid DNA could not be purified from the transconjugants. Nevertheless, complementation could be proved by reversion to the wild type phenotype. As mutant SBW25-F1 showed such a distinctive phenotype (pigmentation, Table 4-2), complementation of the mutation, by a cosmid bearing the genomic region homologous to the transposon insertion site, was readily detectable (Figure 4-6). Complementation of the other mutations in SBW25-A7, -B73 and -D7 could only be confirmed by assaying for direct activity against *Pythium*, as the physiological changes caused by the mutations had not been determined and therefore could not be comparatively assessed.

4.4. Discussion

Transposon mutagenesis has been widely used as an aid to determine the genetic basis of a given phenotype (Loper *et al.*, 1993; Rainey, Brodey & Johnstone, 1993; Carruthers, Conner & Mahanty, 1994; Voisard *et al.*, 1994). Alternative methods, such as chemical mutagenesis have been used (Hill *et al.*, 1994), but it cannot be proved that only a single mutation has occurred in a given survivor (de Lorenzo & Timmis, 1994), thus complicating analysis. However, whilst the molecular techniques required are relatively straightforward, it is the screening of hundreds of mutants for loss of activity that can become problematic, both technically and logistically. The plate based assay developed here, for screening for loss of *Pythium* inhibition, effectively overcame these drawbacks. Other plate assays were assessed for screening mutants but the greatest problem that had to be overcome was the rapid growth rate of *Pythium*. The use of *Pseudomonas* selective agar with antibiotic supplements effectively reduced this problem, allowing the bacteria time to produce anti-fungal compounds whilst fungal growth was still retricted and therefore sensitive to bacterial products. Its use as a primary assay identified a managable number of mutants for *in planta* screening assays.

Typically, other studies emphasize that mutants need only be screened for loss of metabolite production (Voisard *et al.*, 1994) but this relies on there being good evidence to

support the role of such products in disease suppression. For example, *P. fluorescens* CHA0 has been screened for loss of production of pyoverdine, hydrogen cyanide, indole acetic acid, 2,4-diacetylphloroglucinol and pyoluteorin (Voisard *et al.*, 1994) and these mutants subsequently assessed for biological control activity. It was found that some metabolites were important for anti-fungal activity whilst others were not. The problem with this approach is that whilst it confirms or refutes the importance of given metabolites in disease suppression it can not identify novel metabolites. Therefore, some antifungal activities may be overlooked because the metabolites have not been isolated or their role in biocontrol unsubstantiated. It should be noted that this approach has never generated mutants with total loss of biocontrol activity as it is multifactorial (Dowling *et al.*, 1994). Screening random mutants for a reduction of *Pythium* inhibition should theoretically identify a suite of mutations that, when combined, yield a mutant devoid of all anti-fungal activity. The approach described here does not make any assumptions about the nature of the anti-fungal activity and therefore may be used to identify novel biocontrol traits/phenotypes.

The fact that not all the transposon mutants tested in this study were catechol 2,3-dioxygenase active was not surprising. That the transposon inserted into active genes was proved by the observed changes in phenotype. However, *xylE* may not be expressed for a number of reasons. The reporter gene may simply have been inserted in the opposite direction to that of the transcription, of the gene or may directly disrupt the promoter activity. Alternatively, the disrupted gene may not be expressed under the conditions used to test for catechol 2,3-dioxygenase activity (i.e. overnight growth on TSBA). Certain genes may only be induced (or repressed) under specified conditions (Cirvilleri & Lindow, 1994; van Overbeek & van Elsas, 1995) or in the presence of *Pythium* (Smith, L.M. *et al.*, 1997). Whilst such possibilities have not been investigated here, inducible promoters may be useful for controlling expression of introduced genes in the environment (Cirvilleri & Lindow, 1994; de Lorenzo, 1994; van Overbeek & van Elsas, 1995).

Whilst the plate based assay proved to be an effective initial screen for transposon insertion mutants, lack of antifungal activity on plates did not always equate to a reduction in disease suppression in *in planta* assays. However, the lack of correlation between *in vitro* and *in planta* fungal inhibition has been documented previously (Reddy, Hynes & Lazarovits, 1993) and is discussed in the following chapter (5.3.1, p99).

Some mutants selected for subsequent testing in biocontrol assays were chosen on the basis of unusual phenotype. For example, SBW25 produces a halo of white precipitate around colonies on agar plates. A product visually similar to this, tolaasin (a

lipodepsipeptide toxin), has been implicated in the pathogenicity of *Pseudomonas tolaasii* (a close relative of *P. fluorescens* (de Mot *et al.*, 1994)) to mushrooms (Rainey, Brodey & Johnstone, 1993). Therefore, it was presumed that the product from SBW25 may also show antifungal activity. However, the strain that was selected solely on the basis of the loss of this phenotype, G25, was as effective as the wild type in reducing the incidence of fungal infection in the seedling assay and performed as the wild type in the plate based assay.

Production of fluorescent pigment was also considered to be a phenotype associated with anti-fungal activity. These pigments are siderophores, iron chelating compounds which have been implicated in the antagonistic activity of many microbes (Becker & Cook, 1988) and proven to be involved in the suppression of damping-off (Buysens *et al.*, 1996). The three insertion mutants that lacked fluorescence on PSA were not altered in their ability to inhibit *Pythium* growth on this media. However, it was considered that such a phenotype may be important for *in planta* activity and thus these transposon mutants were selected for *in planta* screening. However, of these only the pleiotropic mutant E43 demonstrated reduced disease suppression (Table 4-2). This implied that siderophores were not responsible for the anti-fungal activity of SBW25.

Mutants with altered colony morphologies were also selected for further screening, on the basis that such a phenotype may be indicative of mutations that are detrimental to overall cellular health, such as changes to cell wall, membrane, transcription/translation mechanisms or global regulatory pathways (Gaffney *et al.*, 1994; Bangera & Thomashow, 1996). Mutants of the latter class have been found that have altered production of antibiotics, other secondary metabolites and extracellular enzymes (Sacherer, Defago & Haas, 1994; Sarniguet *et al.*, 1995). The fact that E43 demonstrated changes in multiple phenotypes (Table 4-2) gave further weight to the fact that the transposon had inserted into a site which caused pleiotrophic effects. Given that it performed poorly in the disease suppression assay and its altered FAME profile (Figure 4-4) it is resonable to speculate that the transposon had inserted into a gene encoding a component of a global regulatory mechanism. Consequently, it was not possible to associate the reduction in anti-fungal activity with any one phenotype.

Other traits which have been implicated as important for biocontrol activity are either related to assimilatory pathways (Nelson, 1992) or the ability to colonize roots (Duijff, Gianinazzi-Pearson & Lemanceau, 1997). Whilst the strategy used here may have identifed mutations in the first class, the effectiveness of mutants in root colonization was not investigated.

However, it was considered that such a trait would not be essential for the control of *Pythium* damping-off as soil interactions appear to be more important than those on the plant (chapters 2 and 3).

That a single insertional mutation in a pseudomonad (SBW25-F1) may enhance biological control activity was alluded to by the *in planta* activity of SBW25-F1 (Figure 4-3). Whilst it has been shown that the production of some anti-fungal compounds is increased by inactivation of a single gene in *P. fluorescens* CHA0 (Schnider *et al.*, 1995b; Schmidli-Sacherer, Keel & Défago, 1997), an increase in biocontrol activity was not observed. However, inactivation of *rpoS* in *P. fluorescens* Pf-5 resulted in the overproduction of pyoluteorin and phloroglucinol, thus enhancing suppression of *Pythium* (Sarniguet *et al.*, 1995). Such a discovery emphasizes the need to develop assays which better resolve the search for unknown functions as well as those that are known. The identity of the brown pigment produced by SBW25-F1 on solid media and its role in the potential enhanced biocontrol activity of this mutant *in planta* is not known. It is possible that the colour change is the result of a reaction between product(s) from the mutant and compounds in the media, as has been proposed for the presence of red pigmentation in phloroglucinol producing strains (Bangera & Thomashow, 1996). It can be postulated that the pigmentation of the media is due to the overproduction of a compound that can be implicated in the suppression of *Pythium* infection. The inference is that this overproduction was bought about by the insertional disruption of a regulatory element in *P. fluorescens* SBW25. This implies that until the mechanisms that control the biosynthesis are fully understood, the true potential of these organisms as alternatives to chemical pesticides can not be realized.

It is apparent from the results of FAME analysis that fatty acids are a good indicator of overall cellular physiology. It has been calculated, given the information in (Neidhardt & Umbarger, 1996) and average molecular weight of fatty acids of 220, that fatty acids comprise approximately 6% of the weight of each *Escherichia coli* cell and therefore require a relatively large biosynthetic input. It is probable that pseudomonads contain a similar proportion. Mutations in a single gene can drastically alter the biochemical make up of bacteria and thus it was not surprising to find that they altered FA composition. However, it was unexpected to find the extent to which the relative proportions of individual FA changed. The fact that so many of the mutants had significantly altered FAME profiles from that of the wild type signifies their fundamental role in cellular functioning. It was also surprising to find that non-lethal mutations in a single gene could produce such a large change to cellular physiology as to cause their misidentification by cellular fatty acid analysis.

It is probable that the change in FA composition is caused by a disruption of the central metabolic pathways leading to a build up or lack of a variety of intermediary or precursor compounds. This in turn will affect repression and induction mechanisms and thus force biosynthetic pathways in abnormal directions to compensate for the imbalance.

Analysis of the growth profiles of four of the transposon insertion mutants (A7, B73, D7 and F1) identified some important differences between them and wild type SBW25 (Figure 4-5). It appears that, although B73 and D7 were identical to the wild type in terms of their colony size on PSA, the insertions affected their ability to replicate in PSE. This implies that B73 and D7 may be less competitive in the soil surrounding germinating seeds. A lack of growth in the spermosphere may account for their reduced suppression of *Pythium* infection *in planta*. These insertion mutants are probably defective in the assimilation of nutrient(s) found in the seed exudate. Given that SBW25 suppresses hyphal extension when PSE is used as the sole carbon source (2.3.4, p31), it would appear that the nutrients that the transposon mutants are unable to assimilate are required for hyphal extension in *Pythium.* That the insertion mutant A7 replicates as well as wild type SBW25 in PSE but is still unable to inhibit *Pythium* either *in vitro* or *in situ* implies that is defective in a function other than nutrient assimilation.

Despite the fact that cosmids apparently could not be recovered from complemented mutants, the reversion to the wild type phenotype, in combination with the acquisition of tetracycline resistance, indicated that the cosmids contained DNA that effectively negated the transposon induced mutation. Cosmids may not have been isolated either because the exist at a very low copy number in pseudomonad cells or they may have become integrated into the chromosome by homologous recombination. The recombination function *RecA* was active in SBW25 which may have facilitated this integration. This problem has been observed in other *Pseudomonas* strains in conjunction with a similar cosmid (Carruthers, Conner & Mahanty, 1994; Kraus & Loper, 1995).

It would appear that the anti-fungal activity of *P. fluorescens* SBW25, as with the majority of fluorescent pseudomonads, is multi-factorial. As suggested in chapter 2, it would appear that SBW25 competes with *Pythium* for the limited nutrients in pea seed exudate. This factor was illustrated by mutants B73 and D7 which are defective in terms of replication in PSE. However, one of the mutants (A7) does not appear to be deficient in assimilatory pathways and shows no difference in survival in soil compared to the wild type. Therefore SBW25 probably also produces an antibiotic. The sequence analysis of the isolated

fragments, which is ongoing at the time of writing, should aid in the identification of these anti-fungal attributes.

Table 4-1: Organisms and plasmids used in the course of this study

Species or plasmid	Relevant phenotype	Reference or source
Pseudomonas fluorescens SBW25	Biological control of *Pythium ultimum*	Rainey & Bailey, 1996
Escherichia coli		
HB101	*supE*44, restriction-minus, St^r	Sambrook, Fritsch & Maniatis, 1989
S17/1 λpir		Simon, Priefer & Pühler, 1983
XL1-Blue MR	*supE*44, restriction-minus, Tc^s	Stratagene, UK
XL1-Blue MRF'	*supE*44, restriction-minus, Tc^r	Stratagene, UK
Pythium ultimum	Fungal pathogen of *Psium sativum.*	Zeneca Agrochemicals, UK
Psium sativum var. 'Bohartyr'	Forage pea; susceptible to Pythium damping-off	Nickerson Seeds, Linconshire, UK
Plasmids		
pUTmini-Tn*5xyl*E	Ap^r; mini-Tn*5* carrying promotorless *xyl*E and Km^r, *tnp**, *ori* R6K, *mob* RP4	de Lorenzo *et al.*, 1990; Herrero, de Lorenzo & Timmis, 1990
pIJ3200	Broad host range cosmid; *cos*, Tc^r	Liu *et al.*, 1990
pRK2013	IncP4, Tra^+, Mob^+, Km^r	Simon, Priefer & Pühler, 1983
pBluescript® II SK(+)	Phagemid cloning vector, Ap^r, *ori* f1, *ori* ColE1, *lacZ*	Stratagene, UK

Table 4-2: List of SBW25 transposon mutants selected and the phenotypes leading to their selection.

P. fluorescens SBW25 mutants	Altered phenotype	*xylE* expression[a]	*In planta Pythium* suppression
A7	Reduced *Pythium* inhibition[b]	+	Reduced
A12	Reduced *Pythium* inhibition	+	No change
A24	Reduced *Pythium* inhibition	-	No change
B26	Small colonies[c]	-	No change
B73	Reduced *Pythium* inhibition	-	Reduced
B79	Reduced *Pythium* inhibition	-	No change
C58	Reduced *Pythium* inhibition	-	No change
D7	Reduced *Pythium* inhibition	+	Reduced
D55	Reduced *Pythium* inhibition	+	No change
E43	Small colonies; No fluorescent siderophores produced[d]; No white precipitate produced around colony[e]	+	Reduced
E54	Small colonies	-	No change
F1	Production of diffusable dark brown pigment[f]	+	Increased
F44	No fluorescent siderophores produced	-	No change
F87	No fluorescent siderophores produced	+	No change
G2	Reduced *Pythium* inhibition	+	No change
G4	Reduced *Pythium* inhibition	-	Reduced
G25	No white precipitate produced around colony	-	No change
H8	Reduced *Pythium* inhibition	-	Reduced
H25	Reduced *Pythium* inhibition	+	No change

[a] Determined by lifting colonies onto filter paper and subsequently soaking it in a 1% solution of catechol. Development of a yellow colour indicates the production of XylE.

[b] As determined on PSA.

[c] Colonies were less than 2mm in diameter after 24 hours growth on PSA at 28°C.

[d] No fluorescent pigment produced after 24 hours growth on PSA at 28°C.

[e] No white 'halo' around colony after 24 hours growth on PSA at 28°C.

[f] Extensive coloration of the medium after 48 hours growth on PSA at 28°C.

Figure 4-1: Cartoon illustrating the strategy adopted for cloning genomic regions which code for antifungal activity.

