CONTROL OF *PYTHIUM ULTIMUM* BY ANTAGONISTIC FUNGAL METABOLITES

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No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

R. J. Thompson

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Dedicated to my parents.

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CONTENTS

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ABBREVIATIONS

absorbance	A
active ingredient	a.i.
approximately	approx.
carboxymethylcellulose	CMC
colony forming units	cfu
concentrated	conc.
corn meal agar	CMA
cultivar	cv.
Czapek-Dox medium	CDM
day(s)	d
degrees Centigrade	°C
diameter	diam.
distilled water	DW
dry weight	dry wt.
et alia	*et al.*
ethyl acetate	EtAc
equal to	=
Figure(s)	Fig(s).
fluorescein diacetate	FDA
forma species	f.sp.
formae speciales	ff.spp.
gram(s)	g
hour(s)	h
hydrogen ion, minus log. concentration	pH
hymexazol	Hm
infrared	i.r.
inhibitory unit(s)	IU
kilo (prefix)	k ($x10^{3}$)
less than	<
litre(s)	l
logarithm to base ten	log.
malt extract agar	MEA
malt extract broth	MEB
3-methyl-2-benzothiazolinone hydrochloride	MBTH
micro (prefix)	μ ($x10^{-6}$)
milli (prefix)	m ($x10^{-3}$)
minute(s)	min
moles per litre	M
molecular weight	mol. wt.
more than	>
number	no.
nutrient agar	NA
nutrient broth	NB
page(s)	p(p).
Penicillium claviforme culture filtrate	PCF
pentachloronitrobenzene	PCNB
per cent	%
pounds per square inch	psi
Raolin-Thom medium	RTM

ratio of distance moved by solute to distance moved by solvent	Rf
revolutions per minute	rev min^{-1}
Schmitthenner's medium	SM
species (singular)	sp.
species (plural)	spp.
standard error of the mean	SE
sterile distilled water	SDW
sterile tap water	STW
tap water	TW
tap water agar	TWA
thin layer chromatography	tlc
Trichoderma sp. 1 culture filtrate	TSF
ultraviolet	uv
variety	var.
volume for volume	v/v
wavelength	λ
week(s)	wk
weight for volume	w/v
weight	wt.
Weindling's medium	WM

ABSTRACT

Microorganisms which suppressed the mycelial growth of *Pythium ultimum in vitro* due to the production of extracellular antagonistic metabolites were detected using a cellophane overlay method. An agar plate bioassay was subsequently employed to screen for organisms which produced large quantities of inhibitors in liquid culture. The most potent antagonist was *Penicillium claviforme*, the metabolites of which restricted *P. ultimum* propagule germination in addition to mycelial development. The yield of anti-*Pythium* agents in *Pen. claviforme* culture filtrate (PCF) was optimized by manipulation of the growth medium and cultural conditions, and the principle active component was identified as the polyketide antibiotic patulin.

An extract of PCF was commercially incorporated into sugar beet (*Beta vulgaris*) seed pellets. The treatment caused a delay in germination and abnormal seedling morphology on filter paper, but had no effect on emergence in peat-based compost. PCF significantly improved both emergence and establishment 7d after planting in compost artificially infested with *P. ultimum*. The extract was less effective than the synthetic fungicide hymexazol in reducing a high incidence of disease, but performed equally well in combating a milder pre-emergence infection.

PCF was rapidly inactivated in compost (half-life 11.2h) and seedling protection was extended after the concentration of active compounds had declined to levels sub-inhibitory to *P. ultimum*. The proposition that PCF induced quantitative or qualitiative changes in the spermosphere microflora which augmented its known direct *Pythium*-antagonistic activity was investigated.

Total microbial activity, measured in terms of fluorescein diacetate hydrolysis, in compost adjacent to PCF-treated seed pellets was reduced at 1-3d after planting compared with that in compost surrounding control pellets without additives. This coincided with a decrease in the populations of bacteria and actinomycetes, but numbers of fungi were apparently not affected. A large proportion of fungal isolates recovered from the spermosphere of both PCF-containing and control pellets inhibited the growth of *P. ultimum in vitro*, and all were less sensitive to PCF than was the pathogen. Various possible interpretations of these results are discussed.

It is concluded that the exploitation of antagonistic fungal metabolites as natural fungicides incorporated into seed pellets is a feasible approach to the suppression of *Pythium*-induced seed and seedling diseases. Even if short-lived in the plant growth medium such agents may nevertheless confer a useful degree of protection if sufficiently selective to elicit an integrated mode of control mediated by indigenous microbial antagonists.

CHAPTER 1

INTRODUCTION

1.1 The soil and soil-borne phytopathogenic fungi

1.1.1 Microbial activity in the soil environment

The soil is a highly heterogeneous environment composed of mineral matter, organic matter, water, air and living organisms (Alexander, 1977; Lynch, 1983; Nedwell & Gray, 1987). The mineral fraction is the dominant component in most agricultural soils and consists of particles which are discontinuous and variable in size, ranging from clays with diameters of <0.002mm to large stones. The organic fraction, or humus, is a diverse mixture of materials derived from the residues of plants, animals and microorganisms, as well as some compounds synthesized de novo by the soil microbiota. Inorganic and organic components of soil rarely exist as discrete entities but tend to be aggregated to a greater or lesser extent. The pore spaces within and between the aggregates are filled with either water or air.

Due to the variety of particulate and aggregated constituents, it is recognized that soil should be regarded not as a single microbial habitat, but as a mosaic of numerous, discrete microhabitats within which microorganisms are influenced by a multitude of physical, chemical and biological agencies (Stotzky & Burns, 1982; Campbell, 1983; Lynch, 1988a).

Abiotic factors which have an effect on the activities of the soil microflora include organic substrates, mineral nutrients, soil moisture,

aeration, temperature, pH, redox potential and particulates. The colloidal humus and clay particles play a particularly important role due to the large area and charged nature of their surfaces (Burns, 1983). These tend to concentrate not only water molecules, inorganic ions and organic nutrients, but also the microorganisms themselves, their enzymes and metabolic products and any chemicals added to soil by man.

As a whole, soil is considered to be a nutrient-poor environment for heterotrophic organisms as most potential carbon sources are discontinuous in space and time (Warcup, 1967; Williams, 1985). Soil fungi therefore exist mainly in an inactive state, maintained in this condition by the widespread phenomenon of 'fungistasis'. This term refers to the capacity of natural soil to inhibit the germination of fungal propagules with which it is in contact (Dobbs & Hinson, 1953). It is largely biological in origin, and now appears to be caused by a combination of several factors, including nutrient deficiency at the propagule surface, and the presence of germination inhibitors (Lockwood, 1984). Fungistasis is overcome, and the activities of other groups of microorganisms also stimulated, by the addition of organic nutrients to the soil. This occurs when plants and animals die within it, organic material is added to its surface, or as a consequence of the presence of living plant parts.

The region of intensified microbial activity surrounding plant roots is known as the rhizosphere (Hiltner, 1904, Bowen, 1980; Foster, 1986; Richards, 1987). Rhizodeposits include exudates, secretions, lysates, sloughed-off cell debris and mucigel. Root exudates vary according to plant species, age, vigour and environmental conditions, and include sugars, amino acids, organic acids, vitamins, enzymes and also volatile compounds (Rovira, 1965). A similar range of substances are exuded from

seeds (Vančura, 1988), again resulting in qualitative and quantitive changes in the microflora in the adjacent soil (Slykhuis, 1947). This zone is referred to as the spermosphere.

In favourable microhabitats, particularly in the rhizosphere and spermosphere, microorganisms of different species exist in close proximity and thus have the opportunity to markedly influence each other's growth and activities. Microbial interactions may be beneficial, neutral or detrimental (Stotzky, 1972; Bull & Slater, 1982; Atlas & Bartha, 1987). Those in which at least one of the interactants is adversely affected are termed antagonistic, and can be sub-divided into:

a) competition, in which both individuals suffer indirectly because they share a need for the same limited resource;

b) parasitism and predation, in which one individual suffers directly due to its exploitation as a nutrient source by the other; and

c) amensalism, in which one individual is directly harmed by the metabolic products of another, which itself is not directly benefitted. The amensalistic products which have received most attention are antibiotics, defined as substances produced by microorganisms which, even at very low concentrations, inhibit the growth of other microorganisms (Waksman, 1953).

Overall, the soil microbial population is in a state of dynamic equilibrium and is said to be biologically balanced or buffered (Wilhelm, 1965; Baker, 1980; Cook & Baker, 1983). This balance is maintained by the complex network of interactions and interrelationships within the community, which limit the potential impact of any one constituent species or group. Shifts in balance only occur following natural or

anthropogenic disturbances involving either destruction of, or additions to, the environment.

1.1.2 Soil-borne phytopathogenic fungi

Fungal root pathogens are recognized as a major constraint on crop productivity, but compared with foliar phytopathogens they are poorly understood (Bruehl, 1987). The physico-chemical and biological complexity of the soil environment (section 1.1.1) renders their study *in situ* problematical, and they tend to cause insidious, debilitating, but non-lethal diseases which makes their economic impact difficult to assess.

Two groups of plant pathogens are commonly distinguished: necrotrophs, which rapidly kill all or part of their host; and biotrophs, which coexist with the host tissues for an extended period of time without inflicting severe damage (Dickinson & Lucas, 1982). The majority of root pathogens belong to the former category. They grow inter-cellularly within the host producing cytolytic enzymes and toxins, and then utilize the dead tissues as a substrate. They may be unspecialized opportunistic pathogens (facultative necrotrophs), capable of an active saprophytic existence in the absence of a host. Such organisms often have a wide host range, but their parasitic activities are restricted to damaged, juvenile or senescing tissues. Obligate necrotrophs are more specialized pathogens, with saprophytic abilities limited to mere survival in dead infected host tissues. They have a narrower host range, but are able to attack at many stages of plant development.

Soil-borne phytopathogens are less affected by abiotic environmental factors than are foliar ones, since the soil provides a buffer against rapid and extreme fluctuations of, for example, temperature and humidity.

However they are profoundly influenced by the other microorganisms which abound in the rhizosphere and spermosphere, and which may limit the opportunities for the pathogen's establishment (Wheeler, 1976; Lockwood, 1988). The inherent microbiological balance in soil (section 1.1.1) is an essential bulwark against root-attacking fungi on which successful agriculture depends, and can be considered as a form of natural biological control (Wilhelm, 1965; Cook & Baker, 1983).

The genus Pythium Pringsheim was established in 1858 within the Order Peronosporales, Class Oomycetes. Members are world-wide in distribution and occupy a diversity of habitats. There are approximately 90 species, distinguished primarily by the form of their reproductive structures. A taxonomic account has been given by Middleton (1943) and more recently by Plaats-Niterink (1981), and keys to species and original descriptions by Waterhouse (1967; 1968).

The mycelium of Pythium is coenocytic (non-septate) when young and under appropriate circumstances gives rise to the asexual spores, sporangia, which may be spherical, filamentous or lobate, and the male and female sexual reproductive structures, antheridia and oogonia (Webster, 1980). After fertilization the oospore walls become thickened and require a period of 'after-ripening' before they are capable of germination. Depending on species and environmental conditions, both sporangia and oospores may germinate to form a new thallus either directly, by means of a germ-tube, or indirectly, by producing motile zoospores.

Pythium ultimum was first described by Trow (1901) and is one of the commonest species in soil. It is cosmopolitan in distribution and has an extremely wide host range. The sporangia are globose and zoospore

formation does not occur except in P. ultimum var. sporangiferum (Drechsler, 1960).

Pythium spp. are typical unspecialized necrotrophic pathogens. They infect the root tips of older plants, contributing to many decline and replant diseases, and can cause soft rots of fleshy fruits and vegetables during transit or storage. However pythia are best known for their attack on the immature, succulent tissues of seeds and seedlings (Hendrix & Campbell, 1973; 1983). Seeds may be attacked and rotted before germination, or seedlings infected before or after they emerge from the soil, resulting in pre- or post-emergence damping-off respectively. Post-emergence disease causes greatest damage in glasshouses and nurseries where the humidity amongst a stand of susceptible young plants is high due to overwatering and overcrowding. Under these conditions the fungus spreads rapidly on the soil surface and the hosts are usually penetrated at the base of the hypocotyl. Following invasion discoloured, water-soaked lesions appear and the seedlings collapse shortly after as a result of destruction of the supportive tissues in the cortex (Agrios, 1988).

As with soil-borne pathogens in general, the economic importance of Pythium is difficult to evaluate. Post-emergence damping-off may be obvious when large seedling losses occur and can cause serious financial deficits, particularly in high-value horticultural crops such as tomatoes and peppers (Stephens & Powell, 1982). However, poor seedling stands due to pre-emergence infection are often attributed by growers to low seed vigour (Kadow & Anderson, 1937). In older plants, Pythium spp. act as 'root pruners', reducing nutrient absorption and rendering the host susceptible to secondary pathogens or damage from other stress factors such as drought or frost (Smith & Margot, 1988).

1.1.3 Disease development

Plant disease results from the interaction of a pathogen with its host, but the intensity and extent of this interaction is markedly affected by the environment (Wheeler, 1976).

The virulence of the pathogen, and the influence of the environment upon it, are embraced in the concept of inoculum potential, defined by Garrett (1970) as the energy of growth of a pathogen available for infection at the host surface. It has three components: inoculum density, pathogen vigour and environmental factors.

The major host attributes involved in the initiation of soil-borne disease are their exudates (section 1.1.1) which may affect fungal activity in a number of ways. They can induce the germination of propagules, cause chemotactic movement of motile spores or chemotropic growth of germ-tubes towards the root or seed and stimulate hyphal growth at the organ surface itself (Jackson, 1957; Curl, 1982). The response is usually non-specific and is thought to be due to provision of the sugars and amino acids necessary for germination and pre-penetration growth (Schroth & Cook, 1964).

Root and seed exudates play a particularly important role in the early stages of infection by Pythium spp. (Stanghellini, 1974). Prompt and high percentage propagule germination in response to the favourable stimulus, combined with rapid growth along the nutrient gradient, allows colonization of young susceptible tissues in advance of competitors. Sporangia of Pythium ultimum are capable of 80-100% germination in soil within 1-3h of stimulation and hyphal growth rates may exceed 300$\mu m\ h^{-1}$ (Stanghellini & Hancock, 1971a). Volatile substances emanating from seeds have also been reported to induce the rapid germination of Pythium

sporangia, further increasing the sensitivity of the response (Nelson, 1987).

The degree of stimulation is determined primarily by the quantity rather than the quality of the exudates (Matthews & Whitbread, 1968; Keeling, 1974), and thus factors which increase exudation tend to favour disease. For example, the greater incidence of pre-emergence damping-off occurring when seeds are aged (Hering, Cook & Tang, 1987) or damaged (Flentje & Saksena, 1964; Perry, 1973) has been attributed partly to the concurrent increase in seed exudation.

Most soils inhibit the development of plant diseases to some extent due to the activities of indigenous microorganisms (section 1.1.2). However in some plant growth media, referred to as 'suppressive', pathogen populations and/or disease incidence or severity are significantly lower than in normal ('conducive') media (Hornby, 1983). Suppressiveness may also be induced by certain cultural, biological or chemical treatments (Baker & Chet, 1982). Thus peat media are usually conducive to damping-off but may be rendered suppessive by the addition of composted organic wastes such as hardwood barks or municipal sewage sludge (Hoitink & Fahy, 1986). Microbial interactions are again considered to play an important role in this phenomenon and a distinction has been drawn between suppressiveness due to general antagonism, which is related to the total extent of microbial activity, and that resulting from specific antagonism, which is associated with the presence of select species or groups of organisms (Baker, 1987). For example, in peat media amended with sewage sludge compost which is suppressive to both Pythium and Rhizoctonia solani, the former pathogen is suppressed by high levels of microbial activity (i.e. general antagonism), while propagules of the latter are

specifically eradicated by mycoparasitic Trichoderma spp. (Kuter, Hoitink & Chen, 1988).

Many interacting physical and chemical factors also affect the progression of soil-borne diseases, acting either directly on the pathogen or indirectly via the susceptibility of the host. For instance, high soil moisture promotes Pythium-induced pre-emergence damping-off by favouring mycelial growth (Beach, 1946) and enhancing seed exudation (Kerr, 1964). It also facilitates exudate diffusion from the seed surface, thereby increasing the radial extent of the spermosphere (Stanghellini & Hancock, 1971b).

Other abiotic factors reported as influencing the incidence of Pythium diseases include temperature (Hershman, Varney & Johnston, 1986), pH (Griffin, 1958), soil type (Dunlap, 1936) and aeration (Brown & Kennedy, 1966). Most observations can probably be explained by the concept proposed by Leach (1947) who, after a study in which temperature was the major variable, concluded that the severity of pre-emergence damping-off was determined by the relative growth rates of the host and the pathogen. As pythia are limited to attacking juvenile tissues, conditions optimal for host growth also minimize the duration of susceptibility (Chi & Hanson, 1962).

The initial site of contact between the pathogen and host is known as the infection court (Dickinson & Lucas, 1982). When mature both the outer surface of the root and the seed coat provide an effective physical barrier to infection, which comprises the primary resistance mechanism of the plant. Rather than direct penetration through these protective layers, many pathogens exploit areas of weakness, such as the root hair zone where the necessity for efficient water and nutrient uptake precludes

the presence of a mechanical obstruction. Wounds, caused by man and his machines, or by natural agencies such as pests and extremes of temperature, are also sites where potential infection courts are exposed.

Host penetration by phytopathogenic fungi is frequently associated with the development of germ-tube or hyphal modifications known as appressoria (Emmett & Parbery, 1975). Small, simple appressoria have been observed during the infection of peach rootlets by P. ultimum (Miller et al., 1966). Entry into the plant takes place by means of a narrow hyphal thread or infection peg, arising from the lower surface of the structure.

Penetration involves a combination of mechanical pressure and enzyme action. Extensive production of pectinolytic enzymes is a characteristic of many phytopathogens, including Pythium spp. (Winstead & McCombs, 1961), and facilitates both initial ingress and subsequent ramification through the tissues by dissolving the middle lamellae of the plant cell walls (Bateman & Millar, 1966). They probably act in conjunction with other enzymes such as proteases and possibly cellulases.

The resistance of older tissues to Pythium attack is associated with a decrease in the availability of pectic substances in the cell wall to enzymic degradation, due to the deposition of lignin and other polysaccharides, and may also be related to the presence of inhibitory phenolic compounds (Plaats-Niterink, 1981) which are known to be potent, non-specific enzyme inhibitors. In addition, older plants are capable of active resistance mechanisms should the primary defences be breached (Agrios, 1988). These again may be anatomical, for example deposition of callose, lignin and abscission layers, or chemical, such as production of phytoalexins or fungal wall-degrading enzymes.

The ability of plants to withstand attack by unspecialized necrotrophic pathogens is related to their general state of health. Stressed hosts grown under unfavourable conditions may not only exude greater quantities of organic materials but also possess poorly developed passive defences, and fewer roots so are less able to compensate for any lost or damaged by disease (Bruehl, 1987).

1.1.4 Dispersal and persistence of soil-borne phytopathogenic fungi

In the absence of a suitable host the hyphae of plant pathogens are rapidly lysed in natural soil (Lockwood, 1960; Lockwood & Filinow, 1981). Lysis may be the result of purely internal metabolic changes in response to nutrient stress (autolysis). Alternatively, it may be caused by exposure to enzymes or toxic materials produced by antagonistic microorganisms or to agents of non-biological origin (heterolysis). As the availability of their hosts is discontinuous phytopathogens must therefore disperse either in space or in time (persistence) in order to survive.

Opportunities for the spatial dispersal of fungi in soil are more restricted than in the aerial environment (Hirst, 1965). Their populations are usually localized around the sites of previous substrates and thus the diseases caused by seed- and root-infecting fungi, including *Pythium*, are often patchy in distribution.

Many soil-borne phytopathogens rely largely on temporal dispersal for survival. They produce dormant propagules capable of persistence until stimulated into renewed activity by seed or root exudates which signal the arrival of a potential host.

Fungal resting structures include sexually produced oospores, asexually produced conidia, sporangia and chlamydospores, and multicellular sclerotia (Park, 1965). Many are thick-walled and/or contain stored nutrients. Two types of dormancy have been distinguished (Sussman, 1965): a) constitutive dormancy, which is maintained by internal control factors and ensures that the propagule remains dormant for a period appropriate to the life cycle of the pathogen, and b) exogenous dormancy which is controlled by external factors and prevents propagules from germinating when environmental conditions are unfavourable. Fungistasis is a form of exogenous dormancy and is therefore of great survival value (Dobbs & Hinson, 1953).

Certain phytopathogens are also capable of existence in an active form during inter-host periods, either parasitically in alternative hosts such as weeds, or as saprophytes.

Pythium is known to persist for extended periods in the absence of a host (Hoppe, 1966). Oospores and/or sporangia are thought to be the major structures involved. Among sphaerosporangiate species, such as Pythium ultimum, sporangia are probably equal in importance to oospores, the former for survival in the short term and the latter over a longer time scale (Stanghellini & Hancock, 1971a). Sporangia of P. ultimum are exogenously dormant in soil, whereas the thick-walled oospores are constitutively dormant and are incapable of germination until conversion into thin-walled spores (Lumsden & Ayers, 1975).

Pythium spp. are also able to survive saprophytically as pioneer colonizers of virgin substrates by virtue of their rapid germination and hyphal growth rate, the same attributes which contribute to their success as opportunistic seed and seedling pathogens (section 1.1.3). However,

pythia are not vigorous competitors (Barton, 1961; Lifshitz & Hancock, 1983) and their exploitation of a substrate is dependent on arrival before slower growing, but more antagonistic organisms. The readily assimiliable components are then converted into numerous resting structures. Pythium spp. are therefore more common in cultivated soils, where larger quantities of fresh, uncolonized organic materials are deposited on the surface, than in natural soils (Barton, 1958).

1.2 Chemical control of soil-borne phytopathogenic fungi

1.2.1 Fungicides

Chemicals must satisfy a number of criteria in order to succeed as plant disease control agents (Heitefuss, 1989). These include:

a) provision of effective and consistent control;

b) lack of significant phytotoxicity at the dosage necessary to provide protection;

c) minimal adverse effects on other parts of the agroecosystem;

d) formulations which are reasonably stable and easy to store, transport and apply; and

e) cost-effectiveness.

Although most of these attributes are now attainable for fungicides applied to foliage, for several reasons they are much more difficult to achieve when the pathogen attacks plant organs in the soil (Dickinson & Lucas, 1982; Campbell, 1985). For example, unlike infection of aerial parts, diseases of roots are difficult to detect until well advanced. Furthermore, the efficacy of any chemical applied to soil is markedly

influenced by the physical, chemical and biological components of the system, and in turn the compound is likely to have effects upon non-target soil organisms (section 1.2.2). Finally, the access of soil additives to the roots of established plants is poor. Fungicide solutions or suspensions can be used as drenches around the base of the plant, while solid materials can be applied as dusts or granules to the soil surface and then mechanically incorporated (Rodriguez-Kabana, Backman & Curl, 1977), but these methods are generally imprecise and therefore of variable efficacy. Root dips can be utilized if plants are grown in a two stage process i.e. as cuttings or transplants, but this is only appropriate to high value horticultural crops.

Compared to infections of mature root systems, greater possibilities exist for the control of seed and seedling diseases such as damping-off where the period of host susceptibility is short. Treatment of the seeds themselves offers advantages in terms of efficacy (the chemical being placed directly in the infection court), economy of materials, and reduced environmental impact (Graham-Bryce, 1988). Problems experienced with conventional methods of seed treatment, including low achieved dose and large seed-to-seed variation (Lord, Jeffs & Tuppen, 1971), have been diminished by modern techniques such as seed pelleting and film-coating (Halmer, 1988).

The investment involved in developing new chemicals for plant disease control, with respect to both time and money, is enormous and increasing every year (Green, Hartley & West, 1987). Not only are discovery costs rising as it becomes more difficult to find effective new products, but also registration requirements are becoming ever more stringent as the possible detrimental effects of foreign chemicals on the environment

become more widely appreciated (section 1.2.2). In 1986 it was reported to take 8-10 years from discovery to register a new pesticide, with an expenditure of at least £25 million (Anon, 1986a).

Chemicals used to control soil-borne diseases are either volatile or non-volatile. Volatile agents (soil fumigants) are broad spectrum biocides used to eliminate weeds, nematodes and insect pests in addition to fungi, before a crop is planted (Goring, 1962). This approach is expensive and thus only practical on a fairly small scale and for high value crops. For example, fumigation with methyl bromide or chloropicrin is employed to eliminate Pythium spp. from tree nursery and horticultural seed-beds (Hendrix & Campbell, 1973).

The earliest non-volatile fungicides were based on inorganic materials such as sulphur, copper and mercury (Spencer, 1977). These chemicals were again general biocides and their use as crop protection agents was dependent on their low solubility and limited penetration of the plant.

In the first half of the twentieth century various organic compounds were introduced for plant disease control. These affect many vital cell processes and are active against a wide range of fungi. Those effective against Pythium include thiram (tetramethylthiuram disulphide), captan (N-(trichloromethylthio)-cyclohex-4-ene-1,2-dicarboximide) and drazoxolon (4-(2-chlorophenylhydrazono)-3-methyl-5-isoxazolone) (Bruin & Edgington, 1983). Minimal entry into host tissues is again essential to avoid damage and they are therefore termed surface or protectant fungicides. As they are exposed to wash-off and new plant growth is unprotected, repeated applications are necessary, often at high dosages (Schwinn, 1979). They must also be applied to susceptible tissues before the pathogen reaches

the surface, necessitating costly 'insurance' treatments or reliable early warnings of possible infections.

Within the last two decades a range of systemic fungicides have been developed which are taken up and translocated by plants (Marsh, 1977). These chemicals are protected from physical removal inside the host and can also confer protection on new growth as it occurs, reducing both the concentration and number of treatments required (Brent, 1984). In addition, some of these agents (referred to as curative or therapeutic fungicides) are able not only to protect the host from fungal inoculum arriving on its surface, but also to cure or inhibit established infections (Sbragia, 1975). Modern systemics such as the benzamidazoles and triazoles have dramatically improved the scope for the control of foliar pathogens.

Systemic chemicals are in intimate contact with the host tissues and it is therefore essential that they are selectively toxic to fungal cells whilst not affecting those of plants (Edgington, 1982). This is achieved by targeting a single enzyme or structure unique to fungi. As such features may vary between fungal groups, systemics often exhibit a narrower antifungal spectrum than do the protectant fungicides. Oomycetes in particular possess various unusual properties which enabled them to escape harm from many of the early systemic compounds. For example, Oomycete tubulins show no affinity for benzamidazole fungicides (Bruin & Edgington, 1983). However, a number of chemicals are now available which are highly active against this group *in vitro*, for example etridiazole (5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole), hymexazol (5-methylisoxazol-3-ol), prothiocarb (S-ethyl N-(3-dimethylaminopropyl) thiocarbamate), metalaxayl (methyl N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-

alaninate), and fosetyl-aluminium (aluminium o-ethyl phosphonate) (Cohen & Coffey, 1986).

A problem with most systemic compounds is that they are only passively translocated in the apoplast with the transpiration stream and hence tend to accumulate in the young leaves and shoot apices. This limits their potential for the control of soil-borne diseases. The ethyl phosphonates such as fosetyl-Al are therefore a major advance in showing substantial symplastic movement in the phloem. They can be applied as foliar sprays (Coffey et al., 1984), or even as trunk injections (Darvas, Toerien & Milne, 1984), in order to control important root pathogens such as Phytophthora.

1.2.2 Environmental impact of fungicides

The intrinsic toxicity of an antifungal agent is dependent on its chemical structure. However, the activity of a fungicide in soil is modified by factors such as its persistence and distribution, which determine the degree of exposure of both target and non-target organisms. These characteristics are in turn influenced by the physical, chemical and biological properties of the chemical and of the soil, and processes involving interactions between them (Goring, 1967; Munnecke, 1972; Guth, Gerber & Schlaepfer, 1977; Kaufman, 1977).

Since absolute specificity has not been achieved, fungicides reaching the soil influence the growth and activities of non-target organisms (Anderson, 1978b; Bollen, 1979; Somerville & Greaves, 1987). Side-effects may be positive or negative, and may be caused by the active ingredient itself, or by the solvents and carriers. Standard tests have been recommended to evaluate the likely impact of pesticides on the soil

microflora in the laboratory, based on studies of soil respiration and nitrogen transformations (Somerville et al., 1987). Due to the large variation in microbial activites observed in response to naturally occurring stresses, deviations are only considered to be critical if greater than 50% in magnitude or of greater than 60d duration (Domsch, Jagnow & Anderson, 1983).

Treatment of soil with broad-spectrum biocides (most soil fumigants and many protectant fungicides), initially results in the elimination of a large proportion of the microbial population (Kreutzer, 1965). This is followed by a rapid increase in total microbial activity as the treated soil is reinvaded by species able to take advantage of the new substrates created by the dead biomass. The resulting microflora is therefore dominated by fast-growing opportunistic organisms and is less diverse than the original (Bollen, 1979). There is thus a major disturbance to the microbiological balance. Application of more selective compounds, including most systemics, affects only certain sections of the total population. Sensitive species are replaced by tolerant ones, resulting in lesser changes in community structure.

The effect of fungicides on the general population, or on specific groups of saprophytes, may influence the incidence of plant disease indirectly, in addition to their direct toxicity to the pathogen concerned (Papavizas & Lewis, 1979; Rodriguez-Kabana & Curl, 1980; Griffiths, 1981). If organisms antagonistic to the pathogen are selectively favoured by the treatment, either because they are less sensitive to the fungicide, or are more successful in recolonizing the treated soil, then disease control can be greater or longer-lasting than that achieved by the use of the chemical alone (Richardson, 1954). However, if antagonists are inhibited by a

fungicide, then a so-called 'boomerang effect' may occur, in which disease is initially controlled but then reappears, sometimes causing more serious damage than before treatment. This is a particular problem with the rapidly-growing damping-off fungi (Gibson, 1956; Kraft, Haglund & Reiling, 1969). It has therefore been argued that the use of chemicals specific to the target organism is desirable (Kreutzer, 1963). However, selective control of a dominant pathogen can result in its replacement by another which was previously of lesser importance, a phenomenon known as 'disease trading'. For example, application of benzamidazole fungicides is often followed by an increase in diseases caused by Oomycete phytopathogens (Gibson, Ledger & Boehm, 1961; Warren, Sanders & Cole, 1976).

Fungicides vary in their toxicity to higher organisms. Though in general they are less dangerous than insecticides (Fry, 1977), some, such as drazoxolon, possess considerable mammalian toxicity and even apparently safe chemicals may be decomposed to harmful products. For instance, the ethylenebisdithiocarbamates themselves show only low toxicity to mammals but may give rise to ethylenethiourea, a carcinogen (Erlichman, 1989)

Fungicides also show considerable variation in their suceptibility to inactivation which may be due to physical, chemical or biological processes (Burns, 1975; Hill & Wright, 1978). The most desirable active life of a crop protection agent is one that is sufficient to give adequate disease control without the need for repeated applications, but is not prolonged after it has served its purpose. Fungicides which persist in the soil, such as the mercury-containing compounds, have the capacity to accumulate in food chains and indirectly harm organisms at higher trophic levels (Cremlyn, 1978). These materials have now been banned in many countries. Modern systemic fungicides are generally less persistent in

the external crop environment but have greater potential to leave undesirable residues within plant tissues.

Conversely, fungicides may have too short an active life in the soil, resulting in loss of efficacy against the target pathogen. This problem arises when repeated use of certain compounds induces the development of saprophytic microbial populations capable of rapid decomposition of the chemical, an effect referred to as 'accelerated' or 'enhanced biodegradation' (Walker & Suett, 1986).

The distribution of a chemical in soil, and therefore its availability for uptake by microorganisms or by plant roots, is affected by sorption to soil colloids and also by the mode of application (Wheatley, 1977; Bromilow, 1988). Strongly sorbed chemicals are relatively immobile and have only a localized impact, whereas less strongly sorbed compounds have a wider sphere of influence. Similarly, specific placement, for example by seed treatment, or the use of systemic agents may restrict any side-effects to a limited volume of soil.

1.2.3 Fungicide resistance

The term 'resistance' refers to the situation in which a pathogen becomes less sensitive to a fungicide due to a stable genetic change (Georgopoulos, 1977), in contrast to 'tolerance' which is here used to indicate a transient physiological adaptation (Dickinson & Lucas, 1982). The rate of development of resistant pathogen strains in the field is determined by factors such as the nature of the chemical and of the pathogen, and the intensity of the selection pressure (Dekker, 1982a).

Resistance is not regarded as a major problem with the older protectant fungicides. Most exhibit multi-site activity (section 1.2.1)

and acquisition of resistance would therefore require a number of simultaneous mutations. By contrast, the introduction of many systemic agents was shortly followed by reports of diminished effectiveness (Dekker, 1977; Staub & Sozzi, 1984). For example, dramatic outbreaks of resistance to metalaxyl were observed in populations of Phytophthora infestans after only one year of use (Davidse et al., 1981). The rapidity with which fungi develop resistance to systemics appears to be due to the fact that many of these compounds operate at a single site unique to particular pathogen groups (section 1.2.1) and therefore resistance may arise as a result of mutation at only one or a few genes. Most commonly such mutations occur spontaneously and are then selected for in the presence of the respective chemical, but certain fungicides, for example the benzamidazoles, may themselves be mutagenic (Georgopoulos, 1977).

The chances of resistance arising in soil-borne phytopathgens are considered to be less than in foliar ones since spore populations are thought to be lower, and the soil places a significant constraint on their dissemination (Cohen & Coffey, 1986). Nevertheless, cases of failure of systemic compounds to control organisms such as Pseudocercosporella herpotrichoides (Brown, Taylor & Epton, 1984) and Pythium aphanidermatum (Sanders, 1984) are causing increasing concern.

The degree of selection pressure imposed by a fungicide is dependent on factors such as the rate and frequency of application, the method of treatment, and the persistence of the chemical. Frequent utilization of a given agent at high rates will reduce the initial risk of a mutation conferring resistance, but once it has occurred such a practice will promote the spread of the gene(s) responsible (Dekker, 1977). Certain methods of application, such as slow release granules, and use of more

recalcitrant materials can result in a chemical being present at a lethal or sub-lethal concentration for an extended period of time and increase the risk of resistance, whilst the danger posed by seed treatments is relatively small and well-defined (Smith and Margot, 1988).

Recognition by the agrochemical industry of the extent of the fungicide resistance problem, and of the fact that cross-resistance to chemicals with similar modes of action is a common occurrence, resulted in the establishment in 1981 of the Fungicide Resistance Action Committee (Wade, 1987). FRAC is an inter-company body whose activities are aimed at prolonging the useful life of fungicides liable to encounter resistance problems.

Several strategies have been recommended to prevent or delay the emergence and spread of resistance to systemic fungicides, based on reducing the intensity and duration of the selection pressure (Dekker, 1982a). For example, it is advised that systemics should not be used alone but in mixtures and/or alternating programmes with fungicides showing different modes of action, and that their application should be restricted to critical periods in the development of the disease. Another effective countermeasure is to use fungicides in conjunction with biological methods of disease control (section 1.3.4), which reduces the rate and number of treatments of the chemical necessary to afford adequate protection.

1.3 Biological control of soil-borne phytopathogenic fungi

Biological control has been broadly defined by Cook & Baker (1983) as 'the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man'. The entities mediating biocontrol may be the host plant (section 1.3.1), antagonists of the pathogen, or avirulent or hypovirulent strains of the pathogen itself (sections 1.3.2 and 1.3.3).

1.3.1 Plant breeding for disease resistance

At one time the breeding of resistant crop cultivars seemed to promise an ideal and permanent solution to the problem of plant disease. However in practice it has been found that the introduction of genes conferring resistance to a given disease has regularly been followed by the emergence of specialized physiological races of the causal pathogen possessing new virulence genes which enable it to overcome the resistance mechanism (Lupton, 1984). The phenomenon is largely a consequence of breeding for oligogenic types of resistance which are readily identified and manipulated in breeding programmes, but are based on a limited number of major genes. This trend, combined with modern intensive agricultural systems, imposes a high selection pressure on the pathogen population for the development of new races. The situation is analagous to the appearance of fungicide resistance following exclusive use of single-site chemicals (Georgopoulos, 1977; section 1.2.3), and recommendations for the exploitation of novel plant disease resistance genes again aim to decrease the intensity of the selection pressure. This may be accomplished by using multilines and/or cultivar mixtures to increase the genetic

diversity of the crop, or by restricting the deployment of particularly valuble genes to situations where they are likely to be most beneficial.

The risk of physiological specialization is again not quite so great with soil-borne pathogens due to their more limited opportunities for variation (section 1.2.3). There is evidence for varietal differences in susceptibility to Pythium in certain crops (White et al., 1988), so breeding for resistance may be possible, but the wide host range of species such as Pythium ultimum will make this a difficult task (Hendrix & Campbell, 1973).

There is increasing interest in breeding for more durable types of resistance, particularly for plants which deposit particular compounds or types of compound in the rhizosphere and thereby favour indigenous microbial antagonists of plant pathogens. It has been shown that chromosome 5B of wheat influences both resistance to common root rot caused primarily by Cochliobolus sativus (Larson & Atkinson, 1970), and the numbers of rhizosphere microorganisms antagonistic to the fungus in culture (Neal, Atkinson & Larson, 1970).

1.3.2 Cultural practices

Various cultural methods have been successfully employed for centuries to restrict the development of plant disease, but the underlying mechanisms have only recently been recognized (Papavizas & Lumsden, 1980; Cook & Baker, 1983; Deacon, 1983).

In many cases control is achieved by disturbing the microbiological balance in the soil, either by a destructive or an enrichment method, and then manipulating the simplified microflora so as to favour the activities of naturally occurring antagonists of the pathogen (section 1.1.2).

Because they are indigenous these organisms are likely to be capable of survival in the prevailing environment, which is not always the case with introduced antagonists (section 1.3.3). Alternatively, or additionally, cultural practices may have direct effects on the pathogen or on the host.

Control of the environment

In glasshouse crops management of environmental conditions is an effective means of minimizing seed and seedling diseases caused by Pythium. The factors of particular importance are soil moisture and temperature, and atmospheric humidity (Kadow & Anderson, 1937). Such practices not only create a physico-chemical environment unfavourable to the growth of the pathogen, but also aim to improve plant vigour and hence promote host defence mechanisms (section 1.1.3).

Crop sanitation

Sanitation includes all activities intended to eliminate or reduce the quantity of pathogen inoculum present, and prevent its dispersal. These include removal or destruction of weeds and infected crop residues, and various tillage methods designed to hasten the natural disappearance of the pathogen from such remains. For example, deep burial of fresh wheat stubble is thought to limit the saprophytic multiplication of Pythium spp., which occur mainly in the top 15-20cm of soil. By contrast, the practice of stubble mulching, in which the residues are left on the soil surface to control wind and water erosion, increases Pythium damage to crops subsequently drilled through the mulch layer (Cook, Sitton & Waldher, 1980).

Soil amendment

Organic supplements, such as farmyard manures, composted crop residues and green manures, have traditionally been added to soil to improve its structure and fertility, but have long been known to have the additional effect of reducing inocula of soil-borne plant pathogens during their resting or saprophytic stages (Huber & Watson, 1970). In some cases the decomposition products of such amendments are directly fungitoxic (Lewis & Papavizas, 1971), but more often control is thought to be a consequence of germination of pathogen propagules in the presence of organic nutrients, followed by lysis of the germlings due to antagonism from the saprophytic microflora which is similarly stimulated (Papavizas & Lumsden, 1980). However, this approach is not effective with organisms such as Pythium ultimum which are capable of producing secondary resistant structures prior to lysis (Stanghellini & Hancock, 1971a).

Crop rotation

The traditional practice of crop rotation, in which land is sown with non-host plants during a cycle of intensive cropping, is effective in controlling specialized soil-borne pathogens. Disease reduction usually involves germination-lysis initiated by the root exudates of non-host plants, but in a few cases the break crops in a rotation are known to support populations of specific microbial antagonists (Deacon, 1973). Crop rotation is not successful in restricting damage caused by fungi, including Pythium, which have a wide host range and/or produce long-lived survival structures.

Heat treatment

Sterilization of soil by heat eliminates pathogens, pests and weeds but is costly, hazardous and, as with the use of fumigants and broad-spectrum fungicides (section 1.2.2), destroys much of the saprophytic microflora. This may create a biological void into which opportunistic organisms, such as pythiacious fungi, can grow unchecked (Kreutzer, 1965). A cheaper, safer and more selective treatment can be achieved utilizing aerated steam (Baker and Olsen, 1960). Most pathogens are killed by exposure to steam at 60°C for 30min, but certain antagonists are less thermally sensitive and proliferate, reducing the risk of reinfestation (Baker, 1970).

The use of steam is almost entirely restricted to glasshouses, but heat treatment of soil in the field may be effected through the exploitation of solar energy, by covering it with transparent polythene tarps during the hot season (Katan, 1981). Disease control involves not only physical inactivation of pathogen inocula by the elevated temperatures beneath the tarp but also weakening of surviving propagules and their predisposition to microbial antgonism.

1.3.3 Introduction of selected antagonists

When biological control by manipulation of the indigenous saprophytic microflora does not result in adequate disease reduction, mass inoculation of microorganisms selected for their antagonistic properties against the pathogen concerned may be of greater benefit (Cook & Baker, 1983; Deacon, 1983; Burns, 1985; Papavizas, 1985; Baker, 1987; Weller, 1988; Lethbridge, 1989).

More is known about the mechanisms involved in suppression of plant pathogens by introduced biocontrol agents than by resident antagonists. Examples have been described in which each of the three types of antagonistic interaction described above (section 1.1.1) play a major, though rarely exclusive, role (Baker, 1968; Harman & Hadar, 1983; Whipps, 1986; Lynch, 1987; Campbell, 1989).

Competition for nutrients and for infection sites is involved in the protection of seeds from pre-emergence damping-off by coatings of rapidly growing organisms such as Penicillium oxalicum (Windels, 1981). Competition for iron is one factor operative in the control of minor pathogens and enhancement of plant growth by siderophore-producing plant growth-promoting rhizobacteria (PGPR) (Leong, 1986). Application of purified pseudobactin, a siderophore produced by Pseudomonas fluorescens B10, reduced fungal colonization of the rhizosphere and improved the wet weight of potato plants in a similar manner to bacterization with the PGPR themselves (Kloepper et el., 1980).

Mycoparasitism is implicated in biological control by agents such as Trichoderma spp. which closely coil round the organs of plant pathogens, and produce hyphal wall-degrading enzymes when grown on the cell walls of these fungi as sole carbon sources (Whipps, Lewis & Cooke, 1988). Oomycete walls contain glucan and cellulose, but not chitin (Bartnicki-Garcia, 1968) and the ability of T. hamatum to control Pythium-induced diseases was ascribed to its cellulolytic ability (Chet & Baker, 1981). Isolates of T. harzianum which produce chitinase and glucanase, but not cellulase, control damping-off caused by Rhizoctonia solani but are not effective against Pythium ultimum. Conclusive evidence for necrotrophic

mycoparasitism has been obtained in some cases, by the demonstration of $^{14}CO_2$-release from ^{14}C-labelled host cell walls (Elad, Chet & Henis, 1982).

Biological control achieved by introduction of antibiotic-producing antagonists has been attributed on many occasions to antibiosis in soil, but evidence has been largely circumstantial. However, an active role of antibiotics *in situ* has now been convincingly demonstrated in several instances, using genetic techniques (Fravel, 1988). For example, single-site Tn5 insertion mutants of *Pseudomonas fluorescens* 2-79 defective in production of a phenazine antibiotic were significantly less suppressive of take-all caused by *Gaeumannomyces graminis* var. *tritici* than the parent strain (Thomashow & Weller, 1988). Complementation of the mutants with wild-type DNA from a 2-79 genomic library coordinately restored both antibiotic synthesis and disease suppression.

A bacteriocin, a highly selective antibiotic, is involved in the most commercially significant use of microbial plant disease control agents to date. Crown gall of stone fruit trees caused by *Agrobacterium radiobacter* var. *tumefaciens* can be prevented by prior inoculation with the closely related but avirulent strain 84 (Kerr, 1980; Kerr & Tate, 1984). The antagonist produces a bacteriocin named agrocin 84 which is encoded on a plasmid designated pAgK84. If this plasmid is transferred to a suitable recipient the transconjugant acquires the ability to synthesize agrocin 84 and, in some cases, to inhibit crown gall induction (Ellis *et al.*, 1979).

Techniques such as selection, mutagenesis and gene manipulation offer the opportunity to improve the performance of biocontrol agents by enhancing, or increasing the number of specific antagonistic traits (Papavizas, 1987). Mutagenesis has also been successfully employed to develop fungicide-resistant strains (Papavizas, Lewis & Abd-El Moity,

1982) which could be useful in integrated control programmes (section 1.3.4). Despite the obvious potential presented by the new technologies of protoplast fusion and genetic engineering, their exploitation has so far been restricted by the concern of the regulatory authorities and the general public regarding the possible harmful effects of novel organisms in soil and the transfer of their genetic material to the indigenous microbial population (Beringer, 1988). Furthermore, genetic alterations may decrease the ability of antagonists to survive and compete in the natural environment, an attribute referred to as 'ecological competence' (Schroth, Loper and Hildebrand, 1984).

Biological crop protection agents need to satisfy the same requirements as do chemical ones (section 1.2.1). Their chief advantage is that they are unlikely to have an adverse impact on other parts of the agroecosystem (Deacon, 1983). In general they show a narrow activity spectrum and thus are thought to have at most a slight effect on the ecological balance. In particular they are assumed to cause no harm to the indigenous antagonists which make a vital contribution to the control of soil-borne plant pathogens (section 1.1.2). As they are isolated from natural environments microbial inoculants are considered unlikely to cause toxicity problems, and they are not accumulated in food chains. Since they are self-perpetuating they can also theoretically confer sustained protection, though in practice this is not usually the case (see below).

Another frequently quoted factor in favour of biological disease control agents compared to fungicides is that resistance is less likely to arise in the pathogen population. However this argument is applicable only where the organisms are antagonistic via a combination of different mechanisms or metabolites (Lethbridge, 1989). It is not tenable with

respect to those with specific modes of action, as illustrated by instances of partial failure of crown gall control due to the appearance of agrocin 84-resistant strains of Agrobacterium radiobacter var. tumefaciens (Panagopoulos, Psallidas & Alivizatos, 1979).

The major problem encountered in controlling phytopathogens in soil by the introduction of selected microbial antagonists has been their inconsistent performance when transferred from the laboratory or glasshouse into the field (Kommedahl et al., 1981). This is largely because the web of interrelationships within the resident microbial community (section 1.1.1) ensures that every available niche is already utilized and militates against the establishment of a non-indigenous organism (Garrett, 1965). Two approaches which may help to alleviate this difficulty are a) careful attention to the source and screening of potential biocontrol agents (Cook, 1985; Campbell, 1986), such that only ecologically competent antagonists are selected for field testing, and b) use of chemical or cultural methods to perturb the natural microbiological balance thereby releasing suitable niches for the introduced organisms (section 1.3.4). However, even if such measures are successful in permitting inoculant colonization of the infection court, the expression of their antagonistic traits may be subject to variations in the physico-chemical environment (Howie & Suslow, 1987).

Another disadvantage of introduced antagonists is that they are generally slow acting compared to fungicides (Whipps, 1986). As a result they are really effective only as protective and not curative treatments, so must either be used as an insurance policy, or in conjunction with accurate forecasting.

The storage and application of biocontrol agents also presents more difficulties than do chemicals. In order to compete effectively with fungicides, the end product should be formulated such that it has a shelf-life of a minimum of two years at room temperature and is stable over the temperature range -5° to +30°C (Lethbridge, 1989). In addition, the delivery method must be compatible with existing agricultural practices. Antagonists may be applied to the soil itself, but this requires a large amount of inoculum and often the simultaneous addition of a food base, which can stimulate the growth of the pathogen itself (Kelley, 1976). A more economic and often more effective mode of introduction is with the planting material, as root dips or, in particular, in the form of seed coatings. The versatile modern technologies of seed pelleting and film-coating used to deliver chemicals are also suitable for use with antagonists (Lutchmeah & Cooke, 1985; Clayton, 1988). However, with certain exceptions (Maplestone & Campbell, 1989), organisms applied to seeds generally show little ability to colonize the developing root system (Chao et al., 1986) which may limit the use of such methods to the control of seed and seedling diseases.

1.3.4 Integrated control

It is likely that in the future increasing emphasis will be placed on an integrated approach to plant disease control (Cook & Baker, 1983; Harman & Hadar, 1983; Papavizas, 1985; Baker, 1987; Campbell, 1989; Lethbridge, 1989). This involves the rational use of all available management techniques, which may include fungicides (section 1.2), resistant cultivars (section 1.3.1), cultural practices (section 1.3.2) and introduced antagonists (section 1.3.3). For example, suppression of

Sclerotium rolfsii infection of bean seedlings by Trichoderma harzianum was significantly improved when soil applications of the biocontrol agent were combined with low doses of the fungicide pentachloronitrobenzene (Chet et al., 1979).

The potential for successful implementation of an integrated strategy has been greatly enhanced in recent years by the development of the more specific systemic fungicides (section 1.2.1) and by the induction of fungicide-resistant mutants of antagonists (section 1.3.3). The inoculation of selected organisms, or the manipulation of the indigenous microflora, may decrease the pathogen inoculum potential sufficiently to permit a reduction in the rate or number of applications of a chemical required to achieve adequate protection, or may fill the biological vacuum created by broad spectrum treatments. Conversely, a disturbance, in the form of a fungicide or a cultural procedure, may be necessary to shift the microbiological balance and allow the establishment of a biocontrol agent.

1.4 Use of antibiotics for the control of soil-borne phytopathogenic fungi

Despite the established role of other types of antagonistic metabolites in biological control by microbial inoculants (section 1.3.3), only the use of antibiotics as plant disease control agents in their own right appears to have been studied in any detail.

1.4.1 Past and future applications

There is still considerable controversy regarding the ecological importance of antibiotics in the soil environment and, in particular, whether antibiotic production by indigenous antagonists plays a significant part in the natural biological control of soil-borne plant pathogens (section 1.1.2) (Gottlieb, 1976; Williams & Vickers, 1986). Synthesis of these compounds appears to require plentiful supplies of a suitable carbon source and is therefore probably limited to carbon-rich loci, such as those in the vicinity of fragments of plant debris (Wright, 1956a), in seed coats (Wright, 1956b) and in the rhizosphere (Rangaswami & Vidyasekaran, 1963). Moreover, many antibiotics are known to be rapidly inactivated in soil (section 1.4.2). Nevertheless, indirect evidence suggests that in microhabitats where nutritional and environmental conditions are conducive, some antibiotics can accumulate to sufficient concentrations to exert a biological effect (Brian, 1957a; Park, 1967).

Whether or not this hypothesis is accepted, it has now been shown that antibiosis is an important factor in biological control by certain introduced antagonists (section 1.3.3). Thus the exogenous application of antibiotics extracted from the culture filtrates of these organisms grown *in vitro* is a plausible alternative approach to the management of plant disease (Rodgers, 1989) avoiding some of the problems inherent in the use of the antagonists themselves (section 1.3.3).

The potential for using isolated antibiotics in this way was recognized early (Brown & Boyle, 1944), but work was limited until their commercial production for medical purposes. A considerable amount of research was then conducted, with attention centered on their selectivity

and their systemic properties in the era before the discovery of synthetic systemic fungicides (Brian, 1954; Zaumeyer, 1958; Dekker, 1963).

The first commercially available antibiotics were antibacterial (Bérdy, 1974). Streptomycin, produced by Streptomyces griseus, was found to show activity against a broad range of phytopathogenic bacteria. Its most important agricultural application is in the control of Erwinia amylovora, the cause of fire blight of many rosaceous plants such as apple and pear (Ark & Scott, 1954). It is also effective aginst certain fungi such as Phytophthora infestans (Crosse et al., 1960) and Pseudoperonospora humuli (Horner, 1963). Later, antifungal antibiotics were developed and two of these have been frequently tested as fungicides: cycloheximide, also obtained from S. griseus (Ford et al., 1958) and griseofulvin, produced by Penicillium griseofulvum (Rhodes, 1962).

Subsequently, antibiotics have been screened specifically for activity against plant pathogens, notably by the Japanese. For example, blasticidin S and kasugamycin, isolated from cultures of Streptomyces griseochromogenes and Streptomyces kasugaensis respectively, have been used extensively for the control of rice blast caused by Pyricularia oryzae (Misato & Yoneyama, 1982).