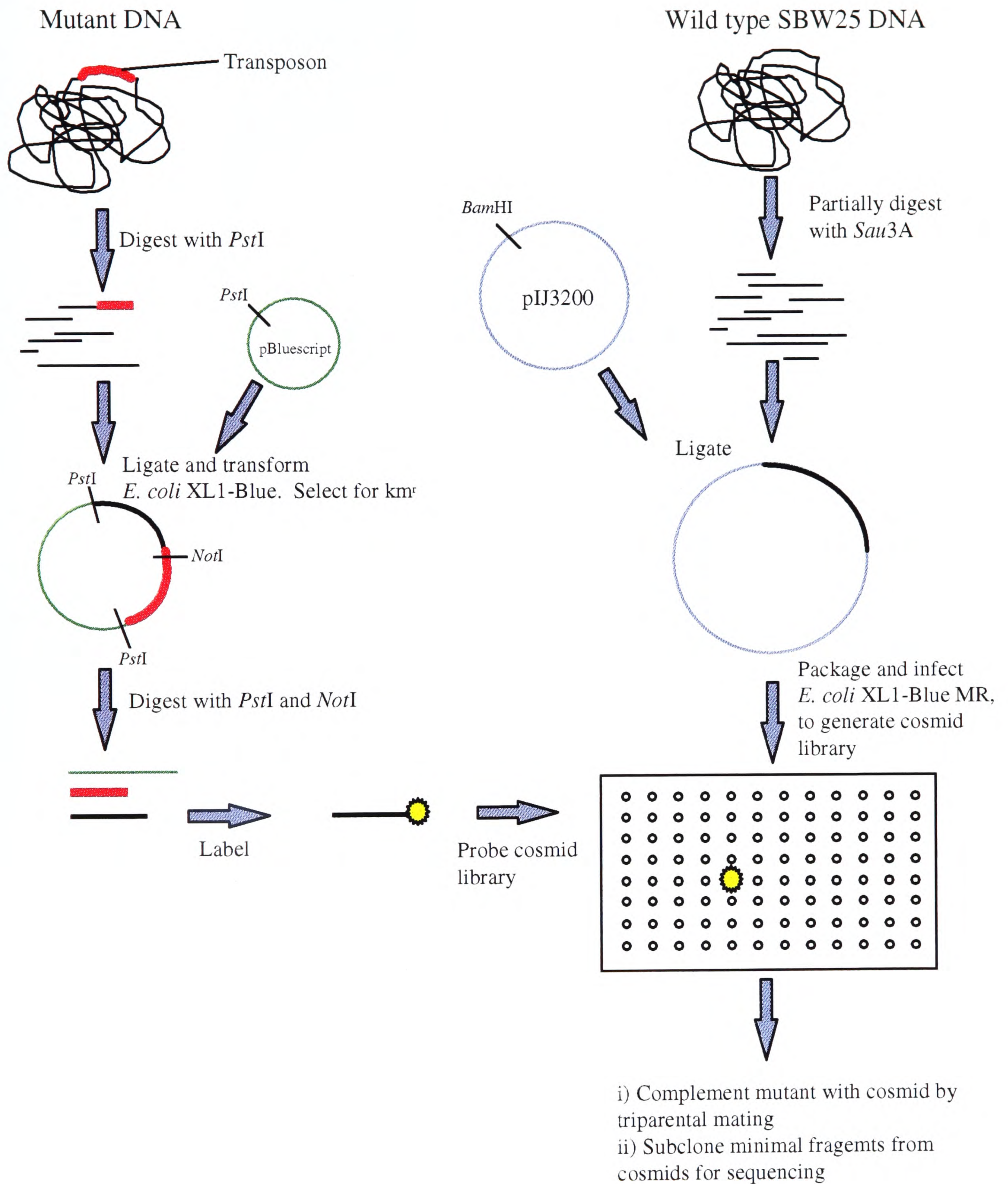


Figure 4-2: Photograph of agar-based screen for mutants of *Pseudomonas fluorescens* SBW25 which have lost the ability to prevent *Pythium* growth, and therefore may be mutants for biocontrol activity. The mutant at the bottom of the picture has been overgrown by *Pythium*.

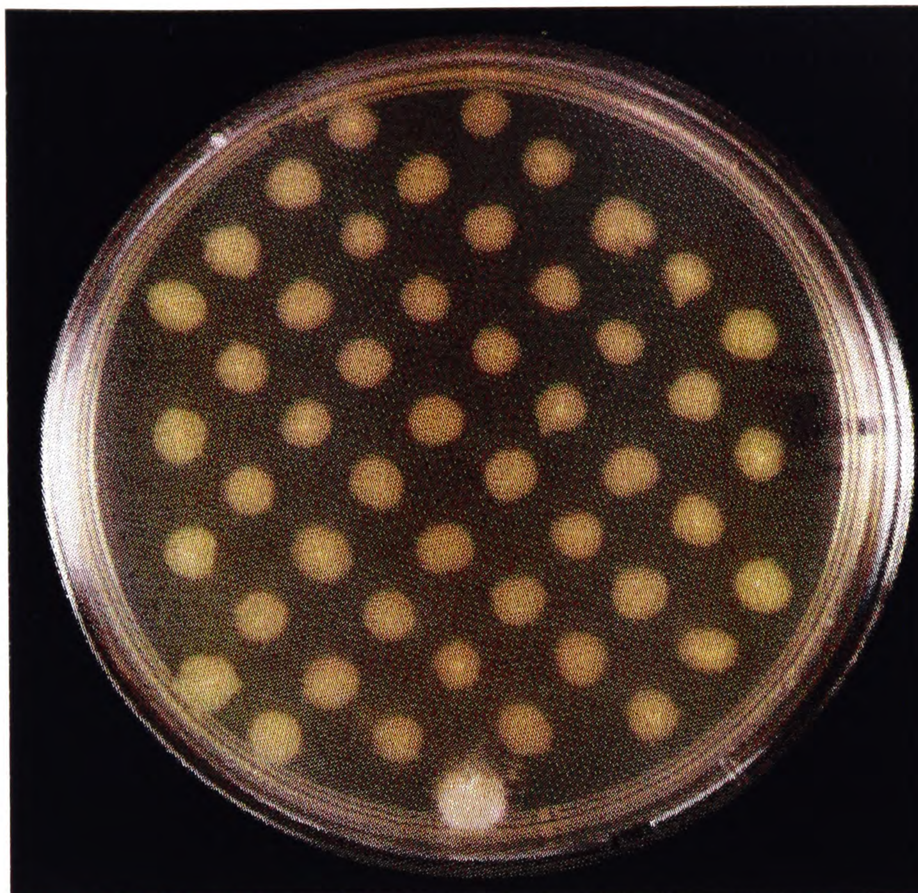


Figure 4-3: Performance of SBW25 transposon mutants in a seedling assay. Control index was calculated using Equation 2-1.

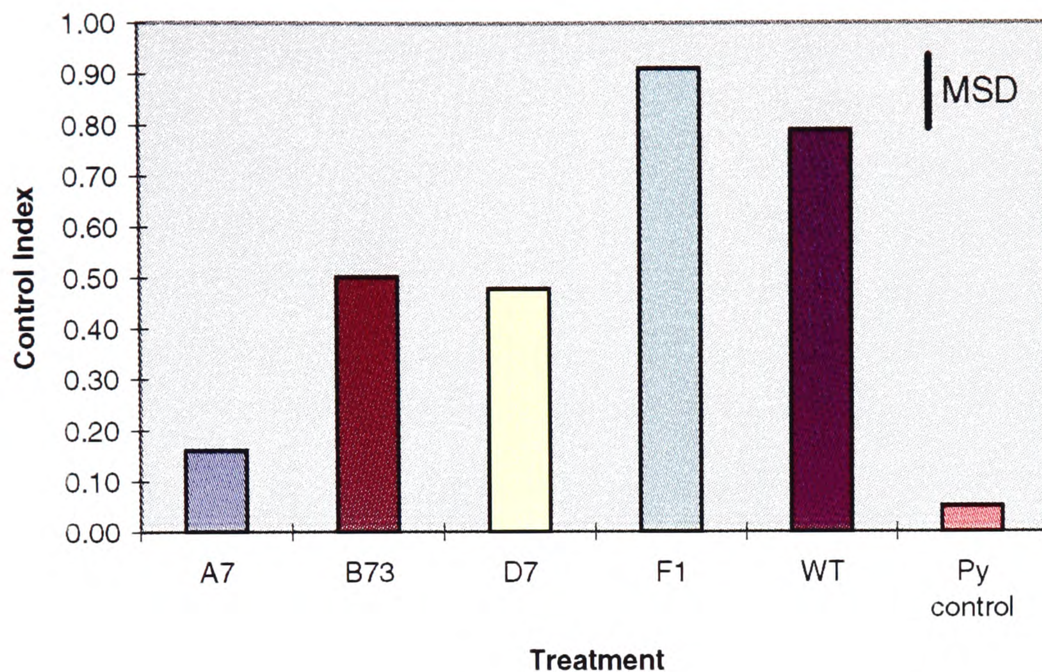


Figure 4-4: Dendrogram indicating the changes in fatty acid profile induced by transposon mutagenesis of Pseudomonas fluorescens SBW25. Based on the Euclidian distance between pairs of mutants and clustered using the unweighted pair group method with arithmetic averages (UPGMA)

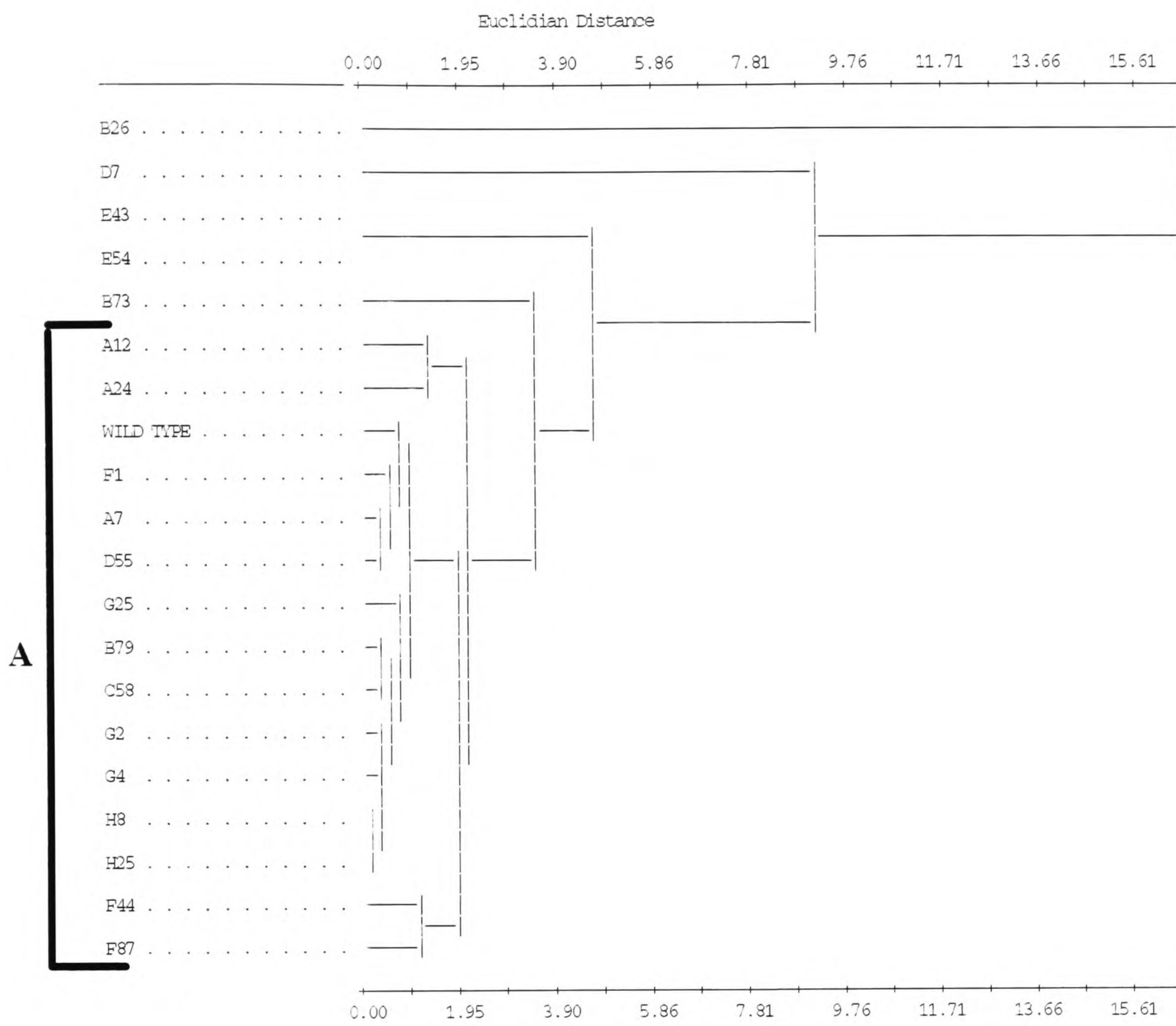


Figure 4-5: Graph indicating the relative growth profiles in pea seed exudate of SBW25 and four transposon derived mutants. Each profile is the average of three readings for each strain.

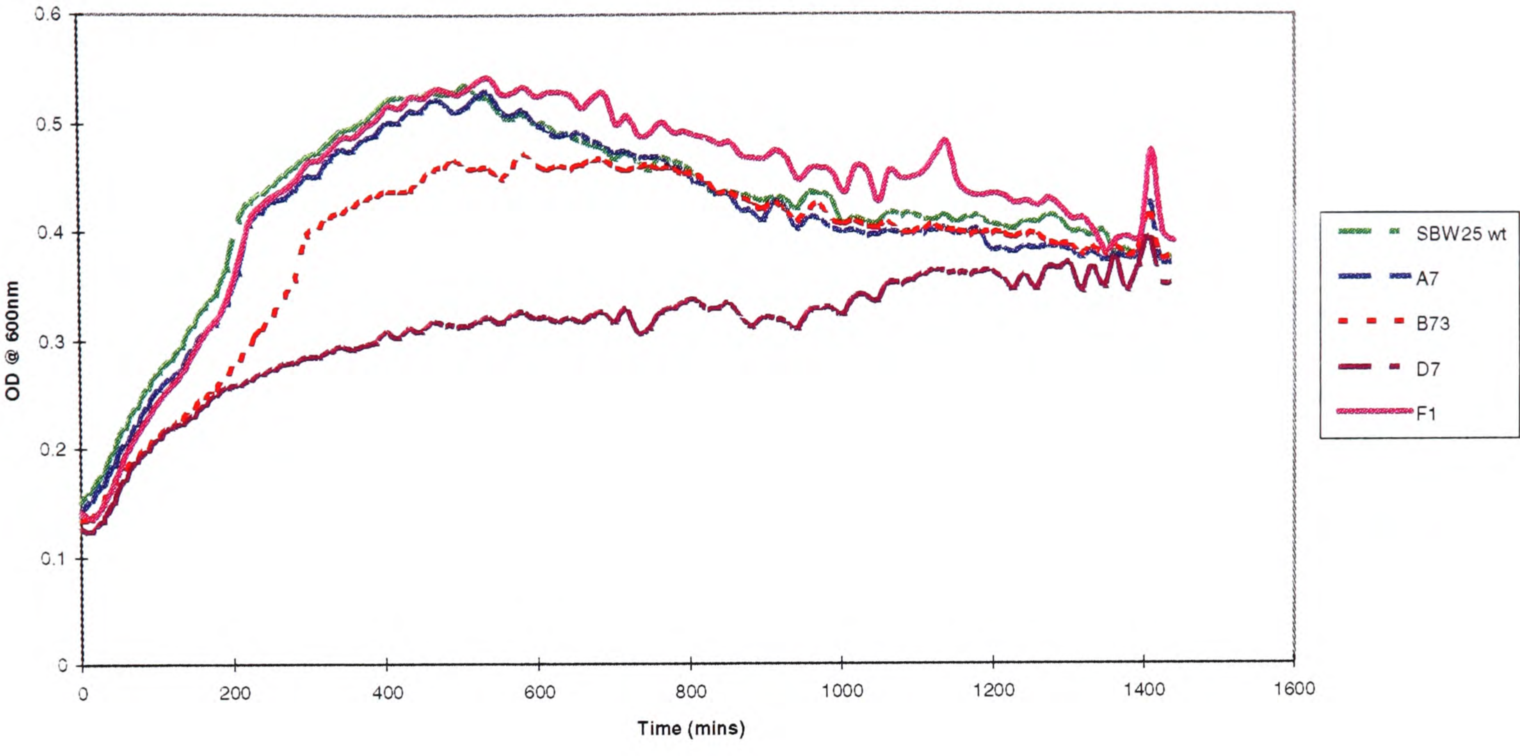
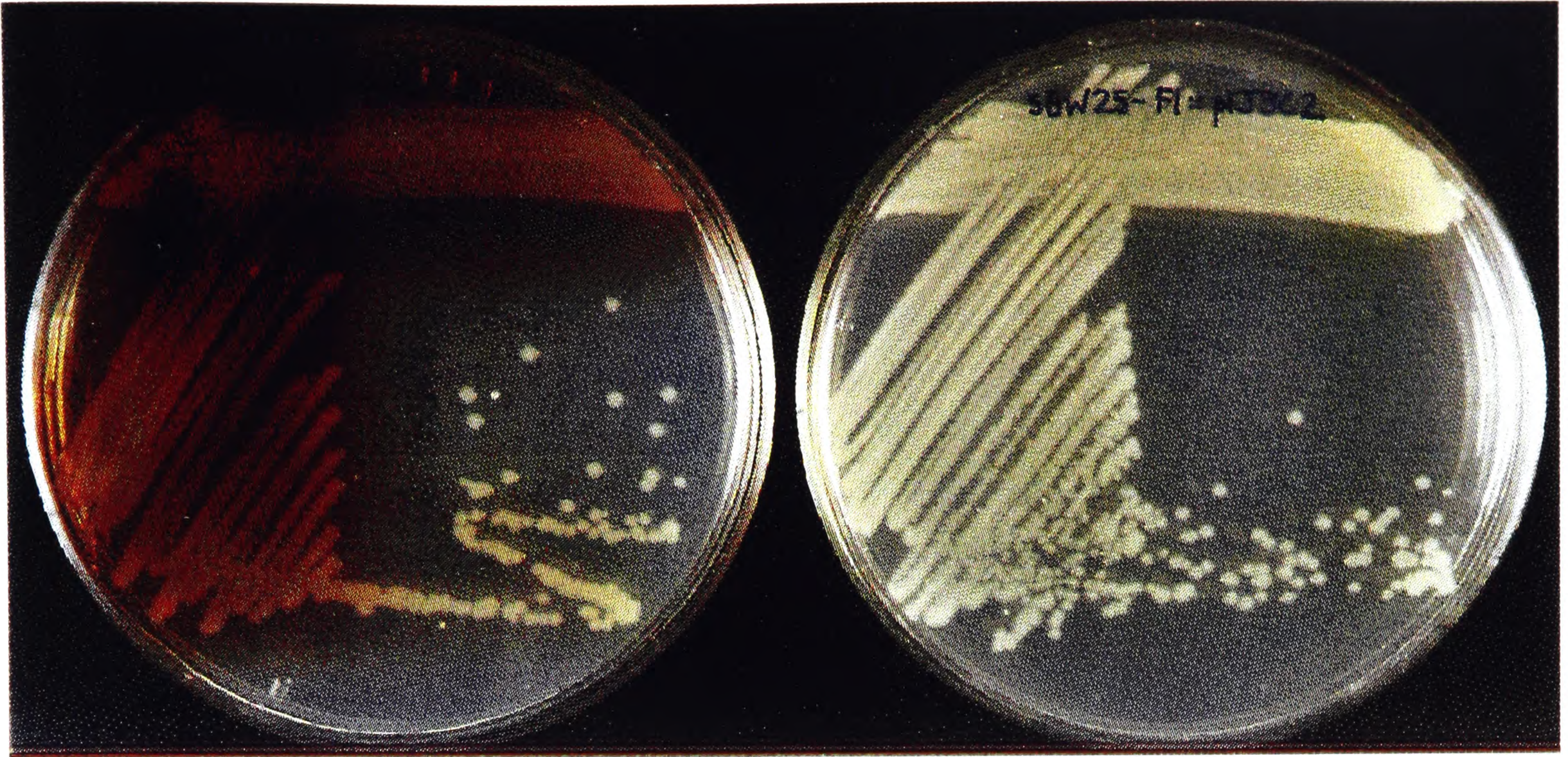


Figure 4-6: Photograph of SBW25-F1 (left), a transposon mutant and SBW25-F1(pIJ3C2) (right), the same mutant with the phenotype complemented by a cosmid containing genomic DNA from the parental strain. Both strains were grown for 48 hours on PSA.



5. IDENTIFICATION OF CONSERVED TRAITS IN FLUORESCENT PSEUDOMONADS WITH ANTI-FUNGAL ACTIVITY

5.1. Introduction

Many fluorescent pseudomonad species are capable of reducing the incidence of plant diseases caused by soil-borne fungi. Few studies have examined the taxonomic and functional diversity of these bacteria. However, it is apparent that a large variety of anti-fungal metabolites are produced by these organisms which include: hydrogen cyanide (HCN) (Voisard *et al.*, 1989), phenazines (phz) (Thomashow & Weller, 1988), phloroglucinols (phl) (Fenton *et al.*, 1992; Keel *et al.*, 1992), pyoluteorin (plt) (Kraus & Loper, 1995), and pyrrolnitrin (prn) (Pfender, Kraus & Loper, 1993). Many pseudomonads produce these antibiotics and attempts have been made to quantify the importance of each in disease suppression (Osburn *et al.*, 1989; Maurhofer *et al.*, 1994). However, the conclusions that stand out from each of these attempts is that the production of characterized antibiotics does not account for 100% of the observed anti-fungal activity (Kraus & Loper, 1992).

A previous study has investigated the prevalence of antibiotic biosynthetic loci in disease conductive and suppressive soils (Raaijmakers, Weller & Thomashow, 1997). PCR amplification of DNA extracted from pseudomonads isolated from these soils, using primers specific for these loci, revealed that the 2,4-diacetylphloroglucinol (phl) biosynthetic locus was more prevalent than the phenazine biosynthetic locus (phz), which was undetectable (Raaijmakers, Weller & Thomashow, 1997). The density of phl positive populations correlated with the suppressiveness of the soil from which they were isolated (Raaijmakers, Weller & Thomashow, 1997). Related studies have revealed that the phl biosynthetic locus can be detected in phenotypically and genotypically diverse of pseudomonad isolates collected from world-wide geographical locations (Keel *et al.*, 1996). However, the transfer of a 6kb genomic region from *Pseudomonas fluorescens* F113 which encoded phl biosynthesis, to non-producing strains, only conferred the ability to produce phl to one of eight strains tested (Fenton *et al.*, 1992). This implied that the ability to produce this antibiotic is dependent on additional factors being present in the host cell. Subsequent

analysis showed that the failure of the test strains to produce phl was due to the presence of a negative regulatory element on the inserted genomic fragment (Fenton *et al.*, 1997). This confirms the importance of gene regulation in determining biocontrol efficacy.

The catabolic versatility of pseudomonads has also been identified as a factor important in the biocontrol capabilities of some strains (Paulitz, 1991). It has been shown that *P. putida* N1R reduced the amount of *Pythium*-stimulatory seed exudates available to the fungal spores (Paulitz, 1991). The high capacity of fluorescent pseudomonads to compete for seed exudates has been implicated in their rapid establishment on roots (Gamliel & Katan, 1992b) and thus may lead to the exclusion of deleterious fungi. The efficient assimilation of iron from soil has also been implicated in biocontrol efficacy (Leong, 1986; Becker & Cook, 1988; Loper & Buyer, 1991) and is attributed to the production of siderophores, low molecular weight iron-transporting compounds (Leong, 1986). However, neither of these features nor the production of antibiotics alone fully explain disease suppression attributes of fluorescent pseudomonads (Hamdan, Weller & Thomashow, 1991; Paulitz, 1991).

Therefore it is relevant to establish what features of these strains, selected for outstanding anti-fungal activity, distinguish them from other fluorescent pseudomonads. It is probable that they are all specialized for survival in soil, and may be capable of the regulated production of antifungal compounds in the soil-root environment, but do they share other common phenotypes? Some important insights may be gained by ascertaining the ecological function of antibiotic production in bacteria. What environmental pressures have led to the evolution of these pathways and what functions do they play in natural environments? It may be that antibiotic production has evolved as a mechanism for survival under intense competition (Mazzola *et al.*, 1992). Concomitantly, it has been suggested that those bacteria that demonstrate non-specific plant growth promotion may have evolved highly efficient signalling systems with the host plants, which lead to specific plant-microbe interactions (Pierson III & Pierson, 1996).

A study which describes the analysis of a collection of *Bacillus cereus* strains identified phenotypic and genotypic differences between those that produced the antibiotic, zwittermicin A, and those that did not (Raffel *et al.*, 1996). Amongst the techniques used to differentiate these strains was Fatty Acid Methyl Ester (FAME) analysis. Further analysis indicated that proportions of certain fatty acids correlated with the production of the antibiotic (Raffel *et al.*, 1996). The method has also been used to discriminate between mosquitocidal strains of *Bacillus sphaericus* and those with no toxic activity (Frachon *et al.*, 1991) and between plant pathogenic and non-pathogenic *Streptomyces* isolates (Ndowora

et al., 1996). In addition, FAME profiling has been successfully used for the taxonomic differentiation of the various pseudomonad rRNA homology groups (Vancanneyt *et al.*, 1996) and therefore it was considered that such a method could be used to analyze the collection of pseudomonads assembled for this study. Ribotyping, the typing of bacterial isolates by rRNA gene restriction patterns (Brosch *et al.*, 1996; Goddard *et al.*, 1997), and metabolic profiling (Grimont *et al.*, 1996) have also been used effectively to differentiate closely related pseudomonad strains. It was proposed that FAME analysis, in combination with other phenotypic and genotypic typing techniques may reveal some characteristics that distinguish BCAs from other fluorescent pseudomonads.

At present, the majority of potential biological control agents have been selected following the screening of large numbers of isolates for their ability to control disease in planta (Campbell, 1989). Seedling assays remain one of the most reliable methods for this as they ensure that any anti-fungal activity observed is an *in situ* phenomenon. However, they are expensive and time consuming to run and the results can often be variable. The identification of traits indicative of biocontrol activity would permit screening of larger numbers of isolates at a greatly reduced cost. Thus, the aim of this chapter was to compare phenotypic and genotypic characteristics of fluorescent pseudomonads with proven biocontrol potential against a random set of strains, and relate the results to the ability to suppress *Pythium* infection of pea seeds. To test the validity of any markers of biocontrol activity identified, a large collection of pseudomonad strains held at the Institute of Virology and Environmental Microbiology, Oxford, were screened for the presence of such markers. Those which were positive were then assayed for their *in planta* suppression of *Pythium* infection. Conversely, biocontrol negative mutants of strains presenting these traits were assayed for their loss in parallel with the loss of anti-fungal activity. In this way a clear link between the ability to suppress *Pythium* and the presence of proposed markers would be forged.

5.2. Materials and Methods

5.2.1. *Bacterial strains and culture conditions*

A collection of fluorescent pseudomonad strains was assembled and are detailed in Table 5-1. The other bacterial and fungal strains used are given in Table 5-2. *E. coli* strains were grown routinely in Luria-Bertani (LB) broth at 37 °C. *Pseudomonas* isolates were cultured on Pseudomonas Agar Base supplemented with 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin, and 50 mg l^{-1} cephaloridine (PSA-CFC; Unipath, UK) at 28 °C. Fungal isolates were grown on

Potato Dextrose Agar (PDA; Unipath, UK) with 320 mg l^{-1} aureomycin (Cyanamid, UK) at 20 °C. Other antibiotics were used, when appropriate, at the following concentrations (mg l^{-1}): ampicillin (A, 50), kanamycin (K, 50), tetracycline (T, 10).

5.2.2. In planta *screening of biological control activity*

An simple assay for quantifying the biological control of *Pythium* was developed and described elsewhere (2.2.5, p27). The soil used in all assays was sterilized Mendip Loam (Minster Brand Products, UK [for soil analysis see 2.2.1, p24]) and *Pythium* oospores (2.2.1, p24) were added to a final dose of approximately 10^4 cfu g^{-1}. Bacteria (approximately 10^8 cfu g^{-1}) were added to the system by applying a 30 ml suspension directly to the soil (60 g). Peas were pressed into the surface of the soil and the assays incubated at 20 °C in the dark. All treatments were set up in triplicate. Seven days later the numbers of infected seedlings were recorded. A control index (CI) was calculated for each strain using Equation 2-1 (p29).

5.2.3. In vitro *screening of fungal inhibition*

All pseudomonad strains were screened for their ability to inhibit the growth or germination of *Pythium* on PSA-CFC (low-iron) media. Each was grown on PSA-CFC overnight at 28 °C. Purified oospores (100 µl; 2.2.1, p24, (Johnson, 1988)) were spread on the surface of fresh agar plates and allowed to dry. A single colony of each strain was then picked from the overnight plates and spotted onto the *Pythium*-inoculated plates. After incubation at 28 °C for thirty hours strains were scored according to the extent of inhibition observed. Bacterial colonies overgrown by hyphae scored zero, hyphae at the edge of the colony - 1, and a distinct zone of clearing around the colony - 2.