As the above examples illustrate, most of the successful applications of antibiotics for plant disease control have been against foliar pathogens. In addition, some penetrate seeds and have been employed for the eradication of seed-borne infections. For instance, soaking pea seeds in cycloheximide was found to reduce the incidence of Ascochyta pisi (Wallen & Skolko, 1951). Despite their relatively rapid inactivation in soil (section 1.4.2), antibiotics have also been utilized for the protection of plants from attack by soil-borne pathogens, albeit in a more

limited number of cases. For example, validamycin A, discovered in culture filtrates of Streptomyces hygroscopicus var. limoneus, is produced commercially for the control of rice sheath blight caused by Rhizoctonia solani in Japan (Trinci, 1984). It is used as a foliar spray, soil drench or dust, or as a seed dressing, and shows both protective and curative action (Wakae & Matsuura, 1975). Seed rots and damping-off diseases would appear to be the most likely soil-borne infections to be suppressed by antibiotic treatment since the period during which hosts are susceptible is short and there are reports of their effective deployment against Pythium spp. on an experimental scale (Gattani, 1957; Howell & Stipanovic, 1980).

The research effort into the use of antibiotics in crop protection diminished following the introduction of the synthetic systemics, but recently there has been renewed interest in their exploitation as natural, potent, specific and biodegradable fungicides (Adams, 1988). However, antibiotics must fulfil the other criteria required of plant disease control agents (section 1.2.1), and a number of problems encountered during early studies need further investigation. Potential difficulties include instability (section 1.4.2), phytotoxicity (section 1.4.3) and the development of antibiotic resistance in both pathogenic and saprophytic organisms (section 1.4.4). The higher production costs of antibiotics have also in the past been a disincentive to commercialization.

1.4.2 Effects of antibiotics in soil

The activity of antibiotics, like that of synthetic chemicals (section 1.2.2), is considerably affected by environmental components. However, they are a heterogenous group of compounds, and therefore show substantial

variation in their stability and behaviour both in soil (Pramer, 1958), and in plant tissues (section 1.4.3).

Many antibiotics are short-lived in natural soils (Williams & Vickers, 1986). This may be the result of physical, chemical or biological processes (Jefferys, 1952; Woodcock, 1978). Basic antibiotics, such as streptomycin, are strongly sorbed by clays and organic matter and although this does not necessarily inactivate them (Pramer & Starkey, 1962), it restricts their influence to the immediate vicinity of the colloidal particles. Conversely, neutral and acidic substances, which are sorbed to a lesser extent, have the potential to exert an effect over a wider volume of soil. Chemical inactivation may involve reactions such as oxidation, hydrolysis and deamination. For example, gliotoxin and penicillin are chemically unstable at high pH. Since they are natural products, most antibiotics are susceptible to microbial attack, indicated by more rapid inactivation in untreated than in sterilized soil. Microbial degradation is a particularly important factor in the loss of compounds such as griseofulvin (Wright & Grove, 1957) and patulin (Gottlieb, Siminoff & Martin, 1952). Repeated addition of some antibiotics to soil stimulates the activity of non-susceptible organisms (Hervey, 1955) and may increase the rate of decomposition (Pramer & Starkey, 1951; Wright & Grove, 1957).

Like synthetic systemic fungicides (section 1.2.1), many antibiotics are specific in their antimicrobial activity and thus do not destroy the ecological balance in the soil (Misato & Yoneyama, 1982). Furthermore, the fact that most are readily degraded also limits any undesirable environmental effects (Misato, 1983). Thus, while some antibiotics inhibit the growth of microorganisms involved in processes essential to soil fertility in laboratory media, the concentration required to affect

these transformations *in situ* is many times greater. For example, Pramer & Starkey (1952) found that while 1μg ml^{-1} streptomycin completely inhibited the growth of an enrichment culture of nitrifying bacteria, the concentration required to obtain comparable inhibition of nitrification in soil was 10,000μg g^{-1}. Similarly, mammalian toxicity, a feature of certain antibiotics (Woodcock, 1977), is less of a problem when the agent is rapidly broken down in the environment.

1.4.3 Effects of antibiotics in plant tissues

Systemic activity of antibiotics in plants depends on the extent and rate of uptake and translocation, and their stability inside the host tissues. These factors vary widely according to the nature and concentration of the antibiotic, the plant species, its physiological state and environmental conditions (Dekker, 1963).

Many antibiotics are taken up by plant roots (Goodman, 1962; Wain & Carter, 1977; Heitefuss, 1989). Of those that are absorbed, some subsequently move more freely than others. Most of the readily translocatable substances are neutral or acidic, whereas variable results are often reported for basic or amphoteric compounds (Crowdy & Pramer, 1955). This may be due to binding to tissue consitituents (Charles, 1953). Some antibiotics are inactivated within days inside the plant (Crowdy *et al.*, 1956), but others may persist for several months (Robison, Starkey & Davidson, 1954).

A number of antibiotics are phytotoxic to a certain extent, but in general microorganisms are much more sensitive than higher plants. Effects reported include inhibition of germination, inhibition of root and shoot growth, foliar lesions, wilting, and suppression of pigment

production (Brian, 1957b). Different plants vary considerably in their susceptibility to any antibiotic at a given concentration (Wright, 1951).

Certain antibiotics, such as penicillin and oxytetracycline, have been reported to stimulate plant growth and seed germination (Nickell, 1953). Even substances markedly toxic at higher concentrations may be stimulatory in very small quantities (Nickell & Finlay, 1954).

1.4.4 Antibiotic resistance in soil microorganisms

As antibiotics are often specific, affecting a limited number of metabolic processes, they can promote the development of resistance within pathogen populations (Dekker, 1983). For example, kasugamycin-resistant strains of Pyricularia oryzae became a serious problem in Japan following intensive spray programmes in paddy fields (Sato, 1983). However, as discussed in relation to synthetic systemic fungicides (section 1.2.3), resistance is less likely in soil-borne pathogens than in those which live above ground, and the rapid degradation of many antibiotics in soil (section 1.4.2) further diminishes the risk.

Perhaps a more serious problem in the potential use of antibiotics to control plant disease is that of resistance aquisition by non-target soil microorganisms. Both direct and indirect evidence indicates that once antibiotic resistance genes have arisen by mutation they can spread within bacterial populations by conjugation, transformation or transduction (Levy & Marshall, 1988). Genetic material may be transferred not only to members of the same species, but also to related ones, so that harmless saprophytic bacteria can act as a reservoir of resistance genes capable of rendering species pathogenic to man or animals non-sensitive.

It is therefore now generally agreed that the alternative use of antibiotics of medical or veterinary importance should be restricted as far as possible, to decrease the intensity of the selection pressure for the emergence and spread of resistance (Swann, 1969). However, such arguments do not preclude the exploitation of those antibiotics which are of no clinical value (Misato, 1983).

1.5 Research objectives

The broad aim of the research project described here is to evaluate the use of the metabolic products of antagonistic microorganisms as natural fungicides to control soil-borne seed and seedling diseases. *Pythium ultimum* was chosen as the test pathogen due to its commercial importance, wide host range and ubiquity.

Four major objectives were identified:

1) to detect organisms which synthesize extracellular metabolites antagonistic to *P. ultimum in vitro* (Chapter 3);

2) to optimize the yield of the active compounds by manipulation of the growth medium and cultural conditions (Chapter 3);

3) to characterize the antagonistic products (Chapter 4); and

4) to assess their utility as *P. ultimum* control agents when incorporated into sugar beet seed pellets (Chapters 5 and 6).

CHAPTER 2

MATERIALS AND METHODS

2.1 Microorganisms

2.1.1 Phytopathogens

Details of the organisms used as test pathogens are given in Table 2.1. The Oomycetes were maintained on corn meal agar (CMA, section 2.2) and the higher fungi on malt extract agar (MEA, section 2.2) at 4°C.

The primary test pathogen was Pythium ultimum Trow. Mycelial inocula for bioassays consisted of 5mm diam. cores cut from the margins of a 2d old culture on CMA incubated at 25°C. To induce the formation of survival propagules, mycelial inocula were transferred into 90mm diam. Petri dishes (one core per dish) containing 20ml sterile tap water (STW) and two sterile rolled oat seeds and incubated at 25°C (Stanghellini & Hancock, 1971a). Propagule suspensions were prepared by homogenizing 10-14d old mycelial mats in 20ml STW with an ultra-turrax probe (Janke & Kunkel KG) for 2min, followed by filtration through muslin to remove hyphal fragments. Propagules were enumerated using a haemocytometer after staining with 10% (v/v) cotton blue-lactophenol (BDH Chemicals Ltd.). One to ten $x10^5$ propagules were obtained from each Petri dish culture.

Table 2.1. Organisms used as test phytopathogens

Organism	Source*	Reference number
Aphanomyces cochliodes	CMI	IMI 241682
Botrytis cinerea	UKC	IMI 100465
Fusarium culmorum	CMI	IMI 159025
Fusarium solani	UKC	IMI 128552
Helminthosporium trucilicum	UKC	i 1318
Phytophthora citricola	CMI	IMI 69664
Pythium aphanidermatum	UKC	-
Pythium debaryanum	CMI	IMI 48558
Pythium mamillatum	CMI	IMI 120409
Pythium ultimum	UKC	IMI 82514
Rhizoctonia solani	UKC	-
Thanatephorus cucumeris	CMI	IMI 144875

* CMI CAB International Mycological Institute, Ferry Lane, Kew, Surrey TW9 3AF.

UKC Biological Laboratory culture collection, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ.

2.1.2 Antagonists

Organisms screened for production of metabolites inhibitory to Pythium ultimum included species and representitives of genera reported to be effective biocontrol agents of Pythium in situ and/or to show antagonism in vitro (Table 2.2), plus isolates obtained by surface dilution plating soil samples (collected from the University of Kent campus) onto soil extract agar (section 2.2).

Successful fungal antagonists were maintained on MEA at 4°C and, depending on rate of growth, experimental inocula consisted of 5mm diam. cores cut from the margins of 2-4d old cultures on MEA incubated at 25°C. Bacterial and actinomycete antagonists were maintained on nutrient agar (NA, section 2.2) at 4°C, and experiments were inoculated with a single colony picked off a 4d old culture on NA incubated at 25°C.

Following optimization of yield (section 3.3), liquid cultures of the two most effective antagonists, Penicillium claviforme Bainier and Trichoderma sp. 1, were grown in Czapek-Dox medium (CDM, pH 4.4-4.6, section 2.2) and Weindling's medium (WM, pH 4.5, section 2.2), respectively. Erlenmeyer flasks (250ml or 500ml) containing 100ml or 200ml medium were inoculated with two or four 10mm diam. cores cut from the margins of 4d (Pen. claviforme) or 2d (Trichoderma sp.1) growth on MEA. Flasks were incubated at 25°C under stationary conditions. Pen. claviforme culture filtrate (PCF) was removed after 18-21d and Trichoderma sp. 1 culture filtrate (TSF) after 10-13d incubation. Culture filtrates were filter sterilized before use in subsequent experiments.

Table 2.2. Organisms screened for antagonistic activity against *P. ultimum*

Organism	Source*	Reference number
Fungi		
Aspergillus flavus	UKC	IMI 910196
Aspergillus terreus	UKC	1897
Chaetomium globosum	UKC	-
Gliocladium roseum	UKC	IMI
Penicillium chrysogenum	UKC	IMI 37767
Penicillium claviforme	UKC	IMI 44744
Penicillium expansum	UKC	IMI 39761
Penicillium frequentans	UKC	IMI 28043
Penicillium griseofulvum	UKC	F 448
Penicillium nigricans	UKC	167032
Penicillium oxalicum	CMI	IMI 39750
Sporotrichum pulverulentum	UKC	IMI 174727
Trichoderma harzianum	JRCS	IMI 298372
Trichoderma harzianum	JRCS	IMI 298373
Trichoderma koningii	UKC	110144
Trichoderma sp. 1	Umi	UKC 17
Trichoderma viride	JRCS	IMI 298376
Trichoderma viride	JWD	T 13

Table 2.2 continued. Organisms screened for antagonistic activity against *P. ultimum*

Organism	Source*	Reference number
Bacteria		
Arthrobacter globiformis	UKC	NCIB 7811
Enterobacter cloacae	NCTC	NCTC 11461
Pseudomonas fluorescens	UKC	NCIB 3756
Actinomycetes		
Streptomyces griseus	UKC	NCIB 8136
Streptomyces venezuellae	UKC	UC 82

* CMI CAB International Mycological Institute, Ferry Lane, Kew, Surrey TW9 3AF.

JRCS Prof. J.R. Coley-Smith, University of Hull, HU6 7RX.

JWD Dr. J.W. Deacon, University of Edinburgh, West Mains Rd., Edinburgh EH9 3JG.

UKC Biological Laboratory culture collection, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ.

Umi Isolated from straw compost (Shah, 1987) in the Agricultural and Environmental Microbiology Group, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, and deposited in the Biological Laboratory culture collection.

NCTC Public Health Laboratory Service Board, 61, Colindale Ave., London NW9 5EQ.

2.2 Microbiological growth media

Recipes for agar and liquid media are given in alphabetical order. All were sterilized by autoclaving at 15psi for 15min.

Alkaline water agar (Ho & Ko, 1980)

Agar No. 3 (Oxoid)	15.0g
1M NaOH	3.5ml
Tap water (TW)	996.5ml

NaOH was added to molten (50°C) water agar after autoclaving and the volume was that predetermined as necessary to adjust the pH to 10.5.

Corn meal agar

Corn meal agar (Oxoid)	17.0g
Distilled water (DW)	1000.0ml

Czapek-Dox medium (Bassett & Tanenbaum, 1958)

$NaNO_3$	3.0g
KH_2PO_4	1.0g
KCl	0.5g
$MgSO_4.7H_2O$	0.5g
$FeSO_4.7H_2O$	0.01g
Glucose	40.0g
DW	1000.0ml

Malt extract agar

Malt extract (Oxoid)	30.0g
Mycological peptone (Oxoid)	5.0g
Agar No. 3 (Oxoid)	15.0g
DW	1000.0ml

Malt extract broth

Malt extract (Oxoid)	30.0g
Mycological peptone (Oxoid)	5.0g
DW	1000.0ml

Nutrient agar

Nutrient broth No. 2 (Oxoid)	25.0g
Agar No. 3 (Oxoid)	15.0g
DW	1000.0ml

Nutrient broth

Nutrient broth No. 2 (Oxoid)	25.0g
DW	1000.0ml

Pentachloronitrobenzene-nutrient agar (Chen et al., 1988)

Nutrient broth (Oxoid)	25.0g
Pentachloronitrobenzene (PCNB, Sigma)	0.1g
Agar No. 3 (Oxoid)	15.0g
DW	900.0ml

PCNB was prepared according to Farley and Lockwood (1968). The chemical (0.1g) was dissolved in 1ml acetone in a stoppered test tube and allowed

to stand for 2h. The solution was then added to 99ml sterile distilled water (SDW) containing 0.05ml Triton X-100, resulting in the formation of a milky suspension which was added to the molten (50°C) nutrient medium after autoclaving.

Raulin-Thom medium (Birkenshaw et al., 1943)

Glucose	50.0g
Tartaric acid	2.7g
Ammonium tartrate	2.7g
$(NH_4)_2HPO_4$	0.4g
K_2CO_3	0.4g
$MgCO_3$	0.27g
$(NH_4)_2SO_4$	0.17g
$ZnSO_4.7H_2O$	0.05g
$FeSO_4.7H_2O$	0.05g
DW	1000.0ml

Schmitthenner's medium (Schmitthenner, 1979)

Sucrose	2.40g
Asparagine	0.27g
KH_2PO_4	0.15g
K_2HPO_4	0.15g
$MgSO_4.7H_2O$	0.10g
Cholesterol (Sigma)	0.01g
Benlate (ICI Garden Products)	0.02g
Pentachloronitrobenzene (Sigma)	0.027g
Neomycin sulphate (Sigma)	0.10g

Chloramphenicol (Sigma)	0.01g
Agar No. 3 (Oxoid)	15.0g
DW	994.0ml

Cholesterol was dissolved in N,N-dimethylformamide (Sigma, 0.005g ml^{-1}), Benlate in SDW (0.02g ml^{-1}), PCNB in chloroform (0.027g ml^{-1}), neomycin sulphate in SDW (0.10g ml^{-1}) and chloramphenicol in methanol (0.01g ml^{-1}). These solutions were added to the molten (50°C) basal medium after autoclaving.

Soil extract agar (Parkinson, Gray & Williams, 1971)

Air-dry soil	1000.0g
Agar No. 3 (Oxoid)	15.0g
TW	1000.0ml

Soil was autoclaved with TW at 15psi for 20min. The liquid was filtered (Whatman filter paper no. 1) and restored to 1000.0ml in volume. The extract was then resterilized and solidified with agar.

Surfactant-potato dextrose agar (Steiner & Watson, 1965)

Potato dextrose agar (Difco)	39.0g
Streptomycin sulphate (Sigma)	0.1g
Tergitol NP-10 (Sigma)	1.0ml
DW	998.0ml

Streptomycin sulphate dissolved in SDW (0.1g ml^{-1}) and tergitol NP-10 were added to the molten (50°C) nutrient medium ater autoclaving.

Weindling's medium (Weindling, 1941)

Glucose	25.0g
Ammonium tartrate	0.05g
KH_2PO_4	2.0g
$MgSO_4$	1.0g
$FeCl_3.6H_2O$	0.02g
DW	1000.0ml

2.3 Detection of antagonistic metabolites

2.3.1 Cellophane overlay method for screening antagonists

Potential antagonists (section 2.1.2) were inoculated onto the centre of a layer of sterile cellophane, thickness 22.6μm (BCL Cellophane, 325 P), overlaying an agar medium in 90mm Petri dishes (Dennis & Webster, 1971). MEA or NA were used to test fungi or bacteria and streptomycetes, respectively. Agar plates were incubated at 25°C for varying lengths of time to allow reasonable growth of the organism to occur, as indicated in Table 3.1. The cellophane and adhering microbial growth were then removed and the agar inoculated with a mycelial core of *Pythium ultimum* (section 2.1.1), upper surface down, in the central position previously occupied by the antagonist. Percentage inhibition of *Pythium* radial growth was determined after a further 24h incubation at 25°C, by comparison of mean colony diam. (excluding the inoculum core) with that of controls growing on MEA or NA pre-incubated with cellophane alone. Each colony diam. was recorded as the mean of two measurements taken at right angles to each other.

2.3.2 Bioassay of antagonistic metabolites

Inhibitory metabolite-containing culture filtrates, extracts or eluates were filter-sterilized (Whatman, pore size 0.2μm), incorporated into molten (50°C) CMA in 90mm Petri dishes, 1ml per 20ml medium, and inoculated with Pythium ultimum (section 2.3.1). Percentage inhibition was determined after 24h incubation at 25°C by comparison of mean colony diam. with that of controls growing on 20ml CMA containing 1ml sterile growth medium or SDW, or an appropriate eluate or extract where indicated. The concentration of metabolites in highly active samples was measured in inhibitory units (IU) ml^{-1}. One IU is defined as the reciprocal of the greatest dilution of that sample which completely suppressed P. ultimum growth under the above conditions. Sample dilutions were bioassayed and dilutions plotted on a logarithmic scale against P. ultimum inhibition, giving a standard curve which was then extrapolated to show the dilution necessary to cause 100% pathogen suppression (Gregory et al., 1952). An example is given in Fig. 2.1.

2.4 Characterization of antagonistic metabolites

2.4.1 Ultrafiltration

Fifteen ml samples of PCF and TSF were separated into three fractions by ultrafiltration using membranes of nominal mol. wt. cut-off points of 1,000 and 10,000 daltons (Amicon, Diaflo UM2 and PM10, respectively). Each sample was first filtered through a PM10 membrane. The metabolites retained on the upper surface were washed off by turning the membrane over and flushing through 15ml DW, thus giving a fraction

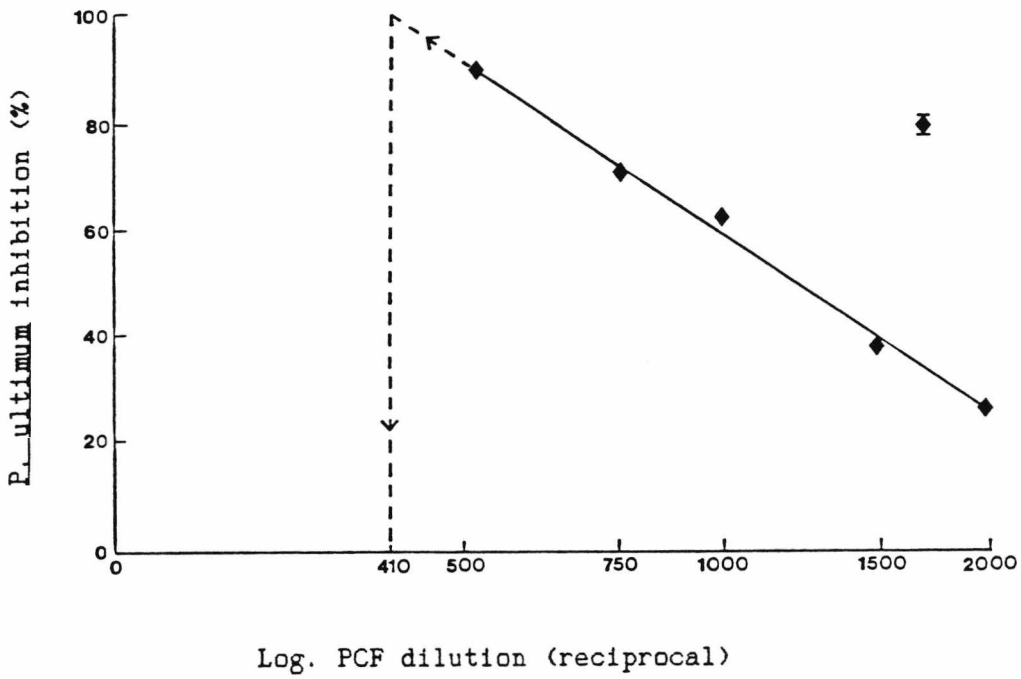


Fig. 2.1. Measurement of P. ultimum-inhibitory activity of Pen. claviforme culture filtrate extract (PCF) containing 410IU ml^{-1}. Bar represents +/- mean SE.

with nominal mol. wt. >10,000 daltons. The filtrate obtained from the first operation was then passed through a UM2 membrane, resulting in a filtrate with nominal mol. wt. <1,000 daltons. Finally, the metabolites retained on the upper surface of this second membrane were washed off in 15ml DW, giving a fraction with nominal mol. wt. 1,000-10,000 daltons. The three fractions were bioassayed (section 2.3.2) and their anti-*Pythium* activity compared with that of an unfractionated sample.

2.4.2 Assay for laminarinase and cellulase

Aliquots of PCF or TSF (1.5ml) were mixed with an equal volume of 1% (w/v) laminarin or 1% (w/v) carboxymethylcellulose dissolved in 0.1M sodium acetate buffer (pH 5.0) and incubated at 50°C for 30min (Kalra, Sidhu & Sandhu, 1986). Reducing sugars were then estimated according to Miller (1959) by mixing with 3ml dinitrosalicylic acid reagent (1g dinitrosalicylic acid, 0.2g phenol, 0.05g sodium sulphite dissolved in 100ml 1% (w/v) sodium hydroxide) and incubating at 100°C for 15min. One ml 40% sodium potassium tartrate was added and samples cooled to ambient temperature under running water. Absorbance was read at 575nm (LKB Ultrospec 4050) using the respective sterile growth medium incubated with enzyme substrate as a blank. Enzyme activities, expressed in $\mu g\ ml^{-1}\ min^{-1}$ glucose equivalents, were calculated by reference to standard curves prepared using known concentrations of glucose (0-200$\mu g\ ml^{-1}$) incubated with enzyme substrate under identical conditions. Samples were diluted as necessary so that absorbance values fell within the linear region of the standard curves.

2.4.3 Thin-layer chromatography

Thin-layer chromatography was conducted according to Braithwaite & Smith (1985). Silica gel 60 tlc plates 250μm thick (Merck 5721, obtained from BDH Ltd.) were activated at 105°C for 45min. PCF or CDM concentrate or extract (section 2.5.1) or purified patulin (Sigma Chemical Co. Ltd.), dissolved in DW, were applied to the plates in 2μl aliquots using disposable micro-pipettes (Drummond Scientific Co.), allowing the spots to air-dry between successive applications. A template was used to position the samples not less than 15mm apart on a start line 15mm from the lower edge. The plates were allowed to dry and then transferred to a chromatography tank lined with filter paper (Whatman, no. 1) and containing 100ml solvent (giving a depth of approx. 5mm), which had been equilibrated for 1h. The two solvent systems used were: a) toluene/ethyl acetate/formic acid (5:4:1, v/v) and b) chloroform/ methanol (4:1, v/v) (Land & Hult, 1987). Plates were developed until the solvent front had reached a stop line scored across the adsorbent layer 150mm above the starting positions, and removed from the tank. When dry the chromatograms were examined under short wavelength (λ_{max} = 254nm) uv light and then sprayed, using a laboratory spray gun (Shandon Southern Products), with one of the following reagents to locate the separated components: a) 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride (MBTH) in DW (Land & Hult, 1987), b) 5% (v/v) conc. sulphuric acid in ethanol (Anon, 1980a) or c) 0.5% (v/v) anisaldehyde in methanol/acetic acid/conc. sulphuric acid (17:2:1, v/v) (Paterson, 1986). In each case chemical treatment was followed by heating the plates at 105°C for 10min.

The Pythium ultimum-inhibitory activity of the separated components was determined using two tlc plates developed concurrently. The first was sprayed with MBTH and was employed as a reference, while the second was used for bioassays. The silica layer at positions corresponding to the spots located on the reference plate was removed using a micro-spatula and fine paint brush. The material present was eluted by standing in 1ml DW for 30min. Eluates were bioassayed (section 2.3.2) in duplicate on a miniaturized scale, using 0.3ml samples incorporated into 5.7ml molten CMA in 55mm diam. Petri dishes. Controls were spots of DW removed from the plate and eluted in the same manner, and the % extraction of material from the chromatograms was calculated by comparison of the activity applied to an undeveloped spot with that removed after drying.

2.4.4 Infrared spectroscopy

Silica gel 60 preparative layer plates 2000μm thick (Merck 5717, obtained from BDH Ltd.) were used without activation as described by the manufacturers (Anon, 1980b). PCF extract (50IU ml^{-1}, section 2.5.1) or patulin (2.5mg ml^{-1}), dissolved in chloroform, were applied in a band (forty 10μl spots) 15mm from the lower edge. Plates were developed in 200ml solvent system b) (section 2.4.3) and the location of the intense yellow band on a reference plate sprayed with MBTH (section 2.4.3) used to determine the position of the bioactive band on the sample plates (section 4.6). The silica layer in these bands was removed and the adsorbed metabolites eluted by standing for 10min in ten successive 1ml aliquots of chloroform. The combined eluates were centrifuged (MSE Centaur 2, 2000rev min^{-1}, 10min) to deposit colloidal silica

particles. The solvent was then removed by vacuum evaporation at 65°C (section 2.5.1), and the residue redissolved in 100µl chloroform. Samples were transferred into wells approx. 3mm diam. and 2mm deep made in the centre of potassium bromide discs, and the solvent evaporated off with a hot air-drier. The i.r. spectrum was determined in a fourier-transfer i.r. spectrometer (Mattson Instruments Inc., Alpha Centauri) using an attachment made by the Chemistry Laboratory Workshop, University of Kent, to concentrate the i.r. beam onto the sample in the central well.

2.5 Production of PCF-containing seed pellets

2.5.1 Preparation of PCF extract

PCF was concentrated and partially purified using a method based on that described by Norstadt & McCalla (1969a). The culture filtate (section 2.1.2) was first concentrated to approx. $^1/_{20}$ of its original volume on a rotary vacuum evaporator (Büchi RE111) in a water bath at 65°C. The concentrate was transferred to a 250ml Erlenmeyer flask and an equal volume of ethyl acetate (EtAc) was added. The flask was shaken on a rotary shaker (200rev min^{-1}) for 10min. Aqueous and EtAc fractions were separated in a glass separation funnel and the lower aqueous layer was extracted with a second equal volume of EtAc. The two EtAc fractions were combined, dried over anhydrous sodium sulphate, the solvent removed by vacuum evaporation and the residue redissolved in DW (sections 2.4.3, 2.5.2 and 2.6) or chloroform (section 2.4.4). In some

experiments CDM extract, prepared in an identical manner, was used for controls.

2.5.2 Incorporation of PCF extract into seed pellets

PCF extract was incorporated into sugar beet (Beta vulgaris L. cv. Accord) seed pellets by Germain's (U.K.) Ltd., King's Lynn, Norfolk (Plate 2.1). In place of the usual thiram steep treatment (section 5.6.2), raw 'seed' (botanically fruit: Perry & Harrison, 1974) was soaked in water for 16h at 25°C prior to pelleting. Twenty ml extract (section 2.5.1), containing approx. 410IU ml^{-1}, was applied to 0.25 unit seed (1 unit = 10^5 seeds, section 6.5.1) to give a target dose of 0.33IU $seed^{-1}$. The method by which a suitable rate of incorporation was calculated is given in the Appendix. The achieved dose was 0.30IU $seed^{-1}$ (section 6.1). Properties of 'PCF-pellets' were compared with those of pellets containing the synthetic fungicide hymexazol (5-methylisoxazol-3-ol, Hm, Sankyo Co. Ltd.) at the recommended rate of 10.5g a.i. kg^{-1} seed, equivalent to 15g kg^{-1} seed of the commercial formulation Tachigaren, ('Hm-pellets') and pellets without additives ('control pellets'), produced in an otherwise identical manner.

In some experiments pellets were prepared using a laboratory method designed to simulate the commercial pelleting process. The required amount of PCF extract in 10µl DW, or an equivalent volume of DW alone, was spotted individually onto pellets without additives which were left wet for 1h and then dried for approx. 1h in a laminar-flow cabinet (Microflo).

(a) (b)

Plate 2.1. Sugar beet (Beta vulgaris) seed, (a) raw and (b) commercially pelleted with 0.30IU Pen. claviforme culture filtrate extract. One division on scale = 1mm.

2.6 Effects of PCF in the seed pellet environment

2.6.1 Seedling growth media

Details of seedling growth media are given in Table 2.3. Moisture content is expressed in terms of container capacity, defined as the volume of water per g dry wt. held in a given plant growth medium in a container of a given depth with saturation at its lower surface and in the absence of evaporation (White & Mastalerz, 1966). Container capacity was estimated in polystyrene seedling wedge cells (Accelerated Propagation Ltd., Vines Cross, Heathfield, Sussex), consisting of forty individual cells each 38 x 38mm at the upper rim, 50mm in depth and with a single drainage hole at the base. The medium was poured into a pre-weighed container and settled by tapping down on a bench surface. The process was repeated until settling was no longer detectable and the medium was level with the top of the wedge cells. The container was placed in a propagator tray to which water was added to a level 10mm below the upper rim and covered with aluminium foil to reduce evaporation. The system was allowed to equilibrate for 24h, the water removed and gravitational water permitted to drain away for a further 24h. The wt. of the container and contents was measured and used to calculate the wt. of the medium at container capacity. The contents of the wedge cell container were removed and mixed. Samples of 5g were oven-dried at 105°C to constant wt. and the total dry wt. of the medium calculated. The difference between the first and second values, divided by the second, gave the container capacity per g oven-dry wt. medium.

Table 2.3. Properties of seedling growth media

	Peat-based compost[a]	Soil[b]
pH[c]	5.6	6.2
Organic matter (%, w/w)[d]	51.0	4.7
Container capacity in seedling wedge cells (ml g^{-1} dry wt.)[e]	6.81	0.80

[a] Fisons plc, Levington compost, Premier Sowing Formula.
[b] From field plots at Shell Research Ltd., Sittingbourne Research Station.
[c] pH of a 2:5 medium:water slurry 10min after mixing.
[d] % dry wt. loss on ignition.
[e] Determined according to White & Mastalerz (1966).

Where sterile media were required they were autoclaved at 15psi for 15min on each of three consecutive days. Sterility was verified by surface dilution plating onto NA and MEA.

2.6.2 Seedling growth conditions

Seed pellets were planted in seedling wedge cells (section 2.6.1), each containing 15.8+/-1.7g peat-based compost (Table 2.3). Four pellets were sown per cell at a depth of 25mm, and 8ml TW or *Pythium ultimum* propagule suspension (section 2.1.1) were then applied immediately to the compost surface to give a water content of 75.0+/-3.5% container capacity (section 2.6.1). Wedge cells containing infested and non-infested compost were restricted to separate containers and were incubated in propagators (Stewart Plastics plc, 245 x 430mm) in a constant environment plant growth room (20°C, Philips warm white 65-80W, 16:8h light:dark cycle). Containers were watered daily to constant weight.

Germination was assessed in Petri dishes (20 pellets per dish) containing sterile filter paper (Whatman, no.1) moistened with SDW and incubated at 25°C in the dark.

2.6.3 Bioassay of PCF activity in seed pellets

PCF-containing pellets were incubated in batches of fifteen in 5ml DW at room temperature (20+/-2°C) for 2h. The extracts were filter sterilized and bioassayed (section 2.3.2), using extracts of pellets without additives as controls.

2.6.4 Bioassay of PCF activity in seedling growth media

Samples of PCF-amended peat-based compost (1.0g) or soil (0.8g) were vortex mixed (Hook & Tucker Ltd., maximum speed) in 5ml DW for 0.5min, and then centrifuged (MSE Centaur 2, 4000rev min^{-1}, 10min). The supernatants were filter sterilized and bioassayed (section 2.3.2), using extracts of the appropriate non-amended medium as controls.

2.6.5 Pathogen population dynamics

Peat-based compost was infested with Pythium ultimum as described in section 2.6.2. Samples of the medium (5g) were taken from the region surrounding seed pellets, or from an equivalent depth in unplanted wedge cells, placed in 250ml Erlenmeyer flasks and shaken with 50ml 0.3% tap water agar (TWA) on a wrist-action flask shaker for 30min (Chen, Hoitink & Schmitthenner, 1987). Serial 10-fold dilutions were made in 0.3% TWA and 1ml aliquots of each of three successive dilutions spread onto the Pythium-selective medium given by Schmitthenner (1979) (SM, section 2.2). Dilution plates were incubated at 25°C for 48h, rinsed with TW to remove debris where necessary, and colonies counted. Results were recorded as the number of colony forming units (cfu) g^{-1} dry wt. compost (dry wt. determined on separate 5g samples).

2.6.6 Microbial activity in the spermosphere

Total microbial activity in peat-based compost and soil was measured by the fluorescein diacetate (FDA) method (Chen et al., 1988). Samples of medium (1g) were removed from the volume adjacent to seed pellets, or from an equivalent depth in unplanted wedge cells, placed in 50ml Erlenmeyer flasks and mixed with 4ml 0.1M sodium phosphate buffer

(pH 7.6) and 80μg FDA (Sigma Chemical Co. Ltd., 40μl of a solution containing 2mg ml^{-1} acetone). Flasks were shaken on a rotary shaker (90rev min^{-1}) at 25°C for 20min. The reaction was then terminated by adding 4ml acetone. Medium residues were removed by filtration (Whatman, filter paper no. 1) and the concentration of fluorescein determined by measurment of absorbance at 490nm (LKB Ultrospec 4050) using a sample terminated at time 0 as a blank. Results are given in relative terms, expressed directly as absorbance values. Linearity of the relationship between absorbance and fluorescein concentration in the region of interest was confirmed by reference to a standard curve prepared using dilutions of a soil sample showing high levels of activity.

2.6.7 Microbial populations in the spermosphere

Samples (5g) were removed from the volume of peat-based compost surrounding seed pellets, transferred to 250ml Erlenmeyer flasks and shaken with 50ml SDW on a wrist-action shaker for 30min. A 10-fold dilution series in SDW was prepared and 1ml aliquots of each of three successive dilutions incorporated into molten (50°C) agar medium selective for each of the three principle groups of soil microorganisms. Surfactant-potato dextrose agar (Steiner & Watson, 1965), pentachloro-nitrobenzene-NA (Chen *et al.*, 1988) and alkaline water agar (Ho & Ko, 1980) (section 2.2) were used to enumerate fungi, bacteria and actinomycetes, respectively. Dilution plates were incubated at 25°C for 7d (fungi and bacteria) or 14d (actinomycetes). Counts are expressed as cfu g^{-1} dry wt. compost (dry wt. determined on separate 5g samples).

For microscopic examination of fungi, spore-bearing mycelium was removed from purified colonies and mounted in 10% (w/v) potassium hydroxide on glass slides. Specimens were teased apart with mounted needles and examined under x40 and x100 magnification. Isolates were identified to genus using the key and descriptions provided by Domsch, Gams and Anderson (1980).

2.7 Statistical treatment of results

Data points given in Figures are the means ($\bar{x}$) of at least three replicate samples (x_i) and, where possible, are shown with bars representing +/- the standard error of the mean (SE), given by [1]:

[1] $$SE = s / \sqrt{n}$$

where s is an estimate of the standard deviation σ, and n is the number of replicates. s can be calculated from [2]:

[2] $$s = \sqrt{(x_i - \bar{x})^2 / n-1}$$

The indirect method by which the inhibitory activity of highly potent culture filtrates and extracts was determined (section 2.3.2) did not permit a simple expression of the variation between replicate samples. Where the character of the data required was relative rather than absolute, solutions were therefore diluted at the start of the experiment such that results fell within the range 0-1IU (0-100% Pythium ultimum inhibition). Variation could then be calculated as SE in the conventional manner.

Treatment means were compared using Duncan's new multiple range test (Steel & Torrie, 1960) in which differences between means are compared with a set of least significant ranges (LSR) of varying sizes, the size

used for any pair of means depending on their closeness after ranking. LSR is given by [3]:

[3] $LSR = SSR \times s_{\bar{x}}$

SSR is the significant studentized range and is extracted from standard tables according to p, the number of means for the range being tested and Rdf, the residual degrees of freedom. $s_{\bar{x}}$ is calculated from [4]:

[4] $s_{\bar{x}} = \sqrt{(\text{residual mean square}) / n}$

Calculation of Rdf and residual mean square (RMS) are given in standard text books (Parker, 1979; Steel & Torrie, 1960). If the difference betwen a pair of means exceeds the value of the respective LSR, then they are considered to be significantly different, and are followed by a different letter. A multiple range test is essential when comparing more than two treatment means because the use of least significant differences (LSD) increases to an unacceptable level the risk of indicating a significant result when in fact no effect is present.

The following example relates to seedling emergence in Pythium-infested compost in Trial 2 (column 1, Table 5.2).

1) Calculation of $s_{\bar{x}}$:

$$s_{\bar{x}} = \sqrt{0.733 / 40}$$

$$= 0.14$$

2) Calculation of LSR, with Rdf = 115:

p	2	3
SSR	2.80	2.95
LSR	0.39	0.41

3) Ranking of the treatment means:

None	PCF	Hm
1.80	2.90	3.15

4) Testing the differences between the treatment means:

Hm - None = 1.35 > 0.41; significant

Hm - PCF = 0.25 < 0.39; not significant

PCF - None = 1.10 > 0.39; significant

therefore Hm and PCF are assigned the same letter, and None a different letter.

CHAPTER 3

PRODUCTION OF ANTAGONISTIC METABOLITES

The first requirement of the project was to detect microorganisms capable of producing extracellular metabolites inhibitory to Pythium ultimum. This was achieved in three stages:

1) A range of possible antagonists were screened using a cellophane overlay technique (section 3.1).

2) Organisms showing potentially useful activity were tested for the production of inhibitory agents in liquid media (section 3.2).

3) The yield of inhibitors produced by the two most promising antagonists was optimized by manipulation of the nutritional and cultural conditions (section 3.3).

3.1 Screening for antagonists

Microorganisms were initially screened by the cellophane overlay method described in section 2.3.1. Triplicate overlay plates were prepared for each potential antagonist. Results are given in Table 3.1.

A large number of the organisms tested retarded the radial growth of Pythium ultimum. It was assumed at this stage that severe inhibition by the test antagonists was due to the production of extracellular metabolites which had diffused through the cellophane into the agar medium. The Penicillium and Trichoderma spp. examined were frequently

Table 3.1. Inhibition of *P. ultimum* radial growth by microbial metabolites produced on agar media

Test antagonist[a]	Duration of incubation of test antagonist (d)[b]	Inhibition of *P. ultimum* radial growth (%)[c]
Aspergillus flavus	4	69.5 (2.0)
Aspergillus terreus	4	75.3 (1.2)
Chaetomium globosum	3	52.8 (3.1)
Gliocladium roseum	4	62.9 (1.2)
Penicillium chrysogenum	4	74.2 (1.2)
Penicillium claviforme	4	100.0 (0.0)
Penicillium expansum	4	43.2 (3.1)
Penicillium frequentans	4	100.0 (0.0)
Penicillium griseofulvum	4	76.4 (5.1)
Penicillium nigricans	4	62.6 (4.1)
Penicillium oxalicum	4	10.3 (3.1)
Sporotrichum pulverulentum	3	22.3 (3.1)
Trichoderma harzianum 372	2	81.9 (2.0)
Trichoderma harzianum 373	2	100.0 (0.0)
Trichoderma koningii	2	100.0 (0.0)
Trichoderma sp. 1	2	39.2 (3.3)
Trichoderma viride 376	2	100.0 (0.0)
Trichoderma viride T 13	2	72.6 (4.1)
Soil isolate F1	4	3.2 (1.2)
Soil isolate F2	2	100.0 (0.0)
Soil isolate F3	4	100.0 (0.0)
Soil isolate F4	3	75.0 (1.2)
Soil isolate F5	4	8.9 (2.0)

Table 3.1 continued. Inhibition of *P. ultimum* radial growth by microbial metabolites produced on agar media

Test antagonist[a]	Duration of incubation of test antagonist (d)[b]	Inhibition of *P. ultimum* radial growth (%)[c]
Arthrobacter globiformis	4	0.0 (2.0)
Enterobacter cloacae	4	2.4 (5.1)
Pseudomonas fluorescens	4	54.2 (3.1)
Soil isolate B1	4	10.2 (4.1)
Soil isolate B2	4	5.7 (4.1)
Soil isolate B3	4	15.4 (3.1)
Soil isolate B4	4	9.3 (5.1)
Soil isolate B5	4	31.7 (3.1)
Streptomyces griseus	4	100.0 (0.0)
Streptomyces venezuellae	4	69.2 (4.1)
Soil isolate A1	4	78.5 (5.1)

[a] Details are given in Table 2.1.

[b] Test antagonists were inoculated onto a layer of cellophane overlaying an agar medium and incubated at 25°C for the time given. The cellophane and microbial growth were then removed and the agar inoculated with *P. ultimum*.

[c] Results were determined after a further 24h incubation by comparison of mean colony diam. with that of controls growing on the agar medium pre-incubated with cellophane alone. SE are given in parentheses.

highly active and seven members of these two genera (including two soil isolates) totally prevented Pythium growth. Actinomycetes were also strongly suppressive, but other bacteria showed only low activity under the test conditions.

3.2 Selection of antagonists

3.2.1 Production of inhibitory metabolites in liquid media

The nine organisms causing greater than 80% inhibition of Pythium ultimum in the primary screen, plus Trichoderma sp. 1 which was obtained at a later date, were tested for production of inhibitory metabolites in liquid media. Once detected active compounds are more readily extracted from solutions than from solid substrata. All fungi were grown in malt extract broth (MEB) and Streptomyces griseus in nutrient broth. Erlenmeyer flasks (500ml) containing 200ml medium were inoculated with four 10mm diam. cores cut from the margins of 2-4d old fungal cultures on MEA, or with a single streptomycete colony picked off a 4d old culture on NA, and incubated at 25°C on a rotary flask shaker (200rev min^{-1}). Samples of the culture filtrates were removed at regular intervals and centrifuged (MSE High Speed 18, 18,000rev min^{-1}, 30min). The supernatants were filter sterilized and bioassayed as specified in section 2.3.2 except that the assay medium used was MEA. Each antagonist was grown in triplicate flasks, and each culture was bioassayed in duplicate. Results are given in Figs. 3.1-3.4.

Most organisms caused lesser inhibition of pathogen radial growth than in the cellophane test. Trichoderma viride 376 was more

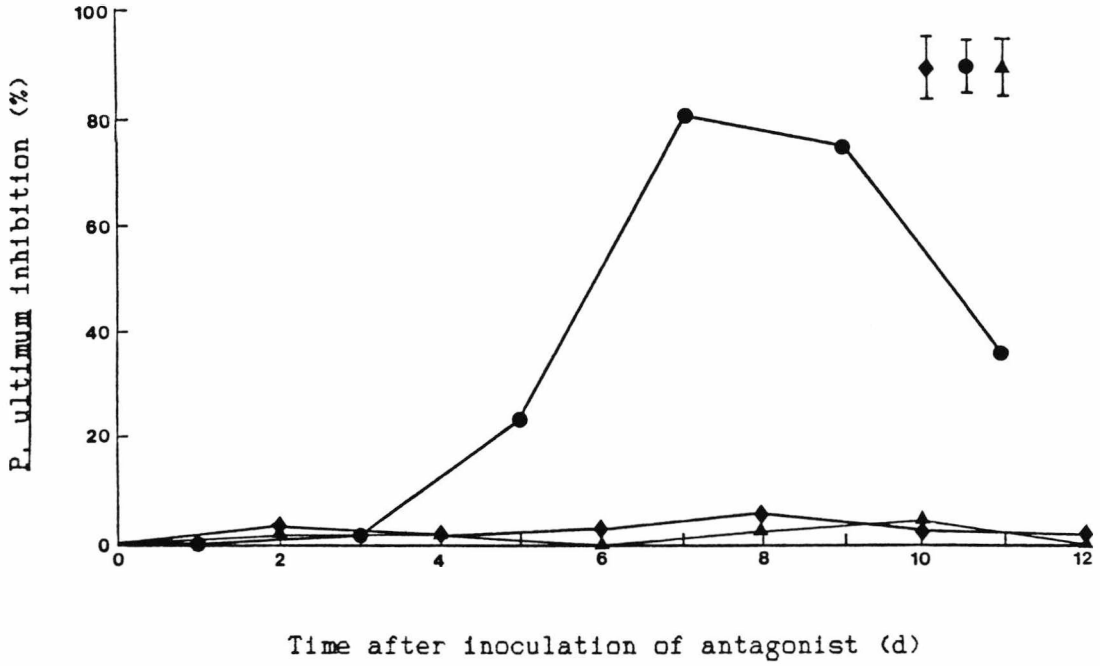


Fig. 3.1. Inhibition of P. ultimum radial growth by culture filtrates of Streptomyces griseus (◆), Penicillium claviforme (●) and Pen. frequentans (▲). Antagonists were grown in malt extract broth (25°C, 200rev min^{-1}). Bars represent +/- mean SE.

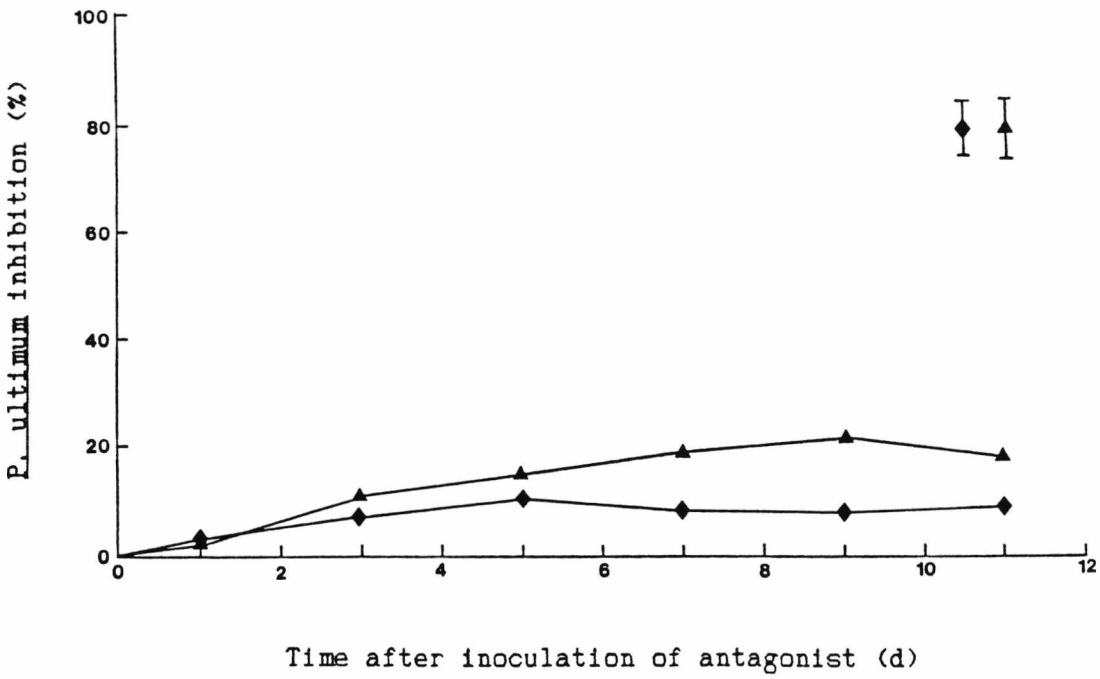


Fig. 3.2. Inhibition of P. ultimum radial growth by culture filtrates of soil isolates F2 (◆) and F3 (▲). Antagonists were grown in malt extract broth (25°C, 200rev min^{-1}). Bars represent +/- mean SE.

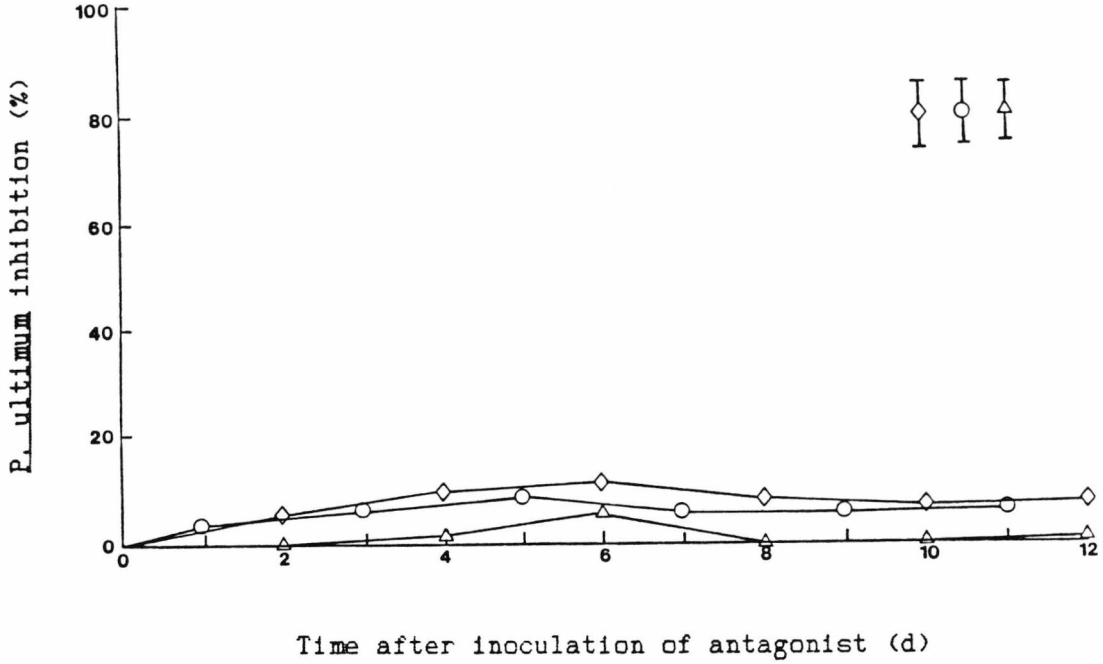


Fig. 3.3. Inhibition of P.ultimum radial growth by culture filtrates of Trichoderma harzianum 372 (◇), T. harzianum 373 (○) and T. koningii (△). Antagonists were grown in malt extract broth (25°C, 200rev min^{-1}). Bars represent +/- mean SE.

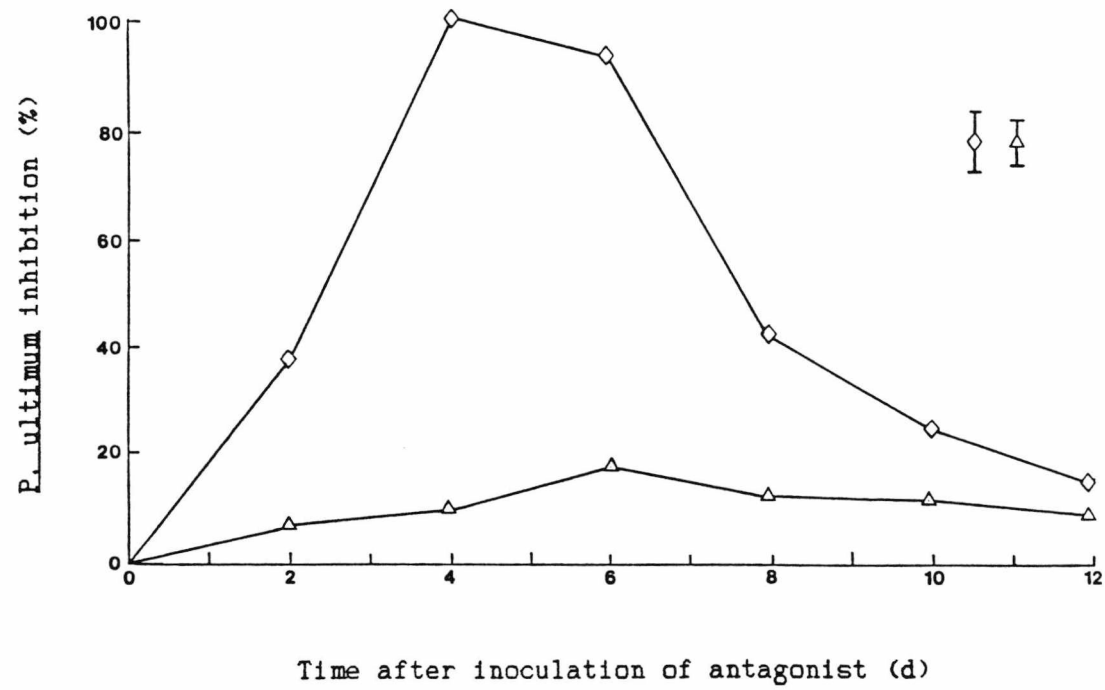


Fig. 3.4. Inhibition of P. ultimum radial growth by culture filtrates of Trichoderma sp. 1 (◇) and T. viride 276 (△). Antagonists were grown in malt extract broth (25°C, 200rev min^{-1}). Bars represent +/- mean SE.

suppressive than was apparent from measurement of Pythium colony diam. alone since the mycelial density was also reduced. Attempts to quantify the results by removing colonies from the agar and determining their dry wt. were unsuccessful due to the small quantities of biomass present. In this case the apparatus for determining fungal lysis on the basis of the amount of light transmitted through seeded agar, as described by Carter & Lockwood (1957), may have been useful.