5.2.4. *Fatty acid profiling*

Phenotypic differences were assessed by fatty acid methyl ester (FAME) analysis. Isolates were streaked on Tryptic Soy Broth (Difco, UK) with 1.2% (w/v) agar (TSBA) in triplicate and incubated at 28 °C for 24 h. Cells (50 mg) were harvested into glass test tubes and the FAMEs extracted as described previously (Thompson *et al.*, 1993a). Essentially, the bacterial cells were saponified in sodium hydroxide (NaOH) in aqueous methanol, methylated in hyrdochloric acid in aqueous methanol and the FAMEs were extracted with a hexane / methyl-tert butyl ether solvent mix. FAME profiles were obtained using a Hewlett-Packard 5890 series II gas chromatograph with a 25 m (length) x 0.2 mm (internal diameter) x 0.33 µm (film thickness) phenyl methyl silicone capillary column and a flame

ionization detector (FID). The analysis was performed under the control of the Microbial Identification System software (Microbial ID Inc., Newark, DE, USA). Samples (1 µl) were injected at 250°C and the column temperature was steadily increased from 170 °C to 270 °C at a rate of 5 °C per min. Peaks were identified by their retention time on the column and the proportion of each in the profile was calculated from the relative peak areas. The mean FAME profile from the replicates was used to construct a library. A dendrogram based on the Euclidean distance (Equation 5-1) between pairs of strains and clustered using the unweighted pair group method with arithmetic averages (UPGMA) was produced. Significant differences in the proportions of individual fatty acids were determined by analysis of variance (Fry, 1993).

Equation 5-1

$$d_{ab} = \frac{\sqrt{(X_{iA} - X_{iB})^2 + (X_{2A} - X_{2B})^2 \cdots\cdots (X_{tA} - X_{tB})^2}}{\sqrt{t}},$$

where d_{ab} is the average Euclidean distance, X_{iA} and X_{iB} are the states of character *i* (*i*=1, 2, ..., *t*) for two individuals and *t* is the total number of characters.

5.2.5. *DNA profiling*

Genomic DNA was isolated from bacteria by an adaptation of the CTAB method as described previously (Ellis, Thompson & Bailey, 1997). Approximately 1 µg of DNA was digested separately with the restriction enzymes *Kpn*I and *Eco*RI (10 Units, Boehringer Mannheim, UK) at 37 °C overnight. DNA fragments were separated by Field Inversion Gel Electrophoresis (FIGE, BIORAD, UK) using a 1% (w / v) pulsed field certified agarose (BIORAD, UK) gel in 0.5 x Tris-borate buffer as recommended by the manufacturers. Electrophoresis conditions [18 °C, 0.4 to 0.8 sec switch time, 180 V forward, 120 V reverse, 20 h] were selected to separate DNA at a size range of 25-50 kb. DNA was transferred overnight to positively charged nylon membrane (Boehringer Mannheim, UK) using 0.4 M sodium hydroxide as the transfer buffer. Membranes were probed with the 4.5 kb *Bam*HI fragment from pAC10 carrying both the 16S and 23S ribosomal RNA genes (Housiaux, Hill & Petersen, 1988a, b). The fragment was purified from agarose and 50 ng was labelled with the non-radioactive ECL system as described by the manufacturers (Amersham, UK). Hybridization and visualization of the genes were carried out according to the manufacturers' instructions. Images were captured using the GelDoc® system (BIORAD, UK) and images normalized to the relative positions of the DNA markers. The genetic

relatedness between the stains was assessed cluster analysis, using UPGMA, of dice coefficients (Equation 5-2) calculated pairwise between strains (Rainey, Bailey & Thompson, 1994).

Equation 5-2

$$S_d = \frac{2n_i}{(n_a + n_b)},$$

where n_a and n_b are the number of hybridizing bands in strain *a* and strain *b*, respectively, and n_i is the number of bands common between the two.

5.2.6. *Metabolic profiling*

Metabolic profiles of all pseudomonad strains were assessed using the BiOLOG® GN (Hayward, CA) microplate system. These microtitre plates are prepared with a total of 95 different sole-carbon-sources in individual wells, together with a redox dye, tetrazolium violet, which indicates respiration via production of NADH (Bochner, 1989). All pseudomonad strains were grown on TSBA at 28 °C for 24 h. Cells were removed from the surface of the agar with a cotton-tipped swab and resuspended into 20 ml 0.85% saline to give an $O.D._{600}$ of 0.35 ($\pm$ 0.05). Each well of the plate was inoculated with 150 µl of this suspension. Plates were incubated at 28 °C for 24 h. Each well was scored on a 4-point scale (0, 1, 2, 3) according to the extent of colour production observed. The data were used to construct a dendrogram of the similarity of the metabolic profiles. Euclidean distances (Equation 5-1) were calculated between pairs of strains and clustered by UPGMA.

BiOLOG® plates were also used to determine the metabolic profile of *Pythium ultimum*. The oospore suspension prepared as described in section 2.2.1, p24, (Johnson, 1988) was used to inoculate the microplates. The nutritional similarity index (*NSI*) between *Pythium* and each of the strains was calculated by an adaptation of the niche overlap index (Wilson & Lindow, 1994), but included those carbon sources utilized only by either of the two organisms (Equation 5-3).

Equation 5-3

$$NSI = \frac{2n_b}{n_{bca} + n_{py}}$$

where n_b is the number of carbon sources utilized by both organisms and n_{bca} and n_{py} are the total number of carbon sources used by the potential biocontrol agent and *Pythium* respectively.

5.2.7. *Relative growth rates in an 'ecological' substrate*

The growth rates of each of the strains in Table 5-1 were compared in a medium consisting of exudate from pea seeds. Pea seed exudate (PSE) was produced by adding SDDW (50 ml) to pea seeds (50 g) and shaking at 20 °C for 1 h. The exudate was then filtered through Whatman No.1 paper before sterilizing by filtration though a 0.22 µm filter. PSE was stored for up to one week at 4 °C. Each strain was grown overnight in LB (10 ml) at 28 °C with agitation. The cells were washed three times with an equal volume of distilled water before resuspension in the same. These suspensions (10 µl) were each used to inoculate triplicate aliquots of PSE (300 µl) in honeycomb well plates. These plates were placed in an automated microplate reader (Bioscreen C, Lifesciences Ltd., UK) and incubated at 28 °C with agitation. The optical density at 600 nm wavelength was recorded every 15 min over a 24 h period. The mean generation time of each strain was then calculated for the period of exponential growth (Equation 3-1).

5.2.8. *Distribution of the ability to produce known anti-fungal metabolites*

5.2.8.1. Cyanide

Each strain was tested for the production of cyanide, phenazines, and siderophores. Cyanide production was assessed by a method described elsewhere (Voisard *et al.*, 1989). Briefly, each strain was grown overnight in 200 µl LB in a microtitre plate. The wells were covered with indicator paper (Whatman 3MM soaked in 5 mg ml^{-1} copper ethylacetoacetate and 5 mg ml^{-1} 4,4-methylene-*bis*-*N,N*-dimethylalanine in chloroform and air dried) and plates incubated at 28 °C. Production of cyanide caused the indicator to turn blue.

5.2.8.2. Phenazine

All pseudomonads in the collection were assessed for the production of phenazine as described by (Thomashow & Weller, 1988). Isolates were grown overnight on TSBA before

being streaked onto Potato Dextrose Agar (PDA, Unipath, UK) and incubated at 28 °C for 4 d. Dark green pigmentation or crystalline deposits in the centre of colonies was indicative of phenazine production.

5.2.8.3. Siderophores

Siderophore production was quantified using chrome azurol S (CAS) medium (Schwyn & Neilands, 1987). Glycerol (5% v/v) was used as the sole carbon source and pseudomonad selectivity was provided by the addition of 10 mg l^{-1} cetrimide, 10 mg l^{-1} fucidin, and 50 mg l^{-1} cephaloridine (CFC; Unipath, UK). When the iron(III) is removed from the chrome azurol S complex by high affinity siderophores its colour changes from blue to orange. The diameter of the orange halo around colonies was indicative of the relative level of siderophore production of each strain.

5.2.8.4. 2,4-diacetylphloroglucinol and pyoluteorin

The presence of genes encoding phl and plt biosynthesis was determined by hybridization of the membranes described above (section 5.2.5, p95) with genes cloned from strains producing these antibiotics (F113 and Pf-5, respectively). Plasmids bearing the cloned genes (Table 5-2) were extracted from *E. coli* hosts using the standard alkaline lysis protocol (4.2.7.8, p75) and labelled as described above (5.2.5, p95). The stringency level was approximately 60% for all probes. Presence of DNA homologous to each of the probes was indicated by one or more hybridizing bands in the genomic digests.

5.2.9. *Data analysis and statistics*

Data analysis was performed using Unistat® version 4 for Windows™. All data sets were subjected to pairwise correlations to test for links between the different characteristics. All data were ranked and the Spearman rank correlation coefficient (ρ; Zar, 1984; Anon., 1996) calculated. The significance of each value was calculated by means of the 1-tail student's t-test (Zar, 1984; Anon., 1996). Stepwise multiple regressions (Zar, 1984; Anon., 1996) were performed to determine the importance of each of the factors assessed in this study to the ability to suppress *Pythium*-induced damping-off in peas.

5.3. Results

5.3.1. *Comparative analysis of anti-fungal activity*

The efficacy of each strain for the *in situ* control of *Pythium* infection of pea seeds is shown (Figure 5-1 and Table 5-3) and is contrasted with the *in vitro* inhibition of *Pythium* growth on agar plates. Three distinct levels of control were observed on plates. Firstly, no control was observed and the bacterial colony was completely overgrown by the *Pythium*. Secondly, *Pythium* grew to the edge of the bacterial colony but not over it and lastly, a zone of clear agar was seen around the edge of the colony where no *Pythium* growth was observed. No apparent correlation between *in vitro* and *in situ* suppression was observed. Indeed, some strains, such as F113, did not inhibit *Pythium* on plates but its control index in the disease suppression assay was 0.87, whist others, such as PH6, generated a zone of inhibition on agar but its control index was 0.33 (Table 5-3, Figure 5-1).

5.3.2. *Fatty acid profiling*

Fatty acid profiling of these twenty-nine pseudomonads revealed a large degree of divergence within the collection (Figure 5-2). By comparison with the commercially available database (MIDI, Newark, DE, USA) all but one of the strains were named as rRNA homology group I pseudomonads (Table 5-1). The exception to this was PGSB 8456, which did not match with any entries in the database. It was thought that the prolific production of an orange pigment by this strain was co-extracted with FAMEs and consequently interfered with the GC analysis. No correlation was detected between the similarity of total FAME profiles of strains and their ability to suppress disease. No clusters were identified which contained only strains with good biocontrol activity. However, further analysis of the data revealed correlations between the proportions of individual fatty acids and the control index. The clearest of these is illustrated in Figure 5-3. It seemed that any strain that had a C17:0 cyclopropane fatty acid content of greater than 5% was an effective biocontrol agent of *Pythium*. However, the exception to this association was strain 76/10.

On this basis, the proportion of C17:0 cyclopropane fatty acid in each of the strains was selected as a variable for further correlation and stepwise multiple regression analysis as described below (5.3.5).

5.3.3. *DNA profiling*

Ribotyping, as described here, demonstrated that there was a large degree of genetic variation in this collection of pseudomonads (Figure 5-5) and that the majority of the strains were genetically distinct (Figure 5-4). However, PGS12 and PGSB 8456 could not be differentiated by this method. In addition, both strains also had a very distinctive orange pigmentation when grown on solid media. Two other strains that were also very similar, CHA0 and Pf-5, which differed by the presence of a single extra band in the *Kpn*I-digested DNA. These two strains had similar FAME profiles (Figure 5-2).

Whilst there was no direct correlation between taxonomic relatedness and absolute biocontrol activity, a small cluster of strains was identified which exhibited similar anti-fungal activities (Figure 5-5). These pseudomonads all had a control index of greater than 0.7 and produced an anti-fungal compound which could diffuse through agar, as identified by a zone of clearing around the colony. The rationale for selecting these strains was that they appeared to have a similar mode of action against *Pythium* and a similar level of disease control and therefore may have evolved from a common ancestor. These strains shared greater than $63.8 \pm 6.8\%$ similarity as determined by ribotyping. Only two strains within this cluster, 2Ps4 and 1335, did not exhibit this combination of antifungal activity. Both showed some activity on plates, although there was no zone of clearing for 2Ps4, but performed poorly in disease suppression ($CI < 0.5$).

5.3.4. *Phenotypic analysis of isolates*

All the phenotypic analyses performed during the course of this study gave further evidence of the relative diversity of these group of organisms. Metabolic potentials, as determined by BiOLOG® profiling, were also used to cluster these isolates and the resulting dendrogram is shown in Figure 5-6. No apparent link between clusters of isolates and their anti-fungal activity was observed. Strains utilized between 33 (PH6) and 60 (B10 and PGSB 5589) of the 95 different carbon sources available.

The similarity of the metabolic profiles of the isolates to that of Pythium was ascertained by calculation of the nutritional similarity index (NSI). The theoretical value was between 0 and 1. However, Table 5-3 indicates that the values obtained for these isolates were all between 0.560 (strain PH6) and 0.857 (strain 54/96). The majority of strains (>85%) exhibited NSI of greater than 0.7, demonstrating the high level of nutritional overlap between *Pythium* and pseudomonads.

The use of an automated plate reader with both incubation and shaking functions facilitated concurrent and accurate assessment of the growth of each isolate in pea seed exudate. There was again a large amount of variation between strains (Table 5-3) with mean generation times varying between 2.227 h (strain UWC1) and 6.596 h (strain Q2-87), but the MGT for most isolates (>90%) was less than 4 h.

Siderophore production, as determined by the colour change of chrome azurol S in an agar medium, was also highly variable (Table 5-4). Whilst some strains (C7) appeared not to produce any compounds with affinity for ferric iron others produced significant quantities, creating a large zone of orange (up to 180 mm^2, UWC1).

The capacity of each strain to produce some of the better known anti-fungal metabolites was assessed by a variety of means. Cyanide and phenazine production were assessed phenotypically by screening for the products *in vitro*, whereas genotypic analysis for the presence of genes homologous to the 2,4-diacetylphloroglucinol and pyoluteorin biosynthetic genes was directly assessed by Southern hybridization (Table 5-4). Neither of these methods determined whether the compounds were actually produced *in situ*. The majority of the collection (>62%) were not positive for any of these well-characterized anti-fungal compounds, despite the apparent potential of these isolates to control *Pythium* infection.

5.3.5. Correlation and regression analysis of data

The matrix indicating the correlations between all characteristics analyzed is shown (Table 5-5). The correlation between cyanide production and the proportion of C17 cyclopropane fatty acid was the highest at 0.7524 ($p<0.001$). Only these two traits showed significant correlations with the control index.