Of the ten candidate antagonists only Penicillium claviforme and Trichoderma sp. 1 caused greater than 80% inhibition in the culture filtrate test and were selected for further evaluation. The culture filtrates of these two fungi differed in both maximum anti-Pythium activity and in the time after inoculation at which this occurred. Thus Pen. claviforme culture filtrate (PCF) gave a maximum degree of inhibition of 81.1% at 7d after inoculation, whilst for the Trichoderma sp. culture filtrate (TSF) comparable figures were 100.0% and 4d.

3.2.2 Selection of bioassay medium

Mycelial growth of Pythium ultimum on the high-nutrient MEA was luxuriant, bearing little resemblance to that which would occur under the oligotrophic conditions existing in soil (section 1.1.1), even in the richer spermosphere environment. The effect of the antagonist culture filtrates on P. ultimum growth were therefore compared on three different media: MEA, CMA and 1.5% TWA, spanning a range of nutrient concentrations from high to low, respectively.

Antagonists were grown as described in section 3.2.1. The culture filtrates were harvested 7d (PCF) or 4d (TSF) after inoculation, diluted 1:2 in MEB and bioassayed as specified in section 2.3.2, 1ml per 20ml

test medium. Each treatment was replicated three times. Results are recorded in Table 3.2.

The assay medium did not significantly affect the mean radial diam. of controls growing on media containing 1ml sterile MEB, but did influence colony morphology. On MEA the pathogen produced profuse aerial mycelium whilst on CMA colonies were closely adpressed to the medium and the margins more regular. On TWA mycelial growth was sparse and the colony margins poorly defined. The assay medium also had an effect on the degree of inhibition resulting from the addition of antagonist culture filtrates. PCF caused a greater reduction of radial growth when incorporated into TWA than in either CMA or MEA, and also in CMA compared to MEA. TSF was more inhibitory when added to TWA than in CMA or MEA, but no significant differences occurred between the latter two test media.

CMA was selected for all subsequent bioassays because its intermediate nutrient content was thought to simulate most closely conditions in the spermosphere, and because P. ultimum colonies on this medium were regular and well defined.

3.2.3 Effect of antagonist culture filtrates on Pythium propagules

In the absence of a plant host Pythium spp. survive in soil by means of sporangia and/or oospores (section 1.1.4). The effect of the culture filtrates on the germination of these propagules was therefore evaluated.

Samples (0.1ml) of suspensions containing approx. 4.5 $x10^4$ Pythium ultimum propagules ml^{-1}, prepared as described in section 2.1.1, were spread onto the surface of CMA assay plates containing 0.25, 0.50, 1.00

Table 3.2. Growth of *P. ultimum* on malt extract agar (MEA), corn meal agar (CMA) or tap water agar (TWA) containing *Pen. claviforme* culture filtrate (PCF) or *Trichoderma* sp. culture filtrate (TSF)[a]

Test medium	*P. ultimum* radial diameter (mm)[b]		
	Control	PCF	TSF
MEA	39.3 x	23.0 x	21.3 x
CMA	40.0 x	15.7 y	20.0 x
TWA	42.3 x	7.7 z	10.3 y

[a] Antagonists were grown in MEB (25°C, 200rev min^{-1}) and culture filtrates removed after 7d (PCF) or 4d (TSF). Samples (1ml) were incorporated into 20ml molten test medium and inoculated with *P. ultimum*.

[b] Results determined after 24h incubation at 25°C. Data in any one column followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

or 2.00IU plate^{-1} PCF or TSF. Plates were incubated at 25°C for 24h, then germination compared with that on control plates spread with STW. Each treatment was replicated three times.

The minimum concentration of PCF of those tested which completely inhibited propagule germination was 0.5IU. TSF was less active in this respect, a concentration of 1IU being the lowest to totally prevent germination.

3.3 Optimization of yield

Experiments were conducted to determine the optimum nutritional and cultural conditions for production of Pythium ultimum-inhibitory metabolites by the two antagonists. In each case Erlenmeyer flasks (500ml) containing 200ml medium were inoculated with four 10mm diam. cores cut from 4d (Penicillium claviforme) or 2d (Trichoderma sp. 1) cultures on MEA. Samples of culture filtrates were removed at intervals, centrifuged (MSE High Speed 18, 18,000rev min^{-1}) and the supernatants bioassayed as detailed in section 2.3.2. Each treatment was replicated three times and was bioassayed in duplicate.

3.3.1 Manipulation of medium

Liquid cultures were grown in MEB or defined media, incubated at 25°C and shaken at 200rev min^{-1}. The defined media tested were Czapek-Dox medium (CDM) or Raulin-Thom medium (RTM) for Penicillium claviforme, and Weindling's medium (WM) or RTM for Trichoderma sp. 1 (section 2.2). Results are given in Figs. 3.5 and 3.7.

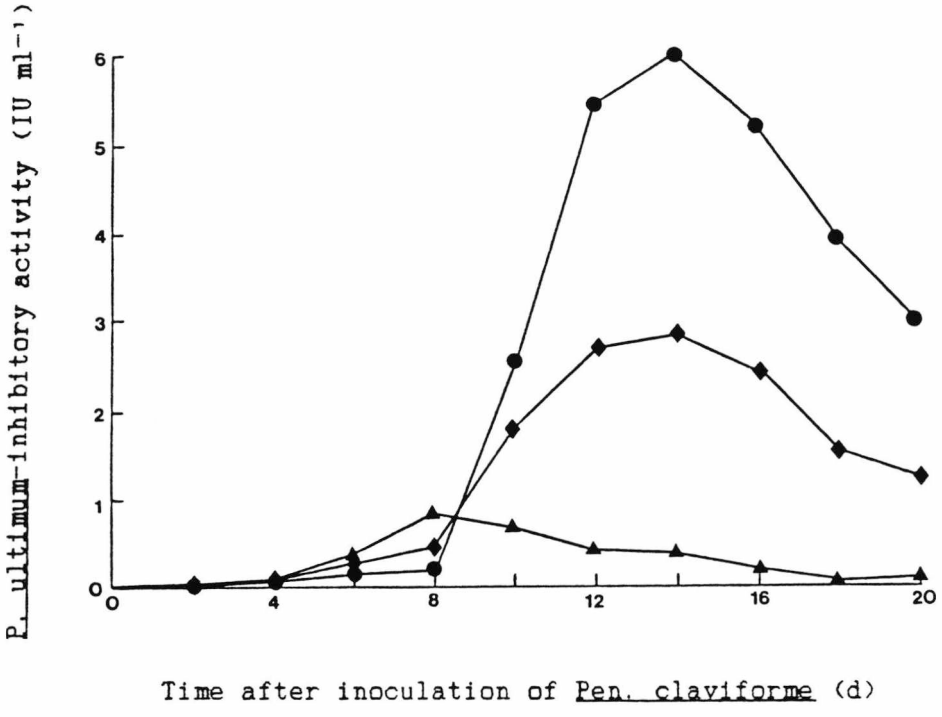


Fig. 3.5. P. ultimum-inhibitory activity of culture filtrates of Pen. claviforme grown in malt extract broth (▲), Czapek-Dox medium (●) or Raulin-Thom medium (◆) (25°C, 200rev min^{-1}).

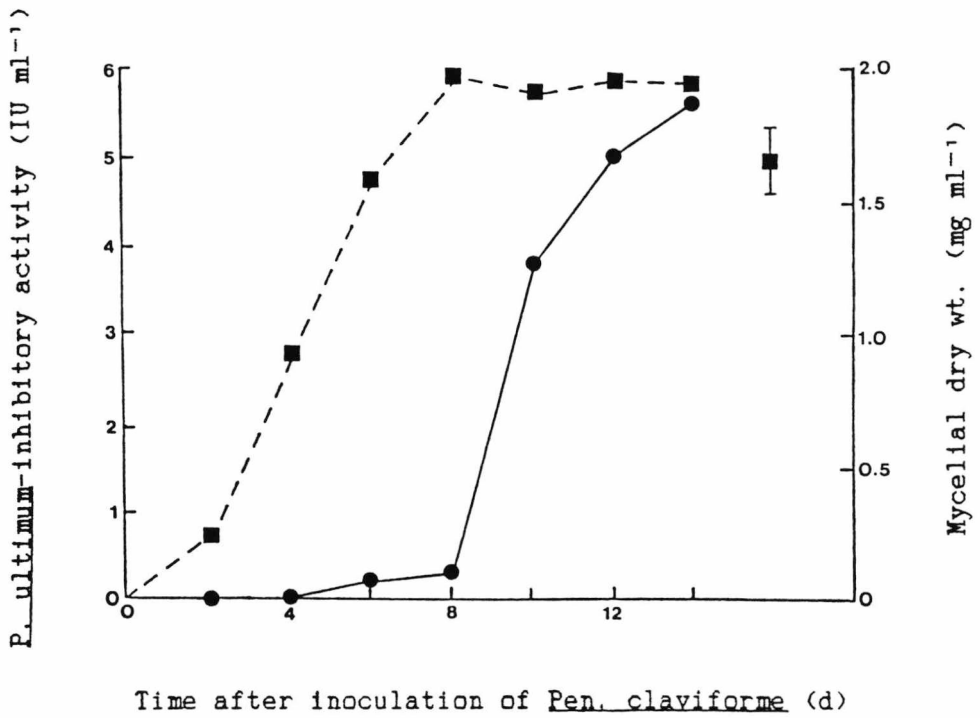


Fig. 3.6. P. ultimum-inhibitory activity (●) and mycelial dry wt. (■) in cultures of Pen. claviforme grown in Czapek-Dox medium (25°C, 200rev min^{-1}). Bar represents mean SE.

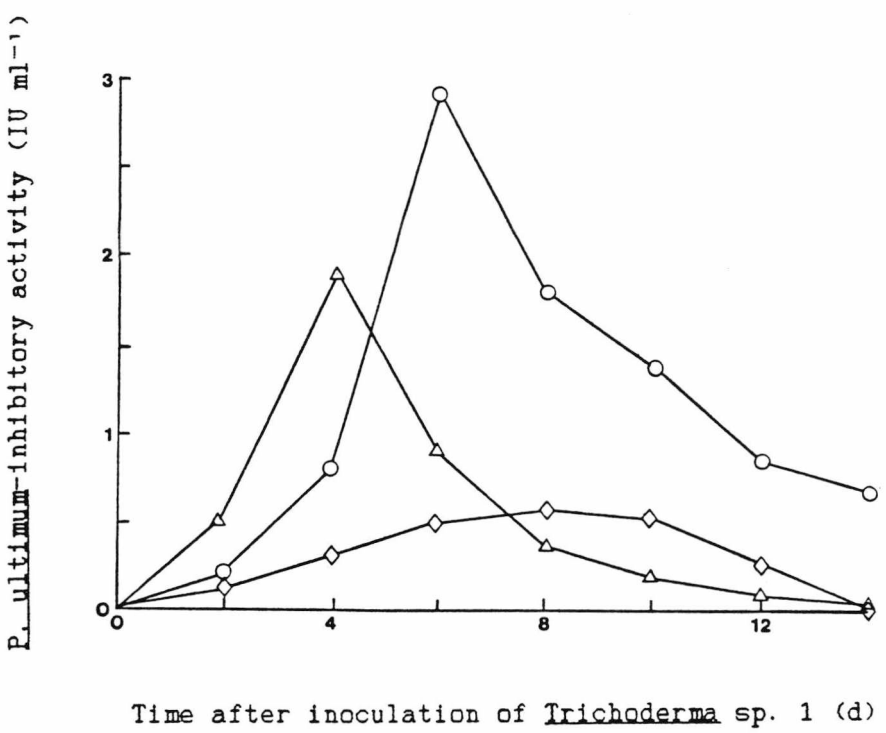


Fig. 3.7. P. ultimum-inhibitory activity of culture filtrates of Trichoderma sp. 1 grown in malt extract broth (△), Weindling's medium (○) or Raulin-Thom medium (◇) (25°C, 200rev min⁻¹).

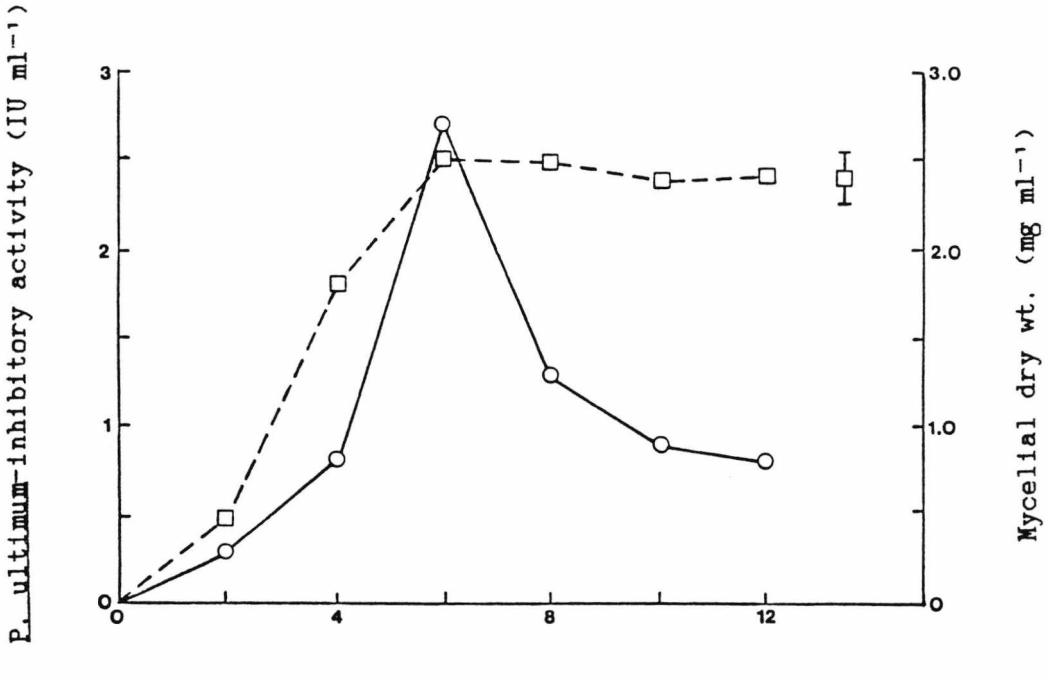


Fig. 3.8. P. ultimum-inhibitory activity (○) and mycelial dry wt. (□) in cultures of Trichoderma sp. 1 grown in Weindling's medium (25°C, 200rev min⁻¹). Bar represents mean SE.

In separate experiments, Pythium ultimum-inhibitory activity of PCF or TSF grown in CDM or WM was measured concurrently with antagonist dry wt. The latter was determined by filtering (Whatman filter paper no. 1) the biomass from three replicate flasks and oven-drying it at 105°C to constant wt. Results are shown in Figs. 3.6 and 3.8.

In cultures of Pen. claviforme growing in MEB the concentration of P. ultimum inhibitors began to increase at 4d reaching a maximum of 0.9IU ml^{-1} 8d after inoculation and then slowly declining (Fig. 3.5). In CDM initiation of their synthesis was delayed but the yield was markedly improved. Thus activity was low until 8d, but then rose rapidly to a maximum of 5.9IU ml^{-1} after 14d of incubation. Yield then fell to 3.0IU ml^{-1} at the end of the experiment. In RTM, a sharp increase in potency occurred after 8d but the maximum yield (2.8IU ml^{-1}) was lower.

In CDM, maximum mycelial dry wt. (1.97mg ml^{-1}) was attained 8d after inoculation, coinciding with the beginning of the rapid build-up in inhibitor concentration (Fig 3.6).

In cultures of Trichoderma sp. 1 growing in MEB (Fig. 3.7), production of anti-Pythium metabolites commenced before that in cultures of Pen. claviforme and reached greatest concentrations (1.9IU ml^{-1}) after 4d of incubation, when activity in filtrates of Pen. claviforme were still very low. The use of one of the defined media, WM, caused a delay in initiation of synthesis and increased production of the inhibitory agents, but to a lesser extent than Penicillium cultures in CDM. Maximum titres (2.9IU ml^{-1}) occurred 6d after inoculation. In RTM, the yield of inhibitors was inferior to that in either MEB or WM.

In WM, mycelial dry wt. of the Trichoderma sp. (Fig. 3.8) increased more rapidly than that of Pen. claviforme in CDM and reached a higher value of 2.51mg ml^{-1} 6d after inoculation. Increases in biomass and in concentration of Pythium-inhibitory metabolites were coincidental during the first 6d of incubation.

3.3.2 Manipulation of aeration

Penicillium claviforme and Trichoderma sp. 1 were grown in CDM or WM, respectively and incubated at 25°C with shaking at 200 or 100rev min^{-1}, or as stationary cultures. Results are presented in Figs. 3.9 and 3.10.

The aeration regime affected both the mode of growth of the two antagonists and their production of Pythium ultimum-inhibitory agents.

In cultures shaken at 200rev min^{-1}, Pen. claviforme (Fig. 3.9) grew as small, dark, irregularly-shaped and non-sporulating pellets 2-3mm in size. Synthesis of Pythium inhibitors commenced at 7d and increased to a maximum yield of 5.4IU ml^{-1} 12d after inoculation. In cultures shaken at 100rev min^{-1} mycelial pellets were again produced but were round, white and 4-5mm in diam. Inhibitor production remained low throughout the experiment, the maximum yield (1.3IU ml^{-1}) was obtained after 16d of incubation. In stationary cultures, discontinuous mycelial growth occurred on the surface of the medium around the inoculum cores. Coremia were first observed after 5-6d and spores were produced at the tips of these fruiting structures following a further 3-4d incubation. The onset of production of Pythium-inhibitory metabolites was less sharp than in cultures shaken at 200rev min^{-1}, and occurred 3d later at 10d. The time at which maximum inhibitor titres were attained in the culture

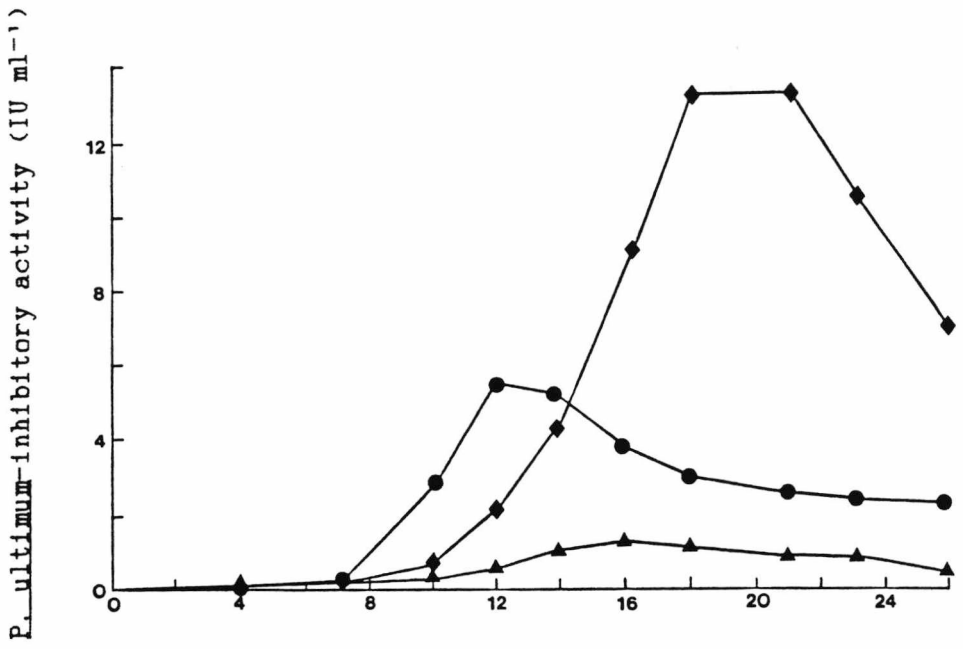


Fig. 3.9. P. ultimum-inhibitory activity of culture filtrates of Pen. claviforme grown in Czapek-Dox medium at 25°C, and shaken at 200rev min^{-1} (●), 100rev min^{-1} (▲) or incubated under stationary conditions (◆).

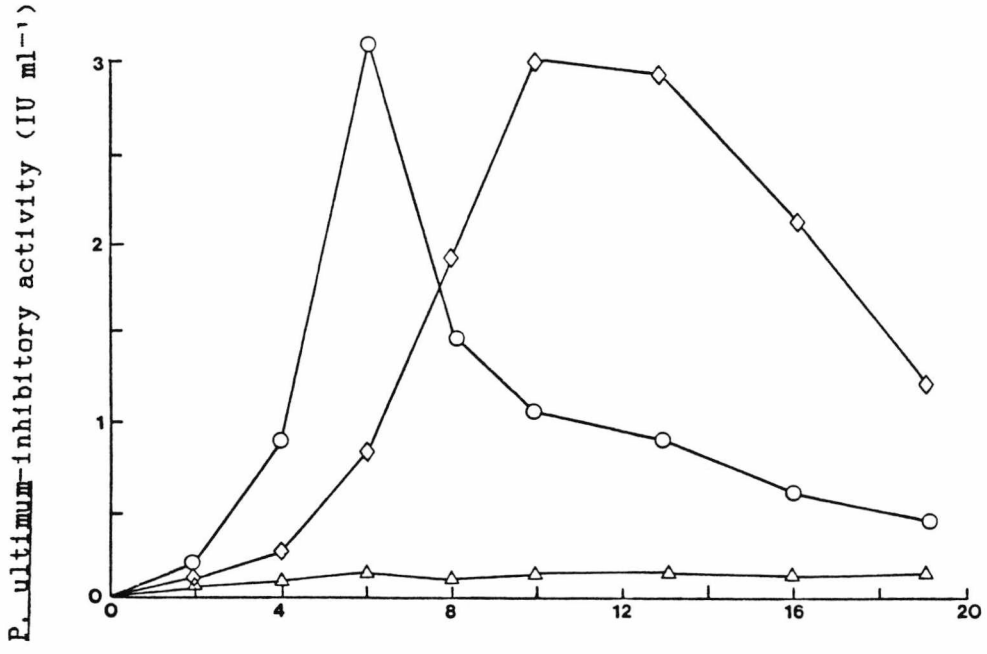


Fig. 3.10. P. ultimum-inhibitory activity of culture filtrates of Trichoderma sp. 1 grown in Weindling's medium at 25°C, and shaken at 200rev min^{-1} (○), 100rev min^{-1} (△) or incubated under stationary conditions (◇).

filtrates was also delayed (in this case from 12 to 18-21d after inoculation) but their potency was considerably improved, to 13.4IU ml^{-1}.

In cultures of Trichoderma sp. 1 (Fig. 3.10) shaken at 200rev min^{-1} growth occurred as short, non-sporulating filaments dispersed throughout the medium. A sharp peak of Pythium-inhibitory activity occurred 6d after inoculation, which reached 3.1IU ml^{-1} before rapidly declining. In cultures shaken at 100rev min^{-1} the fungus grew as large non-sporulating mycelial masses around the inoculum cores. As with Pen. claviforme, production of Pythium-inhibitory agents was low under these conditions and did not show a maximum. In stationary cultures the Trichoderma sp. grew on the surface of the medium as an unbroken mycelial mat, which, in contrast to Pen. claviforme under identical cultural conditions, did not sporulate. Maximum anti-Pythium activity (3.0IU ml^{-1}) was similar to that of cultures shaken at 200rev min^{-1}, but occurred 4d later. The peak was also less sharp so that high yields were obtained over a longer period of time.

3.3.3 Manipulation of temperature

Penicillium claviforme and Trichoderma sp. 1 were grown in CDM or WM, and incubated at 20°C, 25°C or 30°C under stationary conditions. Results are given in Figs. 3.11 and 3.12.

Little difference in Pythium ultimum-inhibitory activity was observed between cultures of Pen. claviforme incubated at 20°C or 25°C (Fig. 3.11). Maxima of 12.7 and 13.2IU ml^{-1} occurred at 20d after inoculation. However, production of inhibitory agents was substantially reduced at 30°C to a maximum of 4.0IU.

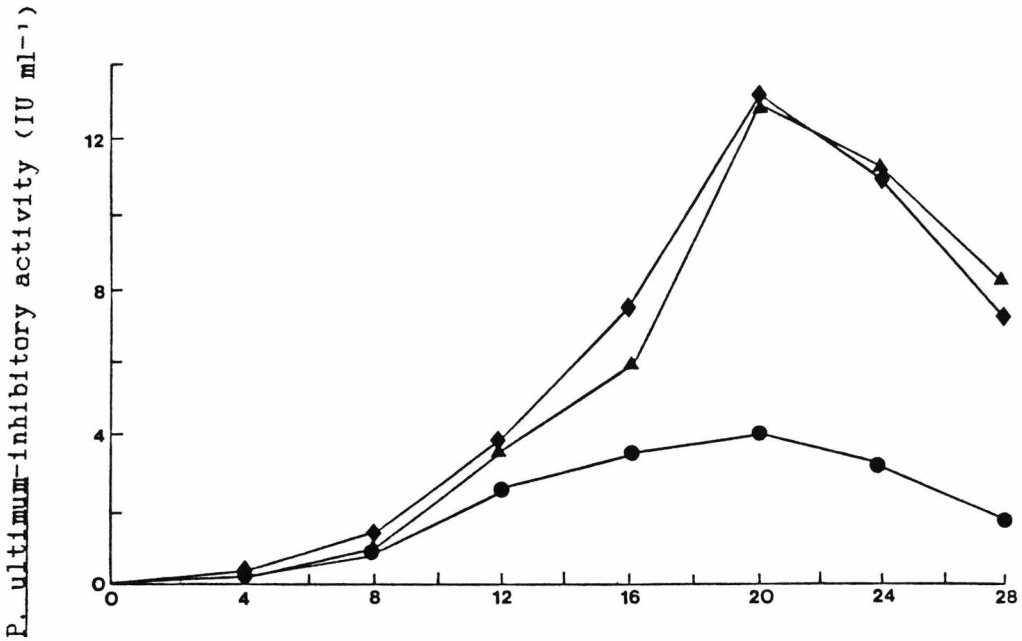


Fig. 3.11. P. ultimum-inhibitory activity of culture filtrates of Pen. claviforme grown in Czapek-Dox medium and incubated at 20°C (▲), 25°C (◆) or 30°C (●) under stationary conditions.

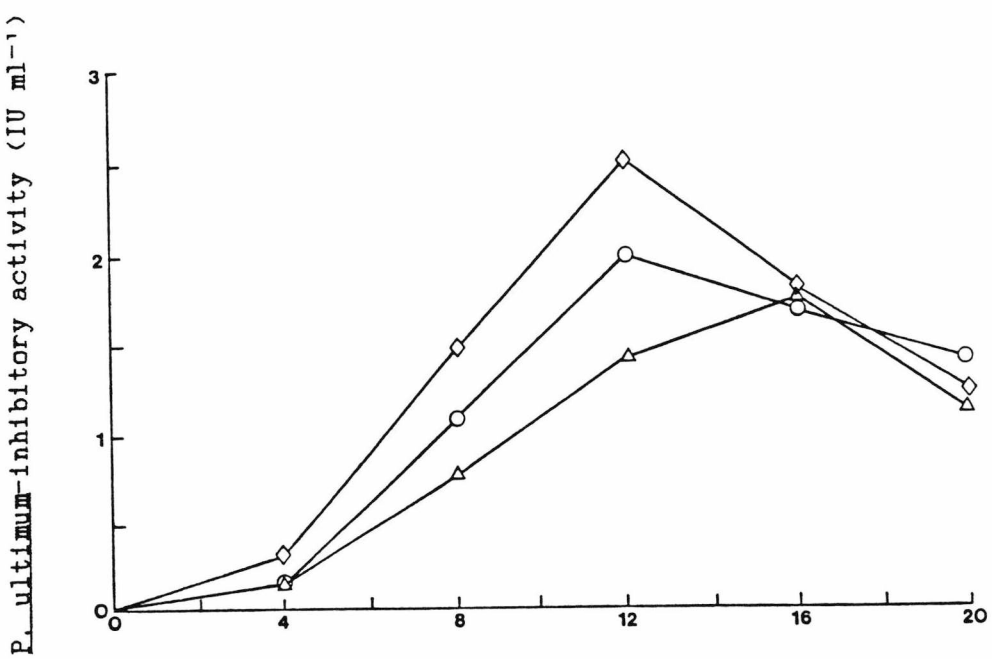


Fig. 3.12. P. ultimum-inhibitory activity of culture filtrates of Trichoderma sp. 1 grown in Weindling's medium and incubated at 20°C (△), 25°C (◇) or 30°C (○) under stationary conditions.

Filtrates of Trichoderma sp. cultures incubated at 25°C showed greatest potency (2.5IU ml^{-1}) against P. ultimum after 12d of incubation (Fig. 3.12). Greatest inhibitor concentrations were reduced to 1.7 and 2.0IU ml^{-1} when the antagonist was incubated at 20°C or 30°C, respectively.

As a result of these investigations, the following protocol was adopted for production of inhibitors in subsequent work: Penicillium claviforme was grown in CDM, and PCF was harvested after 18-21d incubation at 25°C under stationary conditions. Trichoderma sp. 1 was grown in WM and TSF removed after 10-13d incubation at 25°C without shaking. Under these conditions, centrifugation of the culture filtrates was not necessary. Samples were bioassayed directly, after filter sterilization.

3.4 Discussion

3.4.1 Screening and selection of antagonists

The purpose of a primary screen for plant disease control agents is to detect organisms (Campbell, 1986) or chemicals (Ryley & Rathmell, 1984; Shepherd, 1987) possessing potentially useful properties among large numbers of others with lesser activity. It must therefore be simple, rapid, cheap and rigorous. It should have small sample requirements and results must be clearly defined so that only low replication is necessary. Provided overall numbers are reduced, it is not critical if the screen accepts some agents which are excluded at a

later stage (false positives), but it is important that it does not reject agents which would be of real value in the field (false negatives). *In vitro* tests satisfy some of these criteria and have been employed to search for both biological and chemical crop protection agents, but are subject to criticism on several counts relating to the vast disparity between laboratory media and soil *in situ*.

Firstly, the validity of *in vitro* assays for the selection of biological control agents according to their production of inhibitory metabolites, such as the cellophane technique used in the present study (section 3.1), has been challenged on the basis of differences in the expression of antagonistic traits in the two environments. The production of antifungal compounds by antagonists is known to be markedly affected by environmental variables, particularly the quantity and nature of available nutrients (Fravel, 1988). Microbial growth on artificial media containing high concentrations of uniformly dispersed and soluble nutrients is generally unrestricted and under these conditions generation of the precursors necessary for synthesis of inhibitory agents can be maximal (Zähner & Kurth, 1982). By contrast, soil microorganisms in their natural habitat have to compete for fluctuating levels of unevenly distributed and frequently insoluble nutrients. In these circumstances production of the desired metabolites may be low or non-existent (Williams & Vickers, 1986). However, reservations on these grounds do not apply to the detection of organisms which release useful products in culture, which are then extracted and exploited as fungicidal chemicals in their own right, as in the present work. The use of a rich nutrient medium in the primary

screen was therefore justifiable, in order to promote production of antifungal metabolites.

More serious objections to the utilization of *in vitro* screening methods for plant disease control agents in the context of the current study, are those relating to differences in the behaviour of active compounds and susceptibility of pathogens in artificial and natural environments. The toxicity of antifungal chemicals, whether produced *in situ* by biocontrol agents or applied exogenously, may be considerably modified by the physico-chemical properties of the soil, such as its organic matter, clay and moisture content, or they may be rapidly degraded by the indigenous microflora (sections 1.2.2 and 1.4.2). Because of the particulate and aggregated nature of soil (section 1.1.1), target organisms may also be physically separated from chemicals in adjoining microhabitats and thus escape their toxic effects (Katan & Lockwood, 1970). Conversely, physico-chemical and in particular nutritional stresses in soil may enhance the sensitivity of pathogens to fungicidal compounds (Bruehl, 1987). *In vitro*, pathogens may show greater sensitivity on less nutritious media to both antagonists (Whipps, 1987) and antagonistic compounds (Howell & Stipanovic, 1980), as was also observed in the present work (section 3.2.2). This phenomenon is equivalent to that of nutrient counteraction of toxicity reported by Vaartaja & Agnihotri (1969). These authors found that the activity of four antifungal antibiotics against *Pythium* spp. in both agar and soil was diminished by the addition of nutrients such as yeast extract, sucrose or asparagine. The results presented in Table 3.2 suggest that the toxicity of TSF was reduced by lower concentrations of nutrients than PCF. Under natural conditions, the nutrients supplied by

seed and root exudates can attenuate the effects of antifungal compounds (Richardson, 1966), further complicating the interpretation of results obtained in the absence of the host plant.

Other disadvantages of *in vitro* screening procedures are that they preclude the detection of agents which control disease indirectly (section 6.5.4), and do not allow assessment of systemic properties or curative activity (Sbragia, 1975).

Because of these problems, it is now recognized in the agrochemical industry that, although *in vitro* assays have a role in fungicide screening, they cannot be relied upon exclusively (Shepherd, 1987). Tests using whole plants must be conducted concurrently or at an early stage of evaluation.

Not all of the organisms reported by other investigators to be effective *in vitro* antagonists or biocontrol agents of *Pythium* inhibited *Pythium ultimum* in the initial cellophane screen (Table 3.1). There are several possible explanations. Firstly, dissimilarities may have been due to the use of different species or strains of antagonists. Even closely related organisms show considerable variation, both quantitative and qualitative, in the metabolites they produce (Dennis & Webster, 1971). Similarly, discrepancies could have arisen from the use of different species or strains of the test pathogen, which may vary in their susceptibility to the same antagonists (Papavizas & Lewis, 1989). Thirdly, results could be attributable to different nutritional or cultural conditions employed in the test affecting the quantities or types of products released. Finally the mechanism of antagonism may have been other than the production of diffusible metabolites, for example mycoparasitism involving wall-bound enzymes.

The cellophane technique is one of many methods which have been described for the isolation of microbial antagonists of plant pathogens (Dhingra & Sinclair, 1985). It was chosen because it excludes those organisms which are dependent on physical contact with the pathogen in order to exert their effects. However, this does not necessarily infer that such antagonists produce diffusible inhibitory metabolites. Hsu & Lockwood (1969) presented evidence that suppression of Glomerella cingulata on agar by certain streptomycetes was caused by an induced deficiency of nutrients in the medium surrounding the colonies.

A mode of action based on nutrient deprivation could have been one of the reasons why some organisms which were effective in the cellophane assay failed to show activity in liquid media (Figs. 3.1-3.4). Other possibilities are the greater dilution of metabolites in the culture filtrate test, or the different cultural environment. The reverse situation was found in the case of Trichoderma sp. 1 which gave rise to highly inhibitory culture filtrates, but showed relatively low levels of antagonism when later tested on cellophane. This observation illustrates the somewhat arbitrary nature of the in vitro selection process in that, given slightly different growth conditions, some of the other organisms could have shown greater activity than Penicillium claviforme or Trichoderma sp. 1, and been chosen for further evaluation in their place.

Several techniques are available for testing the fungicidal activity of chemicals in vitro, which are applicable to both microbial culture filtrates and synthetic chemicals (Dhingra & Sinclair, 1985). The incorporation of the agent under investigation into an agar medium inoculated with a pathogen, followed by measurement of growth relative

to controls, (the so-called 'poisoned-food' method), proved to be a simple and sensitive means of assessing the effect of microbial products in the current study. More commonly used procedures based on the diffusion of the active agent into agar from a central well or filter paper disc, followed by measurement of zones of inhibition, were found to be unsatisfactory in the case of P. ultimum due to its rapid growth rate (Fig. 4.1) such that inhibition zones, where present, were only transient.

Mycelial growth of soil-borne fungal phytopathogens after propagule germination is often necessary before plant infection can occur. The effect of fungicidal compounds on this phase of their life cycle is therefore relevant to their influence on disease development in situ (Vaartaja, 1974). However, their activity on the germination of survival propagules is also important. The concentration of PCF required to totally inhibit P. ultimum propagule germination was found to be approx. half that for mycelial growth (section 3.2.3). This observation is in agreement with reports in the literature that quantities of toxic substances necessary to prevent the germination of fungal propagules are frequently lower than those for growth (Brian & Hemming, 1945). TSF was therefore atypical in being approximately equally inhibitory to the two processes.

3.4.2 Optimization of yield

When a microbial metabolite with potentially useful properties is discovered, optimization of the growth environment for maximum yields is necessary to allow effective exploitation. It is essential that the producer organism grows on readily available and inexpensive media giving yields which are both consistent and sufficiently high for the product to be economically viable (Stanbury & Whitaker, 1984). This is especially true where it has to compete with synthetic alternatives, as in the agrochemical industry.

Many microbial products of industrial importance are so-called secondary metabolites, defined as compounds which have no clear role in the maintenance, growth or reproduction of the organism (Bu'Lock, 1961). This contrasts with primary metabolites which have clearly ascribed functions in microbial physiology. In batch cultures using nutritionally rich media, secondary metabolites are typically produced only after most of the increase in biomass has occurred. This was observed in the case of the *Pythium ultimum*-inhibitory agent in PCF (Fig. 3.6), and evidence is presented in Chapter 4 that the major active component of the culture filtrate was the polyketide secondary metabolite patulin which is produced by a number of fungi in the genera *Aspergillus*, *Byssochlamys* and *Paecilomyces*, in addition to *Penicillium* Engel & Teuber, 1984).

Bu'Lock *et al*. (1965) introduced the terms trophophase and idiophase to refer to the periods of growth and of secondary metabolite production, the latter indicating the obscure nature of the products. However, though patulin synthesis by *Penicillium urticae* occurs during a well defined idiophase (Bu'Lock *et al*, 1965), in other cases the two

periods cannot be clearly distinguished. For example, gliotoxin was reported to be produced during active growth of Gliocladium fimbriatum (Weindling, 1941), and this pattern was also observed with the anti-Pythium agents in TSF (Fig. 3.8) which again appeared to be antibiotic in character (Chapter 4). Furthermore, it is now apparent that primary and secondary metabolites are formed from common intermediates using the same or similar enzymes. For instance, the first step in the production of patulin involves the condensation of acetyl-CoA and malonyl-CoA units in a series of reactions closely related to those occurring during fatty acid synthesis (Betina, 1984). Thus the elaboration of secondary metabolites cannot be justifiably described as idiosyncratic. The terms general (primary) and special (secondary) metabolites, may be more appropriate (Martin & Demain, 1980), but have not yet been widely adopted.

Commercially, the most important group of microbial secondary metabolites are the antibiotics (section 1.1.1; Rose, 1979). Others include certain pigments, enzymes and plant growth regulators. In addition to these useful compounds, secondary metabolites also include chemicals potentially harmful to man such as mycotoxins (Betina, 1984) and bacterial toxins, which elicit toxic responses when administered to animals.

Secondary metabolites show immense diversity in their structure and many possess unusual chemical groupings such as the lactone ring in patulin. In 1979 more than 10,000 different secondary metabolites had been described, of which approximately 3,000 showed antibiotic activity (Rose, 1979). Synthesis of a given secondary metabolite is often limited to a small number of microbial species. Conversely, some

microorganisms produce more than one such compound. A strain of Micromonospora is known to produce 48 different aminoglycoside antibiotics (Bérdy, 1974), and secondary metabolite profiles have even been used for taxonomic purposes (Frisvad & Filtenborg, 1983).

Several hypotheses have been advanced for the production of secondary metabolites (Turner, 1971). A currently favoured explanation is that they are formed from the low mol. wt. precursors of cellular constituents when nutritional or environmental factors prevent further growth (Bu'Lock, 1961). The removal of these intermediates prevents them accumulating intracellularly and causing metabolic disruption, and also allows the synthetic processes to remain operational during times of stress. Thus it is the process of secondary metabolism which is thought to be of value to the microorganism rather than the chemical nature of the products themselves. The antibiotic activity of certain secondary metabolites is now generally viewed as incidental. Strictly, antibiotic production in natural habitats is associated with the exhaustion of a temporarily high-nutrient environment, following growth and generation of metabolic precursors, rather than with the rich environment itself (section 3.4.1).

Secondary metabolites may also be involved in differentiation (McMorris, 1978). In stationary cultures of Penicillium claviforme the onset of Pythium inhibitor synthesis was coincident with that of sporulation (section 3.3.2), but the agents were also produced under cultural conditions where spores were not formed.

Many factors may be involved in determining the types and quantities of secondary metabolites released by microorganisms. In general, they operate via the same regulatory mechanisms as those which function in

primary metabolism (Demain & Martin, 1980). Each of these factors may be manipulated during the optimization process (Bunch & Harris, 1986). Considerable experience has been acquired in the pharmaceutical industry. However, because of their great variety, information gained for the production of one metabolite is often not applicable to others, or even to the synthesis of the same metabolite by a different organism. Both Pen. claviforme and Penicillium expansum are known to produce patulin, but the two fungi showed appreciably different levels of activity in the cellophane test under the same growth conditions (Table 3.1). The formulation of general guidelines for the manipulation of new systems is therefore not possible, and initial investigations are frequently conducted on an empirical basis.

The composition of the growth medium is usually the first factor to be examined during optimization. Alteration of the nature of the major nutrients can have a considerable effect on the production of secondary metabolites (Woodruff, 1961). This may be due to direct effects on the synthesis or action of the synthetase enzymes involved, or on the balance of metabolism and thus the supply of precursors. Production of Pythium inhibitors by Pen. claviforme was improved 6-fold by growing the antagonist in CDM, containing glucose and sodium nitrate to supply carbon and nitrogen respectively, compared to the undefined MEB (Fig. 3.5). However in RTM, containing a different nitrogen source, yield was improved only 3-fold. Concentrations of anti-Pythium agents were also enhanced (1.5-fold) when the Trichoderma sp. was grown in WM compared to MEB (Fig. 3.7), but in this case titres in RTM were markedly inferior. The importance of the nature of the carbon source on inhibitor production was observed again in a later experiment (section 4.4) where

the replacement of glucose with cellulose decreased the potency of both antagonist culture filtrates.

The concentration of various trace metals can also greatly affect the production of secondary metabolites (Weinburg, 1982). For example, synthesis of patulin is dependent on the provision of iron in the growth medium (Brack, 1947).

The establishment of the biosynthetic pathway of the metabolite of interest, and the characterization of the enzymes involved, can provide the basis for a more rational approach to medium manipulation. It may be possible to 'direct' metabolism in favour of the desired product (Perlman, 1973). This may be achieved by including appropriate precursors in the medium. For example, production of benzylpenicillin by Penicillium chrysogenum is stimulated by the addition of phenylacetic acid, or compounds which can be metabolized to phenylacetic acid (Queener & Swartz, 1979). Similarly, the feeding of analogues of these precursors may result in the synthesis of variants of the secondary metabolite, some of which may possess useful properties (Sebek, 1980). Other methods by which fermentations may be directed towards the requisite metabolite include the addition of specific inducers or derepressors of the respective synthetase enzymes, or metabolic poisons which channel intermediates away from unwanted products (Perlman, 1973).

Other parameters, such as aeration, temperature and pH, may also influence secondary metabolite production (Woodruff, 1961), and are usually investigated after the constitution of the growth medium has been optimized. Again, effects may be direct or indirect.

Agitation of the culture vessels was found to affect both the timing and extent of production of anti-Pythium metabolites by Pen. claviforme

(Fig. 3.9). When cultures were shaken at 100rev min^{-1} inhibitor titres were lower than when shaken at 200rev min^{-1}. These results could have been due to a decreased oxygen concentration in the medium itself specifically affecting inhibitor biosynthesis, or oxygen may have become sufficiently depleted in the centre of the larger pellets formed under these conditions that autolysis occurred (Metz & Kossen, 1977), thus reducing the total metabolic activity of the culture. Inhibitor yields were greatest in stationary cultures, when profuse growth occurred at the surface of the medium exposed to atmospheric oxygen concentrations.

Production of P. ultimum-inhibitory metabolites by Trichoderma sp. 1 was also depressed when cultures were shaken at 100 compared to 200rev min^{-1} (Fig. 3.10). It is likely that the growth of the fungus in large mycelial masses surrounding the inoculum cores in the former case substantially impeded both the inward diffusion of oxygen and the outward movement of metabolic products. Formation of inhibitors by the Trichoderma sp. in stationary cultures was not increased over that in cultures shaken at 200rev min^{-1}, but these incubation conditions were advantageous in prolonging the time period during which yields were maximal.

Production of anti-Pythium agents by Pen. claviforme was similar in the temperature range 20-25°C, but was decreased by approximately two thirds when the antagonist was incubated at 30°C (Fig. 3.11). Synthesis of inhibitors by Trichoderma sp. 1 was lower at both 30°C and 20°C compared to 25°C (Fig. 3.12). These observations are consistent with reports that secondary metabolite formation often occurs only over a narrow range of incubation temperatures (Weinburg, 1982).

Yields of antagonistic metabolites from Pen. claviforme and from Trichoderma sp. 1 were improved 14-fold and 0.5-fold respectively, even by the limited number of manipulations conducted. More extensive investigations would be necessary to determine the optimum growth conditions for large-scale production, but the method adopted in the present work was simple and inexpensive in terms of both materials and equipment, important prerequisites for commericial exploitation.

Enhancement of inhibitory fungal metabolite production by physiological manipulation is only possible up to a limit imposed by the genetic constitution of the organism. Genetic modification of antagonists may further increase the yield of the desired products, an approach which has been highly successful in the pharmaceutical industry (Ball, 1984). Strain improvement programmes involve the generation of altered genotypes followed by screening in order to detect the small proportion of these genotypes which result in elevated yields of the metabolite. Other characteristics may also be sought such as the ability to grow on cheaper substrates or the loss of genes coding for unwanted compounds (Stanbury & Whitaker, 1984).

Traditionally, the modified genotypes are generated by parasexual breeding or mutagenic treatments such as uv-, X- or gamma-irradiation, or chemical agents such as ethyl methane sulphonate or nitro-nitroso-methylguanidine. For example, mutation and selection of Pen. chrysogenum strains made an important contribution to increases in penicillin yields from 100-200 units ml^{-1} in 1944 to approximately 10,000 units ml^{-1} in 1970 (Moss & Smith, 1977). More recently new techniques such as protoplast fusion (Ball, 1983) and genetic engineering using phage or plasmid vectors (Skatrud et al., 1989) have

been exploited. The possible problems with respect to the transfer of the novel genotypes in the natural environment which have limited the use of these technologies for improving biocontrol agents (section 1.3.3), are of lesser importance when applied to microorganisms confined to fermentation vessels. Similarly, the loss of ecological efficiency which may follow genetic manipulation is of lesser consequence when the producer organism does not have to survive and grow in the competitive soil environment. However, because the genetic regulation of the synthesis of antibiotics and other secondary metabolites is highly complex (Hopwood & Merrick, 1977), there are still major difficulties to be overcome before the full potential of these techniques can be realized.

CHAPTER 4

CHARACTERIZATION OF ANTAGONISTIC METABOLITES

During the development of microbial metabolites for crop protection purposes characterization of effective compounds is necessary at an early stage in order to allow accurate assessment of the likely consequences of their introduction into the plant growth environment. Product identification is also the first step in the elucidation of the biosynthetic pathway, an understanding of which may allow rational physiological or genetic manipulation of the producer organism in order to procure greater yields (section 3.4.2).

In the present work initial investigations into the nature of the Pythium ultimum-inhibitory agents in Penicillium claviforme culture filtrate and Trichoderma sp. 1 culture filtrate (PCF and TSF, section 2.1.2) were aimed at differentiating between the three broad types of extracellular antagonistic microbial metabolites which have been described, namely siderophores, cell wall-degrading enzymes and antibiotics (section 1.3.3). Subsequently more discriminatory techniques were employed to identify the active components.

4.1 Preliminary observations

PCF was fungicidal to Pythium ultimum at all concentrations greater than 1IU per bioassay plate, i.e. no growth of the pathogen occurred on

prolonged incubation or on removal of inoculum cores after 24h and transfer onto CMA without the culture filtrate. The effects of TSF were less consistent. At concentrations greater than approx. 2IU plate^{-1} the filtrate was always fungicidal, but between 1 and 2IU plate^{-1}, Pythium growth sometimes began after 2-3d incubation. This growth was usually in the form of aerial hyphae arising from the core itself, although in some cases radial extension on the surface of the assay medium was observed. Similar responses took place when inoculum cores were removed from bioassay plates after 24h and placed on fresh agar without the filtrate. Aerial growth from inoculum cores accompanying severe inhibition of surface spread was also noted by Dennis & Webster (1971).

Microscopic examination of P. ultimum mycelial mats grown in 1% MEB containing 0.50 or 0.75IU PCF per petri dish did not reveal any morphological differences to controls. Similarly, 0.50IU TSF did not induce any morphological symptoms, but at 0.75IU dish^{-1} the culture filtrate caused some of the leading hyphae to become swollen and vacuolated. However, older mycelium taken from the centre of the mats was not affected. Addition of 0.50 or 0.75IU dish^{-1} PCF or TSF to 2d old Pythium mycelial mats grown in 1% MEB without culture filtrates did not result in any changes in pathogen morphology.

On control bioassay plates (CMA plus sterile antagonist growth medium) mycelial growth of P. ultimum was first observed after 6h incubation and thereafter took place at a constant rate of approx. 2.17mm h^{-1} (Fig. 4.1). In bioassay dishes containing 0.50IU PCF, the lag phase was prolonged by approx. 2.5h and the subsequent growth rate was reduced to approx. 1.17mm h^{-1}. Incorporation of TSF into the assay

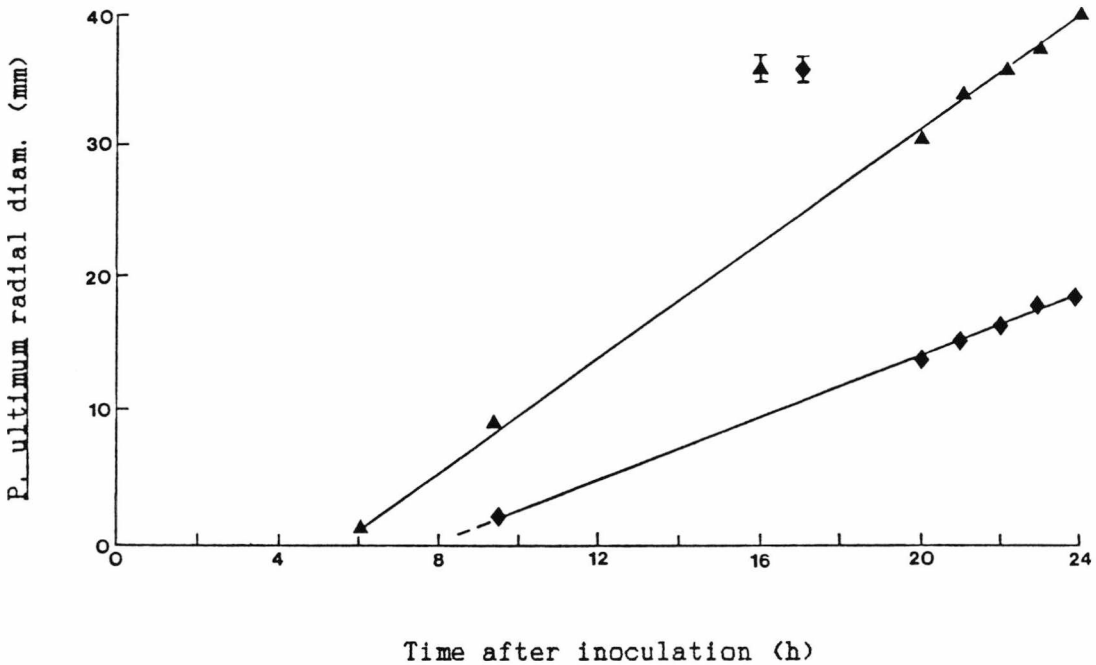


Fig. 4.1. Growth of P. ultimum on corn meal agar containing sterile Czapek-Dox medium (▲) or 0.50IU Pen. claviforme culture filtrate (◆). Bars represent +/- mean SE.

medium gave similar results. The lag phase before growth commenced lasted approx. 9.5h and the ensuing growth rate was approx. 1.31mm h^{-1}.