Small but significant negative correlations were found between siderophore production vs. mean generation time in PSE and siderophore production vs. Nutritional similarity index (*NSI*). A small positive correlation existed between *NSI* vs. MGT. All comparative data were subjected to stepwise multiple regression to test whether a combination of factors could be used to define the ability of an individual strain to protect pea seeds against *Pythium* infection (*CI*). These tests revealed that the only variable that could be rigorously used to predict activity was the ability to produce cyanide. However, the strong correlation of C17:0 cyclopropane fatty acid with both *CI* and HCN production implied that FAME analysis could serve as a marker in the primary screening of collections to identify potentially effective pseudomonad BCAs.

5.3.6. Confirmation of the validity of identified markers

To determine the relevance of this observation, a collection of more than 500 fluorescent pseudomonads isolated from sugar beet phytosphere and soil at the Oxford University Field Station, Wytham (Ellis *et al*, 1998) was screened for the presence of a high proportion of C17:0 cyclopropane fatty acid. Thirteen numerically dominant genotypic groups were identified that were isolated repeatedly, both within and between seasons (Ellis *et al*, 1998). Of these, one group was identified with a C17:0 cyclopropane fatty acid content of approximately 15%. A single strain, *P. chloroaphis* S34/10, was assayed for the ability to suppress *Pythium* infection in the assay described above (5.2.2). Its biocontrol efficacy was as good as strains selected for biocontrol efficacy by more traditional (seedling assay) methods. The control index was determined to be 0.83. Thus, FAME analysis was successfully utilized to select an effective pseudomonad BCA.

Furthermore, biocontrol-negative mutants of two of the strains (CHA0 and F113) from the original collection in Table 5-1 with a high proportion of C17:0 cyclopropane fatty acid were obtained. Several classes of mutant were obtained (Table 5-2) and each subjected to FAME analysis. Each mutant, irrespective of whether the mutation affected a global regulatory gene or a biosynthetic gene, contained a significantly lower quota of C17:0 cyclopropane fatty acid than its corresponding wild type strain (Table 5-6).

5.4. Discussion

The first impression from the data presented here was that this group of bacteria was extremely diverse, despite all sharing the common feature of being able to suppress *Pythium* infection *in planta* to some extent. FAME profiling identified the majority of them all as members of the rRNA homology group I pseudomonads (Table 5-1), as described by Palleroni (Palleroni, 1984). Analysis of other characteristics detected differences that portrayed their apparent degree of relatedness. This versatility and variation appears to be a trait of the *Pseudomonas* genus in general and therefore it was not entirely surprising to find it in this collection. Nevertheless, as most of them were isolated from plant-associated habitats some similarities could have been expected.

The relatively limited genetic similarity between some strains is comparable to that seen in other studies utilizing similar methods for the assessment of relatedness (Rainey, Bailey & Thompson, 1994; Goddard *et al.*, 1997). This implies that the level of similarity between the strains in this collection is no greater than that of a group of pseudomonads selected randomly from a soil or plant tissue sample. It would therefore appear that anti-fungal

activity is not a feature of a specialized group of pseudomonads, but rather a general property of this genus.

Whilst both sequencing of ribosomal RNA operons (Bereswill *et al.*, 1994; Christensen *et al.*, 1994) and macro-restriction fragment profiling (Claus, Rotlich & Filip, 1992; Rainey, Bailey & Thompson, 1994; Romling *et al.*, 1994) have provided useful data on genetic diversity, the protocols are extremely time-consuming. PCR- based methodologies (Louws *et al.*, 1994; Jaunet *et al.*, 1995; Smith, J.J. *et al.*, 1995) and restriction fragment length polymorphisms (Legard, Aquadro & Hunter, 1993) have also been used for typing of *Pseudomonas* isolates. The chosen method for this study was an restriction fragment length polymorphism (RFLP) based system, which relied on the ribosomal RNA operon (*rrn*) as a probe. The genetic organization of these genes in fluorescent pseudomonads is probably highly conserved (Rainey & Bailey, 1996). Due to this conservation, RFLP analysis provided more insight into the divergence between isolates than methods analyzing less well conserved regions. Five hybridizing bands were detected when both *Kpn*I- and *Eco*RI- digested SBW25 DNA were probed with pAC10. This corresponded to the number of operons placed on the physical genetic map generated for this bacteria (Rainey & Bailey, 1996), indicating that these enzymes did not cleave within the ribosomal RNA operon. The variation observed between the isolates in this study was therefore an assessment of the DNA flanking the *rrn* loci and indicative of differences in genome organization. Assessment of ribotypes by digestion of genomic DNA with two restriction enzymes, such as *Eco*RI and *Kpn*I , produced the same groupings, proving that these results are not artefacts of the chosen method. Thus, ribotypes presumably signify divergent clonal groups.

Cluster analysis based on phenotypic traits (FAME and carbon source utilization) augmented the perception of diversity within the group. However, all methods clustered the majority of isolates into different groups indicating that no one data set gave a true representation of the relatedness of these strains. Such lack of conformity between clusters from phenotypic and genotypic data has been recorded previously (Rainey, Bailey & Thompson, 1994; Natsch *et al.*, 1997) and was tentatively attributed to intragenomic recombination and/or point mutations making phylogenies based on RFLP data incorrect. However, data from the FAME analysis of the transposon mutants indicated that significant changes in phenotype may also be attributed to these types of genomic alterations (4.3.2, p75, Table 5-6). Alternatively, the contradictions may merely reflect the notoriously complex and diverse nature of the fluorescent pseudomonad group (Rainey, Bailey & Thompson, 1994). The taxonomy of these organisms is continually changing as more

information is accumulated, and therefore relationships drawn up on the basis of phenotypic and genotypic traits may be artificial.

The lack of correlation between *in vitro* inhibition of fungi and the ability to suppress disease caused by those fungi *in planta* has been documented previously (Reddy, Hynes & Lazarovits, 1993). This may be attributed to the differential expression of a variety of genes *in situ* and *in vitro*. Bacterial cells will always respond differently to artificial media and natural growth conditions. This observation indicates that potential biocontrol agents must be screened for their *in planta* activity, as *in vitro* antibiosis may purely be an artefact of the unnatural culture conditions employed. It must be remembered that the potential to produce anti-fungal metabolites is not the only prerequisite for control efficacy and that ecological fitness is just as important, if not more so.

Despite the significant change in cellular physiology caused by mutational disruption of the phl biosynthetic coding region of F113, as determined by FAME analysis, the ecological fitness of this mutant was not affected (Carroll *et al.*, 1995). This was surprising considering that a change in the fatty acid composition will invariably change the fluidity of the membrane. However, *cfa* (encoding cyclopropane fatty acid synthase) in *E. coli* carries a σ^{38} (*rpoS*) prompter, and therefore is expressed at the log to stationary transition phase (Wang & Cronan Jr, 1994) and thus these changes may only effect bacteria that are not actively growing.

The question that is now posed is: what is the relationship between cyclopropane fatty acid biosynthesis and the ability to suppress Pythium infection of pea seedlings? It has been shown previously that the C17:0 cyclopropane fatty acid is found in higher proportions in the *P. fluorescens* subgroup of the rRNA homology group I pseudomonads than in other subgroups (Vancanneyt *et al.*, 1996). It may therefore be more than coincidence that it is this group of bacteria that have been isolated most frequently when screening for biological control agents. There are several pieces of circumstantial evidence that may aid in determining the link between these coexisting phenotypes.

Firstly, searching sequence data bases has revealed that *phlC* (DAPG biosynthetic locus) has homology to *fadBA* (*E. coli* fatty acid synthesis). This may indicate that both FA and phl (a polyketide compound) synthesis are dependent to some extent on a shared biosynthetic pathway. The similarities between chain elongation in FA and polyketide synthesis are well documented (Hopwood & Sherman, 1990; Cane, 1994) Whilst the biosynthetic pathways are understood, the mechanisms controlling polyketide metabolism are at best sketchy (Cane, 1994) and may be linked to changes in cellular physiology. For

example, production of the polyketide phytotoxin, coronatine, in *Pseudomonas syringae* is temperature dependent and controlled by a two-component regulatory system (Ullrich *et al.*, 1995).

Secondly, evidence exists which suggests that fatty acid derivatives may act as autoinducers in bacteria closely related to *Pseudomonas* (Clough, Schell & Denny, 1994; Clough *et al.*, 1997). If such systems existed in the fluorescent pseudomonads under investigation in this study, a change in the level of expression of such a compound could cause the major changes in metabolism seen in F113 and CHA0. Thus, a possible theory is that the primary function of 'antifungal metabolites' is for the regulation of cellular activity of the producer. The fact that it also has activity against competing organisms may just be coincidence or a perfect example of the efficiency of adaptation and evolution. The non-specific nature of pseudomonad antibiotics bears out this theory. Polyketides, such as phl, are general antibiotics, with activity against other bacteria, such as *Erwinia* spp. (Cronin *et al.*, 1997), as well as fungi and therefore, contradictory to some beliefs (Bosnall, Weller & Thomashow, 1997), it is not surprising that its production in raw soils is greater than that in gnobiotic studies (Bosnall, Weller & Thomashow, 1997). Whether cyanide is produced solely as an antibiotic is also questionable. A major effect that has been identified is that it increases exudation from plant tissue (Åström, 1991) and this may be the major role for its production. Cyanide production by pseudomonads appears to be fairly common (Castric, 1977) but it does not appear to affect population sizes of soil bacteria (Piotrowska-Seget & Sroka, 1996). It has been noted that all pseudomonads that contained DNA homologous to the phl biosynthetic genes produced cyanide (Keel *et al.*, 1996). However, the reverse is not true. This implies that HCN production will be a more widespread phenotype than phl production.

Thirdly, the link between metabolite production and transcriptional regulatory factors has been well documented (Natsch *et al.*, 1994; Schnider *et al.*, 1995a). Assuming that these regulators from different bacteria may show differences in their efficiency of regulation, it is possible that the difference between 'potent' and 'poor' BCAs may be due to effectiveness of sigma or other transcription factors. Over expression of the housekeeping sigma factor (σ^{70}) has been shown to enhance pleiotrophic antibiotic production in CHA0 (Schnider *et al.*, 1995a). This indicates that a regulator that has the ability to enhance expression could also have the same effect.

Finally, such regulatory systems have also been linked to density dependent expression of antibiotics such as phz (Pierson III & Pierson, 1996), which effectively draws together the

observations above. If it is considered that phl may be a cell density-dependent autoinducer, analogous to homoserine lactones (Salmond *et al.*, 1995) in terms of its function, this would explain the both the reduction in disease suppression (Keel *et al.*, 1992), either directly or indirectly, and the general changes in cellular metabolism, when the gene for its production is mutated. The interactive effects of genotype on production of two different compounds have been documented (Rodriguez & Pfender, 1997).

The production of anti-fungal compounds has been shown to be under the control of a global regulatory system in a number of strains (Laville *et al.*, 1992; Gaffney *et al.*, 1994; Corbell & Loper, 1995) and it appears that cyclopropane FAs are also. Thus, the correlations between the proportions of C17:0 cyclo and the production of anti-fungal metabolites may indicate that presence of the fatty acid signals an efficient switch to the secondary metabolism pathways or perhaps just a switch to polyketide synthesis pathways. Either way, screening for pseudomonads with high cyclopropane FA contents may aid in the search for effective BCAs.

The observations here, that the disruption of a single gene by the insertion of a transposon can affect the basic biochemical composition of a cell, augments the information in the previous chapter (4.3.2, p75) on FAME analysis of mini-Tn*5*-induced insertional mutants. The implication from this is that great care should be taken when attempting to prove the role of a given gene in a process. As no single gene product acts completely autonomously, it is probable that any mutation in a single gene will have unknown knock-on effects on many other pathways and thus any one of these may effect the process under examination.

Strains showing anti-fungal activity on plates and biocontrol probably produce antibiotics whilst those which show no effect on agar may prevent the infection process rather than growth of fungi. However, PSA-CFC contains very little iron and therefore the most prevalent activity could be due to siderophore production (Becker & Cook, 1988), but there is no significant correlation between *in vitro* activity and siderophore production.

Niche overlap indices, as determined by nutritional profiling, have been used to identify strains that are capable of coexistence in the same habitat (Janisiewicz, 1996), and has been reversed to identify organisms that are not compatible (Wilson & Lindow, 1994). However, nutritional profiles of fungi and bacteria have not previously been compared for the purpose of biological control. The ability of pseudomonads to utilize a wide range of carbon sources has been well documented (Palleroni, 1992; Schroth, Hildebrand & Panopoulos, 1992) and is reflected in these results but the nutritional versatility of *Pythium*

was unexpected. The relatively high similarity between the metabolic potentials of the fungi and bacteria may reflect the range of nutrients available in soil and plant-associated habitats.

The problem with comparing metabolic profiles of distantly related organisms is that BiOLOG® plates provide no information on the relative affinity of each organism for any given substrate and therefore the relevance to the ability to utilize carbon sources at ecological concentrations is speculative. However, the profiles do identify the potential for metabolism and thus comparisons do identify the potential for competition.

Rather than examine the role of a variety of factors in a single strain, as was done in the previous chapter and by other workers (O'Sullivan & O'Gara, 1992), this study attempted to determine key factors related to biocontrol efficacy by comparison of their prevalence in a number of strains. The variation between strains was surprising large for all of these factors and, with the exception of cyanide production, stepwise regression analysis revealed that none could be used to explain the *Pythium* suppression observed in seedling trials. This implies that either the adaptations are of a more general nature or that biocontrol efficacy is strain specific with no commonality between them.

That such large variation in attributes exists within such a small collection of bacteria implies that there are more as yet undiscovered bacteria that may prove to be more effective biocontrol agents. In order to find them screening strategies must be improved and focused on isolation of novel organisms, not on finding bacteria with known traits.

However, the fact that the proportion of C17:0 cyclopropane fatty acid could be used as a marker for biocontrol efficacy was proved by its use to select a strain with high biocontrol efficacy from a large collection of collection of environmental isolates. The implications of this are that, using this method, effective biological control agents could be selected from any given environment. At present such a task is unmanageable because of the implausibility of *in planta* screening of a large number of strains. Such selection will mean that strains used will be naturally adapted for the local conditions, rather than trying to produce a globally useful BCA.

Table 5-1: *Pseudomonas* strains used in this study.