Addition of 1µM $FeCl_3$ to the bioassay medium, to assess the possible contribution of siderophores to P. ultimum inhibition, did not influence the radial growth of the pathogen in the absence of antagonist culture filtrates (Table 4.1). The same compound also had no significant effect on the degree of suppression resulting from the incorporation of either PCF or TSF.

4.2 Effect of biological, physical and chemical treatments on antagonistic activity of metabolites

A series of experiments were conducted to investigate the stability of the Pythium ultimum-inhibitory components of PCF and TSF to certain biological, physical and chemical treatments. The culture filtrates were treated as described below and then bioassayed as in section 2.3.2. All treatments were replicated three times and bioassayed in duplicate.

4.2.1 Effect of protease

PCF or TSF were incubated with commercial proteases of streptomycete (Sigma, type XIV) or fungal (Sigma, type XI) origin at room temperature (20+/-2°C) for 2h. Enzymes were dissolved in sterile 0.1M potassium phosphate buffer (pH 6.0), and diluted 1:10 in the culture filtrates to give final concentrations of 0.01, 0.10 or 1.00mg ml^{-1} (type XIV) or 0.50mg ml^{-1} (type XI). It was established in preliminary tests that the

Table 4.1. Growth of *P. ultimum* on corn meal agar containing *Pen. claviforme* culture filtrate (PCF) or *Trichoderma* sp. culture filtrate (TSF) with or without the addition of $FeCl_3$[a]

	P. ultimum radial diameter (mm)[b]		
	Control	PCF	TSF
Without $FeCl_3$	39.3 x	9.6 x	11.3 x
With $FeCl_3$	38.3 x	10.3 x	10.3 x

[a] $FeCl_3$ (1μM) was dissolved in distilled water and autoclaved separately, before adding to the molten (50°C) bioassay medium at the same time as the antagonist culture filtrates.

[b] Results determined after 24h incubation at 25°C. Data in any one column followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

enzyme solutions did not directly affect the growth of Pythium ultimum at the concentrations used. Results are shown in Figs. 4.2 and 4.3.

The activity of neither culture filtrate was reduced by any of the protease treatments tested.

4.2.2 Effect of heat

Samples of the two culture filtrates were exposed to temperatures of 62°, 80°, or 97°C for 1min in a water bath, or 121°C for 15min in an autoclave. Results are given in Figs. 4.4 and 4.5.

The anti-Pythium activity of PCF was not diminished by any of the temperature regimes tested (Fig. 4.4). The inhibitors present in TSF were stable at temperatures of up to 80°C. but their activity was reduced by 88.1% after 1min exposure to 97°C and by 94.9% after 15min exposure to 121°C (Fig. 4.5).

4.2.3 Effect of enzyme inhibitors

TSF was incubated with 1mM copper sulphate (Chesters & Bull, 1963b), 30mM dithiothreitol (Reese & Mandels, 1980), 30mM thiourea (Bollag et al., 1987) or 0.001% Triton X-100 (Couche, Pfannenstiel & Nickerson, 1986) at room temperature (20+/-2°C) for 2h. Chemicals were dissolved in SDW and diluted 1:10 in the culture filtrate to achieve the final concentrations indicated. It was established in preliminary experiments that none of these compounds directly affected Pythium growth at the concentrations tested. Results are given in Fig. 4.6.

The inhibitory components of TSF were not affected by treatment with dithiothreitol, or Triton X-100, but their activity was reduced by 18.9% by copper sulphate and completely destroyed by thiourea.

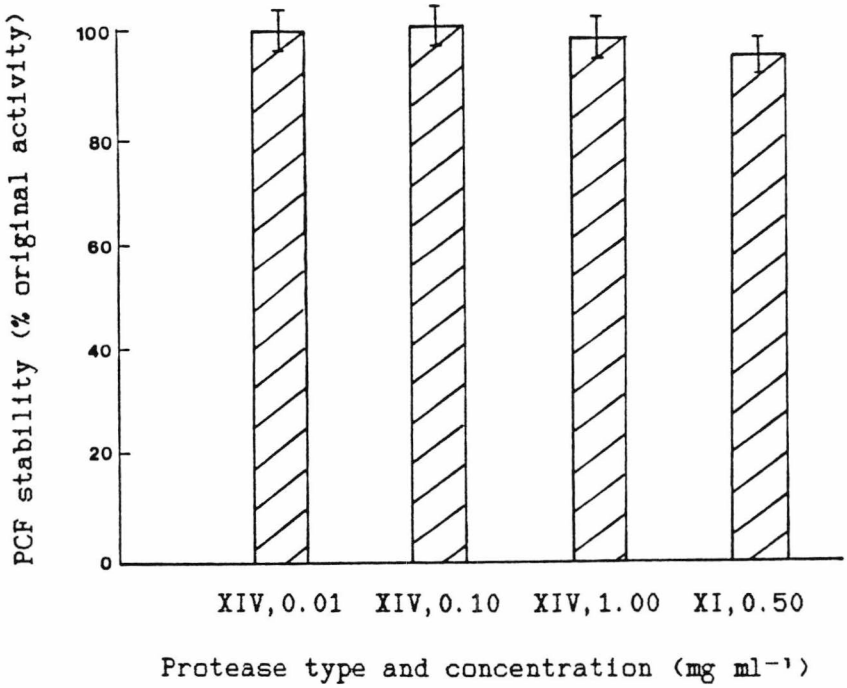


Fig. 4.2. Stability of P. ultimum-inhibitory components of Pen. claviforme culture filtrate (PCF) to protease treatment prior to bioassay. Enzymes were dissolved in sterile 0.1M potassium phosphate buffer (pH 6.0), diluted 1:10 in PCF and the mixtures incubated at room temperature (20+/-2°C) for 2h. Bars represent +/- SE.

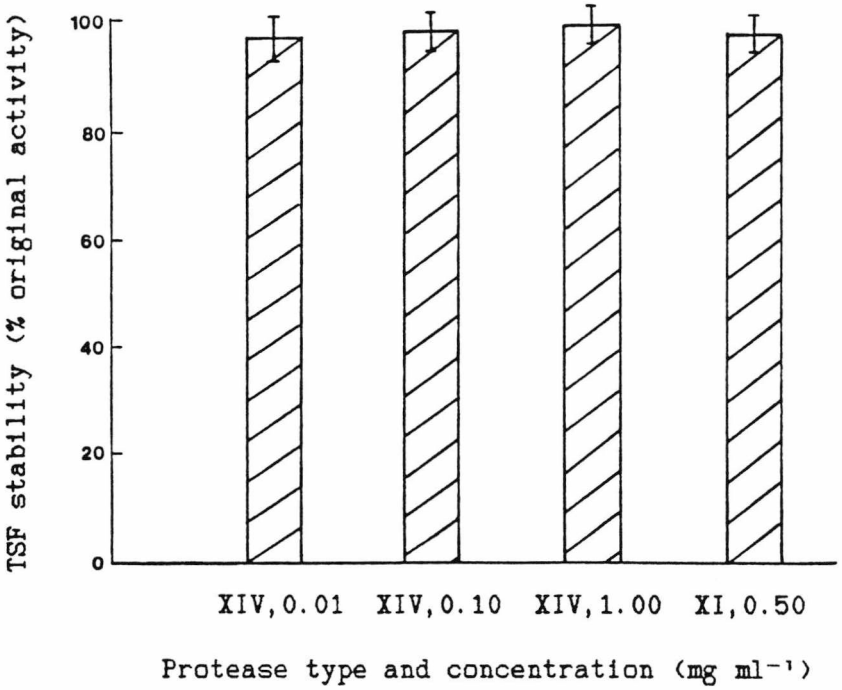


Fig. 4.3. Stability of P. ultimum-inhibitory components of Trichoderma sp. culture filtrate (TSF) to protease treatment prior to bioassay. Enzymes were dissolved in sterile 0.1M potassium phosphate buffer (pH 6.0), diluted 1:10 in TSF and the mixture incubated at room temperature (20+/-2°C) for 2h. Bars represent +/- SE.

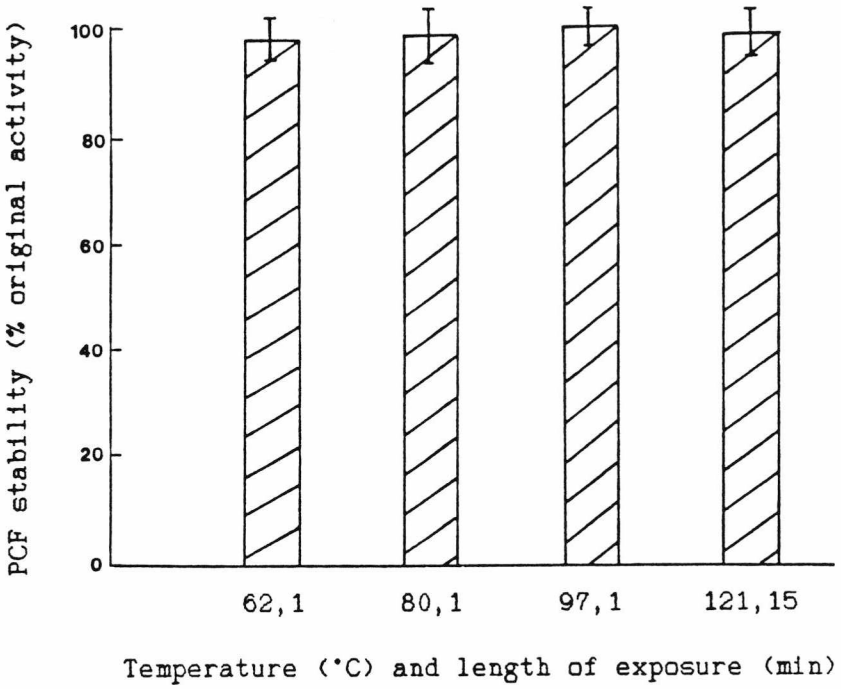


Fig. 4.4. Stability of P. ultimum-inhibitory components of Pen. claviforme culture filtrate (PCF) to heat treatment prior to bioassay. Bars represent +/- SE.

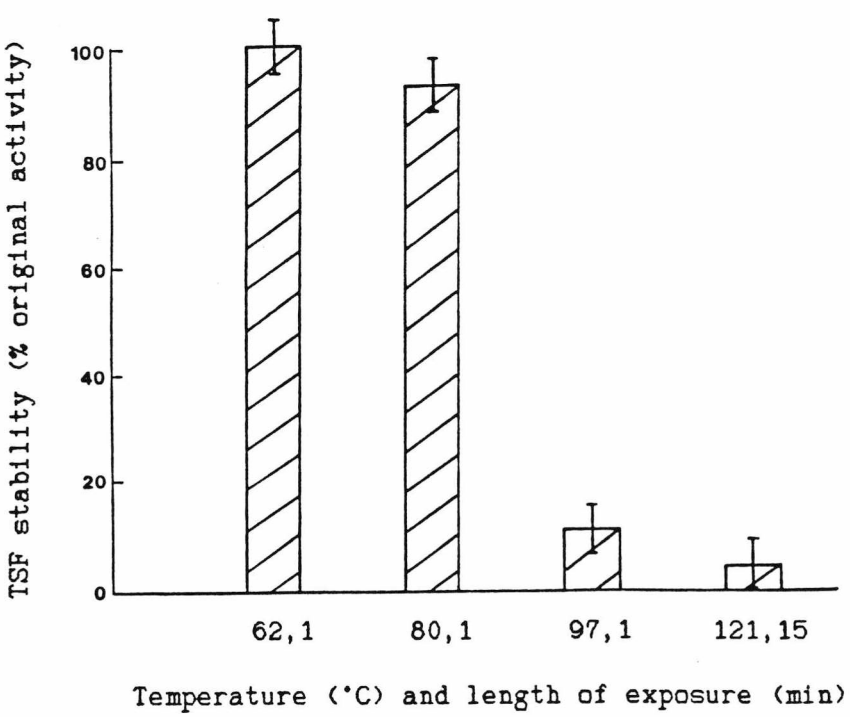


Fig. 4.5. Stability of P. ultimum-inhibitory components of Trichoderma sp. culture filtrate (TSF) to heat treatment prior to bioassay. Bars represent +/- SE.

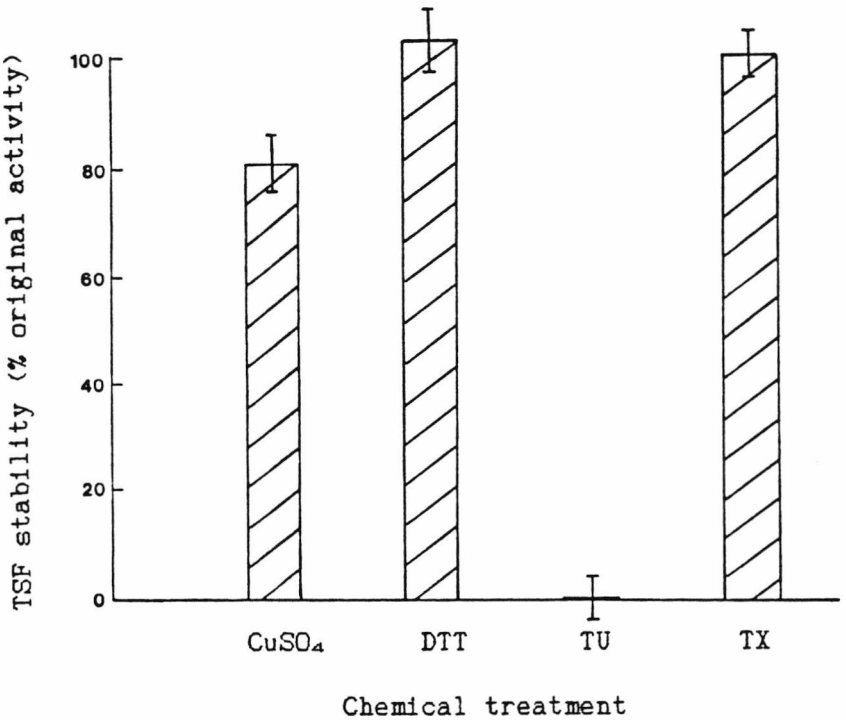


Fig. 4.6. Stability of P. ultimum-inhibitory components of Trichoderma sp. culture filtrate (TSF) to treatment with copper sulphate (1mM), dithiothreitol (DTT, 30mM), thiourea (TU, 30mM) or Triton X-100 (TX, 0.001%) prior to bioassay. Chemicals were dissolved in sterile distilled water, diluted 1:10 in TSF and the mixtures incubated at room temperature (20+/-2°C) for 2h. Bars represent +/- SE.

4.2.4 Effect of storage at different temperatures

Separate aliquots of PCF or TSF were stored at room temperature ($20^{+}/_{-}2°C$), 4°C or $^{-}20°C$. At intervals over the ensuing 7d period samples were removed and bioassayed. Results are presented in Figs. 4.7 and 4.8.

The anti-*Pythium* components of PCF were stable under all the conditions of storage tested (Fig. 4.7). The inhibitory activity of TSF had declined by 96.2% after 2d at room temperature and by 85.8% after 7d at 4°C, but the agents were stable for up to 7d when stored at $^{-}20°C$ (Fig. 4.8).

4.2.5 Effect of storage at different pH values

PCF was diluted 1:20 in sterile 0.1M citrate phosphate buffer at a pH of 5.5, 6.4 or 7.4, or in sterile 0.1M sodium phosphate buffer (pH 8.1) and stored at room temperature ($20^{+}/_{-}2°C$). Samples were removed at intervals during the subsequent 14d and bioassayed. Results are given in Fig. 4.9.

The *Pythium ultimum*-inhibitory components were stable at pH 5.5 or 6.4, but after 14d their activity had decreased by 53.6% at pH 7.4 and by 85.3% at pH 8.1.

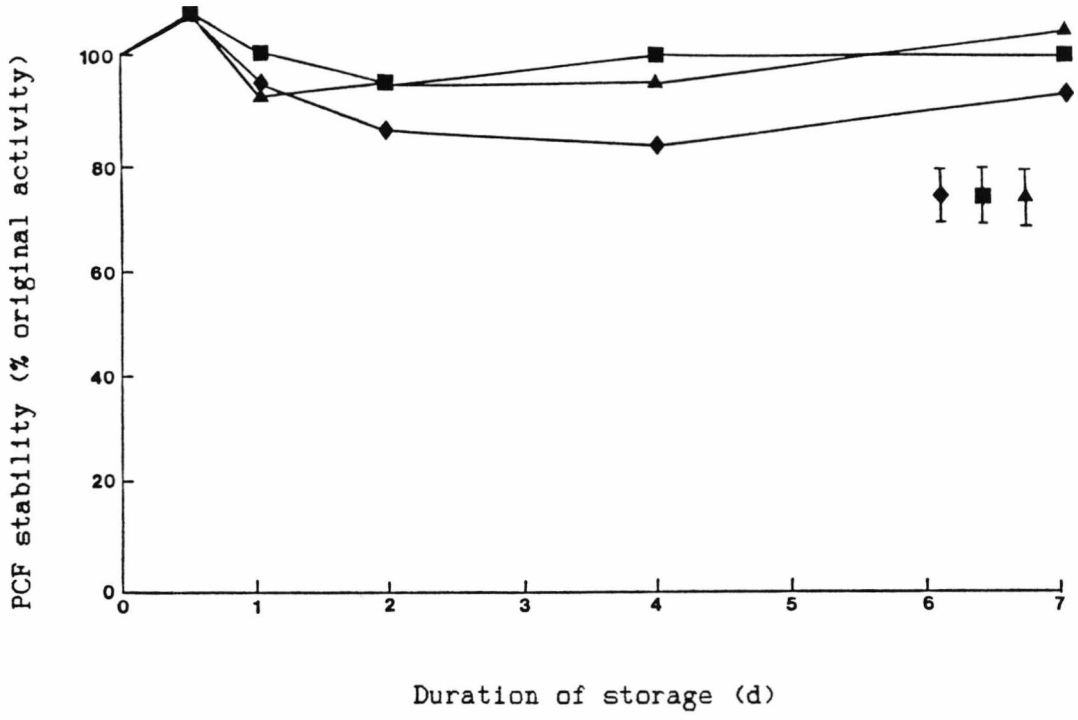


Fig. 4.7. Stability of *P. ultimum*-inhibitory components of *Pen. claviforme* culture filtrate (PCF) to storage at room temperature (20+/-2°C, ◆), 4°C (■) or -20°C (▲) prior to bioassay. Bars represent +/- mean SE.

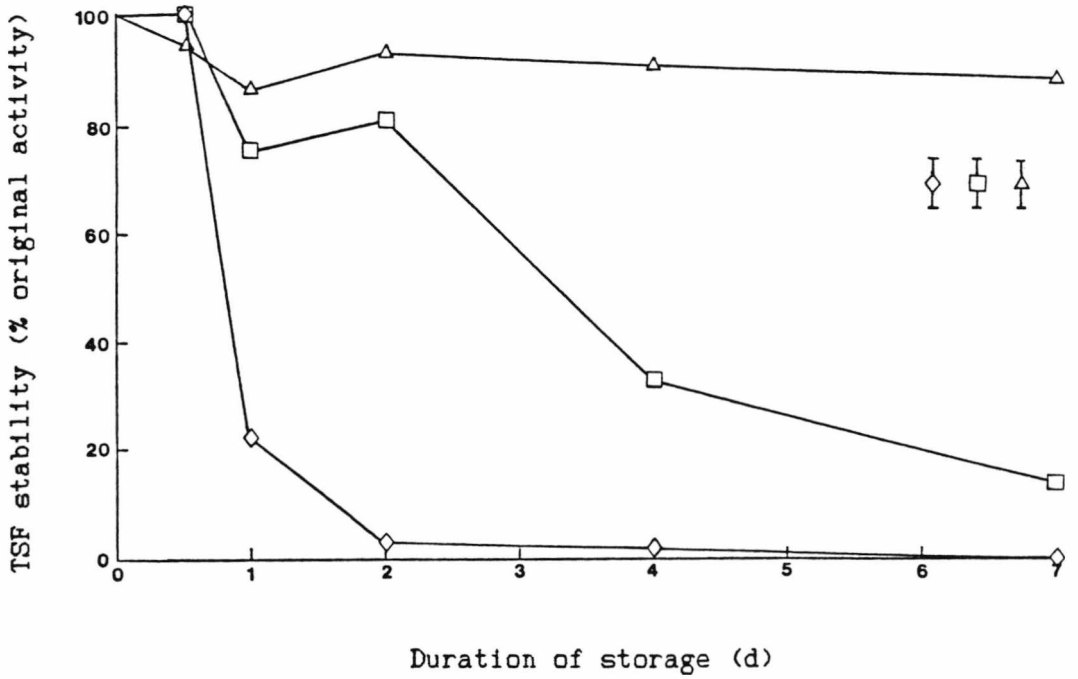


Fig. 4.8. Stability of *P. ultimum*-inhibitory components of *Trichoderma* sp. culture filtrate (TSF) to storage at room temperature (20+/-2°C, ◇), 4°C (□) or -20°C (△) prior to bioassay. Bars represent +/- mean SE.

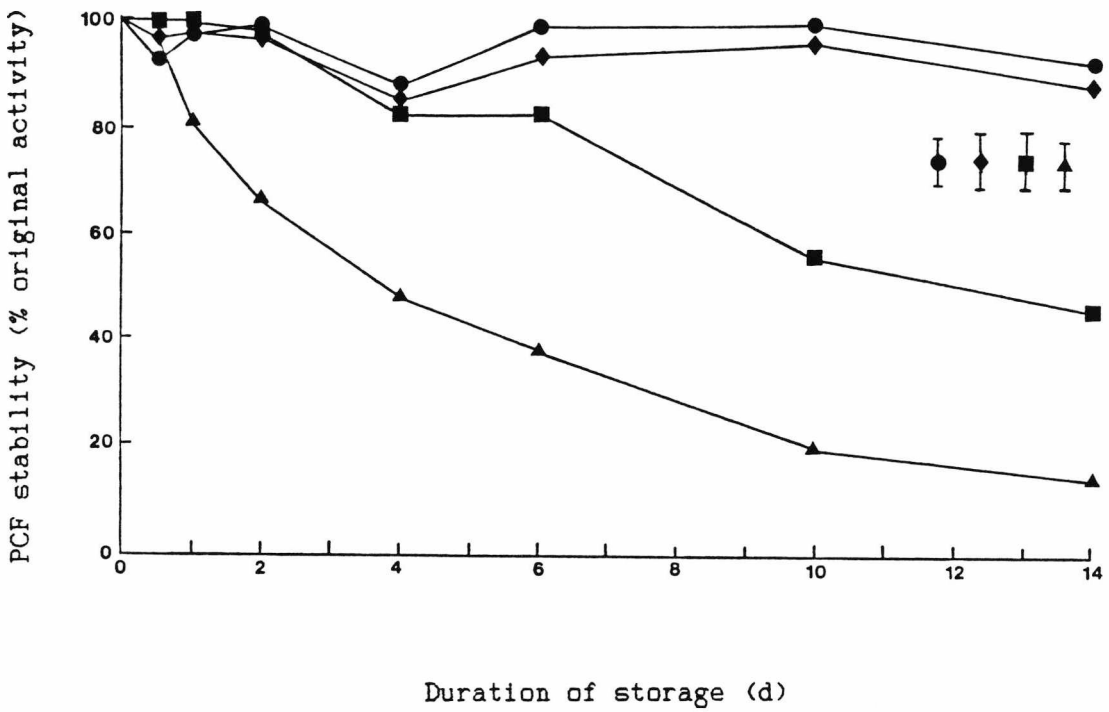


Fig. 4.9. Stability of P. ultimum-inhibitory components of Pen. claviforme culture filtrate (PCF) to storage in 0.1M citrate phosphate buffer at pH 5.5 (●), 6.4 (◆) or 7.4 (■), or in 0.1M sodium phosphate buffer at pH 8.1 (▲) prior to bioassay. PCF was diluted 1:20 in sterile buffer solutions and the mixtures stored at room temperature (20+/-2°C). Bars represent +/- mean SE.

4.3 Antagonistic activity of metabolites fractionated according to molecular size

The relative Pythium ultimum-inhibitory activity of molecules in PCF and TSF of 'low' (<1,000 daltons), 'intermediate' (1,000-10,000 daltons) and 'high' (>10,000 daltons) mol. wt. was assessed by ultrafiltration followed by bioassay, as described in section 2.4.1. The antagonists were cultured in triplicate and each fraction was bioassayed in duplicate. Results are shown in Figs. 4.10 and 4.11.

The low mol. wt. fraction of PCF retained >94.1% of the anti-Pythium activity of the unfractionated filtrate in all samples after 8d of incubation (Fig. 4.10). The intermediate and high mol. wt. fractions both gave low levels (<0.4IU ml^{-1}) of P. ultimum inhibition throughout the experiment.

Both the low and high mol. wt. fractions of TSF appeared to contribute to the anti-Pythium activity of the unfractionated sample (Fig. 4.11). After 19d incubation they caused 44.4 and 58.3% of total Pythium inhibition, respectively. The activity of these two fractions was still increasing at the end of the experiment, 9d after that of the unfractionated sample had begun to decline. The intermediate mol. wt. fraction showed <0.2IU ml^{-1} inhibitory activity throughout the investigation. The activity of the unfractionated filtrate was always greater than the sum of that of the three individual fractions.

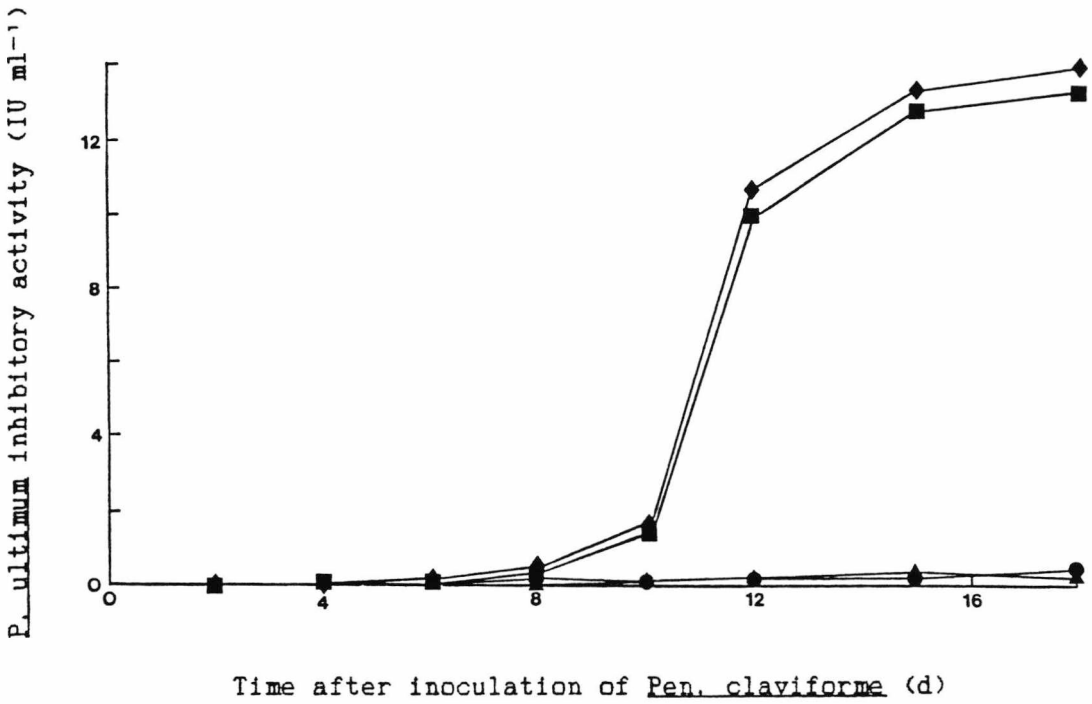


Fig. 4.10. P. ultimum-inhibitory activity in unfractionated Pen. claviforme culture filtrate (◆), and in fractions of nominal mol. wt. <1,000 daltons (■), 1,000-10,000 daltons (●) and >10,000 daltons (▲), obtained by ultrafiltration.

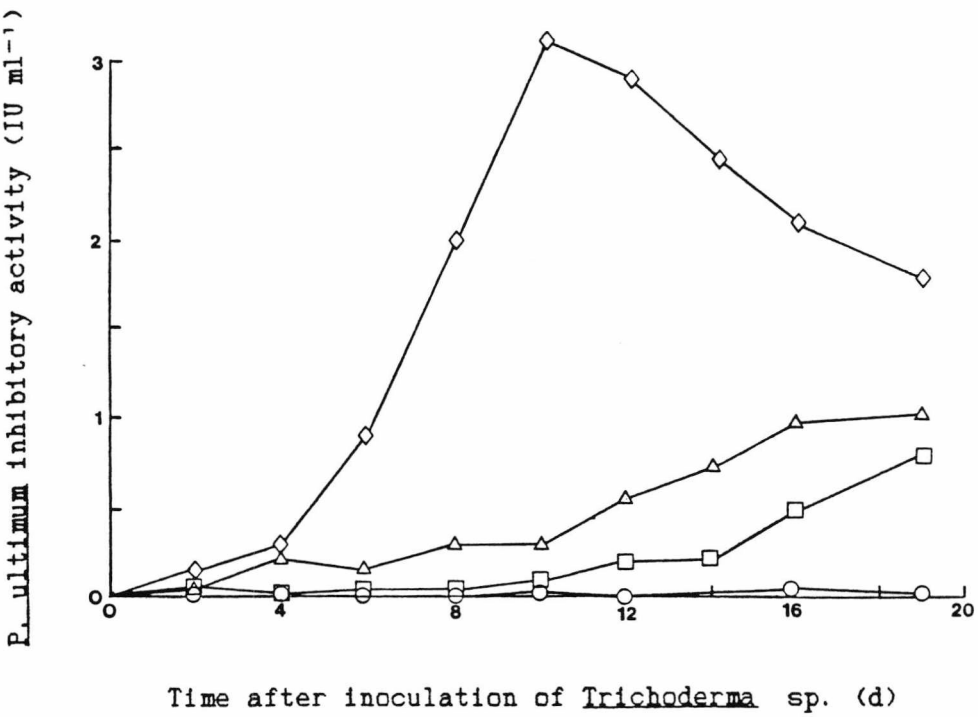


Fig. 4.11. P. ultimum-inhibitory activity in unfractionated Trichoderma sp. culture filtrate (◇), and in fractions of nominal mol. wt. <1,000 daltons (□), 1,000-10,000 daltons (○) and >10,000 daltons (△), obtained by ultrafiltration.

4.4 Relationship between antagonistic activity and the production of laminarinase and cellulase

The possible role of laminarinase and cellulase as active components of PCF and TSF was investigated by conducting concurrent assays for *Pythium ultimum* inhibition (section 2.3.2) and for enzyme production (section 2.4.2). The enzyme assay procedure involved colorimetric measurement of reducing sugars released during incubation of the culture filtrates with the respective enzyme substrates, and it was thus necessary to use antagonist growth media containing a non-reducing carbon source. Consequently, the glucose in CDM or WM was replaced in this experiment by carboxymethylcellulose (CMC, 10g l^{-1}), a water-soluble derivative of cellulose which was not hydrolysed under the test conditions. The two antagonists were grown in triplicate flasks and each culture was assayed in duplicate. Results are given in Figs. 4.12 and 4.13.

Penicillium claviforme produced *P. ultimum*-inhibitory metabolites in lower concentrations when grown in CDM containing CMC in place of glucose as a carbon source (Fig. 4.12, compare with Figs. 3.9, 3.11 or 4.10). Anti-*Pythium* activity increased during the first 14d after inoculation, reaching a maximum of 1.3IU ml^{-1}, and then gradually declined to 1.0IU ml^{-1} after 26d. In the same medium the fungus also produced hydrolytic enzymes, but activities did not correlate with the production of *Pythium* inhibitors, particularly during the early incubation period. Thus activity of laminarinase and cellulase increased rapidly during the first 8d, reaching a maximum of 15.5 or

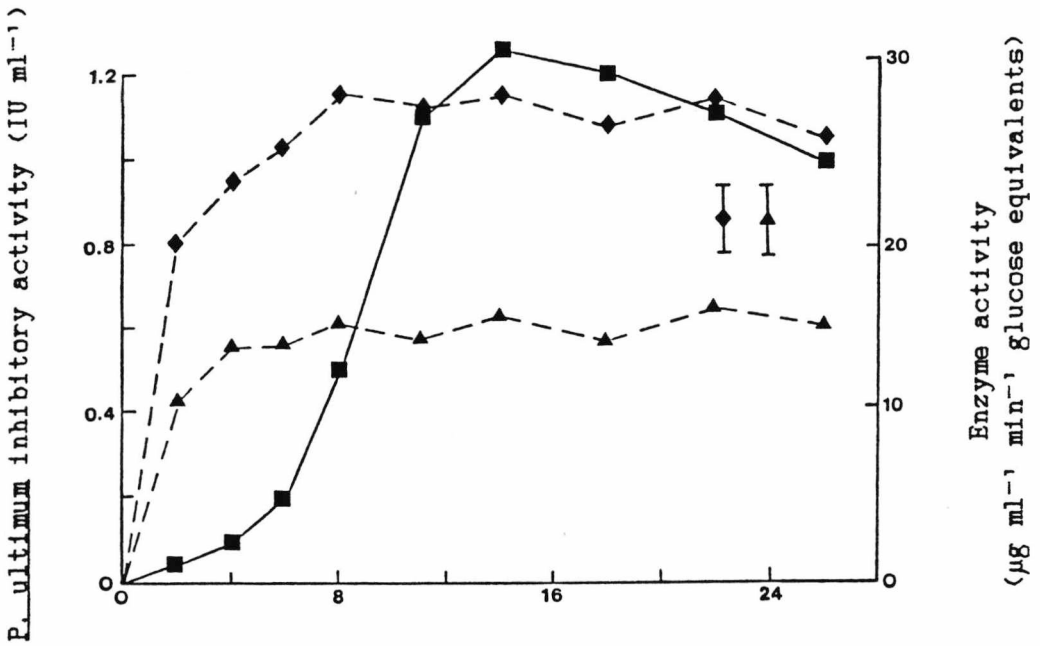


Fig. 4.12. Activities of *P. ultimum*-inhibitory metabolites (—■—), laminarinase (– ▲ –) and cellulase (– ◆ –) in *Pen. claviforme* culture filtrate. Bars represent +/- mean SE.

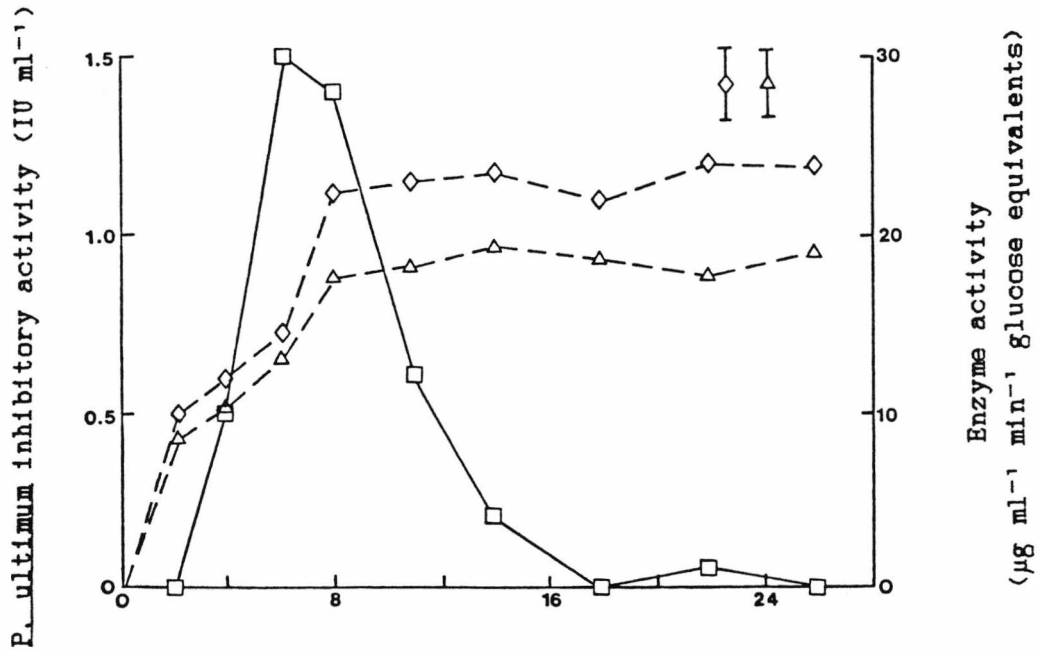


Fig. 4.13. Activities of *P. ultimum*-inhibitory metabolites (—□—), laminarinase (– △ –) and cellulase (– ◇ –) in *Trichoderma* sp. culture filtrate. Bars represent +/- mean SE.

28.0μg ml^{-1} min^{-1} glucose equivalents, respectively, and then levelled off for the remainder of the experiment.

The production of anti-Pythium agents by Trichoderma sp. 1 was also reduced in growth media containing CMC in place of glucose (Fig. 4.13, compare with Figs. 3.10, 3.12 or 4.11), but greatest yields were proportionally depressed to a lesser extent than in cultures of Pen. claviforme. Inhibitor concentrations reached a maximum of 1.5IU ml^{-1} at 6d after inoculation, declined over the next 12d to undetectable levels, and remained at <0.1IU ml^{-1} until the end of the experiment. The antagonist also produced both laminarinase and cellulase in the CMC medium, but again the activites of these enzymes did not correlate with that of P. ultimum inhibitory metabolites. In this case discrepancies were most marked in the later stages of incubation. Enzyme production by the Trichoderma sp. showed a similar pattern to that by Pen. claviforme, increasing rapidly during the first 8d of incubation and then levelling off. However the maximum activity of laminarinase (19.4μg ml^{-1} min^{-1}) was higher, and of cellulase (24.2μg ml^{-1} min^{-1}) was lower than in culture filtrates of Pen. claviforme.

At approximately this point in the project the decision was taken to focus attention on the development of PCF rather than TSF as a plant disease control agent, for the following reasons:

1) the approx. 5-fold greater yield of metabolites suppressive to the mycelial growth of P. ultimum in culture filtrates of Pen. claviforme compared to those of Trichoderma sp. 1 when the antagonists were grown under conditions optimal for inhibitor production (section 3.3.3);

2) the higher potency of PCF against Pythium propagules (section 3.2.3);

3) the greater stability of PCF to storage at both room temperature and at 4°C (section 4.2.4);

4) the broader spectrum of activity of PCF against other damping-off pathogens (section 5.4).

4.5 Effect of reduction in iron concentration and addition of calcium carbonate to antagonist growth medium

Bassett & Tanenbaum (1958) observed a marked effect on the production of patulin by Penicillium patulum caused by reducing $FeSO_4$ in, and adding $CaCO_3$ to the fungal growth medium. The antibiotic concentration was determined spectrophotometrically at a wavelength of 276nm, the absorption maximum of patulin (Tanenbaum & Bassett, 1958). The effect of the same medium modifications on the Pythium ultimum-inhibitory activity and absorbance at 276nm of culture filtrates of Pen. claviforme was evaluated.

The antagonist was grown in standard CDM containing 10mg l^{-1} $FeSO_4.7H_2O$ (section 2.2), in CDM in which the concentration of $FeSO_4$ had been reduced to 1mg l^{-1} and in CDM to which 2g l^{-1} sterile $CaCO_3$ had been added prior to inoculation. Production of Pythium inhibitors in the three media was estimated by bioassay as given in section 2.3.2. Absorbance at 276nm was measured concurrently (LKB Ultrospec 4050). Treatment samples were diluted as necessary so that absorbance fell within the linear region of a standard curve prepared by taking readings

for dilutions of a highly active culture filtrate. Each treatment was replicated three times and assayed in duplicate. Results are presented in Fig. 4.14.

In standard CDM increases in anti-Pythium activity and in absorbance at 276nm were coincidental. Values of both parameters were low until 8d after inoculation, rose rapidly between 10d and 13d, and more slowly from 13d to the termination of the experiment.

The concentration of Pythium inhibitors and absorbance at 276nm were reduced in the low iron medium compared to standard CDM in all samples later than 4d. After 20d incubation, the two parameters were depressed by 50.8 and 56.3%, respectively.

Inhibitor production and absorbance were also decreased by the addition of $CaCO_3$. The effect was greater than that caused by reducing the iron content such that at the end of the experiment values were diminished by 67.7 and 63.0%, respectively.

4.6 Thin-layer chromatography

The technique of thin-layer chromatography was employed to investigate:

1) the presence of patulin in PCF (section 4.6.1); and

2) the contribution of the antibiotic to the Pythium ultimum-inhibitory activity of the culture filtrate (section 4.6.2).

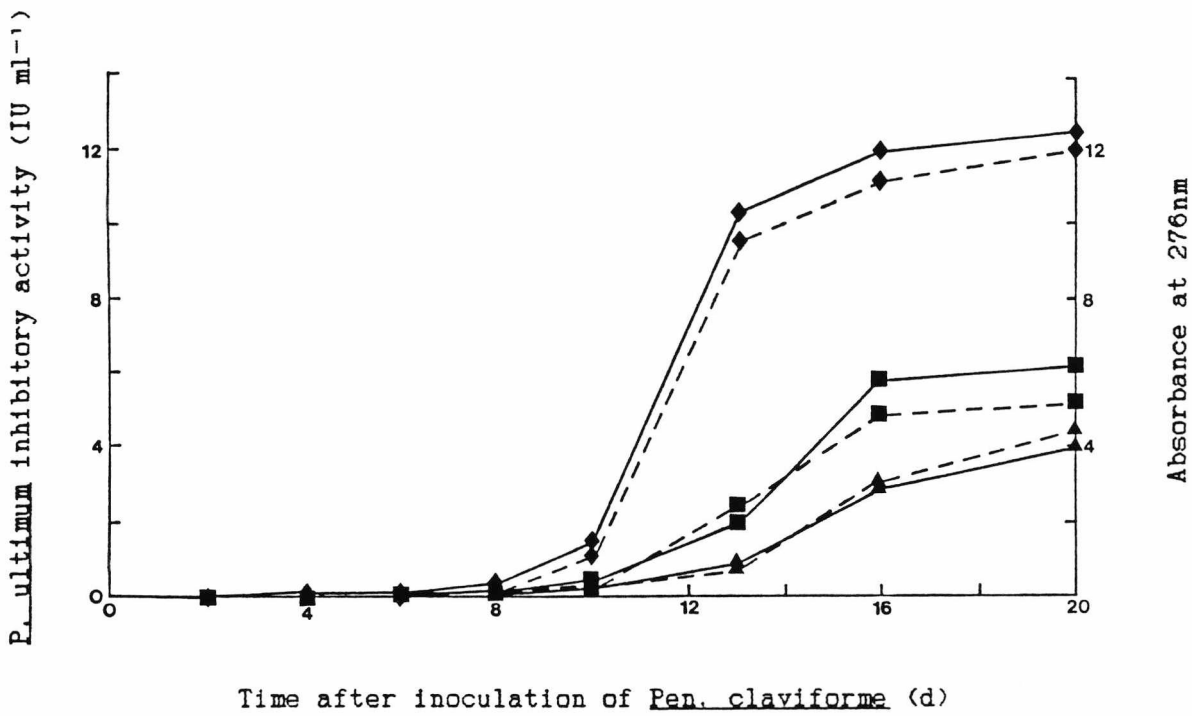


Fig. 4.14. P. ultimum-inhibitory activity (——) and absorbance at 276nm (A_{276}, — — —) of culture filtrates of Pen. claviforme grown in standard Czapek-Dox medium containing 10mg l^{-1} $FeSO_4.7H_2O$ (CDM, ◆), CDM containing 1mg l^{-1} $FeSO_4.7H_2O$ (■) and CDM plus 2g l^{-1} $CaCO_3$ (▲). Mean values for A_{276} SE were smaller than the point marker.

4.6.1 Separation of PCF components and location of patulin

Tlc plates were spotted with 2, 4 or 6µl samples of PCF concentrate (35IU ml^{-1}) or extract (38IU ml^{-1}) (section 2.5.1), CDM concentrate or extract (section 2.5.1) or pure patulin (2.5mg ml^{-1}). The chromatograms were developed and the separated components located as described in section 2.4.3. Rf values were calculated using the formula:

Rf = distance moved by solute/distance moved by solvent

and are the mean of measurements for three spots from each of three experiments. Results are summarized in Table 4.2.

4.6.1.1 Development in toluene/ethyl acetate/formic acid (solvent a))

A single constituent of PCF concentrate at Rf 0.51 fluoresced deep blue under short wavelength uv light.

Five components of PCF concentrate were located with MBTH (reagent a), Plate 4.1, Table 4.2), which gave respectively: i) a faint brown spot at Rf 0.51, ii) a faint brown spot at Rf 0.45, iii) an intense yellow spot at Rf 0.42 which co-chromatographed with patulin and stained an identical colour, iv) a large red spot at Rf 0.35 and v) a blue spot at Rf 0.27. Spots iii) and iv) were visible immediately following chemical and heat treatment but spots i), ii) and v) appeared only after 24h by which time the silica layer itself had acquired a blueish tinge (Plate 4.1). Spots i) and v) were absent after chromatography of PCF extracted in ethyl acetate. No components of CDM concentrate or extract were visualized at any position except the starting spot.

Four and three components of PCF concentrate were detected immediately after treatment with ethanolic sulphuric acid (reagent b))

Table 4.2. Mobility and locating reactions of components of concentrated Pen. claviforme culture filtrate on thin-layer chromatograms

Rf[×]		Locating reactions[ˣ]			
Solvent a)	Solvent b)	Short λ uv	Reagent a)	Reagent b)	Reagent c)
0.51 (0.01)	0.90 (0.02)	Blue	Brown (24h)	Pale brown	-
0.45 (0.02)	0.80 (0.02)	-	Brown (24h)	Pale brown	Blue-brown
0.42 (0.02)	0.76 (0.02)	-	Yellow	Dark brown	Brown
			Co-chromatographing with patulin		
0.35 (0.01)	0.65 (0.03)	-	Red	Dark brown	Brown
0.27 (0.02)	0.47 (0.02)	-	Blue (24h)	-	-

[×] The solvent systems used were: a) toluene/ethyl acetate/formic acid (5:4:1, v/v) and b) chloroform/methanol (4:1, v/v). SE are given in parentheses.

[ˣ] The colour reagents used were: a) 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride in distilled water, b) 5% (v/v) conc. sulphuric acid in ethanol and c) 0.5% (v/v) anisaldehyde in methanol/ acetic acid/ conc. sulphuric acid (17:2:1, v/v), followed in each case by heating at 105°C for 10min. Spots were visible immediately after chemical and heat treatment except where indicated.

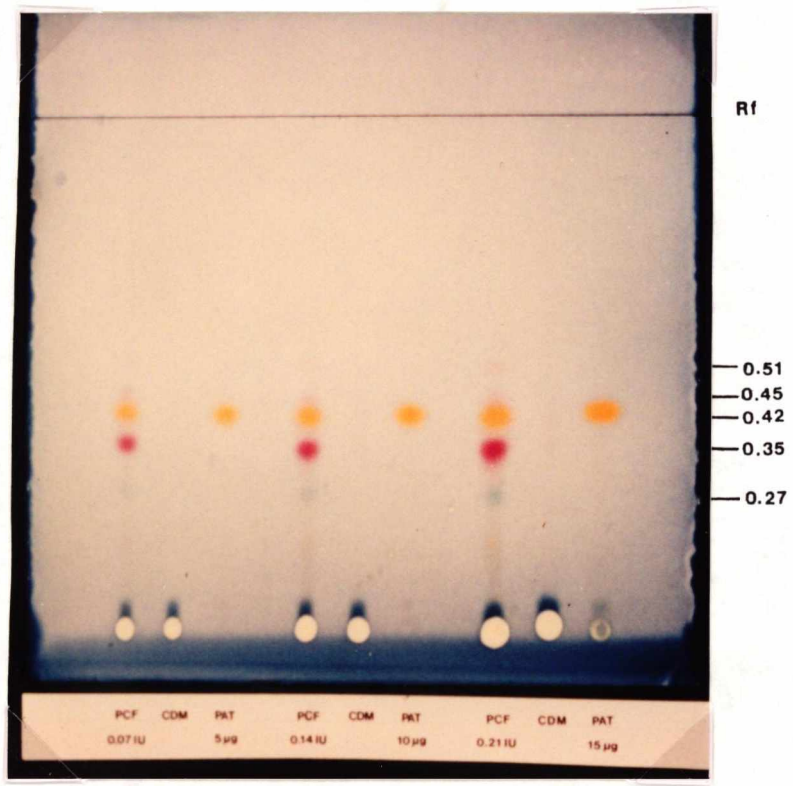


Plate 4.1. Tlc plate spotted with concentrated Pen. claviforme culture filtrate (PCF), concentrated Czapek-Dox medium (CDM) or patulin (PAT), developed in toluene/ethyl acetate/formic acid (5:4:1, v/v) and sprayed with 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride in distilled water followed by heating at 105°C for 10min. Photograph taken 24h after chemical and heat treatment.

and anisaldehyde (reagent c)), respectively (Table 4.2). The constituent with an Rf value of 0.42 gave the same colour reactions as patulin and again no components of CDM concentrate were located at any position except the starting spot. All spots were smaller and fainter than those obtained after spraying with MBTH.

4.6.1.2 Development in chloroform/methanol (solvent b))

Each of the constituents of PCF concentrate observed on tlc plates after development in solvent system a) and treatment with MBTH were also present following development in solvent b), but Rf values were greater in all cases (Table 4.2). The component staining intense yellow again showed the same mobility as patulin and CDM concentrate gave a spot only at the starting position.

4.6.1.3 Development in two dimensions

Two tlc plates were spotted with 4µl PCF concentrate (32IU ml^{-1}) or patulin (2.5mg ml^{-1}) and developed concurrently in toluene/ethyl acetate/formic acid. The chromatograms were removed from the tank and allowed to dry, then rotated through 90° and developed in chloroform/methanol. After drying the plates were sprayed with MBTH.

The large red-staining spot of PCF concentrate was resolved into two components after development in a second dimension (Fig. 4.15), but all other spots appeared to consist of single compounds. The yellow-staining spot once more co-chromatographed with patulin, shown by tracing the patulin reference plate and overlaying on the PCF plate.

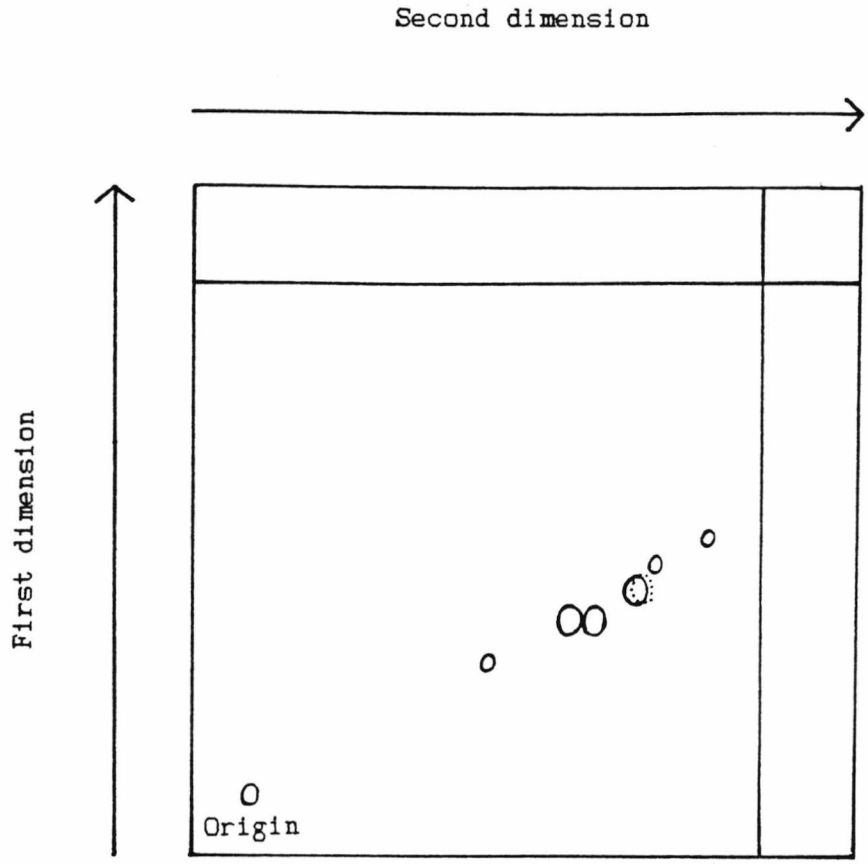


Fig. 4.15. Tlc plate spotted with concentrated Pen. claviforme culture filtrate (——) or patulin (·····) and developed in toluene/ethyl acetate/formic acid (5:4:1, v/v) in the first dimension and chloroform/methanol (4:1, v/v) in the second. Components were located by spraying with 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride in distilled water followed by heating at 105°C for 10min. Chromatograms were traced 24h after chemical and heat treatment.

4.6.2 Bioassay of PCF components

A total of 18µl PCF concentrate (35IU ml^{-1}), CDM concentrate or patulin (2.5mg ml^{-1}) was applied to the start line of each of two tlc plates. An additional 18µl of each material was applied above the stop line and removed for bioassay before development in order to determine % extraction from the chromatograms (section 2.4.3). The plates were developed and sprayed as specified in section 2.4.3. The compounds present in the five spots given by PCF and the single spot given by patulin (section 4.6.1), together with the undeveloped spots, were bioassayed as described in section 2.4.3. The experiment was repeated three times. Results are given in Table 4.3.

85.7% and 85.6% of the applied Pythium ultimum-inhibitory activity was extractable from undeveloped spots of PCF and patulin, respectively. No inhibitory compounds were extracted from undeveloped spots of CDM. 100% of the anti-Pythium activity of PCF extractable from an undeveloped spot was recovered from the position at Rf 0.42 and 14.1% from that at Rf 0.45. The components of all other spots caused only low levels of inhibition. 96.7% of the extractable activity of patulin was recovered from the position at Rf 0.42. The quantity of patulin estimated to cause P. ultimum inhibition equivalent to 1IU PCF was approx. 50µg.

Table 4.3. Recovery of *P. ultimum*-inhibitory agents present in concentrated *Pen. claviforme* culture filtrate (PCF) and patulin from thin-layer chromatograms

Rf[a]	*P. ultimum* inhibition (% extractable activity)[b]	
	PCF	Patulin
0.51	1.9 (3.3)	-
0.45	14.1 (4.1)	-
0.42	100.0 (4.3)	96.7 (2.7)
0.35	4.1 (4.5)	-
0.27	3.3 (3.7)	-

[a] Tlc plates were developed in toluene/ethyl acetate/formic acid (5:4:1, v/v) and components located on a reference plate by spraying with 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride in distilled water followed by heating at 105°C for 10min.

[b] Anti-*Pythium* activity of material recovered from positions corresponding to the located spots was compared with that extractable from undeveloped spots (85.7 (2.4) % and 85.6 (2.2) % for PCF and patulin, respectively). SE are given in parentheses.

4.7 Infrared spectroscopy

Infrared spectroscopy was conducted as described in section 2.4.4 with the objective of confirming the identity of the Pythium ultimum-inhibitory constituent of PCF extract located at Rf 0.42. Results are recorded in Fig. 4.16.

A number of the transmittance peaks in the infrared spectrum of the bioactive material in PCF coincided with those in the spectrum of patulin showing that this antibiotic was a major component. However, other peaks were obscured or shifted, indicating that additional compounds were present at lower concentrations.

4.8 Discussion

4.8.1 Role of siderophores

Siderophores are extracellular, non-protein, high-affinity Fe^{3+}-transport agents (Neilands, 1981) which have been implicated in the biocontrol of certain soil-borne plant pathogens by specific fluorescent pseudomonads termed plant growth-promoting rhizobacteria (PGPR) (Leong, 1986; section 1.3.3). For example, Loper (1988) demonstrated conclusively, using single-site Tn5 insertion mutants, that the production of siderophores contributes to the control of Pythium ultimum-induced pre-emergence damping-off of cotton by Ps. fluorescens 3551. Siderophores may be produced by many other microorganisms including fungi (Emery, 1980). They have mol. wts. in the range 500-1000 daltons, and therefore could have been active constituents of the

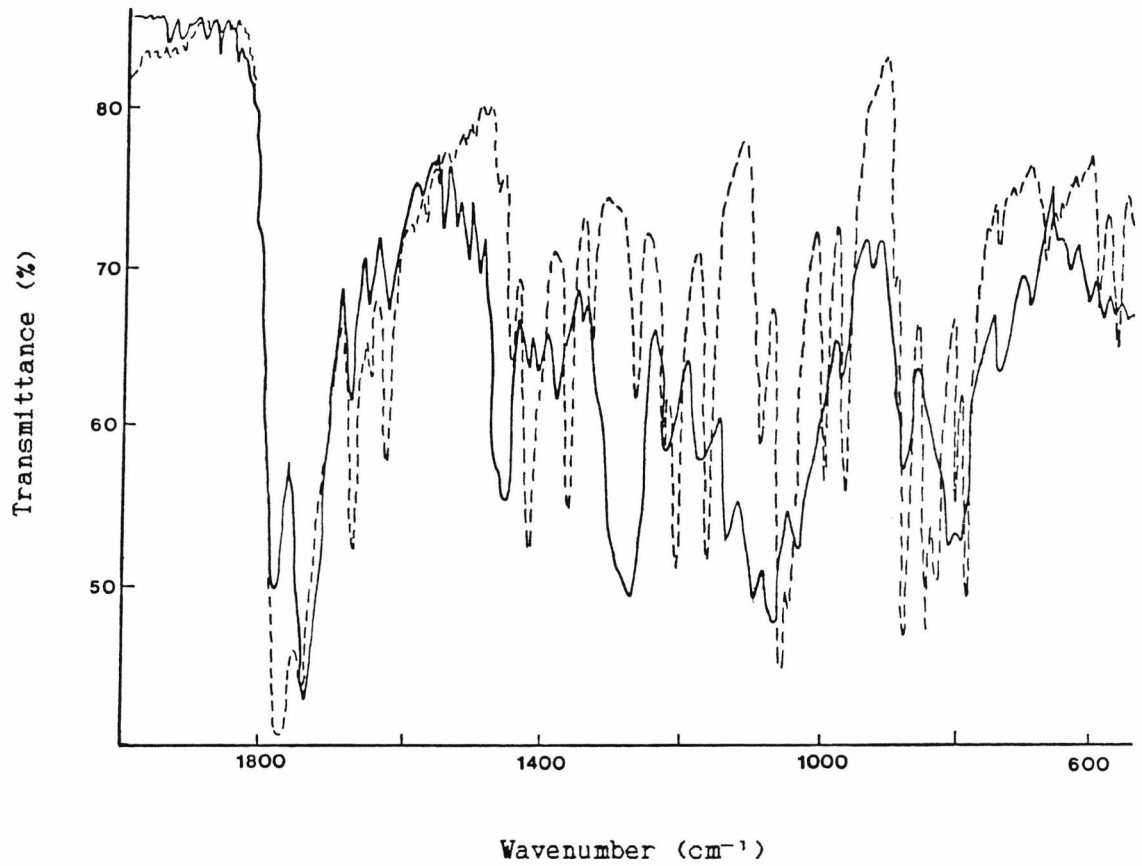


Fig. 4.16. Infrared spectrum of P. ultimum-inhibitory agents present in concentrated Pen. claviforme culture filtrate (——) or patulin (– – –). Components were separated on plc plates developed in chloroform/methanol (4:1, v/v) and located on a reference plate by spraying with 0.5% (w/v) 3-methyl-2-benzothiazolinone hydrochloride in distilled water followed by heating at 105°C for 10min.

low mol. wt. fractions of PCF and TSF which inhibited the growth of P. ultimum. However, no evidence was obtained to support this contention.

Firstly, siderophores are produced only under iron-limited conditions, when they function to scavenge Fe^{3+} from the environment and transport it into the producer organism following attachment to complementary membrane-associated receptors. Thus six PGPR strains were shown to inhibit the growth of Pythium spp. on unamended King's medium B, but not when the medium was supplemented with $FeCl_3$ (Becker & Cook, 1988). In the present study, the antagonist growth media contained plentiful supplies of iron salts (section 2.2) and hence, even if the fungi were capable of producing siderophores, they were unlikely to have done so. Furthermore, when Penicillium claviforme was grown in a medium containing a reduced concentration of $FeSO_4$, the anti-Pythium activity of the culture filtrate was less than that in the standard medium (Fig. 4.14), whereas the converse would be more likely if inducible iron transport agents were the active components.

Siderophores are also effective agents of plant disease control only when Fe^{3+} is in short supply. Under these conditions pathogens which produce siderophores in lower quantities, or with lesser affinity for the mineral, are unable to obtain sufficient amounts for growth. Thus the bacterial siderophore pseudobactin was found to significantly increase the growth of potatoes due to the control of minor pathogens, but ferric pseudobactin was ineffective (Kleopper et al., 1980). Similarly, in vitro, the mycelial growth of P. ultimum was inhibited by the synthetic ferric complexing agent ethylenediaminedi(o-hydroxyphenylacetic acid) (EDDA) (Loper, 1988), and this effect was

annulled by the addition of 1μM FeCl (sic) to the agar medium. In the current work, the addition of 1μM $FeCl_3$ together with PCF or TSF failed to ameliorate the P. ultimum-inhibitory activity of either culture filtrate (Table 4.1), again indicating that the active constituents do not act by rendering Fe^{3+} unavailable to the pathogen.

4.8.2 Role of cell wall-degrading enzymes

The dominant component of the Pythium hyphal wall is a 1,3-β-glucan also containing 1,6-β-linkages, together with a smaller proportion of cellulose (1,4-β-glucan) (Cooper & Aronson, 1967). Lipids and proteins are also present, the latter characteristically rich in hydroxyproline (Novaes-Ledieu, Jiménez-Martínez & Villanueva, 1967). The enzymatic degradation of these walls therefore requires agents capable of hydrolysing 1,3-β- and also 1,4-β-glucosidic bonds (Eveleigh, Sietsma & Haskins, 1968). Both are multi-component systems, the former collectively known as laminarinases since the assay substrate used in early work was the algal 1,3-β-glucan laminarin (Reese & Mandels, 1959), and the latter as cellulases (Burns, 1983). Recent work suggests that a large number of enzymes may be involved in fungal wall degradation in addition to those hydrolysing the principle components (Ridout, Coley-Smith & Lynch, 1986).

The production of laminarinases is common amongst saprophytic fungi, including both Penicillium and Trichoderma species (Chesters & Bull, 1963a). Likewise, representitives of these two genera are known to be capable of synthesizing cellulases (Mandels, 1975). Indeed, Trichoderma viride (synonymous with Trichoderma reesei) is considered to be one of the most promising microbial sources of cellulase for industrial

purposes and has been extensively studied in relation to the saccharification of cellulosic waste materials (Knapp & Legg, 1986). Mycoparasitic Trichoderma species have also been observed to secrete hydrolytic enzymes when grown on the walls of plant pathogenic fungi. Elad, Chet & Henis (1982) found that isolates of Trichoderma harzianum differed in the quantities of chitinase, laminarinase and cellulase they produced during growth on mycelia of Sclerotium rolfsii, Rhizoctonia solani and Pythium aphanidermatum, and that these levels correlated with the ability of the isolates to control the respective pathogens in glasshouse trials. The authors therefore suggested that the synthesis of such enzymes could serve as a basis for the screening of Trichoderma strains as potential biocontrol agents.

However, the production of both laminarinases (Ridout, Coley-Smith & Lynch, 1988) and cellulases (Nisizawa, Suzuki & Nisizawa, 1972) is subject to catabolite repression by glucose. Consequently, given the high concentrations of this sugar in the growth media routinely used for the culture of Penicillium claviforme and Trichoderma sp. 1, it appeared unlikely that such enzymes were involved in the the inhibition of Pythium ultimum by either antagonist culture filtrate. The data presented in sections 4.1-4.4 also do not implicate cell wall-degrading enzymes as major active components.

No morphological abnormalities were observed during microscopic examination of P. ultimum grown in the presence of PCF (section 4.1), but high concentrations of TSF did cause younger parts of the mycelium to become swollen which could have been indicative of cell wall damage. However, neither culture filtrate appeared to be lytic towards the pathogen.

Enzymes are proteins and are therefore subject to inactivation by proteases which hydrolyse peptide bonds between adjacent amino acid residues. However, the anti-*Pythium* agents in neither PCF or TSF were affected by treatment with a non-specific streptomycete protease at concentrations of up to 1.00mg ml^{-1}, or a fungal protease at up to 0.50mg ml^{-1}, for 2h prior to bioassay (Figs. 4.2 and 4.3).

Enzyme activity is critically dependent on the maintenance of the correct conformation of the active site (Wynn, 1979). They are thus susceptible to physical treatments such as heat which disrupt their secondary or tertiary structure. The *P. ultimum*-inhibitory components of PCF were not affected by temperatures of up to 121°C for 15min (Fig. 4.4). Those of TSF were stable to heat at up to 80°C for 1min but showed an 88.1% decrease in activity when subjected to a temperature of 97°C for 1min prior to bioassay and a 94.9% reduction after exposure to 121°C for 15 min (Fig. 4.5). By comparison, Sharma & Nakas (1987) found that the laminarinase produced by *Trichoderma longibrachiatum* was completely denatured at 60°C within 10min. However, fungal cellulases are reported to be remarkably heat stable and must be heated to 100°C for 10-20min for complete inactivation (Mandels & Reese, 1965).

Enzymes may also be reversibly or irreversibly inhibited by certain chemical agents which act on the active site itself or on amino acid residues responsible for maintaining molecular three-dimensional structure (Lehninger, 1975). The anti-*Pythium* activity of TSF was reduced by 18.9% after incubation with 1mM copper sulphate for 2h before bioassay (Fig. 4.6). Both laminarinases (Chesters and Bull, 1963b) and cellulases (Mandels & Reese, 1965) are reported to be inhibited by heavy metal ions, including Cu^{2+}, which act by binding to the sulphydryl

groups of cysteine residues to form mercaptides (Webb, 1966). The Pythium-inhibitory metabolites were not affected by treatment with 30mM dithiothreitol, a chemical which was found to inactivate the cellulase produced by T. reesei (Reese & Mandels, 1980), and which reduces disulphide linkages in proteins (Cleland, 1964). However their activity was completely destroyed by 30mM thiourea, a compound which inactivates peroxidases, possibly by binding to the iron atom in the enzyme active site (Bollag et al., 1987). Finally, the P. ultimum inhibitors in TSF were unaffected by treatment with 0.001% Triton X-100, a neutral detergent to which cellulases are generally susceptible (Mandels & Reese, 1965) and which acts by disrupting hydrophobic interactions within the molecule. The apparent resistance of the anti-Pythium metabolites to certain of the chemical inhibitors tested may in part have been due to the use of crude culture filtrates containing impurities with which the chemicals can also react (Mandels & Reese, 1965). The susceptibility of enzymes to such inhibitors therefore usually increases with purification. Formaldehyde, an example of a compound which may inactivate proteins such as cellulases by reaction with amino groups (Reese & Mandels, 1980), was also tested but itself totally prevented the growth of P. ultimum at a concentration of 30mM.

Ultrafiltration is a technique for separating dissolved and suspended materials on the basis of their molecular size (Williams & Wilson, 1981). Hydrostatic or centrifugal pressure is used to filter an aqueous medium through a semi-permeable membrane containing pores of known dimensions. Molecules smaller than the pore size of the membrane pass through with the solvent while larger ones are retained. Membranes are referred to in terms of nominal mol. wt. cut-off points, defined as

the approx. mol. wt. at which the membrane retains 90% of a globular solute (Anon, 1987). In practice, retention may differ from the nominal value because it is dependent on the shape in addition to the mol. wt. of the solute, and may also be affected by factors such as surface charge and concentration. Ultrafiltration is therefore not a suitable method for the determination of absolute mol. wt., but in the present experiments it did allow the assessment of the relative contribution of 'low', 'intermediate' and 'high' (section 4.3) mol. wt. fractions of the two antagonist culture filtrates to the total anti-Pythium activity.

P. ultimum inhibition by PCF appeared to be due largely to metabolites of nominal mol. wt. <1,000 daltons (Fig. 4.10). The slight inhibition caused by the intermediate and high mol. wt. fractions could be due to the active low mol. wt. molecules leaking through the membrane, or could indicate the presence of additional inhibitory components of lesser potency or at lower concentrations.

At least two molecular species apparently made a significant contribution to the anti-Pythium activity of TSF, with nominal mol. wts. <1,000 and >10,000 daltons (Fig. 4.11). These metabolites appeared to be synergistic in effect. However, the results could also be interpreted in terms of loss of active agents by adsorption to the membrane. Ultrafiltration membranes are made of inert polymeric materials which do not affect the passage of ionic solutes, but may adsorb certain molecules with hydrophobic surface groups (Anon, 1987). The lack of correlation between maximal yields of inhibitors in the unfractionated sample compared to any of the three fractions could also indicate adsorptive losses of active constituents.

Enzymes vary widely in mol. wt. but are generally greater than approx. 12,000 daltons (Lehninger, 1975). For example, Huotari *et al.* (1968) studied an exo-1,3-β-glucanase from basidiomycete QM806 with a mol. wt. of 51,000 daltons and Wood & McCrae (1978) characterized four endo-1,4-β-glucanases produced by *Trichoderma koningii* with mol. wts. ranging from 13,000-48,000 daltons. The results presented in section 4.3 therefore suggested that enzymes do not play a major role in the inhibition of *P. ultimum* by PCF but, taken in isolation, did not exclude their involvement in the activity of TSF.

The production of laminarinase and cellulase by *Pen. claviforme* and *Trichoderma* sp. 1 was studied in growth media in which glucose had been replaced by carboxymethylcellulose (CMC) as a carbon source (section 4.4). However, whilst laminarinases are constitutive (Bull & Chesters, 1966), cellulases are only produced in the presence of specific inducers which include CMC (Halliwell & Lovelady, 1981). The results obtained from this experiment therefore indicated only the potential of the two antagonists to synthesize these enzymes and not their presence or absence in the normal glucose media.

Both *Pen. claviforme* and *Trichoderma* sp. 1 produced laminarinase and cellulase when grown in a 1% CMC medium (Figs. 4.12 and 4.13). Despite the fact that the *Trichoderma* sp. was isolated on the basis of its cellulolytic ability (Shah, 1987), its maximum cellulase activity (24.2μg ml^{-1} min^{-1}, Fig 4.13) was considerably lower than that reported for other members of this genus. For example, the data presented by Sandhu & Kalra (1985) indicate maximal extracellular cellulase activites of approx. 50.4μg ml^{-1} min^{-1} during growth of *T. longi-brachiatum* in a 1% CMC medium. However, the synthesis of hydrolytic enzymes such as

enzymes such as cellulases by a given organism is dependent on a variety of cultural factors in addition to carbon source such as temperature, pH and aeration (Goksøyr & Eriksen, 1980). For instance, total cellulase activity of mixed cultures of T. reesei and Aspergillus phoenicus was two-fold greater at 27° than at 36°C (Duff, Cooper & Fuller, 1987). Thus it is likely that enzyme yields from the two antagonists could have been improved by physiological manipulation.

Environmental conditions also affect enzyme activity. The temperature, buffer type and pH used in the assay procedure were those employed for the estimation of cellulase (Kalra, Sidhu & Sandhu, 1986) and laminarinase (Sharma & Nakas, 1987) produced by T. longibrachiatum, but may have been sub-optimal for the enzymes released by Pen. claviforme and Trichoderma sp. 1.

A corollory of the fact that cellulases are inducible is that, if indeed they were important P. ultimum-inhibitory agents in the present work, then the culture filtrates might have been expected to show greater activity when the antagonists were grown in CMC media than in those containing glucose. However, the reverse was found to be the case, the production of inhibitors being greater when the fungi were supplied with a readily available glucose carbon source. The observation that the synthesis of neither laminarinase or cellulase was commensurate with that of anti-Pythium metabolites provided further evidence that such enzymes do not make a major contribution to the inhibitory activity of PCF or TSF.

4.8.3 Role of antibiotics

The experiments discussed in section 4.8.2 indicated that at least two products were involved in Pythium ultimum inhibition by Trichoderma sp. 1 with nominal mol. wts. of <1,000 and >10,000 daltons. In entirety, results suggested that both compounds were antibiotics rather than enzymes or siderophores (sections 4.8.2 and 4.8.1). A range of metabolites with antibiotic properties have been reported in culture filtrates of Trichoderma spp. (Weindling, 1934; Godtfredsen & Vangedal, 1965; Ooka et al., 1966; Pyke & Dietz, 1966; Meyer & Reusser, 1967; Dennis & Webster, 1971; Simon et al., 1988; Watts et al., 1988), but those released by Trichoderma sp. 1 were not studied further for reasons outlined at the end of section 4.4.

Evidence was obtained that the anti-Pythium agents produced by Penicillium claviforme were antibiotics of nominal mol. wt. <1,000 daltons. Members of the genus Penicillium are known to synthesize an extensive array of secondary metabolites (Pitt, 1979) and for many years Penicillium and Aspergillus spp. were the most important fungal sources of antibiotics (Bérdy, 1974). Metabolites which have been detected in culture filtrates of Pen. claviforme IMI 44744 include the antibiotic patulin (R.R.M. Paterson, C.A.B. International Mycological Institute; personal communication) which has a mol. wt. of 154 daltons and has been shown to be inhibitory in vitro to Pythium spp. including P. ultimum (Anslow et al., 1943; Gilliver, 1946). It therefore appeared possible that this compound could contribute to the activity of PCF observed in the present work.

Patulin was first recorded under this name by Birkenshaw et al. (1943) as a metabolite of Penicillium patulum. It was later found to be

produced by a number of other fungi (section 3.4.2) including several Penicillium spp. such as Pen. claviforme, Pen. expansum, Pen. griseofulvum and Pen. urticae (the latter two organisms are now regarded as synonymous with Pen. patulum) (Wilson, 1976). It has been described under various names depending on the fungus from which it was isolated, for example clavacin, clavatin, claviformin, expansin(e), leucopin, mycoin c, penicidin and tercinin. Culture filtrates of Pen. claviforme were first shown to possess antibacterial properties by Wilkins & Harris (1942) and the isolation of the active agent, referred to as claviformin, was reported by Chain, Florey & Jennings (1942). The synonymity of claviformin with patulin was established by Chain et al. (1944).

Bassett & Tanenbaum (1958) used spectrophotometric measurements, at a wavelength of 276nm in the case of patulin, to estimate the concentration of metabolites in cultures of Pen. patulum, though the method is only presumptive. In the present studies absorbance at 276nm in standard Czapek-Dox medium inoculated with Pen. claviforme was closely related to anti-Pythium activity (Fig. 4.14) suggesting that patulin could indeed be an important active component of the culture filtrate.

The patulin molecule contains an α,β-unsaturated 5-membered lactone ring and a 6-membered hemi-acetal ring (Fig. 4.17). Its biosynthesis by Pen. urticae is one of the best characterized fungal secondary metabolic pathways. The first intermediate is 6-methylsalicylic acid, formed from acetyl- and malonyl-CoA. The pathway then proceeds as indicated in Fig. 4.18. Ring cleavage of gentisaldehyde is catalysed by an oxygenase enzyme which requires iron (Bu'Lock, 1961; 1965) and Bassett & Tanenbaum

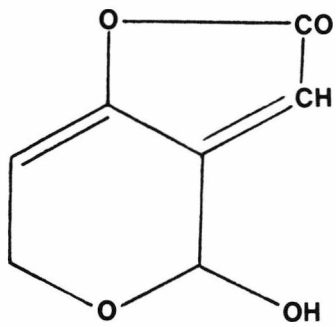


Fig. 4.17. The chemical structure of patulin (Woodward & Singh, 1949).

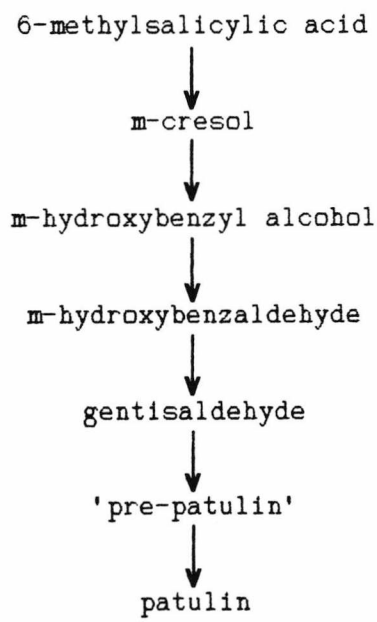


Fig. 4.18. The major biosynthetic pathway of patulin in Pen. urticae (Forrester & Gaucher, 1972).

(1958) found that reducing the content of ferrous sulphate in the growth medium of Pen. patulum to one tenth that in the standard medium caused a pronounced shift in the distribution of fungal metabolic products. The maximal yield of patulin decreased from 16.4mM in the standard medium to only a trace in the low iron medium whereas the concentration of phenols derived from gentisaldehyde increased from 4.8 to 6.2mM. The same modification in medium compostion also suppressed production of patulin (as determined by absorbance at 276nm) by Pen. claviforme in the current work (Fig. 4.14), but the effect was not as great as that observed by the earlier researchers. After 20d, yields were reduced by only 56.3%. It is possible that the medium became contaminated with extra iron during preparation, for example by the ingredients or glassware, but subsequent analysis by atomic absorption spectroscopy revealed that the DW at least was not an additional source of the metal. An alternative explanation is that the respective enzyme in Pen. claviforme IMI 44744 has a lesser requirement for iron than that of Pen. patulum.

The above authors also reported that adding calcium carbonate to the growth medium of three out of four strains of Pen. patulum reduced the maximum concentration of patulin by approx. two thirds, a phenomenon which they attributed to the trapping of aromatic acids and the open chain molecular species of patulin ('pre-patulin') as their calcium salts, rendering them unavailable to the appropriate biosynthetic enzymes. A similar effect was observed in the case of Pen. claviforme where inclusion of calcium carbonate in the medium decreased presumptive patulin production by 63.0% (Fig. 4.14).

In addition to their effect on absorbance at 276nm, a low concentration of iron and the presence of calcium carbonate also reduced

the production of P. ultimum-inhibitory agents measured directly by bioassay. However, these two factors did not have the same effect on the two parameters under investigation. Thus after 20d incubation in the low iron medium the yield of patulin was suppressed to a lesser extent that was that of total anti-Pythium compounds. By contrast, the addition of calcium carbonate had a slightly greater effect on patulin than on inhibitor production. These observations could signify that other components of PCF besides patulin influence its total P. ultimum-inhibitory activity, either in a positive or negative manner.

Thin-layer chromatography is a method for separating and purifying materials from a mixture based on their differing mobilities when carried through a stationary solid phase, in the form of a thin adsorbent layer, by a mobile solvent phase (Braithwaite & Smith, 1985). It has been widely used to detect and characterize fungal secondary metabolites (Gorst-Allman & Steyn, 1984), including patulin (Scott, 1974).

100% of the P. ultimum-inhibitory activity of PCF extractable from tlc plates was recovered from a location at Rf 0.42 (Table 4.3). The component at this position showed the same mobility and colour reactions as authentic patulin in two different solvent systems and using two different chemical locating reagents (section 4.6.1). These results indicated that not only was patulin present in the culture filtrate, but was also responsible for the major proportion of its anti-Pythium activity. The only other located component which caused significant inhibition was at a position (Rf 0.45) very close to the highly active agent and therefore difficult to separate from it. Resolution of

compounds showing similar mobility was improved by development in a second dimension in a different solvent.

Of the two chemical locating reagents tested, 3-methyl-2-benzothiazolinone hydrochloride (MBTH) was found to be more useful than anisaldehyde, giving a more clearly defined spot at the position of interest. MBTH reacts with patulin to give the phenylhydrazone derivative (Pohland & Allen, 1970a) which is intense yellow in colour. The detection limit is approx. 0.05µg patulin per spot (Scott & Kennedy, 1973). MBTH also located a greater number of additional constituents of PCF (Table 4.2) and treatment of chromatograms with ethanolic sulphuric acid, a general method for detecting organic substances, did not result in the appearance of any further spots. The identity of these low-activity compounds was not further investigated but other major end products of the patulin biosynthetic pathway, which usually accumulate to significant levels in the culture medium of producer fungi, are gentisyl alcohol and gentisic acid (Forrester & Gaucher, 1972). A single component of PCF at Rf 0.27 fluoresced under short wavelength uv light (Table 4.2). At the same wavelength both patulin and the Pythium-inhibitory agent present in PCF quenched the fluorescence of an indicator incorporated into the preparative layer chromatography plates used to prepare samples of the material for infrared spectroanalysis, but the band thus demarcated was broader than that obtained by spraying with MBTH, making it more difficult to remove the desired compound alone from the chromatograms.

The anti-Pythium constituents of PCF were soluble in ethyl acetate, shown by comparison of tlc plates after chromatography of either PCF concentrate or PCF extract (section 4.6.1.1). This was confirmed by

bioassay of the aqueous fraction obtained during the extraction process (section 2.5.1) which showed only 8.2% of the activity of the original PCF concentrate, compared with 88.4% in the ethyl acetate fraction after evaporating off the solvent and redissolving in distilled water. Ethyl acetate extraction was therefore useful to further concentrate and partially purify the culture filtrate. This solvent has been used by several authors to remove patulin from fungal cultures (Birkenshaw et al., 1943; Norstadt & McCalla, 1969a; Forrester & Gaucher, 1972; Land & Hult, 1987) and also from fruit juices, flour and grains (Scott & Somers, 1968).

The Rf values of patulin and the Pythium inhibitor in PCF in the present studies did not coincide with those reported previously for patulin. For example, the mean Rf obtained in toluene/ethyl acetate/formic acid was 0.42, compared with 0.30 recorded by Frisvad & Filtenborg (1983), 0.35 by Paterson (1986) and 0.32 by Land & Hult (1987). This is not unexpected due to the many variables which can affect the rate of migration of a given compound on tlc plates such as temperature, solvent purity and size of the chromatography tank. Thus, while Rf values can give a useful indication of the possible identity of a compound under rigourously standardized conditions, and specific staining reactions may supply further information, confirmation of identity requires spectroanalytical studies such as the determination of uv, i.r., mass or nuclear magnetic resonance spectra.

Absorption of electromagnetic radiation in the infrared region causes changes in vibrational energy sub-levels within a molecule and gives rise to a spectrum which is absolutely specific and hence permits conclusive identification of the compound (Whiffen, 1971).

Infrared spectrometer attachments are obtainable from commercial sources which allow the analysis of very small quantities of material. However, such apparatus was not available during the current work. The existing equipment was therefore modified to enable the examination of the agents under study. The infrared spectrum obtained for patulin (Fig. 4.16) was consistent with that recorded by the suppliers (Keller, 1986) showing that the attachment was functioning as intended. The spectrum of the Pythium-inhibitory component of PCF was coincident with that of patulin at some wavenumbers, but not at others, indicating that compounds besides patulin were present. These impurities are likely to have been metabolites located close to the Pythium inhibitor on the chromatography plate, such as that detected at Rf 0.45, or substances not visualized at all due to lack of reaction with, or existence at concentrations below the detection limit of, the locating reagents tested. Resolution cannot be enhanced by development in a second dimension in preparative layer chromatography where the larger amounts of sample are applied across the whole width of the plate, but improvement may have been achieved by removing the bioactive band and rechromatographing on a fresh plate. However this possibility was not investigated because of a shortage of costly materials. Thus infrared spectroscopy confirmed that patulin was a major active component of PCF but, as in the experiments described in sections 4.3, 4.5 and 4.6, did not exclude the potential involvement of additional compounds of lesser potency or present in smaller quantities, but with similar Rf values.

In addition to patulin, the metabolites palitantin, roquefortine C, terrein and terrestric acid have been recorded in culture filtrates of Pen. claviforme IMI 44744 (R.R.M. Paterson, C.A.B. International

Mycological Institute; personal communication). Of these products only terrestric acid showed similar mobility to patulin in a toluene/ethyl acetate/ formic acid solvent system, with Rf values of 0.32 and 0.35, respectively (Paterson, 1986). However, in the present research no PCF components were detected at a position just below that corresponding to patulin, using either the anisaldehyde locating reagent employed by the above author, or with MBTH (Table 4.2). Therefore terrestric acid was present in PCF at only low concentrations, if at all. Nevertheless, in the absence of an authentic source of the compound to use as a standard on tlc plates, or to test for anti-Pythium effects, a minor contribution to the P. ultimum-inhibitory activity of the culture filtrate could not be discounted.

The physical, chemical and biological properties of patulin have been reviewed on a number of occasions (Abraham & Florey, 1949; Singh, 1967; Ciegler, Detroy & Lillehoj, 1971; Scott, 1974; Wilson, 1976; Mirocha, Pathre & Christensen, 1979; Engel & Teuber, 1984). The metabolite is soluble in water and several organic solvents, including ethyl acetate and chloroform used in the handling of PCF during the course of the current investigations (section 2.5.1). It is resistant to thermal destruction and steam-sterilized fungal culture filtrates were used in early examinations of its antibiotic spectrum (Anslow et al., 1943). Likewise, the anti-Pythium properties of PCF were not diminished even when it was autoclaved at 121°C for 15min (section 4.2.2). Patulin is stable under acidic, but not under alkaline conditions. Similarly, PCF retained the capacity to inhibit P. ultimum for up to 14d when stored in buffer at pH 5.5 or 6.4, but gradually lost activity at pH 7.4 and more rapidly at pH 8.1 (section 4.2.5).

Patulin is active against both Gram-positive and Gram-negative bacteria, and inhibits the growth of a number of fungal species, including plant pathogens (see also sections 5.6.2 and 5.6.4). In addition, it has adverse effects on some plants (see also section 5.6.1). Most studies on its acute toxicity to animals have been conducted with mice, in which the LD_{50} by oral administration is 35mg kg^{-1} (Broom et al., 1944). The compound has been classified as a mycotoxin, but the incriminating evidence is indirect (Scott, 1974; Wilson, 1976). Patulin itself has not been identified in toxic feed nor have the effects of feeding it to large farm animals been assessed. Patulin caused the production of malignant tumours at the site of subcutaneous injection in rats (Dickens & Jones, 1961), but no signs of carcinogenicity were observed following oral administration at sublethal doses (Enomoto & Saito, 1972). The antibiotic also induced the formation of petite mutants of Saccharomyces cerevisiae (Mayer & Legator, 1969), and damaged liver, kidney, lung and HeLa cells in culture (Umeda, 1971). Patulin caused vomiting in humans when given orally as tablets, but intravenous perfusion was apparently well tolerated and had beneficial effects against certain infectious diseases (Rosnay, Martin-Dupont & Jensen, 1952). It was also of some value in the treatment of the common cold (Hopkins, 1943).

The precise mode of action of patulin has not yet been confirmed (Wilson, 1976). It inactivates a number of enzymes containing sulphydryl groups, inhibits aerobic respiration at a step prior to the terminal electron transport chain and increases cell membrane permeability. It also inhibits DNA, RNA and protein synthesis in dividing cells, but which of these are the primary effects is uncertain.

In summary, patulin should be considered as a compound which, in addition to its toxicity to many microorganisms, and some plants and animals, may also be harmful to man. However, further studies are required to assess the hazard it presents at concentrations likely to occur in the environment if it were to be implemented in crop protection.

CHAPTER 5

EFFICACY OF PCF AS A SEED PELLET FUNGICIDE

I. EFFECTS ON HOST, PATHOGEN AND HOST-PATHOGEN INTERACTION

A number of criteria are involved in determining the potential utility of a given agent, whether chemical or biological, in crop protection (section 1.2.2). For convenience, the evaluation of Penicillium claviforme culture filtrate (PCF) in relation to these requirements is described and discussed under the following headings:

1) direct effects of PCF on the plant host, the pathogen and the disease resulting from the interaction between the two following introduction into a sugar beet-Pythium ultimum pathosystem in seed pellets (this chapter),

2) additional properties of PCF which could influence its performance (Chapter 6).

However in practice, all these features are closely interrelated.

PCF extract was prepared as given in section 2.5.1. The minimum rate of incorporation into seed pellets was calculated to be 0.10IU pellet^{-1} (Appendix).

5.1 Effects of PCF on sugar beet seedling development

In order to assess any detrimental effects of PCF on sugar beet seedling development in the proposed dosage range, seed pellets containing 0.05, 0.10, 0.30 or 0.50IU pellet^{-1} were prepared by the laboratory method described in section 2.5.2. Percentage germination was determined after 3 and 7d incubation on filter paper as described in section 2.6.2 and compared with that of control pellets spotted with DW. Each treatment consisted of three replicates of 20 pellets. Results are shown in Fig. 5.1.

In a separate experiment, percentage emergence in peat-based compost was evaluated after 4 and 7d under the conditions specified in section 2.6.2. Each treatment comprised 20 replicate wedge cells, each containing four pellets. Results are given in Fig. 5.2.

Germination of control pellets on filter paper began after 1-2d of incubation and reached a value of 91.7% at 7d (Fig. 5.1). Few pellets in any treatment germinated after 7d. At a rate of 0.50IU pellet^{-1}, PCF delayed the onset of germination and reduced the number of developing seedlings at 7d by 43.8% compared to the controls. The treatment also caused marked stunting and curling of seedling radicles, and in some cases necrosis of the root tips. Differentiation of plumule tissues did not appear to be affected. At a rate of 0.30IU pellet^{-1}, PCF reduced germination by 67.3% after 3d incubation and again caused abnormal seedling morphology, but these effects were temporary and after 7d both % germination and appearance were similar to those of controls. Application of 0.30IU equivalents pellet^{-1} patulin (15μg, section 4.6.2) resulted in phytotoxic symptoms closely resembling those

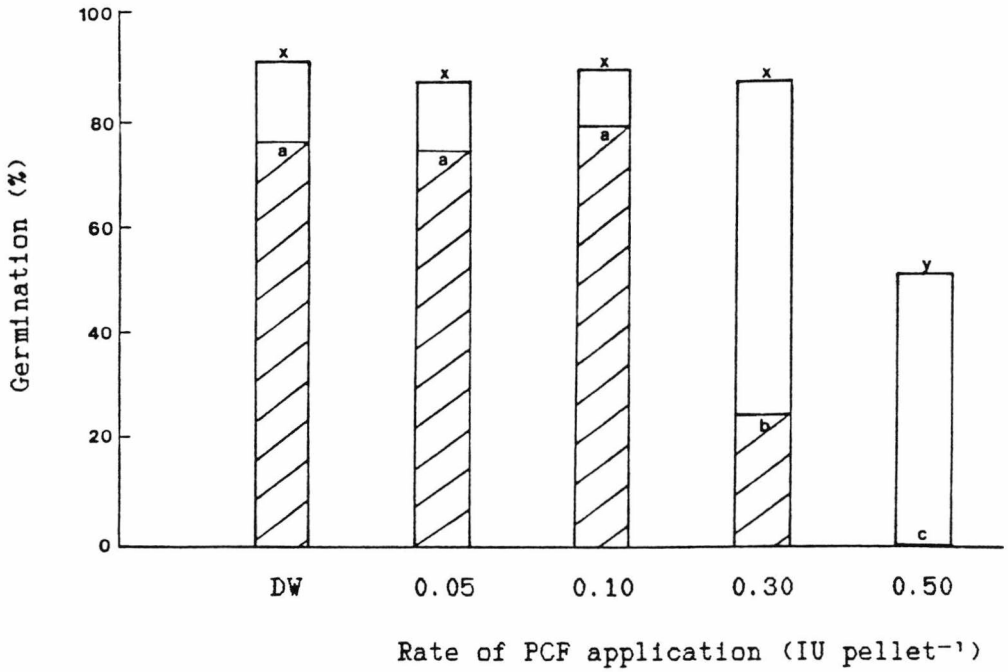


Fig. 5.1. Germination on filter paper of sugar beet seed pellets treated with distilled water (DW) or Pen. claviforme culture filtrate extract (PCF) after 3d (▨) or 7d (□) incubation. DW or PCF were applied individually to pellets without additives. For any one assessment time bars headed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

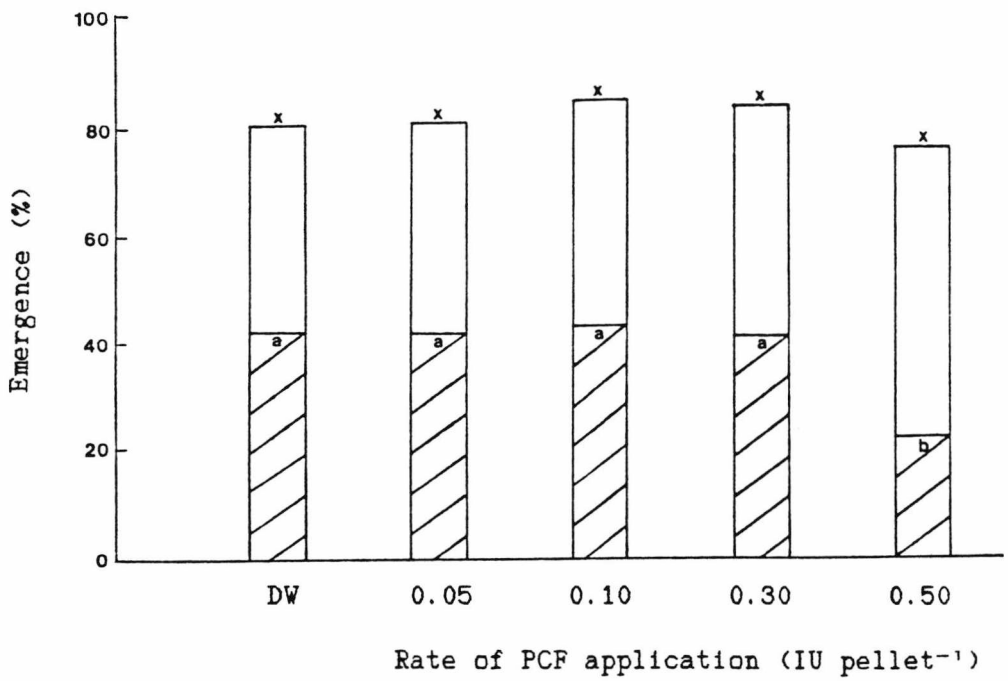


Fig. 5.2. Emergence in peat-based compost of sugar beet seed pellets treated with distilled water (DW) or Pen. claviforme culture filtrate extract (PCF) after 4d (▨) or 7d (□) incubation. DW or PCF were applied individually to pellets without additives. For any one assessment time bars headed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

described above for 0.30IU pellet^{-1} PCF. At dosages of 0.10 or 0.05IU pellet^{-1} PCF, no adverse effects were observed. Indeed, germination of pellets containing 0.10IU pellet^{-1} was slightly greater (by 4.6%) than that of controls after 3d incubation, but differences were not significant.

Emergence of control pellets in peat-based compost commenced after 2-3d incubation and reached a value of 80.6% at 7d (Fig. 5.2). The number of pellets emerging after this time in any treatment was low. At a rate of 0.50IU pellet^{-1}, PCF decreased seedling emergence by 47.3% compared to controls after 4d incubation, but both shoot and root systems were normal in appearance and after 7d % emergence was not significantly different to that of controls. The incorporation of PCF at doses of 0.30IU pellet^{-1} and, to a greater but still non-significant extent, at 0.10IU pellet^{-1}, stimulated emergence (by 4.2 and 5.8%) after 7d incubation. In no case were any morphological irregularities observed.

It was decided to aim for approx. 0.30IU PCF in pellets to be produced commercially as this dose had no long-term deleterious effects on seedling development but was three times the minimum value calculated as necessary to inhibit Pythium ultimum in compost (Appendix) and thus allowed for any loss of activity during the pelleting process or in the seedling growth medium after planting. In practice, the concentration of the PCF extract incorporated into the pellets was such that the target dose was 0.33IU seed^{-1} (Appendix) and the achieved dose was 0.30IU seed^{-1} (section 6.1).

Percentage germination and emergence of commercially prepared PCF-pellets (section 2.5.2) was assessed as before, in conjunction with

evaluation of their susceptibility to P. ultimum attack (section 5.2, trial 1). In germination and emergence tests treatments consisted of three replicate Petri dishes each containing 20 pellets, and 40 replicate wedge cells each containing four pellets, respectively. Results are presented in Fig. 5.3. Similar effects were observed when the experiment was repeated 28d later (section 5.2, trial 2).

The consequences of commercial incorporation of 0.30IU seed^{-1} PCF corresponded to those arising from treatment with the same dose of the extract in the laboratory. Germination on filter paper was reduced compared to controls during the early stages of incubation (for example by 73.6% after 3d) and seedling radicles were stunted and deformed (Plate 5.1). However these effects became less pronounced as incubation proceeded and after 7d both % germination and seedling morphology matched those of controls.

Emergence of commercially prepared PCF-pellets in compost was 5.5% greater than that of controls at 7d, but again differences were not significant. No seedlings showed any morphological symptoms of phytotoxicity.

5.2 Control of P. ultimum-induced disease by PCF treatment

Experiments were conducted to determine a suitable Pythium ultimum inoculum density for use in tests of the efficacy of PCF as a seed pellet fungicide. Sugar beet seed pellets without additives were planted in seedling wedge cells containing non-infested or P. ultimum-infested peat-based compost as described in section 2.6.2.

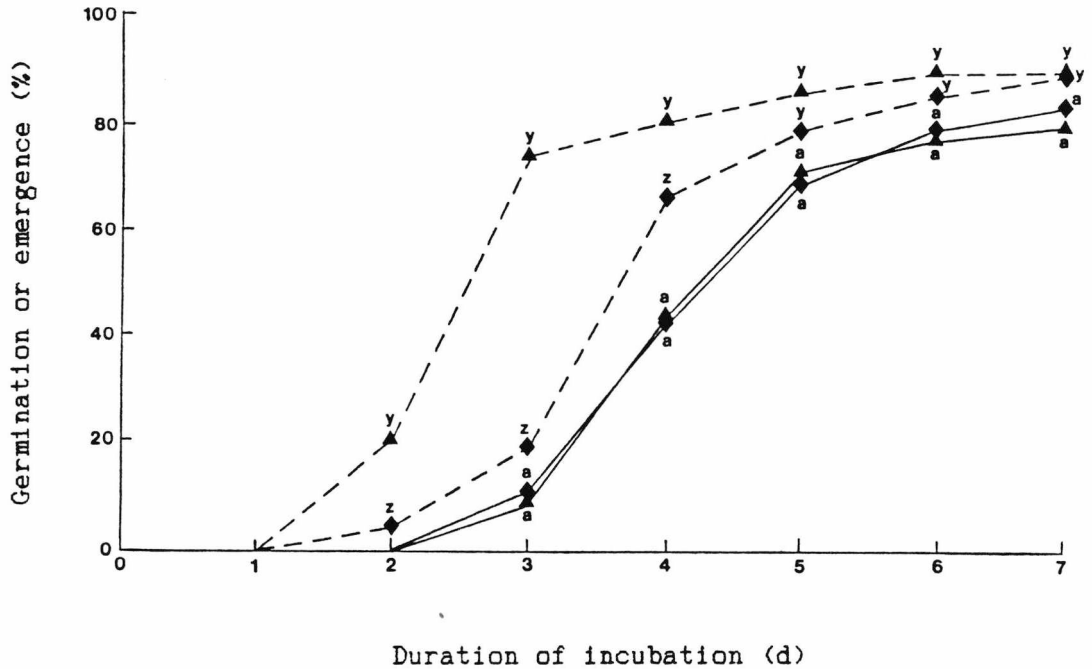
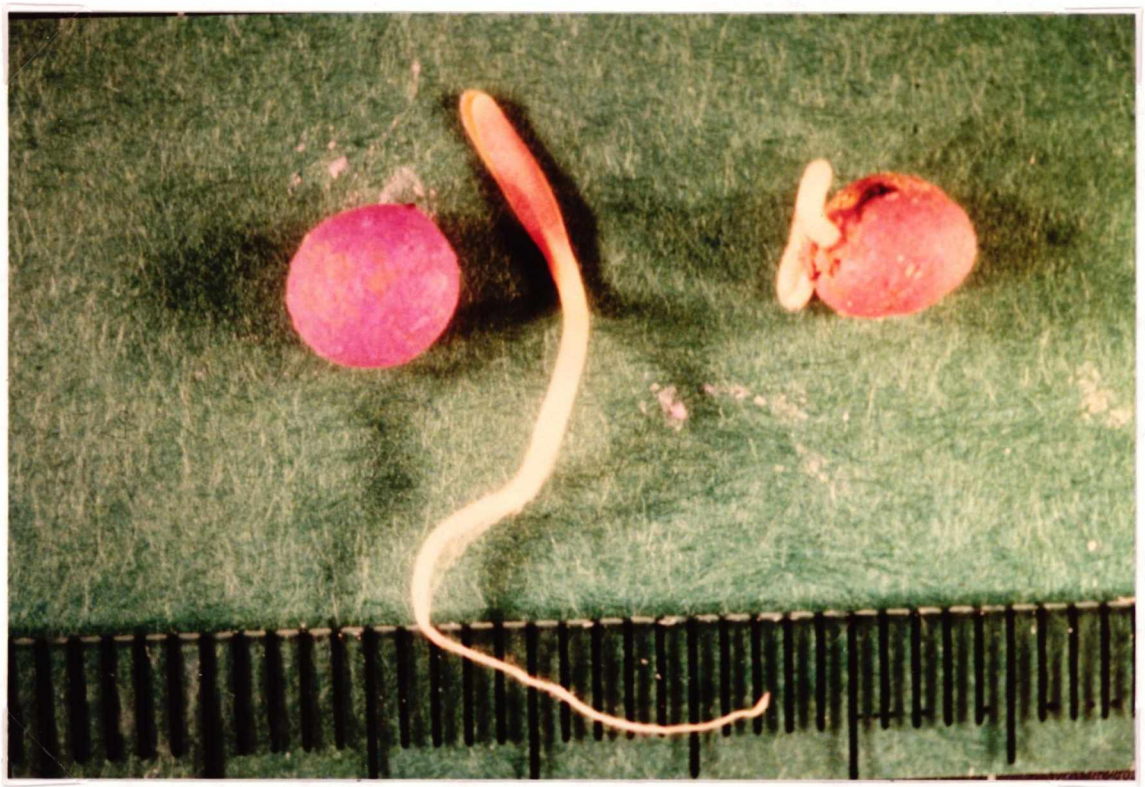


Fig. 5.3. Germination on filter paper (— — —) and emergence in peat-based compost (——) of sugar beet seeds commercially pelleted without additives (▲) or with 0.30IU seed^{-1} Pen. claviforme culture filtrate extract (◆). For any one data set and assessment time values marked with the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).



(a) (b)

Plate 5.1. Sugar beet (Beta vulgaris) seedlings developing from seeds commercially pelleted (a) without additives and (b) with 0.30IU Pen. claviforme culture filtrate extract after 4d incubation on filter paper. One division on scale = 1mm.

The water content of the compost was found in preliminary tests to be a critical factor in determining disease development. Addition of an inoculum of up to 5.0 $x10^5$ propagules wedge $cell^{-1}$ in 5ml TW resulted in only a low incidence of infection whereas application of 10ml liquid $cell^{-1}$ greatly reduced seedling emergence even in control treatments. An intermediate volume of 8ml liquid $cell^{-1}$ was selected for use in all subsequent experiments, which gave a compost water content of approx. 75.0% container capacity (section 2.6.2).

Planted wedge cells were infested with P. ultimum at inoculum densities of 3.0 $x10^3$, 3.0 $x10^4$ or 3.0 $x10^5$ (experiment 1) or 5.1 $x10^5$ or 5.1 $x10^6$ (experiment 2) propagules $cell^{-1}$. Each treatment consisted of 20 replicate wedge cells containing four pellets. Percentage emergence and establishment were assessed after 7d incubation under the conditions specified in section 2.6.2. Emergence refers to the total number of seedlings which appeared above the compost surface while the term establishment is used here to denote the number of seedlings which both emerged and survived to day 7 after planting. Results are given in Fig. 5.4. The incidence of P. ultimum-induced pre-emergence disease was calculated from the difference in seedling emergence in non-infested and infested compost divided by that in non-infested compost. The incidence of post-emergence damping-off was determined from the difference in emergence and establishment in infested compost divided by emergence in infested compost.

Percentage seedling establishment in non-infested compost (81.3 and 78.8% in experiments 1 and 2) was equal to % emergence, i.e. no post-emergence disease occurred.