Isolate	Source	Habitat	MIDI ID
SBW25	Bailey & Thompson, 1992; Bailey *et al.*, 1995	Sugar beet phylloplane, Oxford	*P. fluorescens* A
54/96	Zeneca Agrochemicals	Sugar beet, Belgium	*P. fluorescens* A
1335	Zeneca Agrochemicals	Sugar beet, Belgium	*P. chloroaphis*
76/10	Zeneca Agrochemicals	Sugar beet, Belgium	*P. chloroaphis*
2-79	Thomashow & Weller, 1988	Wheat, Washington	*P. marginalis*
Q2-87	Bangera & Thomashow, 1996	Wheat, Washington	*P. fluorescens* C
CHA0	Voisard *et al.*, 1989	Tobacco, Switzerland	*P. putida* A
Pf-5	Kraus & Loper, 1992	Cotton rhizosphere, Texas	*P. putida* A
2Ps4	van Elsas, NL	Soil, NL	*P. cichorii*
GE1	van Elsas, NL	Soil, NL	*P. fluorescens* A
P1	van Elsas, NL	Grass rhizosphere, NL	*P. chloroaphis*
R2f	van Overbeek & van Elsas, 1995	Grass rhizosphere, NL	*P. putida* A
R12T	van Elsas, NL	Grass rhizosphere, NL	*P. fluorescens* A
PH6	Fuhrmann, 1991	-	*P. putida* A
C7	Latour *et al.*, 1996	Flax rhizosphere, Chateaurenard	*P. savastanoi*
M114	Fenton *et al.*, 1992	Soil, Ireland	*P. fluorescens* B
F113	Fenton *et al.*, 1992	Soil, Ireland	*P. savastanoi*
A1	Fukui *et al.*, 1994a	Potato periderm, California	*P. putida* A
CR30	Fukui *et al.*, 1994a	Field soil, California	*P. marginalis*
ML5	Osburn *et al.*, 1989	Sugar beet spermosphere, California	*P. chloroaphis*
PGS12	Fukui, USA	-	*P. chloroaphis*
R20	Osburn *et al.*, 1989	Lima bean rhizosphere, California	*P. putida* A
UWC1	Cardiff, UK	-	*P. savastanoi*
PGSB 1500	PGS, Belgium	Sugar beet rhizosphere, Belgium	*P. fluorescens* B
PGSB 5589	PGS, Belgium	Sugar beet rhizosphere, Belgium	*P. savastanoi*
PGSB 8456	PGS, Belgium	Sugar beet rhizosphere, Belgium	No match
7SR1	Buyer & Leong, 1986	-	*P. aeruginosa*
A214	Buyer & Leong, 1986	-	*P. viridiflava*
B10	Buyer & Leong, 1986	-	*P. cichorii*

Table 5-2. Organisms and plasmids used in this study.

Organism or plasmid	Relevant characteristic	Source
Fungi		
Pythium ultimum	Causal agent of damping-off disease	Zeneca Agrochemicals
Plants		
Psium sativum var. 'Bohartyr'	Forage pea; susceptible to *Pythium* damping-off	Nickerson Seeds, Linconshire, UK
Plasmids (probes used)		
pAC10	16-23S rRNA operon from *Pseudomonas aeruginosa* PAO	Housiaux, Hill & Petersen, 1988a, b
pCU203	phl biosynthetic locus from F113	Fenton *et al.*, 1992
pJEL5786	plt biosynthetic locus from Pf-5	Loper, pers comm.
Pseudomonad mutants		
F113-16Z1	Tn*5*-*lac* induced *lem*A mutant of F113	Fenton *et al.*, 1997
F113-G22	Tn*5*-*lac* induced phl$^-$ mutant of F113	Fenton *et al.*, 1992
F113-SF1	Tn*5*-*lac* induced *gacA* mutant of F113	Fenton *et al.*, 1997
CHA89	Km^r marker-replacement *gacA* mutant of CHA0	Laville *et al.*, 1992
Other pseudomonads		
Pseudomonas chloroaphis S34/10	Greater than 10% C17:0 Cyclopropane Fatty Acid	Ellis, Thompson & Bailey, 1998

Table 5-3: Nutritional, antifungal, growth and disease suppression potentials of *Pseudomonas* strains listed in Table 5-1.

Isolate	NSI[a]	In vitro activity[b]	*CI*[c]	MGT in PSE[d]
SBW25	0.810	2	0.75	3.095
54/96	0.857	2	0.84	2.493
1335	0.757	2	0.47	2.368
76/10	0.772	1	0.50	3.667
2-79	0.850	1	0.82	2.892
Q2-87	n.d.	0	0.24	6.596
CHA0	0.727	2	0.84	2.775
Pf-5	0.708	2	0.83	2.670
2Ps4	0.772	1	0.44	3.281
GE1	0.838	2	0.83	3.504
P1	0.757	1	0.24	2.897
R2f	0.757	2	0.57	3.091
R12T	0.838	0	0.72	3.325
PH6	0.560	2	0.33	2.563
C7	0.797	2	0.65	5.350
M114	0.777	1	0.76	4.109
F113	0.803	0	0.87	3.963
A1	0.604	0	0.68	3.080
CR30	0.832	2	0.46	2.362
ML5	0.704	1	0.91	2.903
PGS12	0.807	2	0.84	2.943
R20	0.661	0	0.76	2.550
UWC1	0.685	0	0.61	2.277
PGSB 1500	0.836	2	0.79	2.974
PGSB 5589	0.803	2	0.55	3.712
PGSB 8456	0.737	2	0.86	2.507
7SR1	0.767	2	0.55	3.472
A214	0.777	2	0.55	3.095
B10	0.772	1	0.82	3.317

[a] Nutritional similarity index calculated from BiOLOG® profiles of strains compared to BiOLOG® profiles of *Pythium* using Equation 5-3

[b] Level of inhibition of *Pythium* growth on PSA.

[c] Control index calculated from disease suppression assays using Equation 2-1.

[d] Mean generation time of each strain for growth in pea seed exudate.

Table 5-4: Metabolite production potential of *Pseudomonas* strains listed in Table 5-1.

Isolate	HCN[a]	Phenazine[b]	phl[c]	plt[d]	Siderophores[e]
SBW25	-	-	-	-	33.3
54/96	-	-	-	-	76.0
1335	-	-	-	-	107.6
76/10	3	-	-	-	40.4
2-79	-	+	-	-	38.64
Q2-87	-	-	+	-	n.d.
CHA0	4	-	+	+	110.3
Pf-5	3	-	+	+	81.5
2Ps4	-	-	-	-	107.1
GE1	-	-	-	-	139.9
P1	-	-	-	-	76.5
R2f	-	-	-	-	95.0
R12T	-	-	-	-	36.7
PH6	-	-	-	-	110.3
C7	3	-	-	-	0
M114	2	-	-	-	136.2
F113	3	-	+	-	10.7
A1	-	-	-	-	165.3
CR30	-	-	-	-	78.5
ML5	4	-	-	-	36.7
PGS12	3	+	-	-	104.3
R20	-	-	-	-	81.2
UWC1	-	-	-	-	180.8
PGSB 1500	-	-	-	-	92.3
PGSB 5589	-	-	-	-	52.4
PGSB 8456	5	+	-	-	71.8
7SR1	-	-	-	-	62.1
A214	-	-	-	-	13.7
B10	-	-	-	-	70.9

[a] Determined as (Voisard *et al.*, 1989). Numbers indicate the intensity of the blue colour that developed as a result of cyanide production.
[b] Determined by production of green pigment on Potato Dextrose Agar (Thomashow & Weller, 1988)
[c] Determined by hybridization to cloned fragment from F113 (Fenton *et al.*, 1992)
[d] Determined by hybridization to cloned fragment from Pf-5 (Loper, pers. comm.)
[e] Area of orange halo produced on CAS agar (mm^2) (Schwyn & Neilands, 1987). Minimum significant difference (MSD) = 34.0.

Table 5-5: Spearman rank correlation coefficients for the traits listed in Table 5-3 and Table 5-4. Coefficients are only given where $p<0.05$ (95% significance level). Numbers in parentheses indicate the actual level of significance as determined by the 1-tail student's T-test. Keys for traits are listed inTable 5-3 and Table 5-4.

	CI	*In vitro*	MGT	Siderophore	*NSI*	C17:0 cyclo	HCN	phz	phl	plt
CI	-									
In vitro	ns[a]	-								
MGT	ns	ns	-							
Siderophore	ns	ns	-0.3751 (0.025)	-						
NSI	ns	ns	0.3304 (0.043)	-0.3862 (0.021)	-					
C17:0 cyclo	0.4330 (0.011)	ns	ns	ns	ns	-				
HCN	0.5497 (0.001)	ns	ns	ns	ns	0.7524 (<0.001)	-			
phz	ns	ns	ns	ns	ns	ns	ns	-		
phl	ns	ns	ns	ns	ns	ns	ns	ns	-	
plt	ns	ns	ns	ns	ns	ns	ns	ns	ns	-

[a] Correlation not significant at the 95% level.

Table 5-6: Proportion of cyclopropane C17:0 fatty acid in F113, CHA0 and their biocontrol negative mutants listed in Table 5-2.

Strain or Mutant	Proportion of C17:0 cyclo (%)[a]
F113	6.05
F113-16Z1	0.99
F113-G22	2.80
F113-SF1	0.45
CHA0	18.24
CHA89	3.25
MSD[b]	0.78

[a] As determined by FAME profiling

[b] Minimum significant difference calculated from triplicates of each value.

Figure 5-1: Graph illustrating the relation ship between *in vitro* inhibition of *Pythium* and the control of *Pythium* infection of peas in disease suppression assays. Colours of bars indicate the degree of *in vitro* inhibition whilst the height is proportional to the level of control observed.

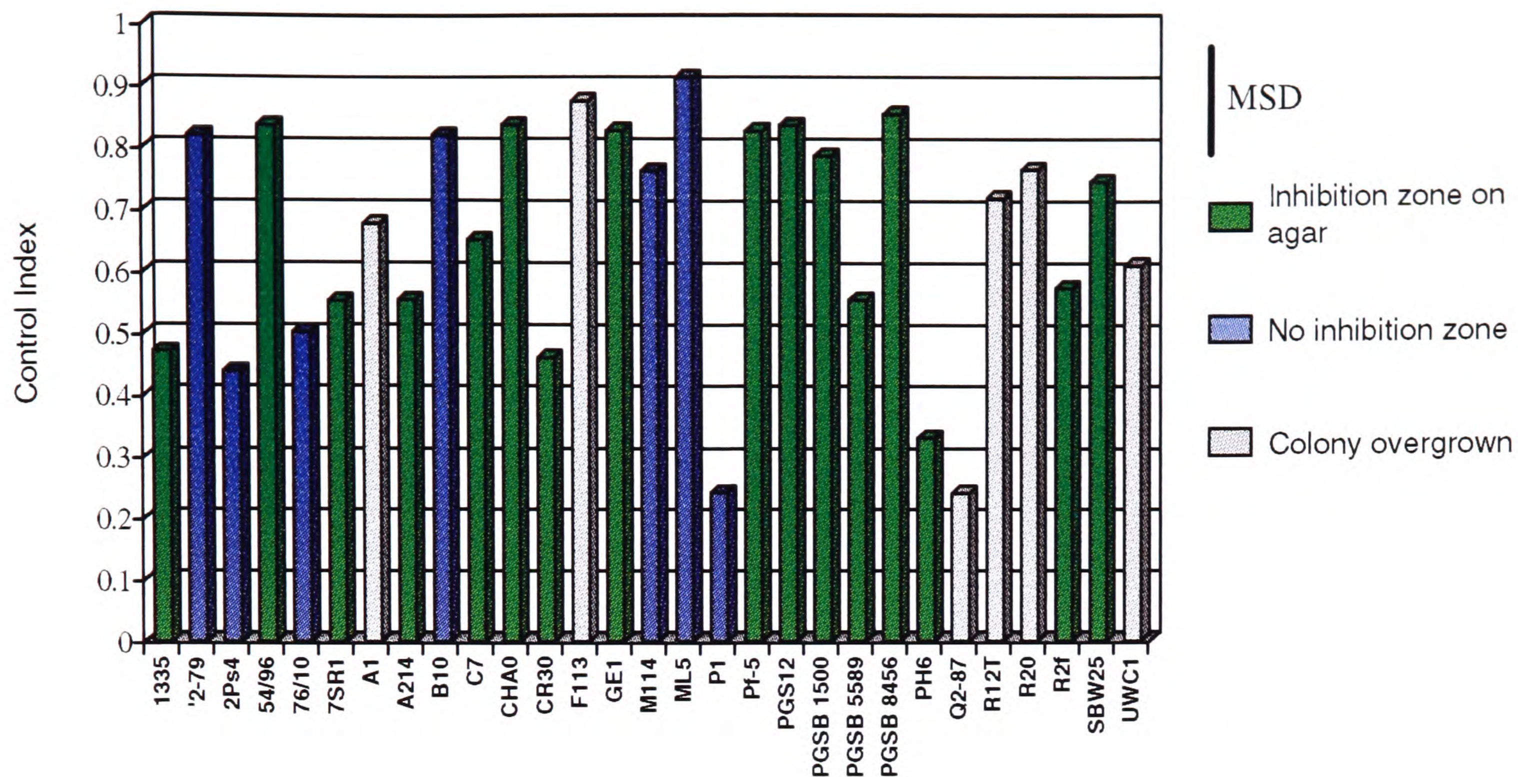


Figure 5-2: Dendrogram indicating the relatedness of the biological control strains listed in Table 5-1, according to the analysis of whole cell fatty acids. Euclidean distances are calculated on the basis of the relative proportions of individual fatty acids of pairs of strains.

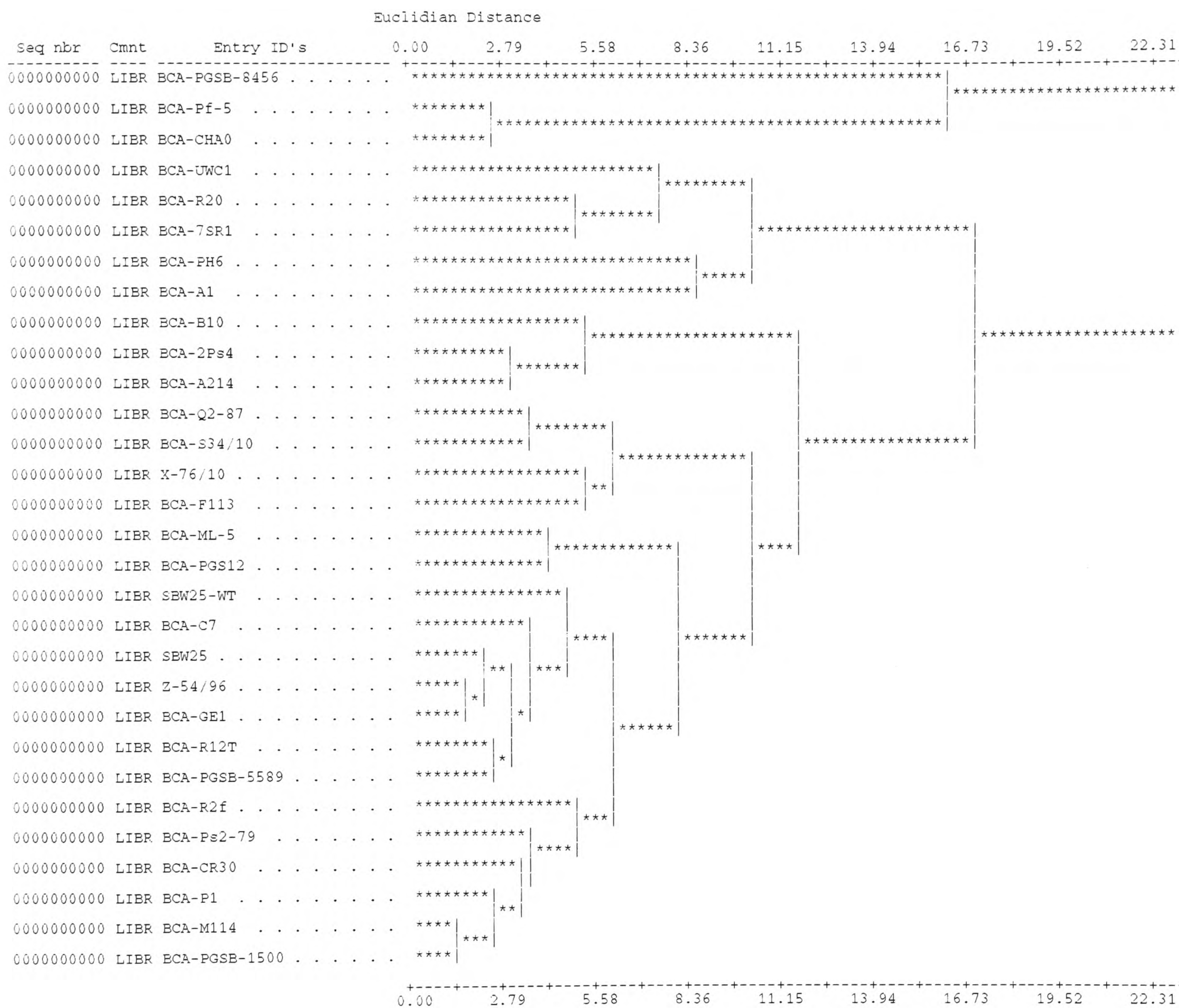


Figure 5-3: Relation ship between the ability to suppress damping-off and the proportion of 17-carbon cyclopropane fatty acid in individual *Pseudomonas* isolates.