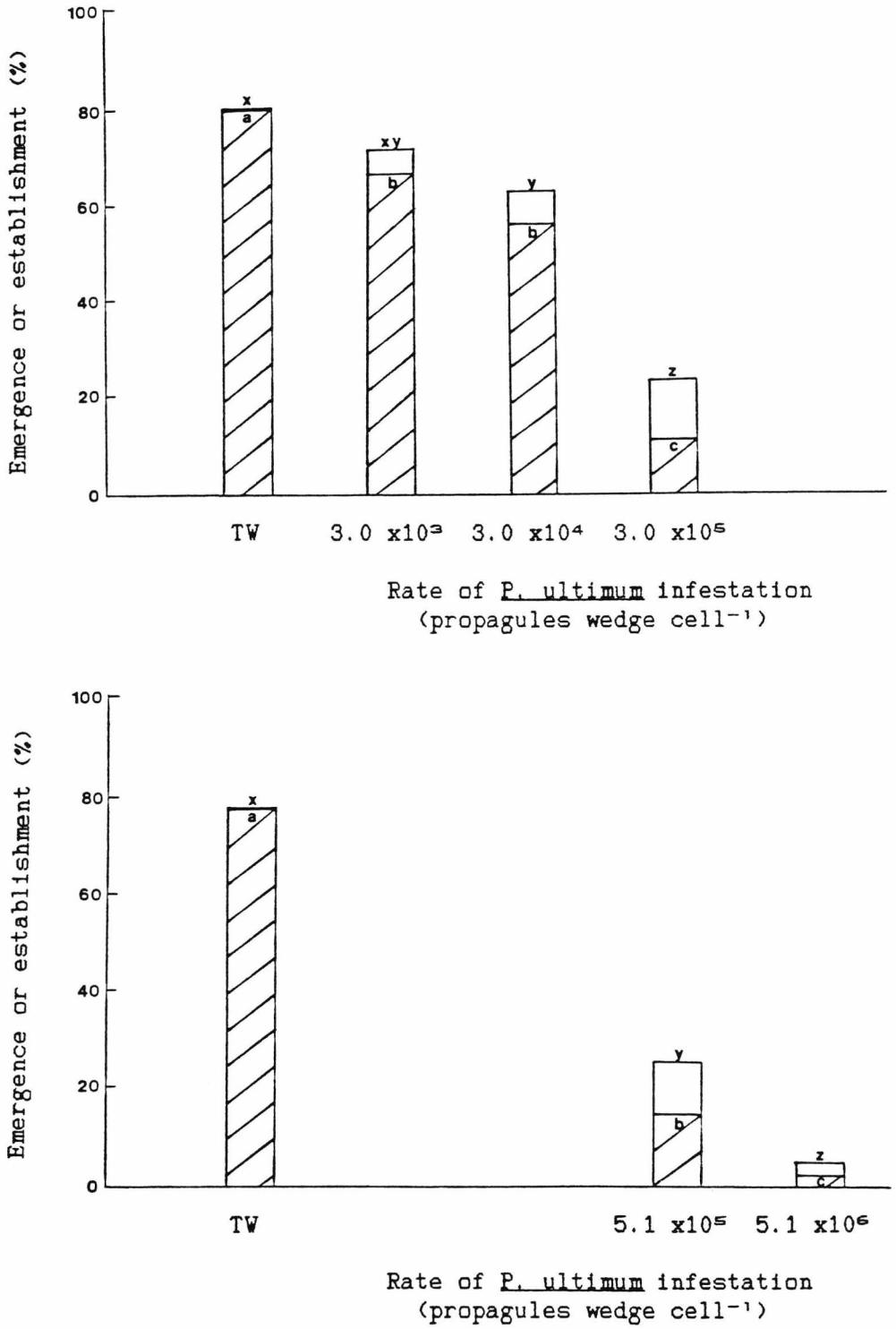


Fig. 5.4. Emergence ( ) and establishment ( ) of sugar beet seedlings developing from pellets without additives after 7d incubation in non-infested or *P. ultimum*-infested peat-based compost in seedling wedge cells. Tap water (TW) or *P. ultimum* propagule suspension were applied to the compost surface immediately after planting. Data are expressed as a percentage of pellets planted. For any one data set bars headed by the same letter are not significantly different according to Duncan's new multiple range test ($P=0.05$).

Percentage establishment in Pythium-infested compost was closely related to the inoculum density of the pathogen, ranging from 67.5% at 3.0 x10^{3} propagules wedge $cell^{-1}$ to 2.5% at 5.1 x10^{6} propagules $cell^{-1}$. The greater seedling losses at higher infestation rates were due to increases in both pre-emergence (from 10.8 to 93.6%) and post-emergence (from 7.4 to 50.0%) infection. Where post-emergence damping-off occurred, seedlings were attacked at the base of the hypocotyl which became constricted and discoloured. Young plants then collapsed without development of any other symptoms. In some cases extensive mycelial growth subsequently took place over host tissues lying on the compost surface. No seedlings were killed after 7d of incubation.

In order to evaluate the performance of PCF commercially incorporated into sugar beet seed pellets in protection against P. ultimum-induced disease, control pellets, PCF-pellets or Hm-pellets (section 2.5.2) were planted in seedling wedge cells containing non-infested or P. ultimum-infested compost as described in section 2.6.2. One trial was initiated 7d after pellet production, and a second 28d later. Infested media received an inoculum of 3.6 x10^{5} (trial 1) or 3.9 x10^{5} (trial 2) propagules $cell^{-1}$. The six treatments were arranged in six randomized blocks and each consisted of 40 replicate wedge cells containing four pellets. Wedge cells were incubated under the conditions detailed in section 2.6.2. Percentage emergence and establishment were assessed 7d after planting. Results obtained for trials 1 and 2 are recorded in Tables 5.1 and 5.2, and expressed in terms of disease incidence in Table 5.3. A non-randomized set of treatments planted during trial 1 (for photographic purposes only) is illustrated in Plate 5.2.

Table 5.1. Emergence and establishment of sugar beet seedlings developing from pellets without additives or containing Pen. claviforme culture filtrate extract (PCF) or hymexazol (Hm) after 7d incubation in non-infested or P. ultimum-infested peat-based compost in seedling wedge cells (trial 1)

Compost treatment[a]	Pellet additive	Emergence (%)[b]	Establishment (%)[b]
Non-infested	None	80.0 x	80.0 x
	PCF	84.4 x	84.4 x
	Hm	85.0 x	85.0 x
Infested	None	30.6 x	13.1 x
	PCF	58.1 y	45.0 y
	Hm	80.6 z	78.8 z

[a] Tap water or P. ultimum propagule suspension at a rate of 3.6 $x10^5$ propagules wedge $cell^{-1}$ were applied to the compost surface immediately after planting.

[b] Data is expressed as a percentage of pellets planted. For each column and compost treatment values followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

Table 5.2. Emergence and establishment of sugar beet seedlings developing from pellets without additives or containing Pen. claviforme culture filtrate extract (PCF) or hymexazol (Hm) after 7d incubation in non-infested or P. ultimum-infested peat-based compost in seedling wedge cells (trial 2)

Compost treatment[a]	Pellet additive	Emergence (%)[b]	Establishment (%)[b]
Non-infested	None	78.1 x	78.1 x
	PCF	80.0 x	80.0 x
	Hm	81.3 x	81.3 x
Infested	None	45.0 x	10.6 x
	PCF	72.5 y	39.4 y
	Hm	78.8 y	75.6 z

[a] Tap water or P. ultimum propagule suspension at a rate of 3.9 $x10^{5}$ propagules wedge $cell^{-1}$ were applied to the compost surface immediately after planting.

[b] Data is expressed as a percentage of pellets planted. For each column and compost treatment values followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

Table 5.3. *P. ultimum*-induced disease of sugar beet seedlings developing from pellets without additives or containing *Pen. claviforme* culture filtrate extact (PCF) or hymexazol (Hm) after 7d incubation in infested peat-based compost

Trial	Pellet additive	Pre-emergence infection (%)*	Post-emergence damping-off (%)*
1	None	61.7	57.1
	PCF	31.1	22.6
	Hm	5.2	2.3
2	None	42.4	76.4
	PCF	9.4	45.7
	Hm	3.1	4.0

* Values were calculated from the data given in Tables 5.1 and 5.2 as described in section 5.2.

Plate 5.2. *P. ultimum*-induced disease of sugar beet (*Beta vulgaris*) seedlings developing from seeds commercially pelleted without additives or with *Pen. claviforme* culture filtrate extract (PCF) or hymexazol (Hm) after 7d incubation in infested peat-based compost.

In both trials the inclusion of either PCF or Hm in seed pellets resulted in greater seedling emergence in non-infested compost compared to control pellets without additives (the former by 5.5 and 2.4%, and the latter by 6.3 and 4.1%), but all differences were non-significant (Tables 5.1 and 5.2). All emerged seedlings became established and were normal in appearance throughout the experiments.

In trial 1, the incorporation of PCF significantly increased both emergence (from 30.6 to 58.1%) and establishment (from 13.1 to 45.0%) in P. ultimum-infested compost compared to the controls (Table 5.1). However the culture filtrate extract was significantly less efficacious than Hm, the addition of which resulted in 80.6 and 78.8% emergence and establishment, respectively. Expressed in terms of disease incidence, PCF reduced pre-emergence and post-emergence Pythium attack by 49.6 and 60.4% whilst the comparable figures for Hm were 91.6 and 95.6% (Table 5.3).

In trial 2, pre-emergence P. ultimum infection of control seedlings was milder than in trial 1 (Table 5.3) and under these less stringent conditions PCF improved emergence (from 45.0 to 72.5%) as effectively as Hm (78.8%, Table 5.2). However post-emergence damping-off was more severe in the second trial than in the first and the increase in seedling establishment given by PCF treatment (from 10.6 to 39.4%) was again less than that achieved by Hm (75.6%). In terms of disease control, PCF decreased pre-emergence losses by 77.8% compared to 92.7% for Hm, and suppressed the post-emergence phase by 40.2% compared to 94.8% for the synthetic fungicide.

At the end of the two experiments PCF and Hm gave established plant stands 3.4-3.7 and 6.0-7.1 times those of controls, respectively (Tables 5.1 and 5.2).

5.3 Effects of PCF on P. ultimum population in the spermosphere

Preliminary tests were performed to determine the % recovery of Pythium ultimum from infested compost, and thus the dilutions of the medium necessary in subsequent investigations into the effects of PCF on the pathogen population in sugar beet seed pellet spermospheres. A suspension of P. ultimum propagules was prepared (section 2.1.1) and its concentration determined by both direct microscopic counts and by surface dilution plating onto Schmitthenner's Pythium-selective medium (SM, section 2.2). The suspension was applied to the surface of peat-based compost in seedling wedge cells planted with seed pellets without additives. After 1d incubation under the conditions given in section 2.6.2 Pythium densities in compost samples removed from the surface layers or from the volume surrounding seed pellets was estimated using the procedure outlined in section 2.6.5. The experiment was replicated three times and each dilution was plated in triplicate. Results are given in Table 5.4.

Only 22.7% of the total propagules in the inoculum suspension germinated on SM. A similar figure was obtained when the test was repeated using CMA. Of the germinable propagules applied to the compost, 71.8% were recovered from the surface, and 23.3% from the spermosphere of seed pellets, 1d after infestation.

Table 5.4. Recovery of *P. ultimum* from peat-based compost planted with sugar beet seed pellets without additives

Sampling location	Recovery of *P. ultimum*[a]	
	% applied propagules[b]	% germinable propagules[c]
Surface	16.3 (3.8)	71.6 (16.8)
Spermosphere	5.3 (3.6)	23.4 (15.7)

[a] An inoculum suspension was applied to the compost surface immediately after planting and propagules recovered 1d later by surface dilution plating onto Schmitthenner's medium. SE are given in parentheses.

[b] Total applied propagules were determined by direct microscopic counts on the inoculum suspension.

[c] Germinable propagules were determined by plating the inoculum suspension onto Schmitthenner's medium and constituted 22.7% of the total applied.

A study was made of the population dynamics of P. ultimum in the presence and absence of PCF. Wedge cells containing peat-based compost were left unplanted, or planted with control pellets or pellets containing 0.30IU pellet^{-1} PCF prepared by the laboratory method (section 2.5.2). The compost was immediately infested with the pathogen at an inoculum density of 3.2 x10^{5} propagules cell^{-1} and the containers incubated as specified in section 2.6.2. At intervals over the following 10d period the Pythium population was estimated in 5g samples of compost removed from the wedge cells as described in section 2.6.5. Each treatment was replicated three times and each dilution was plated in triplicate. Results are presented in Fig. 5.5.

In unplanted compost the Pythium population slowly declined from 5.3 x10^{3} cfu g^{-1} dry wt. compost 1d after infestation to 2.4 x10^{3} cfu g^{-1} dry wt. by day 10. In the spermosphere of control pellets without additives numbers of recoverable propagules remained in the range 5.0-5.8 cfu g^{-1} dry wt. from day 1 to day 5 and then started to increase, reaching a maximum value of 19.8 x10^{3} cfu g^{-1} dry wt. at 9d after infestation. In the spermosphere of PCF-pellets population densities were stable (4.5-5.8 x10^{3} cfu g^{-1} dry wt.) until day 8, but showed a small rise to 7.7 x10^{3} cfu g^{-1} dry wt. at the end of the experiment. Values were significantly lower than those in the spermosphere of control pellets at all sampling times after 6d, and significantly greater than in unplanted compost only at 10d.

Numbers of P. ultimum colonies on SM arising from propagules recovered from unplanted compost or from the spermosphere of control pellets reached a maximum after approx. 30h of incubation. In

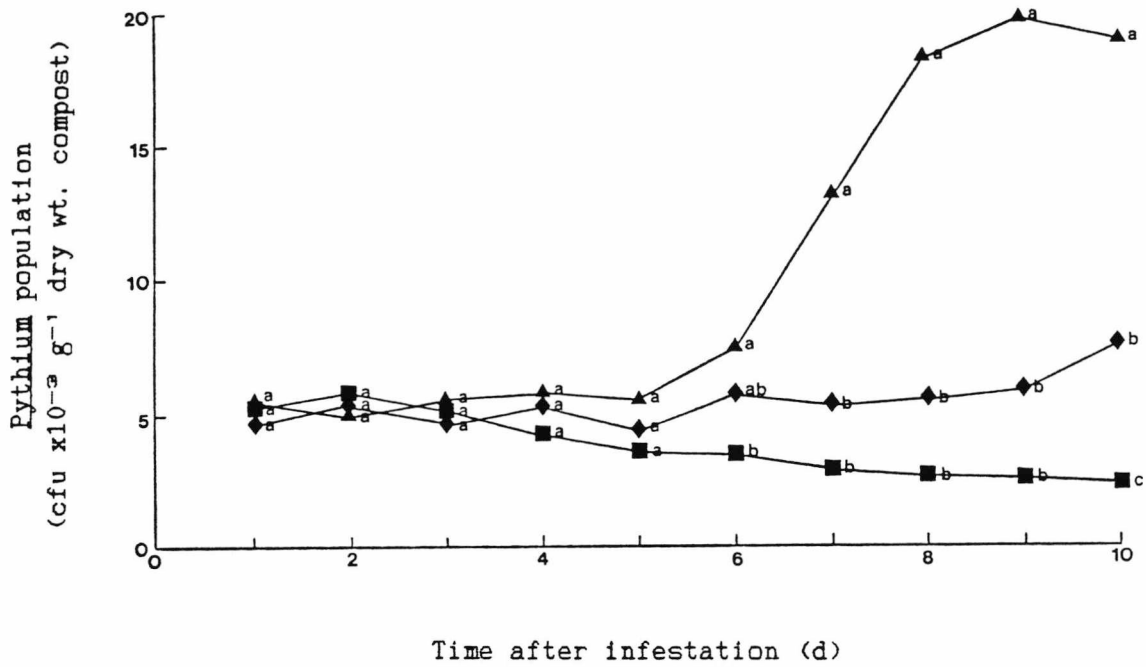


Fig. 5.5. Pythium population dynamics in infested peat-based compost either unplanted (■) or planted with sugar beet seed pellets treated with distilled water (▲) or 0.30IU pellet^{-1} Pen. claviforme culture filtrate extract (◆). An inoculum suspension was applied to the surface of the compost immediately after planting and propagules recovered by surface dilution plating onto Schmitthenner's medium. For any one assessment time values marked with the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

comparison, Pythium counts from the spermosphere of PCF-pellets continued to increase until approx. 48h.

No Pythium spp. were detected in non-infested compost, in the presence or absence of seed pellets, at dilutions as low as 10^{-1}.

5.4 Activity of antagonist culture filtrates against other seedling pathogens

The effects of PCF and Trichoderma sp. 1 culture filtrate (TSF) on the in vitro mycelial growth of Pythium ultimum was compared with their activity against a range of other fungi capable of causing seedling disease.

Bioassay plates each containing 1.0IU PCF or TSF were prepared (section 2.3.2) using MEA as the test medium. Plates were inoculated with a 5mm diam. core cut from the margins of a 2d culture of the phytopathogens grown on CMA (Oomycetes) or MEA (higher fungi). Details of the organisms tested are recorded in Table 2.1. Percentage inhibition was determined after 24h incubation at 25°C as described in section 2.3.2. Results are given in Figs. 5.6 and 5.8. In a separate experiment the relative activity of PCF against the Oomycete fungi was assessed by bioassay on CMA containing 0.5IU $plate^{-1}$ of the culture filtrate as shown in Fig. 5.7. Aphanomyces cochlioides was tested in comparison with P. ultimum at a later date. Because of the large differences in growth rates of these two organisms they were cultured in 140mm diam. Petri dishes and measurements taken after 48h incubation. All tests were performed in triplicate.

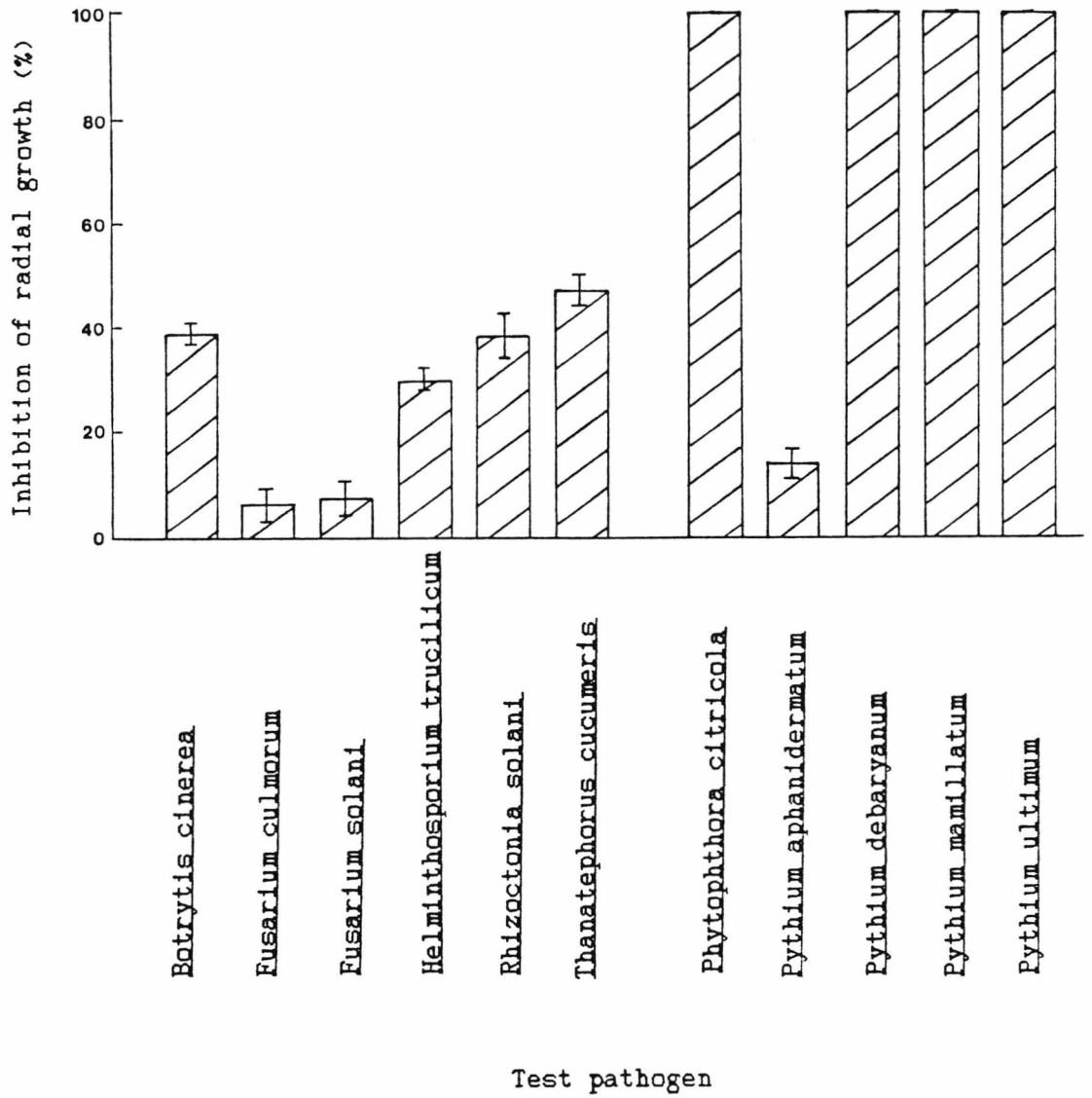


Fig. 5.6. *In vitro* activity of *Pen. claviforme* culture filtrate against seedling pathogens. Test fungi were inoculated onto malt extract agar containing 1.0IU plate^{-1} culture filtrate and % inhibition determined after 24h incubation at 25°C. Bars represent +/- SE.

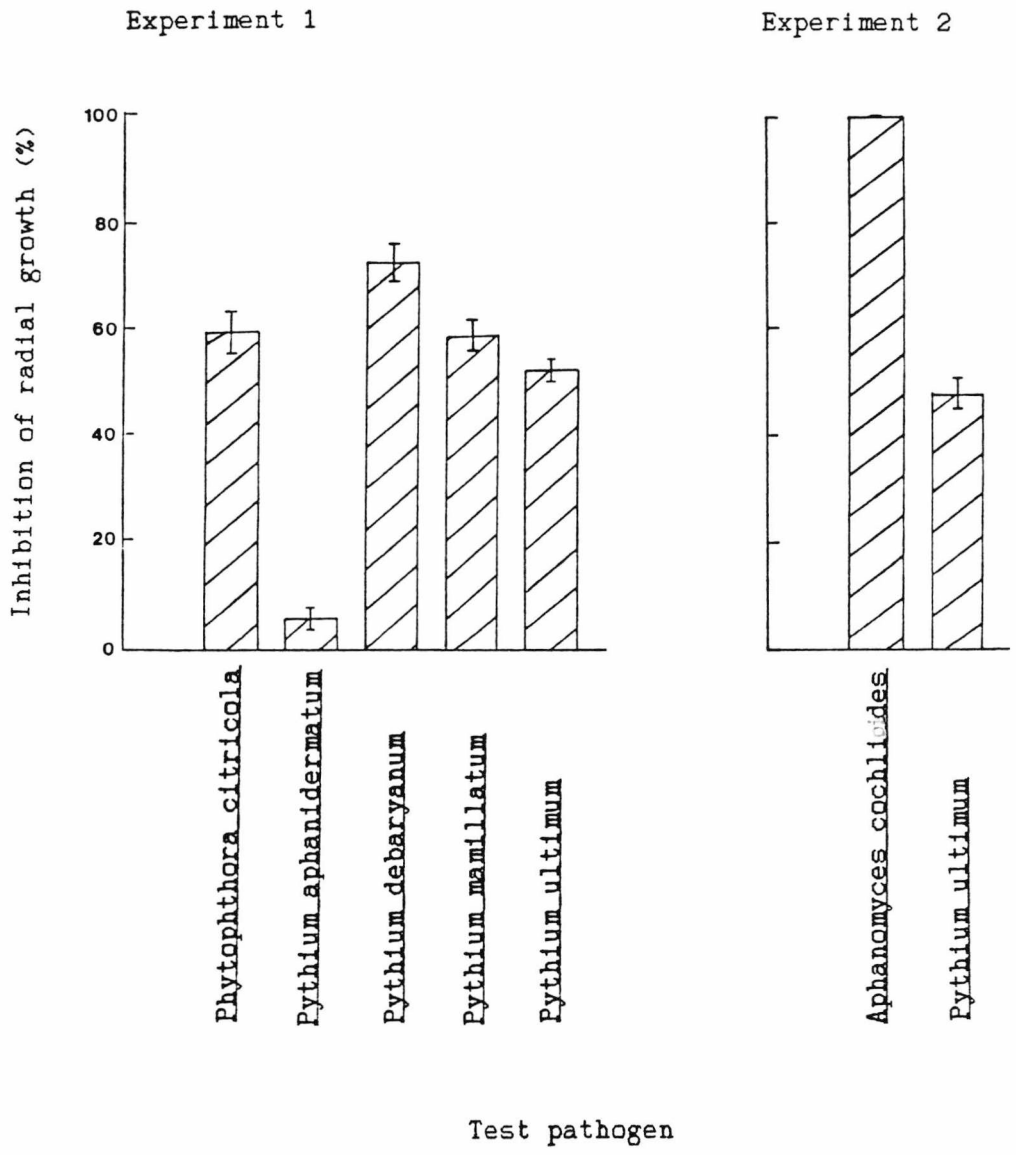


Fig. 5.7. *In vitro* activity of *Pen. claviforme* culture filtrate against Oomycete seedling pathogens. Test fungi were inoculated onto corn meal agar containing 0.5IU plate^{-1} culture filtrate and % inhibition determined after 24h (experiment 1) or 48h (experiment 2) incubation at 25°C. Bars represent +/- SE.

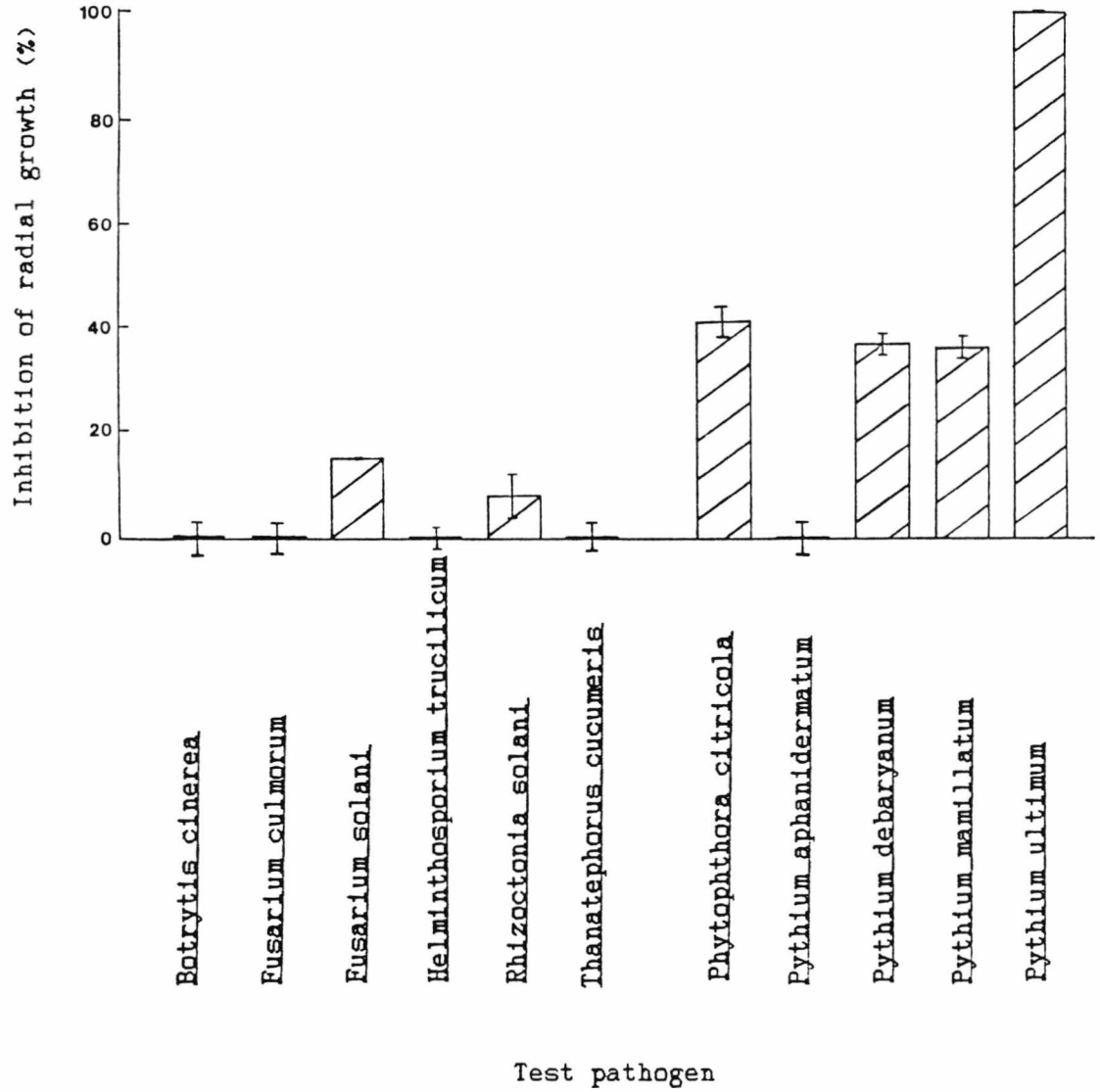


Fig. 5.8. *In vitro* activity of *Trichoderma* sp. 1 culture filtrate against seedling pathogens. Test fungi were inoculated onto malt extract agar containing 1.0IU plate^{-1} culture filtrate and % inhibition determined after 24h incubation at 25°C. Bars represent +/- SE.

PCF inhibited the mycelial growth of a number of seedling pathogens in addition to P. ultimum (Fig. 5.6). In general the higher fungi were less sensitive than the Oomycetes. Of the higher fungi tested, the two Fusarium spp. were the least susceptible, showing only 6.7-7.7% inhibition after 24h growth on MEA containing 1.0IU plate^{-1} of the culture filtrate, while the other organisms were suppressed by 30.0-47.7%. Of the Oomycetes examined during the second experiment (Fig. 5.7), Phytophthora citricola and Pythium mamillatum were inhibited by 0.5IU plate^{-1} PCF in CMA to a similar degree as was P. ultimum (59.6 and 58.4% compared to 52.1%). The growth of Pythium debaryanum was retarded to a greater extent (72.7%), but Pythium aphanidermatum was much less sensitive, showing only 5.8% inhibition under the same conditions. A. cochlioides, a member of the Order Saprolegniales, was found to be more than twice as susceptible as P. ultimum (Peronosporales).

The antifungal spectrum of TSF was narrower than that of PCF (Fig. 5.8). Again the higher fungi tested were less sensitive than the Oomycetes, only Rhizoctonia solani and Fusarium solani responding and even these showing a low degree of inhibition (8.1 and 15.4%). The latter was the only pathogen against which TSF was more active than PCF. Amongst the Oomycetes, P. mamillatum, P. debaryanum and Phy. citricola were suppressed to a lesser extent than P. ultimum (36.8-41.4% compared to 100%) and the culture filtrate had no effect on the growth of P. aphanidermatum.

5.5 Potential of PCF to induce resistance in P. ultimum

An attempt was made to generate PCF-resistant strains of Pythium ultimum using the method of mycelial adaptation (Davidse, 1981). Bioassay dishes containing 0.9IU of the culture filtrate were prepared (section 2.3.2) onto which mycelium of the pathogen was successively transferred at twice weekly intervals. For convenience, this culture is referred to as PCF^{R}, though the use of the superscript R (for resistant) was speculative. Concurrently, a second P. ultimum culture, designated PCF^{S} (S for sensitive), was successively transferred onto CMA containing CDM. Each week, strain PCF^{S} was also freshly transferred onto the PCF-containing medium to confirm its retention of inhibitory activity. After 4wk the growth rates of the two strains were measured on CMA containing either CDM (CDM-CMA) or 0.5IU $plate^{-1}$ PCF (PCF-CMA). Both were then successively transferred onto CDM-CMA for a further 4wk, after which their growth rates were reassessed as above. Each growth rate determination was performed in triplicate. Results are recorded in Table 5.5. Analagous experiments with hymexazol were unproductive due to mycelial lysis when incubation was prolonged beyond 24h even at concentrations as low as 0.06IU per bioassay dish.

After 4wk culture on PCF-containing CMA the growth rate of P. ultimum PCF^{R} on PCF-CMA was significantly greater than that of strain PCF^{S} previously grown in the absence of the culture filtrate. On CDM-CMA the growth rate of the two strains was not significantly different. After a further 4wk culture of both the above strains on CDM-CMA there were no significant differences in their growth rates on either type of medium.

Table 5.5. Growth rates of *P. ultimum* strains cultured in the presence or absence of *Pen. claviforme* culture filtrate (PCF) on corn meal agar (CMA) containing Czapek-Dox medium (CDM) or PCF

Assessment	*P. ultimum* strain[a]	Growth rate (mm h^{-1})[b]	
		CDM-CMA	PCF-CMA
1	PCF^S	2.33 x	1.08 x
	PCF^R	2.25 x	1.83 y
2	PCF^S	2.25 x	1.17 x
	PCF^R	2.08 x	1.33 x

[a] Strain PCF^S was grown on CMA containing CDM (CDM-CMA) for 4wk (assessment 1) or 8wk (assessment 2). Strain PCF^R was grown on CMA containing 0.9IU $plate^{-1}$ PCF for 4wk (assessment 1) followed by 4wk on CDM-CMA (assessment 2).

[b] Growth rates were determined on CDM-CMA or CMA containing 0.5IU $plate^{-1}$ PCF (PCF-CMA). For each column and assessment time data followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

5.6 Discussion

5.6.1 Plant growth effects

The inclusion of PCF in sugar beet seed pellets at rates of 0.30 or 0.50IU seed^{-1} reduced germination during the early stages of incubation on filter paper and caused aberrant seedling morphology (section 5.1). The component of the culture filtrate responsible for most of its activity against *Pythium ultimum* was the fungal metabolite patulin (Chapter 4). There are a number of reports of phytotoxic effects exerted by this antibiotic after exogenous application to plants such as wheat, pea and tomato, but no data appear to have been recorded for sugar beet. For example patulin has been found to inhibit seed germination (Timonin, 1946; Wallen & Skolko, 1951; Wright, 1951; Gattani, 1957; Norstadt & McCalla, 1963; 1971), root growth (Wright, 1951; Iyengar & Starkey, 1953; Norstadt & McCalla, 1963) and shoot growth (Iyengar & Starkey, 1953; Norstadt & McCalla, 1963), and also to cause foliar necrosis (Klemmer *et al.*, 1955), wilting (Klemmer *et al.*, 1955; Gattani, 1957) and loss of geotropic response (Norstadt & McCalla, 1971). The severity of the symptoms varies according to the concentration of the antibiotic and the plant species.

Patulin has also been implicated in adverse effects on plant growth following elaboration *in situ* by soil microorganisms. For instance it has been isolated from soil and wheat straw residues in areas where the practice of stubble mulching (section 1.3.2) is associated in certain seasons with stunted crops and decreased yields (Norstadt & McCalla, 1969b), and the concentrations detected (up to 75μg g^{-1}) were high enough to inhibit seedling development *in vitro* (Norstadt & McCalla,

1963). It was proposed that the large quantities of organic matter which accumulate on the soil surface under this management system provide a suitable substrate for growth and patulin production by Penicillium urticae, the populations of which were found to be significantly greater in stubble mulched than in ploughed soil (Norstadt & McCalla, 1969b). Similarly, the morphological abnormalities shown by the roots of apple seedlings growing in 'apple sick' soil were attributed to the activities of phytotoxin-producing fungi such as Penicillium claviforme (Câtská et al., 1988). Root inoculation of seedlings in control soil with this fungus, which produced patulin in vitro, resulted in similar phytotoxic symptoms.

In the present studies, the morphological effects of PCF on sugar beet seedlings germinating on filter paper included both stunting and curling of the radicles, but not of the plumules which emerged from the pellets approximately 2d later. These observations are consistent with the finding of Norstadt & McCalla (1963) that root growth of wheat seedlings was more sensitive to patulin than that of shoots. The same authors also reported that patulin obtained from culture filtrates of Pen. urticae inhibited root extension but did not cause the root curling and twisting observed in tests of the culture filtrate itself, and concluded that an additional fungal metabolite was responsible for these symptoms. However, in the current work the application of patulin to control pellets without additives elicited a similar response to that of incorporation of an equivalent amount (in terms of P. ultimum-inhibitory activity) of PCF. Thus the presence of this antibiotic in PCF could account for both types of formative effects.

Despite their apparent severity, the consequences of PCF incorporation on sugar beet pellet germination and early seedling development on filter paper were only temporary, and after 7d both % germination and appearance were similar to those of controls. Likewise, the phytotoxicity of certain synthetic fungicide seed treatments may decrease with continued seedling growth and fail to reduce crop yield (Crosier, 1969). Furthermore, PCF had no deleterious effects when seed pellets were grown in peat-based compost. Again a parallel can be drawn with synthetic chemical treatments where symptoms may be accentuated on paper substrata compared to compost or soil due to accumulation of the toxicant in the medium (Crosier, 1969). These observations indicate the need a) to prolong phytotoxicity testing of microbial metabolites which show potential as crop protection agents beyond germination and early development, and b) to conduct assessments in the medium in which the plant is to be grown, in order to obtain results which are relevant to proposed use conditions. Phytotoxicity is a general problem in the development of fungicides due to the similarity in biochemistry of pathogen and host (Green et al., 1987). Dosage rates are of necessity a compromise between maximum benefit in terms of disease control and minimum injury to the plant (Piening et al., 1983).

In cases where microbial products do cause significant crop damage, there are several strategies by which the effects may be reduced to acceptable levels. For example, chemical modification of the natural compound may be advantageous. Thus the semicarbazone, acetate and oxime derivatives of cycloheximide were found to be effective in the control of Puccinia graminis on wheat (Wallen, 1958) but did not show the phytotoxicity often associated with application of the antibiotic

itself. The formulation of the metabolite may also affect the severity of symptoms. Foliar injury on cucumbers and beans caused by several antibiotics including patulin was reported to be prevented by the addition of chlorophyllin to the spray solution (Ark & Thompson, 1958). Finally, the technique of seed pelleting permits the accurate placement of potentially harmful chemicals within the pellet structure so as to minimize negative effects on the seed developing within it. For instance, the toxicity of gamma-HCH to sugar beet may be reduced by restricting the insecticide to the outer layers of the pellet coating (Durrant, Dunning & Byford, 1986).

Low concentrations of PCF (0.10IU seed^{-1}, equivalent to 5μg seed^{-1} patulin) in sugar beet seed pellets were found to be slightly stimulatory both to germination on filter paper at 3d and to emergence in non-infested compost at 4 and 7d. The higher rate of 0.30IU seed^{-1} PCF (15μg seed^{-1} patulin equivalents) adopted for later experiments also consistently increased emergence above that of the controls after 7d incubation. None of these effects were statistically significant, but are interesting in relation to the results recorded by certain other investigators. The data presented by Wright (1951) show that 1μg ml^{-1} patulin incorporated into an agar medium at pH 4.0 enhanced root growth and germination of wheat (by 24 and 13.6%) and white mustard (by 15 and 5.4%) after 4-5d growth, whereas 5-25μg ml^{-1} had the opposite effect. Nickell & Finlay (1954) found that 1μg ml^{-1} patulin in a basal liquid medium improved the fresh wt. of aseptic Lemna minor cultures after 8wk by 90% compared to the controls, even though concentrations of 5-20μg ml^{-1} were markedly inhibitory. Lastly, Norstadt & McCalla (1971) reported that 50mg kg^{-1} patulin applied as a soil drench three times

weekly increased the early growth of wheat seedlings in terms of dry wt. per plant (for example by 73.9% after 27d) and tillering, but that the effect was only short term.

Several possible explanations for plant growth promotion by antibiotics at low concentrations have been proposed, including interference with hormone metabolism (Nickell & Finlay, 1954). It is thus noteworthy that patulin has been found to modify responses to the plant hormone indole acetic acid in certain systems (Iyengar & Starkey, 1953; Norstadt & McCalla, 1963).

The synthetic fungicide hymexazol (Hm), incorporated into sugar beet pellets at the recommended rate of 10.5g a.i. kg^{-1} seed, also had a stimulatory effect on seedling emergence in non-infested compost of the same order as that of PCF. In recent commercial field trials a lower dosage of 3.5g a.i. kg^{-1} seed Hm was frequently associated with earlier emergence compared to the recommended rate (Dewar et al., 1988), but was less effective in controlling soil-borne seedling pathogens. Hm has also been reported to have growth promoting effects on the early growth of rice and other crop species (Tomita et al., 1973).

5.6.2 Disease control

Patulin was examined as a potential crop protection agent on several occasions during the initial period of interest in antibiotics as selective, systemic fungicides and bactericides (section 1.4.1). For example, it was tested against Ustilago tritici (Timonin, 1946), Ascochyta pisi (Wallen & Skolko, 1951), Agrobacterium tumefaciens (Klemmer, Riker & Allen, 1955), Pythium sp. (Gattani, 1957), Pseudoperonospora cubensis (Ark & Thompson, 1957), Erisiphe graminis and

Ustilago nada (Norstadt & McCalla, 1971). Similarly, the culture filtrates of patulin-producing fungi have been investigated in this context. Thus Luijk (1938) and Gregory et al. (1952) studied the effect of the culture filtrates of Penicillium expansum and Penicillium patulum, respectively on Pythium debaryanum. Finally, Grossbard (1949) examined the possibility of inducing patulin formation in situ by Pen. patulum inoculants using various soil amendments as a means of controlling Phytophthora parasitica. In these studies the antibiotic was frequently effective in suppressing the target pathogen, but also often had detrimental effects on the respective host plant. However, in many cases assessments were short term or conducted on artificial media. Thus the significance of the symptoms observed may have been exaggerated (section 5.6.1).

In the current work patulin, as the active component of PCF, had no adverse effects on sugar beet establishment in peat-based compost when applied as a seed pellet fungicide (section 5.1) and it was therefore worthwhile to proceed further in the evaluation of the culture filtrate extract for the management of seedling diseases. Compatibility with modern seed treatment technologies, which permit their efficient introduction directly into the infection court (section 1.2.1), is an important prerequisite for the adoption of any new agent for this purpose.

The original aim of seed pelleting was to convert small, light or irregularly-shaped seeds into spheres of a sufficient size and uniformity to allow them to be precision drilled, and thus to achieve optimum plant density without the costly alternative of oversowing followed by thinning of established seedlings (Halmer, 1988). Pelleting

of sugar beet was implemented in Europe in the mid-1960's when the discus-shaped, high-value monogerm varieties became available, and in many countries the entire seed volume sown is now processed in this way (Durrant, Dunning & Byford, 1986). Sugar beet is the major seed species pelleted worldwide, but a proportion of an increasing range of other types are now treated including fodder beet and fodder Brassica spp., carrot, parsnip, celery, sweetcorn, radish, lettuce, tomato, pepper and smaller quantities of various flower species such as members of the tiny-seeded genera Petunia and Lobelia.

Pellets are produced using rotary drums to continuously roll the seed mass whilst simultaneously adding a powdered blend of the coating material and a binder, together with water, to gradually build up seqential layers around the seed (Halmer, 1988). This process is followed by drying and grading to the desired size range. Until 1984 the principle component of the pellet was a clay, applied in Britain by Germain's (U.K.) Ltd., under the name 'Filcoat'. However, studies showed that this material reduced emergence in wet soils (Durrant & Loads, 1986), and in 1985 it was replaced by the lighter-weight, more porous 'EB3' pellet containing a high proportion of 'wood flour' (Dewar et al., 1988).

It was soon realized that pellet coatings also provide an ideal vehicle for the delivery of chemicals to the immediate environment of the seed. This has developed into the concept of the 'seed package' containing all the crop protection and enhancement compounds necessary to optimize germination and early seedling growth (Clayton, 1988). For example, all sugar beet seed pellets currently contain methiocarb, which affords some protection against soil-inhabiting arthropod pests, and

hymexazol to suppress soil-borne seedling pathogens (Dewar et al., 1988), but the micronutrient manganese has been omitted since 1987 following trials which showed that it failed to relieve deficiency symptoms and reduced seedling emergence and establishment (Fletcher & Prince, 1987). Dyes may also be incorporated to facilitate precision drilling, and bird or mammal repellants to minimize harm to seed-eating wildlife (Greig-Smith, 1988). In addition, all seed receives a pre-treatment thiram steep (0.2% (w/v), 25°C, 8-16h) which advances germination and controls the seed-borne fungus Phoma betae (Fletcher & Prince, 1987)

The P. ultimum-inhibitory components of PCF survived the stresses of commericial seed pellet production, loosing only approx. 9.1% activity during the entire process (section 6.1).

The plant growth containers and medium used for the evaluation of PCF-containing seed pellets were those employed by the pelleting company to test synthetic pellet treatments. Sugar beet itself is a field-grown crop, but the numbers of glasshouse-produced plants grown in containers, either for the whole of the cropping period or for transplanting, is rising rapidly (Bunt, 1988) for reasons including lower unit costs, convenience and ease of mechanization. The test system was therefore of relevance to the wide range of horticultural crops which are susceptible to Pythium attack (Middleton, 1943).

The trend towards container production has been accompanied by the development of new types of growing media due largely to the difficulties in obtaining loam of the requisite quality, and in adequate quantities, to satisfy the demands of the modern horticultural industry (Bunt, 1988). They are also cheaper, lighter to handle, and allow a

greater degree of standardization. The major component of loamless container media is peat, which may be mixed with other relatively inert substrates such as sand, vermiculite and perlite, together with suitable quantities of superphosphate, potassium nitrate, lime and micronutrients (Sheard, 1975). In the current work the growing medium is referred to as a compost, in accordance with the name under which it is marketed. However, traditionally this term is used to describe plant material which has undergone biological decomposition before being dug into garden soil, and the term 'container mix' may thus be preferable (Bunt, 1988).

Microbial populations, including those of plant pathogens, are generally low in peat compared to a mineral soil (section 6.5.4), and in Britain and other West European countries pasteurization of container media is not considered necessary. The absence of detectable numbers of Pythium in Levington compost (section 5.3) was consistent with this view. However, media may become contaminated with damping-off fungi such as Rhizoctonia solani and Pythium spp. within the glasshouse environment (Stephens et al., 1983) and can then become a major problem since factors such as reduced microbial competition, availability of juvenile host tissues, and high moisture and temperature combine to provide an ideal environment for their proliferation (Favrin, Rahe & Mauza, 1988). Control is conventionally by means of fungicide drenches, though these may show appreciable variability in efficacy (Stephens & Stebbins, 1983).

In the evaluation of plant protection agents, a high and uniform level of infection in control treatments is essential in order that effective products can be recognized (Shepherd, 1987). This creates

considerable difficulties in testing for activity against soil-borne pathogens in the field where distribution is notoriously uneven (Capper & Campbell, 1986; section 1.1.4) and where populations may show substantial seasonal fluctuations (Ali-Shtayeh, Lim-Ho & Dick, 1986a). It is therefore common practice in assessments conducted in controlled environments to artificially infest the plant growth medium with a large and known number of pathogen propagules, whilst recognizing that results will not always be comparable with those obtained under field conditions.

In the experimental work described here the compost was infested with P. ultimum by the simple and convenient means of a suspension of propagules applied to the surface immediately after planting (section 2.5.2). This approach ensured that the disease challenge was uniform in a horizontal dimension, but resulted in an irregular vertical distribution of the pathogen (section 5.3). The more common inoculation method of physically mixing propagules with the medium prior to sowing may have given a more homogeneous pattern, but would not necessarily approximate more closely to that occurring under commericial use conditions, where the presence of inoculum is likely to be sporadic depending on the source of contamination. The experimental drench method could simulate the situation occurring when Pythium is introduced into the glasshouse system with the irrigation water (Gill, 1970).

Similarly, the numbers of propagules involved in a disease outbreak during commercial production are likely to be highly variable. In the absence of information on this subject in the literature, a suitable P. ultimum infestation rate was determined on an empirical basis, so as

to give a measurable level of both pre- and post-emergence infection under the test conditions.

In the two trials of commercially-produced seed pellets the P. ultimum inoculum densities were similar but resulted in markedly different disease incidences in the controls (Table 5.3). The frequency of pre-emergence infection was lower and of the post-emergence phase higher in trial 2 compared to trial 1. As all other test conditions were equivalent, it appeared that overnight storage of the propagule suspension at 4°C prior to infestation in the second trial decreased pathogen vigour, and thus its inoculum potential (section 1.1.3), during the early stages. However, this was followed by a severe attack later in the experiment such that seedling establishment after 7d corresponded to that in trial 1 (Tables 5.1 and 5.2).

The incorporation of PCF into the pellets significantly increased emergence and establishment in both trials (Tables 5.1 and 5.2), but its efficacy was greater in response to a lower disease challenge i.e. against post-emergence damping-off in trial 1 and against pre-emergence infection in trial 2 (section 5.2). These results illustrate the importance of rigorous standardization of all test conditions during evaluation if valid comparisons are to be made between different experiments. In addition to the inoculum potential and strain of the pathogen, factors relating to the test plant, such as age and cultivar, the incubation conditions (for example temperature, humidity and light) and the disease control agent itself (formulation, method of application) can all have a substantial influence on activity in a given pathosystem (Kato, 1986).

PCF increased emergence in trial 2 as effectively as hymexazol (Table 5.2), but in the case of emergence in trial 1, and establishment in both experiments, the culture filtrate extract was less successful than the synthetic fungicide (Tables 5.1 and 5.2). The experimental data thus demonstrated the necessity of including the appropriate contemporary product in tests of any putative new crop protection agent to allow a meaningful appraisal of market potential (Rodgers, 1989).

Hymexazol possesses proven efficacy against the seedling pathogen Aphanomyces cochlioides (Dewar et al., 1988), but its reported activity against Pythium spp. has not yet been verified in the field (P. Halmer, Germain's (U.K.) Ltd., personal communication). The consistently high performance of this fungicide in combating P. ultimum in the trials described here fully substantiated the claims of the manufacturer (Tomita et al., 1973).

Results presented in section 6.3 indicate that the half-life of PCF in compost was short. Hence its efficacy in situ may be improved by strategies aimed at prolonging the time over which effective concentrations are maintained in the infection court, such as increasing its rate of incorporation into the seed pellets or by slowing down its release into the spermosphere afer planting. The margins for exploitation of the former method before encountering significant detrimental effects on seedling establishment may be limited (Fig. 5.2), but the latter may have greater potential (section 6.5.2). Stability in plant growth media may also be enhanced by chemical modification. This is an approach which has proved fruitful in the development of antibiotics used in human chemotherapy. For example, the semi-synthetic agent ampicillin is many times more stable under the acid conditions of

the digestive tract than the natural compound penicillin G (Queener & Swartz, 1979).

A raw sugar beet seed treatment was not included in the disease control trials discussed above but certain of the results considered in section 5.6.3 suggested that the pellet coat itself may have suppressed seed infection by P. ultimum. This phenomenon was also reported by Harman & Hadar (1983) who found that the thickness of pea seed pellet coats was directly related to the incidence of seed rot. The observations could be explained by the coating material physically restricting the ingress of the pathogen, or attenuating the outward diffusion of seed exudates. Osburn & Schroth (1988) attributed the reduced colonization of osmoprimed sugar beet seed by P. ultimum to the decreased exudation which followed the pre-treatment.

5.6.3 P. ultimum population dynamics

Only 22.7% of the Pythium ultimum propagules counted microscopically in an inoculum suspension germinated to form colonies when plated directly onto SM. A similar figure was obtained on CMA (section 5.3). The low % germination could be due, at least in part, to the presence in the suspension of both exogenously dormant structures (sporangia and thin-walled oospores) and constitutively dormant, thick-walled oospores (section 1.1.4). All three types were counted microscopically, but only the first two are detected by plating assays (Stasz & Martin, 1988).

Following the introduction of Pythium-selective media by Singh & Mitchell (1961), field studies have been made of the abundance of the fungus in various soils, in the presence and absence of crop plants (Ali-Shtayeh, Lim-Ho & Dick, 1986b). However, little quantitative

information is available on Pythium population dynamics within the confines of plant growth containers where soil moisture, aeration and temperature differ considerably from those encountered under field conditions (White & Mastalerz, 1966; Gugino, 1973). The use in the current work of a growing medium based on peat, with its unique physical and chemical properties (Puustjarvi & Robertson, 1975) diminishes further the comparison with studies conducted in the field.

In artificially-infested, unplanted peat-based compost the P. ultimum population gradually declined over the 10d experimental period, suggesting a lack of sufficient nutrients to support saprophytic multiplication. Peat is an accumlation of the less readily decomposable constituents of plant materials which forms where anaerobic and acidic conditions caused by waterlogging create an environment unfavourable for microbiological activity (Kavanagh & Herlihy, 1975). Thus although the proportion of organic matter in container media is high, much of it is refractory and unavailable to pioneer colonizers (Nedwell & Gray, 1987) such as Pythium.

In the spermosphere of control pellets without additives P. ultimum numbers increased 3.3-fold between 5 and 9d after planting, presumably reflecting invasion of the seed and conversion of its readily assimiliable components into further propagules, which were then liberated into the medium as the surrounding host tissues were broken down. Given that this pathogen can colonize the pericarp of raw sugar beet seed within 4h of planting in soil (Osburn et al., 1989), and that resting structures are often formed within 36h of host penetration (Stanghellini, 1974), the 5d delay before an increase in densities

occurred in the present experiment suggested that the pellet coat itself may have impeded seed infection (section 5.6.2).

Addition of PCF to the pellets prevented the build-up in P. ultimum numbers observed in the absence of additives, at least until day 8, but did not cause a reduction in the population as might be expected if antibiosis was the principle cause of suppression (Baker, 1968). One explanation of these results is that some propagules survived the direct fungicidal effect (section 4.1) of the culture filtrate extract but were nevertheless debilitated such that their inoculum potential was impaired. Such a phenomenon was postulated to be involved in the control of Cochliobolus sativus by methylmercury decyandiamide which had little direct effect on the pathogen in soil (Chinn, 1971). Some support for this interpretion in the present work was obtained from the observation that the in vitro germination of Pythium propagules recovered from the spermosphere of PCF-pellets was delayed compared to those not exposed to the extract. This subject is discussed further in section 6.5.4. After 8d a small increase in P. ultimum densities took place. This could be attributable to differential sensitivity of the different types of progagule present. The viability of thick-walled oospores of P. ultimum was found by Stasz & Martin (1988) to be unaffected by a number of chemical treatments to which thin-walled oospores and sporangia were sensitive. Following conversion to thin-walled structures (section 1.1.4), the former type could therefore give rise to further fully viable propagules. However, after 7d sugar beet seedlings were no longer susceptible to Pythium attack (section 5.6.2), so this input of infective inoculum did not result in further disease losses.

5.6.4 Antifungal spectrum

Many antibiotics are specific in their antimicrobial activity (Misato & Yoneyama, 1982; Cook & Baker, 1983). In the context of their exploitation as plant disease control agents this feature is clearly advantageous in minimizing harmful effects on non-target organisms (sections 1.2.2 and 1.4.2), but can cause problems relating to a) performance, b) the economics of commercialization and c) resistance (section 5.6.5).

'Disease trading' (section 1.2.2) is due to differential fungicide sensitivity of pathogens themselves, or of pathogens and their respective antagonists (Bollen, 1979). It is a particular hazard in the treatment of diseases which may be caused by one or more of a number of pathogens. For example, damping-off of sugar beet, commonly referred to as 'black leg', may be incited by the seed-borne fungus Phoma betae or by several soil-borne pathogens, of which Rhizoctonia solani, Aphanomyces cochliodes and Pythium spp. are the most widespread (Dunning & Byford, 1982; Fletcher & Prince, 1987). The elimination of one of these organisms by a selective compound may be followed by another moving in to fill the vacated ecological niche.

The in vitro antifungal spectrum of PCF was not restricted to Pythium ultimum (Fig. 5.6). Of particular interest with regard to sugar beet was the high degree of activity against A. cochliodes (Fig. 5.7). However, the higher fungi tested were generally inhibited to a lesser extent than the Oomycetes.

These results were consistent with the generalization that there is considerable variation within the fungi in their sensitivity to patulin, but that Pythium spp. are amongst the most susceptible (Singh, 1967).

A notable discrepancy with published information however was the lesser sensitivity of Pythium aphanidermatum to PCF compared to Pythium debaryanum, Pythium mamillatum and P. ultimum, whereas Anslow, Raistrick & Smith (1943) reported that these fungi showed similar susceptibility to patulin. This disagreement could have arisen from the use of different strains of the test pathogen, which may show considerable variation in their sensitivity to a given control agent (Cook & Zhang, 1985). The same explanation could apply to the small differences in activity of PCF against the synonymous organisms Rhizoctonia solani and Thanatephorus cucumeris (Webster, 1980).

The range of phytopathogens inhibited by TSF was narrower than that of PCF (Fig. 5.8) and its deployment as a seedling fungicide would thus be more prone to disease trading effects.

A second important hindrance with respect to the implementation of selective crop protection agents is that of their limited market size. The vast expense involved in the commercialization of new fungicides (section 1.2.1) has resulted in the situation where only broad-spectrum compounds, with potential for extensive use on major crops, can now provide an adequate return on the investment of the company concerned (Green, Hartley & West, 1987). This is plainly an undesirable state of affairs which requires attention from the regulatory authorities.

5.6.5 Resistance

The development of resistance in phytopathogens, including soil-borne organisms, has become a major problem in the chemical control of plant diseases (section 1.2.3). It is therefore desirable to obtain information regarding the risks associated with the use of a new product before its launch so that effective countermeasures can be recommended (Dekker, 1982a).

The potential of fungi to become resistant to fungicides has been studied on laboratory media amended with the compound, with or without the use of mutagens (Dekker, 1982b). In the present work, a strain of Pythium ultimum ('PCF^{R}') showing reduced sensitivity to PCF compared to the parent strain was readily obtained using such methods (section 5.5). However, the property was rapidly lost during subsequent growth in the absence of the culture filtrate, suggesting that it was due to a temporary, physiological adaptation (i.e. tolerance, section 1.2.3) rather than mutation. Similar observations were made by Grover & Moore (1961) after continuous exposure of Sclerotinia fructicola and Sclerotinia laxa to cycloheximide. Because of their instability tolerant strains rarely cause concern in the field (Delp, 1980).

In order to fully evaluate the likelihood of emergence of resistance to PCF additional methods of inducing mutant strains would need to be tested (Davidse, 1981). In particular, studies involving sexually produced spores, in which the potential for genetic variation is much greater, would be required. It is noteworthy in this context that the absence of a perfect state in Rhizoctonia solani is probably an important contributory factor in the lack of resistance problems arising

during the use of validamycin A against rice sheath blight (section 1.4.1; P.J. Kuhn, Shell Research Ltd.; personal communication).

Many antibiotics appear to be single-site inhibitors and thus resistant mutants can often be easily obtained in the laboratory (Dekker, 1983). However, whether such strains would survive and cause failure of disease control *in situ* is dependent on several additional factors (section 1.2.3), of which their fitness relative to that of sensitive organisms is of critical importance (Georgopoulos, 1977; Davidse, 1981; Dekker, 1982b). For example, kasugamycin-resistant isolates of *Pyricularia oryzae* (sections 1.4.1 and 1.4.4) showed reduced virulence compared to sensitive strains in the absence of the antibiotic (Ito & Yamaguchi, 1979), and when field applications were discontinued the proportion of the former in the total population declined (Sato, 1983). Decreased fitness has also been reported in mutants of the soil-borne pathogen *Fusarium oxysporum* f.sp. *narcissi* resistant to pimaricin (Dekker & Gielink, 1979). This antibiotic is registered in the Netherlands for the control of narcissus bulb and root rot caused by *F. oxysporum* ff.spp.

In the current studies the mycelial growth rate of *P. ultimum* PCF^R in the absence of PCF was not impaired after 4wk continuous culture on CMA containing the culture filtrate at sub-lethal concentrations. If resistant mutants had been obtained, the assessment of other parameters of fitness, such as sporulation, spore germination and virulence, would have increased the predictive value of the results (Dekker, 1982b). However even then their accuracy may have been limited (Staub & Sozzi, 1984).

In cases where *in vitro* studies do suggest that the build-up of resistance to an antibiotic proposed as a plant disease control agent may be a problem in practice, there are various methods by which the risk may be minimized. These strategies are analagous to those advised for vulnerable synthetic fungicides (section 1.2.3). For example, rice blast is now successfully controlled using either a combination of kasugamycin with tetrachlorophthalide, or by alternating the antibiotic treatment with another chemical (such as probenazol) with a different mode of action (Dekker, 1983).

CHAPTER 6

EFFICACY OF PCF AS A SEED PELLET FUNGICIDE

II. ADDITIONAL PROPERTIES AFFECTING PERFORMANCE

The previous chapter reported the effects of Penicillium claviforme culture filtrate (PCF) on the plant host, the pathogen and the host-pathogen interaction following introduction into a sugar beet-Pythium ultimum pathosystem in seed pellets. This chapter describes investigations into further properties of PCF which are likely to influence its utility as a seed pellet fungicide.

6.1 Shelf-life of PCF

The shelf-life of PCF was evaluated both in solution and after incorporation into sugar beet seed pellets.

PCF extract containing 410IU ml^{-1} (section 2.5.1) was stored in DW at room temperature (20+/-2°C) under sterile or non-sterile conditions. Pythium ultimum-inhibitory activity was determined at weekly intervals over an 8wk period as given in section 2.3.2. Each treatment was bioassayed in triplicate. Results are shown in Fig. 6.1.

Anti-Pythium activity slowly declined when the extract was stored in a sterile environment and more rapidly under non-sterile conditions. After 8wk, activity had decreased by 21.9 and 60.7% in the two

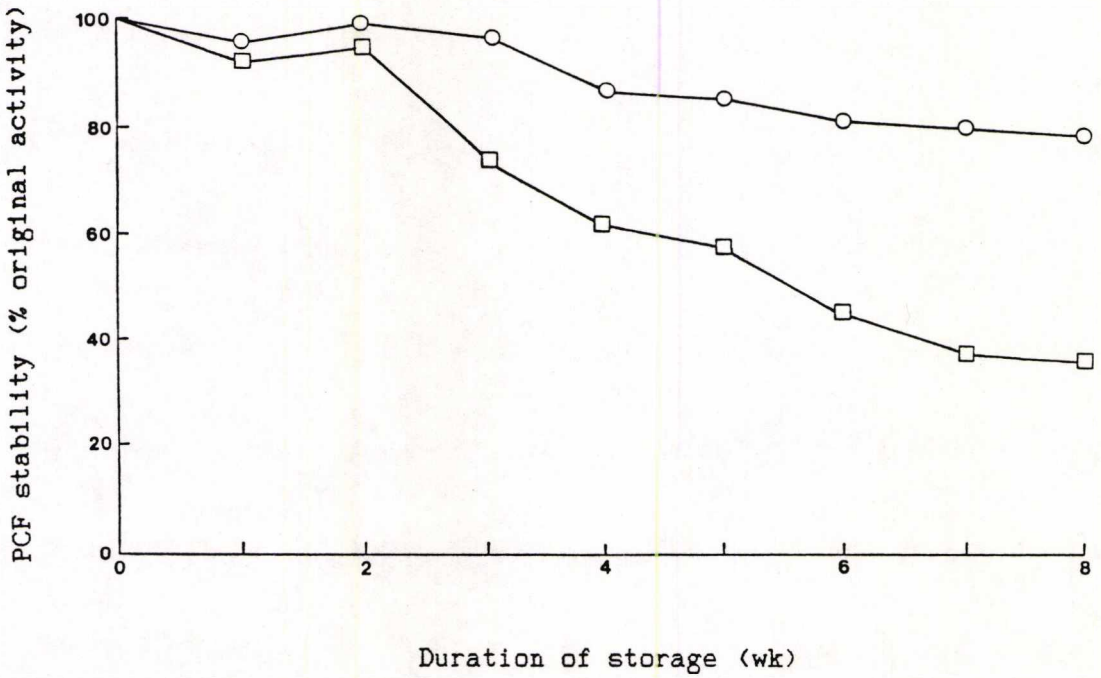


Fig. 6.1. Stability of P. ultimum-inhibitory components of Pen. claviforme culture filtrate extract (PCF) to storage in solution at room temperature (20+/-2°C) under sterile (○) or non-sterile (□) conditions prior to bioassay.

treatments, respectively. The yellow-brown colour of the freshly-prepared extract darkened with time in both cases.

The stability of PCF following inclusion in sugar beet seed pellets was also investigated. In order to determine the % recovery of the inhibitory agents, pellets containing 0.33IU pellet^{-1} PCF were prepared by the laboratory method (section 2.5.2) and incubated in batches of fifteen in 5ml DW at room temperature (20^{+}/$_{-}$2°C). At intervals during the subsequent 4h period duplicate samples from each of three replicate pellet extracts were removed and bioassayed (section 2.3.2), using extracts of pellets without additives as controls. Results are given in Fig. 6.2.

After 1.5h incubation 97.0% (0.32IU pellet^{-1}) of the applied anti-*Pythium* activity was recovered in the pellet extracts. Percentage recovery did not increase on further incubation. For routine purposes, an incubation period of 2h was adopted for pellet extraction (section 2.6.3), and where an absolute value for activity was required, a correction factor of 1.03 (0.33/0.32) was used.

The *P. ultimum*-inhibitory activity of commercially prepared PCF-pellets (section 2.5.2) was determined 1wk after production and at intervals thereafter, as described above. Results are presented in Fig. 6.3.

The anti-*Pythium* activity recovered from PCF-pellets 1wk after production was 0.29IU pellet^{-1}. Thus the absolute value at this time (here referred to as the 'achieved dose') was 0.29 x 1.03 = 0.30IU pellet^{-1}.

The *P. ultimum*-inhibitory components of PCF were considerably more stable after incorporation into seed pellets than when the extract was

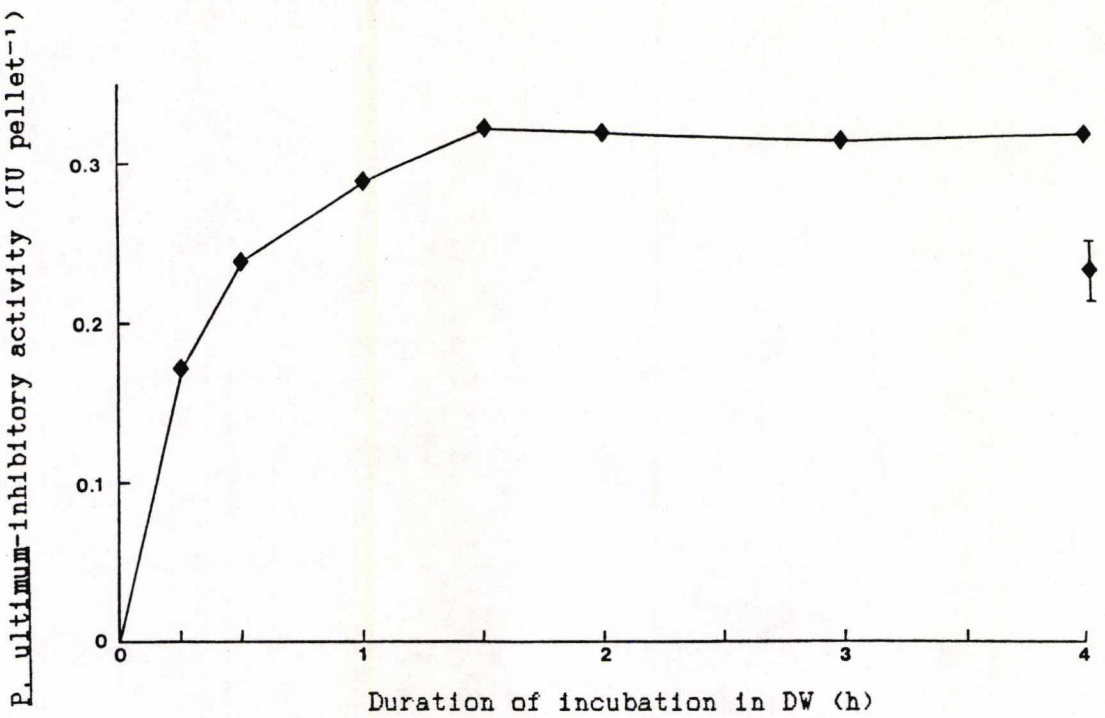


Fig. 6.2. P. ultimum-inhibitory activity extracted from sugar beet seed pellets containing 0.33IU pellet^{-1} Pen. claviforme culture filtrate extract. Inhibitory agents were recovered by incubating the pellets in distilled water (DW) followed by bioassay of the extract. Bar represents +/- mean SE.

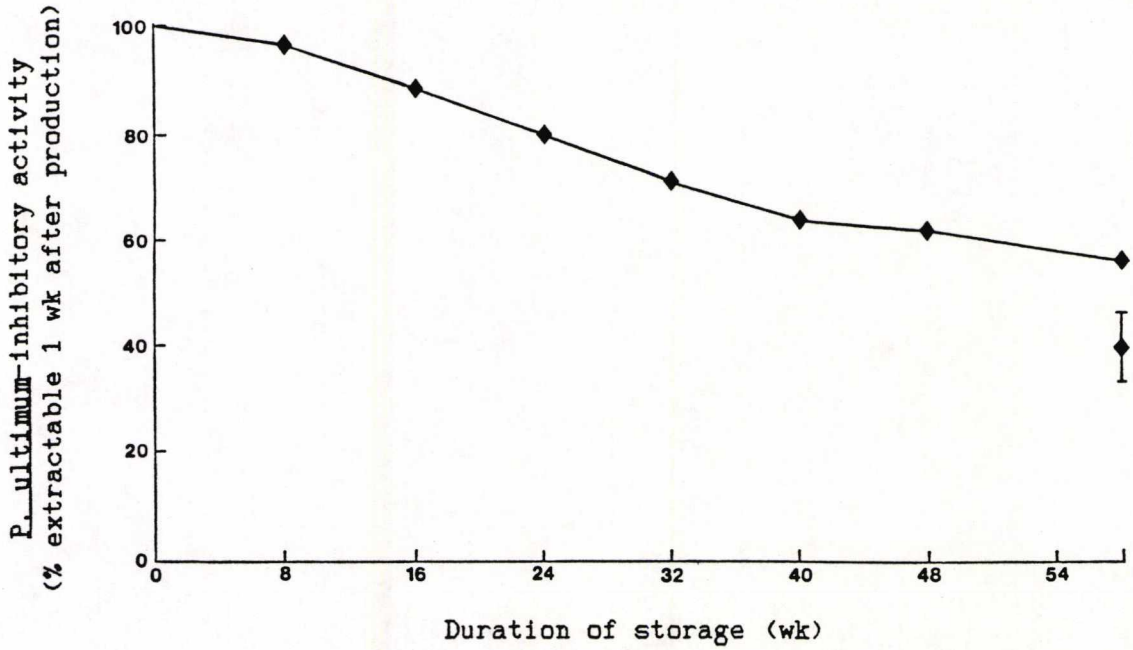


Fig. 6.3. P. ultimum-inhibitory activity extracted from commercially produced sugar beet seed pellets containing Pen. claviforme culture filtrate extract after storage at room temperature (20+/-2°C). Inhibitory agents were recovered by incubating the pellets in distilled water for 2h, followed by bioassay of the extract. Bar represents +/- mean SE.

stored as a solution. The half-life in the pellet formulation was approx. 14 months.

6.2 Rate of release of PCF in seedling growth media

An assessment was made of the rate at which the Pythium ultimum-inhibitory constituents of PCF were liberated from sugar beet seed pellets planted in peat-based compost.

Laboratory-prepared pellets (section 2.5.2) containing 0.30IU PCF pellet^{-1} were planted in compost contained in seedling wedge cells and watered in as described in section 2.6.2. Immediately after burial and wetting, and at intervals during the subsequent 24h period, pellets were recovered and adhering compost removed as far as possible with a fine paint brush. Following this pellets were dried for approx. 1h in a laminar-flow cabinet (Microflo) and any remaining compost particles brushed off. Residual anti-Pythium activity in three replicate batches of fifteen pellets was then determined as given in section 2.6.3. Each pellet extract was bioassayed in duplicate. Results are shown in Fig. 6.4.

87.7% of the applied P. ultimum-inhibitory activity was recovered from pellets removed from compost immediately after planting. The inhibitory components were rapidly released from pellet coatings under the conditions of the experiment, 50% of the added activity being discharged in 3.5h.

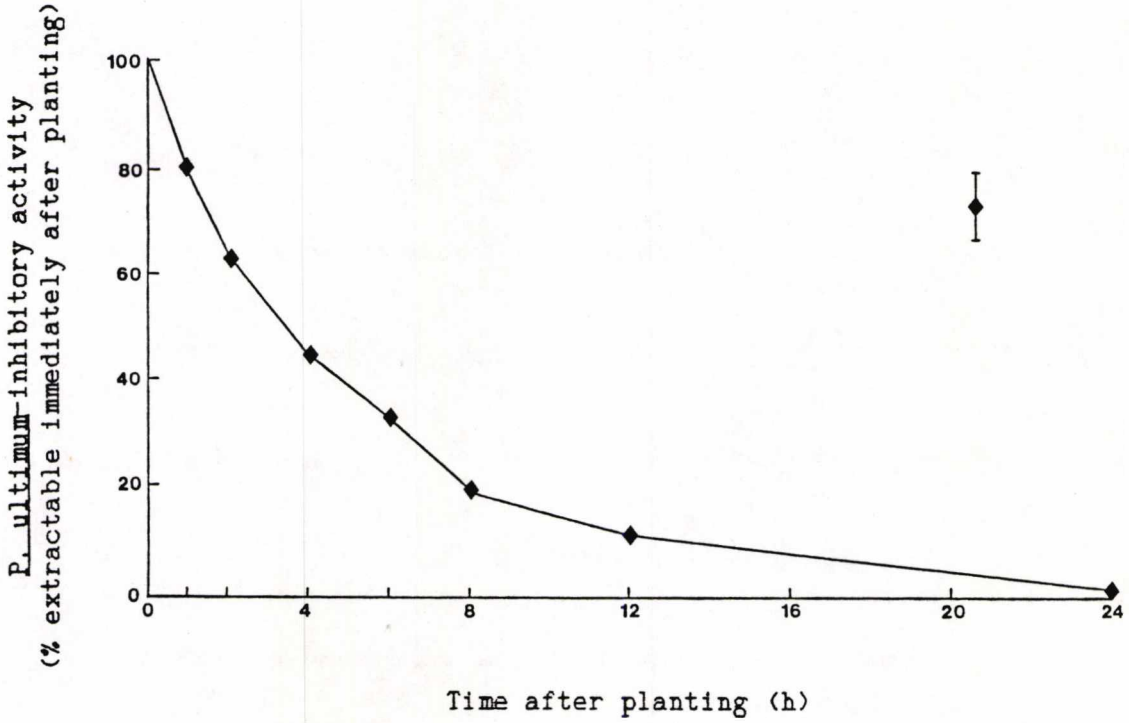


Fig. 6.4. P. ultimum-inhibitory activity extracted from sugar beet seed pellets containing Pen. claviforme culture filtrate extract planted in peat-based compost. Pellets were removed from the compost and the residual inhibitory agents recovered by incubation in distilled water for 2h followed by bioassay of the extract. Bar represents +/- mean SE.

6.3 Persistence of PCF in seedling growth media

The persistence of the Pythium ultimum-inhibitory components of PCF was studied in the peat-based compost used in previous experiments (Chapter 5) and in an agricultural soil (Table 2.3).

PCF extract (150IU, section 2.5.1) was added to 20g sterile or non-sterile compost or soil in the volume of liquid required to adjust the water content to 75.0% container capacity (section 2.6.1, dry wt. determined on separate 5g samples) and mixed in thoroughly. Mixtures were incubated at room temperature (20°/-2°C) in glass beakers covered with parafilm to minimize water loss. Immediately after PCF amendment, and at intervals during the following 7d period, samples were removed after re-mixing and anti-Pythium activity measured by bioassay as described in section 2.6.4. Each treatment was replicated three times and was bioassayed in duplicate. Results are given in Fig. 6.5.

79.0 and 81.4% of the applied P. ultimum-inhibitory activity was recovered from sterile and non-sterile compost, and 86.3 and 87.7% from sterile and non-sterile soil, immediately after PCF addition. The inhibitory components were rapidly inactivated in both non-sterile media with no detectable lag period, but the half-life was longer in compost (11.2h) than in soil (4.8h). Persistence was much greater in sterilized media. In this case the anti-Pythium agents were more stable in soil than in compost, showing 10.1 and 20.4% loss of activity after 7d, respectively.

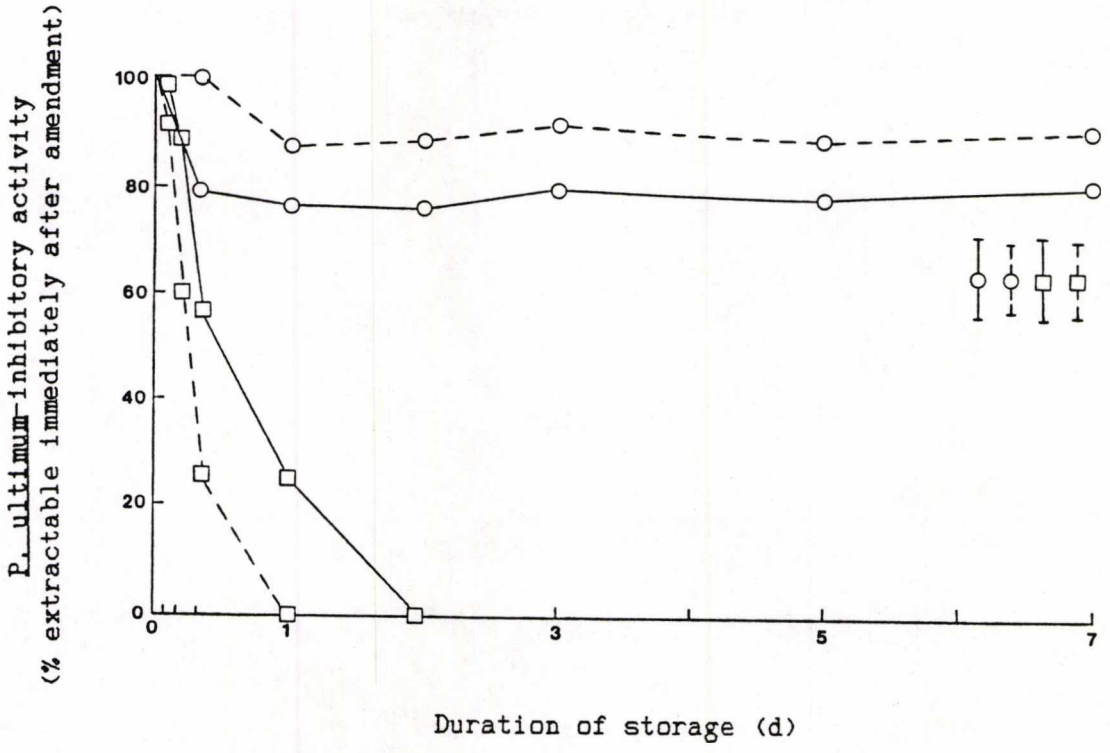


Fig. 6.5. P. ultimum-inhibitory activity extracted from sterile (○) or non-sterile (□) peat-based compost (——) or soil (– – –) amended with Pen. claviforme culture filtrate extract after storage at room temperature (20+/-2°C). Inhibitory agents were recovered by vortex mixing the media in distilled water, followed by centrifugation and bioassay of the supernatant. Bars represent +/- mean SE.

6.4 Effects of PCF on the spermosphere microflora

The influence of PCF incorporated into sugar beet seed pellets on the indigenous microbial population in seedling growth media was evaluated with respect to:

1) effects on total microbial activity; and

2) effects on particular sections of the microbial community resulting in changes in the microbiological balance in the spermosphere.

6.4.1 Total microbial activity

Overall microbial activity was measured in terms of the extent of hydrolysis of fluorescein diacetate (FDA) to fluorescein under standardized conditions (section 2.6.6).

In a preliminary experiment, FDA hydrolytic activity was compared in unplanted, non-sterile or sterile peat-based compost and soil, as described in section 2.6.6. Media were wetted to 75.0% container capacity immediately before the assessment. An additional sterile sample terminated at time 0 was used as a blank. Each of the four treatments was replicated three times and was assayed in duplicate. Results are given in Fig. 6.6.

In non-sterile media FDA hydrolysis was 2.0-fold greater in soil than in compost. In the former treatment the bright yellow colour of fluorescein was obvious, but in the latter was less readily discernable by eye.

Activity was reduced in both sterile media compared to the corresponding non-sterile treatments, but to a lesser extent in soil (by 75.0%) than in compost (by 96.2%).

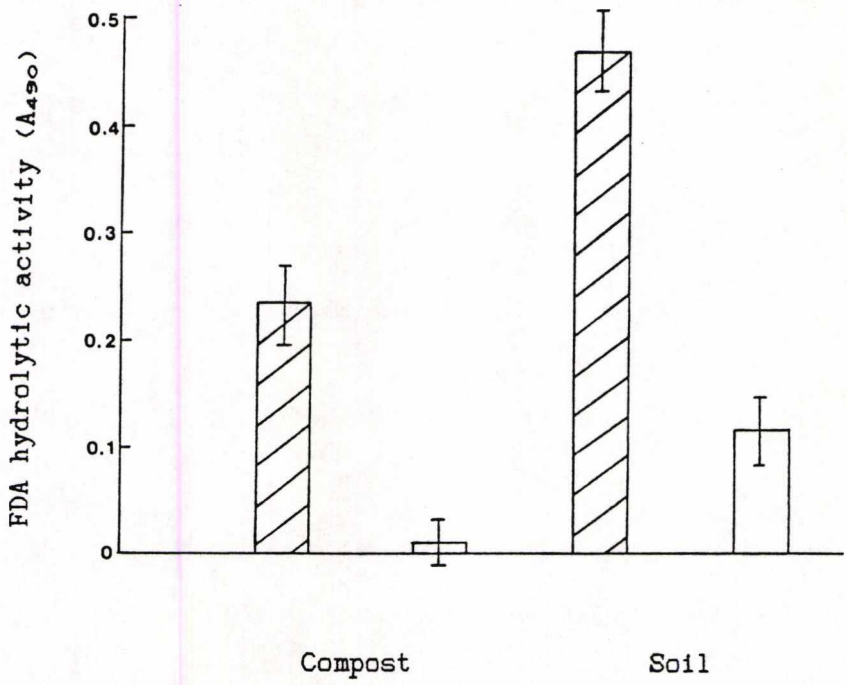


Fig. 6.6. Fluorescein diacetate (FDA) hydrolytic activity in unplanted, non-sterile (▨) or sterile (□) peat-based compost and soil, as determined by spectrophotometric measurement of fluorescein concentration at 490nm after incubation under standardized conditions (25°C, 90rev min^{-1}, 20min). Bars represent +/- SE.

These data suggested that non-biological processes made an appreciable contribution to the FDA hydrolysis which occurred in non-sterile soil. However, it appeared that in non-sterile compost micro-organisms were primarily responsible for the observed reaction. In the interpretation of the following experiments conducted in this medium, FDA hydrolytic and microbiological activities were assumed to be directly related.

To monitor the effect of PCF on microbial activity in peat-based compost, wedge cells were left unplanted, or planted with seed pellets (section 2.6.2) treated in the laboratory with DW or PCF extract (0.30IU $pellet^{-1}$, section 2.5.2) or with an equivalent volume of CDM extract (section 2.5.1). The compost was wetted with TW and containers incubated as described in section 2.6.2. Samples of medium were removed at intervals and microbial activity determined as specified in section 2.6.6. An additional unplanted sample terminated at time 0 was employed as a blank. Each treatment was replicated three times and was assayed in duplicate. Results are presented in Fig. 6.7. The experiment was subsequently repeated, using the second and third treatments only, in compost infested with Pythium ultimum at a rate of 3.5 $x10^5$ propagules $wedge\ cell^{-1}$ (section 2.6.2). Results are shown in Fig. 6.8.

In non-infested, unplanted compost, total microbial activity remained stable throughout the experiment (A_{490} values of 0.202-0.235, mean 0.217, Fig. 6.7). In the spermosphere of control seed pellets treated with DW activity was not significantly different to that in the unplanted medium at any sampling time, although the mean A_{490} reading (0.235) was greater. The addition of CDM to the pellets had no significant effect. However, the inclusion of PCF significantly

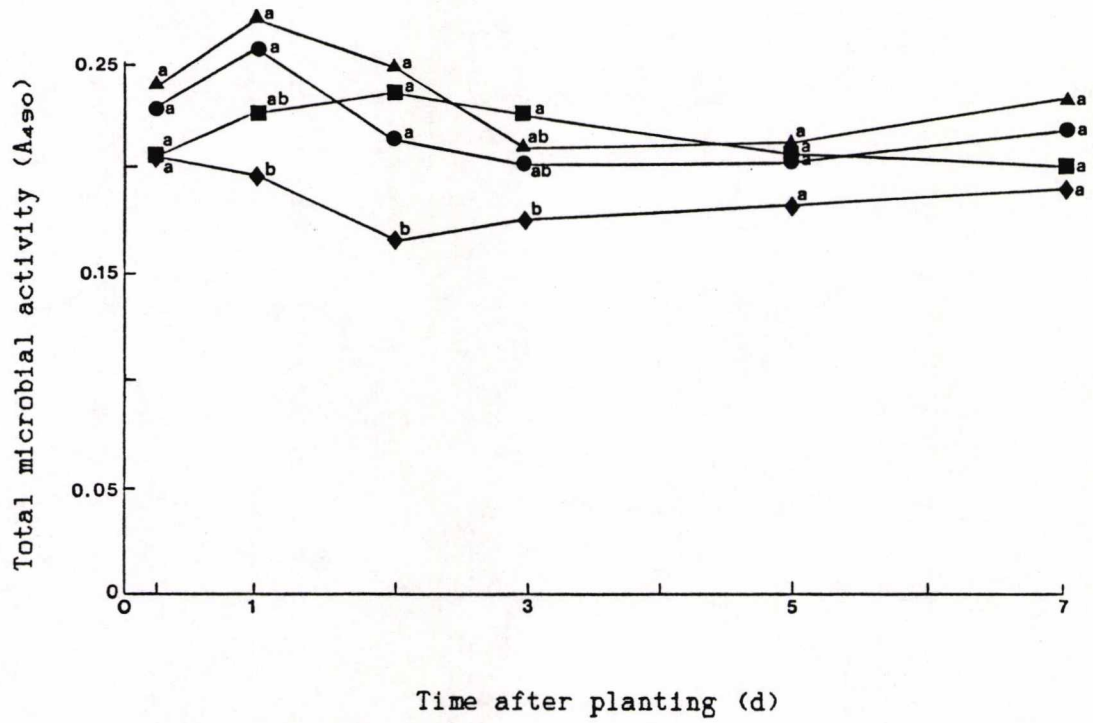


Fig. 6.7. Total microbial activity in non-infested peat-based compost either unplanted (■), or planted with sugar beet seed pellets treated with distilled water (▲), Czapek-Dox medium extract (●) or Pen. claviforme culture filtrate extract (◆). Activity was assessed in terms of the extent of hydrolysis of fluorescein diacetate to fluorescein under standardized conditions (25°C, 90rev min^{-1}, 20min). Fluorescein concentration was measured spectrophotometrically at 490nm. For any one assessment time values marked with the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

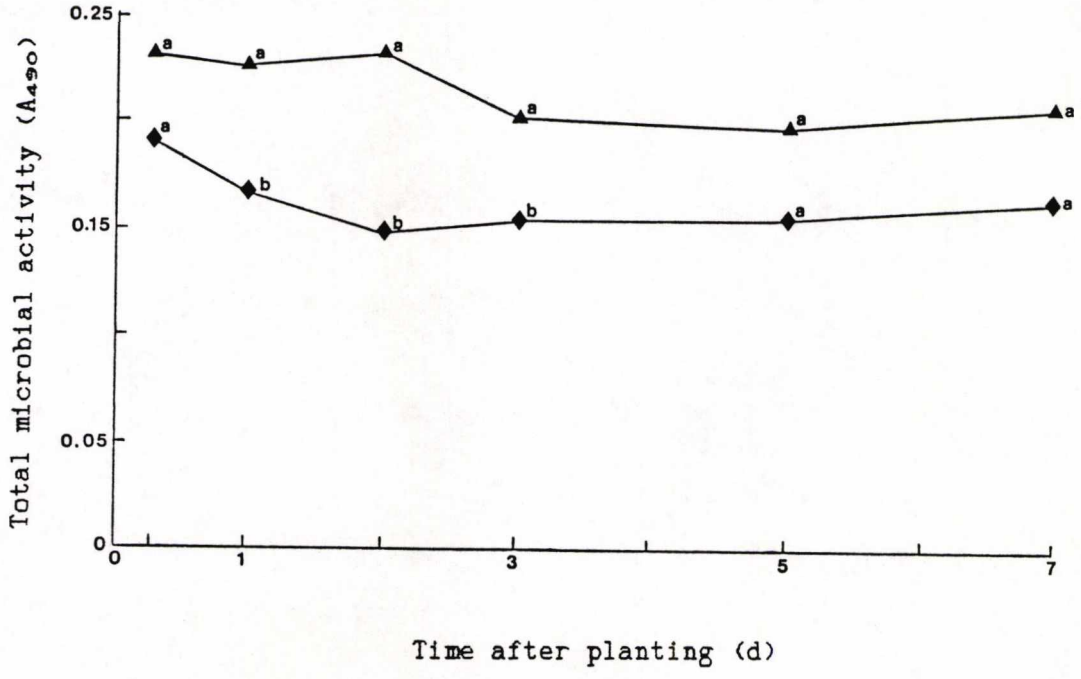


Fig. 6.8. Total microbial activity in P. ultimum-infested peat-based compost planted with sugar beet seed pellets treated with distilled water (▲) or Pen. claviforme culture filtrate extract (◆). Activity was assessed in terms of the extent of hydrolysis of fluorescein diacetate to fluorescein under standardized conditions (25°C, 90rev min^{-1}, 20min). Fluorescein concentration was measured spectrophotometrically at 490nm. For any one assessment time values followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

depressed microbial activity at both 1d (by 27.6%) and 2d (by 31.8%) after planting, compared to control pellets without additives.

In P. ultimum-infested compost, microbial activity in the spermosphere of pellets containing PCF was again significantly less than in that of control pellets at 1d and 2d, and also in this case at 3d after planting (26.6, 35.6 and 23.7% reduction, respectively, Fig. 6.8).

6.4.2 Microbial numbers

The effect of PCF on counts of the three major groups of soil micro-organisms in sugar beet seed pellet spermospheres was examined by dilution plating in selective media (section 2.6.7).

Pellets were treated in the laboratory with DW or PCF extract (0.30IU pellet^{-1}, section 2.5.2) and planted in peat-based compost, which was then wetted and incubated as given in section 2.6.2. At the time of planting, and at 2 and 5d later, samples of medium were removed and microbial populations enumerated as detailed in section 2.6.7. Each treatment was replicated three times and each dilution was plated in duplicate. Results are recorded in Table 6.1.

In the spermosphere of pellets without additives counts of fungi, bacteria and actinomycetes were similar at the three sampling times (4.9-5.3 x10^5, 3.5-3.7 x10^5 and 6.0-6.9 x10^4, respectively). At 2d after planting, the presence of PCF in the pellets had no significant effect on the numbers of fungi isolated from from the spermosphere. However populations of bacteria were reduced by 91.4%, and of actinomycetes by 55.2%, compared to those in the vicinity of pellets without additives. At 5d after planting, there were no significant

Table 6.1. Numbers of microorganisms isolated from the spermosphere of sugar beet seed pellets without additives or containing Pen. claviforme culture filtrate extract (PCF) planted in peat-based compost

Time after planting (d)	Pellet additive[a]	Microbial populations[b] (cfu g^{-1} dry wt. compost)		
		Fungi ($x10^{-5}$)	Bacteria ($x10^{-6}$)	Actinomycetes ($x10^{-4}$)
0	None	5.0	3.7	6.9
2	None	5.3 x	3.5 x	6.7 x
	PCF	4.8 x	0.3 y	3.0 y
5	None	4.9 x	3.5 x	6.0 x
	PCF	4.7 x	3.8 x	5.8 x

[a] Pellets were treated with distilled water or with 0.30IU PCF

[b] Counts of fungi, bacteria and actinomycetes were determined by dilution plating in surfactant-potato dextrose agar, pentachloronitrobenzene-nutrient agar and alkaline water agar, respectively. For each column and sampling time data followed by the same letter are not significantly different according to Duncan's new multiple range test (P=0.05).

differences between the two treatments for any of the microbial groups studied.

6.4.3 Generic composition of the spermosphere mycoflora

The composition of the fungal community in the spermosphere of sugar beet seed pellets was studied further. Twenty colonies were removed at random from isolation plates prepared from compost surrounding pellets without additives ('control spermospheres') and from those containing 0.30IU pellet^{-1} PCF ('PCF-spermospheres') at 2d after planting (section 6.4.2). Isolates were purified by successive transfer on MEA and their generic identity established by microscopic examination (section 2.6.7). Results are given in Table 6.2.

Both the range and the frequency of fungal genera isolated from the vicinity of the two pellet treatments was similar. In both cases the mycoflora appeared to be dominated by Deuteromycetes (15 out of 20 control spermosphere isolates and 16 out of 20 PCF-spermosphere isolates) with some Zygomycetes (3 out of 20 organisms from both spermosphere types). *Penicillium* and *Trichoderma* spp. were the most commonly isolated genera (6-7 out of 20 and 3-4 out of 20 from control and PCF-spermospheres, respectively).

6.4.4 In vitro antagonistic activity of fungal spermosphere isolates against P. ultimum

Twenty fungal isolates from control spermspheres and from PCF-spermospheres (section 6.4.3) were tested for the ability to retard the mycelial growth of *Pythium ultimum* *in vitro* using the cellophane overlay method described in section 2.3.1. The assay medium was MEA and overlay

Table 6.2. Fungal genera isolated from the spermosphere of sugar beet seed pellets without additives or containing Pen. claviforme culture filtrate extract (PCF) planted in peat-based compost

Genus	Frequency*	
	Control spermospheres	PCF-spermospheres
Alternaria	0	1
Aspergillus	1	0
Cladosporium	1	2
Fusarium	1	1
Gliocladium	2	1
Mucor	2	1
Penicillium	6	7
Rhizopus	1	2
Trichoderma	3	4
Verticillium	1	0
Others (non-sporulating)	2	1

* Colonies were removed at random from surfactant-potato dextrose agar isolation plates prepared from compost surrounding pellets treated with distilled water (control spermospheres) or with 0.30IU PCF pellet^{-1} (PCF-spermospheres) at 2d after planting.

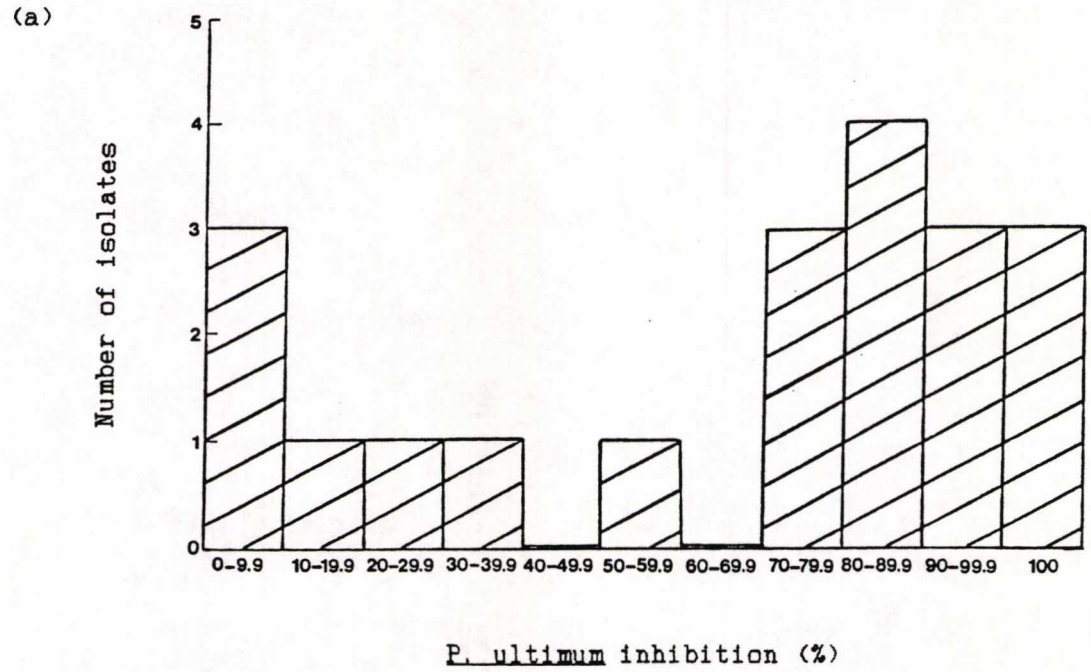
plates were incubated for 2-4d, depending on the rate of growth of the test organism, before removal of the cellophane and adhering microbial growth and inoculation with P. ultimum. Each isolate was tested in triplicate. Results are presented in Fig. 6.9.

A high proportion of the fungi obtained from compost proximal to pellets treated with either DW or with PCF showed anti-Pythium activity. Of the 20 control spermosphere isolates, three totally prevented pathogen growth and 14 caused greater than 50% inhibition. Amongst the PCF-spermosphere isolates the corresponding figures were 3 out of 20 and 13 out of 20, respectively. The Penicillium and Trichoderma spp. isolated were frequently highly antagonistic. Many of the Gliocladium, Aspergillus and Fusarium spp. also showed a substantial degree of inhibitory activity, while the Zygomycetes generally had only a small effect.

6.4.5 Sensitivity of fungal spermosphere isolates to PCF

Twenty fungal isolates from control spermospheres and from PCF-spermospheres (section 6.4.3) were tested for sensitivity to PCF on bioassay plates containing 1, 2, 5 or 10IU of the culture filtrate (i.e. at concentrations one, two, five or ten times that which completely suppressed the mycelial growth of Pythium ultimum, section 2.3.2). MEA was used as the test medium and all assessments were conducted in duplicate. Results were recorded as the minimum inhibitory concentration of those tested and are given in Fig. 6.10.

None of the fungi isolated from compost surrounding pellets treated with either DW or with PCF were inhibited by PCF at concentrations of less than 2IU per bioassay plate. One of the non-sporulating isolates



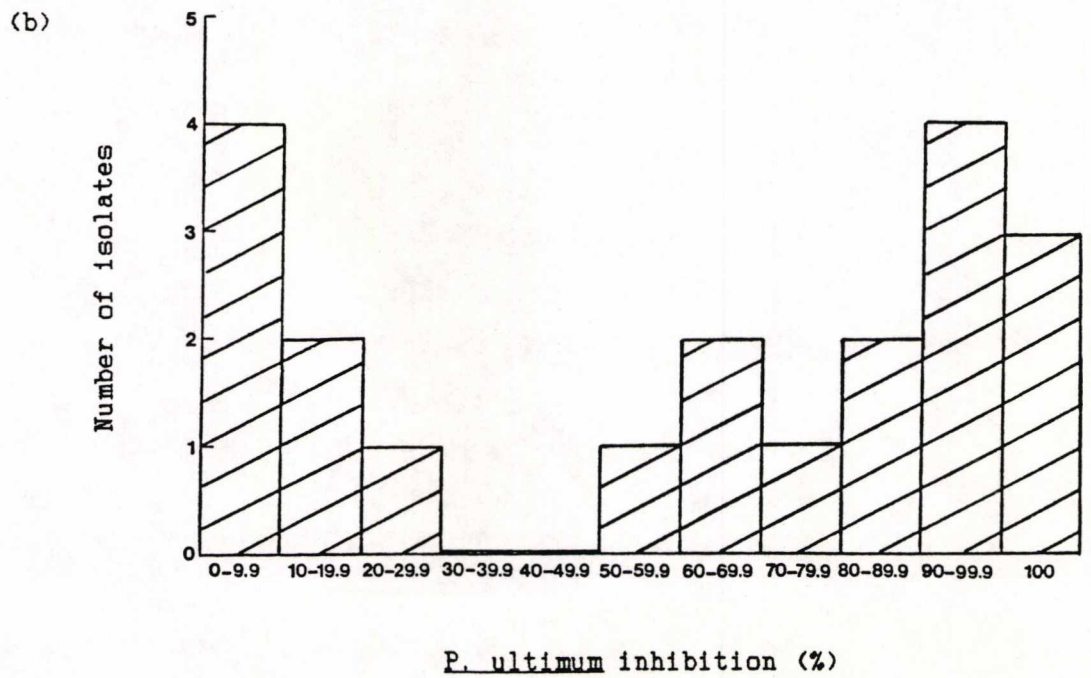
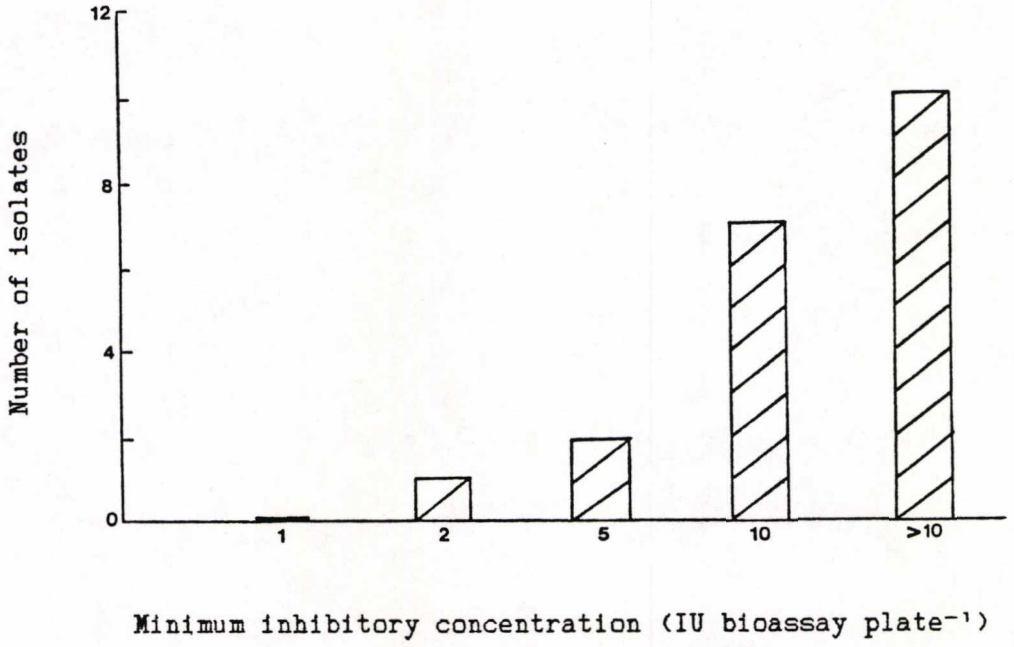


Fig. 6.9. *In vitro* inhibition of *P. ultimum* radial growth by fungi isolated from the spermosphere of sugar beet seed pellets treated with distilled water (a) or 0.30IU pellet^{-1} *Pen. claviforme* culture filtrate extract (b), planted in peat-based compost. Fungal colonies were removed at random from surfactant-potato dextrose agar isolation plates prepared 2d after planting.

(a)



(b)

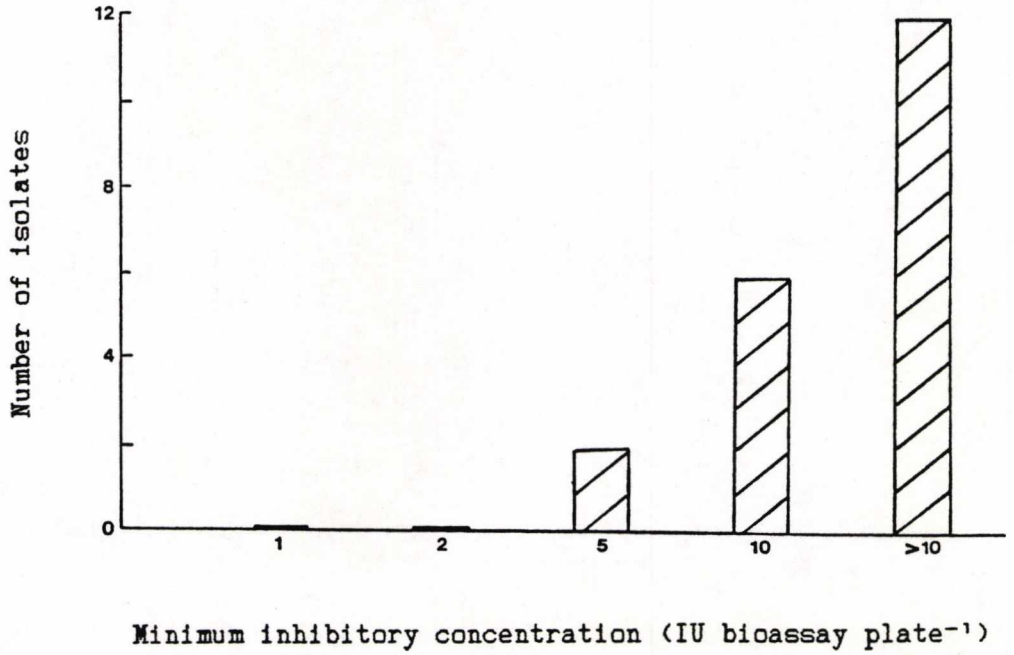


Fig. 6.10. Minimum inhibitory concentration of Pen. claviforme culture filtrate (PCF) in vitro against fungi isolated from the spermosphere of sugar beet seed pellets treated with distilled water (a) or 0.30IU pellet^{-1} PCF extract (b), planted in peat-based compost. Fungal colonies were removed at random from surfactant-potato dextrose agar isolation plates prepared 2d after planting.

was the most susceptible of the organisms tested, its growth being retarded by 2IU plate^{-1} PCF. The Zygomycetes were inhibited by 5-10IU plate^{-1}, but all other fungi were suppressed only by 10IU plate^{-1} or were not affected by any of the concentrations tested.

6.5 Discussion

6.5.1 Shelf-life

PCF extract gradually lost *Pythium ultimum*-inhibitory activity during 8wk storage in aqueous solution at room temperature, and its yellow-brown colour darkened with time (section 6.1). Similar observations have been recorded for patulin, the principle active component of the culture filtrate (Chapter 4). Thus, uv spectroscopy showed that the antibiotic was completely decomposed after 3 months in DW (Katzman *et al.*, 1944) and, while crystalline patulin is a colourless, neutral substance (Scott, 1974), solutions become yellow and acidic after standing for 2wk at room temperature (Birkenshaw *et al.*, 1943).