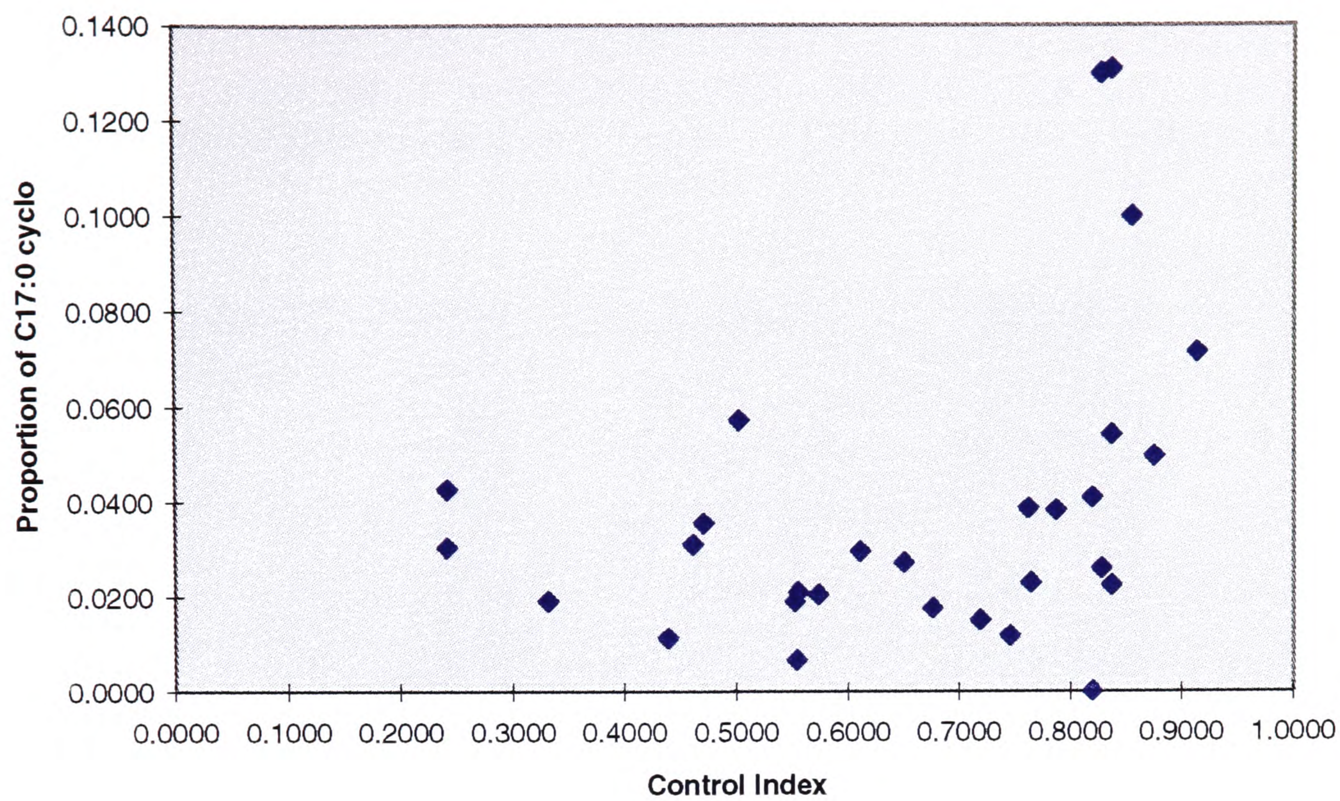
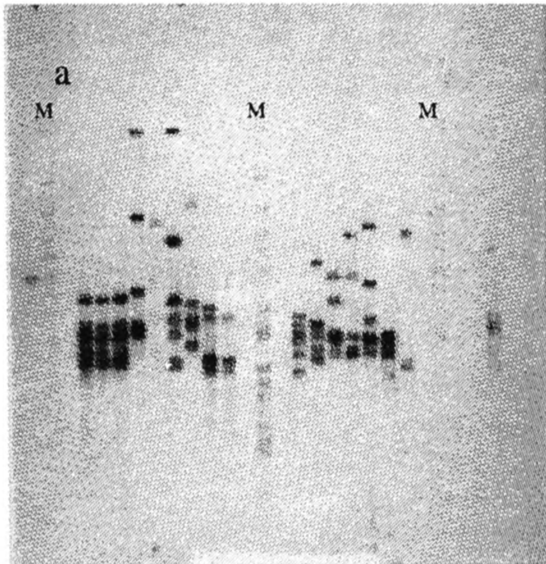
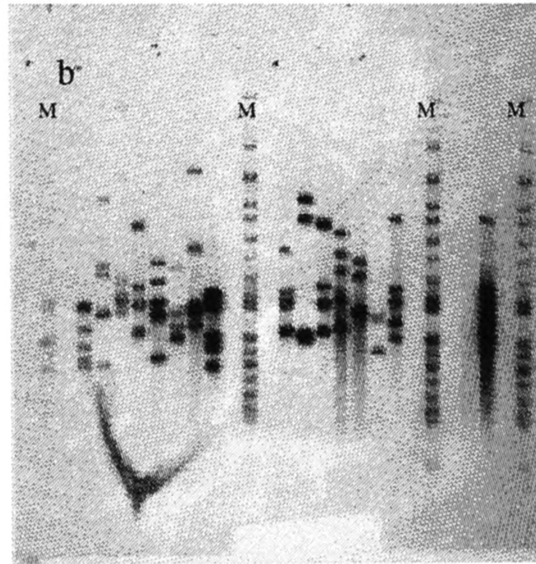
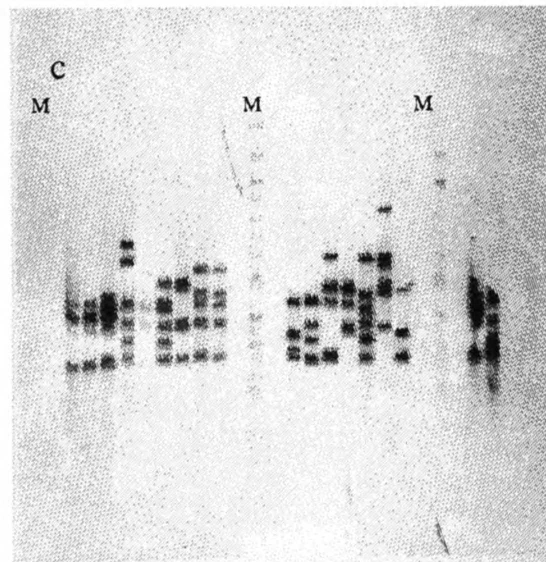


Figure 5-4: Autoradiograms of size-fractionated, restricted DNA probed with part of the ribosomal RNA operon from *Pseudomonas aeruginosa* PAO. The DNA in gels (a) and (b) was digested with *Kpn*I and the DNA in gels (c) and (d) was digested with *Eco*RI. 'M' signifies λ DNA size marker. The other lanes on the gels are, reading from left to right: gels (a) and (c); M, 54/96-1569, 54/96-1882, 54-96-3296, 76/10, 1335, 2-79, Q2-87, CHA0, Pf-5, M, 2Ps4, GE1, P1, R2f, R12T, PH6, C7, M, 54/96, SBW25 and gels (b) and (d); M, A1, CR30, ML5, PGS12, R20, F113, M114, UWC1, M, PGSB 1500, PGSB 5589, PGSB 8456, 7SR1, A214, SBW25, M, 54/96, SBW25, M.







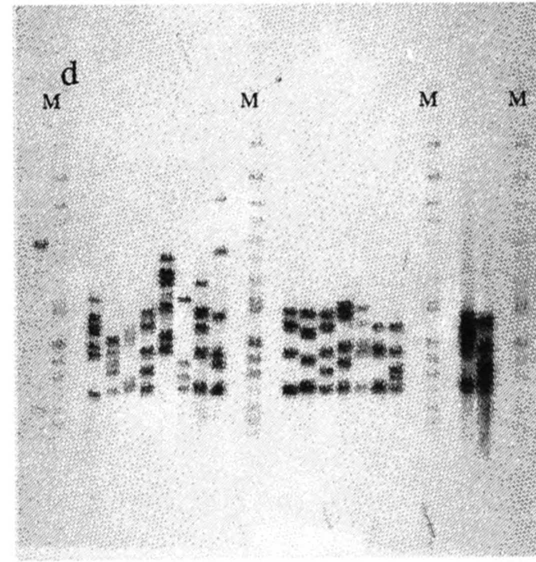


Figure 5-5: Dendrogram indicating genetic relatedness between the strains listed in Table5-1 as determined by RFLP analysis of the rDNA operon on two enzyme digests of genomic DNA. The scale shows percentage similarity as determined by Dice coefficients. Bars at branch points indicate confidence limits for the branch. Those strains marked in red produce an inhibition zone of *Pythium* on agar and control the infection of peas with a control index greater than 0.7.

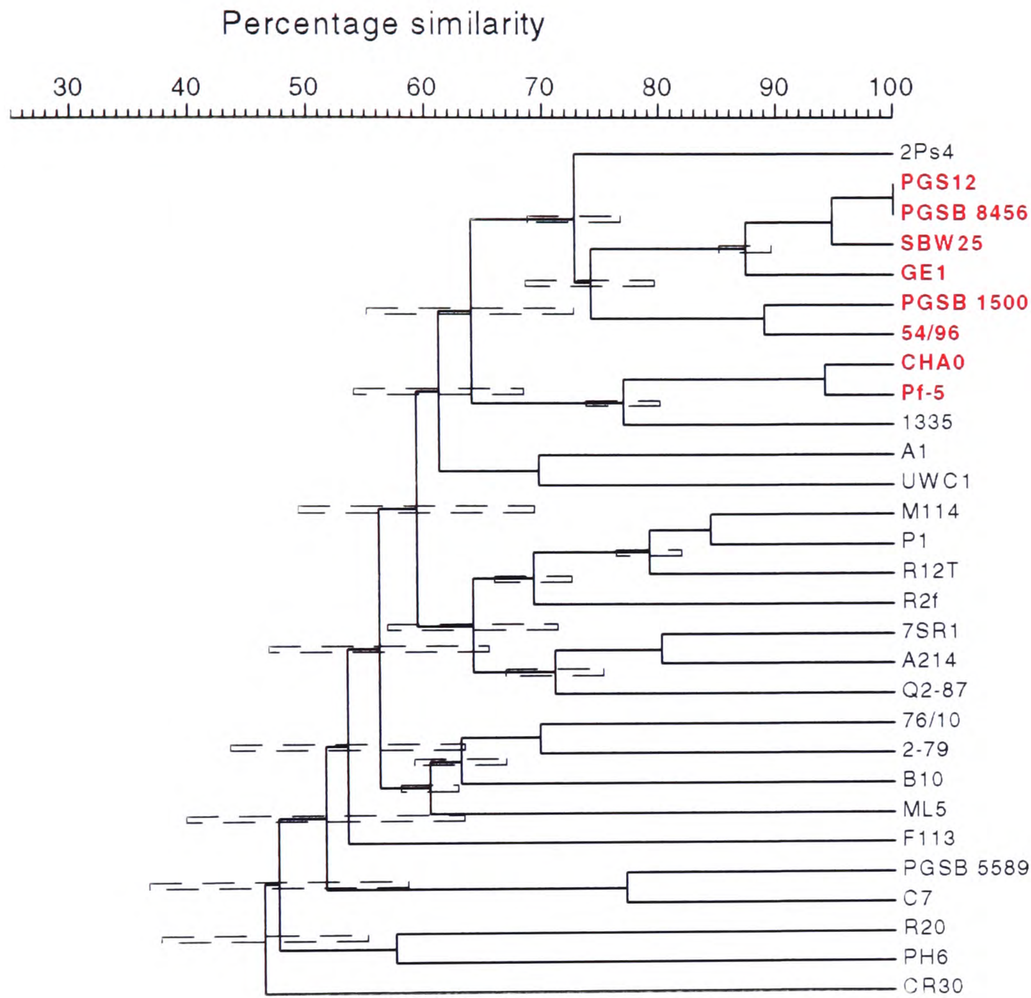
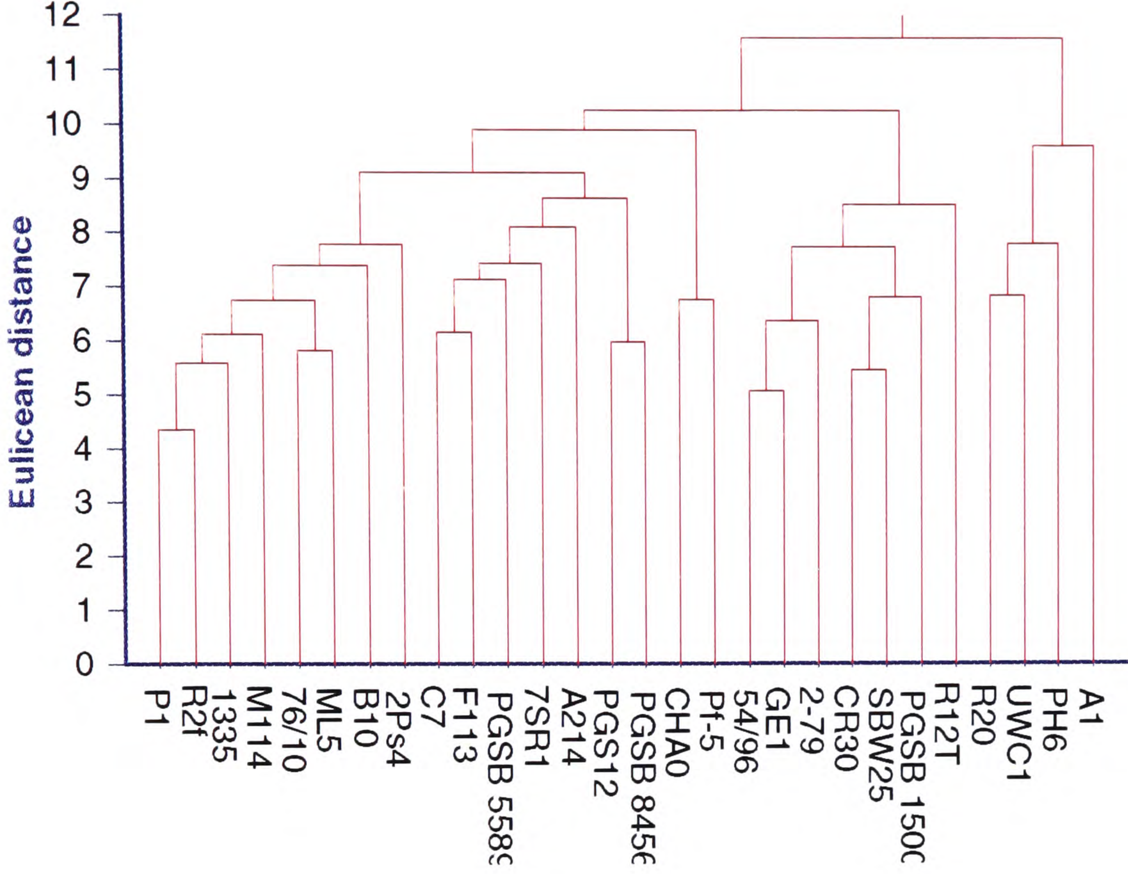