If necessary, the shelf-life of PCF in solution might be improved by storage at low temperatures. Incubation at 4°C or -20°C was found to have no detrimental effects on anti-*Pythium* activity at least for 1wk (section 4.2.4). Similarly, the use of buffers or solvents other than water may be advantagous. Jefferys (1952) showed that patulin retained its antibiotic properties for up to 40d in McIlvaine buffer over a pH range of 3.3-6.3, and Pohland & Allen (1970b) reported that patulin was

completely stable over long periods of time in chloroform, benzene and methylene chloride.

However, the requirement for a prolonged shelf-life is reduced if the proposed use of the antibiotic is as a seed pellet fungicide due to the practice of pelleting seeds annually at the beginning of the growing season (P. Halmer, Germain's (U.K.) Ltd.; personal communication). Water extraction of PCF-containing sugar beet seed pellets followed by bioassay of the extract indicated that in the pellet coating the culture filtrate retained greater than 80% of its P. ultimum-inhibitory activity for 24wk (Figure 6.3). A lack of substantial reduction in potency during the initial period after production was also suggested by the similarity of phytotoxic symptoms in germination tests, and of improvement in seedling establishment in Pythium-infested compost, during trials of PCF-pellets conducted 4wk apart (sections 5.1 and 5.2).

The mean achieved dose of PCF in commercially produced seed pellets, calculated from a sample of ten, was 0.30IU $pellet^{-1}$ (section 6.1). However, it is likely that this rate varied considerably between individuals due to the wide range in sugar beet seed size and density (Durrant, Dunning & Byford, 1986). Hence, although an accurate loading per unit weight of seed can be reliably achieved since loss of the active ingredient after treatment is essentially eliminated, the amount per pellet is less uniform (Longden, 1975). This problem is reduced in many countries by applying pellet additives to a unit (10^5 seeds) rather than a weight of seed (Durrant, Dunning & Byford, 1986). Because of the probable pellet-to-pellet variation in dose, and also the slow decline in activity over time in the commercially-prepared pellets, the effects of PCF on the pathogen population (section 5.3) and the saprophytic

spermosphere microflora (section 6.4) in compost were investigated using pellets treated in the laboratory with a known amount of the culture filtrate immediately prior to the experiments.

6.5.2 Rate of release

PCF was rapidly liberated from sugar beet seed pellets in both DW (section 6.1) and in moist peat-based compost (section 6.2). Processes involved in the release of toxicants from pesticide granules prepared by an agglomeration process, similar in principle to that used to produce seed pellets, include both leaching and mechanical breakdown (Furmidge, Hill & Osgerby, 1966). In the case of the seed pellets themselves, the rupture of the coating at germination is also likely to affect the discharge of residual additives.

Various strategies are available by which the release of chemicals from seed pellets may be manipulated, such as altering their position within the coating and varying the nature of the filler and binding agents used during their manufacture. For example, the % of furathiocarb released from sugar beet pellets after 8h was increased 2.4-fold by placing the insecticide in the penultimate rather than a deeper layer of the coating, and 1.7-fold by placing it in the outermost layer with a water-soluble instead of a water-insoluble binding agent (Elmsheuser et al., 1988).

Methods to delay or retard the discharge of active ingredients are especially useful when, as with PCF (section 6.3), their half-life in the plant growth medium is short (Lewis & Cowsar, 1977). However, the physical and chemical properties of the toxicant itself may restrict the extent to which this is possible (Furmidge, Hill & Osgerby, 1968). In

particular when the compound, like PCF, is highly soluble in water the degree of control which may be achieved is limited. Greater opportunities may be presented in this respect by the technique of film-coating with synthetic polymers (Clayton, 1988).

6.5.3 Persistence

The *Pythium ultimum*-inhibitory components of PCF showed greater persistence in sterile than in non-sterile seedling growth media (section 6.3), indicating that microbial degradation was an important factor in their inactivation. The half-life of the metabolites in non-sterile media of a matter of hours was comparable with values of 24h or less recorded for patulin in soil (Gottlieb, Siminoff & Martin, 1952; Norstadt & McCalla, 1968). Many of the antibiotics used on a commerical scale as crop protection agents also have only a limited existence in the environment. For example, validamycin A (section 1.4.1) has a half-life of less than 4h in soil following application to control rice sheath blight (Wakae & Matsuura, 1975).

The observation that the *Pythium* inhibitors in PCF were less stable in non-sterile soil than peat-based compost was consistent with the higher levels of microbial activity in this medium (section 6.4.1). Contrary to results recorded for certain other antibiotics (Wright & Grove, 1957), no lag period in the inactivation of PCF was detected in either medium, perhaps reflecting the simple structure of the patulin molecule (Fig. 4.17) which may render it a readily available substrate to non-sensitive microorganisms. The identity of the degradation products of PCF was not studied, and the ultimate fate of patulin in soil is also unknown (Pohland & Allen, 1970b)

Some inactivation of PCF also occurred in sterile media, suggesting that chemical or physical processes were also involved. By contrast, the quantities of patulin extractable from sterile soil were reported to be constant for up to 7d (Gottlieb, Siminoff & Martin, 1952). PCF was found to be chemically stable *in vitro* at pH values spanning those of both soil and compost (Table 2.3 and section 4.2.5), but it is possible that the anti-*Pythium* components were sorbed to a certain extent by colloids in the media. This explanation would be compatible with the lesser stability of PCF in sterile compost, with its high proportion of organic matter, than in soil, and also the lower % recovery from the former medium immediately after addition under both sterile and non-sterile conditions. Sorption to organic material is one of the main factors affecting the behaviour of synthetic pesticides in soil (Bromilow, 1988) and there is some evidence that it also plays an important role in the inactivation of certain antibiotics such as cycloheximide (Gottlieb, 1976).

6.5.4 Role of the spermosphere microflora

PCF was released from sugar beet seed pellets within hours of sowing in peat-based compost (section 6.2) and equally rapidly lost *Pythium ultimum*-inhibitory activity in this medium (section 6.3). Yet its incorporation into the pellets was effective in decreasing not only pre-emergence *Pythium* infection, but also post-emergence disease (Table 5.3) which occurred at least 3d after planting. Thus it appeared that an additional, indirect effect could be involved in the PCF-induced control of the pathogen, supplementing the known short term direct activity of the culture filtrate on its mycelial growth and propagule germination

(sections 3.2.1 and 3.2.3). A chemical control treatment which also includes biological components in this way is a form of integrated protection (section 1.3.4).

Indirect modes of action of antifungal agents mediated *via* the resistance of the host plant are an area of considerable current interest (Wade, 1984). For example, fosetyl-aluminium shows only low fungitoxicity in agar but is very effective against certain Oomycete phytopathogens in plant tissue where it may act by stimulating the synthesis of phytoalexins. However, such an effect appeared unlikely in the current work given the juvenility of the host plant and thus its lack of both active and passive resistance mechanisms (section 1.1.3).

Alternatively, chemicals may provoke integrated control by their influence on microbial antagonists of the pathogen (section 1.2.2). This phenomenon is consistent with the key role of the associated saprophytic microflora in limiting infection, particularly by soil-borne organisms (section 1.1.2), and can be considered as a type of induced suppressiveness (section 1.1.3).

Application of certain types of fungicides to soil often causes an initial depression and then a flush of microbial activity (section 1.2.2), most commonly measured in terms of soil respiration or total numbers of organisms. Similarly, treatment with some antibiotics is reported to stimulate microbial activity (Waksman & Woodruff, 1942; Nissen, 1954; Hervey, 1955; Wright & Grove, 1957; Souilides *et al*, 1962). *Pythium* spp. are poor competitors, as shown by the promotion of both their saprophytic (Katan & Lockwood, 1970) and pathogenic (section 1.2.2) development following elimination of opponents with selective chemical treatments. Conseqeuently, it was conceivable that enhanced

antagonism from a PCF-activated spermosphere microflora could have contributed to an integrated control of P. ultimum by the culture filtrate in the present studies. This would have corresponded to so-called general antagonism proposed as a basis for the observed properties of a number of suppressive soils and compost-amended container media (section 1.1.3).

Total microbial activity was here assessed by measurement of the formation of fluorescein from fluorescein diacetate (FDA, section 6.4.1). The ability to hydrolyse FDA appears to be widespread in the microbial kingdom (Medzon & Brady, 1969), and a close correlation has been observed between this activity and respiration in samples collected from different depths in an agricultural soil (Schnürer & Rosswall, 1982).

Total microbial activity in non-sterile peat-based compost was found to be less than that in a field soil. Likewise, respiration has been reported to be lower in peat in situ than in soil (Kavanagh & Herlihy, 1975) due to its anaerobic condition and high acidity (section 5.6.3). Although the pH of container media is raised during preparation by liming (Sheard, 1975), the microflora generally remains restricted compared to soil unless organisms are accidentally or intentionally introduced (Hoitink & Fahy, 1986).

Microbial activity was not significantly greater in the vicinity of control seed pellets than in unplanted compost at any time during the 1wk experimental period. Since evidence has been presented (Osburn et al., 1989) that exudation from raw sugar beet seed is sufficient to stimulate bacterial activity within 4h of planting in soil, these data again suggested a reduction in the quantities of exudate diffusing into

the surrounding medium as a result of the pelleting treatment (section 5.6.2).

The incorporation of PCF into the seed pellets significantly diminished microbial activity at 1-2d after planting, but the magnitude of the response (27.6-31.8%) was less than the 50% value considered to be critical in presently recommended tests for the side-effects of synthetic pesticides on soil microorganisms (section 1.2.2). Furthermore, the duration of the perturbation (2-3d) was well within the 30d recovery period which often follows disturbances caused by natural stresses (Domsch, Jagnow & Anderson, 1983). However, more extensive testing would be required to fully investigate the possible consequences of PCF treatment on the microflora of plant growth media. For instance, current protocols require assessment of effects on specific transformation processes essential to soil fertility, in addition to total activity (Somerville et al., 1987). Patulin, in culture filtrates of Penicillium patulum, has been found to inhibit the growth of Rhizobium meliloti and Rhizobium trifolii in vitro (Gregory et al., 1952). Hence PCF may have an adverse effect on nitrogen fixation in situ and thus be unsuitable as a seed treatment for legumes. Recommendations also include testing at both normal and ten times normal field rates, and in at least two different soil types. However, even with such additional data, considerable difficulties remain in extrapolating the results to use conditions (Heitefuss, 1989).

At no point in the present experiments was microbial activity greater in PCF- than in control spermspheres. The investigation was subseqently repeated in compost infested with P. ultimum as the stimulation of activity in pesticide-treated soils has been attributed

largely to the decomposition of killed biomass by surviving or reinvading organisms (Jenkinson & Powlson, 1976). In the presence of the susceptible pathogen the duration of significant depression of microbial activity was prolonged by 1d compared to that in non-infested compost, but the degree of response was similar. It was therefore concluded that elevated general antagonism was not the cause of the extended PCF-induced control of P. ultimum.

Again by analogy with suppressive plant growth media (section 1.1.3) integrated control of plant diseases initiated by chemicals and mediated via the saprophytic microflora may also involve qualitative changes in the microbiological balance in favour of organisms specifically antagonistic to the pathogen (Bollen, 1979; Papavizas & Lewis, 1988). The earliest recorded example of this type of interaction was the control of the root pathogen Armillaria mellea in citrus orchards after treatment with carbon disulphide at concentrations which are sub-lethal in sterile soil (Bliss, 1951). The protective effect of the fumigant was attributed in part to the antagonistic activities of Trichoderma spp. which were isolated more frequently from treated than from non-treated soil.

The participation of resident antagonists has also been implicated in disease control by certain non-volatile fungicides, including both synthetic chemicals and antibiotics (Richardson, 1954, Stǎnková-Opocěnská & Dekker, 1969; Pinckard, 1970; Chinn, 1971; Wakae & Matsuura, 1975; Langerak, 1977; Malajczuk et al., 1983). For instance, Langerak (1977) proposed that treatment of narcissus bulbs with methoxy ethyl mercury chloride, thiram or pimaricin protected the roots from infection from Fusarium oxysporum ff.spp. after levels of the compounds had fallen

below those toxic to the pathogens by selectively favouring the development of saprophytic Penicillium and Trichoderma spp. These fungi were antagonistic to F. oxysporum ff.spp. in vitro and afforded protection against root rot in soil when bulbs were coated with their spores.

The effect of PCF on the populations of the three major soil microbial groups following its incorporation into seed pellets was investigated by dilution plating (section 6.4.2). This method has substantial limitations since it indicates only those organisms capable of proliferation to form a visible colony under one particular set of nutritional and environmental conditions supplied in the laboratory, which are usually far removed from those obtaining in the field (Stotzky, 1972). The enumeration of fungi and actinomycetes in this way can be especially misleading since colonies arise predominantly from spores and other structures of low metabolic activity rather than from active biomass (Griffin, 1972). Nevertheless, the technique does have comparative value, provided that the test procedure is rigorously standardized, and has proved a useful tool in the study of side-effects of synthetic agrochemicals on non-target soil microorganisms (Anderson, 1978a).

In samples of peat-based compost which had not been exposed to PCF numbers of bacteria per g dry wt. (3.5-3.7 $x10^{6}$) were at the lower end of the range typically recorded in agricultural soils (10^{6}-10^{9} g^{-1}, Lethbridge & Lynch, 1987). The proportion of actinomycetes (1.7-1.9%), which generally comprise 10-33% of the total bacterial population (Atlas & Bartha, 1987), was especially low. Fungal counts in compost (4.7-5.3 $x10^{5}$ g^{-1} dry wt.) were comparable to those reported in soils

(10^4-10^6 g^{-1}, Lethbridge & Lynch, 1987). Thus, although the qualification regarding the comparison of absolute numbers of organisms between the two types of media due to their disparate physico-chemical properties (section 5.6.3) is again applicable, the proportion of fungi appeared to be greater in peat-based compost, perhaps due to its lower pH to which this group as a whole are less sensitive than are bacteria (Stotzky, 1972).

At 2d after planting, the numbers of bacteria isolated from the spermospheres of pellets containing 0.30IU $pellet^{-1}$ PCF were reduced by 91.4% and of actinomycetes by 55.2% compared to those in control spermospheres, coinciding with the depression in total microbial activity (section 6.4.1). By contrast, the fungal count was not affected by the treatment. These results agreed with published information on the antimicrobial spectrum of patulin. The antibiotic is reported to inhibit the growth of most bacteria to a certain extent whereas fungi vary in their sensitivity (Singh, 1967). However, the apparent stability of the fungal population may have been an artefact of the dilution plating technique, relecting the fact that spores are generally less vunerable to physical and chemical stresses than is active biomass (Park, 1965). By 5d after planting there were no significant differences between PCF- and control spermospheres in the counts of any of the microbial groups examined, consistent with the recovery in microbial activity by this time. These data showed some similarities with those recorded by Gottlieb et al. (1952) who evaluated the response of the soil microflora to the addition of 50μg g^{-1} patulin (approximately 1IU PCF equivalent, section 4.6.2). At 4d after application numbers of fungi were increased 11.2-fold compared to those

in soil without the antibiotic while bacteria and actinomycetes were less affected (2.5-fold increase and 50.1% decrease, respectively). At 7d the population of fungi was 57.2-fold greater in patulin-treated soil whereas those of bacteria and actinomycetes were similar to those in control soils.

In further investigations into the potential of spermosphere microorganisms to function as a component of the proposed PCF-induced integrated control of P. ultimum studies were focussed on the saprophytic fungi, rather than the bacteria or actinomycetes, because the compost appeared to favour their presence in control spermospheres and they appeared insensitive to the culture filtrate in PCF-spermospheres. The mycoflora of compost adjacent to the two pellet treatments was studied 2d after planting, when the concentration of PCF itself had declined to levels sub-inhibitory to Pythium.

The fungal populations of both control and PCF-spermospheres was dominated by Deuteromycetes with some Zygomycetes (section 6.4.3). This is typical of aerobic soil dilution plates which promote the isolation of species which sporulate profusely (Griffin, 1972). Penicillium and Trichoderma spp. were particularly common and the former have been found in the container medium under study by other investigators (P.L. Waller, Fisons plc, Levington Research Station; personal communication). Penicillium and Trichoderma, together with Acremonium and Mortierella, are also among the most abundant fungi in peat in situ (Kavanagh & Herlihy, 1975).

The generic composition of the fungal community in the two spermosphere types appeared to be similar, but a sample of 20 isolates from each may have been too small to detect any subtle qualitative

changes. Kuter et al. (1983) examined 200-300 fungal isolates from composted hardwood bark-amended container media suppressive or conducive to Rhizoctonia solani and found no differences in the range of taxa present, but were able to detect variations in their relative abundance.

A large proportion of the fungi isolated from both control and PCF-spermospheres 2d after planting were capable of inhibiting the growth of P. ultimum in vitro in the absence of physical contact (section 6.4.4). These organisms thus possessed the potential to suppress Pythium infection in situ given the requisite nutritional and environmental conditions. A high degree of antagonism was shown particularly frequently by Penicillium and Trichoderma spp. which are amongst the fungi most commonly used as introduced biocontrol agents of Pythium (Harman & Hadar, 1983). Antagonists with a mycoparasitic mode of action were not enumerated.

No quantitative differences were found in the prevalence of potential P. ultimum antagonists in control and PCF-spermospheres. A number of scenarios can be proposed to account for this observation which nevertheless allow for a possible role of such organisms in PCF-initiated Pythium control.

Firstly PCF could itself have stimulated antagonistic activity. Langerak (1977) reported that the antibiotic pimaricin (section 5.6.5) enhanced the in vitro synthesis of antibacterial metabolites by Cylindrocarpon destructans isolated from narcissus roots and that pimaricin and C. destructans acted synergistically in controlling narcissus bulb rot in the field.

Secondly PCF may have rendered the spermosphere environment more conducive to antagonistic activity. Nelson, Kuter & Hoitink (1983)

found that the induction of suppressiveness to R. solani in composted hardwood bark-amended container media was dependent not only on the introduction of Trichoderma harzianum but also on undefined factors associated with compost age which affected the antagonistic activity of the inoculants. In the present work, the decrease in numbers of bacteria in the spermosphere caused by the addition of PCF to the pellets could conceivably have resulted in greater quantities of seed exudates available for generation of antibiotic precursors by the putative fungal antagonists.

A third possible interpretation of the results is that sub-lethal concentrations of PCF debilitated surviving P. ultimum propagules. This could have reduced their inoculum potential directly (section 5.6.3) or predisposed them to levels of microbial antagonism to which fully viable propagules were not susceptible. It is now recognized that sub-lethal doses of fumigants stress A. mellea, manifested for example by a delay in resumption of mycelial growth, and that this is a prerequisite for biocontrol by Trichoderma spp. (Munnecke et al., 1981). Similar mechanisms are thought to function in the control of soil-borne pathogens by solar heating (section 1.3.2), especially in deeper soil layers where the increases in temperature alone are not enough to account for the observed suppressive effects (Katan, 1981).

A fourth factor which was likely to have been involved in the phenomena seen in the present work was the relative sensitivity of saprophytic spermosphere microorganisms to PCF compared with that of P. ultimum. All fungal isolates tested from both control and PCF-spermospheres were less susceptible to the culture filtrate than was the pathogen (section 6.4.5). This suggested that even fungi not capable of

producing metabolites which directly inhibit Pythium may nevertheless reduce disease by virtue of their relative PCF-insensitivity such that they are able to colonize affected volumes of medium and prevent reinfestation by the pathogen. The lesser sensitivity of Trichoderma spp. to fumigants such as carbon disulphide compared to that of A. mellea, combined with their rapid growth rate, is thought to be an important attribute with respect to their participation in the control of this fungus in fumigated soils (Munnecke, Kolbezen & Wilbur, 1973).

A supplementary, indirect effect of a fungicide is indicated where protection is longer lasting than the original chemical and/or occurs at sub-inhibitory concentrations. More definitive evidence may be obtained if it can be shown that disease control is reduced or eliminated in sterile compared to non-sterile plant growth media (Bollen, 1979). Attempts to conduct such an experiment in the present work were unsuccessful since both control and PCF-pellets failed to germinate in autoclaved peat-based compost, perhaps due to the release of phytotoxic substances from the large quantities of organic matter present. Even had seedling growth taken place, the greater persistence of the P. ultimum-inhibitory agents in sterile media (section 6.3) may have ameliorated any reduction in effectiveness due to the absence of the proposed biological component of the treatment, complicating the interpretation of results.

In summary it can be stated that the data collected demonstrated the existence of microorganisms in the sugar beet pellet spermosphere with the physiological potential to reinforce the potent but transient antagonistic effect of PCF on P. ultimum. Thus the indigenous microflora may have contributed to the observed protection of seeds and

seedlings from Pythium infection which followed the incorporation of the culture filtrate extract into the pellet coatings.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

There is evidently a continuing need for agents which effectively combat plant pathogenic fungi. In 1967 it was estimated that 11.8% of potential world crop yields were lost to fungal diseases even with the control measures in use at the time (Agrios, 1988). The trends of intensification and towards genetic uniformity in modern agricultural and horticultural systems have generally aggravated disease problems such that new resistant crop cultivars are often quickly rendered obsolete (section 1.3.1) amd traditional cultural methods of control (section 1.3.2) are no longer alone sufficient to attain the required levels of productivity.

At present the attack on fungal phytopathogens depends primarily on treatment with chemical control agents (Brent, 1984; section 1.2.1), which are largely of synthetic origin. Modern fungicides, where available, are often highly effective (Barnes, 1984), but a number of problems accompany their development and large-scale application.

The aspect currently attracting most public concern is that of perceived detrimental effects on the environment and on the consumer (section 1.2.2). However, major efforts are made by both the regulatory authorites and the manufacturing companies to ensure that agrochemicals are as safe as possible. In Britain the Food and Environment Protection Act 1985, implemented through the Control of Pesticides Regulations 1986 (Anon, 1986b), requires the submission of extensive data covering

environmental, toxicological and residue studies on each new product, in addition to proof of efficacy. This information is evaluated by the independent Advisory Committee on Pesticides, and only if this panel of experts is fully satisfied will the approval necessary to market the compound be recommended (Anon, 1988). All pesticides are now subject to a full review 10 years after the date of the first approval.

A second problem inherent in the reliance on chemicals for plant disease control is that of the rapidly rising expenditure involved in introducing new fungicides (section 1.2.1). This not only results in higher food prices but also reduces the commercial viability of developing selective compounds (section 5.6.4) or those active against pathogens other than those predominating in major world crops. The escalation in development costs arises partly from the more demanding registration requirements and also because it is becoming harder to find chemicals with advantages over existing agents. In 1985 the discovery rate was estimated to be approx. one marketed product per 16,000 compounds screened, compared with one in 5,500 in 1969 (Green, Hartley and West, 1987). New chemicals must show significant improvements in terms of efficacy, safety, systemic properties or mode of action (Ryley & Rathmell, 1984).

Fungicides which act at novel biochemical sites in the pathogen are of critical importance (Baldwin & Rathmell, 1988), both to counter pathogens for which there is at present no satisfactory treatment (particularly root and vascular diseases), and to deal with the third difficulty associated with the use of certain chemical control agents, that of fungicide resistance (section 1.2.3). Such compounds are needed to replace existing materials which are no longer effective, or

preferably to extend their active life *via* the use of mixtures or alternating programmes.

Against this background microbial metabolites, and in particular antibiotics, could make a valuable contribution to crop protection. This has been appreciated in Japan for some years where they are in commerical use not only as fungicides (section 1.4.1), but also as insecticides and herbicides (for example tetranactin and bialaphos, respectively; Misato, 1983).

Antibiotics are frequently selective and are biodegradable. Both attributes diminish the risk of any adverse effects on the environment or on the consumer (section 1.4.2). Furthermore, antibiotics often possess useful systemic properties (section 1.4.3) and may show curative in addition to protective activity (Heitefuss, 1989). Many are also highly potent. For example, blasticidin S (section 1.4.1) is 100 times as active against rice blast as the organomercury fungicides it replaced (Green, Hartley & West, 1987), though the comparison with the latest synthetic fungicides may be less impressive (Powell & Griffin, 1989). Perhaps the paramount feature from the viewpoint of the agrochemical industry however is the enormous variety of microbial products (section 3.4.2) which can therefore increase the diversity of structures available during screening for useful biological activity (Baldwin & Rathmell, 1988). Moreover, many effective antifungal antibiotics have been found to exhibit novel modes of action (Baldwin, 1984). Thus validamycin A (section 1.4.1) is not fungicidal *in vitro* but has a so-called paramorphogenic effect on growing mycelium, decreasing the hyphal extension rate without affecting the specific growth rate (Trinci, 1984). In the plant it may exert control by inhibiting penetration or

reducing the rate of spread of the pathogen. Like some synthetic chemicals (section 6.5.4) antibiotics may also operate by stimulating host defence mechanisms (Crosse et al., 1960).

After nearly 50 years of intensive research new antibiotics are still being discovered in the pharmaceutical industry at a rate of 250-300 per year (Bérdy, 1980). This is due to a number of developments, some of which may also be relevant to the search for metabolites active against plant pathogens. For instance, screening techniques with greater specificity and sensitivity have been introduced, such as those for compounds operating on particular enzymes or receptor sites of known pharmacological importance (Fleming, Nisbet & Brewer, 1982). This principle has been extended into the field of antibiotic fungicides where, for example, use of a chitin synthetase enzyme system resulted in the detection of neopolyoxin A which was effective against Sphaerotheca fuliginea in glasshouse tests (Ōtake, 1983). However, it must be recognized that such methods suffer from the same limitations as any other in vitro assay procedures (Baldwin, 1984; section 3.4.1). Other factors which have contributed to the continuing discovery of novel pharmaceutical antibiotics include the sceening of rare or fastidious microbial species, and organisms isolated from unusual sources (Vandamme, 1984), the adoption of diverse media and cultural conditions to provoke broad gene expression (Hütter, 1982) and advances in detection and separation techniques which permit the isolation of minor components of culture filtrates (Bérdy, 1980). Again, many of these innovations could be equally applicable to the examination of microbial cultures for fungicidal activity.

In the current work, metabolites which effectively protected sugar beet seeds and seedlings from Pythium ultimum-induced disease were found after screening only 23 cultures of microbial genera reported as biocontrol agents of the pathogen (sections 5.2 and 3.1), confirming the validity of the approach. However, the major active component of the culture filtrate, the antibiotic patulin (Chapter 4), is a well-characterized compound and has been evaluated previously as a plant disease control agent in various pathosystems (section 5.6.2). In view of the preceding discussion the lack of novelty was perhaps not surprising given that the producer fungus, Penicillium claviforme, was obtained from a culture collection and was grown in a common mycological medium under standard conditions.

Together with their desirable properties, there are certain disadvantages of microbial metabolites with regard to their deployment as plant disease control agents which must be taken into consideration. For instance, their active life in the environment may be too short to achieve the duration of protection required (Brian & Hemming, 1945), though in the present studies the rapid inactivation of the P. ultimum inhibitors in the plant growth medium (section 6.3) did not preclude significant suppression of disease. Several antibiotics showing promising activity against phytopathogens in vitro have been found to be too phytotoxic for general use (Heitefuss, 1989), but again this was not a problem in the current investigations when the anti-Pythium metabolites were tested in situ (section 5.6.1). Contrary to popular belief, natural products, including antibiotics, may also be harmful to animals and man. Hence the safety testing of those proposed as agrochemicals needs to be just as rigorous as that of synthetic agents

(Powell & Griffin, 1989). The mammalian toxicity and possible carcinogenicity of patulin (section 4.8.3) is likely to prevent its practical exploitation in the current regulatory climate despite its rapid decomposition. However, the short term danger associated with this particular microbial product does not detract from the potential value of others in crop protection (Paterson, Simmonds & Blaney, 1987).

Another problem which may be encountered during the treatment of plant diseases with antibiotics is that of resistance arising within the pathogen population (sections 1.4.4 and 5.6.5). Unfortunately this phenomenon appears to be associated with the use of compounds with a specific mode of action (section 1.2.3). The selection pressure accompanying the application of rapidly degraded metabolites such as patulin however would appear to be minimal.

Finally, the cost of large-scale antibiotic production is an all-important factor which was not addressed in the present work and is notoriously difficult to estimate (Stowell & Bateson, 1983). Fermentation is generally a more expensive method of manufacture than chemical synthesis (Lethbridge, 1989), but a fermentation plant has the advantage of greater versatility (Misato, 1983). Thus the same equipment can be used to obtain a variety of metabolites depending on the organisms cultured and the growth conditions. Moreover, the raw materials for antibiotic production are usually based on renewable, agricultural resources rather than petrochemicals (Misato & Yoneyama, 1982). Optimization of the yield of a given process is essential to maximize its economic viability (Stanbury & Whitaker, 1984) and recent advances in techniques for the genetic modification of microorganisms have considerably improved the prospects in this area (section 3.4.2).

In some cases it may be beneficial to exploit microbial metabolites in ways other than as the natural products themselves (Powell & Griffin, 1989). Thus it may be more cost-effective to produce those of relatively simple structure by chemical synthesis. For example cellocidin, used to control rice leaf blight in Japan, is synthesized from 1,4-dihydroxybutyne or fumaric acid (Green, Hartley & West, 1987). Naturally-occurring antibiotics can also be used as 'leads' for the production of semi-synthetic derivatives which may show improvements relating to fungicidal activity (Rodgers, 1989), stability (section 5.6.2), phytotoxicity (section 5.6.1) or mammalian toxicity (Bérdy, 1974). Lastly, antibiotics have been valuable in revealing potential targets for inhibition by antifungal agents (Baldwin, 1984). In the future it may be possible to design molecules to selectively act at these sites, but as yet the requisite knowledge is not available (Baldwin & Rathmell, 1988).

Although they are tested for possible side-effects as meticulously as many pharmaceutical products, crop protection chemicals, whether natural or synthetic, are used in far greater quantities and are intended to kill certain living organisms (Lisansky, 1989). Thus it is clearly prudent to restrict their implementation to the minimum compatible with efficient food production (Anon, 1983). There are several means by which fungicide inputs may be reduced whilst productivity is maintained, for example by the development of reliable disease prediction systems (Jones, 1983), and by advances in formulation (Zweig, 1977) and application (Coffee, 1981) technology. The mass introduction of microbial antagonists of plant pathogens (section 1.3.3) can be an effective alternative to chemicals in certain situations, such

as those where the population of indigenous organisms is low and where the cropping environment can be controlled (Lynch, 1988b, Campbell, 1989).

However, it is now widely agreed that the strategy with the greatest potential for obtaining maximum benefit from crop protection measures at minimum financial, social and environmental cost, is that of integrated control (Dickinson & Lucas, 1982; Jones, 1987; Agrios, 1988). The concept in its broadest sense recognizes not only the value of combining the most suitable of a range of management techniques (section 1.3.4), but also the pre-eminent importance of natural regulatory agents in the agroecosystem (Green, Hartley & West, 1987; Heitefuss, 1989). Additionally, the proponents of integrated crop protection programmes argue that the aim of totally eliminating phytopathogens should be replaced with the more realistic policy of keeping their populations below the threshold values which cause economic damage (Andrews, 1983; Papavizas & Lewis, 1988). At the same time it is acknowledged that the successful adoption of such an approach will need a greater understanding of the complex interactions and interrelationships between the pathogens and their hosts, antagonists and abiotic environment than is currently possessed. Nevertheless, some results obtained in the present work and discussed in section 6.5.4 suggested that significant control of certain soil-borne pathogens may be attained from chemicals present at only low levels providing they are sufficiently selective so as not to harm indigenous microbial antagonists, and thus support, rather than eliminate, the natural suppressive qualities of the plant growth medium.

Some of the fungi isolated from peat-based compost during the present studies which were highly inhibitory to P. ultimum in vitro (section 6.4.4) have been shown to be effective as introduced biocontrol agents, in the absence of any chemical treatment (Annen, 1989). Hence the next logical step in the context of the current research objectives (section 1.5) would appear to be to study the products of these organisms as fungicidal agents in their own right, with the aim of finding compounds with advantages over patulin in terms of novelty, efficacy, stability and mammalian toxicity.

The high percentage of the fungal isolates capable of producing anti-Pythium metabolites supports the view of many experienced microbiologists in the pharmaceutical industry that microorganisms constitute a rich stock of novel compounds with potentially useful biological activity (Hall, 1989). This vast natural resource has barely begun to be tapped in the agrochemical sector. The 'power of the microbe' (Foster, 1964) should not be underestimated.

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APPENDIX

Rate of incorporation of PCF into seed pellets

A minimum rate of inclusion of PCF in seed pellets was calculated on the basis of achieving a concentration in the water added to the compost at planting sufficient to cause total inhibition of *Pythium ultimum* mycelial growth:

Quantity of water added to each wedge cell

= 8ml

Quantity of PCF per wedge cell necessary to achieve a concentration of 1.00IU ml^{-1} in water = 8 x 1.00 = 8.00IU

However, during the *in vitro* estimation of anti-*Pythium* activity (section 2.3.2), culture filtrate extracts were diluted $x^1/_{20}$ in CMA, therefore quantity of PCF per wedge cell necessary to achieve total inhibition of *P. ultimum* mycelial growth

= 8 / 20 = 0.40IU

As 4 pellets were planted per wedge cell, the minimum quantity of PCF required per pellet = 0.40 / 4 = 0.10IU

The validity of this figure was ascertained using hymexazol (section 2.5.2) as a standard. The recommended rate of the fungicide, as the commercial product Tachigaren, in sugar beet pellets is 15g kg^{-1} seed, which is equivalent to approx. 0.15mg $seed^{-1}$ for the cultivar Accord (calculated by weighing 10 samples each of 20 seeds). The quantity of Tachigaren estimated by bioassay (section 2.3.2) to cause *P. ultimum*

inhibition equivalent to 1.00IU PCF was approx. 1.70mg. Therefore the recommended dose of the fungicide is equivalent to 0.15 / 1.70 = 0.09IU.

Tachigaren reduced P. ultimum colony density in addition to diameter, which was not taken into account during determination of its activity, so the above figure was greater than the true value, but nevertheless allowed the conclusion that the calculated rate of incorporation of PCF in the pellets was in the correct range.

Following evaluation of any adverse effects of PCF on sugar beet seedling development (section 5.1) it was decided to aim for a rate of 0.30IU per pellet.

Thus the minimum quantity of PCF required for 0.25 unit seed

= 25,000 x 0.30 = 7500IU

For a volume of 20ml extract, minimum concentration of PCF required

= 7500 / 20 = 375IU ml^{-1}

Actual concentration of prepared extract was 410IU ml^{-1}, therefore target dose per pellet = 410 x 20 / 25,000 = 0.33IU.

Abstract of a poster presented at
111th Meeting of the Society for General Microbiology,
University of Warwick, 11-14th April 1988.

CONTROL OF PYTHIUM ULTIMUM WITH ANTAGONISTIC FUNGAL METABOLITES

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The exploitation of a biocontrol agent requires that the inoculant survives in the soil and that it expresses its beneficial properties. These are problems that may be avoided by the direct use of antagonistic microbial products such as those described here for the control of damping-off disease caused by Pythium ultimum.

Microorganisms which inhibit the mycelial growth of P. ultimum due to the production of extracellular metabolites were isolated using a cellophane overlay method. Subsequently an agar plate bioassay was used to screen for antagonists which produce high concentrations of inhibitors in liquid culture. The most promising isolate was Penicillium claviforme, culture filtrates of which not only restricted P. ultimum mycelial development but also propagule germination. Conditions of optimal production of the inhibitory agents (designated PCF and which probably include the antibiotic claviformin) were determined and concentration was achieved by vacuum evaporation followed by solvent extraction. [Activity is expressed as inhibitory units (IU) ml^{-1}, defined as the smallest volume required to completely inhibit the mycelial growth of P. ultimum in the agar plate bioassay.]

Concentrated PCF was incorporated into sugar beet (Beta vulgaris) seed pellets by Germain's (U.K.) Ltd. at a target dose of 0.33IU $seed^{-1}$. The treatment caused a delay in germination on filter-paper, but had no effect on emergence in a peat-based compost. PCF significantly reduced pre-emergence damping-off in Pythium-infested compost (pelleted seed 38.3% emergence, pelleted seed + PCF 68.9% emergence), although pellets containing the synthetic fungicide hymexazol (5-methylisoxazol-3-ol) showed 94.9% emergence. PCF also reduced post-emergence damping-off (pelleted seed 21.9% diseased, pelleted seed + PCF 15.6% diseased, pelleted seed + hymexazol 2.2% diseased). Thus the number of healthy plants produced was 16.4% from pelleted seed, 53.3% from pelleted seed + PCF and 92.7% from pelleted seed + hymexazol.

This research suggests that the use of antagonistic microbial products is a way of retaining the advantages of biological over chemical control whilst avoiding some of the problems inherent in the use of the microbial antagonists themselves. Future work is aimed at purifying the active metabolites, improving their efficacy and evaluating any side effects on the indigenous rhizosphere population.

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CONTROL OF *PYTHIUM ULTIMUM* WITH ANTAGONISTIC FUNGAL METABOLITES INCORPORATED INTO SUGAR BEET SEED PELLETS

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(*Accepted* 15 *June* 1989)

Summary—*Penicillium claviforme* Bainier culture filtrate (PCF) contained metabolites antagonistic to the mycelial growth of *Pythium ultimum* Trow *in vitro*. PCF extract incorporated into sugar beet (*Beta vulgaris*) seed pellets had no effect on seedling emergence in peat-based compost and significantly improved both emergence and establishment in compost artificially-infested with *P. ultimum*. Although PCF was less effective than the synthetic fungicide hymexazol in reducing a high incidence of disease, the performance of the two agents in suppressing a milder pre-emergence infection was not significantly different. The half-life of the anti-*Pythium* components of PCF in the seed pellets was *ca* 14 months. It is concluded that antagonistic fungal metabolites incorporated into seed pellets have potential as natural fungicides in the control of *Pythium*-induced seed and seedling diseases.

INTRODUCTION

Seed rot and damping-off due to *Pythium* spp are world-wide in occurrence and are the cause of considerable economic loss, particularly in high-value horticultural and aboricultural industries (Hendrix and Campbell, 1973). These diseases are conventionally minimized by insurance treatments with broad-spectrum protectant fungicides (Bruin and Edgington, 1983), although some may have detrimental effects on non-target organisms (Bollen, 1979). A number of more selective, systemic compounds are now available (Cohen and Coffey, 1986), such as metalaxyl, prothiocarb and hymexazol, but they are costly and may cause the evolution of fungicide-resistant pathogen strains (Sanders, 1984).

Biological methods of disease control obviate many of the difficulties outlined above (Cook and Baker, 1983). Various cultural practices which promote the activities of naturally-occurring microbial antagonists of phytopathogens in the soil have been employed for centuries to limit crop damage. However, they may not confer adequate protection in intensive agricultural or horticultural systems if conditions are favourable for disease development. The mass introduction of selected microbial antagonists has the potential to increase the effectiveness of such strategies, and is currently attracting widespread interest (Campbell, 1989), but this approach too has its problems. The resident microbial population of the soil often prevents the establishment of non-indigenous organisms, and the expression of their antagonistic traits may be subject to variations in the physico-chemical environment, resulting in marked inconsistency in their performance in the field (Kommedahl *et al.*, 1981).

Some of these problems might be circumvented by extracting active products from *in vitro* cultures of effective antagonists and exploiting them as natural fungicides (Adams, 1988), thus combining some of the advantages of both chemical and biological control methods.

An important prerequisite for the adoption of any new seed or seedling disease control agent is compatibility with modern seed treatment technologies, such as seed pelleting and film-coating, which permit the efficient introduction of such products directly into the infection court (Halmer, 1988).

We report the *in vitro* production of fungal metabolites inhibitory to the growth of *P. ultimum* Trow and for the first time present data on their efficacy in controlling *P. ultimum*-induced seed rot and damping-off following inclusion in sugar beet seed pellets.

MATERIALS AND METHODS

Production of P. ultimum inhibitory metabolites

Penicillium claviforme Bainier (IMI 44744) was selected from *in vitro* screening tests as the most promising *P. ultimum* antagonist. Liquid cultures were grown in Czapek Dox 4% glucose medium (CDM), pH 4.4–4.6, inoculated with two 10 mm dia mycelial cores cut from a 4-day-old culture grown on 3% malt extract agar (MEA), pH 5.4, and grown at 25°C under stationary conditions.

Samples of *Pen. claviforme* culture filtrate (PCF) were filter-sterilized (Whatman, pore size 0.2 μm), diluted in CDM and incorporated into molten (50°C) corn meal agar (CMA; Oxoid), pH 6.0, in Petri dishes, 1 ml per 20 ml medium. The solidified agar was inoculated with a 5 mm dia core cut from the growing margins of a 2-day-old culture of *P. ultimum* (IMI 82514) grown on CMA and grown at 25°C for 24 h. Percentage inhibition of *Pythium* linear growth was determined by comparison of colony diameter (excluding the inoculum core) with that of controls grown on 20 ml CMA containing 1 ml sterile CDM. Each colony diameter was recorded as the mean of

two measurements at right angles, and duplicate bioassay plates were prepared for each PCF dilution.

Concentrations of extracellular antagonistic metabolites in PCF are expressed as inhibitory units (IU), defined as the reciprocal of the greatest dilution of culture filtrate that will completely inhibit the growth of *P. ultimum* in an agar plate bioassay (Gregory *et al.*, 1952).

Exploratory experiments had established that the maximum concentrations of *Pythium* inhibitors in PCF occurred after 18–21 days of incubation.

Incorporation into seed pellets

Twenty-day-old PCF was concentrated on a rotary vacuum evaporator (Büchi RE111) and then extracted in two successive equal volumes of ethyl acetate. The extract was dried over anhydrous sodium sulphate, the solvent removed by vacuum evaporation and the residue resuspended in distilled water. Filter-sterilized concentrate, containing *ca* 410 IU ml^{-1}, was incorporated into sugar beet (*Beta vulgaris* cv. Accord) seed pellets by Germain's (U.K.) Ltd, King's Lynn, Norfolk. The target dose was 0.33 IU $seed^{-1}$ and the achieved dose 0.30 IU $seed^{-1}$. Percentage seed germination, seedling emergence and establishment from pellets containing PCF were compared with that from pellets with no additives, and pellets containing the synthetic fungicide hymexazol (5-methylisoxazol-3-ol; Sankyo Co. Ltd.) at the recommended rate of 10.5 g kg^{-1} seed.

Effects on seed germination and seedling emergence

Seed germination was assessed in Petri dishes containing sterile filter paper moistened with sterile distilled water and held at 25°C in the dark. Seedling emergence was evaluated in non-infested peat-based compost, as described below.

Disease control

Seed pellets (4 per cell) were planted 7 days (trial 1) or 35 days (trial 2) after production in polystyrene seedling wedge cells (38 mm; Accelerated Propagation Ltd) at a depth of 2.5 cm. Each cell contained *ca* 17.5 g Levington compost (Fisons premier sowing formula, pH 5.6–5.7). Immediately after planting wedge cells received either 8 ml of a suspension containing 4.5×10^4 (trial 1) or 4.9×10^4 (trial 2) propagules *P. ultimum* ml^{-1} tap water, or an equal volume of tap water alone, giving a soil water content of *ca* 75.6% total container capacity (White and Mastalerz, 1966). The propagule suspension was prepared by homogenizing 10-day old mycelial mats (grown in Petri dishes containing 20 ml sterile tap water and two sterile oat seeds) with an ultra-turrax probe (Janke & Kunkel KG) for 2 min. Propagules were enumerated in a haemocytometer. Wedge cells containing infested and non-infested compost were restricted to separate containers, 40 cells per treatment. Containers were placed in propagators in a plant growth room (20°C, 16 h light, 8 h dark) and watered daily to constant weight. Seedling emergence and establishment from each pellet treatment in infested compost are expressed as a percentage of that in control (non-infested) media. Emergence refers to the total number of seedlings which appeared above the compost surface. Establishment here indicates those seedlings which both emerged and survived to day 7 after planting (i.e. did not succumb to either pre- or post-emergence damping-off).

PCF stability

The *P. ultimum* inhibitory activity of PCF, stored at room temperature (20 ± 2°C) under sterile or non-sterile conditions, was measured every week by agar plate bioassay. Activity in the seed pellets was assessed at the time of planting, and once a week thereafter, by soaking pellets in distilled water for 2 h, followed by bioassay of the filter-sterilized eluant.

RESULTS

Effects of PCF on seed germination and seedling emergence

Incorporation of PCF into seed pellets reduced germination rate on filter paper during the early stages of germination (Fig. 1), and caused stunting

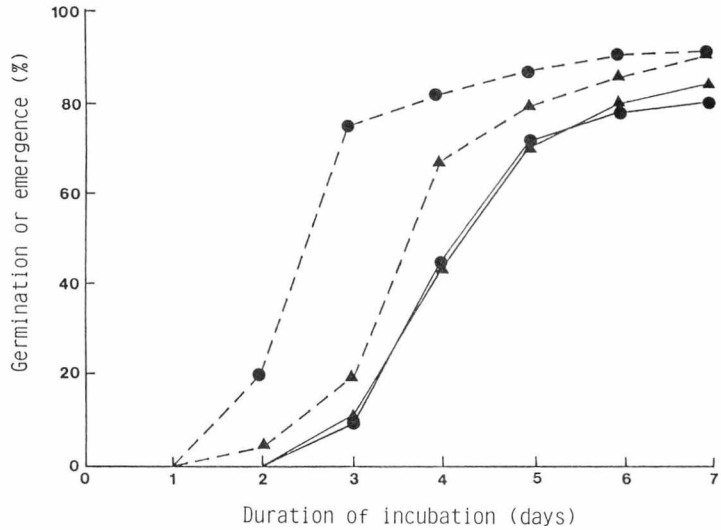

Fig. 1. Germination on filter paper (--) and emergence in compost (—) of *B. vulgaris* seeds pelleted without additives (●) or with PCF (▲).

Table 1. Emergence, survival and establishment of *B. vulgaris* seedlings developing from pellets without additives, or containing PCF or hymexazol, planted in *P. ultimum*-infested compost[a]

Pellet additive	Emergence (%)[b]	Survival (%)[c]	Establishment (%)[b]
Trial 1			
None	36.8 x	42.9	15.8 x
PCF	69.9 y	77.5	54.2 y
Hymexazol	97.0 z	97.7	94.8 z
Trial 2			
None	56.4 x	23.6	13.3 x
PCF	90.9 y	54.4	49.4 y
Hymexazol	98.7 y	96.0	94.7 z

[a] Seed pellets were planted 7 days (trial 1) or 35 days (trial 2) after production in polystyrene cells each containing *ca* 17.5 g peat-based compost infested with *ca* 3.6×10^5 (trial 1) or 3.9×10^5 (trial 2) propagules *P. ultimum*. Results were recorded after 7 days (18°C, 16 h light, 8 h dark).

[b] Data is expressed as a percentage of mean number of plants which emerged in non-infested compost ($83.1 \pm 3.1\%$ in trial 1, $78.8 \pm 1.7\%$ in trial 2), which did not significantly differ between treatments. Each value is the mean of 40 replications each of 4 pellets. For each column, data followed by a different letter are significantly different ($P = 0.05$).

[c] Survival is expressed as a percentage of those plants that emerged in *P. ultimum*-infested compost. Each value is the mean of 40 replications each of 4 pellets.

and abnormal curling of seedling radicles. However, these effects were temporary and after 7 days both percentage germination and morphology were similar to those of controls.

PCF treatment had no effect on rate of, or final percentage seedling emergence in non-infested compost.

Disease control

In trial 1, PCF inclusion in seed pellets significantly increased both percentage emergence and establishment of seedlings 7 days after planting in *P. ultimum*-infested compost compared with plants developing from pellets without additives (Table 1). However, control of both pre- and post-emergence phases of the disease was less than that due to the incorporation of hymexazol.

In the second trial, the incidence of pre-emergence damping-off of control seedlings pelleted without additives was less than in the first (Table 1), and PCF improved emergence as effectively as the synthetic fungicide. However, a greater proportion of seedlings developing from PCF-containing than from hymexazol-containing pellets succumbed to post-emergence *Pythium* attack.

Seedling establishment resulting from each treatment was similar in the two trials. PCF and hymexazol gave plant stands 3.4–3.7 and 6.0–7.1 times those of controls, respectively.

PCF stability

The anti-*Pythium* activity of PCF slowly declined when the extract was stored at room temperature (Fig. 2), particularly under non-sterile conditions (half-life 5.6 weeks). However, the stability of the inhibitory components was considerably enhanced by incorporation into seed pellets which extended the half-life to *ca* 14 months.

DISCUSSION

A number of criteria are used to assess the suitability of potential crop protection agents including phytoxicity, disease control, storage life, effects on other components of the crop ecosystem and cost (Dixon, 1984).

PCF incorporated into seed pellets delayed germination on filter-paper and caused aberrant seedling morphology, but seedlings had recovered completely after 7 days. No phytotoxic symptoms were observed when seed pellets were grown in compost. Similarly, adverse effects of certain fungicide seed treatments may be accentuated on paper substrates compared to compost or soil (Crosier, 1969).

The procedure for infesting compost with *P. ultimum* used in the disease control trials is likely to have resulted in considerably higher inoculum densities in the vicinity of the pellets than that occurring under

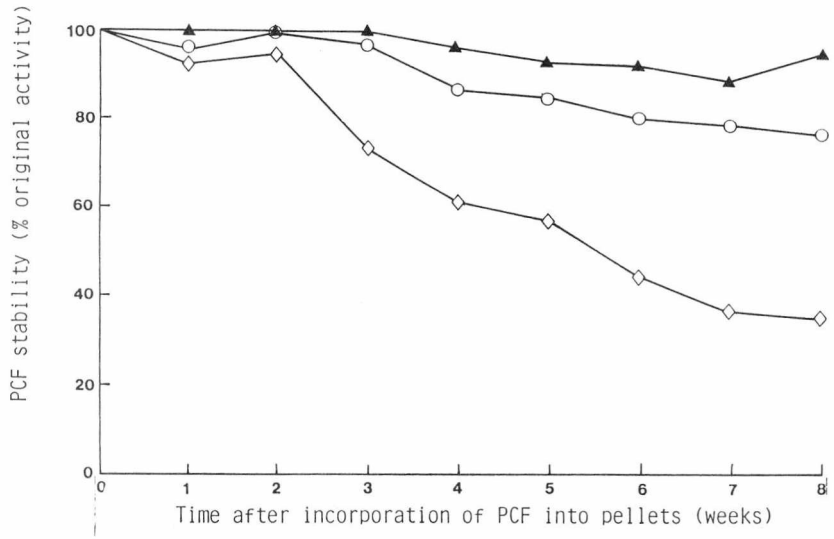

Fig. 2. Stability of *P. ultimum*-inhibitory components of PCF when stored at room temperature under sterile (○) or non-sterile (◇) conditions, or incorporated into *B. vulgaris* seed pellets (▲).

natural conditions. For example, Ali-Shtayeh *et al.* (1986) recovered mean *Pythium* populations ranging from 135 to 5420 propagules g^{-1} dry weight from the surface layers of soil at 24 sites in central England. By comparison, in our experiments means of 2.0–2.1 $\times 10^4$ propagules g^{-1} dry weight were recovered from the surface layers of compost 24 h after infestation. Even in the presence of artificially high pathogen populations PCF significantly reduced damping-off in *P. ultimum*-infested compost. However, except in the case of pre-emergence infection in trial 2 (where overnight storage at 4°C prior to infestation may have decreased the inoculum potential of the pathogen), PCF was less effective than hymexazol. Inferior performance compared to synthetic fungicides was encountered in early attempts to utilize microbial metabolites as plant disease control chemicals (Brian and Hemming, 1945), and is likely to be due in part to instability in soil. Being natural products, such agents are susceptible to degradation by soil microorganism (Pramer, 1958), but this does not necessarily preclude their deployment against seed and seedling pathogens such as *Pythium* spp, where the period during which hosts are susceptible is short (Gattani, 1957). The time over which an effective concentration of PCF is maintained in the infection court could perhaps be prolonged by increasing its rate of incorporation into the pellets, or by strategies designed to slow down its discharge into the spermosphere after planting. For example, the rate of release of insecticides can be manipulated by placing the chemical in different layers within seed pellets, and by altering the nature of the binding agent used during their manufacture (Elmsheuser *et al.*, 1988).

The *Pythium*-inhibitory components of PCF survived the stresses of a commercial seed-pelleting process, and when thus formulated had a half-life of *ca* 14 months. The shelf-life of PCF-containing pellets therefore extends throughout the growing season.

Extracellular antagonistic microbial products implicated in the biocontrol of *Pythium* spp include antibiotics, enzymes and siderophores (Harman and Hadar, 1983). The active agents in PCF have been broadly characterized as antibiotic in nature, and their identity is now being further investigated. Secondary metabolites which have been detected in cultures of *Pen. claviforme* IMI 44744, and which could therefore be involved in *P. ultimum* inhibition, include palitantin, patulin, roquefortine C, terrein and terrestric acid. (R. R. M. Paterson, personal communication).

The costs of discovering and developing new crop protection chemical are enormous and increasing every year (Anon, 1986). When grown in laboratory media based on inexpensive raw materials microorganisms are capable of synthesizing a vast range of compounds which could have potential in combating plant pathogens. The results reported here suggest that the exploitation of such products as seed pellet additives is a feasible alternative approach for the control of *Pythium*-induced seed and seedling diseases.

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