Figure 5-6: Dendrogram indicating relatedness between the strains listed in Table 5-1 as determined by BiOLOG® metabolic profiling. Euclidean distances are calculated on the basis of the differences between profiles. These data were then clustered using the unweighted pair group method of averages.



6. DISCUSSION

The aim of this thesis was to gain an understanding of the molecular and ecological basis for the biological control of *Pythium* by fluorescent pseudomonads. To this end, a fluorescent pseudomonad BCA, *Pseudomonas fluorescens* 54/96, identified as a potential candidate for commercial development, was analysed together with transposon induced mutants in a variety of assays for anti-fungal activity (Chapter 2). The synecology of this organism with *Pythium* was then compared to a similar organism (*P. fluorescens* SBW25) demonstrating a similar degree of anti-fungal activity (Chapter 3). The similarity of the population dynamics of these two strains prompted an examination of the genetic basis for the anti-fungal activity of the second strain, with the intention of comparing with 54/96 (Chapter 4). The comparison of strains was expanded to a larger collection of pseudomonad BCAs which were contrasted by a number of phenotypic and genotypic methods (Chapter 5).

It has been suggested that in order for a microbial inoculant to work successfully it must possess some ecological advantage (van Veen, van Overbeek & van Elsas, 1997). For example, bacteria capable of degrading a xenobiotic will be at an obvious ecological advantage in soil contaminated with that compound. However, the advantage of preventing fungal plant diseases is less apparent. In Chapter 5 it was suggested that antibiosis may be a general stress response to overwhelming competition and therefore the advantage would be survival. Alternatively, bacteria may protect the plant as it is their nutrient source and they are only capable of proliferation in its presence. Whichever the case, these scenarios could be manipulated so as to improve the selective advantage of fungal antagonism, thus improving the degree of biological control observed.

The fungistatic effect induced by 54/96 (Chapter 2) was a clear indication of the direct interaction between the pathogen and its antagonist. It was also clear that the effect was not due to a single mechanism, but several, the implication being that the antagonism was not coincidental but had arisen from necessity. Thus, the evolution of the mechanisms must have provided the bacterium with an ecological advantage. The fact that some fluorescent pseudomonads preferentially activate these mechanisms in plant-associated habitats and not under laboratory conditions (Chapter 5) implied that antagonism may have specifically evolved to enhance the proliferation of pseudomonads in such habitats. Indeed, this specificity has evolved to the point where anti-fungal mechanisms are only active in the

presence of specific plant hosts (Maurhofer *et al.*, 1994; Schmidli-Sacherer, Keel & Défago, 1997).

However, the population densities of pseudomonads in the environment are generally lower than those used to achieve biological control. The inference is, that under natural conditions, the ecological advantage conferred by the anti-fungal mechanisms are insufficient to allow extensive proliferation. Thus, if ecological advantage is not achievable the initial inoculum density must be increased to attain the desired effects (van Veen, van Overbeek & van Elsas, 1997). The use of immobilization/carrier materials has potential for use in the addition of biological control agents to soil and seeds (Harman & Lumsden, 1990) and will aid in maximizing the number of active cells delivered to the target site (van Veen, van Overbeek & van Elsas, 1997). The use of natural complex materials such as clay and peat have received the most attention (van Veen, van Overbeek & van Elsas, 1997). It has been proposed that it is the protective environment that they afford which improves the survival and activity of introduced cells and it may be for this reason that when cells are added to soil several days prior to planting an improvement in biocontrol efficacy is observed (Chapter 2). The bacterial inoculum may bind to clay particles in the loam and stabilize before activity is required thus providing a more homogeneous response when seeds are planted.

In order to be most effective in natural situations one of the key features is that antagonists should demonstrate a degree of specificity towards the pathogen in question. If the antagonistic effects they exert are too wide ranging then other members of the microbial community may be adversely effected, thus altering the general stasis of beneficial plant-microbe interactions. They may adversely effect other beneficial organisms such as the symbionts (mycorrhiza and *Rhizobium*). Alternatively, it may be that some metabolites are phytopathogenic. For example, some species of *Pythium* have been shown to infect other fungi, but being pathogenic to plants they are not suitable for exploitation as biological control agents (Hendrix & Campbell, 1983). It would appear that at least some of the strategies utilized by pseudomonads, such as nutrient and iron assimilation, are non-specific. It has been shown that *P. fluorescens* F113 affects populations of other bacteria such as *Erwinia* on plants (Cronin *et al.*, 1997) and that *P. fluorescens* suppress the growth of *Escherichia coli* under laboratory conditions (Stevenson, 1994).

As it stands, artificial manipulation of certain features, to simulate millennia of evolutionary processes (Stotzky, 1997), would probably be required to ensure pseudomonads were efficacious biological control agents. It would appear that both the ecological advantage

conferred by the anti-fungal mechanisms and their specificity for the pathogen could be improved to enhance the efficiency of these pseudomonad BCAs. It may prove to be more cost effective to search for more specialized organisms.

Interactions between pathogen, BCA and host plant are seldom isolated from other organisms in the system. It has been shown that even symbiotic mycorrhiza and *Rhizobium* spp. are capable of controlling fungal disease (Hassan Dar, Zargar & Beigh, 1997) but these may be direct interactions between the microbes or the symbionts may improve general plant vigour in such a way as it becomes more resistant to the pathogen. Thus, another possibility is to breed plants to improve the effectiveness of microbial inoculants for biocontrol (Smith, K.P., Handelsman & Goodman, 1997). This may be done in a variety of ways. Firstly, an improvement of disease suppression may be observed by altering the plant's response to the presence of the BCAs, for example, by the induction of systemic resistance. Secondly, the plant may be manipulated to support more effective population densities of the pseudomonads, for example, by selectively increasing the exudation of some nutrients from seeds and roots. Thirdly, as some cultivars have been shown to be sensitive to some microbial metabolites (Åström, 1991) greater resistance to anti-fungal metabolites, such as cyanide, could be bred into cultivars to allow the use of higher doses for the suppression of fungi.

Numerous other strategies have been suggested that may improve biocontrol efficacy in field and glasshouse applications. The application of mixtures of strains has been suggested as a way of overcoming the inconsistent performance observed (Pierson & Weller, 1994; Voisard *et al.*, 1994; Janisiewicz, 1996; van Veen, van Overbeek & van Elsas, 1997). Since there are many different mechanisms by which bacteria are antagonistic towards fungal pathogens, it seems logical that the most effective biocontrol strategy would be the application of a cocktail of strains with different anti-fungal activities and environmental preferences. Thus, if conditions prove to be unsuitable for one strain, another may proliferate and provide the required level of plant protection. However, it is not essential that strains of the sane genus or even kingdom are applied as promising results have been achieved by the application of a combination of *Trichoderma* and *Pseudomonas* (Duffy, Simon & Weller, 1986). In addition, mycorrhizal fungi may serve to stimulate some biocontrol bacteria (Nemec, 1997) and thus co-inocualtion of mycorrhiza and BCA holds distinct promise for the future.

Conversely, the fact that bacterial colonization and survival can be affected by the presence of fungal species (Mazzola & Cook, 1991) and other pseudomonads (Fukui *et al.*, 1994a) in

the soil may help explain the inconsistent results achieved with biological control agents in the field. The natural microflora will affect the ability of inoculants to perform and thus should be surveyed before BCAs can be applied effectively. The soil used for seedling assays throughout this thesis had been commercially sterilized before use and therefore few indigenous organisms were present. Thus, bacterial inoculants were unimpeded in terms of competing with existing microbes. The use of non-sterile soil would probably have had a significant impact on the population densities and activities of the introduced organisms (van Veen, van Overbeek & van Elsas, 1997). This impact will also be dependant on physiochemical soil properties, such as water tension, carbon availability, pH and temperature (van Veen, van Overbeek & van Elsas, 1997), and thus will differ between different sites.

Consequently, the selection of BCAs from the site into which they are to be introduced seems to be a logical progression (Weller, 1988). If organisms are selected from the predominant populations then it is probable that they will be naturally adapted for activity and persistence in that particular habitat. Unfortunately, due to the expense of screening programs to find organisms that are sufficiently antagonistic towards the pathogen of interest, the selection of strains from individual sites has not been feasible. However, the identification of potential 'molecular markers' for biological control activity may reverse this preclusion. It has become apparent that C17:0 cyclopropane fatty acid is a marker for anti-fungal activity in fluorescent pseudomonads (chapter 5). FAME profiling may be automated and thus the screening of collections of pseudomonad isolates from different sites may be practicable. If so, the search for 'universal' BCAs will become inefficient and unnecessary.

It has been shown that biological interactions are extremely complex and impossible to unravel unless all aspects of the interactions are studied alongside one another. For example, without an understanding of the dynamics of a population in an environment an indication of total activity would be meaningless. Thus all studies of micobial ecology should be multifaceted and include molecular analysis of the desired traits, and studies of autecology and synecology under a variety of biotic and abiotic conditions.

6.1. Wider implications for molecular microbial ecology

The extrapolation of bacterial behaviour under laboratory conditions to the perception of their activity in the natural environment, especially to those systems where the availability of resources is always limiting, must be viewed with scepticism. In order to fully understand bacteria in their natural habitat they must be studied *in situ* (Whiteley *et al.*, 1996). The

advent of confocal microscopy, together with the design of probes and marker genes for important processes, should aid in this quest (Hansen *et al.*, 1997; van Veen, van Overbeek & van Elsas, 1997). However, microbial ecologists must rethink their theories of cellular activity and physiological status in the environment as they do not correspond to those found under artifical laboratory conditions.

In the same way, the results from Chapter 3 indicate that it is of vital importance to determine the ecological relevance of any experimental design before embarking upon a project. For example, one would not plan to examine an oak tree on a daily basis to determine the impact of a pollutant. A more appropriate time scale, considering the growth rate of the tree, would be to take samples annually or perhaps every decade. In the same way, microbial populations with rapid growth (and death) rates should be sampled frequently if perturbations are to be observed (Thompson *et al.*, 1995a).

The interplay between gene expression and FAME profiles, as illustrated in Chapters 4 and 5, implies that the use of FAME analysis for the characterization of microbial communities (Cavigelli, Robertson & Klug, 1995) could produce some misleading results. For example, a change in environmental conditions could lead to an alteration in gene expression patterns and, by extrapolation from the results presented in this thesis, this may lead to significant changes in FA composition. Therefore differences in FAME profiles may not signify changes in bacterial community composition but merely changes in cellular physiology of the indigenous organisms. It has also been suggested that the physiological status of individual cells in soil populations is not uniform (van Veen, van Overbeek & van Elsas, 1997) and therefore FAME profiles could be expected to exhibit high levels of variability. Despite this, FAME profiling may be able to provide a good estimation of the overall health of the community, if not an estimation of microbial diversity.

6.2. Future Work

The most obvious extension of the work presented in this thesis would be the sequencing of the DNA fragments isolated from 54/96 and SBW25. The information gained from this may provide information valuable to the dissection of the mode of action of these two strains. It would also allow a direct comparison of these taxonomically similar strains to ascertain whether their similarities are coincidental or as a consequence of their shared anti-fungal mechanisms.

Further analysis of the link between the marker 17:0 cyclopropane fatty acid (CFA) and disease suppression may help to shed light on the systems that control the production of

anti-fungal agents. For example, it has recently come to light that the production of some of these compounds is cell density-dependant, using *N*-acyl homoserine lactone (AHL) derivatives as the autoinducer molecules (Laue *et al.*, 1997). A biochemical link exists between AHL and CFA biosynthesis; one of the precursor molecules for both these compounds is *S*-adenosyl methionine (Coa & Meighen, 1993; Moré *et al.*, 1996). Physiological analysis should reveal the basis for the use of CFA as a marker molecule. It is possible that it is an indicator of excess *S*-adenosyl methionine in the cell which in turn may drive the production of autoinducer molecules at a lower cell density thus making these strains more active at lower cell densities.

Once the genes for anti-fungal activity have been identified it would be useful to identify the promoters. These could then be used to promote the expression of marker genes in biological control assays. In this manner, an insight into both the temporal and spatial aspects of the production of anti-fungal activity would be gained. This data could then be combined with that concerning the population dynamics in Chapter 3 to optimize the timing and position of inoculum application.

6.3. Concluding Remarks

A number of approaches for the improvement of the biological control of soil-borne phytopathogens have now been proposed. In order to achieve the desired levels of efficacy and reproducibility it is probable that a combination of these strategies should be applied to the problem and an integrated approach to crop manangement would appear to hold the most promise for successful control of crop disease (Campbell, 1989).

Whilst it is probable that the use of a single bacterial strain on its own will never be satisfactory for the demands of modern agriculture, the development of microbial technology has pinpointed the way forward. The ultimate goal of increasing crop yield with a concomitant reduction in the use of harmful agrochemicals is an achievable one, but it is unlikely that the use of microbial inoculants will be sufficient in the absence of other technologies.

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